The Influence of Probiotics in Reducing Cisplatin Toxicity in Zebrafish (*Danio rerio*)

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Abbreviations

BSA	Bovine serum albumin
CFU	Colony forming units
MRS	De Man, Rogosa and Sharpe bacterial growth medium
OD	Optical density
PBS	Phosphate buffered saline
ROS	Reactive oxygen species
SDS PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
WHO	World health organization
DNA	Deoxyribonucleic acid
gDNA	Genomic DNA
mtDNA	Mitochondrial DNA
mRNA	Micro RNA
RNA	Ribonucleic acid
NO	Nitric oxide
AMPs	Antimicrobial peptides
LMWB	Low-molecular-weight bacteriocins
HMWB	High-molecular-weight bacteriocins
AU	Arbitrary unit
mm	Milli meter
LBS	Lactobacillus selection
TS	Tryptic soy broth

BHI	Brain heart infusion broth
GPx	Glutathione peroxidase
GR	Glutathione reductase
SOD	Superoxide dismutase

1. Title

The Influence of Probiotics in Reducing Cisplatin-Induced Toxicity in Zebrafish (Danio rerio)

2. Introduction

According to the record of World Health Organization (WHO) cervical cancer is the fourth most common type of cancer in women. In India, cervical cancer is one of leading causes of cancer mortality among women 30 to 69 years of age, accounting for 17% of all cancer deaths. Depending on the type and stage of cancer, patients are treated with either traditional therapies (such as surgery, chemotherapy, and radiation therapy) or newer forms of treatment (such as immunotherapy (Zhang et al., 2018), targeted therapy (Baudino et al., 2015), hormone therapy (Abraham et al 2011), gene therapy (Das et al., 2015) and photodynamic therapy (Agostinis et al., 2011). Symptoms of cervical cancer patients are difficult to find because about 70% of cases of cervical cancers are found at an advanced stage.

Chemotherapy is an effective and widespread way of cancer treatment in which one more chemotherapeutic or alkylating agents are used. Cisplatin is one of the best and first metal-based chemotherapeutic drugs (Lind 2008; Eastman 2017). Cisplatin is FDA approved for the treatment of advanced cancer; it is a platinum-based chemotherapy drug as shown in Figure. 1 A cell-based high-throughput screening methodology was implemented, using a library of 1,280 Food and Drug Administration (FDA)-approved drugs, to identify clinical compounds that act synergistically with cis-Diamminedichloroplatinum (CDDP).ClS-DIAMMINE DICHLOROPLATINUM (II) (cisplatin), the first heavy metal coordination compound shown to have antineoplastic activity.

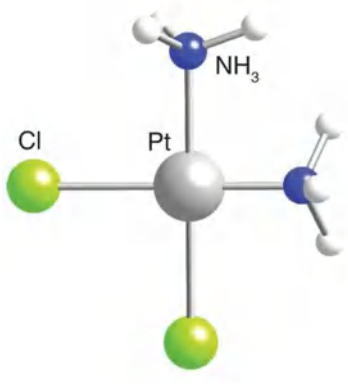

Figure. 1 Structure of Cisplatin - square planar coordination complex

Studies confirmed that cisplatin exerts its anticancer activity by attacking more than one place. It generally binds with genomic DNA (gDNA) or mitochondrial DNA (mtDNA) to create DNA lesions, block the production of DNA, mRNA and proteins, arrest DNA replication, activate several transduction pathways which finally led to necrosis or apoptosis depicted in Figure. 2 (Ghosh, 2019). However, because of drug resistance and numerous undesirable side effects such as severe kidney problems, allergic reactions, decrease immunity to infections, gastrointestinal disorders, hemorrhage, and hearing loss especially in younger patients it is used in combination therapy (Dasari et al., 2014).

When used in other treatments, chemotherapy can:

- Make a tumor smaller before surgery or radiation therapy.
- Destroy cancer cells that may remain after treatment with surgery or radiation therapy.
- Help other treatments work better. Kill cancer cells that have returned or spread to other parts of your body.

Chemotherapy can cause Side effects - chemotherapy not only kills fast-growing cancer cells, but also kills or slows the growth of healthy cells that grow and divide quickly. Examples are cells that line your mouth and intestines and those that cause your hair to grow. Damage to healthy cells

may cause side effects, such as mouth sores, nausea, and hair loss. To overcome the side effects of cisplatin, in this study, probiotic *Bacillus megaterium* was used.

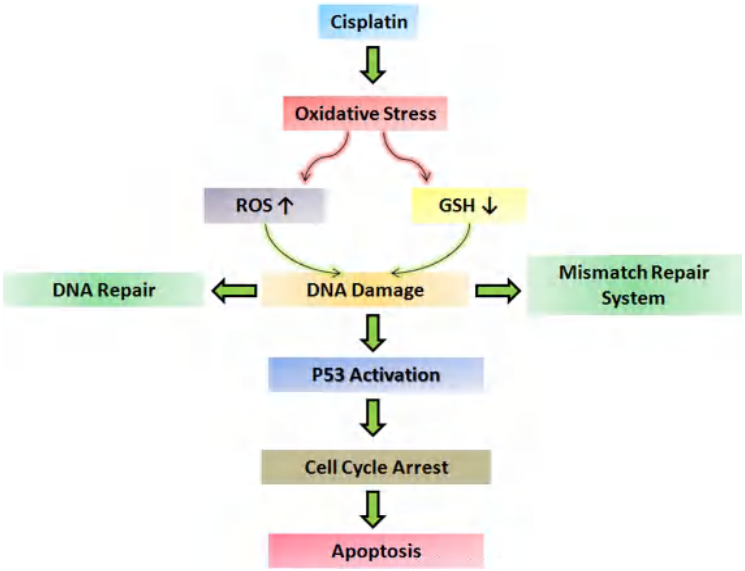

Figure. 2 Overview of molecular mechanisms of cisplatin in cancer treatment.

According to the WHO nutritional guidelines, probiotics can be defined as "live microorganisms when administered in adequate quantities confer a health profit to the host cell" (Nolfo et al., 2013). The use of probiotic applications can stimulate the growth of beneficial microorganisms and out compete potentially harmful bacteria, and thus reinforce the host organism's natural defense mechanisms (Dimitroglou et al., 2011). Evidence indicates that probiotics can stimulate certain aspects of the innate immune system and may influence oxidative stress, although the mechanisms are only partly described (Tovar-Ramírez et al., 2010). Oxidative stress results from either increased exposure or production of oxygen (reactive oxygen species, (ROS)) and nitrogen (nitric oxide (NO)) radicals, by the organism. Free radicals may induce lipid peroxidation, protein degradation, and DNA damage-inducing apoptotic processes (Sies et al., 1986). The rate or amount of ROS and NO production depends on the metabolic rate of the species under consideration and can be increased by environmental stress (Valavanidis et al., 2006).

Review of Literature

Probiotic microorganisms may confer several health benefits to the host as illustrated in Figure. 3. Those effects are the result of three modes of action (Oelschlaeger 2010): i) Immune modulation. Probiotics can exert their modulating effect on the host's defenses, i.e. both the innate and adaptive immune system. This property might be helpful in the prevention and therapy of infectious diseases and the treatment of inflammatory diseases (Sheil et al., 2007). ii) Microbiota modulation. Probiotics can have a direct influence on other components of the microbiota, either commensals or pathogens. Thus, they can be beneficial in dysbiosis or infectious diseases (Claes et al., 2011). iii) Compounds transformation. Probiotics may modify molecular products produced by other microorganisms, i.e. toxins, produced by the host, i.e. bile salts, or from food origin, i.e. indigestible fibers (Guarner et al., 2017). To exert their beneficial effect on the host, probiotics must remain viable and survive under specific conditions found in the gastrointestinal tract. Different stress tolerance assays can be carried out to assess their ability to survive in those conditions. After being ingested, probiotic bacteria must face stressful conditions in the stomach. In this compartment, pH can vary between 1 and 5, and several digestive enzymes are secreted. In the same manner, the passage to the duodenum exposes the bacteria to high pH levels, pancreatic enzymes, and bile salts.

Probiotics as a physiological barrier to prevent the attachment of pathogens

Major probiotic mechanisms of action include enhancement of the epithelial barrier, increased adhesion to intestinal mucosa, and concomitant inhibition of pathogen adhesion, competitive exclusion of pathogenic microorganisms, and production of anti-microorganism substances and modulation of the immune system (Bermudez-Brito et al., 2012). The intestinal barrier is a major defense mechanism used to maintain epithelial integrity and to protect the organism from the environment. Probiotics may promote mucus secretion as one mechanism to improve barrier function and exclusion of pathogens.

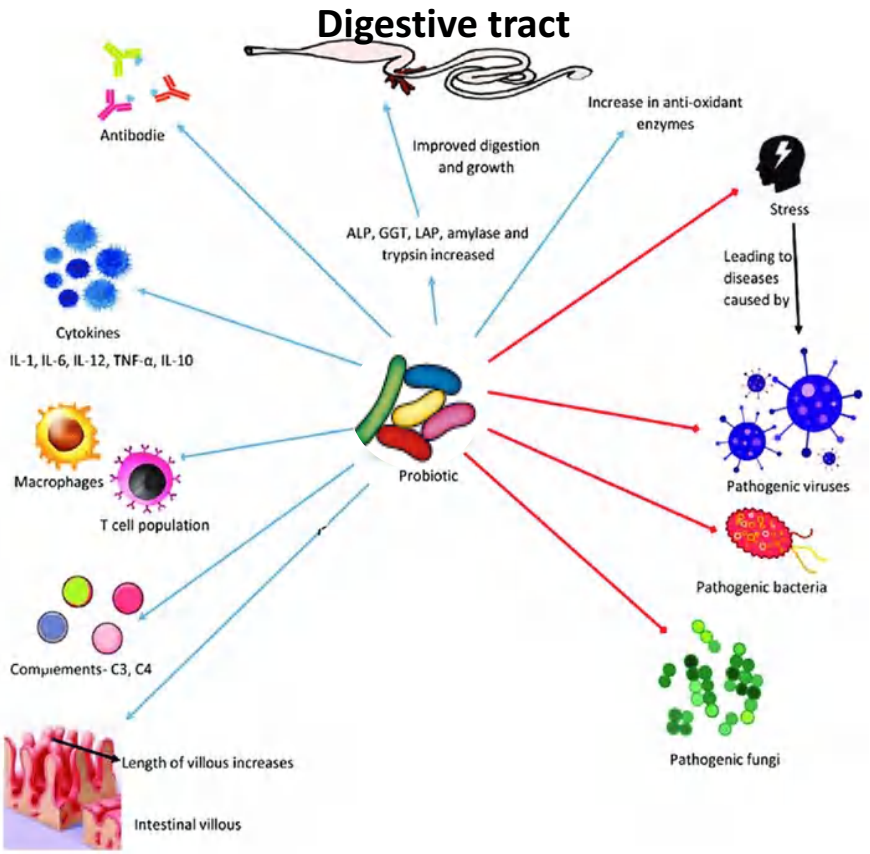

Figure. 3 Beneficial effects of probiotics. Blue arrow indicates additive effects, and red lines indicate inhibitory effect

Cervical cancer prevention with probiotics

Reported mechanisms developed in cancer prevention and probiotic treatment to date, which includes

(i) Gut microbiota modulation;

(ii) Enhancement of the function of the gut barrier;

(iii) Degradation of potential carcinogens and the ability to defend against DNA damage to the intestinal epithelium; and

(iv) Enhancement of the body's immune and inflammatory systems (Yang et al., 2020).

In order to identify and kill cells that may become cancerous, probiotics allow the immune system to operate at its best. Probiotics can alter the intestinal microbial environment, improve the function of the intestinal barrier, provide competitive adherence to the mucosal epithelium, generate antimicrobial

substances, and modulate immune responses by improving the innate and adaptive immune response (Nazir et al., 2018).

Advantages of probiotics

- Probiotics are useful and friendly microbes.
- They are able to compete with the bad microbes and colonize our digestive system.
- They are able to ferment our food to simpler byproducts and could promote our health by many different mechanisms.
- Their amount could be deteriorated due to many factors, such as incorrect diet, alcohol, age and so on. This is why they should be taken through our regular diet.
- In particular cases such as after antibiotic treatments, where they are expected to be affected severely, they should be taken orally in considerable amounts or with food.
- Probiotics promote health while they:

a. Remove the side effects of the pathogens or the harmful microbes.

b. Supply the body with useful byproducts.

c. Reduce the jobs of our digestive system.

d. Reduce the effect of the first attack of harmful compounds, instead of our cells, by their biofilm, which protects our digestive system.

e. Reduce the amount of food needed by our bodies due to the correct digestion and metabolism of any amount of food.

f. Probiotics in some cases could complement the deficiency in our genetic materials by helping us to borrow the products of their genes (such as in case of the lactose fermentation deficiency).

Impact of probiotics on diarrhea caused by Chemotherapy

In people with cervical cancer, diarrhea is one of the most common and problematic side effects associated with chemotherapy or radiotherapy (Stein et al., 2010; Chitapanarux et al., 2010).The prevalence of these adverse effects was as high as 50-80%. Extreme diarrhea triggered by treatment may contribute to loss of fluids and electrolytes and nutritional deficiencies and may adversely affect the quality of life (Andreyevet al., 2012). In addition, owing to anticancer therapy, patients with neutropenia are more prone to diarrhea, which may also lead to dosage changes or discontinuation or delayed care (Maroun et al., 2007). In preventing or treating chemotherapy-or radiotherapy-induced diarrhea, probiotics can be successful (Qiu et al., 2019). Probiotics have positive effects on the frequency and incidence of diarrhea and the need for rescue treatment in patients undergoing radiotherapy (with or without chemotherapy) (Mego et al., 2013). In the study, patients undergoing radiotherapy with or without chemotherapy were given placebo-related probiotics to avoid diarrhea (Mansouri-Tehrani et al., 2016).

Production of antimicrobial substances

Probiotic strains secrete diverse substances with an inhibitory effect on the growth of pathogenic organisms. The main produced components are lactic acid, hydrogen peroxide, low-molecular-weight bacteriocins (LMWB) and high-molecular-weight bacteriocins (HMWB). Low-molecular-weight bacteriocins are antimicrobial proteins that can be divided in three classes; class I antibiotics, class II heat-stable non-cyclic, and class III cyclic antimicrobial peptides (Maqueda et al., 2008). Bacteriocins are bacterial peptides that inhibit or kill microorganisms. Assays for the evaluation of the inhibitory effect of probiotic-culture filtrates are also performed to assess the effect of secreted bacteriocins and other antimicrobial compounds.

In recent decades, many findings have shed new light on the understanding of the antioxidant capacity of probiotics. Oxidative stress defines a condition in which the prooxidant–antioxidant

balance in the cell is disturbed, resulting in DNA hydroxylation, protein denaturation, lipid peroxidation, and apoptosis, ultimately compromising cells' viability depicted in Figure. 4. ROS-mediated oxidative stress are known to play vital role in the development of chronic diseases such as cancer, diabetes, heart disease, stroke, Alzheimer's disease, rheumatoid arthritis, cataract and aging. Antioxidants are molecules which interact with free radicals generated in cells and terminate the chain reaction before damage is done to the vital molecules (Mishra et al., 2015). Consumption of probiotics alone or in food shows that strain-specific probiotics can present antioxidant activity and reduce damages caused by oxidation (Wang et al., 2017).

Usually, an atom is composed of a central nucleus with a pair of electrons orbiting around it. However, some atoms and molecules have unpaired electrons and these are called free radicals. Free radicals are usually unstable and highly reactive because of the unpaired electron in its outermost orbit. The unpaired electron makes the radical highly reactive. Free radicals can be stabilized only either by donating electrons to other molecules or by receiving electrons from other molecules. New radicals are generated as a result which can start a chain reaction. Chain reactions can be very destructive. The chain reaction can damage DNA, lipids; proteins. Potential effects of free radicals constitute oxidative stress. Entities causing oxidative stress are called reactive oxygen species (ROS). ROS includes all the free radicals that contain oxygen. The chain reaction stops only when two radicals react and forms a covalent bond. The chain reaction can also be ceased by antioxidants. Antioxidants remove free radicals by getting oxidized themselves. Oxidative stress can damage or kill cells. Oxidative stress may also be responsible for many diseases. Free radicals are produced continuously by the body as a part of a normal physiological process. Function of antioxidant defense system is to keep ROS at an optimum level. Antioxidants include superoxide dismutase, glutathione peroxidase, glutathione reductase, glutathione, catalase, cytochrome c, uric acid etc. Antioxidants act as free radical scavengers and trap free radicals before they can damage tissues. Therefore, the current study aimed to examine the potential Antioxidant activity of probiotic *Bacillus megaterium*.

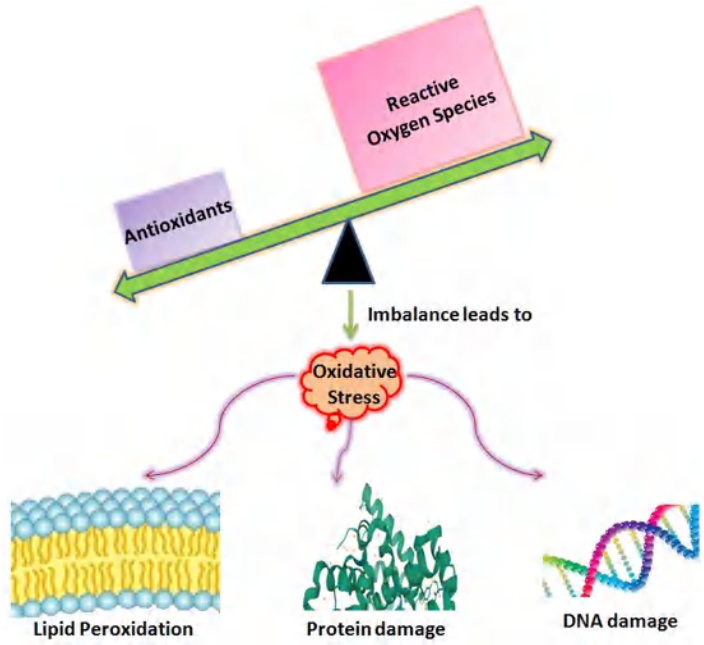

Figure. 4 Antioxidants are required for balancing ROS which cause oxidative stress.

At an intermediate level of complexity, zebrafish is increasingly used as a human-disease model (Lieschke et al., 2007). In addition; recent works have suggested that the zebrafish may be useful for evaluating probiotic activity. Zebrafish (Danio rerio) is a small subtropical freshwater fish that belongs to the Cyprinidae family, of the order of Cypriniformes. The former scientific name was Brachydanio rerio, but it was changed to Danio rerio in 1981. Zebrafish are indigenous from South Asia and can be found in India, Pakistan, Nepal, Myanmar and Bangladesh (Paquet et al., 2009). Their natural habitat is located in water ponds, rice fields, and rivers of slow-moving waters, with slightly alkaline (pH 8) and clear water (McClure et al., 2006). Zebrafish can tolerate a temperature range of 15–35 °C, its optimal temperature is around 28 °C (Schaefer et al., 2006). Likewise, it can survive in water with pH values between 5.5 and 9 (Spence et al., 2008). Zebrafish share high genetic homology with humans, and even though this model cannot replace mammals and higher vertebrate models, it offers a cost-effective and more ethical solution for many biomedical studies. Therefore, this model organism is used in many research fields, such as organogenesis, genetics, toxicology, disease modeling and drug discovery. Assessment of toxicity on a particular organism exposed to a particular toxicant reveal facts regarding the health. Toxicity tests would reveal the organism's sensitivity to a particular toxicant.

Histopathology:

Histology is defined as the study of tissues, and pathology is the study of disease. So, taken together, histopathology literally means the study of tissues as relates to disease. It is the study of diseased cells and tissues using a microscope. Histopathology slides, on the other hand, provide a more comprehensive view of disease and its effect on tissues, since the preparation process preserves the underlying tissue architecture. To visualize different components of the tissue under a microscope, the sections are dyed with one or more stains. The aim of staining is to reveal cellular components; counter-stains are used to provide contrast (Gurcan et al., 2009).

3. Objectives

- To assess different morphological parameters of Zebrafish (*Danio rerio*) after treatment (30 days) with cisplatin and probiotics (individual and combined effects)

- To assess the alternations in antioxidative enzymes of intestine and ovaries homogenate of cisplatin and probiotics treated (individual and combined effects) Zebrafish.

- To distinguish histopathological changes of the tissues of intestine and ovaries of cisplatin and probiotics treated (individual and combined effects) Zebrafish along with ROS scavenging activity.

- To purify partially the bacteriocins extracted from treated probiotics

4. Materials and methods

Experimental strains and culture:

Bacillus megaterium de Bary (**ATCC**® 14581™) was selected as a probiotic strain donated by BioNest, Central University of Hyderabad. Probiotic strain was cultured in a De Man, Rogosa and Sharpe agar (MRS) medium at 37 °C for 48 h before use. The fermentation broth used was centrifuged at 8000 rpm for 5 min at 4 °C. The bacteria precipitation was collected, washed twice with sterile phosphate buffered saline (PBS) buffer and resuspended. The final concentration of strain was 108 CFU mL^{-1}, which were stored at 4 °C for not more than 24 h prior to use.

Experimental Animals

Adult Zebrafish (D. rerio) were purchased from a local supplier (Tirupathi). An adult Zebrafish measures approximately 4 cm in length, and its weight ranges between 0.5 and 0.9 g. They were kept in an aquarium with aeration for the supply of oxygen. Fishes were fed twice daily with commercial feed (Tanganyika, Freeze dried tubifex worms) and another two times with Artemia salina for about 2 months to attain four months. Then those 4 months old adult zebrafish were used for this study. The room temperature or the tank temperature is generally maintained at 25.0 ± 0.5°C, 14L [400 lux]/10D [1 lux] (light on at 07:00) and divided into 4 groups (n = 7-10 each). Fish tanks were cleaned regularly. The control group was fed on the commercial diet.

Probiotic Administration:

The probiotic treated group was fed on the commercial diet mixed with overnight probiotic culture. Several routes for probiotic administration are used in aquaculture systems depicted in Figure. 5. Probiotic is administered as dietary supplements (via live food such as Artemia and rotifers or pellet food).

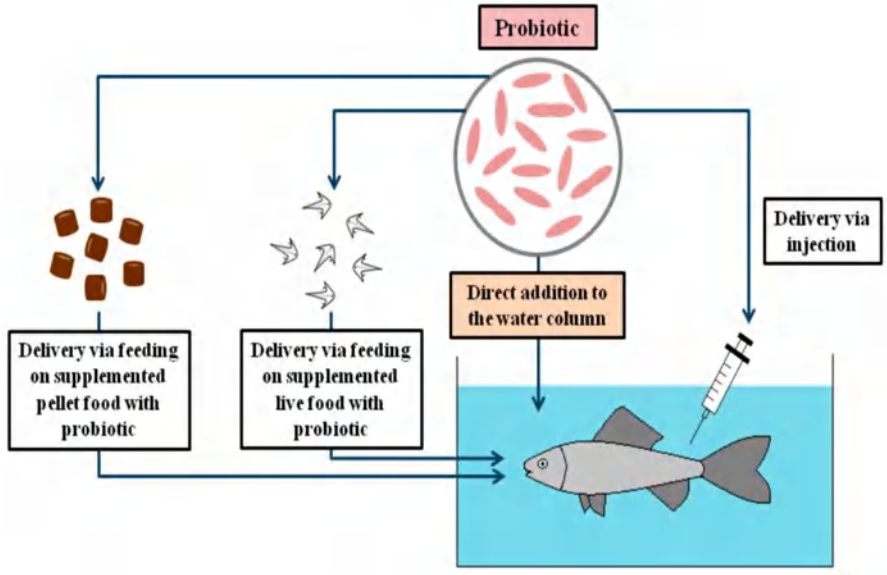

Figure. 5 Routes of Probiotic administration (Jahangiri et al., 2018)

Drug Administration:

Cisplatin treated group was fed on the commercial diet and treated with cisplatin. Cisplatin here used is Celplat 10 (1mg/1mL) which is available commercially. Probiotic + cisplatin group of fishes was fed with commercial diet along with the treatment. Cisplatin is administered orally to the Zebrafish. Firstly fishes were anesthetized by using ice cubes, then by using a micropipette with a tip that is gently inserted into the mouth of Zebrafish. The drug suspension (1 mg/mL) is then gently released into the fish ensuring that the administered suspension does not regurgitate.

Morphological parameters

After 30 days of treatment all fishes were measured for total wet weight in gm and body length in cm and calculated their Body mass index in g/cm^2. Then % of survival was calculated.

After 30 days of treatment ten females from each experimental group were sacrificed by anesthesia (500 mg^{-1} MS-222 [3-aminobenzoic acid ethyl ester]. All fishes were measured for total wet weight (mg). The intestine and ovaries were sampled and immediately flash-frozen using liquid nitrogen and stored at -80 °C for ROS Scavenging and Antioxidant activity. Three-five ovaries from each group were removed and stored in 10% formalin for histological studies.

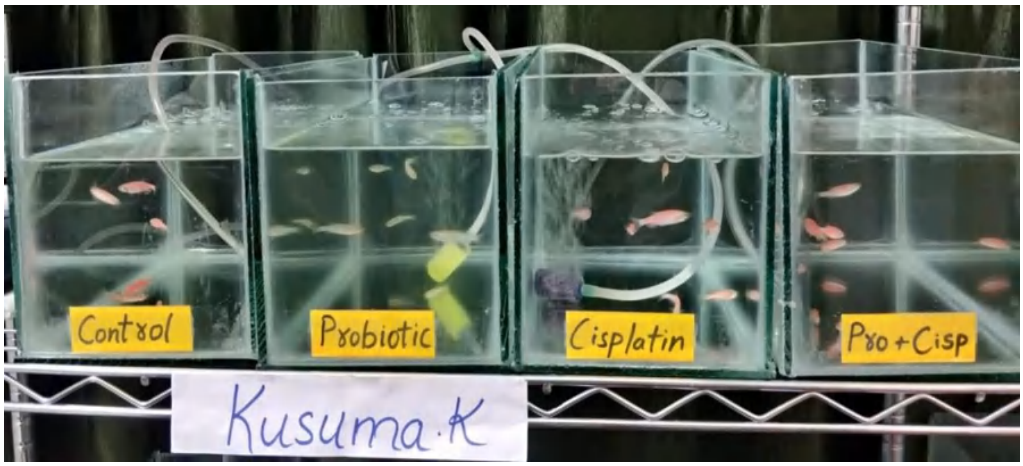

Figure. 6 Maintenance of fishes

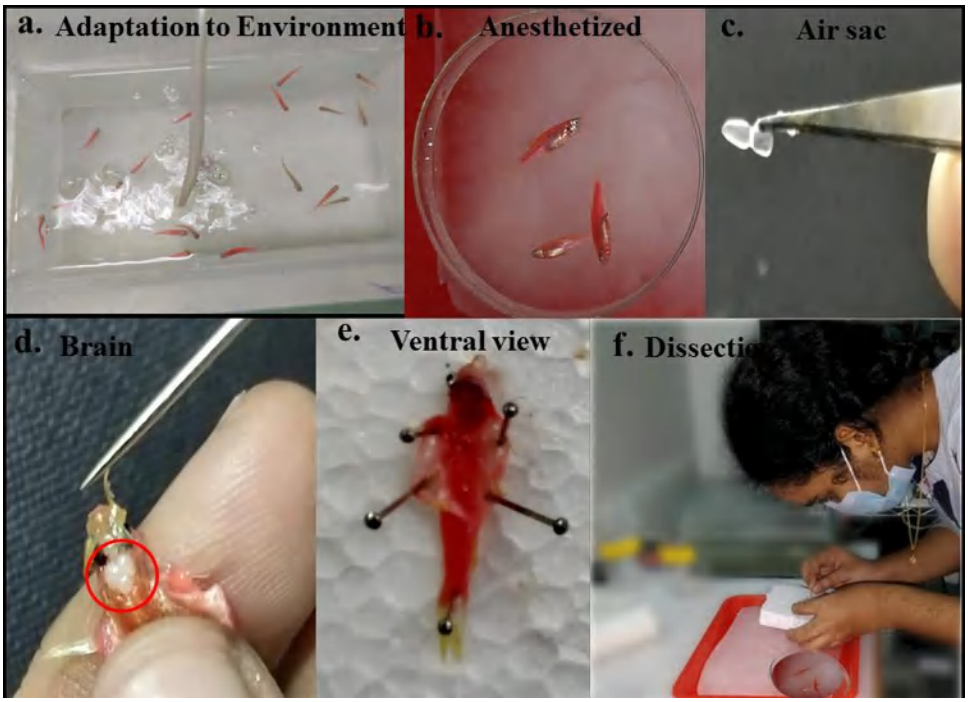

Figure. 7 Experimental model dissection photos a) adaptation to environment b) anesthetized fishes c) air sac of Zebrafish d) brain of Zebrafish e) ventral view of fish during dissection f) during dissection.

Antioxidant enzyme assays:

Lipid peroxidation:

An end product of lipid peroxidation is MDA (Malondialdehyde), were measured in tissue homogenates on the basis of the reaction with thiobarbituric acid (TBA) to form a pink color complex, MDA produced was determined with the absorbance coefficient of the MDA-TBA complex at 550 nm using 1, 1, 3,3-tetraethoxypropane (TMP) as the standard (Okhawa et al. 1979).

Glutathione peroxidase and Glutathione reductase:

By catalysis conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) for the consumption of NADPH, the catalytic activity of glutathione peroxidase and glutathione reductase was measured spectrophotometrically at 340 nm. The specific activities were expressed as the nmol of NADPH consumption per min per mg of protein (Carlberg et al., 1975).

Catalase:

The catalytic activity of catalase was determined by a spectrophotometrical measurement of H_2O_2 breakdown at 240 nm. The specific activity of the enzyme was expressed as μmol of decomposed H_2O_2 per min per mg of protein (Aebi, 1984).

Superoxide dismutase:

The activity of superoxide dismutase was determined in the tissue homogenates by the modified method of NADH-phenazine methosulphate-nitroblue tetrazolium formazan inhibition reaction spectrophotometrically, measured at 550 nm (Kakkar et al. 1984).

ROS scavenging activity:

The reactive oxygen species (ROS) like superoxide anion, peroxide, and hydroxyl radicals can be estimated using the quinol oxidation followed by the formation of corresponding adduct approach in the presence of intracellular ROS upon treatment of quinol with the cells. Initially, the percentage

of ROS activity was investigated with the hydrogen peroxide (H_2O_2) as a standard curve method. Herein, 1 mL of H_2O_2 (different concentration ranging from 288, 144, 72, 36, 18, 9, 4.5, 2.25, and 1.25 µg/mL) was added into a cuvette containing 250 µM of quinol and followed by the incubation of reaction vial for 30-45 min. Subsequently, the radical scavenging activity has been measured using UV-vis spectrophotometer at 305 nm. On the other hand, intracellular ROS was also investigated using a similar procedure in intracellular where approximately 2 mg of selective tissue (intestine and ovaries) has been mashed in PBS buffer. Afterward, the diluted concentration of selected tissue solution was used for the detection of intracellular ROS activity instead of H_2O_2 with the standard quinol solution. Herein, I mL of tissue solution has been added into a cuvette containing 250 µM of quinol and followed by the incubation of reaction vial for 30-45 min. Later, corresponding readings have been measured at 305 nm using a UV-vis spectrophotometer. The percentage of ROS activity has been calculated using the following expression.

The ROS scavenging activity percentage= $\frac{A_{C-A_T}}{A_C} X\ 100$ …… (1)

Where A_C = Absorbance of control, A_T = Absorbance of sample.

Histopathology:

Ovaries and Intestines were excised from different fish groups. They were fixed in 10% formalin solution for 24 h. Subsequently Ovaries and Intestines tissues were processed through graded series of alcohols, cleared in xylene and embedded in paraffin wax. Ovaries were processed by double embedding technique. Sections were cut at 4–6 mm thickness with the help of 820-Spencer rotatory microtome, stained with hematoxylin–eosin (dissolved in 70% alcohol) and were mounted on Glass slides. The photographs at 40X magnification were taken with Magnus SM-100 microscope.

Isolation of probiotic bacterial proteins

Experimental strains and culture

Bacillus megaterium de Bary (**ATCC**® 14581™) was selected as a probiotic strain donated by BioNest from Central University of Hyderabad. Probiotic culture was grown overnight in 10 mL of De Man, Rogosa and Sharpe agar (MRS) medium at 37 °C on a rotary shaker at 150 rpm for 24 - 48 hours. 10 mL of the activated strains inoculated aseptically to 1L MRS medium for the optimization in 1 L bottle with a rubber plug. The culture was shaken at 150 rpm, 37 °C aerobically.

Optimization of Temperature for Bacteriocin Production:

To check Bacteriocin activity at different temperatures 10 mL of active culture *B. megaterium* was incubated at 20, 25, 30, 35, 40, 45, and 50 °C for 24 hours on MRS medium. Antimicrobial activity of all the treated bacteriocins was determined by agar disc diffusion assay.

Optimization of pH for Bacteriocin Production:

To determine the effect of pH, samples of MRS broth were prepared by adjusting pH to 3.0, 5.0, 7.0,7.0,9.0 and 12.0 with 0.1 N or 1 N HCl and 0.1 N or 1 N NaOH before sterilization. The tubes containing 10 mL of pH adjusted MRS broth were inoculated with 50 µL of active culture of the *B. megaterium* and incubated at 37 °C for 24hours.Antimicrobial activity of all the treated bacteriocins was determined by agar disc diffusion assay.

Optimization of salt concentration for Bacteriocin Production:

To investigate the effect of sodium chloride, different concentrations of sodium chloride (2, 3, 4, 5, and 6%) were added to bacteriocin aliquots and incubated at 37 °C for 24 hours. Antimicrobial activity of all the treated bacteriocins was determined by agar disc diffusion assay.

Optimization of Production Media for Bacteriocin Production:

To ensure the maximum bacteriocin production, *B. megaterium* was cultured in various media such as Nutrient broth, Luria broth, MRS broth, LBS (Lactobacillus Selection), BHI (Brain Heart Infusion Broth). Then the flasks were incubated at 37 °C for 48 h in an orbital shaker (150 rpm).

The Growth Curve:

The microbial growth is determined by cell optical density (absorbance at 600nm). A growth curve can be divided into four phases, Lag phase, Log phase, Stationary Phase, Death phase. As inoculum is transferred to the fermentation media, cell growth starts rapidly in the media. The lag phase shows almost no apparent cell growth. This is the duration of time represented for adaptation of microorganisms to the new environment, without much cell replication and with no sign of growth. The length of the lag phase depends on the size of the inoculum. It also results from the shock to the environment when there is no acclimation period. Even high concentrations of nutrients can cause a long lag phase. Once there is an appreciable number of cells and they are growing very rapidly, the cell number exponentially increases. The optical cell density of a culture can then be easily detected; that phase is known as the exponential growth phase. The rate of cell synthesis sharply increases; the linear increase is shown in the semi-log graph with a constant slope representing a constant rate of cell population. At this stage carbon sources are utilized and products are formed. Finally, rapid utilization of substrate and accumulation of products may lead to stationary phase where the cell density remains constant. In this phase, cells may start to die as the cell growth rate balances the death rate. It is well known that the biocatalytic activities of the cell may gradually decrease as they age, and finally autolysis may take place. The dead cells and cell metabolites in the fermentation broth may create toxicity, so deactivating remaining cells. At this stage, a death phase develops while the cell density drastically drops if the toxic secondary metabolites are present. The death phase shows an exponential decrease in the number of living cells in the media while nutrients are depleted.

To evaluate the growth of the bacteria in MRS broth pH 7.0 and temperature 37 °C was maintained. The growth of the bacteria was also monitored by measuring the optical density (O.D) at 600nm.Ten percent of starter culture of the bacteriocin producer at O.D 0.1 was added to 1 liter of sterile MRS broth. The colony forming unit (CFU) and the O.D of the bacteria within 48 hours was monitored. Total bacterial analysis of culture was determined from 0-48 hours of incubation.

Bacteriocin activity:

After incubation, cells were removed from the growth medium by centrifugation at 8000 rpm for 15 min, 4 °C. The cell-free supernatant was adjusted to pH 6.5 using 1 mol/L NaOH and it was used as crude bacteriocin. The agar disc diffusion method was used to detect the antibacterial spectrum of *B.megaterium* (disc of 6 mm diameter) were kept on the plate. Each disc was filled with 30 µL of crude bacteriocin. The plates were incubated at 35 °C for 24 h. The diameter of inhibition zones (mm) around the discs was measured. The antibacterial activity recorded as positive if a transparent halo zone was observed around the disc. Antibacterial activity was expressed as an inhibition zone in mm.

Agar disc diffusion assay

Disc diffusion assay was performed to qualitatively analyze bacteriocin activity. MRS agar medium was autoclaved for 15 min at 121 °C and 15 lbs. Medium was poured in each disposable petri plate and was allowed to solidify. Upon solidification, sterilized discs of 6mm diameter were kept on the plate. To each disc 100 µL of the cell free supernatant was poured and incubated at 37 °C overnight. The appearance of clear zones confirmed the presence of bacteriocin activity.

Bacteriocin Production and Partial Purification:

Step 1: Precipitation by ammonium sulfate

Cell-free supernatant was collected by centrifugation. Ammonium sulfate was slowly added to the supernatant to 40, 50, 60, 70, 80% saturation, Then the mixture was incubated overnight at 4 °C on Magnetic stirrer (Figure. 6a). The precipitated protein was obtained by centrifugation for 20 min at 8000 rpm at 4 °C. The obtained pellet was resuspended in a 10 mL of ice-cold 0.2 M phosphate buffer pH, 7.5. The dissolved bacteriocin was dialyzed (Figure. 8b & c) using a 1000 Da cut-off

membrane with PBS buffer changing collectively to get rid of the excess of ammonium sulfate and then made up the final volume up to 30 mL. The dialyzed fraction was tested for antimicrobial activity by agar disc diffusion assay. Protein concentrations were measured and the specific activity was calculated.

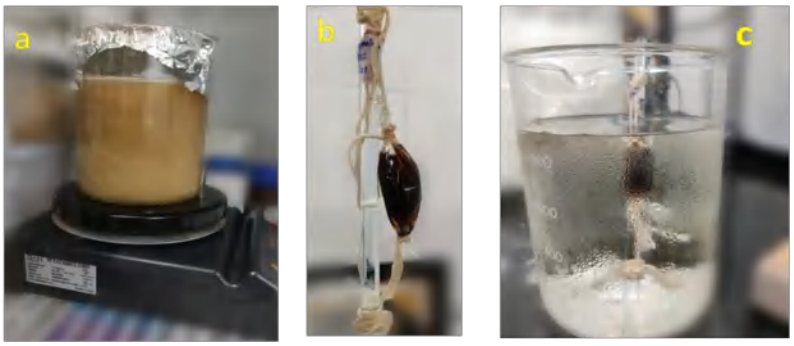

Figure. 8 a) Ammonium sulphate precipitation b) & c) dialysis

Step 2: Chromatography on Sephadox Column:

The dialyzed protein was applied to the column (14 × 25 cm) previously equilibrated with phosphate buffer 0.2 M (pH 7.5). The adsorbed protein was eluted with a stepwise NaCl gradient ranging from 0.0 to 0.7 M prepared in 0.2 M phosphate buffer (pH 7.5) at a flow rate of 0.8 $mL.min^{-1}$. Five mL fractions were collected wherein eluted fractions were monitored at 280 nm for protein and assayed for enzyme activity. Different fractions were eluted with the same buffer and the eluted enzymes were concentrated and kept in an ice bath.

Figure. 9 Set up of a Column Chromatography

Step 3: Protein determination:

Protein content was determined by Lowry's method using bovine serum albumin as a standard protein. Absorbance at 280 nm was used for monitoring protein. The concentration of protein was also determined to calculate the specific activity.

Step 4: Molecular Weight Estimation with Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE):

SDS–PAGE is carried out with a discontinuous buffer system with varied pH and ionic strength (Laemmli, 1970). Laemmli's SDS–PAGE employs two types of gels, stacking gel and the separating gel. Stacking gel pore size is bigger and enables all the protein to stack at the same number but on entering the separating gel the protein sample run depends on their molecular weight. The ability of the discontinuous buffer system to concentrate all of the complexes in the sample into a very small volume greatly increases the resolution of SDS Polyacrylamide gel. SDS–PAGE analysis of purified bacteriocin was performed with 16% resolving gel and 4% stacking gel. The gel was stained with coomassive brilliant blue R-250 in order to determine the molecular size of bacteriocin.

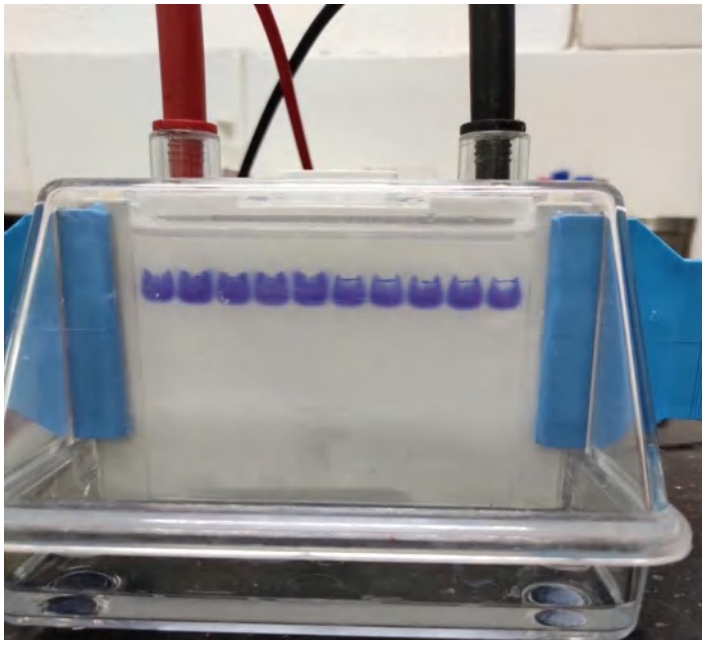

Figure. 10 SDS-PAGE setup

5. Results

All the fishes were active throughout the experiment. No clinical signs of toxicity or mortality were observed in any of the experimental or control fishes. Different morphological parameters were assessed after 30 days of treatment. All fishes were measured for total wet weight in gm. Probiotic group fishes gained the weight represented in Table. 1. Cisplatin group fishes reduced the weight. Probiotic + cisplatin treated group retained the weight. Coming to body length cisplatin treated group increased their body length. According to BMI control, probiotic, probiotic +cisplatin treated group were considered healthy with 0.1 g/cm^2 whereas, cisplatin treated group is considered as unhealthy with 0.06 g/cm^2 of BMI which is depicted in Figure. 11.

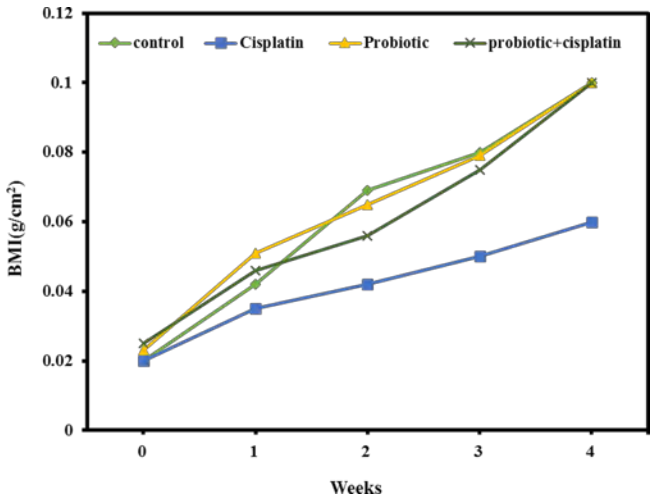

Figure. 11 Body mass index (BMI) of zebrafish. BMI of control, probiotic, cisplatin and probiotic + cisplatin zebrafish over 4 week's period.

Table. 1 Morphological parameters of Zebrafish after treatment.

Parameter	Control	Probiotic	Cisplatin	Probiotic+ Cisplatin
Body weight (g)	0.77 ±0.05	0.8±0.05	0.6±0.05	0.75±0.03
Body length(cm)	2.63±0.05	2.8±0.05	3.4 ±0.05	3.2 ±0.05
BMI(g/cm^2)	0.1±0.05	0.1±0.05	0.06±0.05	0.1±0.05
Survival (%)	100	100	90	100

Probiotics can influence the health of the host in several ways: secreting secondary metabolites that inhibit growth of microbial pathogens and/or directly stimulating immune responses to down regulate gut inflammation. To assess potential health benefits of live probiotics it is important to understand their optimal environment inside the host (oxygen levels, pH, etc.). Probiotic-host interaction was addressed by using Zebrafish (*Danio rerio*). Cisplatin showed symbiotic association with the probiotic i.e.; Growth of probiotic is not inhibited by cisplatin drug which is depicted in Figure. 12.

Figure. 12 Probiotic showing Symbiotic association to different concentrations of cisplatin.

Antioxidant assays:

Antioxidants fight free radicals and protect us from various diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defense mechanisms. It is evident from the results that the toxicity of Cisplatin caused to increase in total Lipid peroxidation activity in Intestine and followed by ovaries tissues represented in Figure. 13. ROS is capable of damaging molecules of biochemical classes including nucleic acids and amino acids. Exposure of reactive oxygen to proteins produces denaturation, loss of function, cross-linking, aggregation and fragmentation of connective tissues. The most destructive effect is the induction of lipid peroxidation. The cell membrane which is composed of poly-unsaturated fatty acids is a number one goal for reactive oxygen molecules, attack leading to cellular membrane damage.

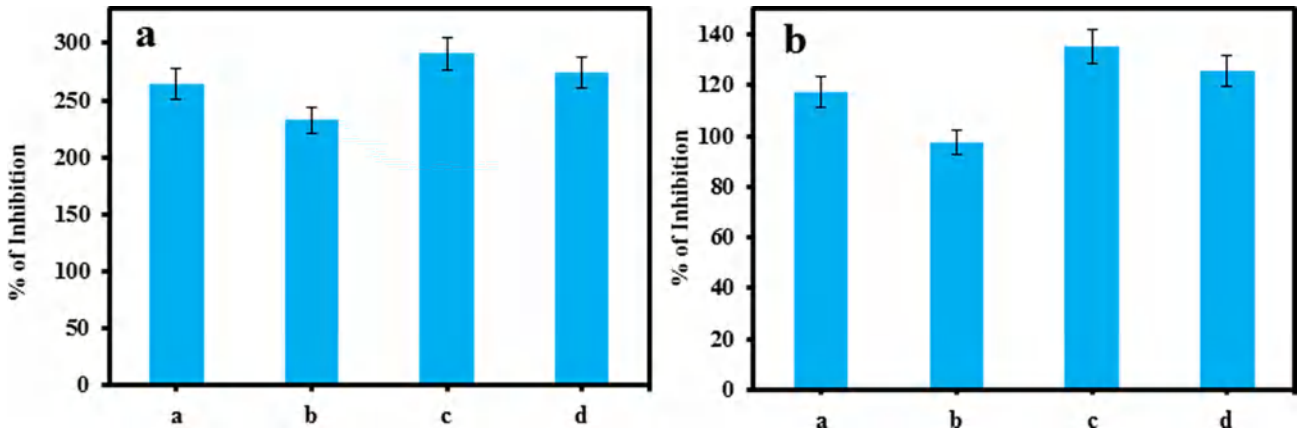

Figure. 13 Lipid Peroxidation activity levels in a. Intestine b. Ovaries tissue of fish.

Glutathione peroxidase plays a major role in the detoxification of hydrogen peroxide; Glutathione peroxidase (GPx) is present in the cytosol of cell. In the present study decreased in activity of GPx was observed in control group, probiotic group when compared with cisplatin treated groups as shown in Figure. 14.

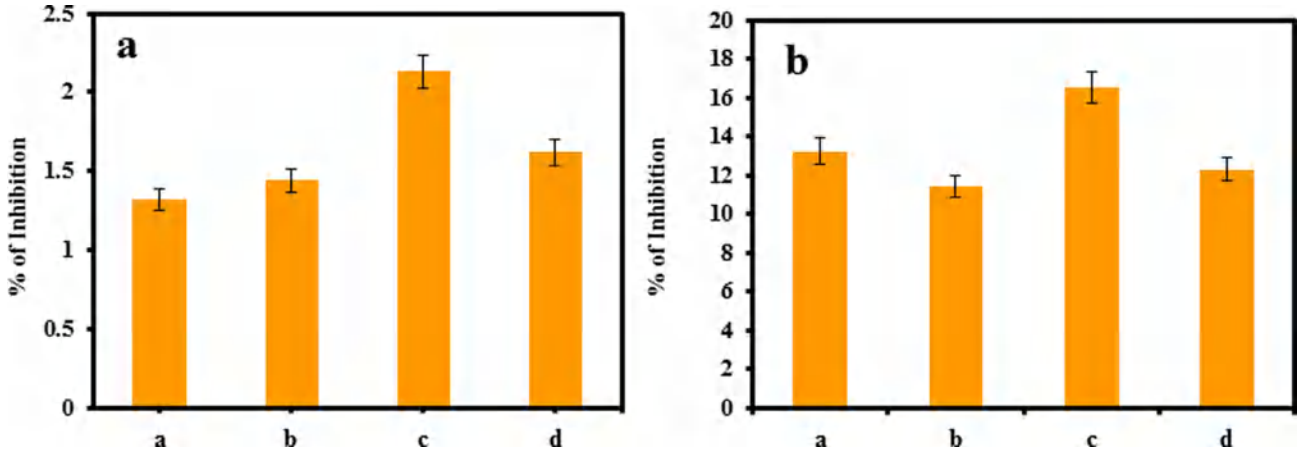

Figure. 14 Glutathione peroxidase activity levels in a. Intestine b. Ovaries tissue of fish.

Glutathione reductase (GR) also serves as an antioxidant. It protects cellular proteins from reactive oxygen species. Glutathione reductase is almost same in the control, probiotic, Cisplatin+ Probiotic treated groups except in cisplatin treated group graphically shown in Figure. 15.

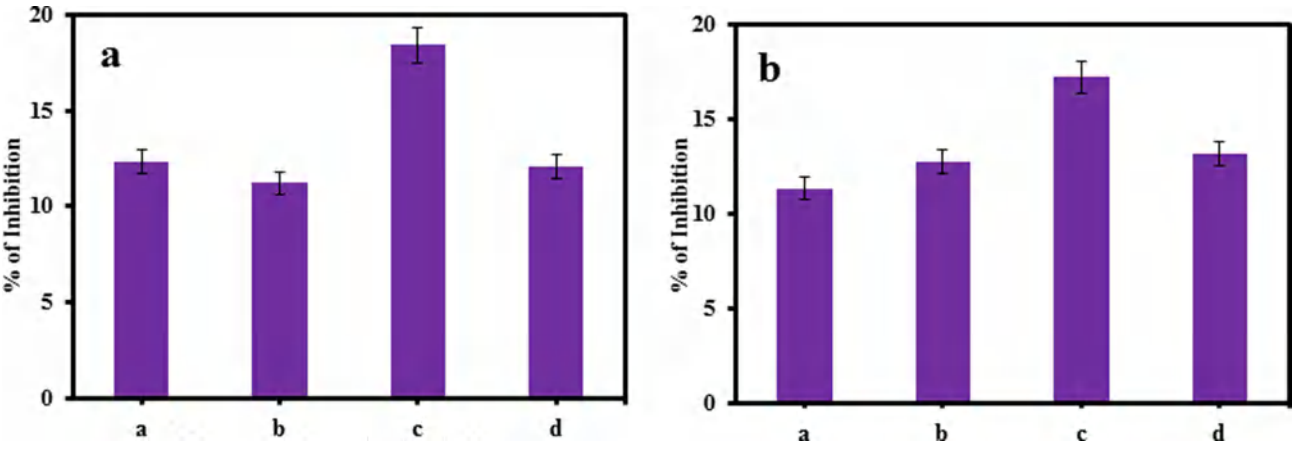

Figure. 15 Glutathione reductase activity levels in a. Intestine b. Ovaries tissue of fish.

There are many enzymes that are able to neutralize hydrogen peroxide. These enzymes include catalase, glutathione peroxidase. Catalase is one of the most important Antioxidant. Catalase is a key enzyme which uses hydrogen peroxide, as its substrate. This enzyme is responsible for neutralization of H_2O_2 into water and oxygen. Catalase activity is almost same in the control, probiotic, Cisplatin+ Probiotic treated groups except in cisplatin treated group as represented in Figure. 16.

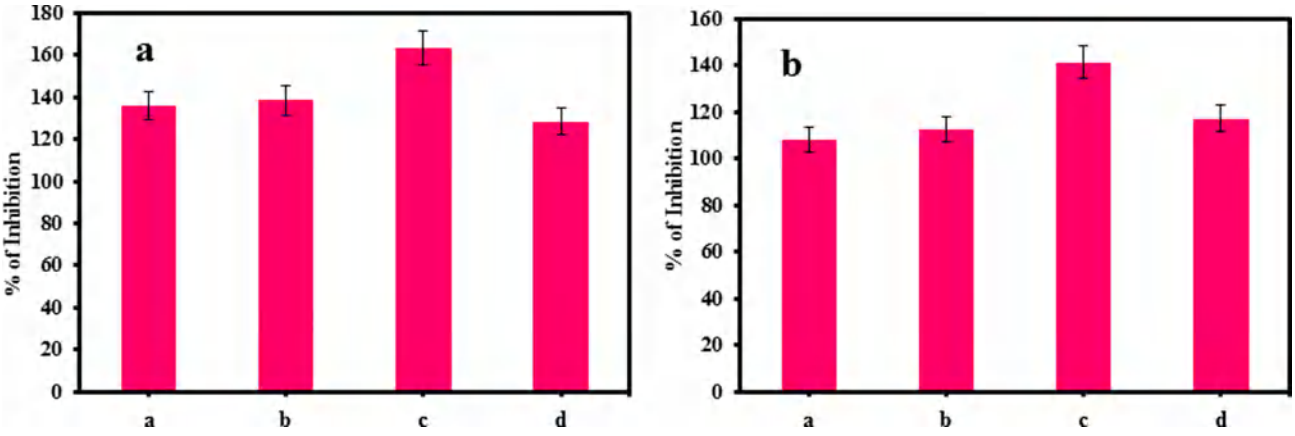

Figure. 16 Catalase activity levels in a. Intestine b. Ovaries tissue of fish.

Superoxide dismutase is an enzyme that helps break down potentially harmful oxygen molecules in cells. This might prevent damage to tissues. Presence of enzymes catalase & superoxide dismutase (SOD) allows toxic forms of oxygen to be neutralized. The enzyme acts as a good

therapeutic active substance against reactive oxygen species-mediated diseases. It removes superoxide anion by converting it to hydrogen peroxide, and thus diminishing the toxic effect caused by this radical. An increase in the percentage inhibition of superoxide is seen in cisplatin treated group depicted in Figure. 17. Probiotics have more ability in removing free radicals. In addition to the anti-oxidant status, further evidences from histopathological studies clearly emphasizing the therapeutic application of probiotics. Thus cisplatin-induced oxidative stress was best prevented by the addition of probiotic.

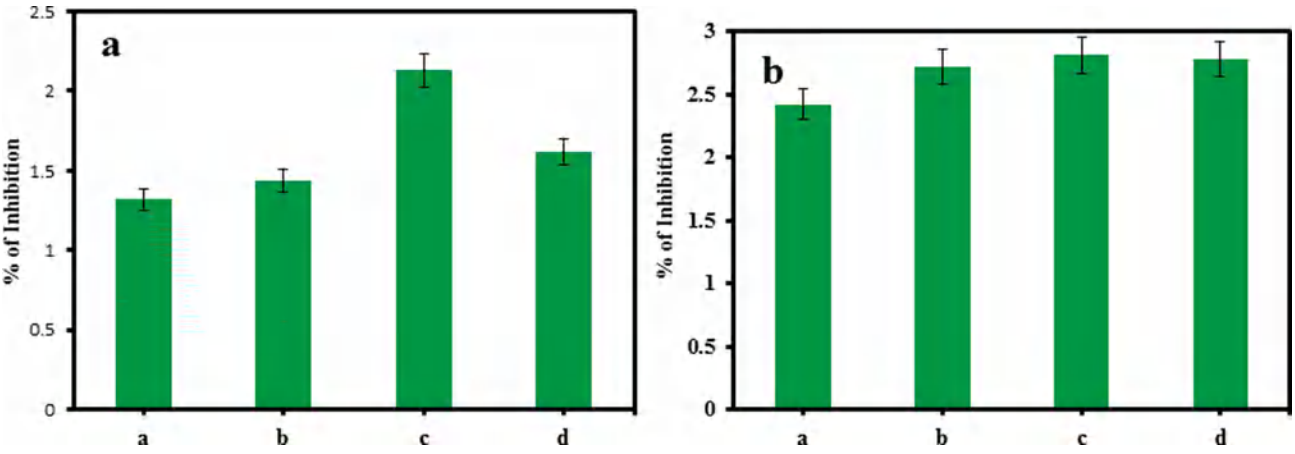

Figure. 17 Superoxide dismutase activity levels in a. Intestine b. Ovaries tissue of fish.

Histological changes in Intestine:

Histopathological changes in Intestine were investigated by hematoxylin–eosin staining Cisplatin intoxicated Intestine exhibited presence of vacuolated spaces as against normal architecture shown by the Intestine of control Fish. Damage is seen in cisplatin treated tissues major intact basement membranes surrounding Intestines is lost. The morphology of the Intestine tissue from fishes treated with cisplatin and probiotic (Figure. 18d) was different from those in the cisplatin group. Treatment with Probiotic decreased the damage caused by cisplatin (Figure. 18d), and the tissue showed morphological features similar to those of the control group.

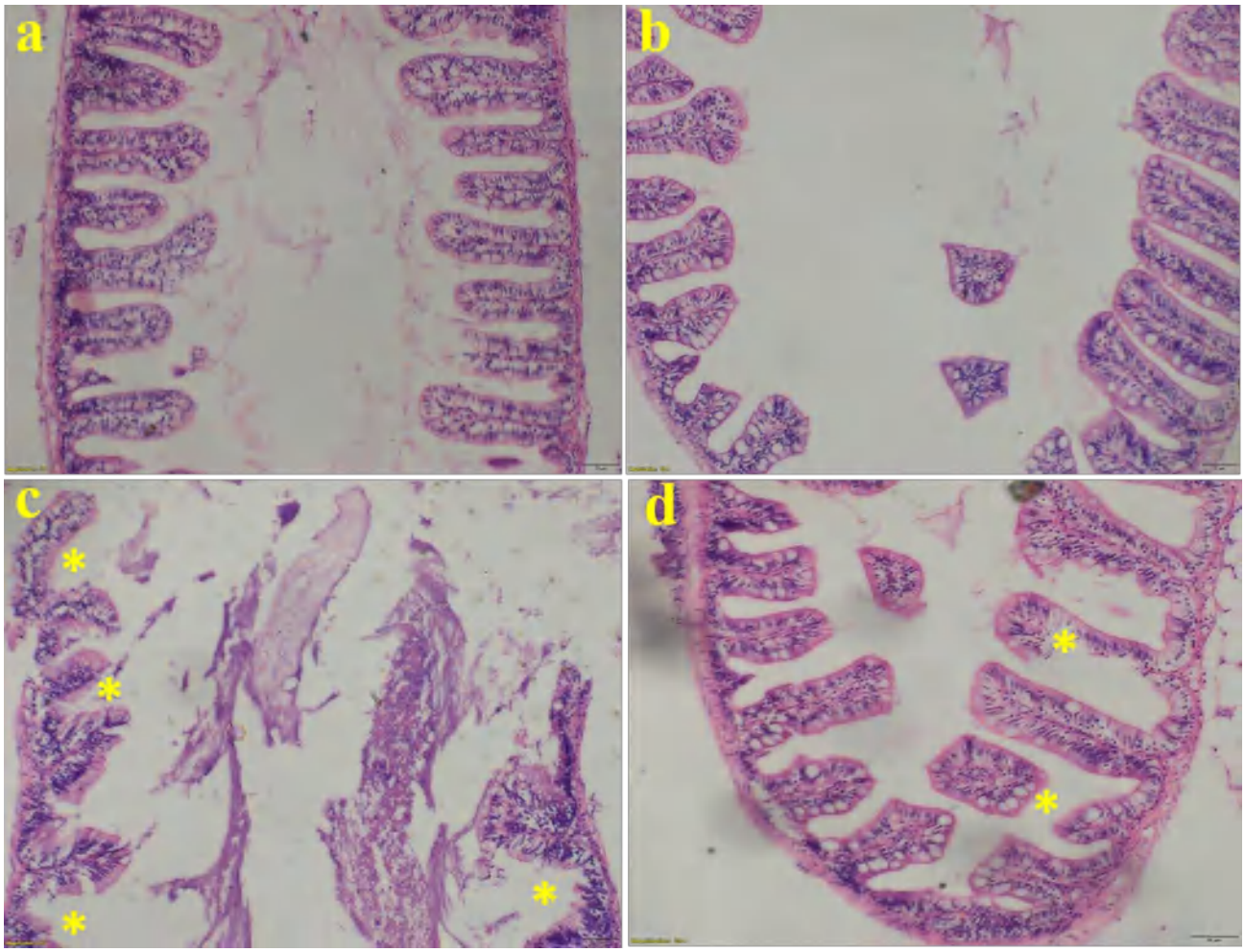

Figure. 18 Histopathological analysis in intestines of Zebrafish after treatment

a) Control, b) Probiotic, c) Cisplatin, d) Cisplatin + probiotic groups.

Histological changes in Ovaries:

Histopathological changes in Ovaries were investigated by hematoxylin–eosin staining. Ovaries from the control group showed normal ovarian morphology with centrally located oocytes and granulosa cells surrounded by normal intact basement membranes. However, Histological examination of the ovarian tissue reveals that Cisplatin toxicities caused abnormal structural changes in the tissue. The cisplatin group damaged the ovarian structure and promoted a reduction in the number of follicles. Probiotic + cisplatin treatment ameliorated minor abnormalities in Ovaries as compared with the Cisplatin group fishes (Figure. 19d). With reference to Histopathological observation, observable difference has been observed between Cisplatin and Probiotic treated tissues.

Therefore, it may be suggested that Consumption of Probiotic along with Cisplatin might inhibit damage and reduce side-effects.

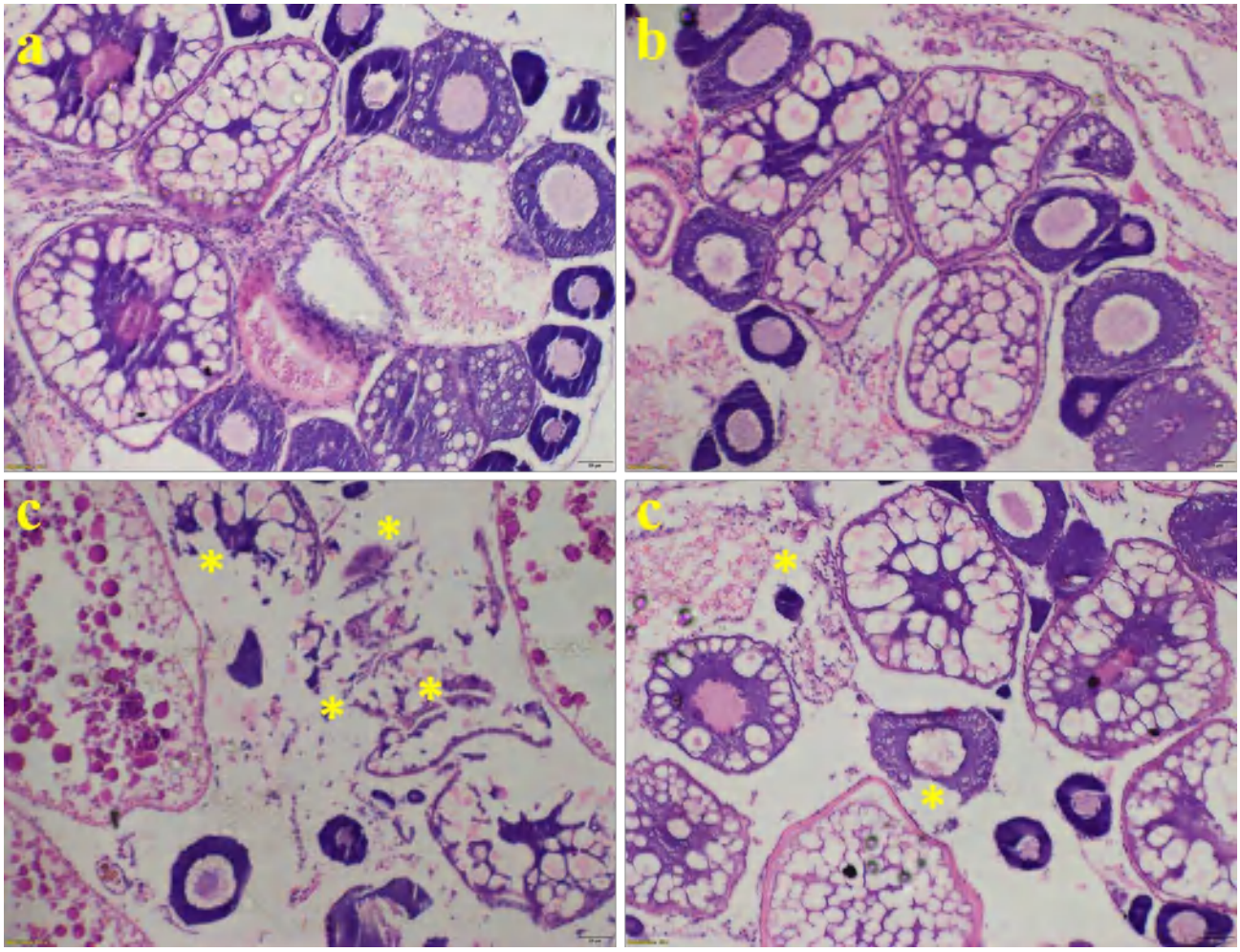

Figure. 19 Histopathological analysis in Ovaries of Zebrafish after treatment

a) Control, b) Probiotic, c) Cisplatin, d) Cisplatin + probiotic groups.

ROS scavenging activity:

An intracellular ROS scavenging activity has been investigated using the quinol and hydroquinone approach. Figure. 20 represents the investigation of intracellular ROS scavenging activity upon the addition of the lowest concentration of tissue solution to the standard quinol solution. As from the image, the ROS scavenging activity has been recorded 60.4 % and 63.7 % in intestine and ovaries respectively upon treatment with a probiotic (yellow color bars). Similarly, after treatment of cisplatin (brick red color bars), approximately 84.3 % and 81.4 % of ROS scavenging activities have been notified in the intestine and ovaries respectively. However, the scavenging activity has

been significantly enhancing in both organs (intestine (69.7 %) + ovaries (78.1 %)) after treatment with the probiotic + cisplatin (cyan color bars) as compared to the neat probiotic. Based on these investigations, probiotic + drug combination could be useful for the ROS radical scavenging applications in real samples. Figure. 21 represents the intracellular ROS-induced brown color quinol-hydroquinone adduct formation. Herein, the brown color quinol-hydroquinone adduct has been formed when quinol was treated with the cells of Zebrafish through intercellular ROS species via as explained earlier mechanism (Figure. 21A). Figure 20B represents the conversion of brown color quinol-hydroquinone adduct from the colorless quinol with the treatment of ROS species from the tissues of Zebrafish.

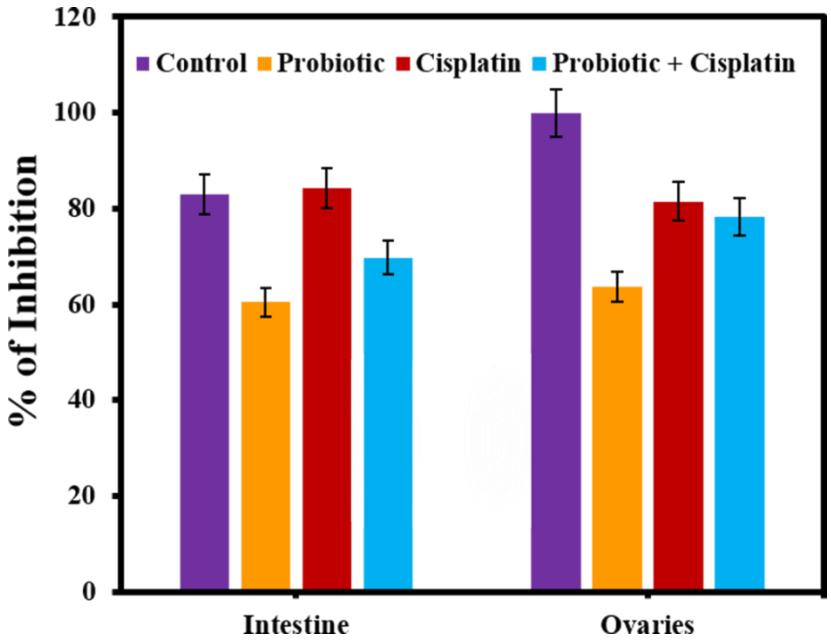

Figure. 20 Intracellular ROS scavenging activities using quinol-hydroquinone approach in intestine and ovaries of Zebrafish.

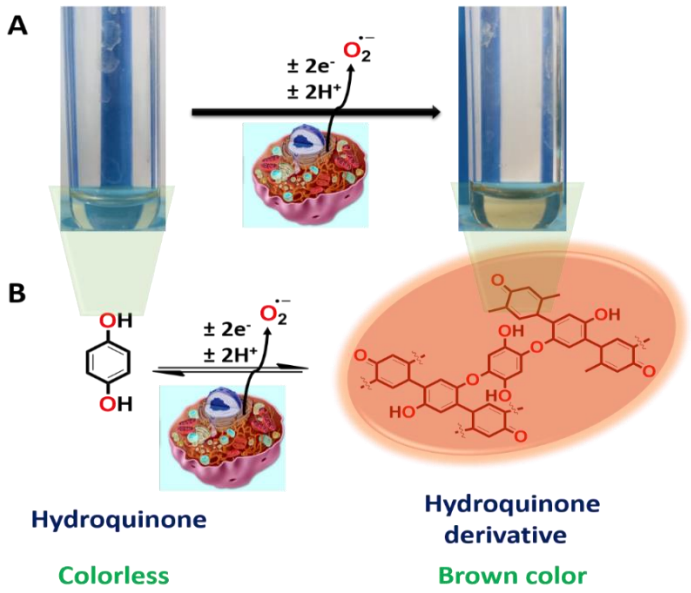

Figure. 21 (A) An intracellular ROS species from the tissues of Zebrafish mediated brown color quinol-hydroquinone adduct formation via color less quinol, (B) overall schematic representation of the conversion of quinol to the formation of brown colored quinol-hydroquinone product.

Intestine	**Control**	**Probiotic**	**Cisplatin**	**Probiotic+Cisplatin**
Lipid peroxidation nmol MDA/g wet wt	264.23	232.36	290.82	274.62
Glutathione peroxidase nmol min^{-1} mg protein^{-1}	16.23	18.78	25.52	16.71
Glutathione reductase nmol min^{-1} mg protein^{-1}	12.32	11.21	18.42	12.1
Catalase µmol min^{-1} mg protein^{-1}	135.74	138.62	163.24	128.51
Superoxide Dismutase Unit inhibition/min/mg tissue	1.32	1.44	2.13	1.62
Ovaries	**Control**	**Probiotic**	**Cisplatin**	**Probiotic+Cisplatin**
Lipid peroxidation nmol MDA/g wet wt	117.27	97.27	135.21	125.54
Glutathione peroxidase nmol min^{-1} mg protein^{-1}	13.25	11.41	16.52	12.32
Glutathione reductase nmol min^{-1} mg protein^{-1}	11.34	12.75	17.23	13.15
Catalase µmol min^{-1} mg protein^{-1}	108.23	112.51	141.34	117.27
Superoxide Dismutase Unit inhibition/min/mg tissue	2.42	2.72	2.81	2.78

Table. 2 Quantification of antioxidant enzymes of Intestine and Ovaries after treatment.

Isolation of probiotic bacterial proteins:

On screening *B. megaterium* results obtained are concave, smooth and milk white, coarser with fine threads around the colony (Figure. 22). The cell morphology shows that the cell is rod-shaped, gram positive and sporous.

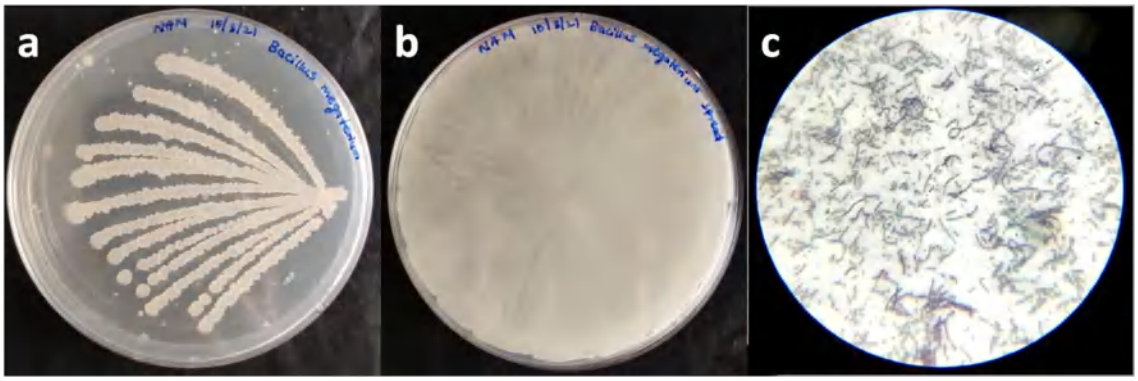

Figure. 22 a) Streak culture) Spread culture) Gram's Staining of *Bacillus megaterium.*

Optimum Temperature of Bacteriocin Production:

Figure. 23a shows the effect of temperature on bacteriocin activity in terms of inhibition zones. A partial loss in the activity was observed with a continuous increase in temperature. Bacteriocin production (7.1 mm and 7.2 mm) was found to be higher at 35-40 °C and lower at 50 °C respectively.

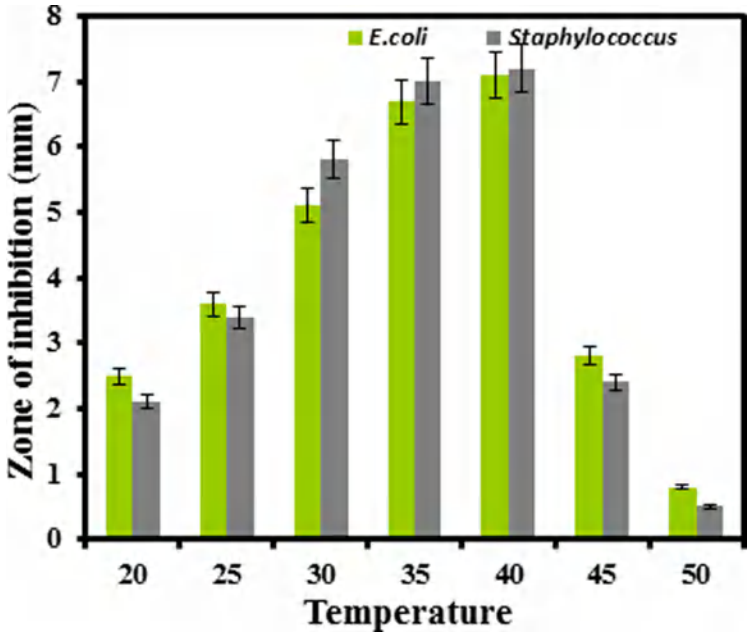

Figure. 23a Optimum temperature for bacteriocin production.

Optimum pH for Bacteriocin Production:

Bacteriocin was active in a wide range of pH, but the maximum activity was observed at pH-7.0. Bacteriocin production (6.9mm & 6.3mm) was found to be higher at 7.0 and lower at 12 respectively (Figure. 23b). Bacteriocin could retain its antibacterial activity partially when there was a shift to acidic or basic range. *Bacillus megaterium* retained its antibacterial activity in an acidic pH range of 3.0 to 6.0, while inactivation occurred at pH 9.0 to 12.0.

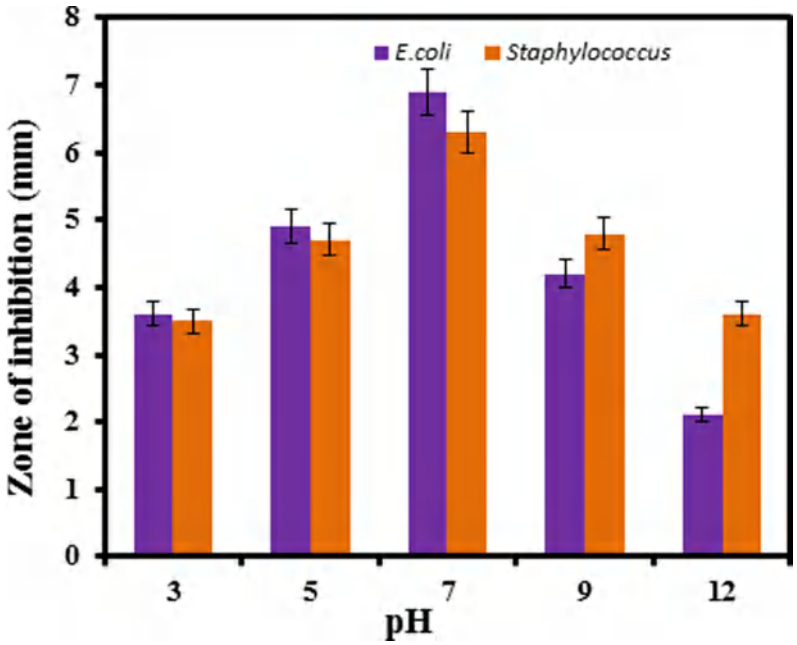

Figure. 23b: Optimum pH for bacteriocin production.

Optimum salt concentration for Bacteriocin Production:

The maximum (5.2 and 5.5mm) bacteriocin production by *B. megaterium* was achieved with 3% NaCl supplementation (Figure. 23c).

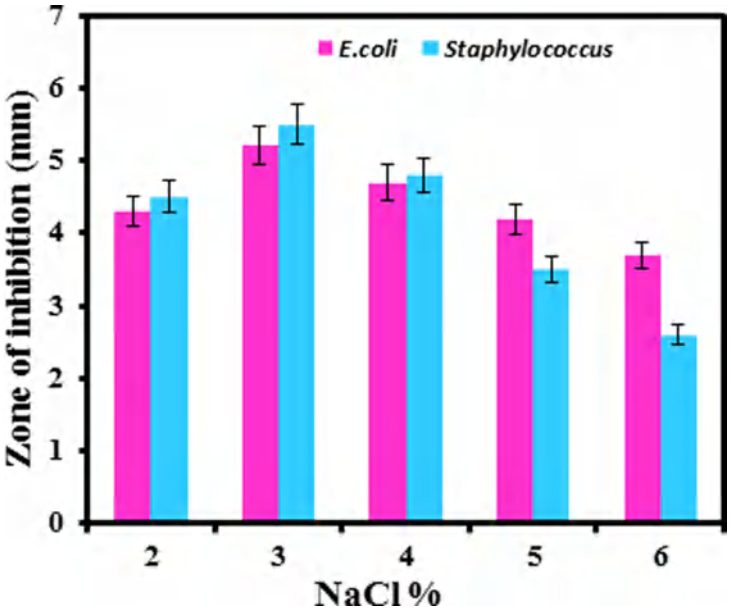

Figure. 23c Optimum salt concentration for bacteriocin production.

Growth Curve and Production of Bacteriocin Study:

According to the growth curve bacteria reached the stationary phase after 18 hours of incubation. According to the inhibition zone from the crude bacteriocin purified from the supernatant, the bacteriocin started to produce at 8 hours of incubation and reached optimum production at 18 hours incubation period. The antibacterial activity remained almost the same between 18 to 24 hours of growth. However, the activity of bacteriocin started to decrease at 28 hours of growth (Figure. 23d). Therefore, the bacteriocin was best harvested between 18 to 24 hours to get optimum production.

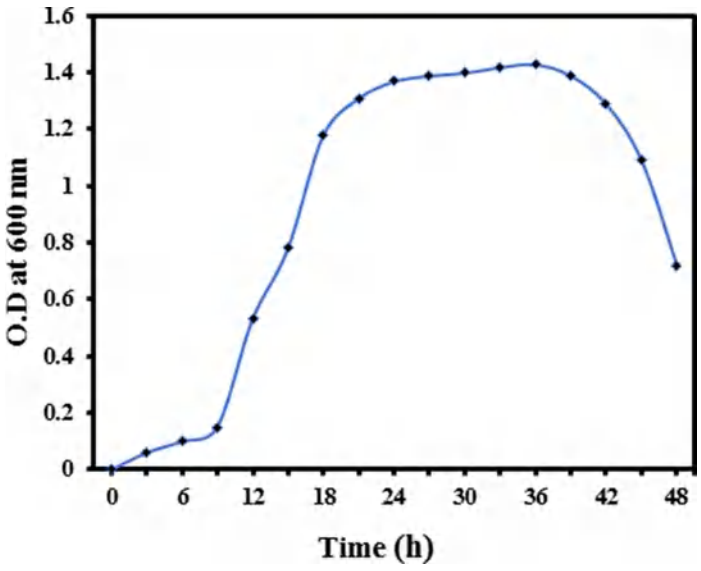

Figure. 23d Growth curve of *Bacillus megaterium*

Optimum Production Media for Bacteriocin Production:

The influence of growth conditions on bacteriocin studies revealed that maximum bacteriocin production by MRS broth favored the bacteriocin production (6.5mm and 6.1mm).The other production media resulted in minimal bacteriocin yield (Figure. 24). MRS medium is a better medium for cell growth and bacteriocin production. Earlier reports also evidenced that MRS medium is a better medium for cell growth and bacteriocin production than the other culture media (Biswas et al., 1991, Ten Brink et al., 1994, Toba et al., 1991).

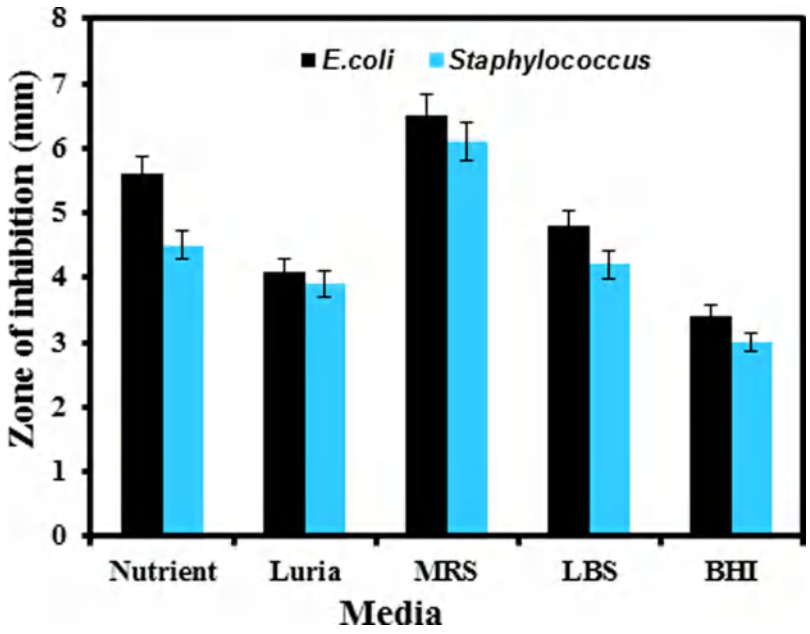

Figure. 24 Optimum production media for bacteriocin production

The influence of growth conditions on bacteriocin studies revealed that incubation temperature and pH Furthermore, bacteriocin production (7.1 mm) was found to be higher at 37 °C, pH -7.0 and lower at 50 °C, pH -12 (2.1 mm) respectively. The decrease in antibacterial activity observed after a longer incubation time could be due to the degradation of the bacteriocin by proteolytic enzymes present in the medium, or else by the low pH.

Purification of Bacteriocin:

Bacteriocin present in the cell-free supernatant was subsequently precipitated by 40, 50, 60,

70, 80% ammonium sulfate by keeping on magnetic stirrer overnight. The results showed that 60% ammonium sulfate enhanced the antibacterial activity by 10.31-fold with a 48% yield. This illustrated that 60% ammonium sulfate could be used as a crude method of bacteriocin purification (Figure. 25).The precipitates were collected by centrifugation and dissolved in double distilled water. The dissolved bacteriocin was dialyzed using a 1000 Da cut-off membrane with PBS buffer changing collectively. The dialyzed fraction was tested for antimicrobial activity by agar disc diffusion assay. Protein content at different steps was determined by Lowry's method (Table. 3).

Purification steps	**Volume (mL)**	**Total activity (U)**	**Total protein (mg)**	**Specific activity (U mg^{-1} protein)**	**Purification (Fold)**	**Recovery (%)**
Cell free Supernatant	1000	187400	4560	41.09	1.00	100
$(NH_4)_2SO_4$ (60%)	500	137650	550	250.27	6.09	73.45
Sephadox column	A	57250	192	561.27	13.63	30.54
	b	34340	154	229.8	5.42	18.32
	c	20620	65	117.23	7.72	11.00

Table. 3 Protein quantity at various steps of purification.

The precipitates (by 60% ammonium sulfate) were further purified by Sepharose column chromatography, which revealed that the fractions with antimicrobial activity were collected between 70 and 100 mL. This might be due to very low concentrations of the protein. However, the antimicrobial activity in these fractions was 520 mg of protein. In conclusion, partially pure bacteriocin could be obtained by purification steps.

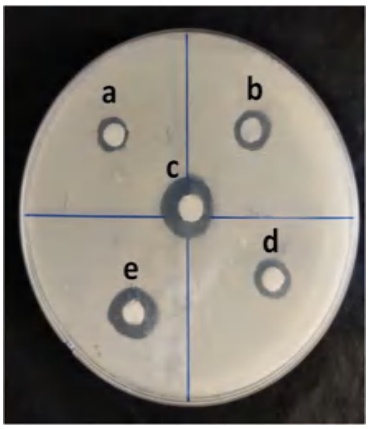

Figure. 25 Antibacterial activity of bacteriocin produced with different concentrations of ammonium sulfate **(a)** 40% ammonium sulfate, **(b)** 50% ammonium sulfate, **(c)** 60% ammonium sulfate, **(d)** 70% ammonium sulfate, and **(e)** 80% ammonium sulfate.

Molecular Mass determination:

SDS–PAGE analysis of 1st single band between 6 and 10 kDa in the stained gel. The molecular mass of the peptide was found to be approximately 7.73 kDa (Figure. 26). Next single band was between 3.5 and 5 kDa in the stained gel. The molecular mass of the peptide was found to be approximately 5.7 kDa. Third single band below 3.5 kDa in the stained gel. The molecular mass of the peptide was found to be approximately 2.23 kDa.

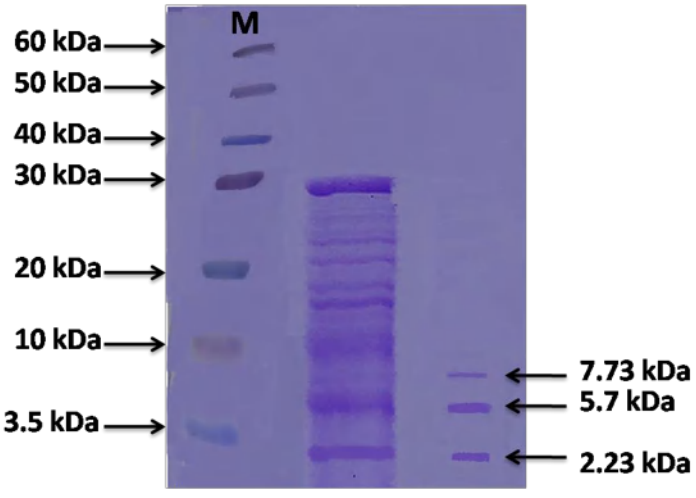

Figure. 26 Molecular weight determination by SDS-PAGE

6. Statistical analysis

Data were expressed as mean±SD from at least three independent experiments. One-way ANOVA was performed for the comparison of results between untreated control and treatments. Values were considered significant when P values were < 0.05 and < 0.01.

ONE-way ANOVA: Tests of Between-Subjects Effects					
Lipid peroxidation (Ovaries)					
Source	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Intercept (Between the groups)	285420.986	1	285420.986	7026.995	.000
Groups (Within the group)	7381.142	3	2460.381	60.574	.000
Error	812.356	20	40.618		
Total	293614.483	24			

ONE-way ANOVA: Tests of Between-Subjects Effects					
Superoxide Dismutase (Ovaries)					
Source	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Intercept (Between the groups)	213.243	1	213.243	2179.727	.000
Groups (Within the group)	5.626	3	1.875	19.170	.000
Error	1.957	20	.098		
Total	220.826	24			

ONE-way ANOVA: Tests of Between-Subjects Effects					
Catalase (Ovaries)					
Source	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Intercept (Between the groups)	350878.196	1	350878.196	4329.318	.000
Groups (Within the group)	3275.355	3	1091.785	13.471	.000
Error	1620.940	20	81.047		
Total	355774.491	24			

ONE-way ANOVA: Tests of Between-Subjects Effects					
Glutathione peroxidase (Ovaries)					
Source	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Intercept (Between the groups)	4285.861	1	4285.861	2727.008	.000
Groups (Within the group)	95.202	3	31.734	20.192	.000

Error	31.433	20	1.572		
Total	4412.496	24			

ONE-way ANOVA: Tests of Between-Subjects Effects					
Glutathione reductase (Ovaries)					
Source	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Intercept (Between the groups)	4427.078	1	4427.078	3834.730	.000
Groups (Within the group)	99.416	3	33.139	28.705	.000
Error	23.089	20	1.154		
Total	4549.584	24			

ONE-way ANOVA: Tests of Between-Subjects Effects					
Glutathione reductase (Intestine)					
Source	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Intercept (Between the groups)	4269.293	1	4269.293	1907.159	.000
Groups (Within the group)	148.225	3	49.408	22.071	.000
Error	44.771	20	2.239		
Total	4462.288	24			

ONE-way ANOVA: Tests of Between-Subjects Effects					
Glutathione peroxidase (Intestine)					
Source	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Intercept (Between the groups)	9383.382	1	9383.382	5323.210	.000
Groups (Within the group)	515.638	3	171.879	97.507	.000
Error	35.255	20	1.763		
Total	9934.275	24			

ONE-way ANOVA: Tests of Between-Subjects Effects					
Lipid peroxidation (Intestine)					
Source	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Intercept (Between the groups)	1562509.192	1	1562509.192	2876.042	.000
Groups (Within the group)	17934.021	3	5978.007	11.003	.000
Error	10865.691	20	543.285		
Total	1591308.903	24			

ONE-way ANOVA: Tests of Between-Subjects Effects

Catalase (Intestine)					
Source	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Intercept (Between the groups)	457973.839	1	457973.839	4020.567	.000
Groups (Within the group)	5682.721	3	1894.240	16.630	.000
Error	2278.155	20	113.908		
Total	465934.716	24			

ONE-way ANOVA: Tests of Between-Subjects Effects					
Superoxide Dismutase (Intestine)					
Source	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Intercept (Between the groups)	55.886	1	55.886	2446.972	.000
Groups (Within the group)	1.713	3	.571	25.004	.000
Error	.457	20	.023		
Total	58.056	24			

7. Discussion

Bacteriocins are proteins or complexes proteins biologically active with antimicrobial action against other bacteria, principally closely related species. The production of bacteriocins depends largely on the pH, source of nutrients and temperature. Various physicochemical factors seemed to affect bacteriocin production as well as its activity. Maximum activity is normally noted at pH 7.0, temperature 37°C and 3% NaCl. MRS medium is a better medium for cell growth and bacteriocin production. The bacteriocin produced by *Bacillus megaterium* was partially purified by Column chromatography and the molecular mass of the bacteriocin was determined by SDS-PAGE analysis. Results inferred that the bacteriocin production contained three distinct bands weighing 7.73 kDa 5.7 kDa and 2.23 kDa which is represented in Figure. 26 in comparison with the molecular mass of standard markers.

The present study was carried out to reduce the side-effects of cisplatin with the use of probiotic strain and also to evaluate the antioxidant potentials of treated fishes in comparison with the control ones. This study provides insights into the mechanisms underlying cisplatin-induced toxicity

and evidence for the potential utility of Probiotic in the safer administration of cisplatin. Cisplatin is a well-known chemotherapeutic drug. It has been used for treatment of numerous human cancers. Its mode of action has been linked to its ability to crosslink with the purine bases on the DNA; interfering with DNA repair mechanisms, causing DNA damage, and subsequently inducing apoptosis in cancer cells. Cisplatin is a highly effective anticancer agent that primarily functions through covalent-binding to purine bases, resulting in DNA cross links, activation of repair pathways, formation of double stranded breaks (DSBs), increased levels of reactive oxygen species (ROS) and apoptosis. However, cisplatin treatment is associated with a variety of side effects, including dose-limiting toxicities. But due to its undesirable side effects it is used in combination therapy. Ovaries are crucial to assess the toxicity of Cisplatin. Zebrafish had few tissue alterations in the Intestine when exposed to Probiotic + Cisplatin. However, in the presence of Cisplatin, the Ovaries, and Intestines were affected, demonstrating the toxicity of Cisplatin. Behavioral alterations like erratic swimming, restlessness and surface gasping are seen which may be on avoidances reaction to the toxic chemicals. The increased gulping activity and opercula movements by the exposed fish may be the reflection of an attempt by the fish to extract more oxygen to meet the increased energy demand to sustain the Cisplatin toxicity. Antioxidant enzymes catalyze reactions to neutralize free radicals and reactive oxygen species**.** These form the body's endogenous defense mechanisms to help protect against free radicals induced cell damage. This study demonstrated that the Probiotic treatment attenuated the cisplatin-induced damage in fish ovary.

The ROS activity was calculated using the standard curve method with H_2O_2 over quinol as depicted in Figure. 27. Here, the figure was plotted % of inhibition as a function of different concentrations of H_2O_2 over quinol. Noteworthy, a significant straight line was observed from 9-244 µg/mL concentration among all concentrations with the regression coefficient $R^2 = 0.982$ (Right side down inset image). The left side inset image represents the corresponding color changing of quinol upon the addition of tissue solution via an intracellular ROS reaction.

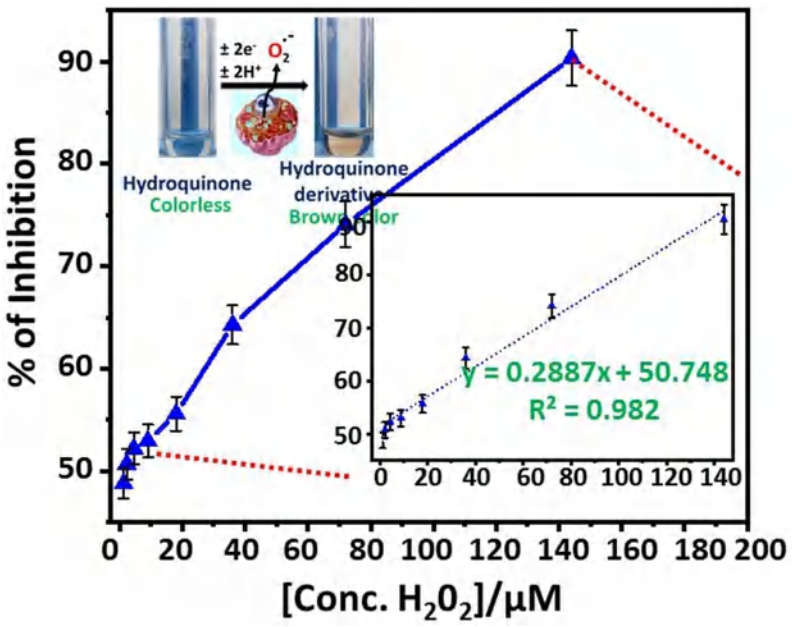

Figure. 27 A standard curve for ROS scavenging activity against quinol. The inset image shows the significant color change before and after addition of intracellular ROS species.

Hydrogen peroxide (H_2O_2) is produced from superoxide anion ($O_2^{\bullet-}$) by superoxide dismutase (SOD) through a dismutation reaction. It is the least reactive molecule among Reactive Oxygen Species and is stable under physiological pH. The hydroxyl radical (•OH), the most reactive and hazardous radical, is formed from H_2O_2 in the presence of metal ions. The hydroxyl radical plays an important role in the reactions of lipid peroxidation. Probiotic is a highly effective antioxidant and protects cells against oxidative stress caused by ROS, such as lipid peroxidation and DNA damage.

It is well known that chemotherapeutic agents cause toxic effects on ovarian function. In this study, treatment with Cisplatin+probiotic administration showed more morphologically normal follicles and cell proliferation, reduced apoptosis, decreased ROS production compared to cisplatin treatment alone. Under normal physiological conditions, cells control reactive oxygen species levels by balancing the generation of reactive oxygen species with their elimination by scavenging system (reduced glutathione-GSH, superoxide dismutase-SOD, and catalase-CAT). But under oxidative stress conditions, excessive reactive oxygen species can damage cellular proteins, lipids and DNA, leading to fatal lesions in cells that contribute to carcinogenesis. Oxidative stress can arise from

overproduction of ROS by metabolic reactions that use oxygen and shift the balance between oxidant and antioxidant. Cancer cells exhibit greater reactive oxygen species stress than normal cells do, partly due to oncogenic stimulation, increased metabolic activity and mitochondrial malfunction. Oxidative stress is the one of most important mechanisms involved in cisplatin toxicity. The mitochondrion is the primary target for cisplatin induced oxidative stress, resulting in loss of mitochondrial protein sulfhydryl group, calcium uptake inhibition and reduction of mitochondrial membrane potential (Dasari et al., 2014). Probiotics produce antimicrobial substances, such as organic acids, fatty free acids, ammonia, hydrogen peroxide, and bacteriocins in vitro (Alvarez-Olmos et al., 2001).Furthermore, probiotic strains must be safe and effective in humans, remain viable for the shelf life of the product, and not have pathogenic properties (Senok et al., 2005).Probiotic therapy has already made its way in the treatment of a number of conditions-Infectious, inflammatory, neoplastic and allergic. There is a long list of potentials of giving probiotics in a number of these conditions. But before bringing probiotics into routine usage, proper evaluation of these products is essential (Gupta et al., 2009).

Conclusion and Future prospectives

It is worth mentioning that Probiotic was used as an adjuvant in the chemotherapy of different tumors (lung, gastrointestinal tract, testis, prostate, cervix, and ovarian cancer) improving the rates of tumor regression as well as increasing survival and the quality of life of human cancer patients. This study provides the basis for a combination of local delivery approaches along with the beneficial effects of probiotic strain which could be a better approach for the treatment of cervical cancer. There is a great deal of evidence that the use of functional foods such as probiotics may play a significant role in combating cancer and in supporting anti-cancer treatments as well. Improving the understanding of the relationship between the immune system, microenvironment and probiotics will provide insights into the growth of probiotic-based therapeutics.

8. Impact of the research in the advancement of knowledge or benefit to mankind

Benefits to Mankind:

Safer Chemotherapy: Cisplatin is a widely used chemotherapy drug, but its toxicity can be severe, leading to side effects that affect the patient's quality of life. If probiotics are shown to mitigate this toxicity, it could lead to safer chemotherapy regimens, allowing patients to undergo treatment with fewer adverse effects.

Personalized Medicine: Understanding the role of probiotics in reducing cisplatin toxicity could contribute to personalized medicine approaches. By considering an individual's gut microbiome composition, doctors could tailor probiotic interventions to enhance the patient's ability to tolerate cisplatin treatment.

Reduced Healthcare Costs: Severe toxicity from cisplatin often requires additional medical interventions and care, increasing healthcare costs. If probiotics can mitigate these toxic effects, it could potentially reduce the need for extensive medical interventions, leading to cost savings for both patients and healthcare systems.

Improved Treatment Compliance: The fear of side effects often leads to reduced patient compliance with chemotherapy. If probiotics can reduce the severity of cisplatin-induced side effects, patients might be more likely to complete their treatment regimens, thus improving treatment outcomes.

Enhanced Quality of Life: Minimizing the adverse effects of cisplatin can greatly improve the quality of life for cancer patients undergoing treatment. Reduced toxicity could mean less pain, nausea, and discomfort, allowing patients to maintain a better overall well-being during their treatment journey.

In summary, the research on using probiotics to reduce cisplatin-induced toxicity in zebrafish not only has the potential to advance our understanding of toxicology and microbiome-host interactions but also holds promise for improving cancer treatment outcomes and patients' quality of life.

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