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1. Introduction

Ocean covers more than 70% of Earth surface, contains diverse biological sources accounting more than 95% of global ecosystems. Amongst, marine microorganisms encompass a wide and diverse assemblage of organisms in global ecosystem, of which it is estimated that only 1% has been cultured or identified. In recent years, researchers focusing on marine actinomycetes due to its excellent metabolic and physiological potential that not only assures high growth at extreme habitats, but also offers the capability to produce secondary metabolites with biological activities that would not be available in terrestrial microorganisms. These naturally occurred marine actinomycetes derived secondary metabolites are trapped through high-throughput screening and fermentation, mining genomes for cryptic pathways and combinational biosynthesis to produce new secondary metabolites related to existing pharmacophores.

In case of marine actinomycetes there have already been several attempts to optimize their isolation and growth from several sources. Development of fermentation process for production of bioactive compound from marine actinomycetes with excellent applications is fascinating now-a-days. Although more than 30,000 diseases have been clinically described, less than one third of disease can be treated symptomatically and fewer can be cured. Natural products play a major role in drug discovery as secondary metabolites by many organisms act as effective drug for many human diseases. Exploitation of marine actinomycetes as a source for discovery of novel secondary metabolites is at an early stage, numerous novel metabolites have been isolated in the past decade. Around 23,000 bioactive secondary metabolites produced by microorganisms and 70% secondary metabolites are produced by actinomycetes, 20% from fungi, 7% from Bacillus spp. and 1–2% by other bacteria. Among actinomycetes, around 10,000 compounds are produced by Streptomyces species and many of these secondary metabolites are potent antibiotics which has made streptomycetes the primary antibiotic-producing organisms exploited by pharmaceutical industry.

The rare actinomycetes produce diverse and unique, unprecedented, sometimes very complicated compounds exhibiting excellent bioactive potency with low toxicity. Various antimicrobial substances from actinomycetes have been isolated and characterized including aminoglycosides, anthracyclines, glycopeptides, beta-lactams, macrolides, nucleosides, peptides, polynyes, polyesters, polyketides, actinomycins and tetracyclines. Adriamycin, also called doxorubicin (DOX), is an antibiotic anthracycline that was isolated from a pigment of Streptomyces peacetius has been employed for more than 30 years.
in the battle against cancer\textsuperscript{18-19}. On other hand, chronic effects may develop several weeks or even months after recurrent administration of drug, provoking heart, liver, brain or kidney injury in chemotherapy given cancer patients as well as animal models. Since cardiomyocytes, as well as neurons, are post-mitotic cells, the vast majority of damage is irreversible and unalterably affects cardiac and brain functions\textsuperscript{20,21}. To overcome this problem, the researchers focusing on the production of bioactive compounds from natural resources with less toxicity. In this connection, the present study aims for the production and characterization of bioactive compound from \textit{Streptomyces variabilis} and evaluate the toxicity against human model system \textit{Danio rerio}. Further, anti-oxidant activity of identified compound was determined through various in vitro assays.

2. Results

2.1. Antimicrobial activity of ethyl acetate extract

Among 21 actinomycetes isolated from coral mucus, \textit{S. variabilis} was dominant in nature and selected for mass culture to produce bioactive compounds. After 7 days culture of actinomycete in ISP2 medium, the fermentation was terminated and centrifuged at 10000 rpm for 10 min. Ethyl acetate was used to extract the bioactive compounds from culture supernatant of actinomycetes. Antimicrobial activity of crude ethyl acetate extract was assessed against selected human pathogens using disc diffusion test. The results showed excellent antimicrobial activity against all tested pathogens (Fig. 1a). The crude extract of \textit{S. variabilis} showed antimicrobial activity against \textit{E. coli} (19mm), \textit{V. cholerae} (21mm), \textit{K. pneumoniae} (17mm), \textit{P. aeruginosa} (24mm), Enterobacter sp. (23mm) and \textit{Streptococcus} sp. (20mm). This finding indicated that crude ethyl acetate extract of \textit{S. variabilis} has potential bioactive compounds and inhibit the growth of clinical human pathogens.

2.2. TLC chromatogram and antimicrobial activity

The crude ethyl acetate extract was chromatographed on silica gel for separation of compounds using methanol: ethyl acetate: chloroform (4:4:2) solvent system. The separated compounds were purified in full column chromatography and tested for antimicrobial activity against selected human pathogens. A total of six fractions (Fig. 1b) were obtained in ethyl acetate extract of \textit{S. variabilis} and assessed for antimicrobial activity. The results showed that fractions 2 and 6 has good antimicrobial activity against all tested pathogens while fraction 1 and 3 did not showed any antimicrobial activity and moderate activity was observed in fraction 5 against few selected pathogens. Among six fractions, fraction 4 (F4) showed excellent antimicrobial activity (Fig. 1c) against \textit{E. coli} (16mm), \textit{V. cholerae} (18mm), \textit{K. pneumoniae} (13mm), \textit{P. aeruginosa} (20mm), \textit{Bacillus} spp. (23mm), Enterobacter sp. (21mm) and \textit{Streptococcus} sp. (26mm). The results confirmed that purified compound (F4) from \textit{S. variabilis} has excellent antimicrobial activity and F4 was selected for further characterization.

2.3. FT-IR

The F4 from \textit{S. variabilis} showing high antimicrobial activity was purified using column chromatography and analyzed for FT-IR characterization. The F4 indicated the presence of =C-H stretch at 760 cm\textsuperscript{-1}, C-H stretch, C-N stretch, C-C stretch and C=O stretch at range of 1089, 1324, 1590 and 1684 cm\textsuperscript{-1} (Fig. 2a). FT-IR results suggested that functional groups present in F4 may possibly influence antimicrobial and anti-oxidant activity.

2.4. GC-MS

GC-MS analysis of F4 showed 15 volatile substances were carefully identified on the basis of their mass spectral properties as compared with NIST database. Among 15 volatile compounds, the compound 1-Hydroxy-1-norresistomycin was obtained at retention value of 38.017 RT and occupied about 97.24% of relative area revealing that unidentified compound was single and pure (Fig. 2b).

2.5. NMR

The F4 obtained from actinomycetes strain \textit{S. variabilis} was characterized using \textsuperscript{1}H NMR and \textsuperscript{13}C NMR to elucidate the structure of compound. \textsuperscript{1}H NMR spectrum of purified compound showed two hydroxyl groups at region of $\delta$ 12.228 and $\delta$ 10.944, aromatic groups at $\delta$ 7.692 – 6.864. The four methoxy signal at $\delta$ 3.814, 3.692, 3.560 and 3.523 and two aromatic methyl group at $\delta$ 2.515 and 2.321 (Fig. 3a). \textsuperscript{13}C NMR spectrum showed two carbonyl carbons at 173 and 136 ppm bearing with double bonded oxygen, seven addition aromatic carbons at region between 108-127 ppm and methyl carbon signals were obtained at region of 31-40 ppm (Fig. 3b). From the above characterization viz. FT-IR, GC-MS, \textsuperscript{1}H NMR and \textsuperscript{13}C NMR, F4 obtained from actinomycetes strain \textit{S. variabilis} was identified as 1-Hydroxy-1-norresistomycin (HN M) and molecular formula was $\text{C}_{12}\text{H}_{14}\text{O}_{7}$ (Fig. 4). The results were compared with previous reports (Kock et al., 2005; Gorajana et al., 2005) and structure of HNM was predicted using Chemdraw (Ver. 15).

2.6. Anti-oxidant activity

The efficacy of HNM in scavenging of oxidative stress and cancer inducing molecules was assessed via. DPPH radical scavenging activity, Hydrogen peroxide scavenging activity, Hydroxyl radical scavenging activity and Reducing power.

2.6.1. DPPH scavenging activity

DPPH radical scavenging is one of the prominent methods to study the efficiency of scavenging activity of natural compounds by scavenging the oxidation through providing hydrogen atoms and formed non-radical DPPH. The color changed from purple to yellow is the primary confirmation of scavenging process and mixture after reaction was read at 517nm. DPPH scavenging activity was increased with increase concentration of HNM (50-500 µg/ml). About 82% of DPPH was scavenged at 500 µg/ml concentration of HNM and 300 µg/ml concentration was identified as IC\textsubscript{50} for purified compound HNM (Fig. 5a). These results indicated that HNM can act as potential DPPH scavenger by donating hydrogen atoms.

2.6.2. Hydrogen peroxide scavenging activity

Scavenging of hydrogen peroxide is an important process of natural drugs and plays a key role in oxidative stress by increasing ROS level in cells. The purified compound was scavenged the $\text{H}_2\text{O}_2$ by dose (50-500 µg/ml) dependent manner and results indicated that $\text{H}_2\text{O}_2$ scavenging activities of purified compound was nearly equal to commercial antioxidant BHT at higher concentration (500 µg/ml) tested. Almost 76% (Fig. 5b) of $\text{H}_2\text{O}_2$ was scavenged at higher concentration of HNM and IC\textsubscript{50} concentration was determined as 400 µg/ml. The conclusion of these findings shows that HNM plays a key role in scavenging ROS inducing molecule DPPH.

2.6.3. Hydroxyl radical scavenging activity
Hydroxyl radicals are one of the most hazardous molecules among radicals that can damage the normal cells and causes cancer. In this study, HNM scavenged about 75% of hydroxyl radical was scavenged at 500 µg/ml (Fig. 6a) and 350 µg/ml of HNM was determined as IC50. This IC50 concentration of HNM was balanced to BHT (350 µg/ml) in scavenging the hydroxyl radicals.

2.6.4. Reducing power

Reducing the power of ferric chloride is also an important assay to prove antioxidant potential of natural drug. In relation to this, HNM reduced 81% (Fig. 6b) power of ferric chloride at 500 µg/ml concentration indicated the HNM was found to be an excellent reducing power ability and also lesser than commercial antioxidant BHT at 500 µg/ml.

2.7. Histopathological study

The mortality of experimental fish D. rerio a human model system was observed after 21 days exposure of 500 µg/L concentration of HNM. The results showed that less number of mortality (>5%) was observed after 21 days. The histopathology of zebra fish organs such as heart, liver, kidney, spleen, intestine and gills were analyzed. There is no evidence for significant morphological changes in control and HNM treated of heart after 21 days (Fig. 7a & 7b). Normal morphological structure such as liver parenchyma, clear hepatic cords and centered nuclei were detected in control as well as HNM exposure liver (Fig. 7c & 7d). The well-structured glomerular capillariae, mesengial cell hypertrophy, matrix, Bowman’s capsule layers and Bowman’s spaces were observed in water (control) and HNM treated kidney of human model system D. rerio (Fig. 7e & 7f). After 21 days, in control HNM treated intestine has well structure of basal lamina, connective tissues, lumen, structured epithelium and vacuoles were observed (Fig. 8a & 8b). But, there are morphological changes such as disorder in ellipsoid cells, increase the number and size of melanomacrophage centers, cloudy swelling was observed in spleen of zebra fish after HNM exposure while there is no apparent changes was detected in control fish (Fig. 8c & 8d). The normal strips of primary and secondary lamellae with epithelial and chloride cells were present in both control and HNM exposure gills of D. rerio (Fig. 8e & 8f).

3. Discussion

Oceans with their diverse life forms cover around 70% of earth’s surface and their milieu of environment was quite differ from nutrient-abundant regions to nutritionally sparse locations where only a few microorganisms can survive. Among microorganisms, actinomycetes have been recognized as potential producers of metabolites such as antibiotics, growth promoting substances for plants and animals, immunomodifiers, enzyme inhibitors and many other compounds of use to man. Isolation of new microbial species from unexplored environments is one of more efficient approaches for development of potential novel bioactive metabolites.

A potent actinomycete S. variabilis was isolated from unexplored marine organism i.e. the mucus of Scleractinia coral A. formosa. Initially, ethyl acetate extract of S. variabilis was screened for antimicrobial activity against selected human clinical pathogens and results showed actinomycetes were produced antimicrobial secondary metabolites. Ethyl acetate extract of actinobacteria showed maximum antimicrobial activity than methanol and ethanol extracts while acetone extracts has potential antimicrobials and proper fermentation process reveals to bring out new class of antibiotics from actinomycetes.

The biomolecules present in ethyl acetate extract was separated through TLC, F4 showed better antimicrobial activity among obtained six fractions. The bio-autography of separated fractions is employed to detect single compound is responsible for antibacterial activity. Fraction VI was selected for compound characterization owing to excellent antimicrobial activity among tested seven purified fractions obtained in TLC.

The F4 obtained from S. variabilis was characterized using FTIR, GC-MS and NMR. The FT-IR analysis of Streptomyces sp. showed the presence of alkyl halides, alcohol and aldehyde groups. IR spectrum analysis indicated the presence of hydroxyl (3398 cm⁻¹), carbonyl (1685 cm⁻¹), aromatic system (1619, 1589 cm⁻¹) and other groups (1166 cm⁻¹)²⁸. In connection to this, IR spectrum of F4 from S. variabilis showed the functional groups that may possibly involve in antimicrobial and anti-oxidant activity and also as valuable support for structure prediction of purified compound.

GC-MS spectral analysis of ethyl acetate extract of Streptomyces ruber showed the presence of Pthalic acid at 21RT with 91% of relative abundance. Among 11 compounds obtained from Streptomyces lavendulae using GC-MS analysis the compound Actinomycin was identified as abundant that showed 7.12% of relative abundance. The present study also reveals the 1-Hydroxy-1-norresistomycin was identified using GC-MS analysis from S. variabilis with 38.07RT with 97.24% of relative area.

The structure elucidation of F4 was predicted by analyzing the ¹H NMR and ¹³C NMR and results confirmed that the compound was 1-Hydroxy-1-norresistomycin. ¹H NMR and ¹³C NMR spectroscopy analysis of bioactive compound obtained from Streptomyces chibaensis showed the presence of three aromatic singlets, hydroxyl group attached to cyclic compound, methyl group and two carbonyl groups. NMR and EI HRMS spectroscopy of Streptomyces strain B8005 were confirmed the structure of 1-Hydroxy-1-norresistomycin and showed the presence of three hydroxyl and aromatic groups in ¹H NMR and aliphatic quaternary and hydroxylated carbon in ¹³C NMR.

The antioxidant activity of HNM was assessed using four different methods and compared with commercial antioxidant BHT. The results indicated HNM has equivalent activity with BHT which reveals HNM has potential antioxidant activity. Increase the concentration of 5-(2,4-dimethylbenzyl)pyrrolidin-2-one could gradually increase DPPH scavenging activity. DPPH assay is a prominent method to assess free radical scavenging ability of natural compounds by donating hydrogen atoms or electrons and neutralize free radicals. Coral associated actinomycetes has potential source of antioxidant showing high hydrogen peroxide and nitric oxide scavenging activity that useful in preventing the progress of various oxidative stress related disorders.

Histopathology is a basic technique in aquatic toxicity to provide helpful information for identifying the effect of drug on various organs of experimental animal and its mechanism of injuries due to enhanced sensitivity of histological monitoring compared with other toxicological parameters such as mortality, behavior and other physiological changes. In this study, histological parameters...
were monitored after 21 days exposure with HNM and results suggested that HNM induced minor morphological changes in spleen. Spleen is the major lymphatic organ where the clearance of blood takes place. The morphological changes in spleen due to the effective interaction of HNM with immune cells and lymph nodes of fish. As well as, there is no morphological changes observed in HNM treated heart, kidney, liver, intestine and gills. Due to potential antioxidant activity of HNM may not cause oxidative damage and morphological changes in major organs of D. reria.

4. Materials and Methods

4.1. Collection of samples

Mucus samples of *Acroporaformosa* were collected from coral reef of Gulf of Mannar, Keelakarai Coast, Southeast Coast of India (Latitude 9.230157 and Longitude 78.785454). All corals appeared to be healthy during collection and mucus samples were collected in eco-friendly manner with sterile swabs that were placed in 15 ml sterile tubes. Mucus samples of 1 cm² coral surface area was taken and placed back inside the empty tubes. All collected samples were transferred to laboratory for further work.

4.2. Isolation of actinomycetes

The mucus swab samples were transferred to sterile tubes with 1 ml autoclave-sterilized artificial seawater (ASW), in a sterile hood. The mucus samples were suspended in ASW by vigorous vortexing for 3 min. Representatives of each colony morphotype were isolated using standard serial dilution and plating techniques in triplicate on starch casein agar prepared in 50% of seawater. To prevent fungal and bacterial contaminants, Cycloheximide (100 µg/L) and Nalidixic acid (20 mg/L) were added respectively to prevent fungal and bacterial contaminants. Cycloheximide (100 µg/L) and Nalidixic acid (20 mg/L) were added respectively to prevent fungal and bacterial contaminants. A total of 21 actinomycete strains were isolated from coral mucus and named as A.f – 1 to A.f – 21. All isolates were identified by comparing their morphological characteristics with those of known strains and the 16S rRNA gene sequence of actinomycetes.

4.3. Isolation of genomic DNA and 16S rRNA amplification

The pure cultures of *A. fischeri* were subjected to DNA extraction using the genomic DNA extraction kit (Qiagen). The DNA concentration was determined using a spectrophotometer. The 16S rRNA gene was amplified by PCR (Polymerase Chain Reaction) using the primer set 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTGCTCAG-3')

4.4. Production of bioactive compounds

*S. variabilis* was cultured in 2L Erlenmeyer flask containing ISP2 medium under aseptic condition and incubated for 7 days at 28°C in rotary shaker at 250 rpm. After maximum antibiotic production was observed, incubation was terminated and culture was centrifuged at 10000 rpm for 20 min to separate mycelial biomass. Ethyl acetate was found to be an excellent polar solvent to extract bioactive compounds from cell free supernatant of actinomycetes. Ethyl acetate was added with supernatant (2×100ml) and mixture was agitated for 1 hr with homogenizer. The ethyl acetate was centrifuged at 6000 rpm for 15 min to remove traces of fermentation broth. The crude extract was used to assess antimicrobial activity against human pathogens using disc diffusion method.

4.6. Characterization of solvent extracts

4.6.1. Thin layer chromatography (TLC)

The ethyl acetate extract of *S. variabilis* showing antimicrobial activity was separated by TLC using methanol: ethyl acetate: chloroform (4:4:2) solvent system. The fractions were separated and purified by loading in column. The adsorbed compound was eluted with methanol and water (1:1) under a flow rate of 4ml/min.

4.6.2. Fourier Transform Infrared Spectrophotometer (FTIR)

FTIR spectrum analysis was carried out to determine the influence of possible biomolecules that involved in antimicrobial activity. The purified fractions were completely dried and blended with KBr to obtain a pellet. FTIR spectra were collected at resolution of 4 cm⁻¹ in transmission mode range between 4000-400 cm⁻¹ using a SAM-AV spectrum (Model – Spectrum RXI).

4.6.3. Gas Chromatography-Mass Spectrometry (GC-MS)

The purified fraction was characterized by GC-MS using Shimadzu QP-2010 Plus with Thermal Desorption System TD 20 instrument at GC column oven temperature 70°C, injector temperature 200°C at split mode ratio 40 with a flow rate of 1.51 ml/min. The MS with ion source temp 200°C, interface temp: 260°C, scan range: 40-1000 m/z, event time 0.5 sec, solvent cut time: 5 min, MS start time: 5 min, MS end time: 40 min, ionization: EI (-70ev).

4.6.4. Nuclear Magnetic Resonance (NMR)

The purified fraction obtained from actinomycetes isolate *S. variabilis* was further characterized by Nuclear Magnetic Resonance (NMR). The purified compound (5 mg) was dissolved in 5ml of deuterated DMSO and analyzed for H NMR and 13C NMR using 500 MHz NMR Spectrometer with solid state attachment (CP-MAS) - Bruker Avance III.

4.7. Antioxidant activity
4.7.1. DDPH assay

Free radical scavenging effect was estimated according to earlier method\(^1\). Briefly, 1 mM solution of DPPH (1,1-Diphenyl-2-Picrylhydrazyl) was prepared in methanol, and 1 ml of this solution was mixed with different concentrations of F4 (50-500 µg/ml). The mixture was vortexed vigorously and left for 30 min at room temperature in dark and absorbance (OD) was measured at 517 nm using UV-spectrophotometer (UV 1800, Shimadzu, Japan). The antioxidant BHT was used as a positive control in all assays.

4.7.2. H₂O₂ scavenging activity

Hydrogen peroxide scavenging activity was measured by incubating the reaction mixture contains various concentrations of purified compound (1 ml), 2.5 ml of phosphate buffer (pH 7.4; 100 mM) and 400 µl of H₂O₂ (5 mM) for 20 min\(^4\). After incubation, reaction mixture was read at 610 nm. For each and every experiment, mixture without sample was considered as blank and ascorbic acid was used as control.

4.7.3. Hydroxyl radical scavenging activity

Hydroxyl Radical Scavenging Activity (HRSA) of F4 was evaluated as described previously\(^5\) with slight modifications. 100 µl of different concentrations of purified compound was dissolved in buffer (0.6 ml of PO₄ buffer (pH 7.4), 10 mM deoxy ribose and 170 mM EDTA) and reaction was initiated by adding 100 µl of ascorbic acid (2 mM) and 100 µl of 10 mM H₂O₂. After incubation at 80-90°C for 20 min, the reaction was stopped by addition of 1 ml of TCA (1%) and heated in a boiling water bath for 5 min. The amount of pink colored chromogen was measured at 532 nm against ascorbic acid as control.

4.7.4. Reducing power

The various concentrations of purified compound (1ml) was dissolved into 0.2 M phosphate buffer (pH 6.6) with 1% of potassium ferricyanide (2.5 ml) and mixed vigorously. After incubation at 50°C for 20 min, reaction was terminated by adding 1 ml of 10% TCA. The reaction mixture was centrifuged at 3000 rpm for 15 min and ferric chloride was dissolved into supernatant. The resultant solution was recorded at 700 nm using UV spectrophotometer against ascorbic acid as control and reducing power was calculated\(^6\).

4.8. Experimental design for acute toxicity assessment and histological study

The experimental animal Danio rerio, a human model system was collected from local fish market and maintained under laboratory conditions. The light illumination was at normal (12 hr light and 12 hr dark) and temperature was maintained at 28±2°C throughout the study period. For acute toxicity experiment, the adult fish (12 numbers) were stocked in 10L tank and exposed to 500 µg/L of HNM for 21 days. The water was changed at every 24 hr and mortality was monitored daily. A control experiment setup was also maintained without addition of HNM. After 21 days of sub-lethal exposure, the experimental and control fishes were sacrificed and heart, kidney, liver, intestine, spleen and gill were dissected out to assess the impact of HNM toxicity in fish by analyzing histological parameters. All organs were stained with hematoxylin and eosin and analyzed under microscope (Carl Zeiss, Axioskop 2 plus).

Conclusions

In conclusion, a potent actinomycete S. variabilis was selected for production of 1-Hydroxy-1-norresistomycin which has excellent antimicrobial and anti-oxidant activity. Further, toxicity evaluation of HNM against Zebra fish showed minimal morphological changes was observed in spleen and there is no apparent changes were observed in major organs of control and HNM treated D. rerio. In conclusion, the compound HNM obtained from S. variabilis isolated from mucus of A. formosa can act as potential anti-oxidant and used to prevent the oxidative stress related disorders since it has minimal toxicity against human model system.

Ethical statements

The zebra fish (Danio rerio) was used as an experimental model organisms and it is not governed by any law. Therefore, the organisms were not placed for Institutional Animal Ethical Committee (IAEC) approval. All the experiments were performed for best practice according to Bharathidasan University IAEC guidelines. It is also informed that, as per the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), use this fish is not subject to approval by IAEC. Further, we did not conduct any experimentation with human subjects.

References

Fig. 1 Antimicrobial activity of ethyl acetate extract of *S. variabilis* (a), TLC chromatogram of ethyl acetate extract (b) and antimicrobial activity of TLC fractions (c).
Fig. 2 FT-IR (a) and GC-MS (b) analysis of purified fraction 4 obtained from ethyl acetate extract of *S. variabilis*.

Fig. 3 $^1$H NMR (a) and $^{13}$C NMR (b) analysis of purified fraction 4 obtained from ethyl acetate extract of *S. variabilis*. 
Fig. 4 Structure of 1-Hydroxy-1-norresistomycin (HNM) produced by *S. varaiabilis* (Kock et al., 2005; Gorajana et al., 2005)
Fig. 5 DPPH (a) and Hydrogen peroxide (b) scavenging activity of HNM

Fig. 6 Hydroxyl radical scavenging activity (a) and reducing power ability (b) of HNM
Fig. 7 Histological changes of Zebra fish organs such as heart (b), liver (d) and kidney (f) stained with hematoxylin and eosin after 21 days exposure of HNM (350µg/ml). a, c and e represents the heart, liver and kidney of Zebra fish (control) respectively.

a) b)  
c) d)  
e) f)
Fig. 8 Histological changes of Zebra fish organs such as intestine (b), spleen (d) and gills (f) stained with hematoxylin and eosin after 21 days exposure of HNM (350µg/ml). a, c and e represents the intestine, spleen and gills of Zebra fish (control) respectively.