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Heart and liver regeneration in zebrafish using silver nanoparticle synthesized from *Turbinaria conoides* – *In vivo*

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ABSTRACT

The present study was aimed to identify the heart and liver regeneration in zebrafish using biosynthesised silver nanoparticles from *Turbinaria conoides.* This work focussed on the bioactivity of AgNP when incorporated into the zebrafish (*Danio rerio*) system. We began investigating the *in vivo* assays: Hepatocyte viability staining after H_2O_2 treatment, cardiomyocyte response to Ca^{++}, cardio vascular heart rate activity by measuring hypertrophy, cardio vascular pathology and cardio vascular regeneration, liver regeneration and liver pathology, molecular pathway target identification and hypothesis on interacting domain ($AgNO_3$) of the vertebrate model organism. The FTIR results of biosynthesized silver nanoparticles from *Turbinaria conoides* showed prominent peaks (range between 620.967 and 2854.14). Further, the results of XRD analysis showed the 2γ intense values (38.11 and 70.57) within the ranges of Bragg's reflection. In addition, the SEM analysis determined the size of nanoparticle (50–100 nm). It can be concluded from the present findings that, the biosynthesised silver nanoparticles from the seaweed extract of *Turbinaria conoides* can be used as potential cardioprotective and liver protective agent.

1. Introduction

Recently, the nanoparticles have been extensively investigated for gene delivery and drug delivery systems to transport the drug to the target cell site, to improve the uptake of poorly soluble drugs, and to increase drug bioavailability (Lam et al., 2006; Spitsbergen et al., 2000). To achieve efficient drug delivery it is important to understand the interactions of nanomaterials with the biological environment, targeting cell-surface receptors, drug release, multiple drug administration, stability of therapeutic agents and molecular mechanisms of cell signalling involved in the pathology of several diseases. It involves studying the interactions of nanostructures with biological systems aiming to elucidate the relationship between the physical and chemical properties of nanostructures, such as size, shape, composition and surface characteristics, and the induction of toxicity (Feitsma and Cuppen, 2008).

The nanotoxicology has focused on *in vitro* assays, using the variety of cell culture systems. A major shortcoming of this approach is that it does not consider the various levels of physiological reactivity in a whole organism; thus, the data from these studies could be misleading and animal experiments may be required (Spitsbergen et al., 2000). On the other hand, the characterisation of *in vivo* toxicity is a daunting task due to the complexity of nanomaterials and the lack of protocol standardization, leading to conflicting results and different conclusions regarding their safety. Therefore, the prediction of important aspects of toxicity is compromised. Furthermore, toxicological assessments will require a research effort to develop new standards to study correlations between nanomaterials properties and biological responses, both *in vitro* and *in vivo* (Stanton,1965).

It is simple and cost-effective to initially study the toxicity of a nanomaterial using *in vitro* approaches, such as cell culture systems. However, unfortunately, *in vitro* assays may have little, if any, correlation to *in vivo* effects, inhibiting their validation. In addition, the use of small mammalian models for large-scale screening for toxicity and bioavailability may be less economically feasible. Alternatively, *Danio rerio*, commonly known as the zebrafish may be considered a well suited vertebrate model to quickly assess the toxicity of submicron materials at low cost (Langenau et al., 2003). Zebra danio is a hardy fish, resistant to disease, handling and transportation, easy to feed and breed, desirable in community tank and easily maintained. It is sensitive to chemical substances. It grows to about 4.5–5 cm and available through out the year. By virtue of its adaptability to laboratory conditions and suitability to toxicological studies, zebrafish has been accepted by the international standard organization Fig. 1. Hence this fish

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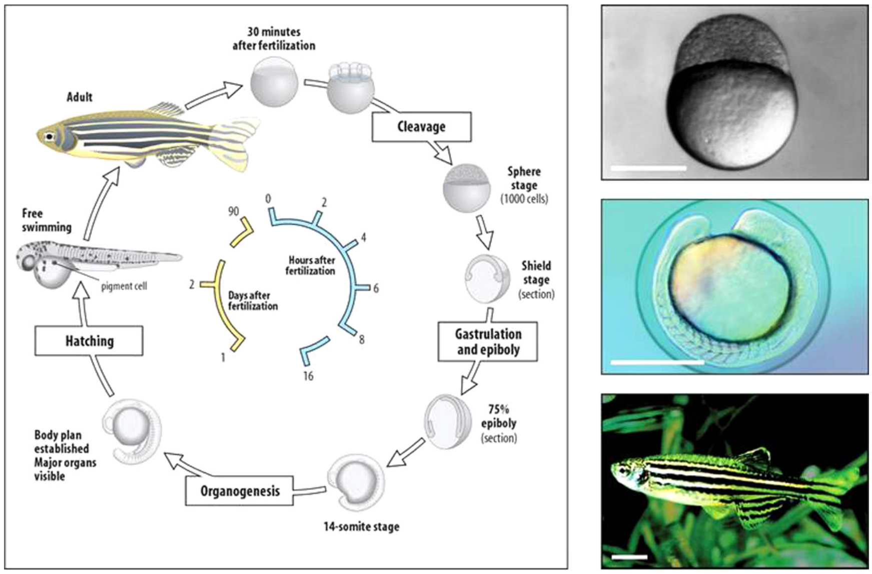

Fig. 1. Zebrafish development (Zhang, 2015).

is selected as the experimental animal for the present investigation.

Seaweeds are macrophytic algae, a primitive type of plants lacking true roots, stems and leaves, grow in saltwater or marine environment. They have recently received significant attention for their potential as natural antioxidants. Antioxidant activity of marine algae may arise from carotenoids, tocopherols and polyphenols (Syed Ali et al., 2013). These compounds directly or indirectly contribute to inhibition or suppression of free radical generation (Gnanadsigan et al., 2011). Investigation on dimethyl sulphoniopropionate (DMSP) has recently revealed that their compound from marine algae could serve as an effective antioxidant (Barman, 1991). It provides blood thinning, anti-inflammatory and antioxidant polysaccharides, which keeps the blood thin and easier for the heart to push through the blood vessels, prevents clots from forming, prevents free radical damage to the blood vessels and keeps plaques from clogging the blood vessels that feed the body. Thus seaweeds not only possess nutrient potentials but also nutraceutical potentials like antioxidant, antimutagenic, anticoagulant, anticancerous and antibacterial activity. Hence, seaweeds can be considered as futuristically promising plants forming one of the important marine living resources of high nutritional value and nutraceutical potentials. Therefore, in this review, we will focus on recent progress in the regeneration of the heart and liver in zebrafish, which have been extensively investigated.

2. Experimental assay

2.1. Biosynthesis of AgNP

Fresh leaves of *Turbinaria conoides* were collected and cleaned. The collected samples were washed thrice with tap water and twice with distilled water. About 10 g of seaweeds were taken and finely cut into small pieces and boiled with 100 ml of double distilled water for 5 min. The boiled extracts were filtered through Whatman no. 1 filter paper. A total of 10 ml of collected filtrate was treated with 90 ml of 2 mM silver nitrate aqueous solution and incubated at room temperature for 10 min, resulting in the formation of brownish black colour indicating the formation of silver nanoparticles. After that, about 1 ml (diluted with 1:20 v/v Milli Q water) of silver nanoparticle solution was monitored in UV–visible spectrophotometer (at 550 nm) at different time intervals (15 min, 30 min, 4, 6 and 8 h). After the incubation period, the solution was centrifuged at 12,000 rpm for 20 min, and their pellets were re-dispersed in Milli Q water. The centrifugation and redispersion was repeated three times to ensure the complete separation of nanoparticles (Syed Ali et al., 2015) from any possible contaminants. Later the pellets were air dried. The air dried pellets of AgNP was taken and mixed thoroughly with 2 ml of DMSO stored at 4 °C for future use.

2.1.1. Characterisation of nanoparticle using FTIR and SEM

FTIR was used to identify the possible functional groups responsible for the reduction of the AgNP and capping of the 104 bioreduced Silver nanoparticles synthesized. The samples were analyzed on a ABB Horizon MB 3000 spectrum instrument in the diffuse reflectance mode operation with the scanning range of 3450–1000 cm^{-1} at a resolution of 0.7 cm^{-1} utilising the DTGS (Deuterium Triglycine sulphate) detector. In order to obtain good signal/noise ratio 512 scans were recorded. The peaks obtained were plotted as % transmittance in y-axis and wave number (cm^{-1}) in x-axis. Combined with the intuitive Horizon MBTM FTIR software, the MB3000 will facilitate easy acquisition, processing and analysis of samples. Structural studies of AgNP were done by AURIGA-Cross beam FESEM (M/s Carl Zeiss, Germany). The lyophilisation was done using VIRTIS BENCHTOP machine. SEM analysis was done using JEOL-MODEL 6390 machine. Thin films of the sample were prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper and then the films on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 m.

3. Screening of cardio and liver regeneration assay using zebrafish

3.1. Animal strains

3.1.1. Zebrafish experimental study

Adult zebrafish (*Danio rerio*) of both sexes were purchased from a local dealer and fed twice a day with live feed artemia and kept at ~28 °C with a 14 h:10 h light–dark cycle. Adult zebrafish weighed 0.3 ± 0.02 g were selected for this study. Before starting the experiment zebrafish were placed in static tanks, and fasted for 24 h prior to experimentation. To determine the sub lethal concentrations (LC50), followed the Organization for Economic Cooperation and Development (OECD) guidelines for testing chemicals (OECD, 1992). The 47.4 µg/l of AgNPs suspension was prepared and dispersed prior to use, using ultrasonicator for 30 min. The zebrafishes were randomly allotted to three groups of 7 no. per group and then exposed to each concentration of AgNPs suspensions for 96 h in a 2 L tank containing 1 L of AgNPs suspensions. In addition, one group of 7 fishes were acted as control without any treatment. Each treatment was carried out in triplicates under the same conditions described above. The water temperature and pH were maintained at 28 ± 1 °C and 6.8–7.3 respectively. The number of dead fishes was recorded at every 12 h of intervals and they were removed from treatment tank immediately to avoid contamination. After the determination of LC50 concentration of 20 mg/L was fixed and exposed once in every day to 10 zebrafishes in a 2 L static test solution tank for 14 days. Further, to maintain the constant concentration of test solution, the fresh water was changed at 24 h intervals and accurate concentration of NPs was exposed. After treatment period, zebrafish were quickly euthanized and liver tissues were collected and stored for further analysis.

3.1.2. Caudal fin regeneration

Eight Zebrafish were transfer into each beaker. Tip of zebrafish fin was cut to initiate angiogenesis in the model. Percent regeneration of the zebrafish was calculated at 7th day to determine the fin regeneration.

3.1.3. Heart regeneration

20 mg/L of *T. conoides* along with one control were used for this test. The selections of the test concentration of *T. conoides* were based from the result obtained from the range finding experiment. Mortality was monitored continuously and the fish were considered dead when operculum movement was no longer detected and the fishes could not response when contacted with a glass rod. The dead fish were immediately removed from the tank. After 24 h the fish were transferred to new tanks containing their respective concentrations of the AECT. The fish were not fed prior to or during the experimental period. During the experiment, the behaviour of the experimental fish was monitored regularly

Animals were anesthetized by immersion into 0.04% tricaine (Sigma, St Louis, MO, USA) and immobilized by squeezing them ventral upwards into a foam holder mounted on a Petri dish. A small incision was made through the body wall and the pericardium using forceps and microdissection scissors, tearing the tissue rather than making a clean cut in order to facilitate healing. Once the pericardial sac was opened, the heart ventricle was exposed by gently squeezing the abdomen. A 0.3 mm diameter copper filament (Goodfellow, UK) linked to a polyamide tube (Parker Hanninfin, Cleveland, OH, USA) was cooled in liquid nitrogen and placed on the ventricular surface until thawing could be observed (a few seconds). An Armaflex cover (Armacell, Germany) was used to protect cooling of the polypropylene tube. Sham operations consisted of touching the exposed ventricular surface with a copper filament at room temperature. After the operation, fish were placed in a tank of fresh water, and reanimation was enhanced by pipetting water onto the gills for a couple of minutes. Fish were swimming normally after half an hour.

For analysis of regeneration, animals were killed at different times post-injury by immersion in 0.16% tricaine (Sigma, St Louis, MO, USA) and hearts were dissected in media containing 2 U/ml heparin and 0.1 M KCl. To assess the extent of damage caused by the procedure, photographs of cauterized hearts were taken between 1 and 24 h post-injury. The damaged area was easily identified from the accumulation of blood at the injury site. Damage was also estimated by examination of sections, as previously described (Poss et al., 2002). The percentage of the ventricular surface area damaged by the procedure was calculated using ImageJ.

3.1.4. Histological staining

Hearts were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight at 4 °C. Samples were then washed in PBS, dehydrated and paraffin wax embedded. Sections (7 µm) were cut on a Leica Microtome, mounted on Superfrost slides and dried overnight at 37 °C. Sections were deparaffinized in xylol, rehydrated and washed in distilled water. Connective tissue was stained using the Masson–Goldner's trichrome procedure (Merk, Darmstadt, Germany). Muscle was stained brick red and connective tissue was stained green. As a more-sensitive assay for collagen deposition, sections were stained using the Picro–Mallory procedure. Muscle was stained light brown and collagen was stained blue.

3.1.5. Liver regeneration (Natalia et al., 2009)

Fish were anesthetized in 0.015% Tricaine solution for 1 min and placed on a wet sponge with the ventral side up, and the scales were removed from the ventral body wall using forceps. Next, the ventral body wall was opened by a 3–4 mm incision. The ventral liver lobe was carefully pulled out of the peritoneal cavity and resected at the very base of the lobe. Special care was taken to resect the whole ventral lobe without damaging other areas of the remaining liver tissue. Removal of the ventral lobe led to a 30% PH. For local, incomplete PH and surface scratch experiments, the ventral lobe was exposed, and a 0.5 mm piece of liver tissue from the tip of the lobe was resected, or a small scratch (0.5–1 mm) was introduced to the liver surface using sharp forceps. The remaining liver was placed back carefully into the peritoneal cavity.

The animals were placed into fresh fish water and monitored for full recovery for 2–4 h at room temperature before transfer to 28 °C water tanks. *T. conoides* – treated animals were subjected to the same procedure excluding liver resection. The postoperative survival rate was ~90%, with most deaths occurring on the day of surgery. Transgenic zebrafish lines were subjected to heat shock (37 °C for 12 h) directly before and every following night after surgery until the fish were analyzed. Transgenic fish were identified by ultraviolet light scanning, and only GFP-positive animals were subjected to surgery. Control wild-type animals were subjected to the same heat-shock protocol. For analysis, animals were anesthetized in tricaine solution, and livers were dissected out and processed. All fish were weighed directly before and after surgery. All animals were placed on dry KimWipe paper before weighing to remove excess water. Dissected livers were also carefully dried on paper (KimWipe) and weighed on an analytical balance.

For bromodeoxyuridine (BrdU) incorporation experiments, 25 µl of BrdU solution (5 mg/ml PBS) was injected intraperitoneally 8 h before liver dissection. Zebrafish livers were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C, washed in PBS, dehydrated, and embedded in paraffin.

Histological staining of 7 µm thick paraffin sections was performed according to standard protocols. Immunohistochemical analysis on paraffin sections was performed as described elsewhere Junghans et al., 2005 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2747679/#B18).

4. Results and discussion

4.1. Characterisation of biosynthesised AgNP

It is based on the principle that molecules containing π-electrons or non-bonding electrons (n-electrons) can absorb the energy in the form of ultraviolet or visible light to excite these electrons to higher anti-bonding molecular orbitals. The reduction of the metal ions occurs fairly rapidly; more than 90% of reduction of $Ag+$ ions is complete within 4 h after addition of the metal ions to the plant extract. The metal particles were observed to be stable in solution even 4 weeks after their synthesis (Fig. 3a). The absorbance spectrum of silver nanoparticles showing maximum absorbance near 450 nm.

Fig. 3(b) illustrate the FTIR spectrum of the bark extract showed a large sharp peak at 3450 cm^{-1} strongly indicates the presence of alcohols and phenols whereas the band broadens with the spectra of the biosynthesised nanoparticles showing the utilisation of these phytochemicals for the reduction of metal ions. The AgNP showed a broad stretch around 1000–1150 cm^{-1}. Therefore, the synthesized nanoparticles were surrounded by proteins and metabolites such as terpenoids having functional groups.

Scanning Electron Microscopy is done for revealing the surface morphology of particles. Here, the bead for the SEM analysis was prepared by placing a drop of the silver nanoparticle suspension on the carbon tape attached to the head of cylindrical bead and it was dried inside a vacuum dryer for a couple of hours. The particles on the top of the bead were scanned by Scanning Electron Microscope and the following image (Fig. 3c and d) was obtained.

Overall, our studies showed that zebrafish heart and liver regeneration is driven primarily by pre-existing cardiomyocytes, rather than by progenitor cells as suggested previously. The tightness and specificity of cardiomyocyte and liver regeneration lineage-tracing system allow us to demonstrate that the vast majority of heart muscle cells, if not all of them, formed during regeneration arise from pre-existing cardiomyocytes and liver regeneration. Even though we cannot formally exclude the possibility that stem or progenitor cells may give rise to cardiomyocytes and liver regeneration during this process, in the light of our results we can conclude that their contribution to newly formed myocardium would be only marginal. To facilitate proliferation, we found that pre-existing cardiomyocytes and liver regeneration undergo limited dedifferentiation. Soon after amputation, cardiomyocytes close to the wound start to disassemble their sarcomeric structure and detach from one another. Furthermore, reduced sarcomeric structure is observed in zebrafish cardiomyocytes re-entering the cell cycle. Similar structural changes are also associated with hibernating myocardium in humans after cardiac injury. Hibernating cardiomyocytes and liver typically show a depletion of sarcomeric structure and an expression pattern of structural proteins closely resembling that in fetal heart cells. Although mammalian cardiomyocytes and liver are unable to regenerate, it is tempting to speculate whether they can complete one of the steps involved in this process. The fact that zebrafish cardiomyocytes and liver differentiate to a limited extent during heart regeneration, rather than undergoing marked changes in gene expression, offers hope that liver and heart regeneration can also be induced in mammals. In this respect, our studies provide a mechanistic insight into previous findings in mice indicating that forced expression of cell-cycle regulators can induce regeneration after cardiac and liver injury.

5. In vivo experiment

5.1. Hepatocyte viability staining after H_2O_2 treatment

The use of zebrafish as an *in vivo* model for the discovery of drug candidate molecules was proposed 58 years ago. This study presents the use of embryos and ZF larvae for the screening of synthetic drugs and natural products (Jones and Huffman, 1957). However, only in the year 2000, was described, the first "screening" using multiwell plates. Since then, over 60 studies have reported the use of ZF for performing whole projects aimed at drug discovery (Rennekamp and Peterseon, 2015).

Liver cells are chosen as sample under study because they are responsible for a number of metabolic processes. The Fig. 2 shows the histopathology of untreated liver cell, liver cell treated with hydrogen peroxide to induce liver damage and liver cells treated with biosynthesized silver particle along with Hydrogen peroxide to test the efficacy of the test samples in the reversal of liver cell damage caused by H_2O_2. In Untreated Liver Cell, the cells are normal with clear nucleus. The treatment with H_2O_2 causes irregular shape of liver cells or death of liver cells. H_2O_2 releases single oxygen which can react with multiple molecules within the cell to disrupt normal cell functions and cause cell death. In Liver cells treated with biosynthesized silver particle along with H_2O_2, the number of viable cells was counted. It is observed that more cells are normal with clear nucleus. On treatment of

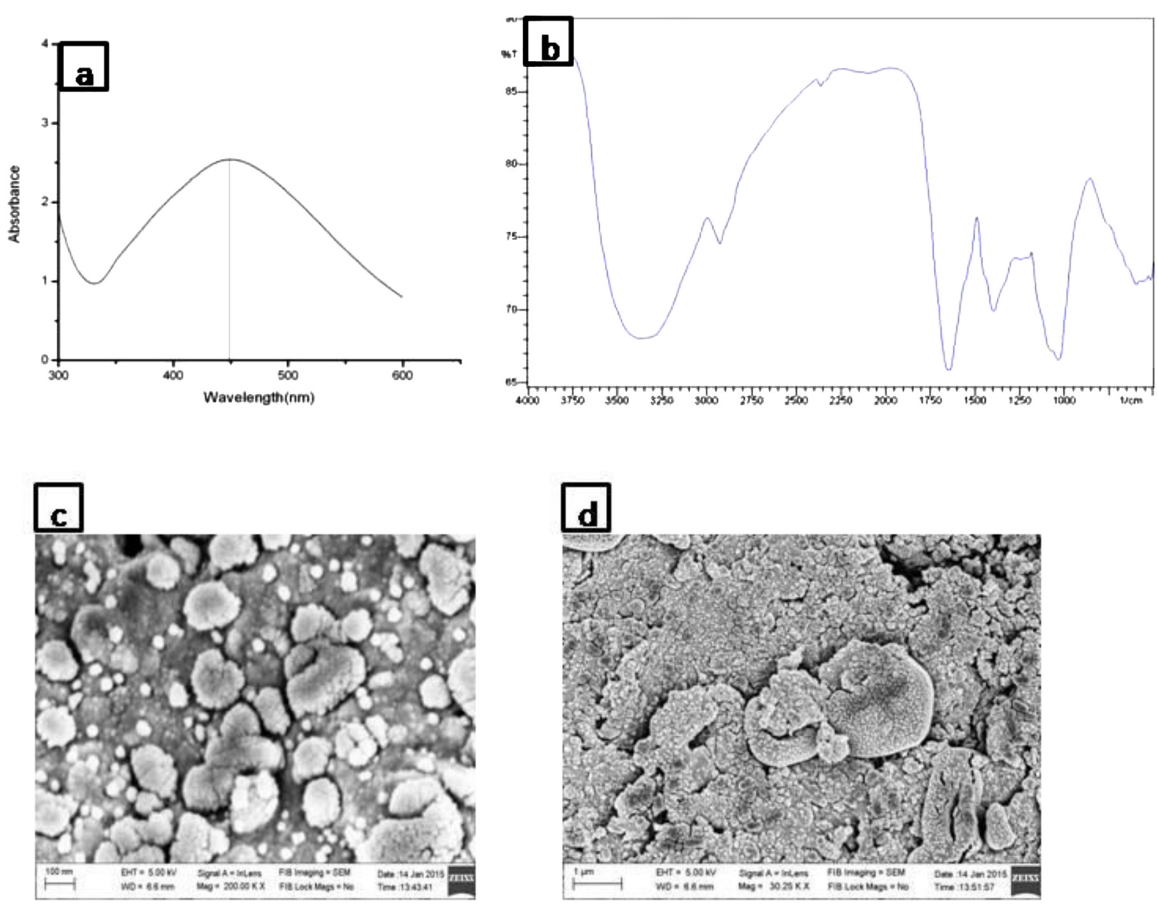

Fig. 2. Caudal fin, Heart and Liver regeneration.

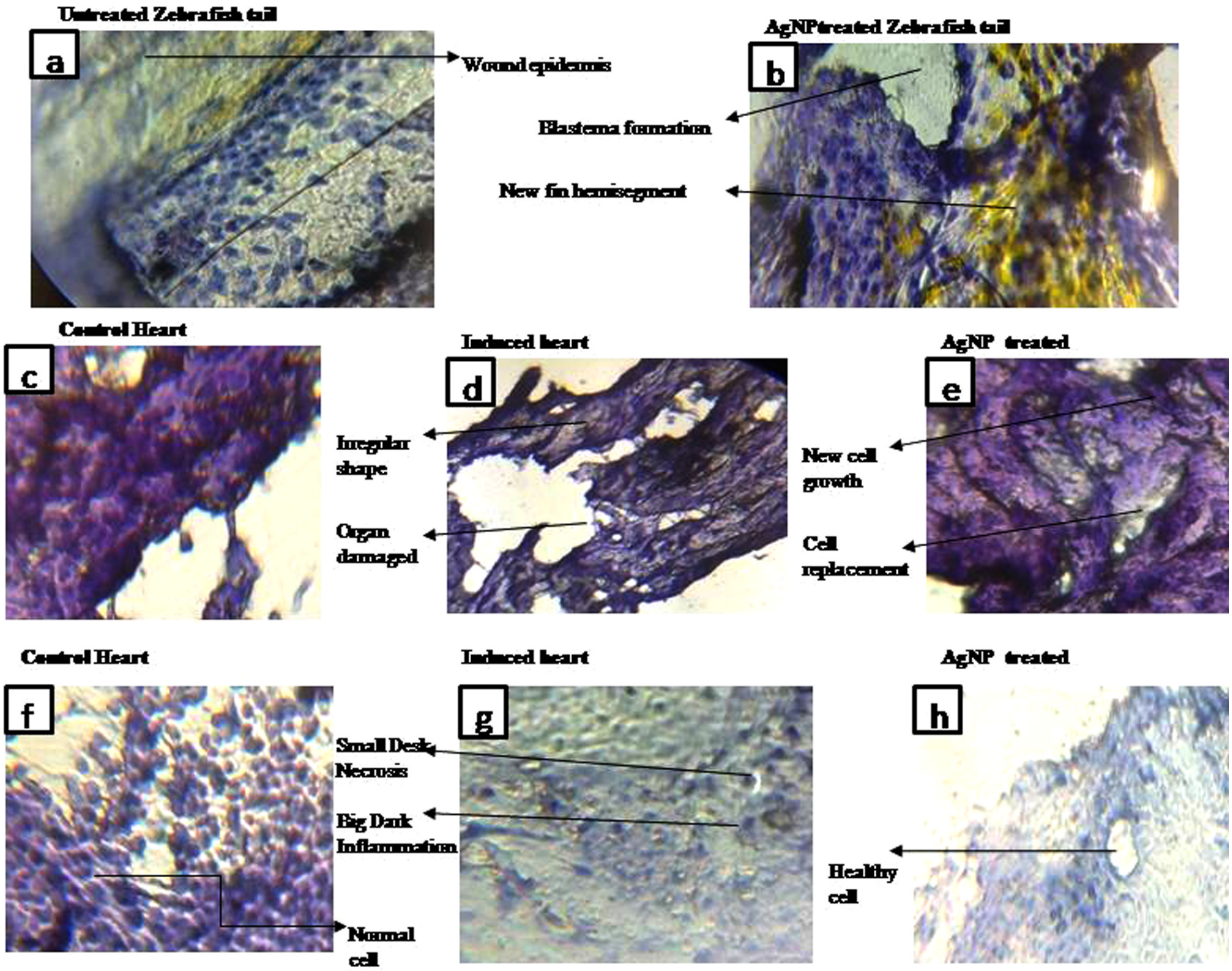

Fig. 3. FTIR and SEM analysis of AgNP.

single cell liver cells with hydrogen peroxide, the survival of the liver cell is based on the ability of the adsorbed drug in the liver to overcome the singlet oxygen created by hydrogen peroxide. Dead liver cells will loose shape and burst open. Alive liver cells remain and will be those counted.

Similarly, Kim et al. (2011) investigated that the standard drug of acetaminophen liver injury resulting in degeneration of cells may be due to the accumulation of non-adipose tissue which results in cell necrosis and cell death and treatment with flavonoid resulted in regeneration of vacuoles and liver cells indicating that the flavonoid treatment decreases the oxidative hepatic injuries such as necrotic aggregations in acetaminophen treated which shows the protective nature of flavonoids against acetaminophen liver injury.

5.1.1. *Zebrafish caudal fin as a model system for studying regeneration*

Zebrafish has an excellent regeneration capacity of the limbs in this case the fins. The caudal fin is cut and visualized for wound healing properties. If larger cells are seen near the amputated region, it is a sign of new cell growth and replacement. In untreated zebrafish tail, the cells are more with large nucleus. In treated tail, no large nucleus is observed near the tail. The cells are affected with acetamine, erythromycin. In zebrafish treated with study sample, more number of large cells are seen near the tail which shows that the silver nanoparticles synthesized can induce regeneration in zebrafish (Fig. 2a and b).

Like that, Rubinstein (2003) and Westerfield (2000) reported that the comparison of anthocyanin extract and Standard Drug of Paclitaxel effect was done with Control and PEG vehicle control for regeneration of tail fin as a parameter for studying regenerative anti-angiogenesis. Control group was kept to know the normal growth of fin.

Cardiovascular pathology helps to understand the three dimensional architecture of the tissue. A highly spaced cell indicates poor function of organ. In Induced Heart, small dark cells indicates necrosis, cells which are being engulfed as small bodies are apoptotic, and presence of inflammatory cells with big dark nucleus indicates inflammation. Cells which are poorly stained and star like shaped are fibroblast indicating new cell formation with loss of organ functionality. The sample treated heart has not showed inflammation, necrosis, apoptosis, scar tissue fibroblast, or spacing showing that the heart is healthy (Fig. 2c, d and e). There are some earlier research findings supported to our study, Lam et al. (2008) used transgenic zebrafish to characterise the pro-angiogenic properties of *Angelica sinensis* (Dong Quai). Liu et al. (2011) used transgenic zebrafish to investigate the angiogenic activity of the crude aqueous extract of *Rehmannia glutinosa*.

Fig. 2 illustrates the clear nucleus without the lake of scar tissue for complete healing. Zebrafish has an excellent regeneration capacity of internal organs as well in control. Irregular dead cells are observed and irregular shape of the cells indicate that the organ is damaged then when visualized for wound healing properties in induced heart and AgNP larger cells are seen near places where cells are dislocated then, it is a sign of new cell growth and replacement. More number of large cells is seen in sample treated fish then the sample can induce regeneration in Zebrafish.

Zebrafish has an excellent regeneration capacity of internal organs.

In induced Liver, the organ is damaged which is then visualized for wound healing properties upon treatment. If larger cells are seen near places where cells are dislocated, it is a sign of new cell growth and replacement. More number of large cells seen in sample treated fish confirmed the ability of the biosynthesized AgNP for regeneration in zebrafish (Fig. 2f and g).

Liver pathology helps to understand the three dimensional architecture of the tissue. Fig. 2(f and g) illustrates the nucleus of control liver, Presence of large cells with normal nucleus indicates healthy cells. In Induced liver, small dark cells indicates necrosis, cells which are being engulfed as small bodies are apoptotic, and presence of inflammatory cells with big dark nucleus indicates inflammation. Cells which are poorly stained and star like shaped are fibroblast indicates new cell formation with loss of organ functionality. Histological study of AgNP sample treated liver tissue showed the presence of large cells with normal nucleus indicating healthy cells. If assay sample treated liver has not inflammation, necrosis, apoptosis, scar tissue fibroblast, or spacing then Liver is healthy.

6. Conclusion

The synthesis and application of metal nanoparticles using either terrestrial or aquatic plant derivatives is extremely studied in the last two decades. The plant metabolites induce the production of metallic nanoparticles in a safe and ecofriendly manner. The plant mediated nanoparticles have the potential to be used in various fields such as pharmaceuticals, therapeutics, sustainable and renewable energy and other commercial products. Moreover, zebrafish is emerging as an alternate to mice model for vertebrate *in vivo* studies. Hence, in future, the study of nanoparticles using zebrafish for mammalian disease model has wide perspective for the discovery of potent drugs in time consuming manner.

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