

**PLANT CONSORTIUM BASED ANTI-SEPTIC FORMULATION AND
POTENTIAL ANTI-MICROBIAL ACTIVITY AGAINST HUMAN
PATHOGENIC MICRO-ORGANISM**

Submitted in partial fulfillment of the requirements for the award of

Bachelor of Technology Degree in Biotechnology

Submitted by,

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SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

Accredited with grade “A” by NAAC

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the Bonafide work of **PREETHIKA.R (37230060)** who carried out the project entitled **“Plant Consortium Based Antiseptic Formulation and Potential Antimicrobial Activity Against Human Pathogenic Micro-Organisms”** under our supervision from OCTOBER 2020 to APRIL 2021

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We (**Ms. PREETHIKA.R** and **Ms. JENNIFER STEFFY JOY.D**) hereby declare that the Project Report entitled **“Plant Consortium Based Antiseptic Formulation and Potential Antimicrobial Activity Against Human Pathogenic Micro-Organisms”** done by me under the guidance of **Dr. Karthick Raja Namasivayam** at center for bio resource Research and Development, and submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

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ABSTRACT

Plant based pharmaceutical are the major source of anti-microbial agents. Because of wide spread of antibiotics resistant pattern, it is necessary to formulate anti- microbial against with high anti-microbial activity. In this present study, plant consortium mixture consists of orange peel powder, neem powder and *Acalypha indica* (Kuppaimeni) powder. Prepared plant consortium showed beatable antimicrobial activity against human pathogenic microorganism. Biocompatibility of the prepared consortium was evaluated on zebra fish model by determination of embryonic or developmental toxicity, gene expression changes and histo- pathological parameters. Prepared consortium reveals that the consortium was not inducing any sign of toxic effect on the developmental parameter and no indication of stress genes histo-pathological changes. The present study would suggest the possible utilization of the prepared plant consortium as an effective and safe antimicrobial agent.

KEYWORDS: Orange peel, powder, Neem powder, *Acalypha indica* powder (Kuppaimeni), Plant consortium, Antimicrobial Activity, Biocompatibility against Zebra fish model

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ABBREVIATION

1	MPC	Medicinal Plant Consortium
2	HR	Hatching Rate
3	HPF	High Power Field
4	LC 50	Lethal Concentration
5	QAMS	Quantitative Analysis Of Multi Components By Single Material
6	LPO	Lipid Peroxidation Assay
7	EDTA	Ethylene Di-Amine Tetra Acetic Acid
8	TAE	TRIS Acetate EDTA
9	TBE	TRIS Broate EDTA
10	ETBR	Ethidium Bromide
11	PCR	Polymerase Chain Reaction
12	INOS	Inducible Nitric Oxide Synthase
13	TNF	Tumor Necrosis Factor

14	MIC	Minimum Inhibitory Concentration
15	HPF	Hours Post- Fertilization

CHAPTER 1

INTRODUCTION

1. AN OVERVIEW

Everybody respects sound, brilliant and wonderful skin. However, it takes a great deal to look after it. One necessities to ensure that the person is eating a solid eating routine, practicing consistently and getting sound rest. In any case, numerous individuals utilize particular sorts of plastic and manufactured excellence items which contain substance intensifies that are amazingly unsafe to the skin. Despite the fact that such items are well known, convenient, and simple to-utilize, these items can taint, gives rashes, sensitivities and can cause numerous other skin issues. Consequently, one should deal with the skin with most extreme consideration since this is the most fragile and delicate piece of the body. Numerous individuals utilize characteristic fixings which are suggested specialists for customary healthy skin. These fixings have their own arrangement of advantages for the skin and can do some amazing things whenever utilized reasonably. Utilizing common plant-based definitions that are wealthy in indispensable skin supplements and cancer prevention agents can help sustain the skin and fix itself

1.1 SCOPE AND PLAN OF THE WORK

Plant based medicinal products are now being extensively utilized in biomedicine due to their high efficacy and safety. Plant based anti-microbial have been used since years. Successful formulation of plant-based products is quite popular to control various diseases. With this objective, the present work is aimed to prepare plant consortium composed of three plant materials with anti-microbial efficacy and biocompatibility.

The objective of the present study is as follow

- To prepare plant consortium with high anti-microbial spectrum.
- To evaluate biocompatibility using zebra fish model.

1.2 BENEFITS OF ORANGE

Orange is one of the useful fruits that is beneficial for health as well as wealth. Due to a higher amount of Vitamin C present in oranges, it proves to be large useful to the skin and can be added in the day today life skin care routine

- The orange peel skin to orange juice, everything is very good when we used on skin. Due to a more amount of antioxidants present in oranges.
- If you suffer from acne disorder , this is a great best for you. orange peels can be grinded and used in making a very beneficial cosmetics. The vitamin C and antibacterial properties in the orange peel help to reduce bacteria and fight the recovering of acne.
- Instead of depending so more money on getting skin facial toner filled with active agents, you can make a very active skin toner from the orange. To do this, immerse the peels of two orange in warm water and leave it overnight. Shake it on the morning and throw out the orange peels. You now have a very effective toner. For better results, use mostly
- orange peels can also help to slow down the aging process because of the presence of vitamin C and antioxidants. When you use the orange peel powder for a face mask or use the orange toner regularly, you'll notice difference in the way of your skin.
- The liquid that comes out while you are peeling the orange is good for skin

1.3 BENEFITS OF NEEM LEAVES

Heals Scars

Neem paste heals scared tissues, help in the removal of scars left out acne, pimples, burns, and wounds. To reduce the appearance of your scars, add a pinch of turmeric to your neem paste and make a paste of the mixture. You'll see the difference with daily usage.

Fights Acne

Neem leaf powder for skin area not only treats acne scars but also treats existing acne. It also prevents newly coming acnes. coming acne and pimples from breaking out. To fight acne against always, take some fresh neem leaves and some fresh Tulasi (basil) leaves and grind them together with a little rosewater. Apply the thick paste on your face and body (if you suffer more body leave it until it dries). Then wash with mild cold water

BENEFITS OF ACALYPHA INDICA

Treats Skin Problems

The leaves of Acalypha Indica (குப்பபேணி) Benefits work extra super effect to treat skin problems such as eczema, sores, wounds, and scabies. They are also much used in the treatment of acne scars due to their antibacterial activity in higher. A powder form made of roots and leaves of the plant is highly used to treat wound infections as it inhibits the growth of bacteria and fungus in the face tissues.

Controls Blood Sugar Level

The polyphenols and steroids present in the plant content, provide anti-diabetic properties in more scale. These compounds lower blood sugar levels in low and play a great role in controlling diabetes

Treatment of Eye Infections

The leaves of the plant are used in some old cultures for the treatment of eye care infections. The leaf sap or ground leaves grinded together with water can be mostly used as eye drops to eye. These acids can effectively be used to exfoliate the skin in good and slough off dead Skin.

CHAPTER - 2

LITERATURE SURVEY

Joe Chappell, ph. D (2015) worked on the medicinal plant consortium (MPC) represents a very large array of academic, governmental and private industry scientists across the US associated with the advancement of plants and plant biology for human health. The MPC wishes to help the NCCIH and other NIH centers/institutes fully appreciate the opportunities of emerging all other capabilities and research endeavors empowering current and future uses of plants for human health.

Elzbieta kurowska, (2007) the peel is something in it that prevents us histamine from being released. Histamines were chemical that causes of allergic reactions. Orange peels prevention of its release makes them a potentially anti-allergic food that causes from allergies to skin.

Kriti malik, H. (2018) in his paper in the world of Ayurveda, neem is a popular medicinal herb that's been part of traditional remedies that date back almost 5000 years. Also known as azadirachta indica in English as well or as well 'neemba' in Sanskrit, the neem tree is a really good example of how nature holds both the problem and the cure. It's home to more than a 130 large different biologically active compounds were No wonder it's such an effective anti-viral and as well anti-bacterial, along with being a powerful immuno- stimulant. Murli Manohar, author of the book 'Ayurveda for all: effective Ayurveda self- cure for common and chronic ailments' suggests that the primary purpose of neem leaves is the treatment of vaata disorders or neuromuscular pains.

The other benefits: which purify the blood, and prevent damage caused by free radicals in the body, remove toxins, treat insect bites and ulcers. Neem leaves generly have more anti-bacterial properties in nature which is as to be why it works wonders on infections, burns more less and any kind of skin problems. It destroys the bacteria activity that causes large infections, stimulates the immune system and encourages rapid healing

Ravi et al., (2014) kuppaimeni plant has amazing health benefits & medicinal uses. From unwanted hair removal to treating cold, cough and skin problems, it has widely used. In my humble opinion, if there is one herb that we should all get to know and use it in our daily lives for its many health benefits and uses, it is kuppaimeni.

Julie blanner et al., (2019), Dried orange peel adds a delightful citrus fragrance to a variety of recipes, including drinks, foods, and even homemade cleaning and beauty products. As much as I love the incredible fragrance and flavour of oranges, I also hate to feel like I am wasting those gorgeous peels. Did you know that the peel of an orange actually contains more Vitamin C in large scale than the orange itself. That's right! There are so many health benefits to citrus fruits, more over in large scale far beyond simply eating those delicious wedges.

Neem has so many incredible medicinal benefits as all known, but it also one of the most important things is that, it is kills cancerous cells. Everyone has they are own cancerous cells in their body, but normally, they are many disorganized in nature. However, if you create many certain situations in the body, they will get more organized normally. As long as they cells are loafing all around by themselves, it is not an issue. If they all are gather in one place together and hit it off, it becomes a problem. This is like a very good shift from petty crime to organized crime. It is a serious problem. If you consume neem every day as a routine, it keeps the number of cancerous cells in the body within a certain limit, so that they will not gang up against your system. (Sadguru., (2018),).

Rajsekhar Saha & Azhar ahmed et al., (2017) studied lot of plants and their preparations have been highly used as medicine since ancient time. The Ayurveda and various ancient Indian erect annual herb with numerous long branches covered with soft hairs. According to the siddha text, 'path Artha gun Chinthamani acalypha cures diseases of the teeth and gums, burns, toxins of plant and mixed origin, stomach pain, diseases due to pitha, bleeding piles, irritations, stabbing pain, wheezing, sinusitis and neutralizes predominance are all of the the kabha factor. In the traditional medicine the preparations from whole plants have been highly used to counter various diseases and disorders. The present review is therefore, an very high effort to give a detailed survey literature on its pharmacognosy, phytochemistry, pharmacological and traditional uses.

Sahira Banu., Et al 2013) plants are most recognized in the pharmaceutical industry for their own broad structural diversity as well as their own wide range of pharmacological activities in nature. The biologically active compounds which present in plants are called phytochemicals so on. These phytochemicals are derived from various parts of plants such as leaves, flowers and roots etc. These phytochemicals are used as a great sources of medicinal agents for usage. They serve as a raw material base for elaboration of more complex semi-synthetic chemical compounds to be known. This paper mainly deals with a collection of plants, the extraction of active compounds from the various parts of plants, qualitative and quantitative analysis of phytochemicals.

Satyajit D.Sarker et al., (2011) studied the strategy combining quantitative analysis of multi-components by single marker (QAMS) with charged aerosol detection (CAD) has certain advantages for quantifying multiple components. However, relevant research was very limited. In this study, a high-performance liquid chromatography method was successfully developed for the multi-component determination of Qishen Yiqi dripping pills. Factors that affect the performance of the approach were sufficiently investigated. After the method verification over, principal component analysis was applied to evaluate the complete quality consistency of three types of samples.

Antimicrobial efficiency of *Melia azedarach* L. a medicinal plant (leaf extracts) was examined using Methanol, Ethanol, Petroleum ether and water, as solvents and tested against eight human pathogens like Bacteria are: *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, Fungi: *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxisporum*, *Rhizopus stolonifera* using in general agar well diffusion method and Minimum inhibitory concentration. All the plants showed their significant activity against the all pathogens, but only alcoholic extract of *azedarach* showed maximum zone of inhibition and minimum inhibitory concentration against all the microorganism(Antarasen et al., (2016)

Zebrafish is well recognized as an ideal model organism for investigating the cellular as well as molecular mechanisms underlying heart formation and regeneration in general. Zebrafish has a closed cardiovascular system in body and a cardiac cycle that is highly reminiscent of human cardiovascular physiology. Zebrafish can be genetically manipulated more, and their genome has been fully sequenced well. Genetic studies in zebrafish have been discovered as novel genes and pathways in cardiovascular development and function, with close correlation with cardiovascular diseases. Zebrafish heart regeneration occurs the following injury through the indifference and proliferation of mature cardio myocytes. Embryo-based small-molecule screens have discovered chemical probes and drug candidates for enhancing cardiovascular development, function, and regeneration in general T.P Zhong., (2018).

(John Collin et al., 2017) The zebrafish, *Danio rerio*, has been very much emerged as an enormously way of powerful model organism for biomedical translational research over recent years in general. This is very largely due to highly significant in homology form with very higher vertebrates, but also because of a opportunities for a genetic manipulation and in vivo imaging. The functional anatomy of the zebrafish and their practicalities of breeding and husbandry are described well. Key methods for genetic and physical manipulation are outlined well. Applications such as disease modeling source, forward genetic screens, and pharmacological screening work that are ideally suited to zebrafish-based studies are been presented.

(Abigdil cline et al. 2015, Aug 15) he experimental the advantages of a zebrafish making this heavy model system very highly amenable to the field of dermatology. Zebrafish skin very highly development is similar to humans and its genome is ~70% orthologous to the human genome as well. Its external developmental process was allowing for genetic manipulation and they are analysis of embryogenesis within a short time frame with all the important internal organs and skin compartments formed within 6 days soon after. Zebrafish models of a cutaneous human diseases offer insight into pathogenesis and the unique platform for testing of a potential therapies. This review details the specific advantages of zebrafish

(Daniel Kohane & Robert langar et al., 2021) Drug delivery technology has been very much emerged as an most important focus of the biotechnological research and commercial enterprise. While. much attention is justifiably focused on the design and effectiveness of drug delivery devices too, the nature of their interaction with surrounding tissues their biocompatibility is crucial. Here we discuss biocompatibility, specifically as it relates to drug delivery systems, which differ from other biomaterial-based devices by possibly containing high quantities of drugs with their own effects on tissues.

Dr. Dakshita Joy Sinha et al., (2014)., Biocompatibility is fundamentally important to ensure the health of patients, dental staff members (including laboratory personnel) and practitioners themselves. Furthermore, the ability of dentists is often linked to biocompatibility issues. Practitioners should understand enough about biocompatibility testing methods to critically judge advertising claims and ask relevant questions of manufacturers. Data: Articles from 1942-2014 were been studied and relevant articles were included in this review.

CHAPTER - 3

AIM AND OBJECTIVES

3.1 AIM

To study and prepare plant consortium based on antiseptic formulation and to check potential antimicrobial activity against human pathogenic microorganism using zebra fish model.

3.2 OBJECTIVES

- To prepare plant consortium based Antiseptic formulation
- To check the phytochemical compounds by phytochemical analysis
- Observing the effective activeness of anti-Bacterial activity based on plant consortium
- Evaluation of bio compatibility of plant consortium using zebra fish model.

CHAPTER - 4

MATERIALS AND METHOD

4.1 PREPARATION OF PLANT CONSORTIUM

The s is made by selecting healthier and newly planted medicinal plant like orangepeel, neem leaves and acalypha indica. The combination of plant is taken in the ratio of 2:1:2. The above-mentioned plants are finely made into powder using mortar grinder, Before the analysis

PHYTOCHEMICAL ANALYSIS OF PLANT

CONSORTIUM Phytochemical Analysis

The Phytochemical examination was carried out for all three crude extracts as per the standard methods (Evans, & Trease., 1994)

1. Alkaloids

Meyers Test: 1ml extract was taken and few drops of Mayer's reagent were added. Formation of cream color precipitate was observed. It indicates presence of alkaloids.

2. Carbohydrates and glycosides

Fehling Test: To 1ml extract few drops of Fehling solution were added and the Formation of a red precipitate was observed. Red precipitate showed the presence of Carbohydrates and glycosides.

3. Phenolic compounds & tannins

Ferric Chloride Test: Few drops of Ferric chloride solution (5%w/v) were added to 1ml of extract and the formation of a bluish black coloration was observed. Bluish color indicated the presence Phenolic compounds & tannins.

4. Proteins & Amino acids

Ninhydrin test: To 1ml extract few drops of Ninhydrin were added and the Formation of a purplish pink coloration was observed. It indicates presence Proteins & Amino acids.

5. Flavonoids

Shinoda test: Add small piece of magnesium ribbon to extract (5 mL) plant material. Then add concentrated hydrochloric acid drop-wise. Formation of pink color indicates presence of flavonoids.

6. Saponins

Froth Test: 2gms of extracts were boiled in 20ml of distilled water in water bath and filtered. To 10ml of filtrate 5ml of distilled water was added and shaken vigorously. Froth indicated the presence if saponins.

7. Terpenoids

Salkowski test: To 5ml of each extract, 2mL of chloroform was added followed by addition of concentrated H₂SO₄ (3mL). A reddish-brown coloration of the interface showed presence of terpenoids.

Consortium prepared with the respective plant material was analyzed for phytochemical analysis

4.3. ANTI-BACTERIAL ACTIVITY

Anti-bacterial activity of the prepared plant consortium was tested against human pathogenic strains *Pseudomonas aeruginosa* and *Staphylococcus aureus* by well diffusion assay and broth micro dilution assay.

4.3.1 WELL DIFFUSION ASSAY

Inoculum of the respective bacterial strain was prepared in nutrient broth. Inoculum thus prepared was uniformly swabbed with sterile cotton swabs on the sterile Muller Hinton agar (MH). 0.1 ml of respective plant material extract and plant consortium was added to the well. Inoculated plates were incubated at 30°C for 24 hours. After the incubation period, the zone of inhibition was always measured.

4.3.2 Broth Micro dilution assay

Clinical and laboratory standard Institute (CLS) protocol was adopted to determine MIC of the Nano composite against tested bacterial strain using double dilution 96 well micro titre plate (Ahmed et al 2018). Known volume of bacterial culture (0.1 ml) was inoculated into each well followed by addition of aliquots of respective plant extract and plant consortium under aseptic condition, incubated at 37°C for 24 hours. Lower concentration of the Nano composite that inhibited the bacterial growth was considered as MIC

4.4 OBSERVERING BIO COMPABILITY OF PLANT CONSORTIUM USING ZEBRA FISH MODEL

4.4.1 Collection and Maintenance

Zebrafish (*Danio rerio*), it was purchased from aquarium. They were acclimatized under laboratory conditions in 35 glass aquaria containing dechlorinated water, aerated continuously through stone diffusers connected to a mechanical air compressor. The zebrafish is a hardy fish and can withstand a pH ranging always between 7.2 and 7.5. The temperature of the water was maintained upto $26 \pm 1^{\circ}\text{C}$ throughout the study. During the acclimation period, the adult fish was fed twice per day with complete readily available fish food. The care and husbandry of the zebrafish used in this study was in conformity with the guidelines that regulate the care and use of laboratory animals by humans for research purposes.

4.4.2 Breeding setup:

The zebrafish we used as spawners had a length of 3.05 ± 0.04 cm and a weight of 1.00 ± 0.5 g. Prior to spawning, the male and female fish were housed separately for a minimum of 1 day. For breeding, male and female fish were taken in the ratio of 2:1 (male: female) to get the maximum number of embryos. Care was taken in collecting the eggs that Nylon net was placed between the fish and the bottom of the aquarium; the spawned eggs fell through the net, thus, preventing the adult fish from eating the eggs. Breeding kept for overnight or 16 hours in darken environment. After 16 hours the darken closure was removed and left the fishes as it to lay the eggs. Then, the eggs were transferred to a petri dish containing E3 medium and checked for fertilization under a microscope, only the fertilized eggs taken for the studies.

4.4.3 Sample preparation and Treatment:

Samples was weigh mg/ml concentration this sample was boiled for 2 hours then the extract were filtered then used in this study.

The concentration of sample used was $0.5 \mu\text{g/ml}$, $1.0 \mu\text{g/ml}$, $1.5 \mu\text{g/ml}$, $2.0 \mu\text{g/ml}$ and $2.5 \mu\text{g/ml}$. The fertilized eggs (12/concentration) were taken for the treatment in 96 well plate. Without closing the lid fully covered, gap was created for aeration and at the same time evaporation also avoided. The eggs were observed for 0, 2, 4, 6, 24, 48, 72 and 96 hours.

4.4.4 Survival Rate and Hatching Rate

Percentage of survival rate was calculated as a ratio of number of healthy (fertilized) developing embryos at various time of exposure divided by total number of incubated embryos initially x 100.

Percentage of hatched embryos (HR) was calculated as a ratio of number of hatched embryos divided by total number of incubated embryos x 100. HR at each exposure-duration was separately considered as an individual variable. Therefore HR between 48 hrs to 72 hrs were considered as separated variable, presented as HR-48, HR-54, HR-60 and HR-66 as picture.



Fig 4.4.4 Fish Grown In Laboratory

4.5 ADULT ZEBRA FISH STUDY

4.5.1 LC50 determination of the sample:

Zebra fishes of interest were weighed and separated in various tanks for the study. The sample concentration tried as 2µg/ml to 10µg/ml and then 10 µg/ml to 50 µg/ml. Finally the doses tried as 20, 40, 60, 80 and 100 µg/ml. Finally the dosages calculated for 2L to analyses for the acute effects. Also control fishes maintained in separate tank. The fishes were observed under treatment for 7 days to check the dose effect.

4.5.2 Dissection of fishes for muscle tissues:

Each fish was slowly anesthetized by adding ice chips into the fish water, until the temperature reaches 12°C. When the fish remains still to any external stimuli then those were taken for dissection. The muscle tissues removed carefully and stored in 10% formalin for histopathology and in PBS for DNA isolation and LPO assay.

4.6 HISTOPATHOLOGY OF MUSCLE TISSUE:

Processing of Sample:

- Samples of muscle tissues were taken out of fixative.
- Washing of these tissues was done with Saline water/ solutions as per procedures adapted.
- *Dehydration*: The removal of water or dehydration was done by transferring tissue in to the 70% alcohol of sufficient quantity and then graded alcohol 90% for one change and two changes of absolute alcohol.
- *Clearing*: The clearing of tissues was done by subjecting to a clearing agent i.e., Xylene.
- *Embedding and Impregnating*: The impregnation agent used was paraffin wax as it facilitated easy penetration into the tissue without causing structural damage and without much shrinkage or crystal formation.
- *Trimming*: The embedded block containing tissue was further trimmed that only one tissue is subject for cutting into sections Mounting of the Block: An iron block of 30 mm was used to hold the block at the correct angle and in position for cutting.

- *Section cutting:* The block containing tissue was cut at 5-8 μm thickness with the help of a microtome
- *Mounting of Sections:* The cut sections were adhered the slides firmly and in a right position. The slide was given an egg albumin coating before affixing the sections over it.
- Staining the slides with Azan, Hematoxylin & Eosin.
The stained sections were observed under light microscope and microphotographs Were taken for pathological observations.

4.7 LIPID PEROXIDATION ASSAY (LPO)

Thio barbituric acid-reactive substances (TBARS) assay is used to detect lipid peroxidation. In this assay, malondialdehyde forms a complex with Thio barbituric acid, which can be quantified spectrophotometrically. The treated cells with various concentration of the test sample were taken for assay. The cells were homogenized with ice cold KCl Buffer then the homogenized tissue sample was collected in Eppendorf Centrifuged at 5000g for 5 minutes, then the pellet was washed and re dispersed in 0.2 ml of 8.1 % SDS, 1.5 ml of 20% acetic acid was added to this suspension and incubated for 10 min, then added 1.5 ml of 0.8 % TBA buffer and 0.7 ml of distilled water to the solution. This reaction mixture was to be incubated for 1 h at 95°C and then cooled to 25°C. 5 ml of n Butanol and Pyridine mixture (15:1) was added and the reaction mixture was again subjected to centrifugation at 5000g for 15 min to remove debris. Absorbance was recorded at 532 nm. The untreated cells were taken as a control. The standard was performed with MDA (*Malondialdehyde*). The sample values compared with standard, and the concentration expressed as μM .

4.8 ISOLATION OF GENOMIC DNA

4.8.1 Requirements:

1. Liquid Nitrogen
2. Digestion buffer
3. PBS (Ice cold)
4. 7.5M Ammonium acetate
5. 70% and 100% ethanol
6. TE buffer(pH 8)
7. Water bath

Procedure

1. Muscle tissues weighing 100mg was taken in a mortar and pestle and was homogenized well by freezing using liquid nitrogen.
2. The ground tissues were dissolved in 1.2ml digestion buffer and incubated overnight(12-18hrs) in a water bath at 55°C.
3. Equal volume of Phenol-Chloroform-Isoamyl alcohol mixed in the ratio 25:24:1 was added and mixed gently and centrifuged at 3000rpm for 5 mins at room temperature.
4. The aqueous phase was carefully removed without disturbing the middle or bottom layer and transferred to a new tube and $\frac{1}{2}$ the volume of 7.5M ammonium acetate and twice the volume of absolute ethanol was added, mixed well and centrifuged at 3000rpm for 5 mins at room temperature.
5. The pellet was rinsed using 70% ethanol and the ethanol was discarded.
6. The pellet was kept for air drying.
7. Once dried, the pellet was dissolved in 50 μ l nuclease free water for further processes.

4.9 AGAROSE GEL ELECTROPHORESIS:

4.9.1Requirements:

1. TAE buffer: 40M Tris-acetate, 1 mM EDTA (or)
2. TBE buffer: 45 mM Tris-borate, 1 mM EDTA
3. Agarose: 0.8%
4. Ethidium bromide: 0.5 μ g/ml
5. Gel loading dye (6X): 0.25% bromophenol blue and 30% glycerol

4.9.2 Preparation of the Gel:

1. Weighed the appropriate mass of agarose in an Erlenmeyer flask. Agarose gels were well prepared using a w/v percentage solution. Most gels ranging between 0.5%-2%. The volume of the buffer always should not be greater than 1/3 of the capacity of the flask.
2. Added running buffer to the agarose-containing flask and mixed. The most common gel running buffers are TAE or TBE.
3. Melted the agarose/buffer mixture. At 30s intervals, removed the flask mixed and repeated until the agarose has completely dissolved.
4. Added ethidium bromide (EtBr) to a concentration of 0.5µg/ml. Allowed the agarose to cool.
5. Placed the gel tray into the casting apparatus and poured the melted agarose then placed comb into the gel mold to create the wells.
6. Allow the agarose to set at room temperature. Remove the comb and place the gel in the buffer system.

4.9.3 Setting up of Gel Apparatus and Separation of DNA Fragments:

1. Added loading dye to the DNA samples to be separated. Gel loading dye is typically made up to 6X concentration.
2. Programmed the power supply to desired voltage (1-5V/cm between electrodes).
3. Added enough running buffer to cover the surface of the gel. It is important to use always the same running buffer as the one used to prepare the gel.
4. Connected the leads of the gel box to the power supply.
5. Removed the lid. Loaded the DNA sample(s) into the gel. An appropriate DNA size marker is should always be loaded along with experimental samples.

6. Replaced the lid to the gel box. The cathode should be closer to the well than the anode. Power turned on.

4.9.4 Observing Separated DNA fragments:

1. When electrophoresis was over, the power supply was turned off and the lid was removed from the tank.
2. The gel was removed and any excess buffer from the well was drained and the gel was placed in a paper to absorb any excess buffer.
3. The gel was exposed to UV light using an UV illuminator. DNA bands should always show up as orange, fluorescent bands. A picture of DNA bands was taken, and the gel discarded.

INFLAMMATORY MARKER EXPRESSION THROUGH PCR AMPLIFICATION

4.9.5 Primers

For the current study iNOS, TNF α and β -Actin primers were used. Inflammatory markers such as TNF- α and iNOS (induced Nitric Oxide Synthase) were designed with NCBI Primer BLAST tool. The sequences are as follows;

1. Nucleotide sequence alignments of iNOS gene were obtained through databases. Based on these alignments, iNOS primers

Forward - 5'ACACTTCGAAAAGCAAGATGG 3' and

Reverse 5' ACGGGCATCGAAAAGCTGTA 3' gene were designed.

2. Nucleotide sequence alignments of TNF- α gene were obtained through databases. Based on these alignments, TNF- α primers

Forward - 5' TCATTTTGGCTGTGGGCCTT 3' and

Reverse - 5' GGCGGTTCAAATCTCACTCAC 3' gene were designed.

3. Nucleotide sequence alignments of β -Actin gene were obtained through databases. Based on these alignments, β -Actin primers

Forward - 5' CCATCGGCAATGAGCGTTTC3' and

Reverse - 5' CATCCTGAGTCAATGCGCCA 3' gene were designed.

Other than the inflammatory markers employed here, β -actin was taken as the control for housekeeping gene.

4.10 PCR AMPLIFICATION:

For PCR amplification TAKARA® 's EMERALD PCR master mix was utilized, and the recipe was made as follows;

Table 1: Components used for PCR amplification

Reagent	Volume (μ l)
EMERALD master mix	12.5
DNA template	2.5
Forward primer	1
Reverse primer	1
H ₂ O	8

The above cocktail for each sample was amplified in the following order,

Table 2: Conditions applied for amplification

Cycle	Temperature (°C)	Time
Initial denaturation	95	2 min
Denaturation	95	1 min
Annealing	Respective to the primer chosen	30 sec
Extension	72	1 min
Final extension	72	5 min

Amplification was carried out in 30 cycles and the resulting PCR product was resolved in 1% Agarose gel electrophoresis, and the results were interpreted.

CHAPTER – 5

RESULTS AND DISCUSSIONS

5.1 PREPARATION OF PLANT CONSORTIUM

The sample is made by selecting healthier and newly planted medicinal plant like orange peel, neem leaves and acahypha indica. The combination of plant is taken in the ratio of 2:1:2. The above-mentioned plants are finely made into powder using mortar grinder, Before the analysis.

5.2 PHYTOCHEMICAL ANALYSIS

From the Phytochemical Analysis its Observed That the Given Plant Consortium Consist of Components

TABLE 3: PHYTOCHEMICAL ANALYSIS OF PLANT CONSORTIUM

Serial no	Phytochemical analysis of sample	Result of sample
1	Tannin	++
2	Phenol	++
3	Flavonoids	++
4	Alkaloids	++
5	Carbohydrates	++
6	Proteins	+-

5.3 ANTIBACTERIAL ACTIVITY

Anti-bacterial activity of the respective plant extract and plant consortium was studied by determination of zone of inhibition and MIC which reveals the prepared plant consortium inhibited both the tested strains as significant manner. Well diffusion assay indicates that the prepared plant consortium inhibited the tested strains. Zone of inhibition was found to be higher than individual plant extract (Table 4). Broth microdilution assay which was used to determine MIC was also reveals the effectiveness of plant consortium against the both the tested strains. The MIC was found to be lower than the individual plant extract.

TABLE 4: ZONE OF INHIBITION AGAINST THE TESTED STRAIN

Treatment	MIC (in microgram)	
	Pseudomonas Aeruginosa	Staphylococcus Aureus
Orange Peel Extract	9.0	9.0
Neem Leaves Extract	12.2	10.0
Acalypha Indica Leaf Extract	8.0	7.0
Consortium	34.2	28.2

TABLE 5: MINIMUM INHIBITION CONCENTRATION (MIC) AGAINST TESTED BACTERIAL STRAIN

Treatment	MIC (in microgram)	
	Pseudomonas Aeruginosa	Staphylococcus Aureus
Orange Peel Extract	97.0	121.2
Neem Leaves Extract	65.0	70.0
Acalypha Indica Leaf Extract	90.0	105.5
Consortium	35.0	30.0

5.4 OBSERVATION OF EGGS IN DIFFERENT HOURS

TABLE 6: LIFE STAGES OF ZEBRA FISH UPTO 96 HOURS

AFTER DRUG TREATMENT

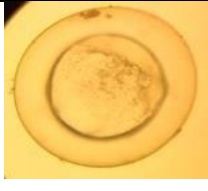
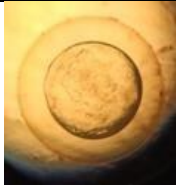
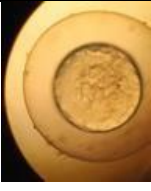
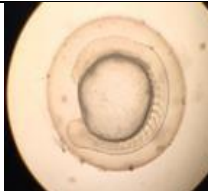
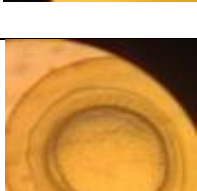
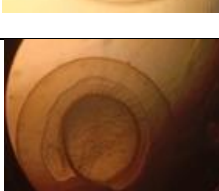
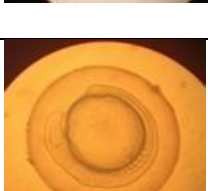
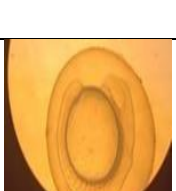
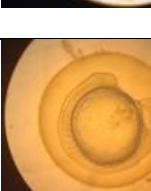
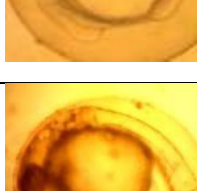
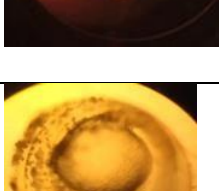
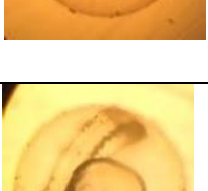
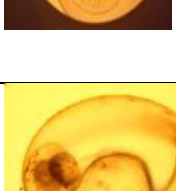
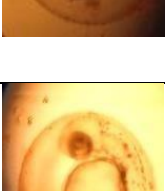
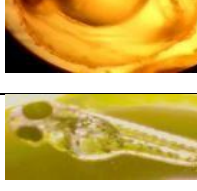
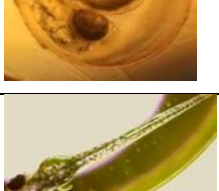
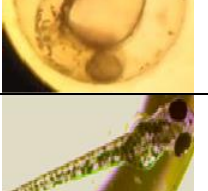
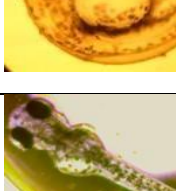
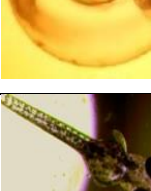
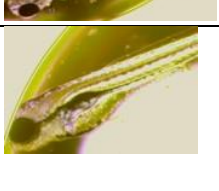
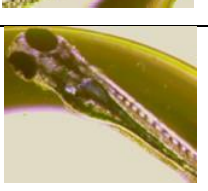
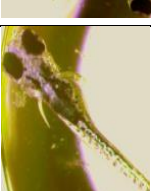
Time (hrs) / Conc. ($\mu\text{g/ml}$)	0.5	1.0	1.5	2.0	2.5	control
2						
4						
6						
24						
48						
72						
96						

Fig 5.4: Life stages of Zebrafish embryo upto 96 hours after drug treatment.

5.5 INTERPRETATION ABOUT THE OBSERVATION:

The eggs were collected and subjected to treatment with nanoparticle treated tea leaves powder and observed at 2,4,6,24,48,72 and 96 hours.

Upto 24 hours: At 2 hours the embryos were in cell division(Oblong) stage and few eggs are at epiboly stage. Further embryos were at somite development stage in 4 hrs. The spinal cord appeared thick at 20 somite stage during 6th hour with initiation of eye lids and ear buds at the spaces.

24 hours: The development of growth of tail region and heart region with pigmentation.

48 hours: Blood circulation and heartbeat were observed at 48 hours without any Yolk Sac and Cardiac edema. Morphological Characters (Tail/Yolk sac/eyes) were normal and with proper movement.

72 hours: Hatching was observed with exact breakage of chorion sac and starts swimming.

96 hours: At 96 hrs embryo developed into larvae and the swimming was normal.

The treatment with sample up to 2.5 µg/ml concentration does not show any abnormal effect in embryonic development. The embryonic development was normal in treated group similar to control.

5.5.1 Survival rate and Hatching Rate

The survival and hatching rates of zebrafish embryos from the control and sample exposed (0.5µg/ml, 1µg/ml, 1.5 µg/ml, 2.0µg/ml and 2.5µg/ml, from 4 hrs to 96 hrs) groups are shown in Figure (A) . From the data in Figure (A), it is clear that the survival rate of the control group was high and that only 2-3 % of the embryos failed to survive beyond 48 hrs post fertilization (hpf). This is the typical survival rate for zebrafish under normal lab conditions. A slight decline in the survival rate was found in the group of embryos exposed to sample and no significant difference in the survival rates was found between controls and sample at 48 hpf. However, within 48 hpf, 85% - 95 % of embryos survived in the presence of sample extract in various

concentrations. The results subjected to two way analysis using Graphpad prism software where it is showed non significant with p value <0.0001. So it is indicated that the sample does not affect the embryonic development and function.

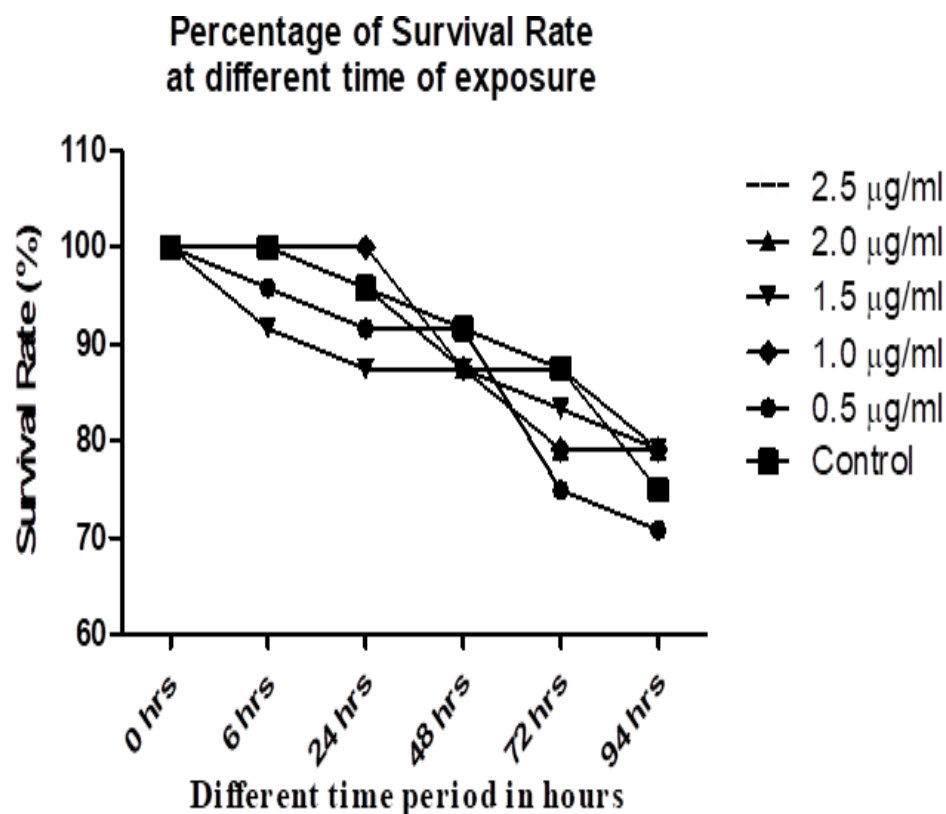


Fig 5.5.1 a) Percentage of Survival Rate at Different time of Exposure

It is showed that the hatching success rate of zebrafish embryos, there was no significant decrease with increasing exposure time (from 48 to 72 hrs), as well as with increasing concentrations of sample between 48 and 96 hrs. The results subjected to two way analysis using Graphpad prism software where it is showed non significant with p value <0.0001. These results clearly indicated that the survival and the hatching rate of sample extract exposed zebrafish embryos not affected with this sample extract exposure.

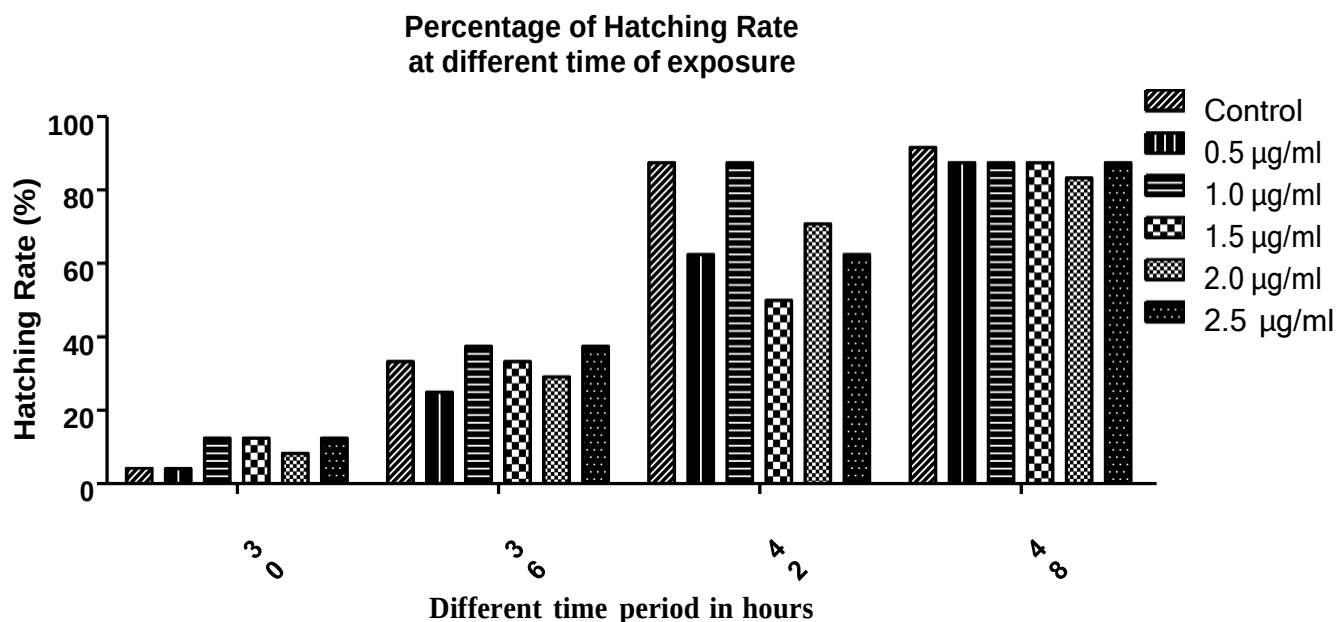


Fig 5.5.1 b) Percentage of Hatching Rate at Different time of Exposure

5.5.2 Heartbeat Count:

TABLE 7: Heartbeat in counts

Conc./ Hours	Control	0.5µg/ml	1.0 µg/ml	1.5 µg/ml	2.0 µg/ml	2.5 µg/ml
48	130	140	142	148	140	138
72	140	136	149	142	146	146
96	136	146	140	148	142	149

The results indicated showed that the sample doesn't have considerable effect on the embryonic development in Zebra fish embryo between 0.5µg/ml and 2.5µg/ml concentration. At this level the product doesn't have toxicity effect. The further analyses have to be done with higher dosage for the toxicity confirmation.

5.5.3 LC 50 Determination:

The sample was subjected to LC50 analysis in the range of 2, 4, 6,8 and 10 µg/ml for 7 days. For each group 12 fishes were taken and duplicated which includes treatment group with different concentration of sample and control. There were no mortality and behavioural changes

observed upto 10 µg/ml. The dosage range was increased to 10, 20, 30, 40 and 50 µg/ml and

observed for 7 days. In all dosages, fishes showed normal behaviors and no mortality was observed between 10 – 50 $\mu\text{g/ml}$. The dosage level further increased to 20, 40, 60, 80 and 100 $\mu\text{g/ml}$ and kept under treatment for 7 days. There was no evident effect in the entire dosage range upto 100 $\mu\text{g/ml}$. The sample did not showed any prominent toxic effect and abnormal behaviors throughout the treatment period in acute phase. Fishes from control group and treated group (various concentration of sample such as 20, 60 and 100 $\mu\text{g/ml}$) were sacrificed after 7 days of exposure. The collected muscle tissues were stored for histopathology analysis and for gene expression studies.

5.5.4 Histopathology:

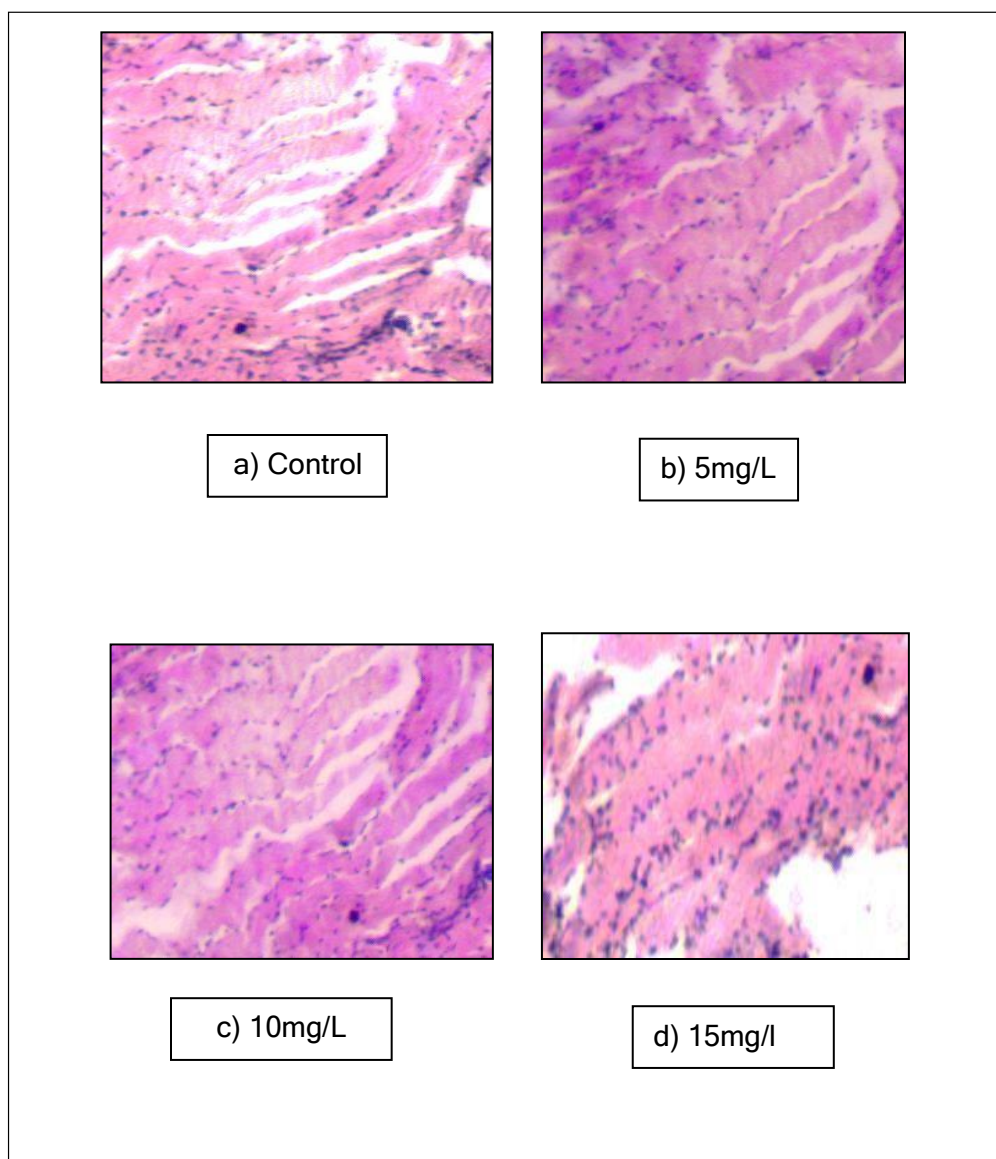


Fig 5.5.4 Histopathology of muscle tissues of treated Zebra fish model.

A - control,

B - 5 mg/L,

C - 10 mg/L and

D - 15 mg/L of dosages.

The histological results observed in muscle tissue of zebrafish in the present study indicate that there was no alteration in muscle architecture, there was no deformation of the muscle fibrils and the neutrophil accumulation was not observed, rather was present in a scattered manner when treated with different concentrations of given sample. So we conclude that the sample does not play any role in affecting the histology of the zebrafish muscles.

5.5.5 Lipid Peroxidase:

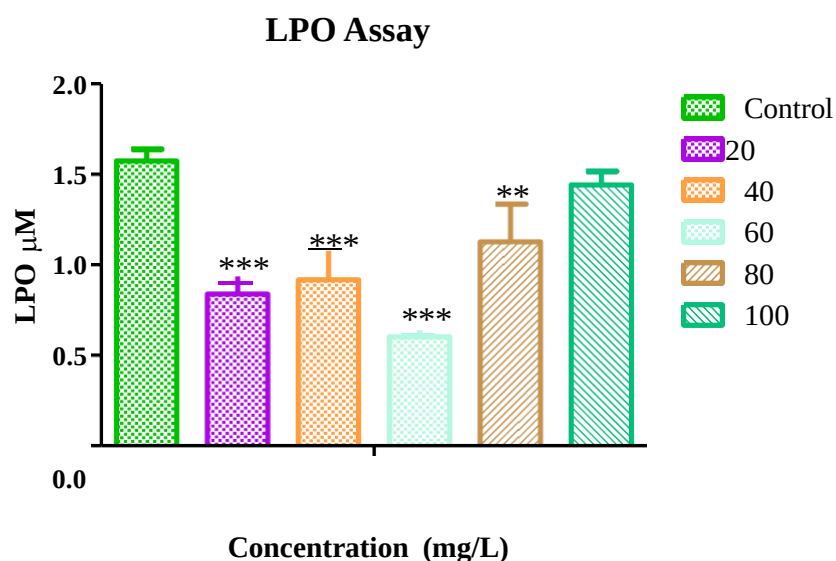


Fig 5.5.5. Lipid peroxidase

Lipid peroxidation assay (LPO) the samples had no prominent increase or decrease in the level of production of the LPO with respect to dosage. These results indicate very small deviation between LPO values of different sample which are negligible. So we can conclude that the given sample did not play any prominent role in inducing or decreasing oxidative stress in the zebra fishes

5.6 GENE EXPRESSION STUDIES OF PLANT CONSORTIUM IN ADULT ZEBRA FISH

A) β Actin

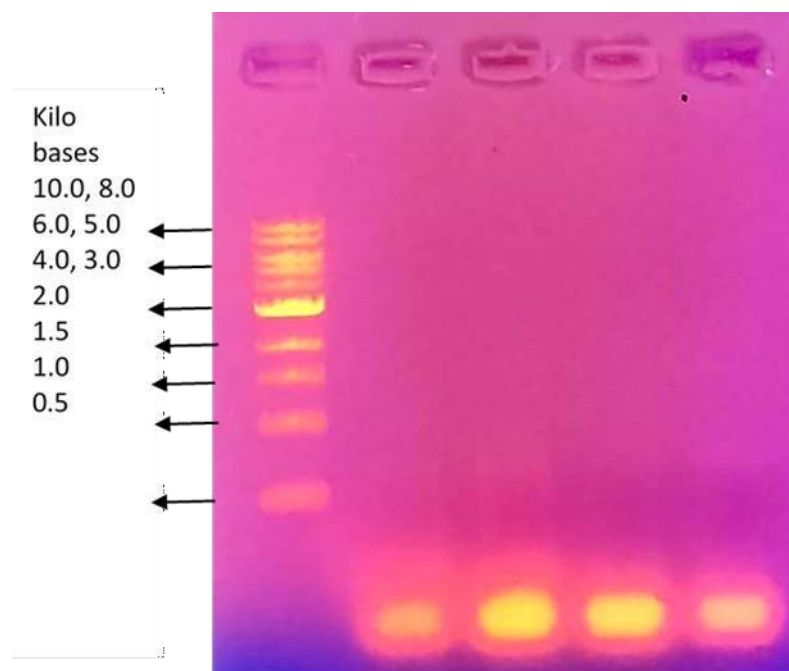


Fig.NO: 5.6 a) β actin gene expression of treated Zebra fish model

Lane 1 - 10 Kb DNA Ladder

Lane 2 - Control Lane3-

Sample (20 mg/L)

Lane4-Sample (60 mg/L)

Lane5-Sample (100 mg)

B) TNF- α

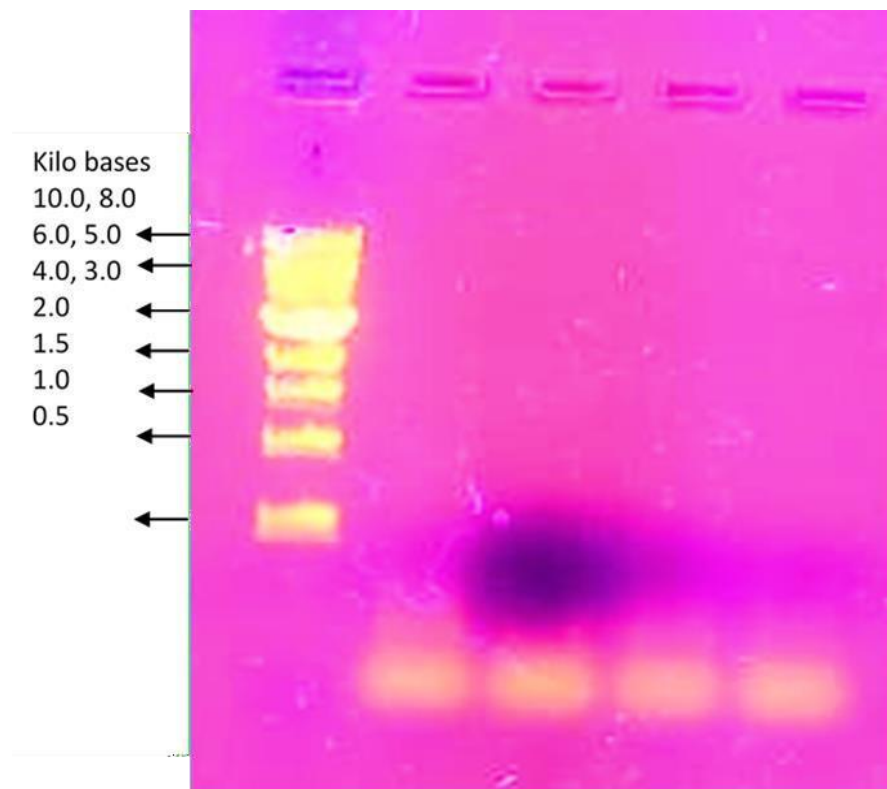


Figure 5.6 b) TNF- α gene expression of treated Zebra fish model

Lane 1 - 10 Kb DNA Ladder

Lane 2 - Control

Lane 3 - Sample (20 mg/L)

Lane 4 - Sample (60 mg/L)

Lane 5 - Sample (100 mg/L)

C) IONS

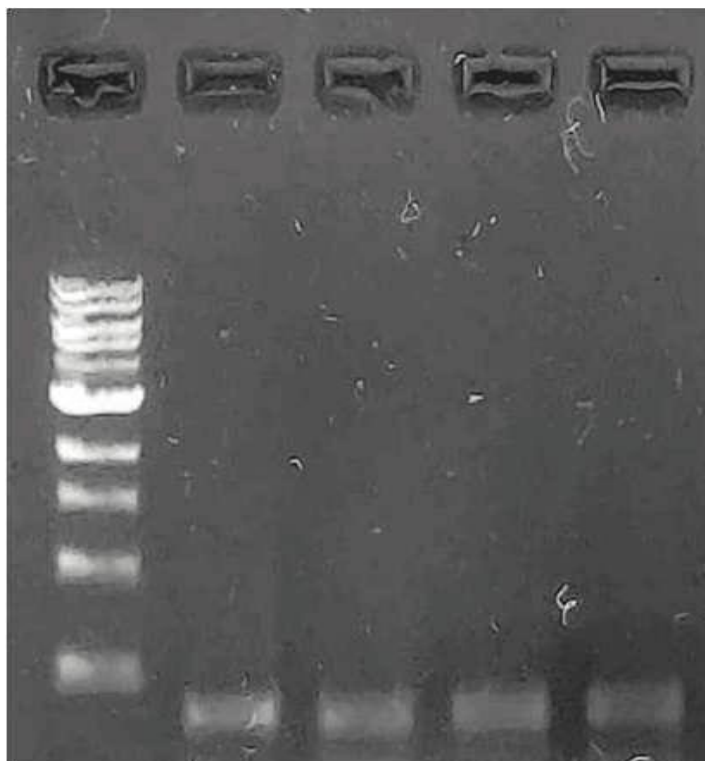


Fig 5.6 c) INOS gene expression of treated Zebra fish model

Lane 1 - 10 Kb DNA Ladder

Lane 2 - Control

Lane 3 - Sample (20 mg/L)

Lane 4 - Sample (60 mg/L)

Lane 5 - Sample (100 mg/L)

This indicates housekeeping gene (β actin) between various treated concentrations and control. The expression of treatment bands exhibits that no effect up to 100mg/L with reference to control. Figure 5&6 indicates the effect of given sample on inflammatory markers — TNF- α and iNOS expression between various treated concentrations and control. From gene expression studies, it confirms the given sample did not induced oxidative stress in Zebra fish up to a high dose of 100mg/

CHAPTER 6

CONCLUSION

SUMMARY AND CONCLUSION

Increasing resistance to the anti-microbial agents by the pathogenic microorganism is the major complications in biomedicine plant-based products have been used since years to treat diseases. In this study, plant consortium was prepared with orange peel powder, neem leaf powder and *Acalypha indica* powder in water-based formulation was evaluated against antibacterial activity and biocompatibility plant consortium was found to be inherited the faster bacterial pathogen and exhibited notable biocompatibility against zebra fish model, no toxic effect on embryonic stages of anti-oxidative enzymes status and histopathological parameters. Further studies will be useful to utilize the prepared consortium as an effective anti-microbial agent.

CHAPTER 7

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