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EFFECT OF STEROL ISOLATE FROM ECHINODERM *STELLASTER EQUESTRIS* ON ZEBRAFISH (*DANIO RERIO*)

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Keywords:

Secondary metabolites, *Stellaster equestris*, Zebra fish, Sterol, NMR, FTIR, Toxicity assessment, Compound A

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ABSTRACT: Expansion of secondary metabolites from marine resources mainly from echinoderms has been a subject of intense research due to massive bioactive nature. Isolation of new secondary metabolites from sea stars has been found to be highly polar. An initial assessment and validation of these compounds are necessary to assess human risk. Zebrafish are used as a part of a diverse phase of the drug discovery development and has proved to be a constructive and cost-effective alternative to various mammalian models. The current study was performed to observe the effect of sterol isolate from *Stellaster equestris* on Zebrafish (*Danio rerio*). The selected active crude extract (which showed enhanced cytotoxic activity) was subjected to purification and was characterized by spectroscopic analysis. The absorption frequency of the FTIR and NMR analysis resembled the absorption frequency of a steroidal compound. The analyzed compound was then evaluated for toxicity using *Danio rerio* using DNA fragmentation assay, Embryotoxicity, Fin regeneration, and Cardiotoxicity assay as the endpoints. The fishes were exposed to 4 different concentrations (50 mg/ml, 100 mg/ml, 200 mg/ml and 400 mg/ml). DNA fragmentation assay revealed intact DNA bands suggesting that the compound is not genotoxic. The extent of fin regeneration was found to be marginally affected by the increase in the concentration of the compound. Embryotoxicity and cardiotoxicity assay in Zebrafish demonstrated the absence of any visible abnormality even with the increase in concentration. Thus, the present study signifies that compound A isolated from *Stellaster equestris* is not toxic.

INTRODUCTION: Marine natural products are a valuable medicinal resource for humans. Research studies on compounds isolated from starfish which possess pharmacological properties have extended rapidly over the years. Starfish has fascinated the researchers as an intriguing source of bioactive marine natural products [1, 2].

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Starfish *Stellaster equestris* belongs to the family Goniasteridae and class Asteroidea which resides in the benthic region and are constantly exposed to various organisms; consequently, they adapt the survival tactic to protect themselves from the external pathogens.

They thus secrete pharmacologically effective secondary metabolites. Since these compounds are secreted as a metabolic product during their survival instinct, a preliminary assessment of these compounds is necessary to evaluate human risk. Target-based drug discovery instigates categorizing target identification and validation. Screening of compounds is important to overcome risk factors.

In-vitro studies using cell lines has its limitations as not all biological processes can be mimicked *in-vitro.* It is important to understand that the metabolism of the compounds may be greatly altered in whole organisms [3, 4, 5]. Screening of compounds with high throughput for biological activity in whole organisms is important and has become feasible using Zebrafish embryos or Zebrafish adults. Zebrafish is a dynamic model organism because of their intrinsic characteristics like sharing of genetic homology with humans, size, short life cycle, ease of breeding and cost-effective for screening drugs [6, 7]. *In-vitro* fertilization and embryogenesis make Zebrafish a simpler and more attractive animal model for investigating reproductive toxicity and teratogenicity [8, 9]. The Zebrafish model is increasingly used for calculating the toxicity and safety of the drug. Various studies on toxicity substantiate that mammalian model and Zebrafish model are outstandingly comparable. In the early stages of drug development, zebrafish can be used to purge potentially unstable compounds rapidly to evaluate and prioritize compounds for future preclinical and clinical studies [10, 11, 12]. Recent research exploits zebra fish as a genetic model in the field of studies such as cancer research, nervous system physiology, and drug discovery. The present study deals with the effect of isolated sterol compound from Echinoderm *Stellaster equestris* on Zebrafish (*Danio rerio*).

MATERIALS AND METHODS:

Sample Collection and Extraction: Sample collection was done using the fishing net from the fishing harbor of north Chennai (Latitude 13º 06'N, Longitude 80º 18'E). Starfish *Stellaster equestris* was collected by bi-catch using fishing nets from the fish landing centers of Chennai coast. The samples were then shifted to the laboratory aseptically by cold storage thermal resistant box. Whole body extract from Starfish *Stellaster equestris* was extracted using appropriate solvents by standard extraction procedures. Extraction procedure was followed with dichloromethane, chloroform, ethyl acetate, and methanol sequentially. Dried extracts were transferred to an amber bottle and stored at 5 °C.

Purification of Crude Extracts: Based on the results of *in-vitro* studies, the IC_{50} values for the extract were defined as 50 µg/ml (Hexane), 50 µg/ml (Dichloromethane) and 35 µg/ml (Chloroform and methanol). These results suggested that chloroform and methanolic extract have stronger cytotoxicity compared to other extracts. Hence, the crude extract of chloroform as a solvent was taken for column chromatography [13]. The silica slurry was made using n-hexane, and the solvent used for elution was hexane: ethyl acetate in various ratios. The crude sample which showed the IC_{50} value was subjected for separation. Test tubes were placed in a test tube rack for the sequential collection of the fractioned compound.

Each fractioned compound was subjected to thin layer chromatography. The TLC was used to separate various compounds present in the crude chloroform extract using ideal solvent hexane: ethyl acetate (7:3) the separated bands were subjected to further isolation. The compound was then isolated using sub column chromatography, where the compound was eluted from active guided fraction in the ratio hexane: ethyl acetate [50:50, v/v] showed a promising yield of the compound which was single with no mixture and a potent yield. The compound A (1000 mg) was lyophilized, and FTIR and NMR characterized the powdered compound.

Spectroscopic Analysis:

Fourier Transform Infrared Spectroscopy: The lyophilized sample was subjected to identification of organic molecular groups, functional groups, side chains and cross-links involved. FTIR analysis was performed at the College of Pharmacy (SRMC& RI).

Nuclear Magnetic Resonance Spectroscopy: The lyophilized sample was dissolved in chloroform and scanned in the range of 4000-500 cm^{-1}. H NMR spectra and ^{13}C NMR spectra were recorded at 400 MHz and 100 MHz respectively. The NMR (BRUKER) spectroscopic analysis was performed at Indian Institute of Technology Chennai, India.

Preparation of Test Compounds: The lyophilized 1mg of the compound A was dissolved in 5 ml of DMSO (1000 mg/ml = 5000 ppm). The working concentrations of the study were defined as 50 ppm (50 mg/ml), 100 ppm (100 mg/ml), 200 ppm (200 mg/ml) and 400 ppm (400 mg/ml).

***In-vivo* Toxicity Assessment - Zebrafish Maintenance ((*Danio rerio*):** Adult Zebrafish and embryos were acquired from an aquarium at Kolatthur, Chennai. The procured embryos and adult fish were carefully preserved in tanks filled with fresh water which is constantly filtered and aerated to maintain the water quality required for a healthy environment for fish. The tests performed using embryos, and adult fish are cardiotoxicity assay of embryo, DNA fragmentation assay and fin generation assay using adult Zebrafish. 25 adult fish were accommodated in a 45-liter tank each which was covered using a muslin cloth to allow appropriate air and light for the circulation and aeration was provided by using a pump motor to make a healthy environment for the fish. The water quality was checked frequently to avoid contamination.

DNA Fragmentation Assay- DNA Extraction by PCI Method: The fishes were exposed to varying concentrations of compound A (50 ppm, 100 ppm, 200 ppm, and 400 ppm) along with benzaldehyde as the positive control and water as negative control. Post-exposure, they were euthanized and incised into tiny pieces using a sterile surgical blade. The tissue fragments were then transferred to an Eppendorf to which 0.5ml of the extraction buffer (800 mM Tris pH 8.5, 200 mM NaCl, 0.5% SDS, 5 mM EDTA) was added and incubated at 65ºC overnight. The supernatant was collected by centrifugation at 10000 rpm for 10 min to which 0.5 ml of PCI (phenol/chloroform/isoamyl alcohol) was added, mixed well and centrifuged. The supernatant was transferred into a fresh Eppendorf to which 0.5 ml of isoamyl alcohol was added. The contents were mixed thoroughly and centrifuged. The supernatant was discarded, and the pellet was washed with 75% ethanol and centrifuged. The resulting DNA pellet was air dried and suspended in 150 µl of TE buffer. The extracted DNA was then subjected to 3% agarose gel electrophoresis at 50 volts. The gel was then visualized under UV trans-illuminator to check the presence of DNA bands.

Fin Regeneration Assay: The fin regeneration assay was performed to assess the degree of regeneration and toxicity induced by the compound. Healthy adult Zebrafish were selected and exposed to varying concentrations of compound A (50 ppm, 100 ppm, 200 ppm, and 400 ppm) along with benzaldehyde as the positive control and water as negative control. The adult Zebrafish were anesthetized by introducing fishes in an anesthetic tub (1g of prilox cream in 500 ml water) for about 5 min, *i.e.* till pasting of response and decline in heart rate was observed. The anesthetized fish were rapidly transferred to a Petri dish which was then shifted under microscopic observation the tip of the caudal fin (~2 mm) was then cut using a sterile surgical blade. The amputated fish were then immediately transferred to a revival bath (freshwater). The fish usually takes the same time to revive as for anesthetization. Different concentration of compounds (50 ppm, 100 ppm, 200 ppm, and 400 ppm, negative and positive control) was added into the separate tanks and incubated for 5 days. After completion of 5 days, the fish were again anesthetized and checked under a stereomicroscope for the extent of regeneration of the caudal fin.

Embryotoxicity Assay: The Zebrafish embryos act as an excellent model for studies aimed at the understanding of toxic mechanisms and the indication of possible adverse and long-term effects. Analysis of acute toxicity in embryos helps in monitoring the visual assessment of morphological characters and also includes the screening for developmental disorders as an indicator of teratogenic effects [14, 15]. The Zebrafish embryos [48 h post fertilized (hfp)] were acquired from Kolathur farm, Chennai. Zebrafish embryos were individually exposed in 12 well culture plates in different concentrations of the compound A with a negative and positive control (50 ppm, 100 ppm, 200 ppm, and 400 ppm). For every concentration, two embryos were added into each well of a 12 well plate, and their developmental changes were observed at periodic time intervals for a week. The phenotypic changes of the embryos were observed. The embryos were observed for malformations (such as tissue ulceration, pericardial edema, body arcuation, yolk sac edema, tail, and head malformations) and the results were documented.

Cardiotoxicity: The systematic evaluation of pharmacologically active compounds using cardiotoxicity assay in Zebrafish embryo has been an endpoint for the assessment of compounds. Zebrafish embryos were individually exposed in

different concentrations of the compound with a positive and negative control (50 ppm, 100 ppm, 200 ppm, and 400 ppm) in 12 well culture plates. The embryo was transferred aseptically under the zoom microscope, and the pulsing heart rate was focussed under the microscope, some pulse per minute was counted for each of the embryos was tabulated. Any variation in the number of beats for each of the concentrations was noted.

RESULTS:

Spectroscopic Analysis:

Fourier Transform Infrared Spectroscopy:

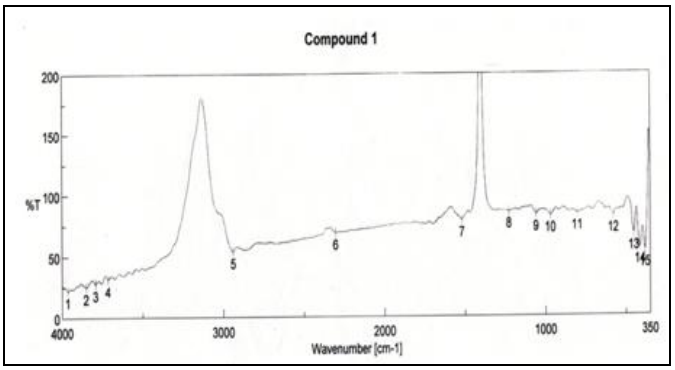

FIG. 1: IR SPECTRA OF THE PARTIALLY PURIFIED COMPOUND A

Absorption bands in **Fig. 1** shows the infra-red region premeditated results from interatomic vibrations, whose frequencies are related to the strength of the atomic bands to particular atomic groups or linkages. This has been accomplished by comparison of the various spectra obtained in the laboratories. On subjection to the IR spectroscopic analysis, the observed absorption bands as mentioned in **Table 1** confirm the position at 3795.22 cm^{-1} that is characteristics of O-H stretching. Absorption at 2938.98 cm^{-1} is assumed to be due to cyclic olefinic –HC=CH structure, 2302.59 cm^{-1} is due to = CH structure and 2938.98 cm-1 are assigned to C-H structure. Other absorption frequency includes 1638.83 cm^{-1} as result of C=C absorption; however, the band is weak. 1519.63 cm^{-1} is a bending frequency for cyclic (CH_2) n and 1226.5 cm^{-1} for $–CH_2$ (CH_3) 2ɣ. The absorption frequency at 1056.8 cm^{-1} signifies cycloalkane. This absorption frequency resembles the absorption frequency of steroidal compounds.

TABLE 1: RESULT PEAK PICKING

S. no.	Position	Intensity
1	3964.93	22.4612
2	3853.08	25.3003
3	3795.22	27.9466
4	3718.08	31.5572
5	2938.98	53.9437
6	2302.59	68.8289
7	1519.63	78.577
8	1226.5	85.5686
9	1056.8	82.9873
10	968.09	81.9876
11	802.242	83.636
12	578.54	81.4432
13	451.261	66.8165
14	412.692	55.4828
15	381.836	52.8252

Nuclear Magnetic Resonance Spectroscopy:

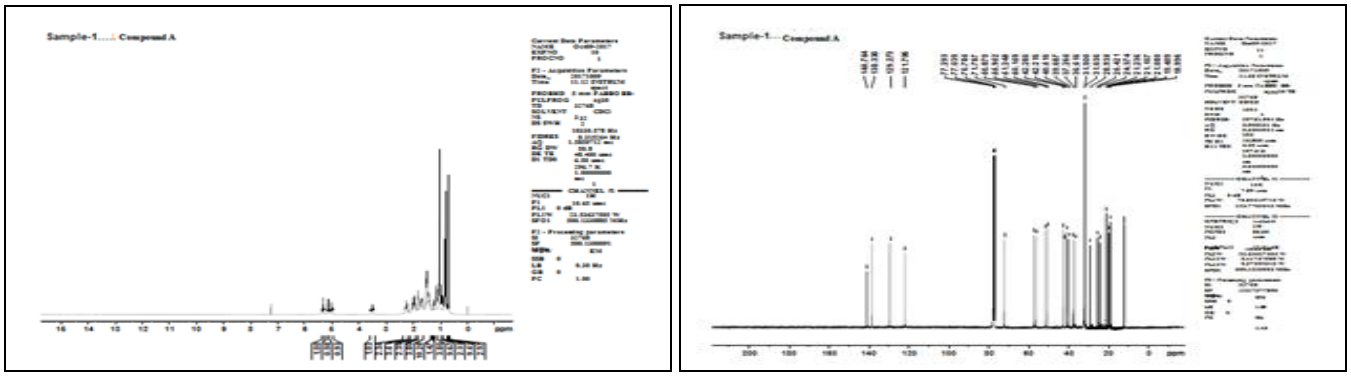

FIG. 2: NMR SPECTRUM OF THE PARTIALLY PURIFIED COMPOUND A

The ^{1}HNMR spectrum of compound Ain **Fig. 2** varied between 0.789-5.126 ppm. This spectrum in **Fig. 2** showed the presence of six high-intensity peaks of the methyl group at δ 0.789, 0.852, 0.961, 1.043, 1.209 and 1.518 ppm. The proton corresponding to H-3 of sterol moiety appeared as a triplet of 3.530, 3.520, 3.510 at δ5.157 ppm to 5.351 ppm corresponds to a peak in the form of a single peak in the region of ethylene protons suggesting the presence of three protons. The ^{13}C NMR spectrum of compound A has given signals at 140.754, and 121.706 ppm which corresponds to

double bond for C5, C6 respectively and hence represents signals at 138.330 and 120.273 ppm which corresponds one more double bond for C22 and C23. The δ value at 71.78 ppm is due to C-3 β hydroxyl group. The signal at δ 31.900 and δ 12.268 ppm correspond to carbon atom C25 and C 29 respectively. The above NMR observation of the isolated compound resembles steroidal compound Stigmasterol.

DNA Extraction by PCI Method: DNA from the exposed fish with the concentrations mentioned above **Fig. 3** along with positive and negative control was extracted by the PCI method. The quality of the DNA was checked using nanodrop. 3% agarose was employed to evaluate DNA fragmentation. Nanodrop result suggested good yield and quality of DNA. DNA fragmentation assay revealed intact DNA bands which suggest that the compound A is not genotoxic to the DNA of fish.

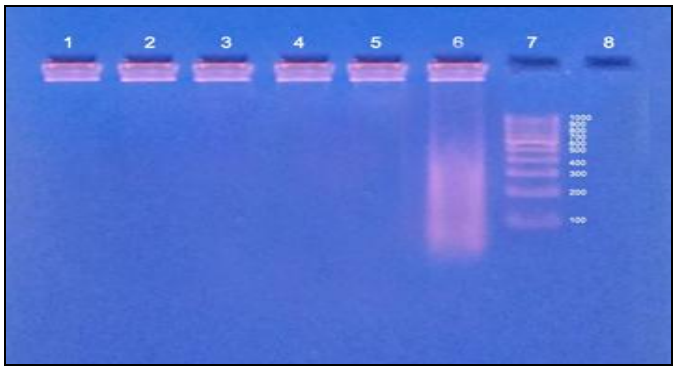

FIG. 3: DNA FRAGMENTATION ASSAY (*DANIO RERIO*)
Legend: 1- Negative control. 2- 50 ppm (50 mg/ml of compound A), 3- 100 ppm (100 mg/ml of compound A), 4- 200 ppm (200 mg/ml of compound A), 5- 400 ppm (400 mg/ml of compound A), 6- Positive control, 7- DNA ladder, 8- Blank

Fin Regeneration Assay: Fin regeneration assay was performed with the concentration mentioned above along with positive and negative control. The results in **Fig. 5** suggest that the extent of regeneration has been marginally affected with an increase in concentration as shown in **Table 2** and **Fig. 4**.

TABLE 2: LENGTH OF FIN REGENERATION

S. no.	Concentration of compound	Length of Fin regeneration (mm)
1	50 ppm	2.4 mm
2	100 ppm	2.0 mm
3	200 ppm	1.8 mm
4	400 ppm	1.6 mm
5	Negative control (water)	2.5 mm
6	Positive control (benzaldehyde)	0.9 mm

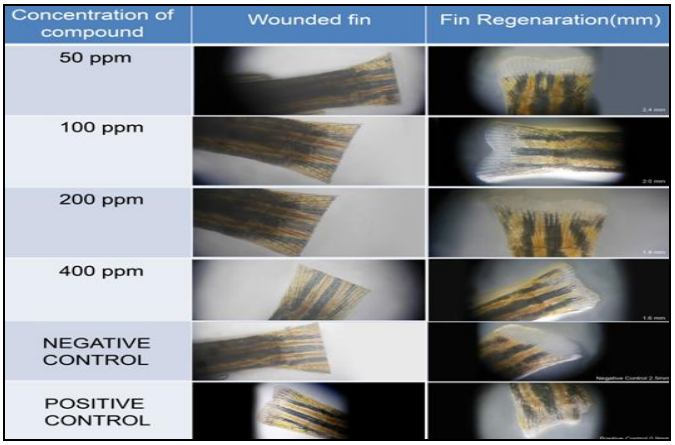

FIG. 4: FIN REGENERATION ASSAY

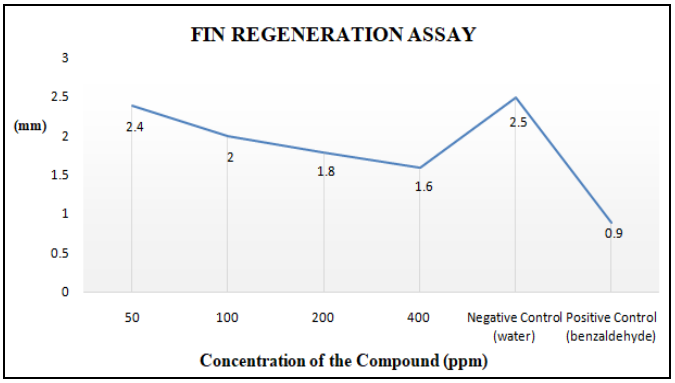

FIG. 5: DATA ANALYSIS OF FIN REGENERATION ASSAY

Embryo Toxicity Assay: The embryo exposed to the compound A exhibit any developmental toxicity during the five day incubation period. It did not affect the survival of the embryos, and no phenotypic abnormalities were observed for the concentration, but the disintegration of embryos occurred on day 5 for the positive control as indicated in **Fig. 6**.

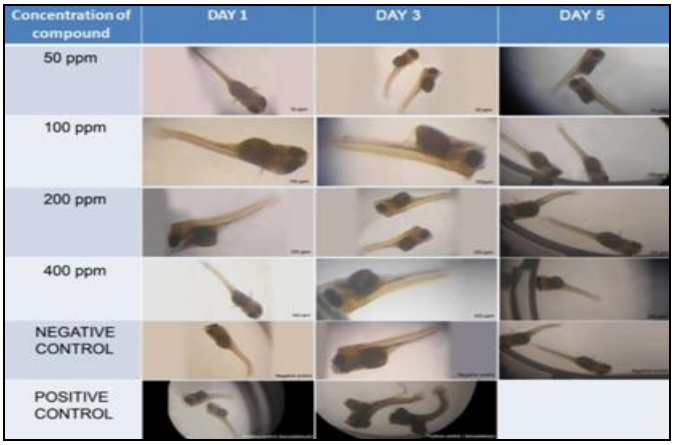

FIG. 6: EMBRYOTOXICITY ASSAY SHOWING DEVELOPMENTAL STAGES OF THE ZEBRAFISH EMBRYOS AT 1, 3 AND 5 DAY INTERVALS FOR CONCENTRATIONS 50 ppm, 100 ppm, 200 ppm, 400 ppm NEGATIVE CONTROL AND POSITIVE CONTROL

Cardiotoxicity: Adult fishes were evaluated for some pulses per minute post 48 hour's incubation with the compound A. The number of pulses indicated a marginal drop **Fig. 7** as a consequence of exposure to the compound. The result suggested that the number of pulse per minute in comparison with the negative control were stable and did not show any change in heart rate. Some pulses were recorded and tabulated as below **Table 3**.

TABLE 3: NUMBER OF PULSES PER MINUTE FOR VARIOUS CONCENTRATION OF COMPOUND A

S. no.	Concentration of compound	No. of pulses/minutes
1	50 ppm	150
2	100 ppm	148
3	200 ppm	146
4	400 ppm	145
5	Negative control (water)	152
6	Positive control (benzaldehyde)	122

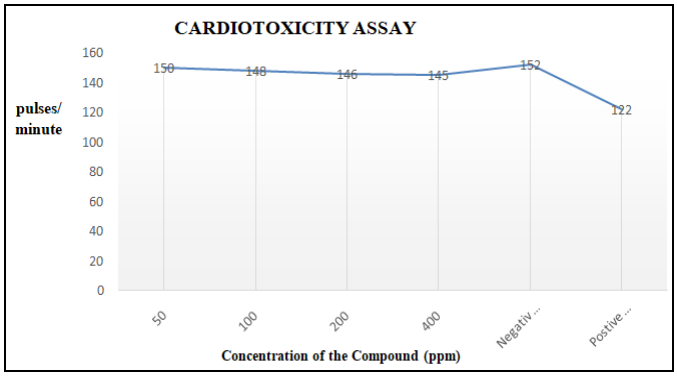

FIG. 7: DATA ANALYSIS OF CARDIOTOXICITY ASSAY

DISCUSSION: The present study has evaluated the *in vivo* toxicity of compound A on *Danio rerio* as a model organism. Whole body extract from Starfish *Stellaster equestris* was extracted by cold percolation method with dichloromethane, chloroform, ethyl acetate, and methanol in a sequential manner. The crude extract of chloroform as a solvent was subjected to column chromatography and TLC and the separated bands were subjected to further isolation and compound A was lyophilized and FTIR and NMR characterized the powdered compound. FTIR demonstrated absorption frequency that resembled the absorption frequency of steroidal compounds and NMR observation of the isolated compound resembled steroidal compound Stigmasterol. With this characterization, the lyophilized compound was dissolved in DMSO (1000 mg/ml = 5000 ppm).The working concentrations were defined at 50 ppm (50 mg/ml), 100 ppm (100 mg/ml), 200 ppm (200 mg/ml) and 400 ppm (400 mg/ml). The *in-vivo* toxicity of the defined concentrations was tested on *Danio rerio* as the animal model. DNA was isolated from exposed fishes, and nanodrop results confirmed good yield and quality of DNA. DNA fragmentation assay revealed intact DNA bands which suggest that the compound is not genotoxic.

The results of fin regeneration assay suggest that the extent of regeneration has been marginally affected with an increase in concentration. The embryos exposed to the compound did not exhibit any developmental toxicity during the five day incubation period. It did not affect the survival of the embryos, and no phenotypic abnormalities were observed for the defined concentrations. The results of cardiotoxicity assay suggested that the number of pulse per minute on comparison with the negative control were stable and did not show any change in heart rate. The present study concludes that compound A isolated from *Stellaster equestris* is not toxic with the results obtained from experiments conducted on *Danio rerio*.

CONCLUSION: The outcome of the present study reflects non-toxicity of secondary metabolites from marine resources and their potential application as anticancer and antimicrobial agents. Zebrafish has proved to be a dynamic *in-vivo* model in assessing the toxicity of sterol and further testing and characterization may help us evolve sterol as an effective bioactive compound.

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CONFLICT OF INTEREST: The author(s) R. Sumitha, Dr. V. Deepa Parvathi and Dr. N. Banu declare(s) that there is no conflict of interest regarding the publication of this article "Effect of sterol isolate from echinoderm *Stellaster equestris* on Zebrafish (*Danio rerio*)."

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