

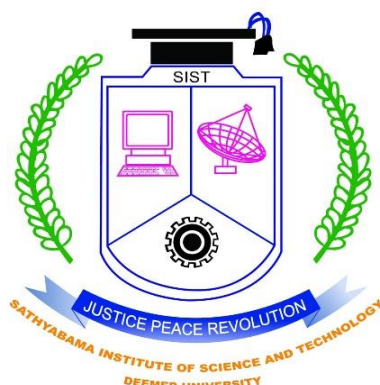
Biocompatible Nanocomposite Preparation for the Enhanced Biological Activities

Submitted in partial fulfilment of the requirements for the award of
Bachelor of Technology degree in Biotechnology

By

RAVIVARMA.M (37230068)

RISHAB.S (37230069)



**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING**

SATHYABAMA

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

**Accredited with Grade "A" by NAAC | 12B Status by UGC | Approved by AICTE
JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI - 600 119**

APRIL-2021



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DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Ravivarma.M (37230068)**, **Rishab.S(37230069)** who carried out the project entitled **"BICOMPATIBLE NANOCOMPOSITE PREPARATION FOR THE ENHANCED BIOLOGICAL ACTIVITIES"** under our supervision from **December 2020** to **March 2021**.

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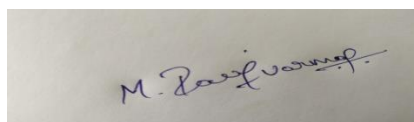
External Examiner

DECLARATION

We **Ravivarma.M (37230068)** and **Rishab.S (37230069)** and hereby declare that the report entitled **“BIOCOMPATIBLE NANOCOMPOSITE PREPARATION FOR THE ENHANCED BIOLOGICAL ACTIVITIES”** done by me under the guidance of **Dr.KARTHICK RAJA NAMASHIVAYAM, Centre for Bioresource Research and Development, Department of Biotechnology, Sathyabama Institute of Science and Technology, Chennai 119** is submitted in the partial fulfilment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

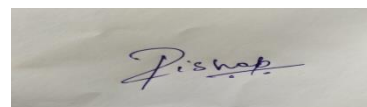
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PLACE: Chennai

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SIGNATURE OF CANDIDATE

ACKNOWLEDGMENT

I am pleased to acknowledge our sincere thanks to the Board of Management of **SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY** for their kind encouragement in doing this project and for completing it successfully.

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We would like to express my sincere and deep sense of gratitude to our project guide **Dr.L.Inbathamizh PhD., and Dr.S.KARTHICK Raja Namashivayam PhD.,** Centre for Bioresource Research and Development, Department of **Biotechnology** Department of Biotechnology their valuable guidance, Suggestions and constant encouragement paved way for the successful completion of my report work.

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We would like to express my gratitude to my loving parents for being the strength in my life and support me during this entire training.

ABSTRACT

Polymeric nonmaterials have gained recent attention in biomedicine due to the high efficiency and biocompatibility. In the present study, chitosan nanocomposite fabricated triphala extract was prepared by in site green science principles and the synthesized nanocomposite was characterized by suitable Analytical techniques like Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM). Highly stable nano structural material thus obtained was evaluated for anti cancer activity against breast cancer cell line (MCF7). Cell viability, MTT assay, apoptosis regulators markers expressions, anti oxidative enzymes status were carried out to determine anti cancer activity. Synthesized nanocomposite induced a good sign of anticancer activity by recording high rate of cell toxicity, improved expression of apoptosis regulators, notable changes in antioxidative enzymes status. The present findings would suggest the possible utilization of nano composite as the pharmaceutical agent.

Key words: Triphala, chitosan, cell toxicity, nanocomposite.

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LIST OF ABBREVIATIONS

FTIR	Fourier Transform Infrared Spectroscopy
MTT	3-(4,5-DIMETHYLTHIAZOL-2-YL)-2,5-DIPHENYL TETRAZOLIUM BROMIDE
g/L	gram per litre
Rpm	Rotation per minute
ML	Milliliter
EDTA	Ethylenediamine tereteaacetic acid
TAE	Tris – acetate- EDTA
DNA	Deoxyribo nucleic acid
Mg	Milligram
PCR	Polymerase chain reaction
Cm	Centimeter
SEM	Scanning electron microscope
EDAX	Energy dispersive X- ray analysis
AFM	Atomic force microscopy
MCF	Michigan cancer foundation
LPO	Lipid Peroxidation

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CHAPTER 1

1. INTRODUCTION

1.1 Nanotechnology:

Nanotechnology is the emerging trends in today's world. The field of nanotechnology is one of the most commercial areas for current research and development in basically all technical disciplines. Nanotechnology is technology to handling and control a substance at the nanometer level=1 billionth of a meter. The level of nanometers is the level of atoms and molecules. This opened the way for creating various nanostructures. For example in current world people need devices able to store information at high densities and high speeds, using little energy. However, as there are limits to mini compound with existing technology, we need technology that used for different approaches to process components and systems with nm level precision .It deals with nanometer scale and nanoparticles which act as segments in nanotechnology (Virji, et al., 2009). The nanotechnology increases to the field of applied science and technology whose theme is the control of matter on the atomic and molecular scale, normally 100 nm or smaller than that, and the production of materials within that size range. Hereafter, "nanotechnology" will likely to incorporate building machines and mechanisms with nanoscale dimensions, referred to these days as Molecular Nanotechnology. Molecular manufacturing primarily highlights the use of precisely developed computer-controlled nanoscale tools to construct vast numbers of improved tools as well as products with vast numbers of precisely designed nanoscale features. Recently, the Foresight Institute has suggested another term to represent the original meaning of nanotechnology: Zetta technology (Kuldeep et al., 2012)

Nanotechnology is not a new technology. The Romans and Chinese were using nanoparticles many years ago. Likewise, every time we light a match, fullerenes are produced. What is new about nanotechnology is our capacity to not only see, and manipulate matter on the nanoscale, but our understanding of atomic scale interactions. Two methods of controlling the structure of substance at the nanometer level are the Top-down and bottom-up methods. In the top-down approaches larger substance are finely processed in the lithography with light or electron beams. In the

bottom-up approaches structures are created by small atoms and molecules. Various different methods are being studied in bottom-up approach. Currently, there is a new interest in combining top-down and bottom-up approaches to develop technologies for assembling and operating complex components and system. When Nanotechnology is comparatively new field of research, it has various applications to a range of industrial field. In the information technology area, researcher are investigating the application of nanotechnology to the development of, for example, Computer devices with new operating principles, high-luminosity devices etc. Nanotechnology is progressively being used in the agri food sector to improve food and nutritional security and sustainability.

1.2 Nanoparticles:

Nanoparticles are defined as a particulate matter with one dimension which is less than 100nm. A fraction of solid particles on earth can be found in size of nanoparticles. In last two decades scientist have proven that colloids and nanoparticles are present everywhere in environment. Nanoparticles constitute a fraction of colloids. The definition of both is based on spatial dimension of object. Colloidal system is a twophase system which is made from dispersing medium and dispersed medium of gas, liquids and solids. The dispersing medium is mainly water and air where the dispersed media is solids of inorganic, organic or biological origin. Aqueous dispersions and aerosols are dominating colloidal systems. Colloids in environment are of natural origin which is formed by millions of years (Christian et al., 2008).

Atmospheric colloids are produced by combustion processes that are emitted as by-products in technological process but these will become the part of terrestrial and aquatic environment. Colloids can be transported to large distances in atmosphere and can be naturally produced as carbon nanotubes. It is generally accepted that definition of small particles uses simple scale to define size in case of nanoparticles.

1.3 Quantum dots:

Most of the quantum dots are dangerous especially for future medical applications. The widely used QDs consist of cadmium selenide or telluride because their quantum confinement region spans the optical spectrum. Scientists have been using gelatin during the production of CdTe QDs, thereby reducing the toxicity of the particles. The peptides could be used as a ligand for coating the surface of QDs for several reasons: 1) peptides mimic the biological environment and are stable at physiological pH; 2) reactive groups (amines, carboxyl, thiol, and peptide tags) can be dialed into the hydrophilic domain of the peptide sequence and enable enzymatic or standard conjugation chemistry to obtain biomolecules of interest; 3) molecular evolution strategies to randomize peptides can be adapted to select high-affinity binders to the QD surface; and 4) there is the possibility of creating multifunctional QDs by mixing peptide sequences in certain molar ratios in a single step for in vitro and in vivo studies (e.g., a QD with biotin and PEG, conjugating receptor ligand and PEG, etc.). In other words, surface functionalization plays the key role in nanoparticle toxicity (Jana et al., 2009). It was found the improved biocompatibility that certain surface coatings provide could arise from decreased cellular uptake of these nanoparticles for cellular degradation.

QD absorption, distribution, metabolism, excretion and toxicity turn on multiple factors derived from both inherent physico-chemical properties and environmental conditions; QD size, charge, concentration, outer coating bioactivity (capping material and functional groups), and oxidative, photolytic, and mechanical stability have each been implicated as determining factors in QD toxicity. Quantum dots also can damage DNA and disrupt normal cell activity caused by factors like the surface coatings (Chang et al., 2006). QDs are excellent candidates for biosensing owing to their unique physical and optical properties and possibility of attaching various biomolecules to their surface. Some new assays of QDs' usage, which may improve the current methods of DNA and protein detection, were performed. Biomolecules are often sure to bound to the surface of QDs either directly (covalently or non-covalently) or attached through stabilizing layer which act as a crosslinker between the ligand and reactive surface of the nanoparticle. Non-covalent direct binding are often achieved by applying electrostatic-based coupling strategy. Compared to the traditional covalent techniques, the electrostatic non-covalent approach is easier,

more reproducible, and more easily achieved. This approach has been used for instance to bind cysteamine-stabilized CdTe QDs with single stranded DNA through electrostatic attraction between positive amino function groups on the surface of CdTe quantum dots and negatively charged DNA.

1.4 Nanorods:

Nanorods are morphology of nanoscale objects. The size of nanorods ranges from 1-100nm. These are synthesized from metals or semiconducting materials and also by chemical synthesis. Nanorods are object of fundamental and applied research. Commonly these are prepared from zinc oxide carbon, gold and other materials. These are bigger than individual atoms and little molecules (Sadri et al., 2021).

1.5 Polymer Nanoparticles:

Nanoparticles are often prepared both from natural polymers for instance protein, polysaccharide or synthetic polymer such polystyrene. The nanoparticles which are prepared from synthetic polymers include heat, organic solvent or high shear force which will harm the drug stability. In contrast, nanoparticles prepared from natural polymers offer gentle also as simple preparation methods without the utilization of organic solvent and high shear force (Kreuter et al., 2001). Over the last few years chitosan nanoparticles, have gained considerable attention in present scenario due to their inherent biological properties. CSNPs are getting used during a sort of different products and applications like starting from pharmaceutical, drug delivery, tissue engineering, and food packaging to bio-sensing, enzymes immobilization, fuel cell manufacturing and waste-water treatment. (Jafarizadeh et al., 2012). Chitosan [poly-(b-1/4)-2-amino-2-deoxy-D-glucopyranose] is a natural biopolymer with film, fiber and micro/nanoparticles forming properties; because of its abundance, low production cost, biodegradable, biocompatible, renewable and non toxic nature. It's chemically inert, non-toxic, natural polysaccharide possessing robust and broad antimicrobial activities due to its polycationic nature (Calvo et al., 1997). Chitosan has been selected as a carrier for drug delivery especially macromolecules due to its physical and chemical properties. From a technical point of view, charge and solubility of chitosan in water is extremely important. A Chitosan nanoparticle is a biopolymer obtained from chitin by deacetylation. Chitin may be a polysaccharide obtained from exoskeletons of crustaceans and sea insects like crab, shrimp etc.

Chitosan may be mostly deacetylated polymer of N acetyl glucosamine that can be obtained through alkaline deacetylation of chitin. It consists of a β -(1, 4)-linked-D-glucosamine residue with the amine groups randomly acetylated. The amine and -OH groups endow chitosan with many special properties, making it applicable in many areas and easily available for chemical reactions. These factors can cause interactions between chitosan and negative charged surfaces in aqueous environment or with charged membrane of microorganisms, moreover, the interactions are gaining the attention in then nanotechnology field (Kim et al., 2005). Chitosan may be non-toxic, biocompatible and biodegradable compound that's approved with food and drug administration (FDA). In today's world pharmaceutical research has focused on the event of nanotechnology systems applicable in various fields of medicine, especially in the field of drug delivery. Currently, the utilization of biopolymers as nanoparticles (NPs) represents an another system with the massive potential of medicine or biological macromolecules within the body. Biopolymer NPs are often used effectively to supply bioactive molecules for in vivo and in vitro applications. Nano biopolymers also find applications within the field of enzyme replacement therapy (ERT). Indeed, the likelyhood of using NPs constituted by biocompatible and biodegradable polymers to deliver enzymes in those tissues where they're lacking or absent represents a huge advantage by overcoming a series of ERT problems. More generally, nanotechnologies are an extended research field characterized by the utilization of materials with sizes ranging between 1 and 1000 nm. The use of biopolymers in medicine has led to the definition of "polymers therapeutics" to explain different classes of nanocompounds currently in use as polymeric drugs, polymer-drug conjugates, polymer-protein conjugates, polymeric micelles, and polyplexes. (Se et al., 2005).

1.6 Bioactive Compound Fabricated Nanocomposite:

Bioactivity of the material is to stimulate a slected biological reaction at the boundary of the fabric. Nanocomposites are ideally bioactive materials due to their biological interfaces and structures that operate at the nanoscale. Nanocomposites attracted the researches an excessive amount due to their unique properties. Several types of nanocomposites are organic, inorganic and bioinorganic nanomaterials. These are utilized in biomedical fields like drug delivery, cancer therapy, medical imaging and chemical sensing.

1.7 Triphala:

Triphala, a well-recognized and highly efficacious polyherbal Ayurvedic medicine consisting of fruits of the plant species *Emblica officinalis* (Amalaki), *Terminalia bellerica* (Bibhitaki), and *Terminalia chebula* (Haritaki), could also be a cornerstone of gastrointestinal and rejuvenative treatment.

Triphala is assessed as a tridoshicrasayana, meaning that the energetics are appropriate for Vata, Pitta, and Kapha or all kinds of patients. Charak explains rasayanas as having the qualities of supporting strength and immunity. As Given these qualities and therefore the tonic energetics. Triphala are used to considered for the utilization within the very young, the infirmed, and therefore the elderly. The Another classical Ayurvedic classifications attributed to the formula are shukrala, digestive, bowel tonic at low dose ,mild laxative at normal doses, purgative at high doses, carminative, expectorant, antispasmodic, and bronchodilator. Especially, myriad other uses are described both within the Ayurvedic medical literature and anecdotally.



Fig-1.7.1 The image Triphala

CHAPTER 2

2. LITERATURE SURVEY

(Jing et al., 2016) explained that the use of nanoparticle hybrid materials in bioapplications like biosensing, bioimaging, delivery of drugs and genes and tissue engineering. In this study the graphene material was used for nanocomposite. These types of nanocomposites are less-toxic, biocompatible and useful for applications.

(Velu et al., 2020) stated that nanomaterials have shown significant breakthrough in bioengineering and medical fields. The fabrication methods used for functioning of medicinal plants is viewed. Further the applications of nanocomposites and techniques are also studied.

(Naskar et al., 2018) described that chitosan is one of the successfully developed biodegradable polymers. It is also chosen as the main source for wound dressing and has been used to design nanoparticles as drug delivery systems in pharmaceutical applications.

(Balochowhan et al., 2013) explained that triphala is used as a medicine in traditional system of India. The plant is antioxidant-rich herbal formulation which possess variety of beneficial purposes. It is an ayurvedic drug which is used in all dosas and act as a good source in digestive capacity and is a polyherbal compound. The plant also helps in improving body's defense system.

(Cheung et al., 2015) described chitosan as a natural polysaccharide from chitin. Chitosan act as a versatile biomaterials due to their nontoxicity, low allergenicity, biocompatibility. They highlighted the recent research trends and other applications of chitosan.

(Namasivayam et al., 2018) indicated the chitosan nanocomposite prepared with chitosan-insecticidal metabolites. Insecticidal metabolites and chitosan nanocomposite was prepared by fungal strain and by ion gelation method. Further the evaluation was carried out by toxicity against zebra fish. Hemolysis was recorded in nanocomposite treatment and this act as good component for pest control against economically important insect pests which does not affect non-target organisms.

CHAPTER 3

3. AIM AND SCOPE

Nanotechnology principles play a major role in biomedicine as drug delivery agent and diagnosis. Polymer based nanomaterial plays a vital role in delivery of pharmacologically active compounds with this background, the present work is aimed to synthesize nanocomposite fabricated with triphala extract objective of the present study is as follows

1. To synthesize chitosan nanocomposite fabricated with triphala extract
2. To evaluate anti cancer activity against breast cancer cell line (MCF-7)

CHAPTER 4

4. MATERIALS AND METHODS

4.1 Synthesis of chitosan nanocomposite fabricated triphala biomass aqueous extract

Ionic gelation method was used for the preparation of chitosan nanocomposite loaded triphala metabolites. In this method, known volume of chitosan was suspended in de ionized water – acetic acid mixture followed by addition of triphala aqueous extract and tri poly phosphate. The triphala consists of mainly three fruits known as **“Terminalia chebula.Fr.R. 33.3% “Emblica officinalis.Fr.R” 33.33% “Terminalia bellerica.Fr.R 33.33%”** .

Suspension was stirred with magnetic stirrer and the collected suspension was centrifuged, collected pellet was used for further studies. Characterization of the composite was done by scanning electron microscopy, fourier transform infrared spectroscopy (FTIR) and atomic force microscopy (AFM) and particle size analysis

4.2 MTT Assay (Mosmann *et al.*, 1983):

MTT assay was used to investigate mitochondrial function as described by Mossman *et al.*, 1983. Briefly, 1×10^4 cells/well were seeded in 96-well plates and exposed to different concentrations of sample (1-10 and 10-100 $\mu\text{g/ml}$) for 24h. At the end of the exposure, culture media were replaced with new media containing MTT solution (0.5 mg/ml) and incubated for 4 h at 37 °C. As a result, formazan crystal was formed, and it was dissolved in DMSO. The plates were kept on a shaker for 10 min at room temperature and then analyzed at 490nm and 630nm using a multi well microplate reader. Untreated sets could also come under identical conditions and served as controls. Inhibition rate was calculated using the following formula:

$$\% \text{ Inhibition Rate} = \{1 - [A_{490} - A_{630} (\text{Treated}) / A_{490} - A_{630} (\text{control})] \times 100\}$$

4.3 Morphological Changes Using Phase Contrast Inverted Microscope:

Observation of morphological changes of apoptotic cells was performed according to the method with slight modifications. Briefly, 5×10^5 cells were incubated for 24 h with or without selected concentrations of sample 0, 30, 40, 50 and 100 $\mu\text{g/mL}$ in a 60mm

diameter tissue culture dishes. The medium was discarded and cells were washed once with PBS. The morphological changes of the apoptotic cells were observed using phase contrast inverted microscope at 200x magnifications.

4.4 Fluorescence microscopy

4.4.1 Propidium Iodide staining:

The grown cells were treated with the sample for 24 hours. The treated cells were washed with PBS twice and fixed with 4 % formaldehyde for 30 minutes. The fixed cells washed with PBS again, then PI stain (1 mg/mL stock; 10 µg/mL standard) added for each wells and incubated for 30 minutes. The excess stains were removed with PBS washing. The cells observed and imaged under the fluorescence microscope.

4.5 ROS estimation:

The NBT reduction assay was performed for ROS estimation with slight modification. 1×10^5 cells/ml seeded and incubated for 24 hours. Test samples were then added onto the appropriate wells and incubated for 24 hours. Washed with PBS twice and 100 µl of 0.1 % NBT solution was pipette to each well of the 24 hours incubated cells approximately 1 hour after which the medium was carefully aspirated then washed with 70 % methanol three times. To the dried well 120 µL of 2M potassium hydroxide (KOH) followed by 120 µL of DMSO were added to each well and then read in a plate reader at 630nm with KOH or DMSO as blank.

4.6 Lipid peroxidation assay (LPO):

Thiobarbituric acid-reactive substances (TBARS) assay is used to detect lipid peroxidation. In this assay, malondialdehyde forms a complex with thiobarbituric acid, which can be quantified spectrophotometrically. The treated cells with various concentration of the test sample were taken for assay. The cells was 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 % TBARS buffer and 0.7 mL of distilled water to the solution. These reaction mixtures were incubated

about 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 .

4.7 DNA isolation: Isolation of genomic DNA:

4.7.1 Requirements:

Liquid Nitrogen, Digestion buffer, PBS (Ice cold), 7.5M Ammonium acetate, 70% and 100% ethanol, TE buffer (pH-8) and Water bath

4.7.2 Procedure:

Cultured Cells were out of its culture medium, Trypsinize adherent cells and collect the cells from the flask. Centrifuge 5mins at 1200 rpm and supernatant were discarded. Resuspend cells with 1 to 10 mL ice-cold PBS. Centrifuge 5 mins at 1200 rpm and discard the supernatant. Resuspend cells in 1 vol digestion buffer. For $<3 \times 10^7$ cells, use 0.3 ml of digestion buffer. Incubate the samples at 50°C for 12 to 18 hr in tightly capped tubes. After incubation Equal volume of Phenol-Chloroform- Isoamyl alcohol mixed in the ratio 25:24:1 is added and mixed gently and centrifuged at 3000rpm for 5 mins at room temperature. 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 were added, mixed well and centrifuged at 3000rpm for 5 mins at room temperature. The pellet was rinsed using 70% ethanol and the ethanol was discarded. The pellet was kept for air drying. Once dried, the pellet is dissolved in 30 μl nuclease free water for further processes.

4.7.3 Agarose gel electrophoresis:

4.7.3.1 Requirements:

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

4.7.3.2 Preparation of the Gel:

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

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

Added loading dye to the DNA samples to be separated. Gel loading dye is typically made at 6X concentration. Programmed the power supply to desired voltage (1-5V/cm between electrodes). Added enough running buffer to cover the surface of the gel. It is important to use the same running buffer as the one used to prepare the gel. Connected the leads of the gel box to the power supply. Removed the lid. Loaded the DNA sample(s) into the gel. An appropriate DNA size marker should always be loaded along with experimental samples. Replaced the lid to the gel box. The cathode (black leads) should be closer the wells than the anode (red leads). Power turned on.

4.7.3.4 Observing Separated DNA fragments:

When electrophoresis was over, the power supply was turned off and the lid was removed from the tank. 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. The gel was exposed to UV light using an UV illuminator. DNA bands should show up as orange fluorescent bands. A picture of DNA bands was taken and the gel discarded.

4.7.3.5 Gene expression studies:

4.7.3.6 Primers:

For the current study β -Actin, Bax and C mycprimers were used. Nucleotide sequence alignments of β -Actin gene were obtained through databases. Based on these alignments, β -Actin primers (Forward – 5' TCAAGGTGGGTGTCTTTCCTG 3') and (Reverse – 5' ATTTGCGGTGGACGATGGAG 3') genes were designed. Nucleotide sequence alignments of BAX gene were obtained through databases. Based on these alignments, BAX primers (forward – 5' CGTGTCTGATCAATCCCCGA 3') and (Reverse – 5' GAGGCCAGAAGGCAGGATTG 3') genes were designed. Nucleotide sequence alignments of C- myc gene were obtained through databases. Based on these alignments, C myc (forward – 5' TCTCCGTCCTCGGATTCTCT 3') and (Reverse – 5' TGAGCTCCCAAATCTCTCCAG 3') genes were designed.

4.7.3.7 PCR:

Initially DNA isolation was done using Nucleospin DNA isolation kit according to the manufacturer's instruction. Then the PCR was performed using Thermo cycler gradient PCR. Amplification reaction were performed in a volume of 25 μ l containing 4.5 μ l of template DNA, 12.5 μ l of 1 X PCR Emerald master mixture, each of the specific primers of volume 1 μ l respectively and made up with remaining volume of distilled water. The amplification program consisted of 1 cycle at 95 °C for 2 minutes for initial denaturation, 95 °C for 1 minute for DNA denaturation, 54 °C (β -Actin) ,59.6 °C (C myc) and 54.3 °C (bax) for 30 seconds for annealing and 72 °C for 1 minute for extension of two strands, 72 °C for 5 minutes for final extension. Same condition followed for 34 cycles. The PCR products were analyzed on a 1.0 % agarose gel containing 1 mg/ml ethidium bromide, 6 μ l of the PCR products with 4 μ l of 6X gel loading dye were loaded into wells and electrophoresis was performed in 1 X TAE buffer at 50 V for 2 hours. The gel visualized under UV and photographed.

4.8 CHARACTERIZATION:

4.8.1 FTIR:

FTIR analysis of samples was performed with a2 technologies portable attenuated total reflectance (ATR) Fourier transform infrared (ATR-FTIR). Sample spectra can be recorded in the infrared range from 4000 cm^{-1} to 400 cm^{-1} with a resolution of 4 cm^{-1} in the absorbance mode for 10 scans at room temperature. FTIR spectra of sample were obtained by placing 1 mg of sample on the sensor of the instrument and spectrum (Kumirska et al., 2010).

4.8.2 SEM:

The size and the morphology of sample were examined in Quanta 400 ESEM (FEI). Vacuum dried small amount of samples were kept on an SEM stub using double-sided adhesive tape at 50 mA for 6 min through a sputter. Afterwards, the stub which containing the samples were placed in the scanning electron microscopy (SEM) Chamber. (Lam TD et al., 2006)

4.8.3 EDAX:

The EDX technique analyse X-rays which are emitted by a material when hit by electromagnetic radiation. In EDX system, a high-energy beam is focused on the sample, an atom within the sample has unexcited electrons in discrete energy levels or electron shells which are bound to the nucleus.

4.8.4 PARTICLE SIZE ANALYSIS:

Mean particle sizes the samples were determined by using Delsa Nano-C (Brea, CA) at room temperature in triplicates.

4.8.5 AFM:

The morphological characteristics of the sample were examined by tapping-mode atomic force microscopy (JPK NanoWizard AFM, Berlin, Germany). One drop of solution was placed onto a freshly cleaved piece of mica and air-dried.

CHAPTER 5

5. RESULTS AND DISCUSSION:

5.1 Synthesis of chitosan nanocomposite:

Ionic gelation method brought about high stable structurally modified nanocomposite. Characterization was primarily carried out by FTIR which shows the specific interaction of functional group of triphala with chitosan.

5.1.1 FTIR:

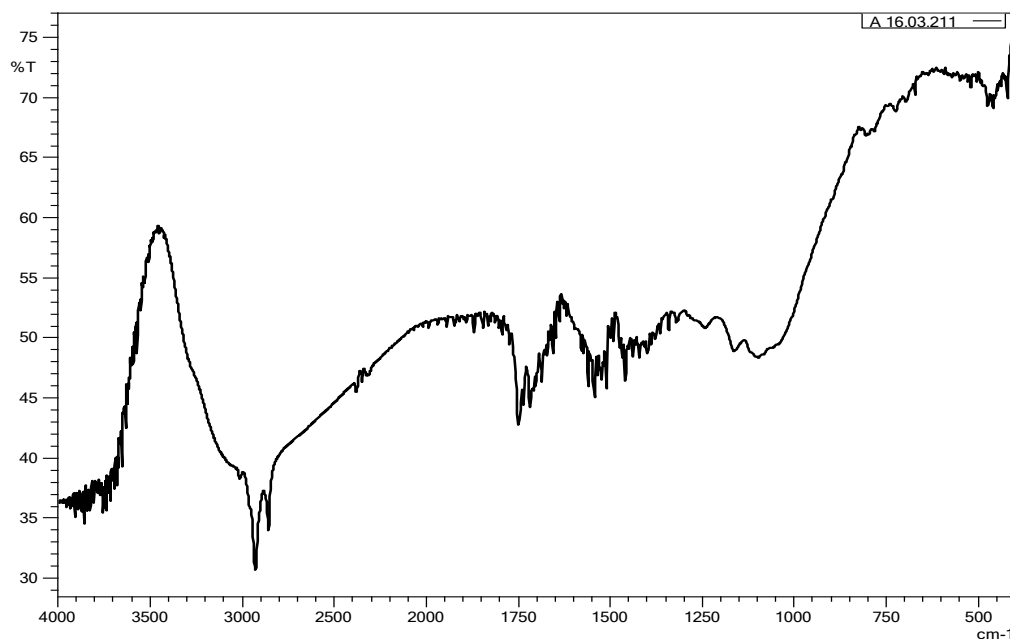


Fig 5.1 FTIR image of Chitosan nanocomposite fabricated Triphala extract

5.2 Sem and Edax:

Particles morphology size and shape was done by scanning electron microscopy equipped with EDAX which reveals the rough dense agglomerated particles with the

size arrange of 70-199 nm (Fig 5.2.4) EDAX spectra that shown in the fig indicates the elemental composition

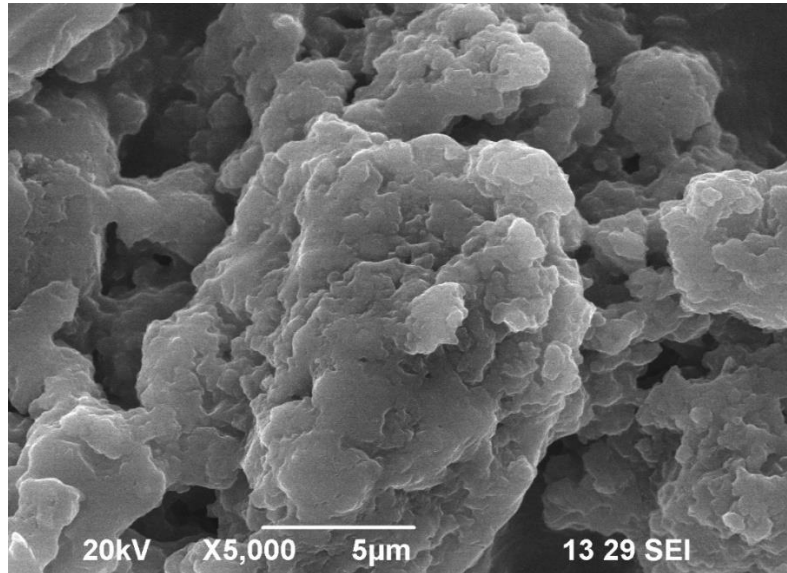


Fig 5.2 SEM image of Chitosan nanocomposite fabricated Triphala extract at 5000 magnification of 5 micrometer

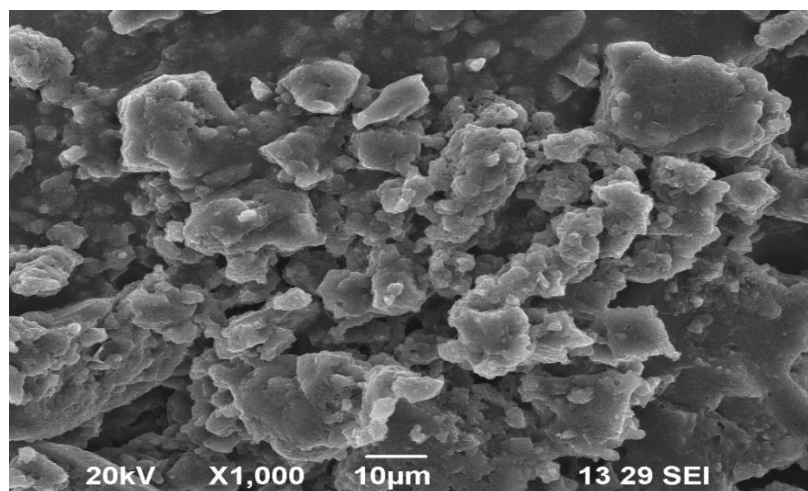


Fig 5.3 SEM image Of Chitosan nanocomposite fabricated Triphala Extract at 1000 magnification of 10 micro meter

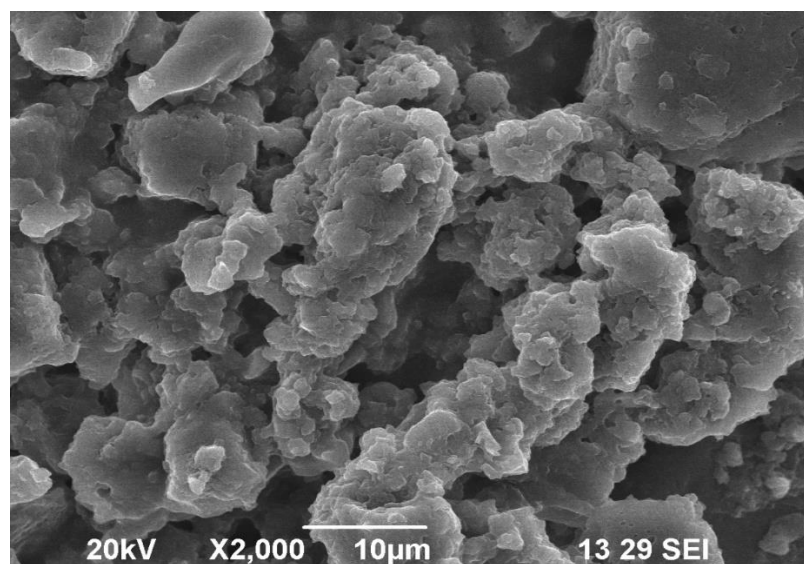


Fig5.4 SEM image of Chitosan nanocomposite fabricated Triphala extract at 2000 magnification of 10 micro metre

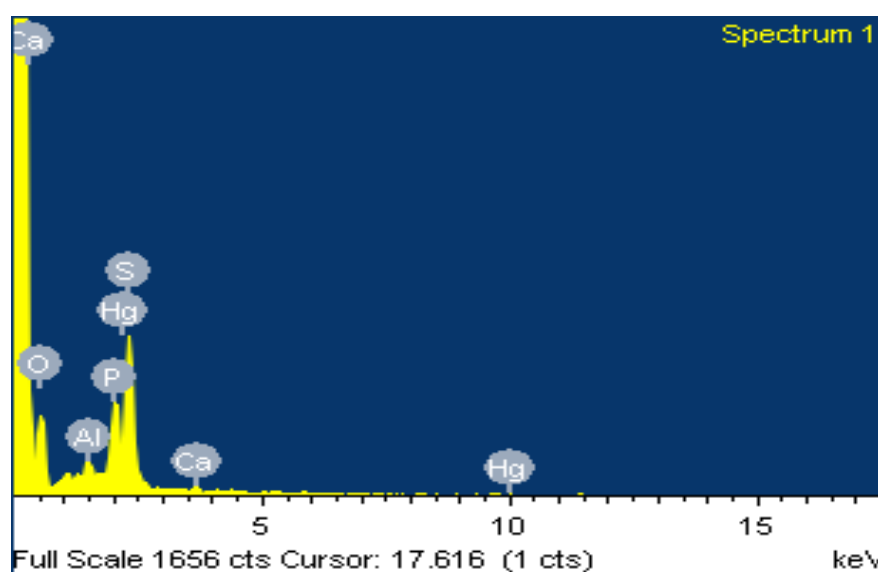


Fig 5.5 shows the spectrum image of chitosan nanocomposite Triphala extract

5.3 PARTICLE SIZE ANALYSIS:

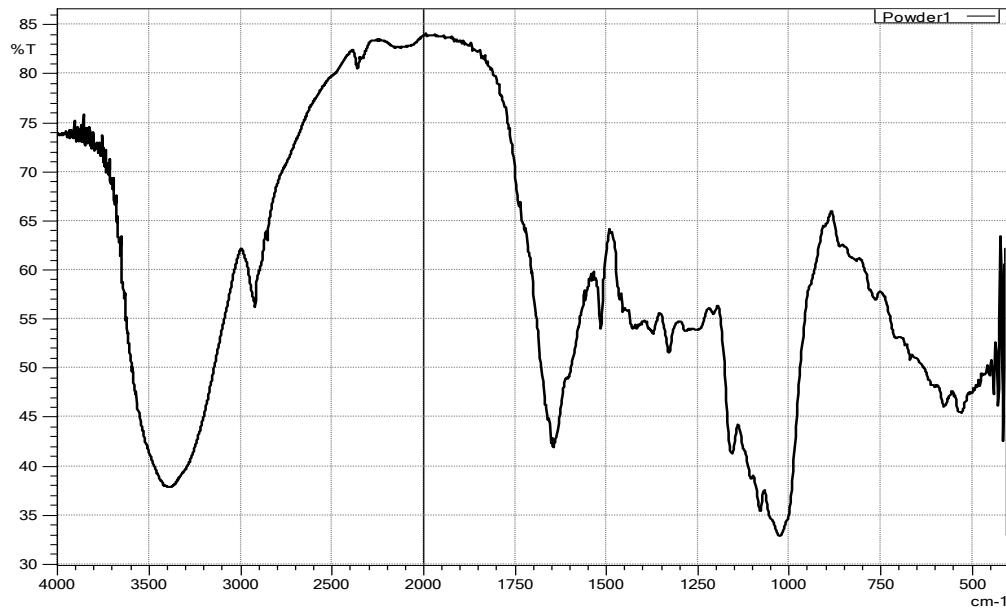


Fig 5.6 The particle size analysis also shows the nano dimensional structure.

5.4 AFM (ATOMIC FORCE MICROSCOPY):

Surface topography was studied by AFM which indicates the white bright spot nano dimensional particles with the distinct roughness

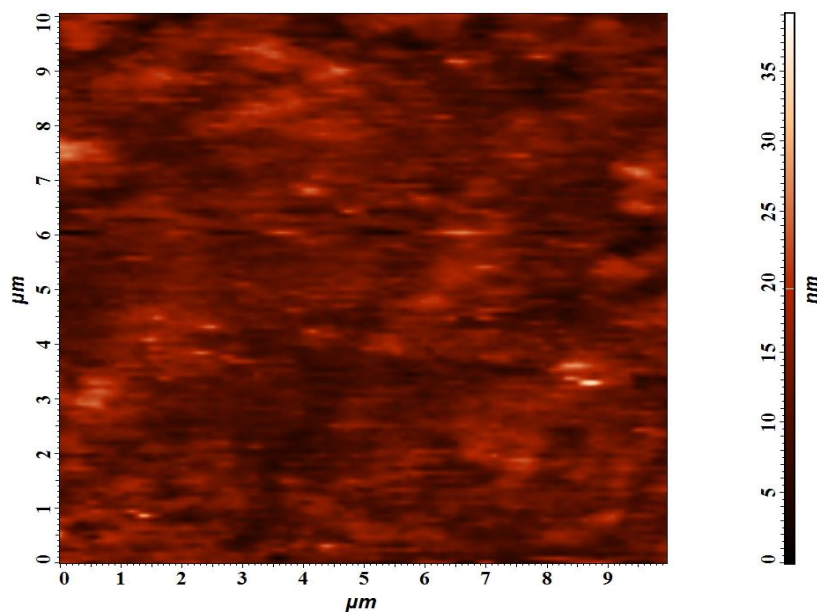


Fig 5.7 The AFM image of surface topography of chitosan nanocomposite fabricated triphala

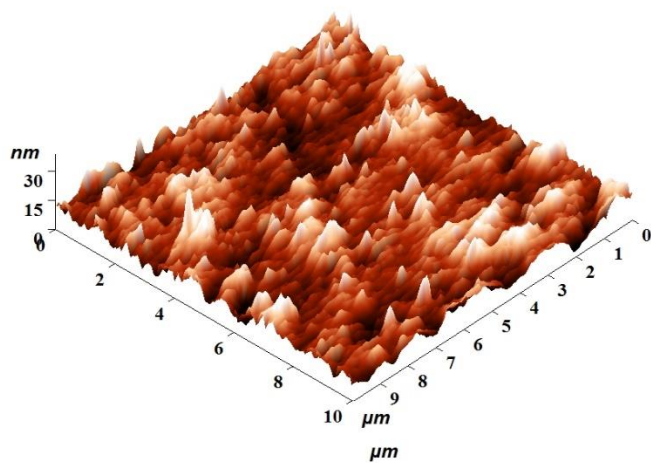


Fig 5.8 The peak image of chitosan nanocomposite fabricated Triphala

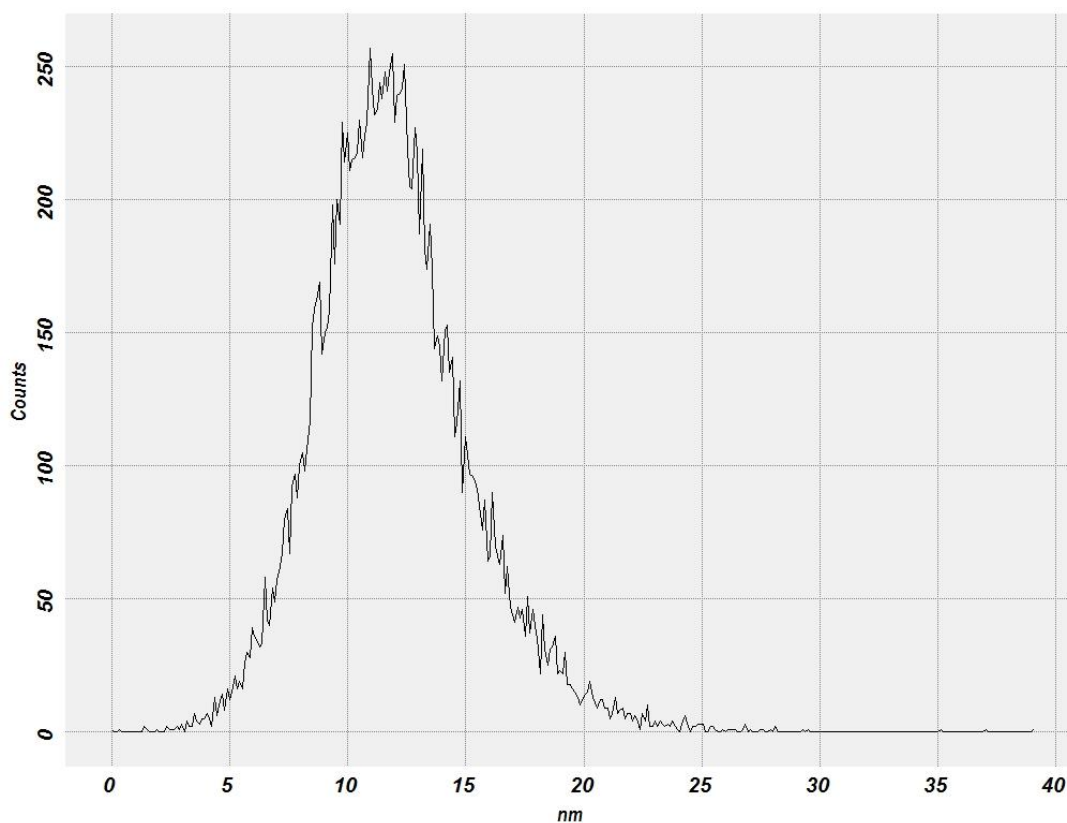


Fig5.9 The peak of chitosan nanocomposite fabricated triphala extract

5.5ANTI CANCER ACTIVITY:

5.5.1Anti cancer activity was studied against breast cancer cell line (MCF 7)

5.5.1.1 MTT Assay

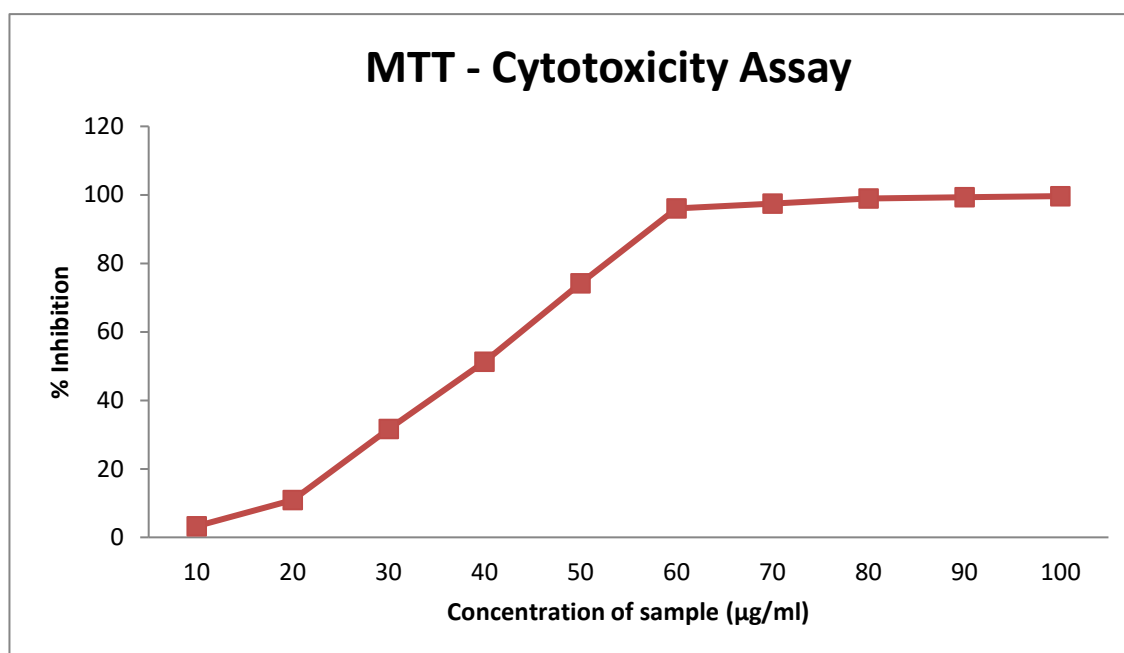


Fig 5.10 *The effect of Sample on cell viability.*

Toxic effect of Sample in Human Breast cancer cells (MCF7) was studied by MTT assay. The varied concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/ml were showed cytotoxicity effect of sample in human breast cancer cell line (MCF7) as shown in the Fig 1. The LC₅₀ range of the sample was found at 40 µg/ml concentration were 51.27%, whereas the viability of cell was declined to 0.37% at 100µg/ml concentration.

5.6 Morphological changes (Phase contrast microscope):

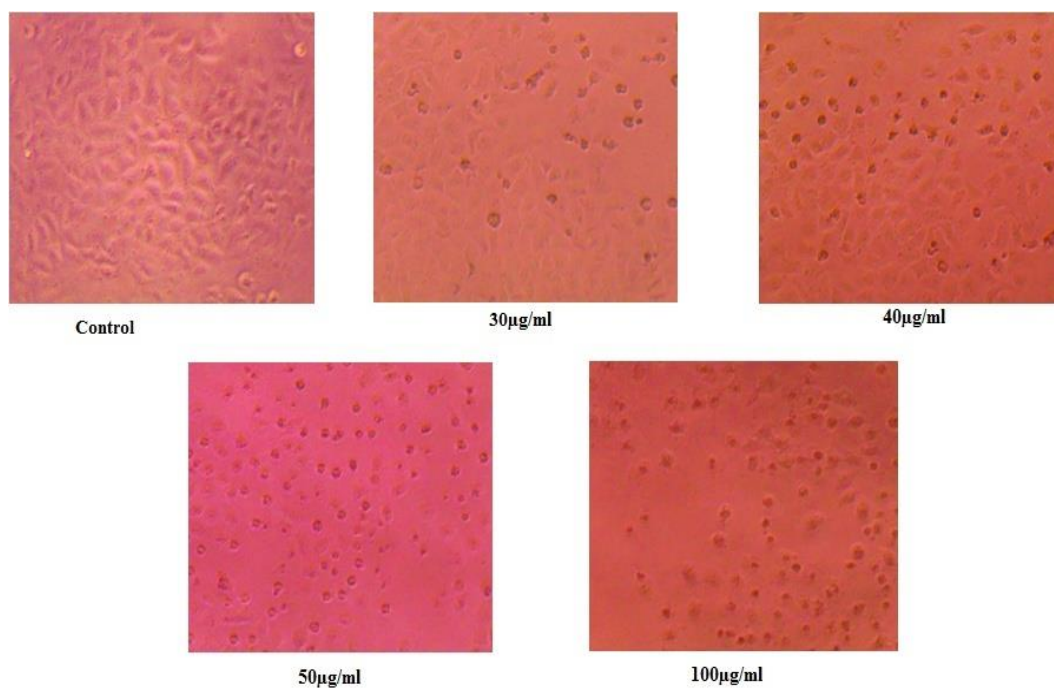


Fig 5.11 The effect of sample on Human Breast Cancer Cells (MCF7)

Morphological changes of apoptotic cells were observed in MCF7 cell, which treated with or without selected concentrations of sample 0, 30, 40, 50 and 100 µg/mL.

5.7 Fluorescence Microscopy:

MCF

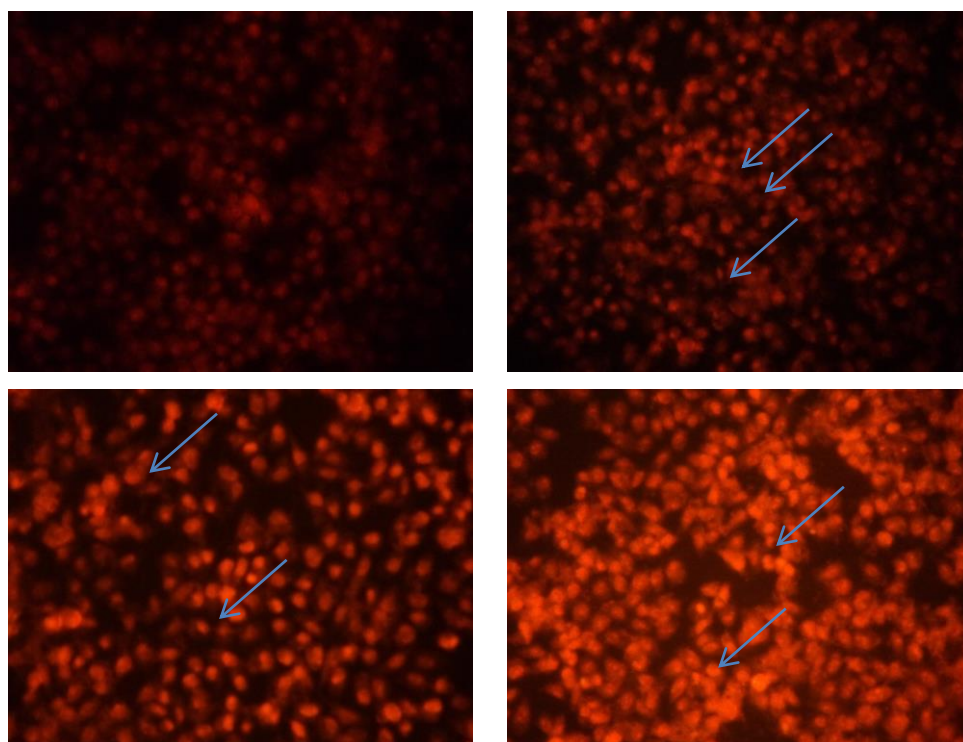


Fig 5.12 The control (untreated) B)30 µg C) µg D) 50 µg of sample treated MCF7 cell line, arrow indicates that the apoptotic cells and the apoptosis effect increased as does depended manner.

5.8 Ros Estimation:

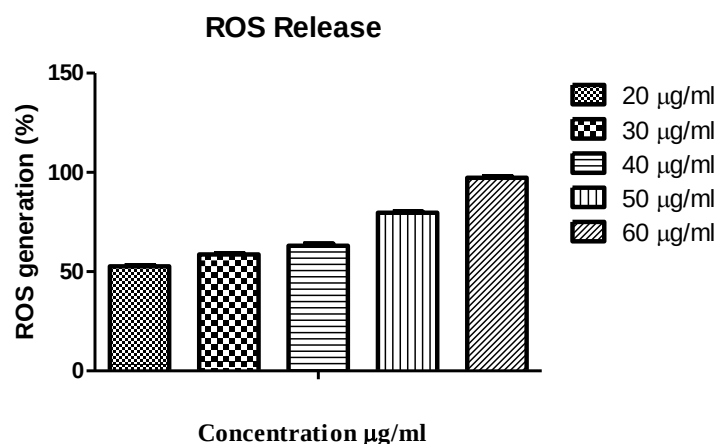


Fig 5.13 MCF7 cells treated with the given sample as 20 µg , 30 µg, 40 µg, 50 µg and 60 µg for 24 h. ROS production was significantly increase with the increasing of drug concentration. The graphical representation denotes that the parallel increase of ROS with the increasing drug concentration ($P<0.0001$).

5.9 Lipid Peroxidation Assay:

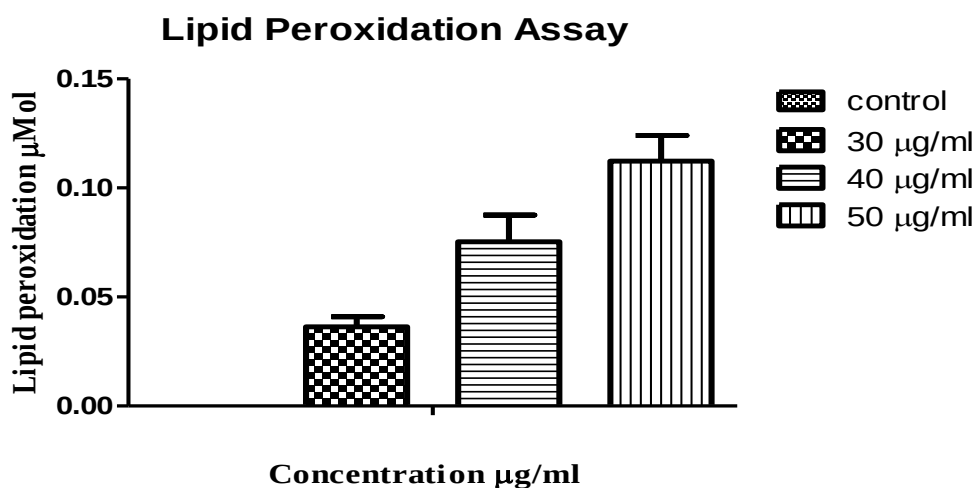


Fig 5.14 Lipid Peroxidation assay (LPO) the sample had markedly increased the level of production of the LPO. The graphical representation shows the significant increase of LPO as dose dependent manner ($P<0.0001$) after treatment with the sample as same as 30 µg, 40 µg and 50 µg for 24 h. These results clearly indicate that the increased oxidative stress in MCF7 cells with the treatment.

5.10 Gene expression studies:

5.10.1 β - actin:

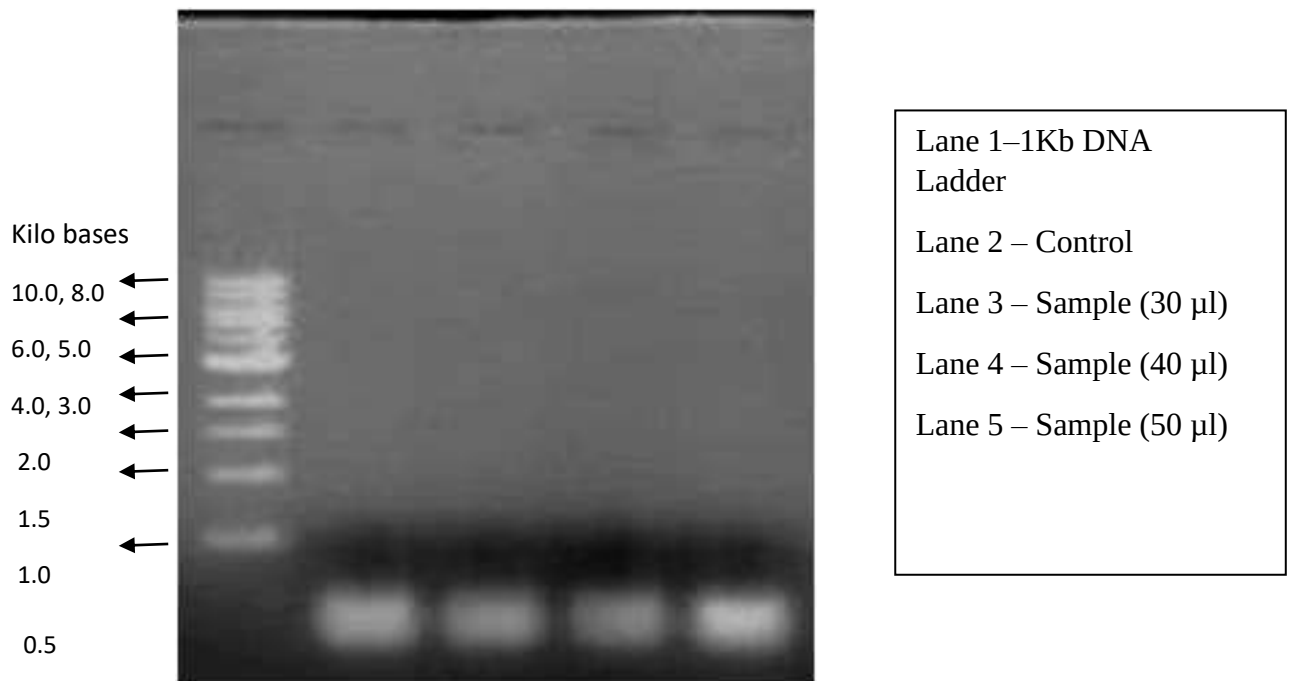


Fig5.15 The band image of Beta- actin observed under U-V

5.10.2 Bax gene:

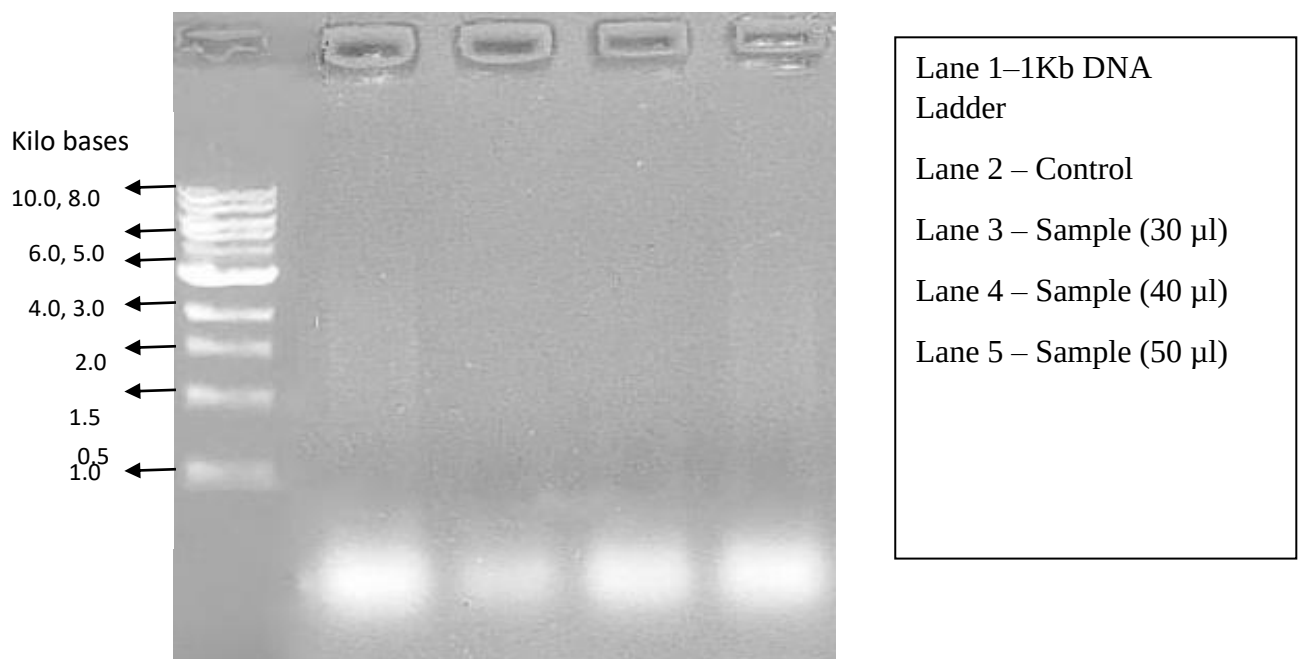


Fig5.16 The band image of Bax gene observed under U-V

5.10.3 C – myc:

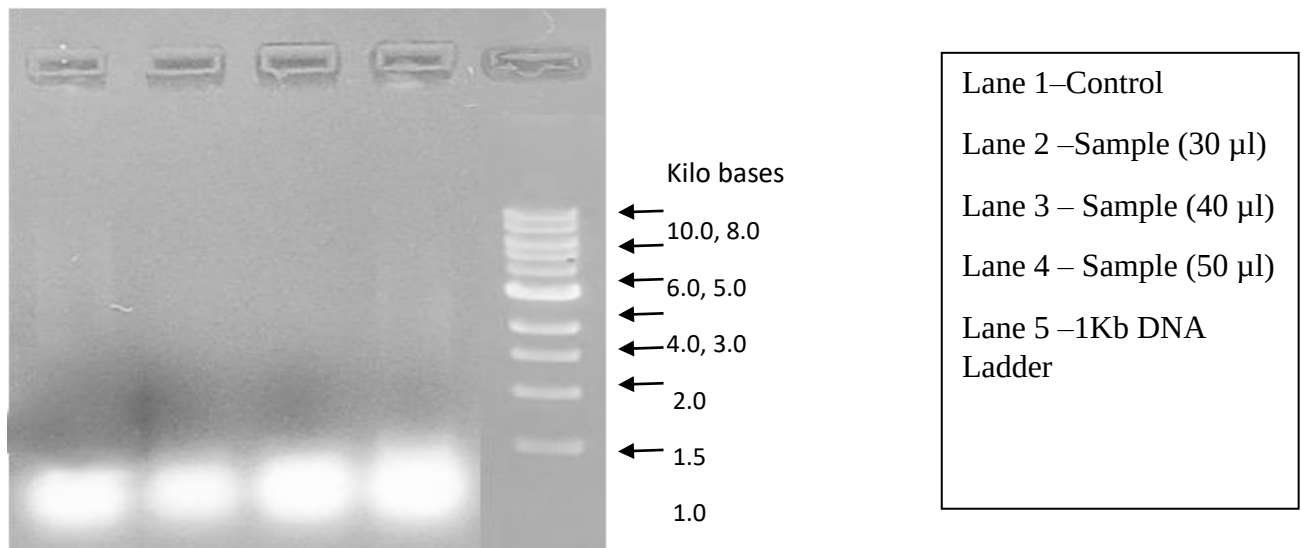


Fig 5.17 The band image of *Cmymc* observed under U-V.

The gel picture that there was increased gene expression levels obtained for the increasing sample concentration for the genes *Bax*, β actin and *Cmymc*. The results indicate that oxidative stress induced apoptotic pathway for PCR product comparing to control.

DISCUSSION:

(Saber-Samandari et al., 2017) this author has done a work on chitosan graft poly nanocomposite scaffold was developed to improve cell viability and proliferation in bone tissue engineering. This works reveals that chitosan has effective mechanical properties, morphology, chemical bonds and pore size. Comparatively in the present study chitosan with triphala has been used for the study against breast cancer and it shows effective result. Hence chitosan nanocomposite can be widely used in nanotechnology.

(Ziąbka et al., 2018) this author has briefly worked in the investigation of silver nanoparticles with modified biocompatibility of polymeric implants. The main goal of this work has been tissue healing process and cytotoxicity test. Hence it was prepared and tested on the rats to check its effectiveness and properties on tissue healing. It exhibits the effective result. Therefore silver can be good nanocomposite agent in ontology. In the present study chitosan nanocomposite were used for the oncology studied which shown the good result.

(Manivasagan et al., 2017) this author has worked on cancer nanotechnology by combining the photothermal therapy and photoacoustic imaging against breast cancer. And the study reveals that chitosan polypyrrole with multifunctional biocompatible shows the effective result against the cell line with its stability, strong infrared absorbance . Hence it been more effective in cancer technology.

In this study, chitosan nanocomposite with triphala extract was evaluate for an anti cancer activity against breast cancer cell line (MCF-7). Synthesize nanocomposite induced a good sign of anti cancer activity. Further structure will be useful to utilize the possible utilization of nanocomposite as an anti cancer agents.

CHAPTER 6

6. SUMMARY AND CONCLUSION

Polymeric nonmaterial have gained recent attention in biomedicine due to the high efficiency and biocompatibility. In the present study, chitosan nanocomposite fabricated triphala extract was prepared by in site green science principles and the synthesized nanocomposite was characterized by suitable Analytical techniques like Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM). Highly stable nano structural material thus obtained was evaluated for anti cancer activity against breast cancer cell line (MCF7). Cell viability, MTT assay, apoptosis regulators markers expressions, anti oxidative enzymes status were carried out to determine anti cancer activity Synthesized nanocomposite induced a good sign of anticancer activity by recording high rate of cell toxicity, improved expression of apoptosis regulators, notable changes in antioxidative enzymes status. The present findings would suggest the possible utilization of nano composite as the pharmaceutical agent. Drug resistance development is a severe problem in biomedicine various synthesised drugs formulation are recommended for drug resistant pathogens and cancer cells. But the success of pharmacological agents are highly depends on certain formulation which enhances the delivery of pharmaceutical active agents. Nano technology principles are now being extensively utilized in the drug formulation. In this study, chitosan nanocomposite with triphala extract was evaluate for an anti cancer activity against breast cancer cell line (MCF-7). Synthesize nanocomposite induced a good sign of anti cancer activity. Further structure will be useful to utilize the possible utilization of nanocomposite as an anti cancer agents.

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