

Mud crab shell derived Chitosan based hydrogel system for stem cell culturing and other applications

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

By

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This is to certify that this Project Report is the bonafide work of Mr. Noel Richard Prakash. L (Reg. No 39230030) and Mr. Sanjay Preeth. R (Reg. No. 39230040) who carried out the project "Mud crab shell derived Chitosan based hydrogel system for stem cell culturing and other applications" under our supervision from August 2022 to May 2023.

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DECLARATION

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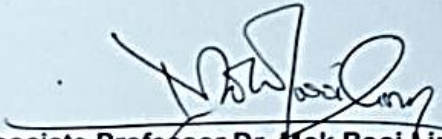


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ABSTRACT

Chitin is an abundant polysaccharide on world, when derived to chitosan it has got more applications. This study was planned with intention to utilize crab shell chitin for several applications, which include chitin cup, drug delivering agent and hydrogel for stem cell cultivation. The produced chitosan was successfully made into biodegradable cup, agent for drug delivery and a hydrogel system for stem cell culturing. This study was done to extract chitin and chitosan from crab shells using a chemical method and to characterize the extracted samples. The yield percentages of chitin and chitosan were found to be 90% and 71.3%, respectively. Various characterization techniques were used to study the samples, including UV-Vis, FTIR, X-ray Diffraction, Scanning electron spectroscopy, Energy Dispersive X-Ray Analysis, and Thermogravimetric analysis. Additionally, the bioactivity of the samples was investigated, including their antioxidant, antibacterial, and anticancer activities. Chitosan showed significant scavenging activity and good antibacterial activity. Chitosan hydrogel was synthesized and examined for stem cell culturing. The extracted chitosan was also used to synthesize a nanocarrier for curcumin delivery, and the nanocarrier was characterized using various techniques. Overall, this study provides valuable insights into the potential uses of chitosan for biomedical applications such as tissue engineering and drug delivery. Overall, this study provides insight into the potential applications of chitosan and its derivatives in the fields of tissue engineering and drug delivery.

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LIST OF ABBREVIATIONS

AF-MSCs	-	Amniotic fluid mesenchymal stem cells
AO/EB	-	Dual acridine orange/ethidium bromide staining
BaCl ₂	-	Barium chloride solution
CaCl ₂	-	Calcium chloride
CD	-	Cluster differentiation
DMEM	-	Dulbecco's Modified Eagle's Medium
DPPH	-	2,2-diphenylpicrylhydrazyl
FBS	-	Fetal bovine serum
FTIR	-	Fourier Transform Infrared Spectroscopy
hADSC	-	Human adipose-derived stem cells
HCL	-	Hydrochloric acid
HLA-DR	-	Human Leukocyte Antigen – DR isotype
LSCs	-	Limbal stem cells
MSC	-	Mesenchymal stem cells
NaOH	-	Sodium Hydroxide
PBS	-	Phosphate buffered saline
PEC	-	Polyelectrolytic complex
PEG	-	Polyethylene glycol
PLGA	-	Poly Lactic-co-Glycolic Acid
rhBMP-2	-	Recombinant human bone morphogenetic protein-2
rhIGF-I	-	Recombinant complex of insulin-like growth factor-I
SEM	-	Scanning Electron Microscopy
TGA	-	Thermogravimetric Analysis
UCMSCs	-	Umbilical cord mesenchymal stem cells
UV-Vis	-	Visible Spectroscopy
XRD	-	X-Ray Diffraction

CHAPTER 1

1. INTRODUCTION

The term "biopolymer" refers to a type of polymer created throughout an organism's normal growth phase. Accordingly, they are also called natural polymers (Rao *et al.*, 2014). Due to their significant benefits including eco-friendliness and biodegradability, which put them ahead of synthetic polymers, biopolymers are expected to challenge them in the future. They may be divided into three categories: microbial biopolymers, synthetic biopolymers, and natural biopolymers (Udayakumar *et al.*, 2021; Hassan *et al.*, 2019). Biopolymers are produced by complex physiological activities inside the cell. The relevance of biodegradable polymers is increasing daily, and present research is focused on creating newer biodegradable polymers (Rao *et al.*, 2014). It has been discovered that the biopolymers are biodegradable and biocompatible, making them helpful in a variety of applications, including edible films, emulsions, packaging materials, and medical implants including artificial organs, tissue regeneration, tissue scaffolds, and dressing materials in the pharmaceutical and food sectors (Baranwal *et al.*, 2022). In nature, polysaccharides are found in abundance. These materials are significant in a variety of sectors because they have distinctive structures and properties that set them apart from conventional synthetic polymers. The two most significant sources of biomass from the various polysaccharides are cellulose and chitin. Chitin is acquired from rest of the animal kingdom, whereas cellulose is made by plants. (Honarkar & Barikani, 2009). Chitin is a naturally occurring crystalline microfilament that forms organized structural elements in the exoskeleton of crustaceans as well as in the cell walls of fungi and yeasts. Crab and shrimp shells are the main commercial sources of chitin today. In commercial procedures, calcium carbonate is first dissolved by acid treatment to remove chitin from crustaceans, then proteins are extracted by alkaline extraction to solubilize them. Moreover, a decolorization process is frequently used to get rid of any remaining pigments and create a colourless product (Younes & Rinaudo, 2015; Rinaudo, 2012;). In the year 1868, Ernst Haeckel first used the phrase "stem cell" in the research literature (Pandey *et al.*, 2022). Undifferentiated cells called stem cells are found throughout the embryonic, fetal, and adult stages of life. They produce specialized cells that underlie the development of tissues and organs. Organs that have undergone differentiation

are where tissue-specific stem cells are located throughout the postnatal and adult phases of life. These cells play a crucial role in organ repair after damage (Kolios & Moodley, 2013). Stem cells may be divided into five types, including totipotent/omnipotent, pluripotent, multipotent, oligopotent, and unipotent, depending on how they were isolated (Pandey *et al.*, 2022). Totipotent or omnipotent stem cells have the capacity to differentiate into all cell types needed for adult development and can produce embryonic tissues. When compared to multipotent stem cells, pluripotent stem cells can develop into all three primary germ layers while multipotent stem cells can only differentiate into certain types of germ line tissues. Adult organ tissues often include oligopotent or unipotent stem cells, which have chosen a cell lineage and are only capable of differentiating into cells from that lineage (Kang *et al.*, 2016; Poliwoda *et al.*, 2022; Ilic & Polak, 2011; Kolios & Moodley, 2013). The capacity to differentiate along many lineages and the capacity for self-renewal are two characteristics of stem cells. There are two main categories of stem cells: adult stem cells and embryonic stem cells. Stromal cells known as MSCs exhibit multipotential differentiation and may self-renew. Several different tissues, including the umbilical cord, endometrial polyps, menstrual blood, bone marrow, adipose tissue, etc. can provide mesenchymal stem cells (Ding *et al.*, 2011; Deng *et al.*, 2016). Mesenchymal stromal cells (MSCs), which are self-renewing non-hematopoietic pluripotent stem cells, are currently the subject of extensive clinical studies. Since Frieden Stein first identified MSCs in bone marrow in the 1970s, MSCs have been isolated from a variety of tissues, including muscle, umbilical cord, liver, placenta, skin, amniotic fluid, synovial membrane and roots, and has been studied extensively. in both preclinical and clinical studies. MSCs are now understood to have a wide range of physiological effects, along with the maintenance of tissue homeostasis and regrowth as well as immunomodulatory actions suitable for therapeutic application (Zhou *et al.*, 2021; Midha *et al.*, 2021; Ullah *et al.*, 2015; Reagan & Kaplan, 2011). It is possible to recognise MSCs by their adherence to the bottom of the flask, ability to differentiate into osteoblasts, chondroblasts, and adipocytes, as well as the expression of specific surface CD90, CD105, CD73, and CD44 markers. The lack of CD34 and HLA-DR are further indicators (Ranjbaran *et al.*, 2018). As Amniotic Fluid may be obtained during amniocentesis, which is typically carried out between the 16th and 28th weeks of pregnancy, the majority of research

examining amniotic fluid mesenchymal stem cells (AF-MSCs) in humans use cells from the mid-trimester of pregnancy (Moschidou *et al.*, 2013). Another interesting source of MSCs has been identified as the human umbilical cord, the majority of which contains Wharton's jelly. Wharton's jelly intervacular, perivascular, and subamniotic regions may all be used to identify mesenchymal stem cells. These cells are also known as umbilical cord connective tissue or matrix-derived MSCs (Messerli *et al.*, 2016). Wharton's jelly-derived mesenchymal stem cells, in contrast to adult MSCs, have recently sparked a great deal of interest for allogeneic or autologous application due to their advantageous qualities, such as their limitless availability, low cost, large quantities, simple and non-invasive isolation procedure, and higher proliferative capacity (Abbaszadeh *et al.*, 2020). Cells seeded on two-dimensional (2D) hard surfaces or animal models are typically used to research biological concerns. Animal models may be difficult to adopt and monitor spatiotemporal cell behaviour, especially with regard to human relevance, whereas 2D cultures may be examined with human cells but are non-physiological with regards to biophysical qualities and cellular structure (Benam *et al.*, 2015). The use of soft materials to enclose and cultivate cells in three dimensions (3D) is growing, as demonstrated by Matrigel, which has enhanced the culture and range of in vivo-like collective cell activities within organoids (Daly *et al.*, 2021). In the rapidly developing field of biofabrication, tissue constructs with a hierarchical design are made. Particulate leaching, freeze-drying, electrospinning, and microengineering are a few examples of traditional biofabrication methods. A revolutionary biofabrication technique that has recently gained popularity is 3D bioprinting, which offers substantially better control over the architecture of the generated tissue structures and great repeatability thanks to the automated deposition process (Ozkerim *et al.*, 2018; Zhang *et al.*, 2016; Zhang *et al.*, 2017). In general, the three primary 3D bioprinting techniques are extrusion-based bioprinting, inkjet bioprinting/droplet bioprinting, and laser-assisted bioprinting (Wang *et al.*, 2015). Our research aims to build methods for using chitosan as hydrogel in stem cell culturing and its application in 3D bioprinting.

CHAPTER 2

2. LITERATURE SURVEY

2.1. Biopolymer

The Greek terms for nature or living things, bio and polymer, are the source of the English word "biopolymer." Large macromolecules known as biopolymers include several repeating components. Typically, living things form the lengthy chains that make up polymeric macromolecules. These monomers units are connected by covalent bonding to create massive structures. DNA, RNA, fatty acids, gelatin, keratin, cellulose, starch, and others are some examples of biopolymers (Singh *et al.*, 2021; Ezeoha & Ezenwanne, 2013; Ali *et al.*, 2013). While more complicated, biopolymers' structures are clearly characterised. They also possess functional qualities, degradability, and renewability traits. Yet, effective functionalization can greatly enhance their qualities despite their weak mechanical properties, decreased producibility, and dependence on several environmental conditions (Bibire *et al.*, 2022; Augustine *et al.*, 2013; Niaounakis, 2015; Rebelo *et al.*, 2017). In contrast to artificial plastics, these polysaccharides have been present on Earth for thousands of years. Biopolymers are structured in a specific structure as opposed to the similar and random configurations of synthetic polymers. In order to create smaller chains, big chains in biodegradable polymers must first be broken down by biological forces (Thomas *et al.*, 2020). Due to the ongoing depletion of petroleum supplies, interest in polysaccharides, one of the most common natural polymers, has increased. Polysaccharide studies in both the public and private sectors will accelerate the growth of present uses and broaden their applicability in new areas. Polysaccharides have been one of the most widely used biomaterials for biomedical applications because to its exceptional benefits, including biocompatibility, renewability, biodegradability, and nontoxicity (Liu *et al.*, 2021; Meier *et al.*, 2017; Calvino *et al.*, 2020).

2.2. Chitosan

Chitin, a naturally occurring polysaccharide, is the source of chitosan. French scientist Henri Braconnot first extracted and described chitin from mushrooms in 1811 (Ghormade *et al.*, 2017). Throughout the entire globe, it is the second-most common biopolymer. The most prevalent polysaccharide in nature (apart from

celluloses) is chitin, which is also found in nematodes, the cell walls of yeast and fungus, and as the primary component of the exoskeleton of insects and crustaceans (Yadav *et al.*, 2015). One of the most promising natural biopolymers for uses including tissue engineering, biocompatible coatings, and drug delivery systems is chitosan, a cationic polysaccharide. Due to these outstanding qualities, interest in using chitosan-based composite coatings has been steadily increasing. (Avcu *et al.*, 2019). For the use of chitosan in biomaterials, substantial research has recently been conducted. A chitosan-mixed dressing has been used in medicinal biomaterials, as documented in several studies. It has been demonstrated that a chitosan composite film with the addition of glycerol has excellent mechanical and antibacterial qualities, may enhance fibroblast attachment and proliferation, and is an effective therapy for many types of skin injury (Li *et al.*, 2017).

2.3. Chitosan-based hydrogels

Out of other polymers, chitosan, naturally occurring cationic copolymer that holds a lot of promise for hydrogel architectures. This polymer's hydrophilic character and ability to be broken down by human enzymes provide it the biocompatibility and biodegradability that are typically required for biological devices (Ahmadi *et al.*, 2015). Hydrogels made of chitosan have the potential to be used as engineering scaffolds for successful tissue restoration. Few studies on chitosan-based hydrogel in bone tissue engineering has found to improve bone regeneration, encourage periodontal tissue engineering, and stimulate angiogenesis and vascularization (Tang *et al.*, 2020; Ressler *et al.*, 2022). Lindborg *et al.*, (2015) have reported that this hydrogel is distinct because it is made of polyelectrolytic complex (PEC) fibres, which are produced when chitosan and hyaluronic acid combine. A hydrogel with an elastic modulus of 264 ± 38 Pa quickly generated with cells dispersed throughout the matrix when hydrated with fetal bovine serum (FBS) containing human mesenchymal stem/stromal cells. A lattice-like meshwork of PEC fibres generating asymmetrical compartments was visible under scanning electron microscopy. In the first 24 hours, human mesenchymal stem/stromal cells were 48% viable; beyond that, cell populations reached a stable state for 14 days. In the matrix, chondrogenic, osteogenic, and adipogenic phenotypes of human mesenchymal stem/stromal cells were

produced. During days 56 and 70, emerging characteristics included chondrogenesis on the surface of hydrogels driven to osteogenic and adipogenic phenotypes. As a result, this matrix may be beneficial for applications like tissue regeneration and clinical diagnostics. Xu *et al.*, (2018) reported that the in-situ hydrogel was very transparent, gelled fast, biocompatible, and had minimal cytotoxicity. In vitro-cultured limbal stem cells (LSCs) displayed the stem marker p63 but lacked the differentiated epithelial markers cytokeratin 3 and 12. In addition, the hydrogel encasing LSCs could be swiftly generated on the corneal alkali burn lesion and was demonstrated to greatly enhance epithelial restoration. Together, the use of this innovative in situ hydrogel-mediated LSC transplantation technology may be a quick and efficient way to cure corneal wounds. According to Linh *et al.*, (2017) the enzymatic crosslinking employing hydrogen peroxide as an oxidant and horseradish peroxidase as a catalyst was used for in-situ gelation. Controlling the concentration of polymers led to gel formation in 30-90 s. Based on the in vitro experiment and subcutaneous implantation into BALB/c-nu/nu nude female mice, platelet-derived growth factor release demonstrated sufficient release kinetics within the anticipated time frame, and human adipose-derived stem cells (hADSC) demonstrated satisfactory compatibility with the hydrogel formulation. The viability of the given hADSC was established by immunohistochemical investigation. Histological examination verified the development of blood vessels and revealed no immunological response. The findings suggest that the hydrogel is a suitable means of cell and growth factor distribution or transportation, supporting vascularization for tissue engineering applications.

2.4. Wharton's jelly MSCs and its applications:

Mesenchymal Stem cell (MSCs) have drawn a lot of attention over the past 30 years due to their intriguing cell biology, extensive therapeutic potential, and role as a key component in the rapidly expanding area of tissue engineering (Pittenger *et al.*, 2019). Spindle-shaped cells having the capacity for multiplication and self-renewal are mesenchymal stem cells, often referred to as mesenchymal stromal cells (Jiang & Xu, 2020). Several illnesses, including both immunological and nonimmunological problems, have a great deal of promise for MSC therapy. Even though a consistent mechanism regulating MSC-based therapy is still being

identified, the evidence at hand has shown many operational theories that support the positive benefits of MSCs (Squillaro *et al.*, 2016). Umbilical cord as it may be obtained painlessly and in large quantities from millions of births globally with no donor site morbidity, Wharton's jelly looks to be one of the most appropriate sources of allogeneic MSCs for MSC treatment. As compared to adult MSCs, Wharton's jelly-derived MSCs are less diverse, have a high proliferation rate, the capacity to maintain their stemness features for several passages in vitro, and are hypoimmunogenic and nontumorigenic (Liau *et al.*, 2019). Wharton jelly contains a significant amount of umbilical cord mesenchymal stem cells (UCMSCs), about 400,000 cells per umbilical cord, compared to 1 mesenchymal stem cell per 10,000 nucleated bone marrow cells. The human umbilical cord, which joins the growing embryo or foetus to the placenta, serves as a conduit between the mother and the foetus throughout pregnancy. It has a term length of 3065 cm, a weight of around 40 g, and an average diameter of 1.5 cm. It begins to form at day 26 of pregnancy from the yolk sac and allantois, and its primary jobs are to nourish the foetus with blood and remove biological waste. The lining of the cord covers Wharton's jelly, which has an exterior layer of umbilical epithelium made up of one or more layers of epithelial cells that are thought to be of amniotic origin and display a cytokeratin pattern matching that of the epidermis of a human being (Stefańska *et al.*, 2020; Arutyunyan *et al.*, 2016; Lim & Phan, 2014).

2.5. 3D Bioprinting

In nature, chitosan occurs abundantly in chitin, which is a polymer made up of N-acetylglucosamine found in crustaceans, most notably crabs and shrimps (Song *et al.*, 2012; Komi *et al.*, 2016). The bioprinting technology allows for the development of devices to deliver medications, including growth factors, proteins, and drugs, by replacing damaged or harmed tissues (Bishop *et al.*, 2017). The use of 3D printing and bioprinting is an effective method for generating biological components or therapeutic delivery systems in a reduced time than conventional methods (Papaioannou *et al.*, 2019; Lazaridou *et al.*, 2022). An innovative approach for automating construct fabrication, ensuring high reproducibility and precision. The biodegradability, biocompatibility, low cost, and non-immunogenic properties of chitosan make it an attractive candidate for use as ink. The viscosity of polymer solutions and printability can be significantly affected by bioinks when

they are rheologically and mechanically characterized in the presence of cells (Schwab *et al.*, 2020; He *et al.*, 2020). A chitosan-based hydrogel scaffold has been prepared for tissue regeneration using 3D bioprinting technology (Lazaridou *et al.*, 2022; Rajabi *et al.*, 2021; Taghizadeh *et al.*, 2022). In addition to their biocompatibility and low cytotoxicity, bioinks made from chitosan exhibit high natural characteristics (Ozkerim *et al.*, 2018). There are a number of issues with chitosan-based hydrogels for bioinks, including poor mechanical properties, sterilization difficulty, and reproducibility issues (Ahmadi *et al.*, 2015; Croisier *et al.*, 2013). The performance and integrity of chitosan could be improved by adding chemical agents, irradiating the material, adding thickening molecules, and even modifying the molecule itself. These modifications can enhance the properties of chitosan, such as improving its mechanical strength, increasing its flexibility, and introducing new functional groups for improved biological interactions (Mikušová *et al.*, 2021; Islam *et al.*, 2020). Butler *et al.* (2020) determined 50% starch and 50% chitosan is the optimal composition for bio-ink that is both printable and biocompatible with Neuro-2a cells and hence confirmed that increasing the chitosan percentage enhances the biocompatibility. There are a large number of 3D printing technologies that can utilize chitosan-based systems due to their wide range of properties (Pahlevanzadeh *et al.*, 2020). A number of modifications relying on chitosan's functional groups and its combination with other substances could enhance its properties. A very promising future for 3D printed products can be predicted by incorporating active substances as drugs or cell cultures (Yang *et al.*, 2022; Mao *et al.*, 2020).

2.6. Applications of 3D bioprinting in Tissue engineering:

Researchers have been searching for alternate methods to make artificial tissues and organs function as the need for tissue transplantation grows. Combining these materials is very interesting for tissue scaffolding and regenerative medicine applications due to the distinctive physicochemical and biological properties of biopolymers and the alluring structural qualities of aerogels like extremely high porosity, ultra-low density, and high surface area. By cultivating appropriate cells along with the growth factor in tissue engineering scaffolds, it has been possible to regenerate skin, cartilage, bone, heart valves, and even blood vessels (O'Brien *et al.*, 2016). In a study, the use of a limbal epithelial cell population that contains

stem cells to grow and expand on a biopolymer substrate comprised of chitosan that has been crosslinked with gelatin. Their findings show that chitosan to gelatin ratio of 20:80 membranes are acceptable for the growth and maintenance of limbal niche-derived stem cells and can be used as an alternate substratum for the transplantation of grown limbal cells (Yahya *et al.*, 2021). Another biopolymer named, fibrin-based scaffold works well as a temporary platform to encourage angiogenesis, connective tissue development, cell migration, and proliferation. In order to cure a critical-size bone lesion, a novel heterologous fibrin biopolymer as a scaffold for mesenchymal stem cells (MSCs) was tested. Fibrin based scaffold determined to be a great scaffold for MSCs, and can be used in studies on regenerative medicine. The fibrin-based scaffold ability to keep viable MSCs at the location of the bone defect has changed the inflammatory environment, speeding up their regeneration (Mata *et al.*, 2013). In a study, order to create concentration gradients, growth factors rhBMP-2 and rhIGF-I can be functionally encapsulated in either PLGA or silk microspheres and then added to alginate or silk scaffolds. The two microsphere systems displayed distinct growth factor loading and release properties depending on the carrier matrices and the microencapsulation procedure, which have an impact on hMSC osteochondral development differentially. Alginate gel scaffolds were outperformed by silk sponge-like scaffolds (Wang *et al.*, 2009).

CHAPTER 3

3. AIM AND SCOPE

3.1 AIM OF THE PROJECT

The project's objective is that to prepare chitosan from crab shell waste which can be effectively used as hydrogel for stem cell culturing and other applications.

3.2 SCOPE OF THE PROJECT

- Extraction of chitin and chitosan from mud-crab shell waste
- Production of chitin cups
- Synthesis of Chitosan nanocarriers
- Preparation of chitosan hydrogel
- Culturing of Wharton's Jelly-Mesenchymal Stem Cells over chitosan hydrogel

CHAPTER 4

MATERIALS AND METHODOLOGY

4.1 COLLECTION OF SAMPLES

The mud crab waste was collected in the local market, Selangor, Malaysia.

4.2 EXTRACTION OF CHITIN

4.2.1 MATERIALS REQUIRED

Hydrochloric acid (HCl), Sodium Hydroxide (NaOH) and acetone. All chemicals used were analytical grade.

4.2.2 PROCEDURE

The mud crab shells were washed, dried and blended and were subjected to demineralization using HCl, deproteinization using NaOH and finally depigmentation using acetone (Metin *et al.*, 2019)

4.2.2.1 DEMINERALIZATION

- i. Initially, 2M of HCl was prepared by taking 14.584mL of HCl and made up to 100mL using distilled water.
- ii. The demineralization was carried out by adding 40g of powdered crab to the prepared HCl solution and heated for 24 hours at 80°C.

4.2.2.2 DEPROTEINIZATION

- i. For deproteinization, 2M of NaOH was prepared prior by adding 8g of salt to 100 mL of distilled water.
- ii. Further, the demineralized powder was then proceeded for deproteinization by adding 2M of NaOH in the ratio of 1:10.
- iii. It was heated at 110°C for 20 hours and the precipitate was collected.

4.2.2.3 DEPIGMENTATION

- i. The obtained precipitate was then washed using acetone for the removal of pigmentation.
- ii. It was dried until the acetone gets evaporated, and the precipitate was collected
- iii. The obtained precipitate is chitin.

4.2.3 YIELD ESTIMATION

The yield of the extracted chitin was estimated using the following equation:

$$\text{Yield\%} = (\text{Initial concentration} - \text{Final concentration}) / (\text{Initial concentration}) \times 100$$

where i) initial concentration is the weight of crab shell powder taken

- ii) final concentration is the weight of chitin obtained

4.3 EXTRACTION OF CHITOSAN

4.3.1 MATERIALS REQUIRED

NaOH of analytical grade and extracted chitin were used.

4.3.2 PROCEDURE

The chitosan was extracted by deacetylating the extracted chitin using NaOH (Metin *et al.*, 2019).

4.3.2.1 DEACETYLATION

- i. Firstly, the extracted chitin was added to 50% of NaOH in the ratio of 1:10 (w/v).
- ii. It was then heated at 150°C for 4 hours.
- iii. The deacetylated chitin was washed repeatedly using distilled water to neutralize the pH.
- iv. Chitosan was obtained.

4.3.3 YIELD ESTIMATION

The yield of the extracted chitosan was estimated using the following equation:

$$\text{Yield\%} = (\text{Initial concentration} - \text{Final concentration}) / (\text{Initial concentration}) \times 100$$

where i) initial concentration is the weight of extracted chitin taken

- ii) final concentration is the weight of chitosan obtained.

4.4 CHARACTERIZATION OF CHITOSAN

The extracted chitosan was then subjected to characterization like UV-Visible Spectroscopy (UV-Vis), Fourier Transform Infrared Spectroscopy (FTIR), Scanning electron microscopy (SEM) and Thermogravimetric Analysis (TGA).

4.5 BIOACTIVITY DETERMINATION OF CHITOSAN

4.5.1 ANTIOXIDANT ACTIVITY

- i. Prepare 0.1 mM DPPH solution as stock.
- ii. Take different concentration of nanocarrier (100µg/ml; 200µg/ml; 300µg/ml; 400µg/ml; 500µg/ml).
- iii. Add 1 ml of prepared DPPH solution to the test tubes.
- iv. Incubate for 30 minutes in dark temperature.
- v. Take reading at 517 nm.

4.5.2 Anticancer activity

4.5.2.1 MTT assay

Anticancer activity of the extracted chitosan was determined by MTT assay as described in Samrot *et al* (2019) and Mosman (1983).

Cell viability assay, MDA-MB-231 viable cells were harvested and counted using haemocytometer diluted in DMEM medium to a density of 1×10^4 cells/ml was seeded in 96 well plates for each well and incubated for 24 h to allow attachment. After MDA-MB-231 cells treated with control and the containing different concentrations were applied to each well. MDA-MB-231 cells were incubated at 37°C in a humidified 95% air and 5% CO₂ incubator for 24 h. After incubation, the drug-containing cells wash with fresh culture medium and the MTT (5 mg/ml in PBS) dye was added to each well, followed by incubated for another 4 h at 37°C. The purple precipitated formazan formed was dissolved in 100 µl of concentrated DMSO and the cell viability was absorbance and measured 540 nm using a multi-well plate reader. The results were expressed at the percentage of stable cells with respect to the control. The half maximal inhibitory concentration (IC₅₀) values were calculated and the optimum doses were analyzed at different time period.

Inhibitory of cell proliferation (%)

$$= \frac{\text{Mean absorbance of the control} - \text{Mean absorbance of the sample}}{\text{Mean absorbance of the control}} \times 100$$

The IC₅₀ values were determined from the dose responsive curve where inhibition of 50% cytotoxicity compared to control cells. All experiments were performed at least three times in triplicate.

4.5.2.2 Dual acridine orange/ethidium bromide (AO/EB) staining

MDA-MB-231 cells were seeded at 5×10^4 cells/well in a 6 well plate and incubated for 24 hours. After treatment with IC 50 concentrations for 24 h, the cells were detached, washed with cold PBS and then stained with a mixture of AO (100µg ml⁻¹) / EB (100µg ml⁻¹) ratio (1:1) at room temperature for 5 min. The stained cells were observed by a fluorescence microscope at 20x magnifications. At the end of treatment, the cells were collected and washed three times with PBS. The plates were stained with acridine orange/ethidium bromide (AO/EB 1:1 ratio; 100 µg/ml) for 5 minutes and examined immediately under fluorescent microscope 20x magnification.

The number of cells showing feature of apoptosis was counted as a function of the total number of cells present in the field.

4.5.3 Antibacterial activity

Antibacterial activity of the extracted chitosan was determined by well diffusion method as described in Samrot *et al* (2019).

4.6 PREPARATION OF CHITOSAN HYDROGEL

4.6.1 MATERIALS REQUIRED

Sodium alginate, acetic acid, calcium chloride (CaCl₂), glutaraldehyde, NaOH of analytical grade and extracted chitosan were used.

4.6.2 PROCEDURE

- i. Hydrogel was prepared by the method proposed by Bagher *et al.*, (2020)
- ii. 2% of sodium alginate solution was prepared by mixing 2g of sodium alginate in 100 mL of distilled water.
- iii. Simultaneously, 2% (w/v) chitosan was prepared in 0.5% (v/v) acetic acid and stirred for 24 hours at 40°C
- iv. Both the solution was then mixed together in the ratio of 2:1 (v/v)
- v. The solvent containing 50mM of CaCl₂ and 10μL of glutaraldehyde with 1M of NaOH was prepared.
- vi. The prepared chitosan-alginate solution was then added to the above solvent drop by drop using a serological pipette.
- vii. Chitosan hydrogel was obtained.

4.7 STEM CELL CULTURING

4.7.1 CULTURING OF STEM CELLS ON CHITOSAN HYDROGEL

4.7.1.1 MATERIALS REQUIRED

Dulbecco's Modified Eagle's Medium: nutrient mixture (DMEM), Fetal Bovine Serum (FBS), Streptomycin, chitosan hydrogel, cell line - Wharton's Jelly (MSC), and Inverted microscope (Nikon Eclipse TS100).

4.7.1.2 PROCEDURE

- i. Stem cell was cultured by using the following method (Bagher *et al.*, 2020)
- ii. DMEM supplemented with 10% (v/v) FBS and antibiotics like streptomycin were prepared and humidified in an incubator at 37 °C.
- iii. The cell line was then cultured at the density of 1×10^4 cells on hydrogel using the above prepared medium.

- iv. The growth of the stem cells was then observed using an inverted microscope at a regular time interval.

4.8 SYNTHESIS OF CHITOSAN NANOCARRIER

4.8.1 MATERIALS REQUIRED

Extracted chitosan, acetic acid, Barium chloride (BaCl_2), Polyethylene glycol solution (PEG). All chemicals are of analytical grade.

4.8.2 PROCEDURE

- i. Chitosan nanocarrier has been synthesized by ionic gelation method (Samrot *et al.*, 2018)
- ii. Chitosan was initially demineralized by mixing 0.2g of chitosan in 50 mL of acetic acid.
- iii. The solution was stirred vigorously for 10 minutes.
- iv. Next, curcumin solution was prepared by adding 0.1 g of curcumin to 25 mL of ethanol.
- v. Both the chitosan solution and curcumin solution were mixed together.
- vi. Further, 25 mL of 0.1% of BaCl_2 solution was made and mixed together with the curcumin-chitosan solution and stirred for 10 minutes.
- vii. Finally, 25 mL of 0.4% of PEG solution was prepared and added to the above mixture drop by drop while stirring and kept aside for 1 hour.
- viii. The mixture was then centrifuged at 5000 rpm for 10 minutes.
- ix. Chitosan nanocarrier was obtained.

4.8.3 CHARACTERIZATION OF CHITOSAN NANOCARRIER

The synthesized chitosan was then subjected to characterization like UV-Visible Spectroscopy (UV-Vis), Fourier Transform Infrared Spectroscopy (FTIR), Scanning electron microscopy (SEM), X-Ray Diffraction (XRD) and Zeta Potential.

4.8.4 DRUG DELIVERY KINETICS

- i. Drug delivery kinetics method was done by using the following method (Samrot *et al.*, 2020).
- ii. Add 10 mg of loaded nanocarrier into the dialysis bag.
- iii. Submerge the dialysis bag into the 200 ml of PBS buffer.
- iv. For every 15 min (3hrs) take the reading using spectrophotometer (425 nm).

- v. Plot the graph against absorbance and time interval.

4.9 BIOACTIVITY DETERMINATION OF CHITOSAN NANOCARRIER

4.9.1 ANTIOXIDANT ACTIVITY

- i. Prepare 0.1 mM DPPH solution as stock.
- ii. Take different concentration of nanocarrier (100µg/ml; 200µg/ml; 300µg/ml; 400µg/ml; 500µg/ml).
- iii. Add 1 ml of prepared DPPH solution to the test tubes.
- iv. Incubate for 30 minutes in dark temperature.
- v. Take reading at 517 nm.

4.9.2 ANTICANCER ACTIVITY

4.9.2.1 MTT assay

Anticancer activity of the extracted chitosan was determined by MTT assay as described in Samrot *et al* (2019) and Mosman (1983).

Cell viability assay, MDA-MB-231 viable cells were harvested and counted using haemocytometer diluted in DMEM medium to a density of 1×10^4 cells/ml was seeded in 96 well plates for each well and incubated for 24 h to allow attachment. After MDA-MB-231 cells treated with control and the containing different concentrations were applied to each well. MDA-MB-231 cells were incubated at 37°C in a humidified 95% air and 5% CO₂ incubator for 24 h. After incubation, the drug-containing cells wash with fresh culture medium and the MTT (5 mg/ml in PBS) dye was added to each well, followed by incubated for another 4 h at 37°C. The purple precipitated formazan formed was dissolved in 100 µl of concentrated DMSO and the cell viability was absorbance and measured 540 nm using a multi-well plate reader. The results were expressed at the percentage of stable cells with respect to the control. The half maximal inhibitory concentration (IC₅₀) values were calculated and the optimum doses were analyzed at different time period.

Inhibitory of cell proliferation (%)

$$= \frac{\text{Mean absorbance of the control} - \text{Mean absorbance of the sample}}{\text{Mean absorbance of the control}} \times 100$$

The IC₅₀ values were determined from the dose responsive curve where inhibition of 50% cytotoxicity compared to control cells. All experiments were performed at least three times in triplicate.

4.9.2.2 AO/EB staining

MDA-MB-231 cells were seeded at 5×10^4 cells/well in a 6 well plate and incubated for 24 hours. After treatment with IC 50 concentrations for 24 h, the cells were detached, washed with cold PBS and then stained with a mixture of AO ($100\mu\text{g ml}^{-1}$) / EB ($100\mu\text{g ml}^{-1}$) ratio (1:1) at room temperature for 5 min. The stained cells were observed by a fluorescence microscope at 20x magnifications. At the end of treatment, the cells were collected and washed three times with PBS. The plates were stained with acridine orange/ethidium bromide (AO/EB 1:1 ratio; $100\mu\text{g/ml}$) for 5 minutes and examined immediately under fluorescent microscope 20x magnification.

The number of cells showing feature of apoptosis was counted as a function of the total number of cells present in the field

4.9.3 ANTIBACTERIAL ACTIVITY

The antibacterial activity of chitosan, curcumin-unloaded chitosan nanocarrier, and curcumin-loaded chitosan nanocarrier was evaluated in vitro against Gram-negative bacteria (*Pseudomonas aeruginosa*) at varying concentrations was done.

CHAPTER – 5

RESULTS AND DISCUSSION

5.1 EXTRACTION OF CHITIN

The extraction of chitin has been done using the chemical method by undergoing demineralization followed by deproteinization and depigmentation (Figure 1) (Metin *et al.*, 2019).

5.1.1 YIELD ESTIMATION

The yield of the extracted chitin was estimated using the formula proposed by Demir *et al.*, (2016). The yield percentage of the extracted chitosan was found to be 90% (Figure 2) which is 2 times higher than the yield obtained by Sarbon *et al.*, (2015) whose yield percentage is said to be 44.57 ± 3.44 %. Also, Doğdu *et al.*, (2021) extracted chitin from *Charybdis longicollis* with a lesser percentage of 25.78 %.

5.2 EXTRACTION OF CHITOSAN

The chitosan was successfully extracted by subjecting the extracted chitin to deacetylation using NaOH (Figure 3) (Metin *et al.*, 2019).

5.2.1 YIELD ESTIMATION

The yield of the extracted chitosan was estimated to be 75% using the formula proposed by Demir *et al.*, (2016). A similar yield percentage of 71.3% was reported by Duraisamy *et al.*, (2022). Chitosan was extracted from Blue Crab *Portunus segnis* and reported to have a yield percentage of 52% (Jabeur *et al.*, 2022) (Figure 4).

5.3 CHARACTERIZATION OF CHITOSAN

5.3.1 UV-VISIBLE SPECTROSCOPY (UV-Vis)

The preliminary test for the identification of chitosan was done using UV-Vis Spectroscopy (Figure 5). The absorption peak around 200 to 300nm depicts the presence of chitosan (Coccean *et al.*, 2022). Similarly, the presence of chitosan in the chitosan treated cellulose is determined by the distinctive peak below 220nm (Kumirska *et al.*, 2010). Also, Hai *et al.*, (2018) observed no absorption peaks in the wavelength range of 240–880 nm for chitosan.

5.3.2 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

The functional group present in the extracted chitosan was analyzed using FTIR analysis between the frequency range of 500 cm^{-1} and 4500 cm^{-1} (Figure 6). The sharp peak at 1408.04 cm^{-1} with an intensity of 27.66 shows the presence of organic compounds like C-H stretching bonds in chitosan. Also, peaks at 3691.75 cm^{-1} , 3261.63 cm^{-1} , and 869.90 cm^{-1} show the stretching of OH bond, stretching of N-H bond and vibration of CH_2 bond respectively. The presence of functional groups like C (carbon), N (nitrogen), and O (oxygen) is indicated by vibration patterns acquired from the FTIR spectrum of chitosan extracted from *Modiolus modiolus*. Zam *et al.*, (2021) depicted the presence of the stretching band for the -OH group and the -NH group in the range of $3000\text{--}3700\text{ cm}^{-1}$, and the OH absorption band is confirmed by the peak at 3448.72 cm^{-1} in the extracted chitosan.

5.3.3 X-RAY DIFFRACTION (XRD)

The structure of the chitosan was examined using XRD analysis (Figure 7). The sharp peaks in the $2\theta = 9.74^\circ$, 19.24° , and 29.49° with the respective intensities of 107, 124, and 213 show the crystalline structure of the extracted chitosan. In general, all kinds of chitosan exhibit two crystalline peaks that are roughly at 10° and 20° in their XRD patterns (Julkapli *et al.*, 2010). Similarly, Dey *et al.*, (2016) depicted that the peaks around 9.63° and 20.53° are associated with crystal-I and crystal-II in the structure of chitosan and its high degree of crystallinity.

5.3.4 SCANNING ELECTRON SPECTROSCOPY AND ENERGY DISPERSIVE X-RAY ANALYSIS (SEM-EDX)

The surface morphology and the elementary analysis of the extracted chitosan was examined using SEM and EDAX analysis respectively (Figure 8). The extracted chitosan had a smooth irregular shape with the elemental composition of carbon (C), oxygen (O) and a trace amount of calcium (Ca). Rasti *et al.*, (2017) explained the microfibrillar structure of chitosan, with some sections of the fibres accumulating in its crystalline particles. An additional element known as iron (Fe) is reported to be present in chitosan along with carbon (C) (Rasti *et al.*, 2017). Earlier, it was depicted that pure chitosan film was found to have a smooth and irregular surface structure with nonporous and a smooth, pore-less texture (Bhuvaneshwari *et al.*, 2011).

5.3.5 THERMOGRAVIMETRIC ANALYSIS (TGA)

The TGA curve of pure chitosan reveals that there are two stages of weight loss, the first of which takes place in the range of 47 to 450 °C due to the loss of water molecules and results in a weight loss of around 9%. Pure chitosan began to break down primarily around 247 °C and finished degrading at about 450 °C with a weight loss of roughly 34% (Kumar and Koh, 2012). The evidence that the degradation of chitosan begins at temperatures lower than 250°C along a complex pathway is made possible by the reduction of the heating rate from 10 to 2°C/min (Corazzari *et al.*, 2015) (Figure 9).

5.4 BIOACTIVITY DETERMINATION OF CHITOSAN

5.4.1 ANTIOXIDANT ACTIVITY

DPPH assay was performed to determine free radicals scavenging ability of chitosan (Remya *et al.*, 2022). Higher scavenging activity was observed in chitosan with the percentage of 90.72% in 400 µg/mL. Similarly, Chen *et al.*, (2021) reported that *P. Americana* chitosan films had a significantly higher antioxidant activity. Luan *et al.*, (2018) reported that there was 100% of antioxidant activities in dipropylene sulfonated chitosan (Figure 10).

5.4.2 ANTICANCER ACTIVITY

5.4.2.1 MTT Assay

The extracted chitosan showed a better antitumor activity with IC₅₀ concentration of 23µg by triggering M1 macrophage polarisation, and changing the immunosuppressive tumour milieu into an immunosupportive state, exerting an anticancer effect and enhancing the effectiveness of cancer immunotherapy (Zeng *et al.*, 2021; Ding *et al.*, 2022) (Figure 11). Adhikari and Yadav (2018) have also reported to exhibit better anticancer activity of chitosan by breaking the cell membrane of cancer cells via cellular enzymatic activity, immune-enhancing pathways, antiangiogenic, antioxidant defense mechanism, and apoptotic pathways.

5.4.2.2 AO and EB staining

Using AO and EB staining, anticancer activity of chitosan was estimated where green fluorescence indicates living cells without apoptosis, yellow fluorescence indicates early apoptotic cell death and orange fluorescence shows indicates late apoptotic cell death with condensed or fragmented nuclei and necrotic cells.

Higher yellow and orange fluorescence were observed indicating distinct apoptotic and necrotic cells on the exposure to chitosan (Figure 12 a, b). Hence, chitosan is determined to have a better anticancer activity.

5.4.3 ANTIBACTERIAL ACTIVITY

Antibacterial activity of chitosan was determined by dissolving it in acetic acid of various concentration like 10 µl, 20 µl, 40 µl and 60 µl. Chitosan was observed to possess antibacterial activity against *Pseudomonas aeruginosa* with the zone of inhibition of 12 mm. The zone of inhibition was observed to be directly proportional to the concentration of chitosan (table 1 and Figure 13). Divya *et al.*, (2017) proposed that chitosan exhibit antibacterial activity by disrupting the cell membrane of bacteria and thus releasing in the intracellular components.

5.5 SYNTHESIS OF CHITOSAN HYDROGEL

The chitosan hydrogel has been successfully synthesized using the methodology proposed by (Bagher *et al.*, 2020) where the produced hydrogel was soft and irregular in shape (Figure 14).

5.6 CHARACTERIZATION OF CHITOSAN HYDROGEL

5.6.1 SCANNING ELECTRON SPECTROSCOPY AND ENERGY DISPERSIVE X-RAY ANALYSIS (SEM-EDX)

SEM analysis can indicate chitosan hydrogel miscibility and polymer mix compatibility with hydrogel matrix constituents. These chitosan hydrogel found to be regular in shape with high porosity ranging between 344.7 to 612.2 nm (Figure 15). Further, EDAX analysis showed the presence of elements like carbon (C) and nitrogen (N). Thirupathi *et al.*, (2022) synthesized hydrogel containing microporous structure with pore dimensions ranging from 10 m to 50 m depending on the water concentration.

5.7 CULTURING OF STEM CELLS ON CHITOSAN HYDROGEL

The high porous structure of the synthesized chitosan hydrogel facilitated the growth of WJ-MSCs via absorption of required nutrients in it. Also, it also showed an excellent adherence of the cell on it with higher cell viability. Due to the degradability nature of these hydrogel, the usage of trypsin can also be avoided (Figure 16). Similarly, Couce *et al.*, (2022) reported that chitosan hydrogels

permitted the preservation of the phenotypic expression of chondrocytes like cell shape and their chondrogenic properties. These cells maintained their spheroid morphology and adhered to the hydrogel surfaces with no abnormality in cell shape cells (Couce *et al.*, 2022). Liu *et al.*, (2020) showed that these chitosan hydrogel can be used in the treatment of Myocardial Infarction by improving the therapeutic efficacy of Bone Marrow-Derived Mesenchymal Stem Cells and reducing the vascular endothelial cell pyroptosis.

5.8 SYNTHESIS OF CHITOSAN NANOCARRIER

The extracted chitosan was further subjected to the synthesis of nanocarrier for drug delivery (curcumin). The chitosan nanocarrier was successfully synthesized using the ionic gelation method (Figure 17) (Samrot *et al.*, 2018).

5.9 CHARACTERIZATION OF CHITOSAN NANOCARRIER

5.9.1 UV-VISIBLE SPECTROSCOPY (UV-Vis)

The synthesized chitosan nanocarrier was tested using UV-Vis analysis (Figure 18). The broad peak at 250 nm corresponds to the π - π^* transition of the synthesized chitosan nanoparticles and the peak at 441 nm in Figure 17b depicts the encapsulation of curcumin inside the chitosan nanoparticles forming a nanocarrier (Thamilarasan *et al.*, 2018; Nong *et al.*, 2016). Similarly, Rajan *et al.*, (2016) synthesized curcumin loaded in BSA–chitosan nanoparticles where the peaks at 422 nm, 419.4 and 419.9 nm depicted the presence of curcumin, BSA-chitosan nanoparticles and PEGylated BSA-chitosan nanoparticles respectively (Rajan *et al.*, 2016).

5.9.2 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

The functional groups of both curcumin unloaded and loaded chitosan nanocarrier was identified using FTIR analysis (Figure 19) where the peak at 1006 cm^{-1} indicates the presence of P=O in nanocarrier and the sharp peak at 1413 cm^{-1} and 1550 cm^{-1} depicts the $-\text{CH}_2$ and N–O bonds respectively (Lustriane *et al.*, 2018; Anand *et al.*, 2018). In curcumin loaded chitosan nanocarrier, the peak at 1417 cm^{-1} shows the enol group of curcumin (Ismail *et al.*, 2014). Curcumin-loaded chitosan nanocarrier synthesized by Samrot *et al.*, (2018) showed distinctive peaks between 1153 cm^{-1} and 1120 cm^{-1} representing $-\text{OCH}_3$ functional group of curcumin and for the drug-unloaded samples, peaks at 1314 – 132 cm^{-1} were

observed showing the C-N stretching vibrations of amine. Ruman *et al.*, (2021) synthesized Sorafenib-loaded folate-coated chitosan nanoparticles for human Hepatocellular and Colorectal Adeno-carcinoma where the band at 1292 cm^{-1} and 1124 cm^{-1} is caused by the presence of the carboxylic acid present in Sorafenib, and the band at 678 cm^{-1} is attributable to the C-Cl stretching of Sorafenib. These bands show that the chitosan nanoparticles have been loaded with drugs (Ruman *et al.*, 2021).

5.9.3 SCANNING ELECTRON MICROSCOPY (SEM) AND ENERGY DISPERSIVE X-RAY ANALYSIS (EDAX)

Through SEM analysis, the size of the loaded nanocarrier was determined to be ranging from 162.2 to 240.4 nm with the composition of elements like carbon (C) and oxygen (O) indicating the encapsulation of curcumin inside the nanocarrier (Figure 20 a, b). Similarly, FE-SEM characterization showed the size ranging from 150 to 210 nm for CSNPs and from 180 to 400 nm for Cur-CSNPs (Bhoopathy *et al.*, 2021). Also, Prasad *et al.*, (2022) examined the morphology of curcumin loaded chitosan nanoparticles where the nanoparticles were found to be spherical in shape and had a smooth surface ranging in size from 114 to 160 nm.

5.9.5 ZETA POTENTIAL

The stability of the unloaded and loaded nanocarrier was estimated using zeta potential whose net charge were -8.51mV and -28.87mV respectively (Figure 21 a, b). These negative net charge facilitates improved stability during blood circulation and least non-specific adsorption to protein like bovine serum albumin (Chen *et al.*, 2017). Iswanti *et al.*, (2019) synthesized a chitosan nanocarrier for the delivery of CpG ODN, whose zeta potential of nanocarrier changed from $+3.30\pm 4\text{ mV}$ to $-11.3\pm 1.5\text{ mV}$ after drug delivery. Contrarily, curcumin-Loaded Chitosan nanoparticles synthesized for the treatment of Celecoxib-induced Toxicity had a positive net charge of $53.5 \pm 3.7\text{ mV}$ (Ebrahimi *et al.*, 2022)

5.9.6 DRUG DELIVERY EFFICACY OF CHITOSAN NANOCARRIER

Drug release was to be slow and steady and even releasing for 3 hours (Figure 22). Samrot *et al* (2018) found the similar pattern of drug release by crab shell derived chitosan. Also, Adhikari and Yadav (2018) have proposed that this chitosan nanocarrier is also good candidate for oral administration drugs due to their sustained drug released.

5.10 BIOACTIVITY DETERMINATION OF CHITOSAN NANOCARRIER

5.10.1 ANTIOXIDANT ACTIVITY

Antioxidant activity of drug loaded of nanocarrier was found to more in drug loaded nanocarrier than the unloaded carrier (Figure 23). Samrot *et al.*, (2023) reported that the percentage of scavenging activity was 47.03% and 45.34% at 500 µg concentration for *A. heterophylla* and *P. chilensis* respectively.

5.10.2 ANTICANCER ACTIVITY

5.10.2.1 MTT Assay

The anticancer activity was found to be more with the drug loaded nanocarrier with IC₅₀ concentration 15 µg than the unloaded nanocarrier whose anticancer activity was determined to be 23 µg (Figure 24). Similarly, Ruman *et al.*, (2021) reported that Sorafenib -loaded chitosan nanoparticles had anticancer activity against HepG2 Cells with the IC₅₀ concentration of 20.3 ± 1.5 µg/mL.

5.10.2.2 AO and EB staining

AO/EB staining was performed to visualize the apoptotic activity of cancer cells induced by synthesized curcumin unloaded and loaded chitosan nanocarrier (Figure 25 and 26). Anticancer activity of loaded nanocarrier was observed to be higher than that of unloaded nanocarrier. Additionally, the activity was found to be increased with increase in concentration. Morphological alterations like cell membrane detachment nuclear fragmentation, and blebbing of cell membrane were also seen under confocal fluorescence (Figure 27 and 28). Similarly, curcumin-loaded chitosan coated hydroxyapatite nanocarrier reported to induce anticancer activity via nuclear condensation, apoptotic body formation, nuclear migration, DNA fragmentation and chromatin coagulation (Hemmati *et al.*, 2021).

5.10.2 ANTIBACTERIAL ACTIVITY

The antibacterial activity of drug-unloaded chitosan nanocarrier and drug-loaded chitosan nanocarrier against Gram-negative bacteria *Pseudomonas aeruginosa* by dissolving in two different solvents (distilled water and acetic acid) were examined (Figure 29 & 30). The antibacterial activity of the curcumin-loaded nanocarrier dissolved in acetic acid was determined to have higher activity with a zone inhibition of 17mm than the chitosan and drug-unloaded nanocarrier. Since chitosan is only partially soluble in water, samples that have been dissolved in

distilled water showed no activity (Table No. 2). Previously, it was reported that curcumin-loaded nanoparticles has the ability to inhibit the growth of gram-positive bacteria such as *Staphylococcus aureus* (Jahromi *et al.*, 2015). Alavi *et al.*, (2022) synthesized curcumin-gelatin- chitosan nanoparticles which showed similar results with zone of inhibition of 24 mm and 18mm against *Escherichia coli* and *Staphylococcus aureus* respectively.

CHAPTER 6

SUMMARY AND CONCLUSION

Chitosan is the second most abundant polysaccharide in the world. Chitosan can be extracted from the wastes of the seafood processing industry like crab shells, shrimps and lobsters. In this study, the shells of mud crabs have been utilized where the yield of the chitin and chitosan was quite higher when compared to previous studies. Through the characterization of the extracted chitosan, it was found that it has a microfibrillar structure, higher crystallinity and thermal stability, and excellent anticancer, antibacterial and antioxidant activity. Hence, it can find its application in drug manufacturing and delivery, stem cell culturing, etc. Here, the extracted chitosan is utilized for two purposes: i) chitosan hydrogel and ii) chitosan nanocarrier. Chitosan hydrogel with its high porosity facilitated a better environment for the growth of stem cells (WJ-MSCs) with higher viability and higher proliferation. Thus, it can be used as an engineering scaffold to achieve successful tissue restoration and wound healing.

Further, chitosan was used for the synthesis of nanocarrier for the delivery of curcumin. The curcumin was well-encapsulated inside the nanocarrier due to the mucoadhesive property of chitosan and had better drug delivery efficacy ensuring better-targeted delivery of the drug and can offer the capability of tissue binding. Hence, it can be concluded that both chitosan hydrogel and chitosan nanocarrier would be great candidates for stem cell culturing with higher cell viability when compared to the conventional method, and can be employed in tissue/organ regeneration and effective targeted drug delivery.

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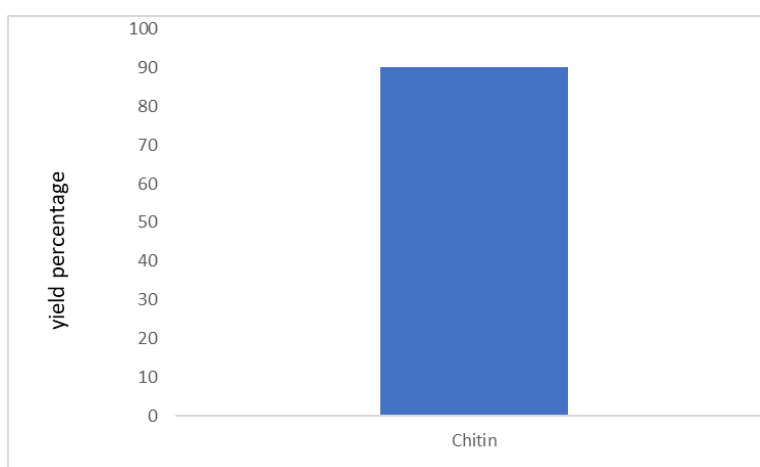


Figure 2. Yield percentage of chitin

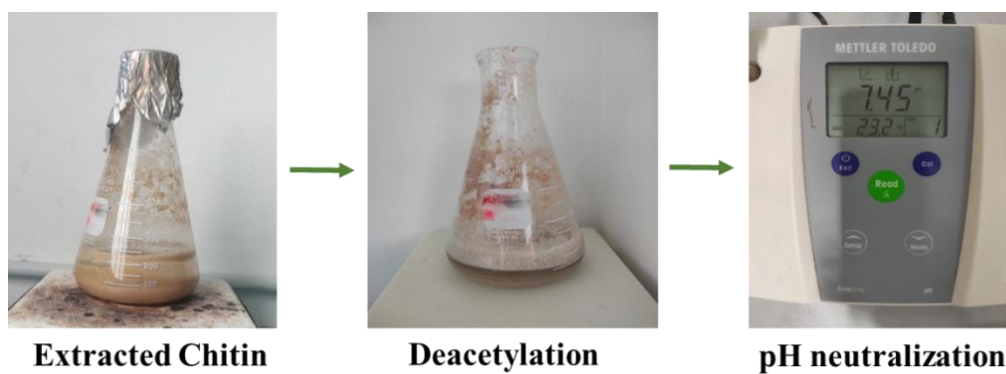


Figure 3. Extraction of Chitosan

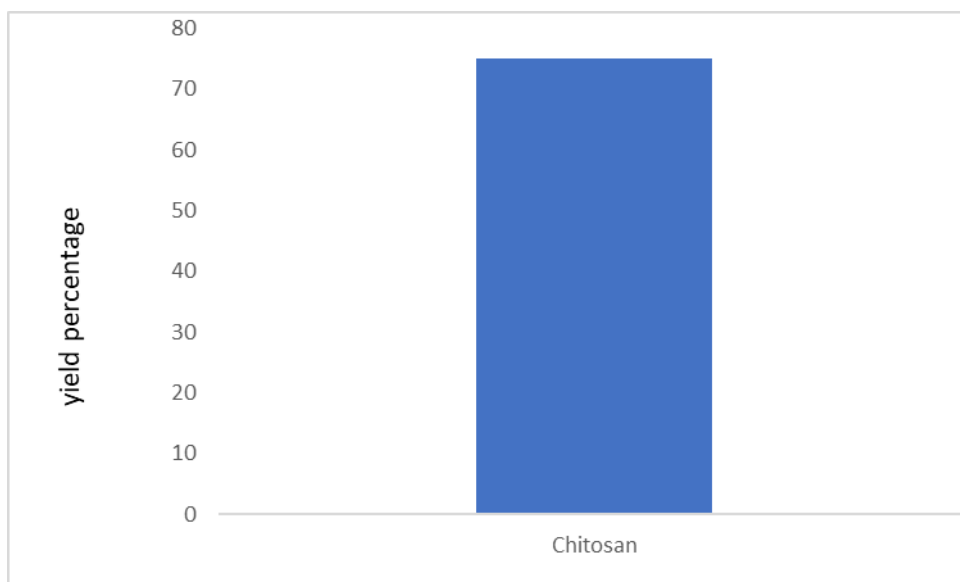


Figure 4. Yield percentage of chitosan

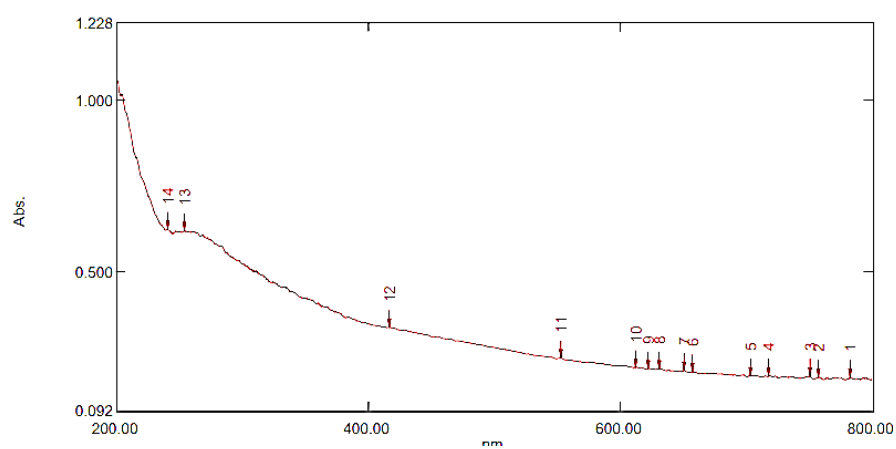


Figure 5. UV-Vis Spectroscopy of Chitosan

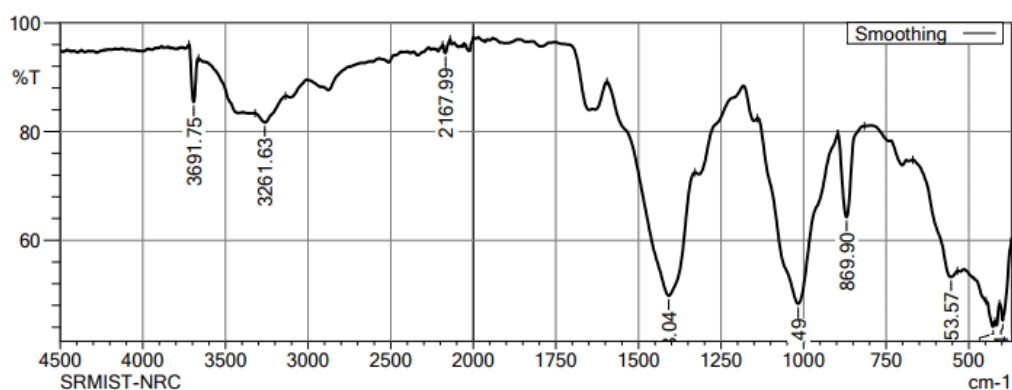


Figure 6. FTIR Analysis of Chitosan

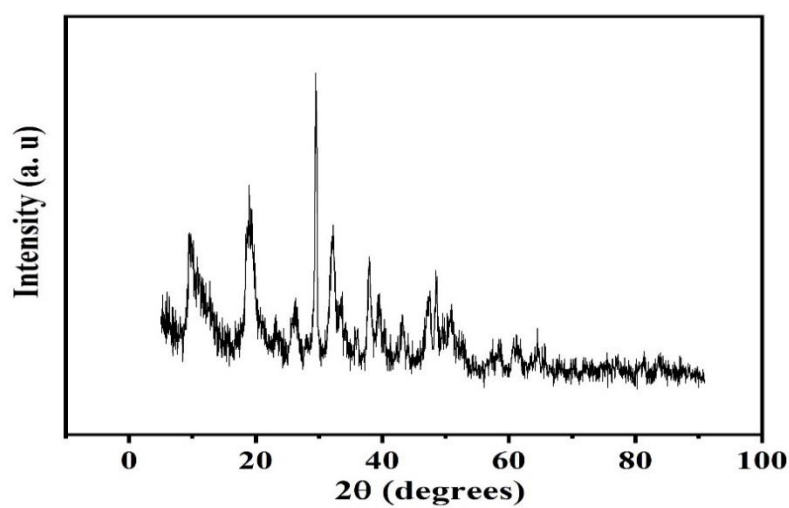


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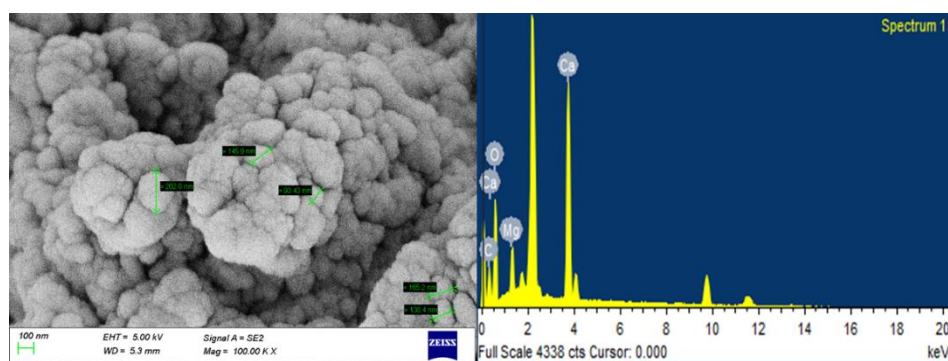


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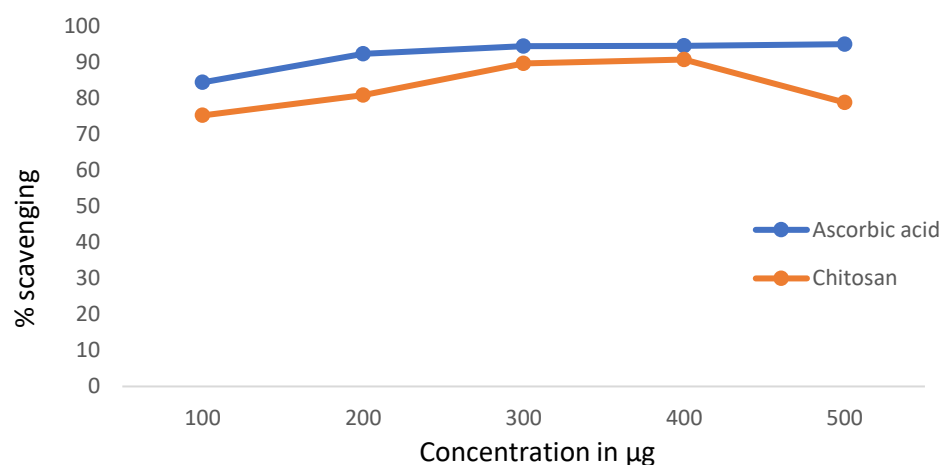


Figure 10. Antioxidant Activity of Chitosan

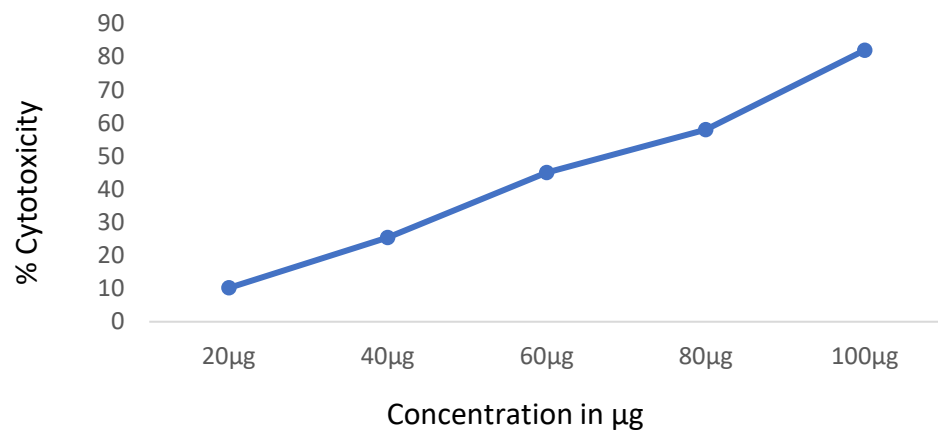


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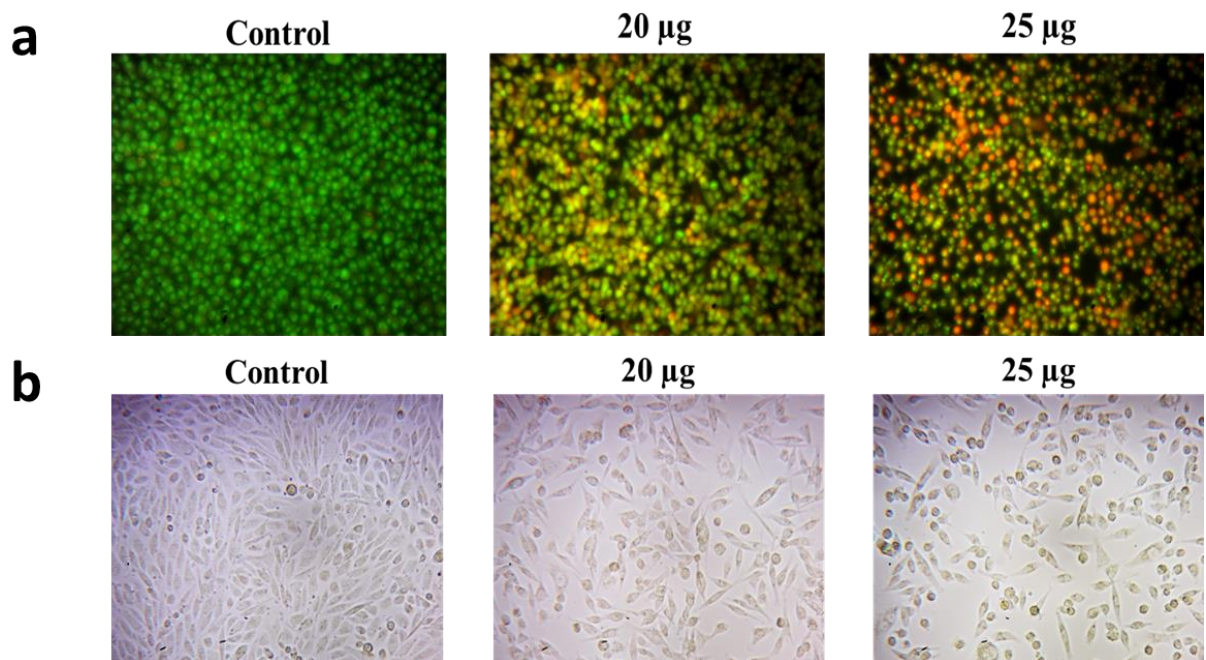


Figure 12: Effect of chitosan on the apoptotic cell death in the Breast cancer MDA-MB-231 cells a) AO/EB Staining of apoptotic cells b) morphological changes

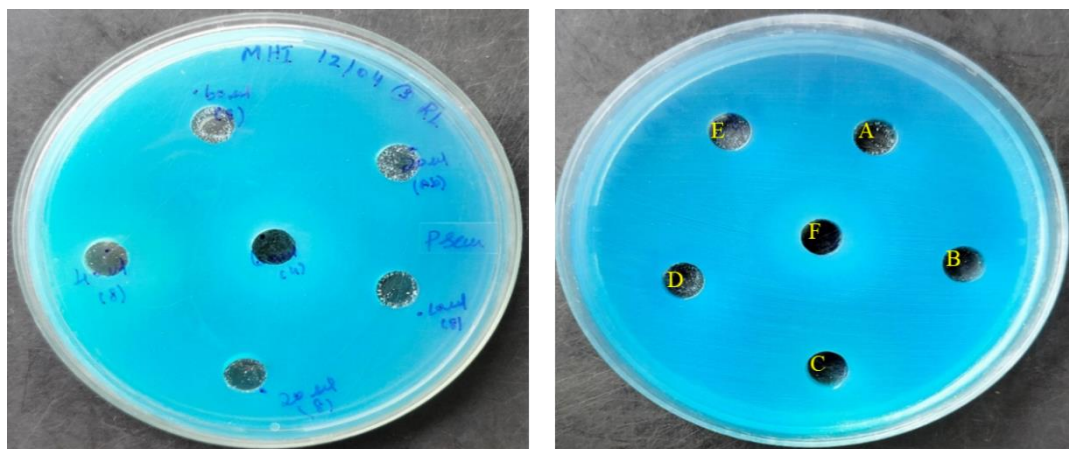


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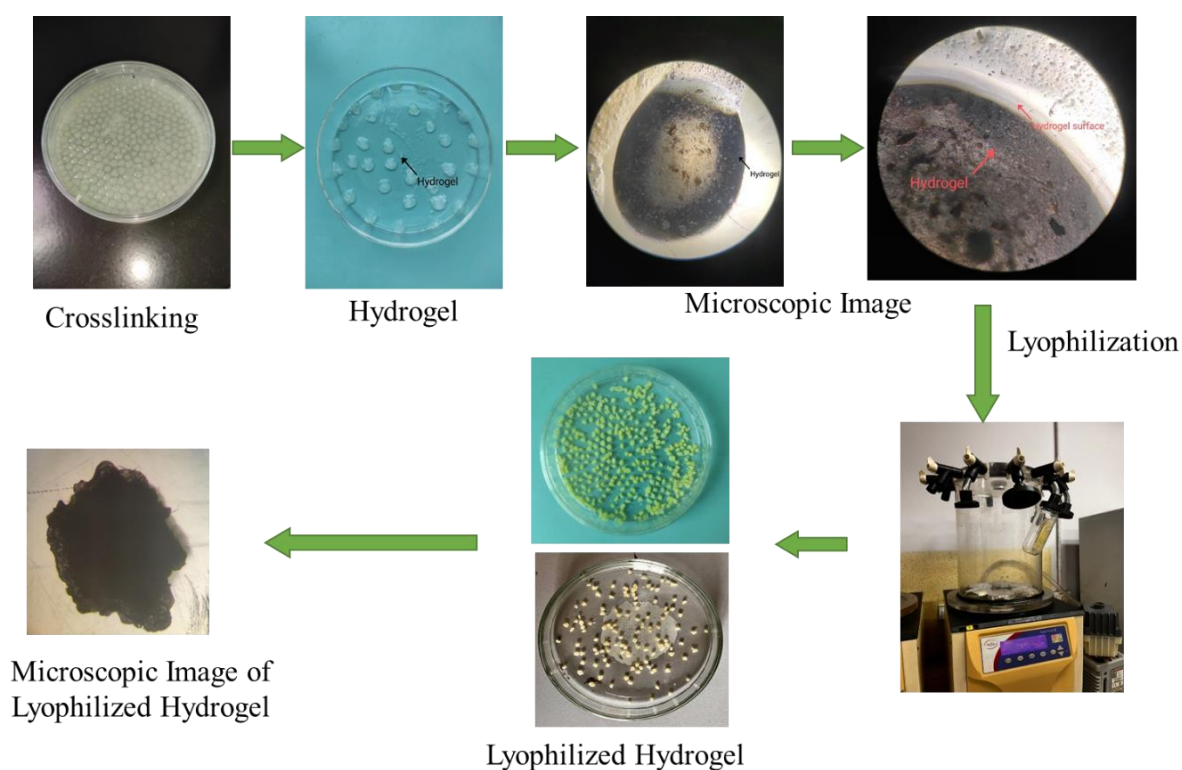


Figure 14. Synthesis of Chitosan Hydrogel and its microscopic image

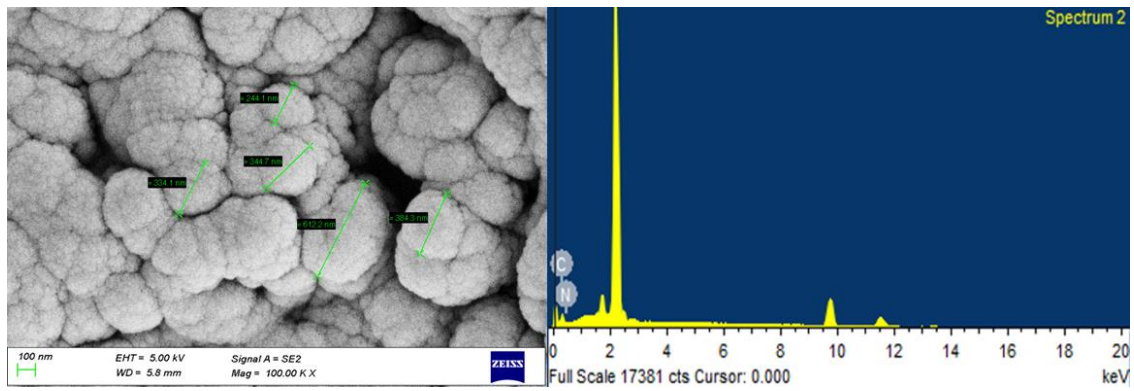


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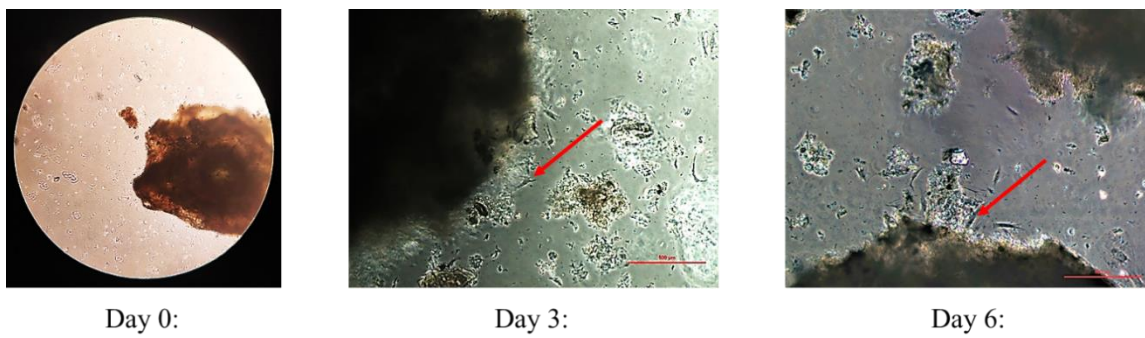


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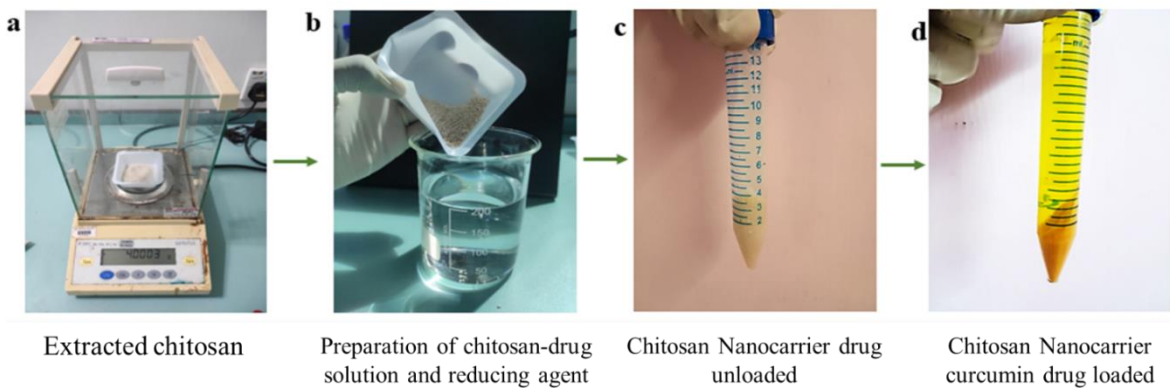


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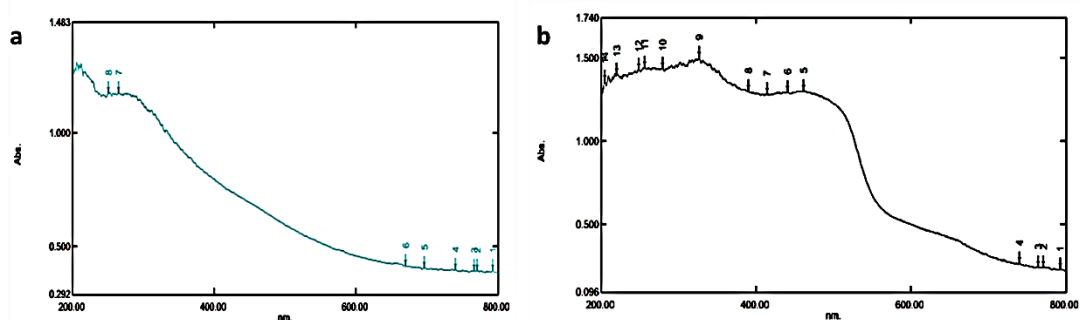


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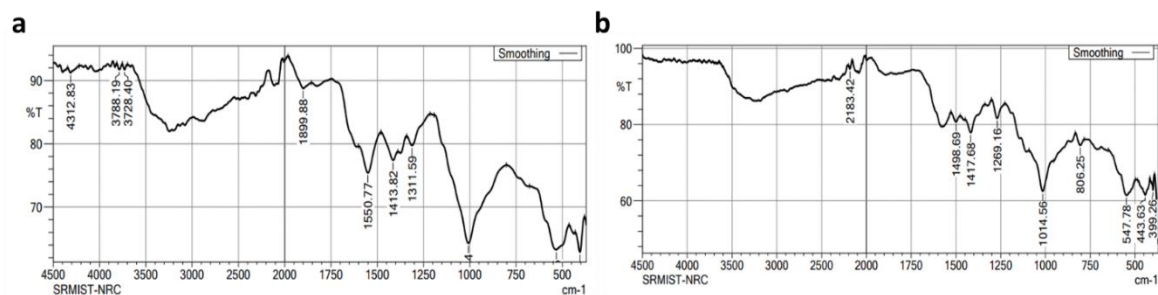


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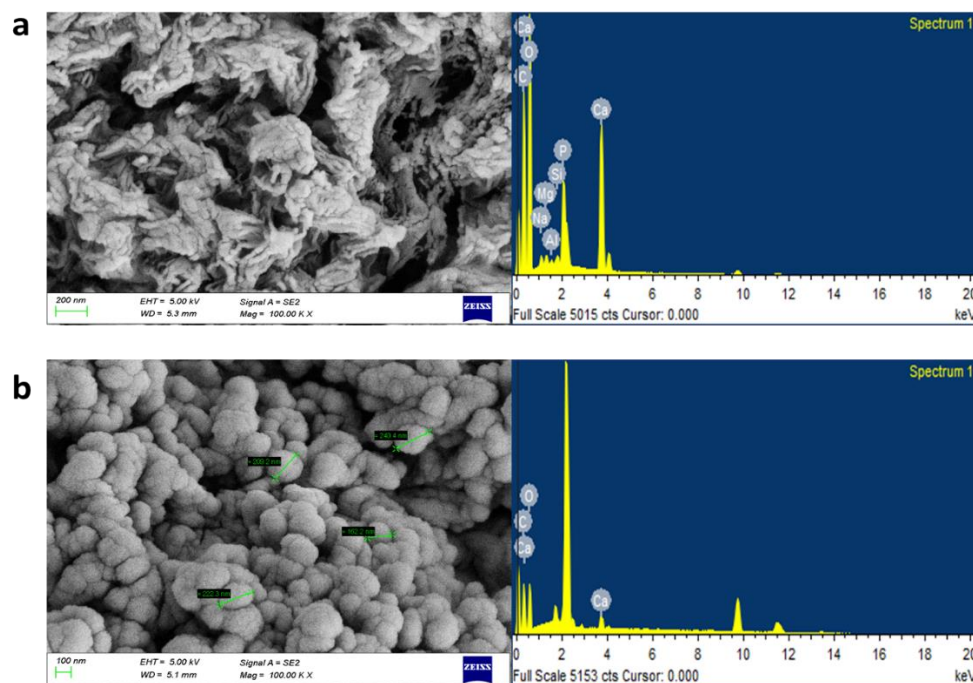


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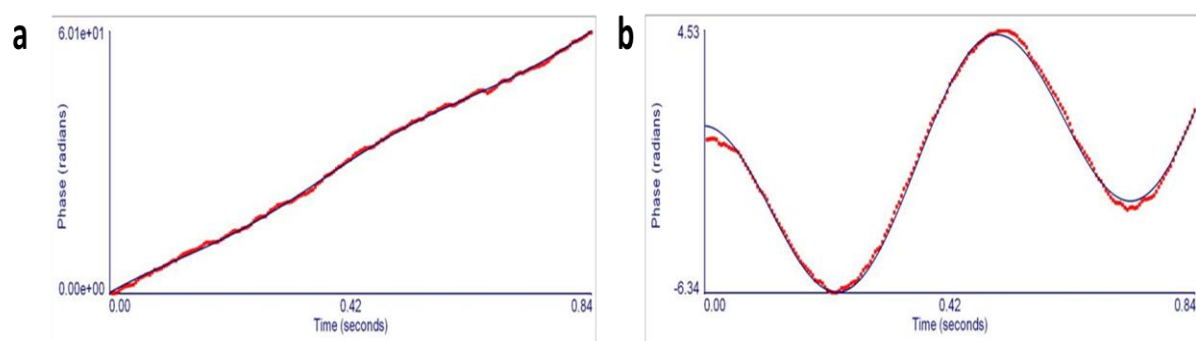


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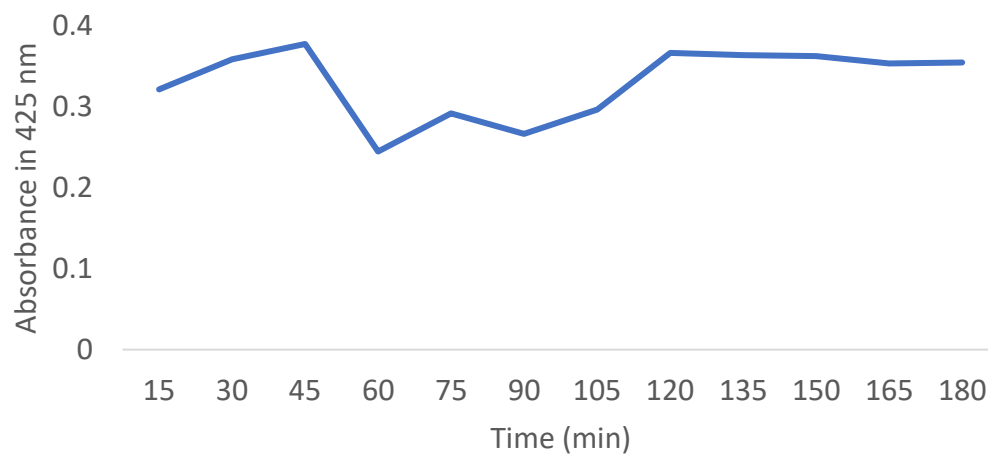


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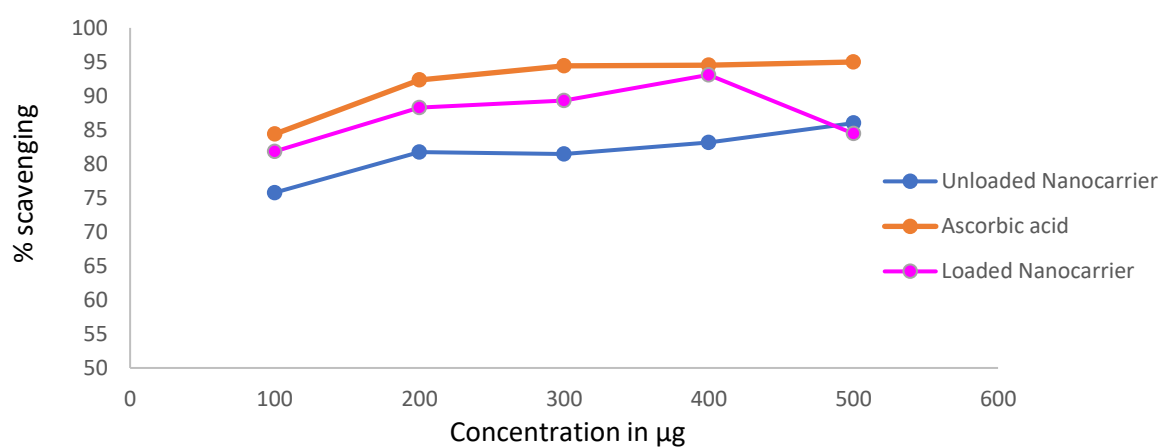


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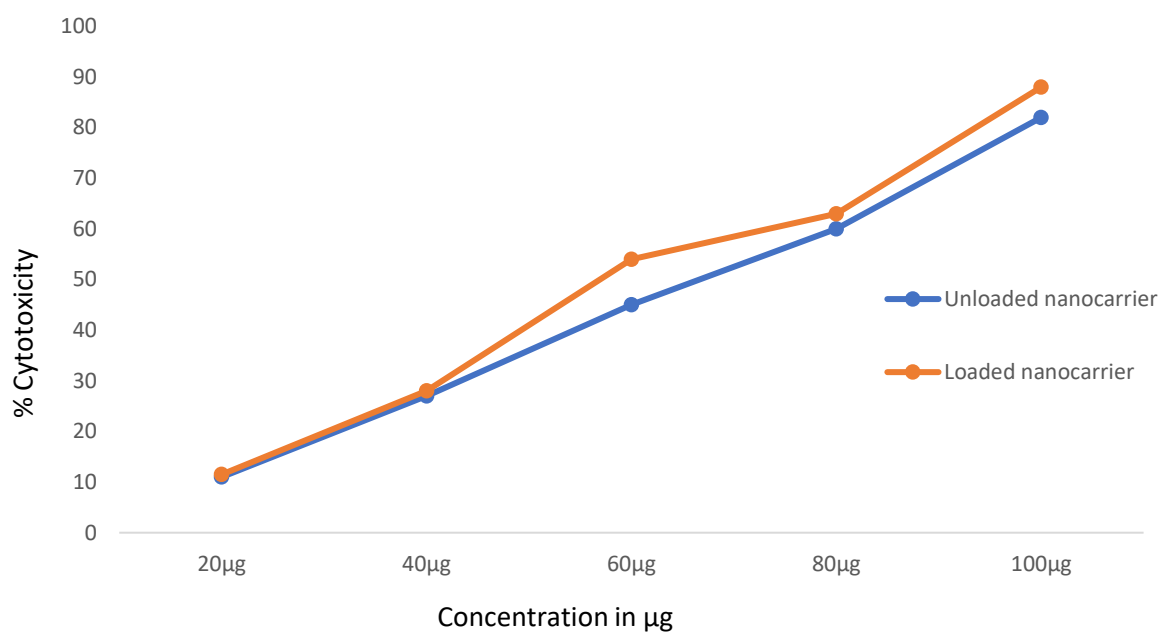


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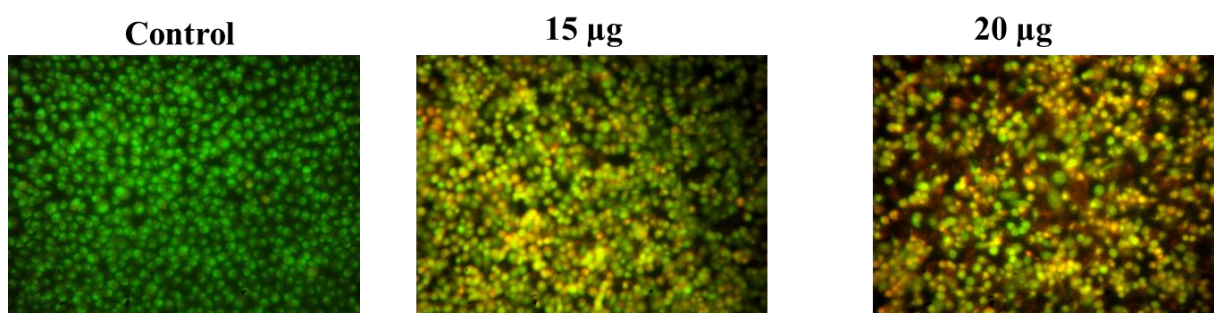


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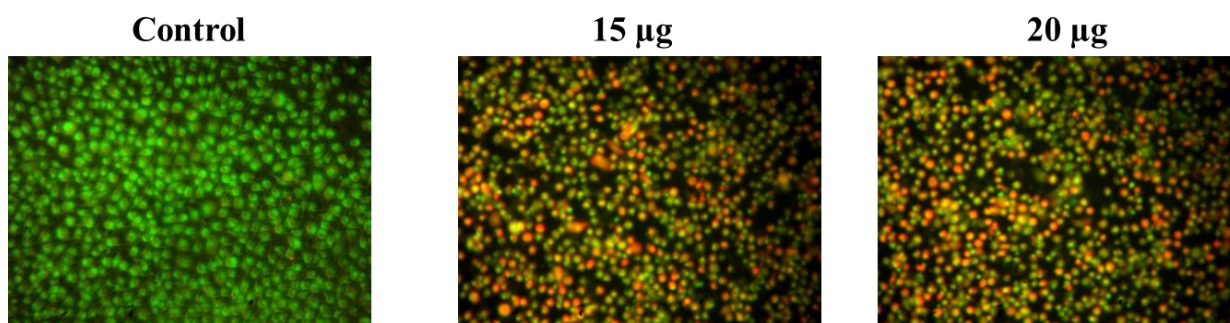


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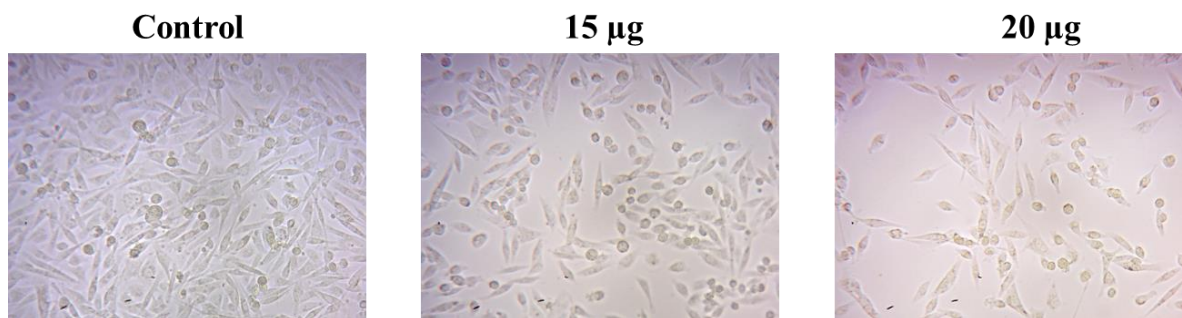


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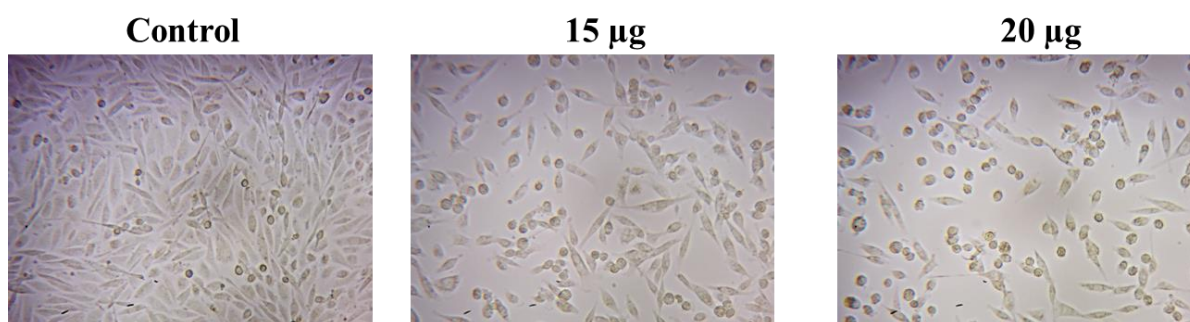


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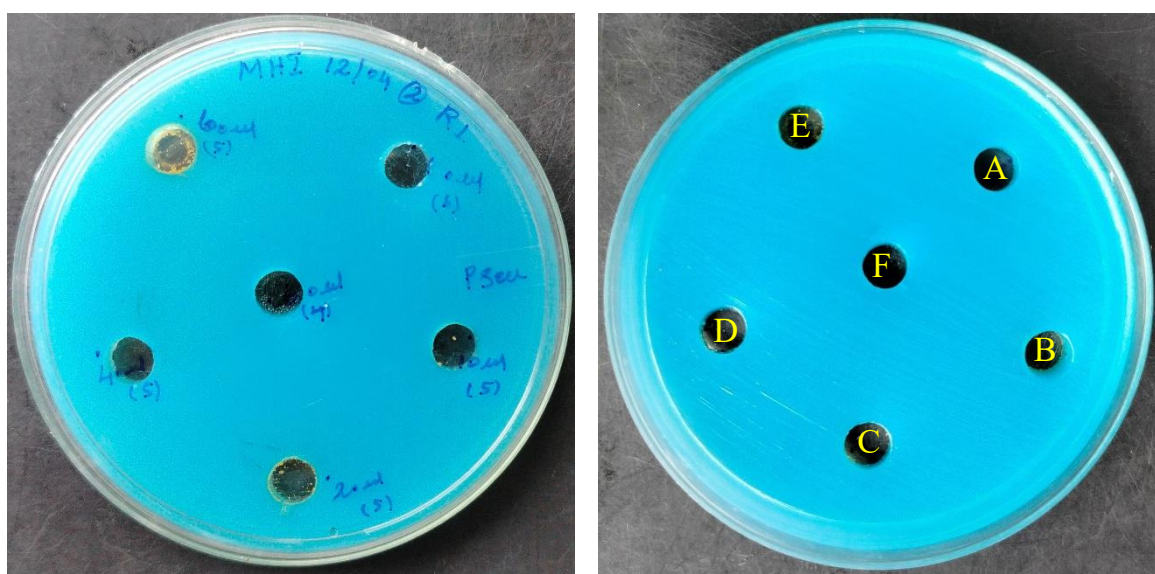


Figure 29. Antibacterial activity of curcumin-unloaded and curcumin-loaded chitosan nanocarrier against *Pseudomonas aeruginosa* using distilled water as solvent. A: 60 µg/10µl Unloaded Chitosan in distilled water; B: 10 µg/10 µl Loaded Chitosan in distilled water; C: 20 µg/10 µl Loaded Chitosan in distilled water; D: 40 µg/10 µl Loaded Chitosan in distilled water; E: 60 µg/10 µl Loaded Chitosan in distilled water; F: distilled water

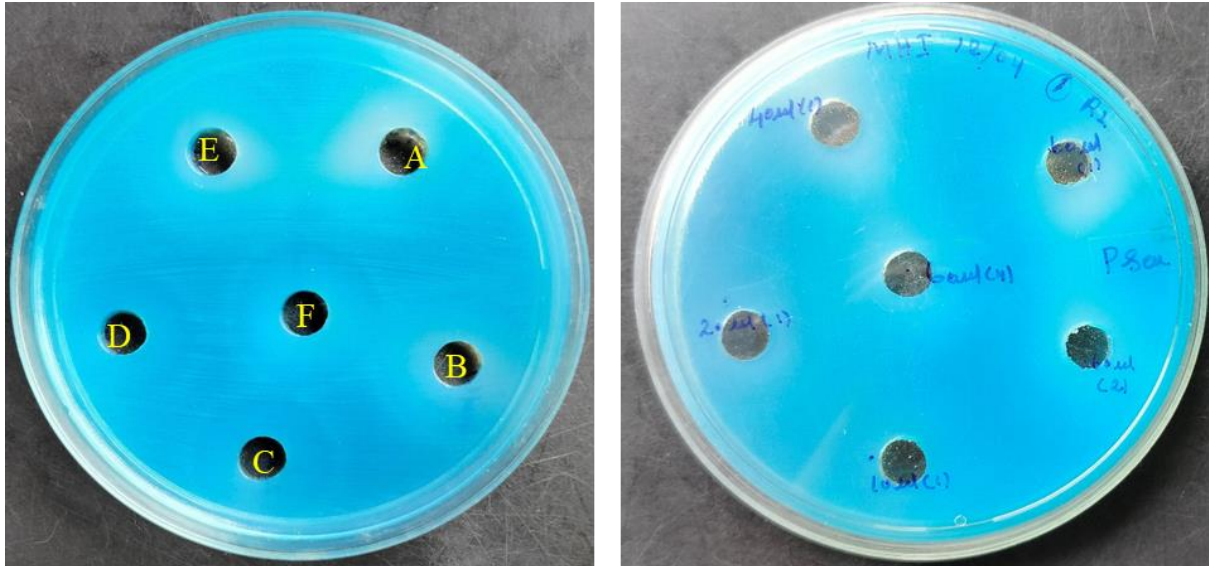


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TABLES

**Table 1. Zone of inhibition of chitosan against *Pseudomonas aeruginosa*
AA – 0.01 % acetic acid**

S. No	Sample Concentration	Zone of Inhibition (mm)
1	A: 60 µg Antibiotic	13
2	B: 10 µg Chitosan in AA	9
3	C: 20 µg Chitosan in AA	10
4	D: 40 µg Chitosan in AA	11
5	E: 60 µg Chitosan in AA	12
6	AA	0

Table 2. Zone of inhibition of curcumin-unloaded and loaded chitosan nanocarrier against *Pseudomonas aeruginosa*

Sample Concentration	Zone of Inhibition (mm)	
	Distilled water as solvent	Acetic Acid as solvent
A: 60 µl Unloaded Chitosan Nanocarrier	-	15
B: 10 µl Loaded Chitosan Nanocarrier	-	12
C: 20 µl Loaded Chitosan Nanocarrier	-	15
D: 40 µl Loaded Chitosan Nanocarrier	-	16
E: 60 µl Loaded Chitosan Nanocarrier	-	17
F: Solvent	-	-