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Optimization studies of Tetracycline Hydrochloride loaded microspheres using mucilage extracted from Abelmoschus Esculentus for tissue recovery from serious accidental injury

Sahabuddin Gori Mallick[2], Sourav Mandal[1*], Krishna Das[2] , Kaif Sekh[3] ,Ayan Hossain[4], Avik Mishra[5]

[1*] Assistant Professor, PhD Scholar, Department of Pharmaceutical Technology, Birbhum Pharmacy School, Dubrajpur, West Bengal,731124
[2] B.Pharm Student, Birbhum Pharmacy School, Dubrajpur ,West Bengal,731124
[3] B.Pharm Student, Birbhum Pharmacy School, Dubrajpur ,West Bengal,731124
[4] B.Pharm Student, Birbhum Pharmacy School, Dubrajpur ,West Bengal,731124
[5] B.Pharm Student, Birbhum Pharmacy School, Dubrajpur ,West Bengal,731124
Corresponding Author Details:
Mr. Sourav Mandal
Assistant Professor/PhD Scholar
Department of Pharmaceutical Technology
Contact no. (+91) 8649828303
Mail id. souravmandalbirbhum@outlook.com

ABSTRACT:

There lies enormous potential in the treatment of infected wounds and the acceleration of healing by the development of antimicrobial biomaterials, which also promote tissue regeneration. The potential of okra (Abelmoschus esculentus) mucilage as a matrix material for the encapsulation of antimicrobial agents (TETRACYLINE HYDROCHLORIDE) to develop microspheres for tissue regeneration purposes was examined here. The physicochemical properties of the isolated okra mucilage were reported. Microspheres were formulated using techniques such as emulsion cross-linking or spray drying with various amounts of TETRACYCLINE HYDROCHLORIDE, an example antimicrobial drug. Microsphere size, shape, encapsulation efficiency, and in vitro drug release profile were determined.

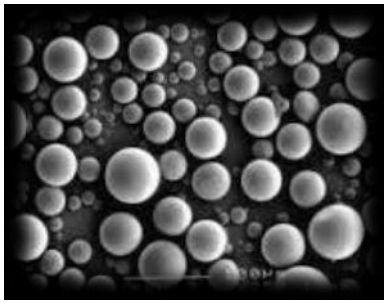
Secondarily, zone of inhibition and minimum inhibitory concentration assays were applied to assess drug-loaded microspheres' antibacterial activity versus relevant bacterial organisms, including Staphylococcus aureus and Pseudomonas aeruginosa. The cytocompatibility of microspheres against mammalian cells, including mesenchymal stem cells or fibroblasts, was assessed through cell viability and growth studies in vitro. Finally, adhesion, migration, and matrix deposition were evaluated in vitro for analyzing the capacity of the okra mucilage microspheres to stimulate tissue regeneration. This work is intended to demonstrate the potential to use okra mucilage as an inexpensive and biodegradable biomaterial for designing antimicrobial microspheres with potential in the promotion of effective tissue regeneration within contaminated surroundings.

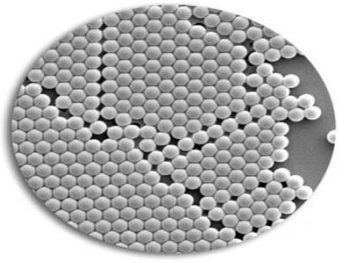
KEYWORDS: Okra mucilage, microspheres, antimicrobial, drug delivery, tissue regeneration, biomaterial, wound healing.

1. INTRODUCTION

1.1 Microspheres

Microspheres are innovative drug delivery systems designed to extend or regulate drug administration for the purpose of treatment and stability, ensuring that medicines are delivered precisely to the intended areas at consistent rates. Microspheres are made up of polymer waxes or substances like natural, semisynthetic, or synthetic polymers.. Microspheres have a wide range of applications due to their ability to release substances in a controlled and sustained manner.[1]





1.1.1 IDEAL CHARACTERISTICS OF MICROSPHERES

1.1.2

- Clinically acceptable shelf life with improved stability following synthesis
- Correct particle size and aqueous vehicle dispersability for injection.
- Drug release with a good regulation over an extended period of time.
- Good Biocompatibility with a controlled biodegradability.[2]

1.1.2 ADVANTAGES OF MICROSPHERES

- Protection of unstable, sensitive materials from their environments prior to use.
- Self-life enhancement by preventing degradative reactions.

1.1.3 LIMITATIONS OF MICROSPHERES

- Controlled release formulations have higher drug loads, hence loss of integrity of the exemption properties of the dosage form can result in possible toxicity.
- Avoid shredding or chewing this form of dosage.[3]

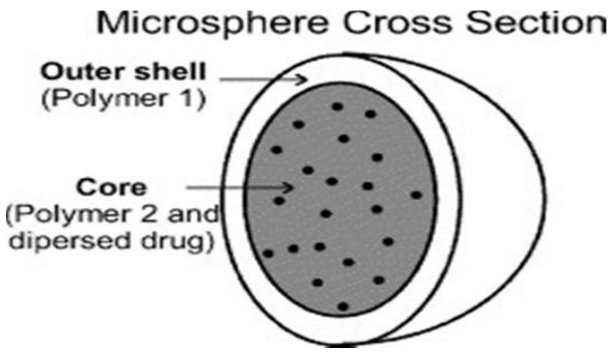


Fig:2 Cross Section Of Microspheres

1.1.4 CHARACTERISTICS OF MICROSPHERES

Sr. No.	Property	Consideration
1	Size	Diameter, Uniformity/distribution
2	Composition	Density, Refractive index, Hydrophobicity/hydrophilicity, Nonspecific binding, Autofluorescence
3	Surface Chemistry	Reactive groups, Level of functionalization, Charge
4	Special Properties	Visible dye/ fluorophore, Superparamagnetic

Table 1 : Characteristics of Microspheres[4]

1.1.5 TYPES OF MICROSPHERES:

Bioadhesive microspheres: In this type of microspheres ,Adhesion is the sticking of drug to the membrane using the sticking nature of water soluble polymers. These microspheres have a prolonged residence time at the site of application and causes close contact with the site of absorption and exerts more therapeutic effect.[5]

Magnetic microspheres: This type of delivery system is quite important which localizes the drug to the disease site. In this delivery system larger quantity of freely circulating drug can be replaced by smaller quantity of magnetically targeted drug. [6]

Floating microspheres: In these little spheres, the density is less than the fluid in the stomach and thus they float.The stomach without altering the rate of gastric emptying. When the system floats on the stomach contents and translates upward. The gastric stay and the fluctuation in plasma concentration result in a gradual release of the drug. The target speed. [7]

Radioactive microspheres: The microspheres employed in the radio emobilisation therapy are bigger than the capillaries so that they can successfully target and treat the affected area.Gets clogged in the first capillary bed when they meet each other. Then they are administered through the arteries supplying blood to the heart. [8,9]

Biodegradable polymeric microspheres: Natural polymers, e.g., starch, are desirable because of biodegradability and biocompatibility. Accordingly, it is a bio-compatible glue. In the presence of contact with mucous membrane, the biodegradable component is dissolved. Which leads to the development of gel. [10]

Synthetic polymeric microspheres: Synthetic polymeric microspheres are widely used in the medical treatment process, and there are other applications as well. [11]

1.1.6 METHODS OF PREPARATION

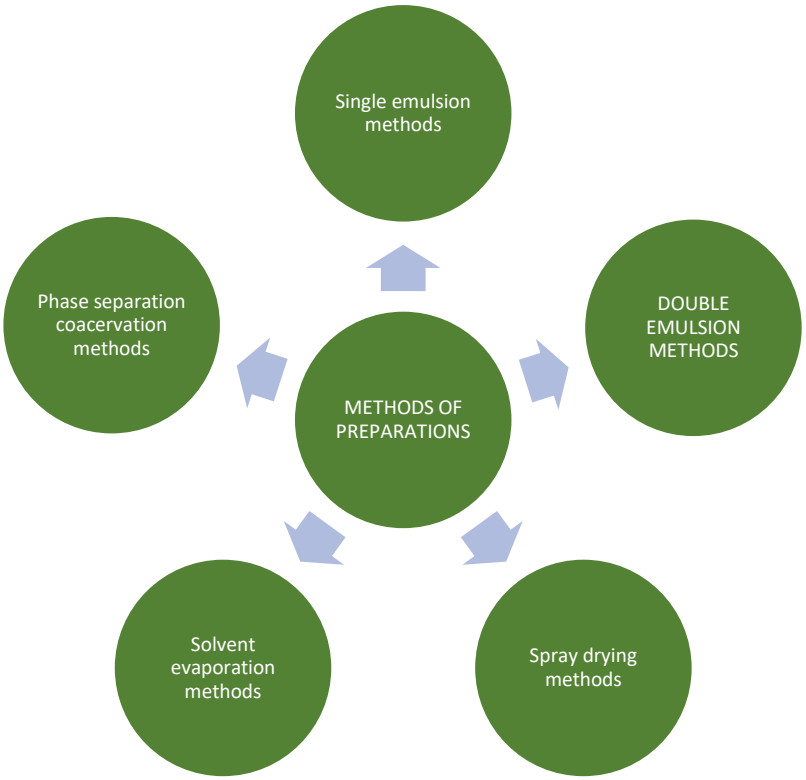


Fig No. 3 :Preparation Of Microsphere

Single emulsion method: Single emulsion was utilized to produce proteins and naturally occurring polymer dietary sources, which were employed as microparticulate carriers. In a liquid phase, the natural polymers are first dissolved or dispersed. The mixture is then transferred into an oil-based, non-aqueous phase. 12-14]

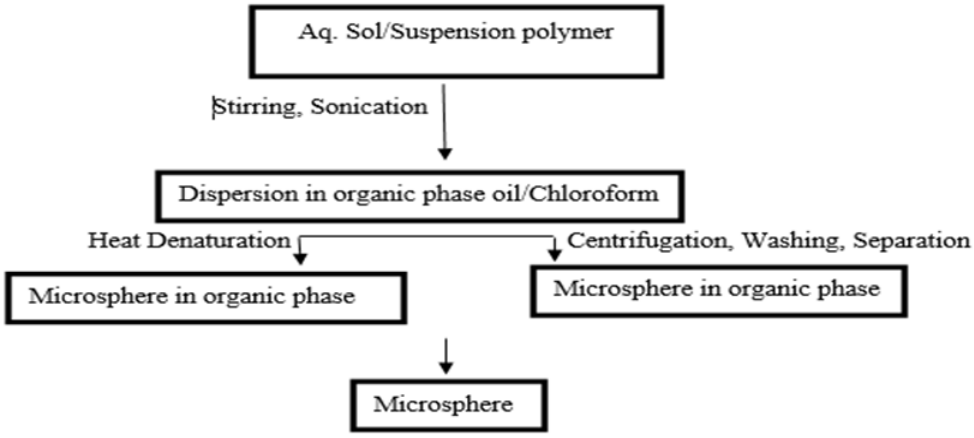


Fig:4 Single emulsion method

Double Emulsion Method: Water-soluble drugs, peptides, proteins, and vaccines are optimal candidates for the double emulsion process of microsphere preparation. It involves the formation of a series of emulsions or a double emulsion free of water. Using this method, microspheres can be prepared from polymers obtained from both natural and synthetic origins. The hydrophobic organic continuous phase has a dispersion of the aqueous solution of the protein. This protein solution can have the functional components.[15]

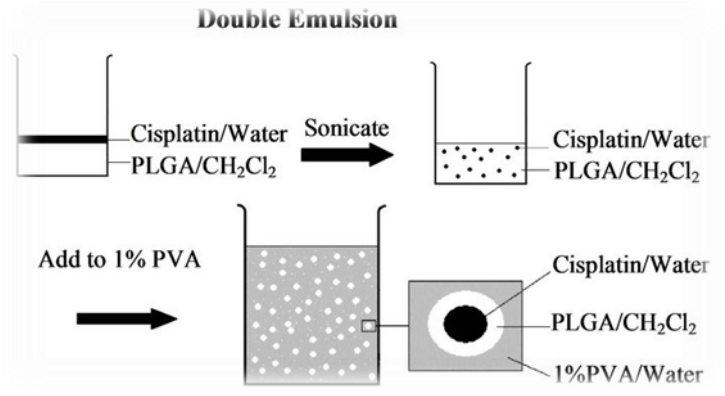


Fig:5 Double Emulsion Method

Solvent evaporation method :This technique, referred to as microparticle formation, entailed elimination of the organic phase by the employment of an organic solvent. It employs water as a compatible organic substance solvent, and extraction via water eliminates the organic phase. The technique lessens the period of elongation of microspheres.[16-18]

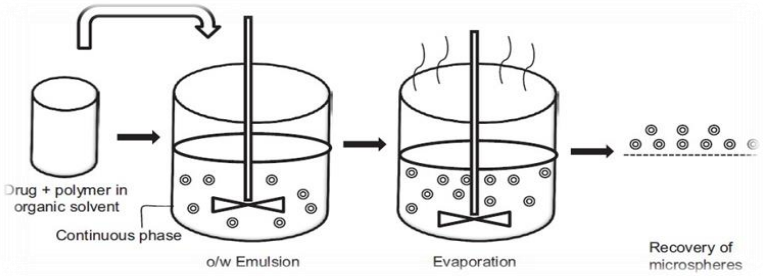


Fig:6 Solvent evaporation method

Phase separation coacervation method :The concept behind this process is to decrease the polymer's solubility during the initial phases of the natural phase in order to influence formation of a polymer-rich phase known as the coacervates.This process entails mixing the drug-containing polymer solution with an incompatible polymer.Phase separation is a result of the initial polymer taking up the drug particles.Addition of a non-solvent is what makes a polymer solidify.The process by which the microspheres of polylactic acid (pla)have been formed. A butadiene-incompatible polymer is being employed.The synthesis rate of coacervate influences the polymer film dispersion, particle size, and agglomeration of the resulting particles.[15]

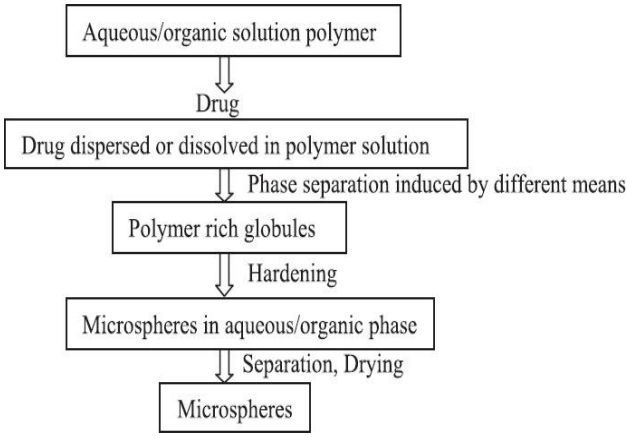


Fig:7 Phase separation coacervation method

Spray drying method : It's a one-step closed system method that is effective for a wide range of materials, including heat-sensitive materials. The medication and polymer coating ingredients are either suspended. It may also be suspended or dissolved within an emulsion or coacervate system. Methylene chloride is utilized to dissolve the drug and polymer.For example, one can make polylactide microspheres in the polymer solution or dissolve them in an appropriate solvent (either aqueous or non-aqueous).The spraying rate, drug solution on the basis of polymers supply rate, nozzle size, temperature of the chambers for drying and collection and the size within the two chambers all influence the microsphere size.[19]

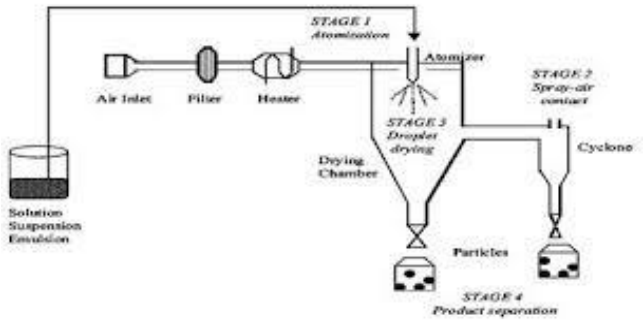


Fig:8 Spray drying method

1.1.7 EVALUATION OF MICROSPHERE

Percentage yield of microspheres: Microspheres that had been completely dried were gathered and accurately weighed. By using the given formula helps to obtain the percentage yield below.

%Yield = mass of microsphere / total weight of medication divided by 100

Optical Microscopy : In this method, an optical microscope were used to determine the particle size of the microspheres. (Meizer OPTIK).100 particles were calculated for the measurement under 450x (10x eyepiece and 45x objective). [20]

Scanning Electron Microscopy : The dried microspheres were weighed and precisely measured accurately. The formula was then used to find the percentage yield, which is found by dividing the weight of the microspheres by the overall weight of the drug and then multiplying by 100.[21]

Thermal Analysis : Thermal analysis methods regularly examine such changes using predefined specimen atmospheres and pressures and programmed temperature ranges for heating and cooling.Some of the most common properties to observe are the slight variations in gas evolution, thermal expansion or contraction, weight loss or gain, young's modulus, and heat and enthalpy.[22]

Entrapment Efficiency : Five milligrammes of the drug were blended with distilled water in crushed microspheres, stirred with an ultrasonic device for three hours, filtered, and then analyzed by uv-vis spectroscopy.[21]

Flow Properties : The hausner ratio, the angle of repose at rest, and the carr's compressibility index may all be employed to study the flow properties.The densities of the tapped and bulk materials were determined using a volumetric cylinder.

Swelling Index : Swelling index = (mass of swollen microspheres –mass of dry microspheres / mass of dried microspheres) 100[23]

Drug Content : After allowing the dust to settle, the mixture should be left alone prior to washing it away. A 1 ml of the filtrate was filled in a volumetric flask, and the volume was filled up with 0.1 n NaOH. The drug was evaluated. Using spectrophotometry upon proper dilution.[24]

1.2 OKRA (Abelmoschus esculentus L)

Abelmoschus esculentus L. Goes by the local names okra or lady's finger. The plant belongs to the malvaceae family, is native to Africa, and is cultivated in different tropical, subtropical, and warm temperate areas throughout the world such as Africa, Asia, South Europe, and America.[25,26]

Fig No.:9 Picture of Ladies Finger (Abelmoschus esculentus L)

Okra, having been originated in Ethiopia and cultivated in North Africa, the Mediterranean,

Arabia, and India, is of great economic importance in the subtropical parts of the globe. [27] Okra may be eaten raw or cooked and added to a range of dishes like soups, salads, and stews.[28]

Okra fruit is rich in moisture, it is rich in nutrients, and it is rich in vitamins and minerals. Okra consists mainly of carbohydrates in the form of mucilage, which is utilized extensively in different industries and as a medicine. The nature and composition of okra's fruits, seeds, and leaves are favorable for different uses.[29-31]

1.2.1 COMPOSITION OF OKRA

Okra is a very nutritious vegetable, and its inclusion in the diet can bring many benefits. The most prevalent macronutrients are dietary fibers (8.16 g/100 g fresh weight), followed by carbohydrates (4.86 g/100 g fresh weight) and proteins (3.55 g/100 g fresh weight).[32,33] Notwithstanding the okra fruits' low fat (0.19 g/100 g) and energy (33 kcal/100 g equivalent to 138 kj/100 g) contents, their seeds hold unsaturated fatty acids like linoleic acid that are indispensable for human diet. The seeds are also rich in α-tocopherol and hold large amounts of minerals like ca, k, cu, fe, p, mg, zn, and mn.[34-37]

The protein content of the okra seed meal, as determined by the research of ofori, tortoe, and agbenorhevi, can vary from 16.80 to 17.40% depending on the variety. Other authors have quoted protein contents in excess of 40%, stating that okra is a good source of the vital nutrient. [38-40]

1.2.2 BENEFICIAL PROPERTIES OF OKRA MUCILAGE TO HEALTH AND ITS RELEVANCE FOR CONSIDERING OKRA AS A FUNCTIONAL FOOD

The search for foods that are nutrient-dense and bioactive compounds with functions that aid in the proper body functioning and the improvement of consumers' health underscores the importance of functional foods.

The whole okra fruit contains less mineral content than the zn and ca content in its mucilage, as indicated by adetuyi and dada [41]. The following section presents further information regarding the composition and physical properties of okra mucilage. Vegetable mucilages' biological activities have been studied comprehensively in animals and humans. The polysaccharides that are obtained from these mucilages have intense biological activities, such as anti-inflammatory and immunomodulation.[37,42,43,44]

Okra mucilage particularly has shown functional health attributes by in vitro and in vivo experiments, including antitumor, antioxidant, antimicrobial, hypoglycemic, and antiulcerogenic activities, and cholesterol and bile acid binding, detoxifying the liver of toxins [45,46]. Matazu et al. [47] formulated a nutraceutical from okra seeds and peels, which are enriched with mucilage, and studied its antidiabetic and antioxidant activities. [48]. The application of okra mucilage as nutraceutical therapy or adjuvant therapy for the cure of this disease is highly promising [48]. As stipulated by Bonciu [42], some vegetables are real natural nutraceuticals, which contribute to the cure of different diseases. This reality was attested to by chukwuma et al. [42], who contrasted the anti-hyperglycemic effect of amadumbe (colocasia esculenta) and okra mucilage and noted increased inhibition of glucose uptake by okra because of the greater viscosity of its mucilage.

Apart from exerting an effect on free radical sequestration, these polysaccharides are able to raise the levels of superoxide dismutase (sod), promoting the antioxidant pathway [49,50,51]. Okra mucilage carbohydrates, particularly the pectic polysaccharide fraction wop-2 (a rhamnogalacturonan i backbone with type ii arabinogalactan side chains substituted partly at o-4 of rhamnopyranosyl), possess antioxidant activity that can help reduce lipid peroxidation reactions responsible for the destruction of beta cells [52,53].

1.2.3 EXTRACTION METHODS

Okra mucilage can be prepared using several methods that mostly depend on the utilization of distilled water or organic solvents. Heat is also used in several processes. Farooq, malviya, and Sharma [54] removed the mucilage from the okra while stirring it constantly in distilled water at 60 °c for approximately 4 hours. In a step-by-step process, the mucilage was removed using acetone.

Sengkhamparn et al. [55] collected the material from the cell wall under different conditions, such as hot buffer, chelating agent, dilute alkaline, and concentrated alkaline. Polysaccharide fractions obtained using different methods, except concentrated alkaline, were found to have galactose, rhamnose, galacturonic acid, and arabinose. Arabinose was also found in polysaccharide that was derived using the dilute alkali method. Polysaccharide derived using concentrated alkali was found to have xxxg-type xyloglucan and 4-methylglucunoxylan.[56]

Okra mucilage extraction is easy in an aqueous solution since the polysaccharides in it are soluble and the process has the benefit of yielding a high yield. Cahyana and Kam [57] investigated the effect of parameters such as time, temperature, and water-to-okra fruit ratio on the yield of extraction and antioxidant and anti-α-glucosidase activities. The different methods of extraction used did not significantly affect the yield, although the fruit was treated with water at 4–5 °c for 12 hours. 1:6 (fruit:water) ratio showed the maximum antioxidant activity. The effect of parameters such as time and temperature on the yield or biologic activity of the mucilage polysaccharides depends on the method used for extraction. Ultrasound-assisted extraction had fine extraction yields (9–10%) at 55–65 °c temperatures for 20–30 min [58].The extraction was carried out in a closed system using a flow rate of 0.5 mL/min.

1.2.4 APPLICATIONS OF OKRA MUCILAGE

Plant mucilages have rheological characteristics that qualify them as thickeners and food stabilizers and are generally accepted by consumers as being of natural origin. Some mucilages in the pharmaceutical industry can be used as raw materials for the production of natural coatings, as they consist of pectin, galactans, and glucuronic acid.[59,60]

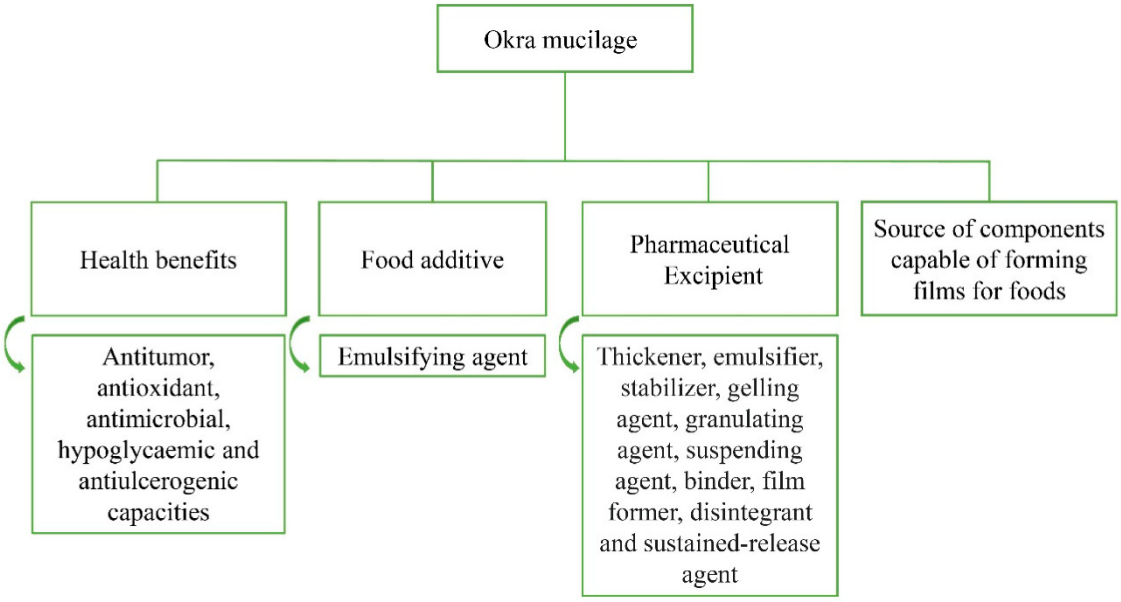


Fig No.:10 Okra mucilage utilization.

1.3 SODIUM ALGINATE

Sodium alginate is a linear polysaccharide of natural origin, is biodegradable, biocompatible and non-toxic to the body, gives strength and elasticity to the tissue, and can be industrially applied since it possesses gelling, viscous and stabilizing properties and water-retaining ability. [61]Alginate can be prepared from the cell wall of brown algae of many species: laminaria hyperborea, ecklonia maxima, ascophyllum nodosum, eisenia bicyclis and macrocystis pyrifera ecc., and from several species of bacteria: azotobacter and pseudomonas.[62] Among them, alginate derived from brown algae is of immense importance for the food, pharmaceutical, cosmetic industries, and so on.[63]

Brown algae are alkalized with the use of sodium carbonate, sodium hydroxide or aluminum hydroxide when the extraction of the alginates is being conducted in several stages after dry shredded algae obtained during collection. [62]The extract is precipitated with sodium chloride or calcium and subjected to filtration process, the precipitate thus obtained (sodium/calcium alginate) is hydrolyzed to alginic acid by treatment with diluted clorhydric acid, and the alginic acid is converted to a dry sodium alginate powder.[63]The alginate that is acquired to be utilized should be treated chemically to eliminate impurities (e.g., heavy metals, endotoxins, proteins, carbohydrates and polyphenols) and subsequently converted into powder.[64] For utilization in the biomedical and pharmaceutical sectors, alginate should be safe for the body and biocompatible, which means it should possess a high degree of purity. A purified alginate resulting from a multi-step extraction procedure is impurity-free or has negligible impurities and can be ingested orally without causing an immune response.[65]

1.3.1Chemical Structure of Alginate

As per phillips g.O. And williams p.A. And lee k.Y. And mooney d.J.'s information, up to 1958, data on the chemical composition of alginate indicated that sodium alginate is comprised mainly of only β-d-manuronic fractions, but subsequently it was noted that α-l-glucuronic acid fractions also occur in its composition. [66]The proportion in which the two fractions exist in its composition differs depending on the natural source where it was derived.[67] Sodium alginate is regarded as a polyanionic copolymer which is structurally the same as the sodium salt of alginic acid, an acid that contains a number of consecutive series of the two uronic acids: the β-d-manuronic acids (m) and α-l-glucuronic (g), linked linearly to one another by 1–4 glycosidic bonds.[68,69,70]

It has the formula $(C_6H_7NaO_6)_n$ and a mean molecular weight of 216.121 g/mol [17]. Through a partial hydrolysis reaction in an acidic environment, the alginate molecule can be split into three successive fractions: manuronics (mmmmm), glucuronics (ggggg) and a mixture of manuronic fractions with glucuronics (mgmgmg).[71,72]

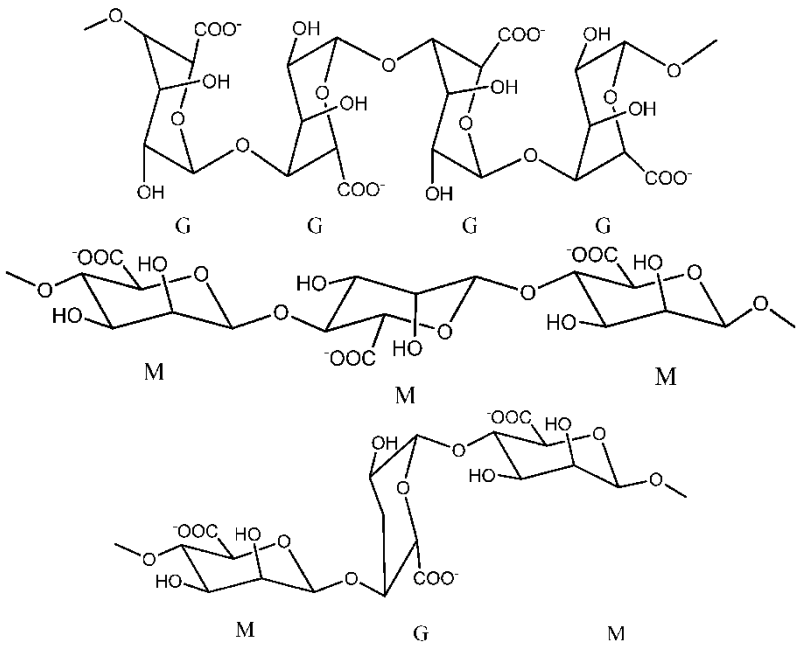


Fig No.: 11 Structure of sodium alginate

1.3.2 Physico-Chemical Properties of Sodium Alginate

1.3.2.1 Molecular Weight: Industrial sodium alginate contains a molecular weight between 32,000 and 400,000. It contains long m and g chains in the structure and a polydispersion index that ranges from 1.5 to 3 (mw/mn). Raising the molecular weight of alginate raises the gelling rate and physical properties of gels (tensile strength, elasticity, viscosity).[73,74]

1.3.2.2 Solubility: Sodium alginate dissolves in cold water at a slower rate, thereby giving rise to a viscous and thick solution. It will not dissolve in chloroform, alcohol content more than 30% solutions, ether, and alcohol. [74] Unlike sodium alginate, calcium alginate is insoluble in water and in organic solvents, but dissolvable in sodium citrate.[75]

1.3.2.3 Stability : Sodium alginate is compatible with most anionic materials and with few cationic materials, and it is more stable against outside influences when in the dry powder form than in solution form. In the short term, sodium alginate can withstand high temperatures and can be sterilized, but high-temperature exposure over a long period during sterilization can lead to a loss in its viscosity.[76]

1.3.2.4 Viscosity : Viscosity of alginate depends on its molecular weight and concentration, and its gelling capacity depends on glucuronic acid in the structure [2,5,25,26]. It has been indicated through research that sodium alginate solutions are non-Newtonian fluids, though pseudoplastic fluids that behave in a complex manner when dispersed in water and water diluted to various concentrations.[77]

1.3.2.5 Mucoadhesion : Alginate has high adhesive potential due to free hydroxyl and carboxyl groups present in its molecule. In the biological environment, there are electrostatic repulsive forces between alginate and mucin because of the negative charge of sialic acid, sulfate groups within the mucus matrix and anionic carboxylic groups of alginates[78]. This property is useful in the delivery of medication to mucous membranes since it increases the contact time and adhesion of the drug to the target site, as well as enhances the absorption of drugs.[79]

1.3.3 Advantages of Sodium Alginate

- Biocompatible: Safe to use in the body; non-toxic and well-tolerated.
- Biodegradable: Naturally breaks down without harming the environment.
- Gel-forming ability: Forms gels easily with calcium ions; useful in food, medicine, and cosmetics.

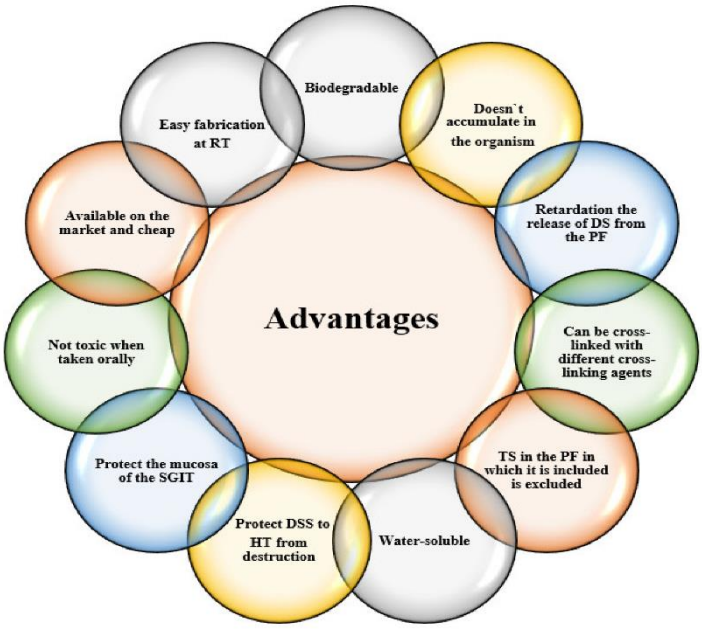


Fig No.:12 Advantages of using alginate

1.4 TETRACYCLINE HYDROCHLORIDE

Tetracycline hydrochloride is bacteriostatic and works against a wide range of bacteria, both gram-positive and gram-negative bacteria. In 1947, streptomyces aureofaciens was used to produce the parent compound chlortetracycline. Its use in animal production and to avert human diseases has made it a rising global threat to terrestrial and aquatic biodiversity. [80]

However, efficacy and possible hazards of antibiotics of the same structural class are similar.[81,82] Aside from their function in treating infections, antibiotics have been useful in animal husbandry, enhancing feed efficiency and growth in livestock.[83] In China, Canada, and the United States, it is also commonly used in animal feeds as an additive that encourages growth, but it is prohibited in the European Union.[84]. The consumption of antibiotics has increased remarkably across the globe. From 2000 to 2015, defined daily dose had increased by 65% due to a 39% increase in antibiotic consumption.[85] About 180,000 tons of antibiotics are used in China for animal and human use.[86] Twenty different antibiotics and tcs are produced from diverse streptomyces species and marketed in the market.[87]

1.4.1 Mechanism of Action

The tetracycline molecule has been modified by numerous semisynthetic structural changes since the 1950s, giving various tetracycline compounds with varied pharmacokinetic profiles and antibacterial activity.[88] Encapsulated tetracycline the associated substances minocycline, doxycycline, oxy-, and chlortetracycline.[89] Minocycline was introduced in 1972, tetracycline in 1953, doxycycline in 1967, and oxytetracycline and chlortetracycline in 1948 [90]. Of these, the others are semisynthetic, while chlortetracycline and oxytetracycline are natural products.Tetracyclines act on bacteria by attaching to an exposed organism's 30s ribosomal subunit.Tetracycline suppresses bacterial protein synthesis by interfering with aminoacyl-trna's ability to bind to the messenger rna molecule/ribosome complex after ribosomal binding [91]. Tetracycline can suppress protein formation in mitochondria by attaching to the 70s ribosomes found there [92,93].

1.4.2 Enzymatic degradation of TC

The removal of tc from the environment has been successfully achieved using enzymatic preparations. Laccase is an enzyme that has been widely used in the oxidation of diverse phenolic and non-phenolic substrates, such as environmental pollutants. Laccase can be used to deliver an environmentally acceptable solution for wastewater treatment and bioremediation [94]. Laccases, which are a type of multicopper oxidase, can be found in various organisms such as insects, fungi, and bacteria [95]. The catalysis of various substrates, including tetracycline, is supported by laccases, which are glycoproteins composed of four Cu atoms arranged in an active site [96].

Three steps constitute the catalysis mechanism of laccase: reduction of type i cu, type i cu to type ii and type iii clusters electron transfer, and reduction of oxygen to water [97]. In the final step, o2− is liberated as a second water molecule when all four cu centers get oxidized. Eventually, a single molecule of oxygen is converted into two water molecules and four substrate molecules are oxidized to yield four radicals. In another report, suda suggested that the primary process for biodegradation of tcs is the oligomerization of oxidized tcs through radical–radical coupling by laccase in the presence of 1-hydroxybenzotriazole (hbt) [98]. 40 u l−1 enzyme activity, phanerochaete chrysosporium crude lignin peroxidase oxidized 95% of 50 mg l−1 tc and otc [99].

1.5 Wound healing

Etiology of skin lesions is a breakdown in the integration of the skin. Curing is influenced by systemic mediators, local wound factors, injury types and underlying illness. All these factors play on each other to determine whether there is an illness process of treatment, which is commonly called chronic wound healing, or whether there is physiological or acute wound treatment. Approximately 1% are Europeans that suffer from chronic lesions, in certain cases it is hard to treat with an interdisciplinary approach. Not only do chronic lesions have adverse impacts on the quality of life of patients involved, but they also remove a heavy economic burden: approximately 2% healthcare budgets are allocated to treat old lesions [10]. The orderly process of wound healing is a series of overlapping, which is ongoing in the incremental steps of increasing complexity [100].

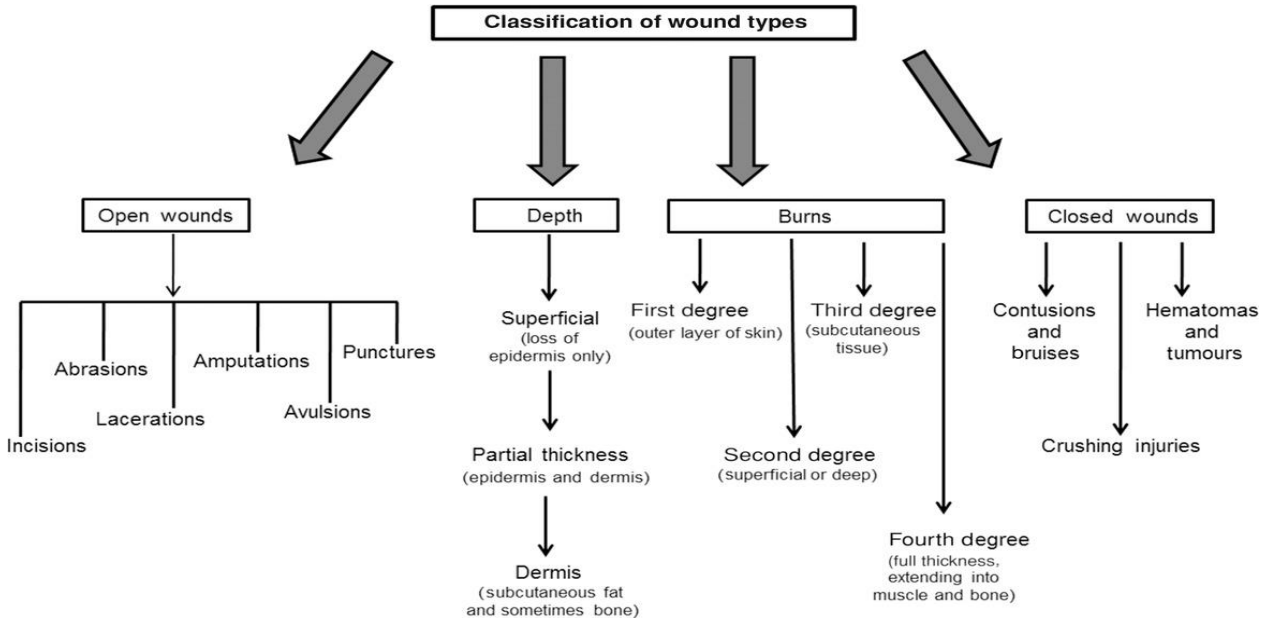


Fig No.:13 Classification of wound healing(https://doi.org/10.1002/jps.24068)

1.5.1 Classification

From the management point of view, diagnosis, selection of therapy, need, and risk assessment, wound classification is critical and avert infection that may result in the process of healing. 7-9 to date, there is no universal and standardized classification within this region. This can be attributed to the vast diversity of wounds, their complexity, most of their etiology, and a mix of sub-classes that lie beneath for wounds. Nonetheless, publications, journals, scientific papers and associate websites offer a number of current categorizations in this area. Based on the wholesome qualities, such groups have been proposed to categorize minimum quantities of lesions [101].

1.5.2 Phases of wound healing

A severe wound is treated in a general order of events. This cascade of events is in a cautious regulated process repeatable from wound to wound. Although the processes of the wound healing overlap, they are outlined in a linear process for ease of explanation. The five phases of wound healing are is : 1. Hemostasis, 2. Swelling, 3. Dissemination, 4. Remodeling or maturity [102].

1. Hemostasis (immediate): A series of serin protease reactions intended to remove blood loss, early events following injury are intended to produce hemostasis within the first few minutes of the first few minutes. Apart from hemostasis, platelet activation also initiates the release of growth factors like platelet-type development factors (PDGF) and immunological mediator, which stimulate the immune system and initiate the inflammatory stage of wound healing [103].

If the blood tissue is spilled in the open wound site as a result of trauma, the extrissure clotting cascade is brought in, which is secreted to mediators like serotonin which results in regional vasoconstiction and initiates the phase of hemosthesis [104]. Following platelet activation and aggregation upon subdorothellial collagen, growth factor and cytokines are secreted by platelets that result in the creation of a hemostatic plug [105]. By releasing scaffold proteins like fibronectin, vitronctin, and thrombospondins, it not only stops bleeding, but also acts as a pre-existing matrix for migration of cells in order to facilitate immune cells, keratinocytes and fibroblast migration [106].

2. Inflammation (0–3 days): During the initial 72 hours of tissue injury, inflammatory phases and early hemostasis are comparable to one another [107]. To avoid unwanted tissue damage and clear pathogens and exotic debris, this period is typified by a intricate pattern of most chemical signals that ultimately leads to neutrophil and monocyte invasion in the wound bed [108].

Local signals play a subordinate role in the recruitment of inflammatory cells into the wound site [109]. Molecular patterns (Damps) for damage are given jointly to these products . PAMP is also unleashed in the environment of a wound when a wound is infected with a pathogen [110]. These danger signals are detected by pattern recognition receptors (eg, toll-like receptors) of tissue-dwellers in the vicinity, which activate local cells. Several genes encoding crucial chemical mediators, which are inflammatory reactions inducers are later activated by these cells [111].

3. Proliferation: (3–10 days): This occurs. 14 days after injury and reaches. 4 days after injury. A cascade. of cells are released in the body like. individuals who are liable for migration and. spread in the form of inflammation. Ceratinocytes located at the basal layer of the wound edge move and differentiate into epithelial stem cells from nearby hair follicles or sweat glands, which are employed in recrycation [112,113].

These three major MAP kinage pathways that govern keratinocyte discrimination are initiated by a chain of stimuli like calcium inflex, EGF and TNF. Approximately two to three days post-injury, keratinocytes are matured and granular tissue, which substitutes homeostasis matrix and forms a barrier between the surrounding environment and wound [114].

4. Remodeling and maturity (month to month): remodeling or maturity is the last phase of wound healing. The process remains almost identical in permanent month or even, each lesion [115]. Fibroblast cells discharge the wound bed and the collagen is transformed into a more structured matrix in the process of maturity. Two weeks following the injury, the granulated tissue is replaced with scar tissue. Mechanical stress and fibroblast in Myofibroblast result in tightening of the wound [116]. Cross-link to enhance collagen fibril tensile strength or mechanical stress, which is induced by vitamin C. Smarted muscle actin (SMA) by fibroblast, and activation of cytokines like the contraction of the wound, and the TGF-β transform into myofibroblasts.

Following the injury, the tensile strength of the recovered tissue (scar tissue) only gets 70–80% of the initial tensile strength [117,118].

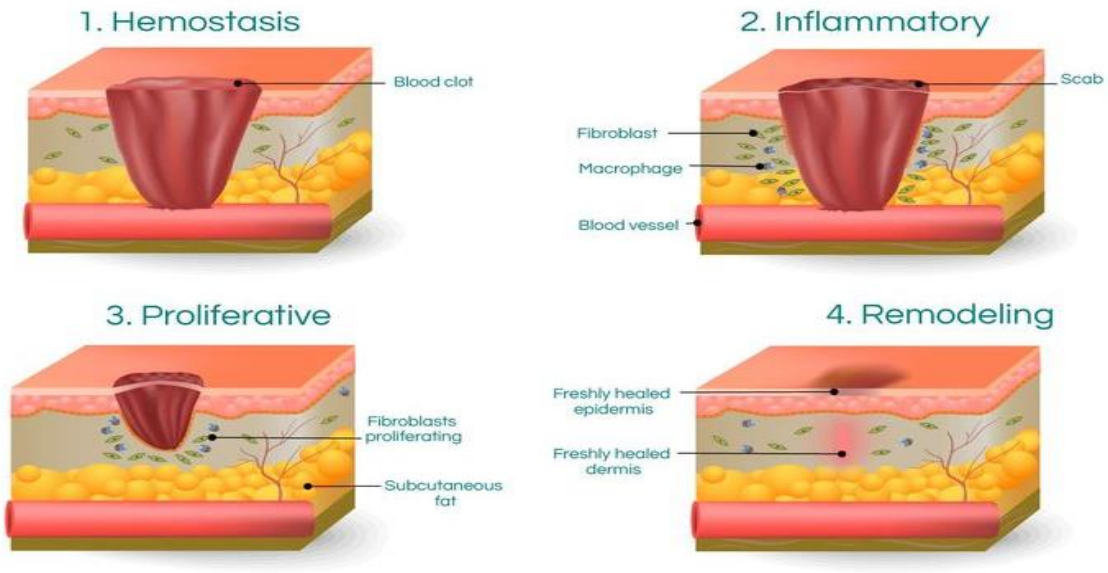


Fig No.:14 Phases of wound healing (https://i.pinimg.com/736x/b4/14/de/b414de3ee000efe7399566abe1835ad0.jpg)

1.5.3 Skin layers involved in wound healing:

The skin has three main layers involved in wound healing: **epidermis**, **dermis**, and **hypodermis**. The **epidermis** is the outermost layer (0.5 mm on eyelids to 1.5 mm on soles), primarily made of keratinocytes and melanocytes arranged in stratified squamous epithelium. It includes Merkel and Langerhans cells, with keratinocytes being the most prevalent [129]. The epidermis typically has four layers based on keratinocyte size and health. The basal layer lies on the basement membrane, while other cells undergo apoptosis or differentiate into the stratum spinosum [119].

Epidermal appendages, developed from the acaroderma, include nails, apocrine glands, eccrine ducts, and pilosebaceous units. Keratinocytes from pilosebaceous units aid in wound epithelialization [120].

The **dermis**, beneath the epidermis, is a thick, fibrous, and elastic tissue with two layers: the superficial papillary dermis and deeper reticular dermis [121]. Most dermal structures are mesodermal, except veins derived from the neural crest like melanocytes. Dermis includes fibroblast-derived cells, neurons, glands, hair roots, mast cells, muscles, and blood/lymphatic vessels [122].

The **hypodermis** (subcutaneous fat) starts forming in the fifth month of gestation. It undergoes adipocyte remodeling (adiponectin), acts as an endocrine organ via aromatase enzymes converting androstenedione, and releases leptin for weight regulation [123].

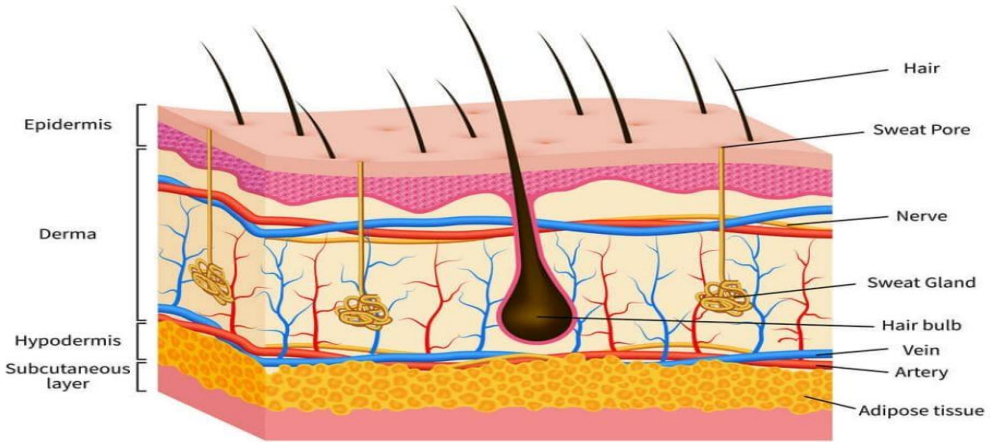


Fig No.:15 Human Skin layers(https://images.app.goo.gl/nQ98d41HX14BhXqW6)

1.5.4 Factors effecting of wound healing:

Wound healing is influenced by both local and systemic factors. Local factors directly affect the wound site, while systemic factors relate to the overall health of the individual.

Local Factors:

- **Ischemia:** Healing requires ATP, mainly produced via oxidative phosphorylation. Ischemia limits oxygen and glucose delivery, slowing or halting healing due to hypoxia or low glucose availability [124].
- **Oxygen:** Transient hypoxia can trigger healing, but sustained hypoxia impairs it. Hypoxia stimulates repair mechanisms via cytokine and growth factor release from macrophages, keratinocytes, and fibroblasts [125].
- **Infection:** Skin damage allows microbial entry. Colonization involves bacteria without tissue damage, while contamination implies harmful organisms causing local tissue reactions. Infections lead to collagen breakdown and impaired healing, especially in elderly patients [126,127].

Systemic Factors:

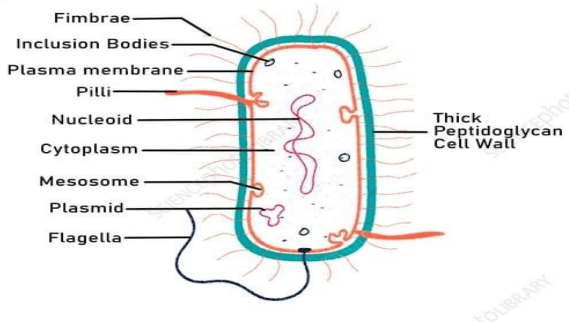
- **Age:** Older adults heal slower due to increased comorbidities like diabetes, pulmonary disease, and vascular conditions, which independently hinder recovery [128].
- **Diabetes Mellitus:** Hyperglycemia affects wound healing via toxic by-products like sorbitol and increased vascular permeability, disrupting nutrient and oxygen delivery [128].
- **Hypothyroidism:** Low thyroid hormone levels impair fibroblast activity and collagen synthesis, reducing wound tensile strength. Animal studies confirm delayed healing under hypothyroid conditions [129].

1.6 GRAM POSITIVE BACTERIA

Gram-positive bacteria are those that respond positively to gram stains, a test that has been working for many years, they have to quickly classify the bacteria into two large groups according to the nature of the cell wall. Microbiologist use gram stain to divide bacteria into two broad groups: gram-positive (+) and gram-negative (-). The walls of the gram-positive bacterial cell are heavy. However, the walls of the gram-negative bacterial cells are thin.

Methicillin-resistant staphylococcus strains occur in most countries in high frequencies in medical institutions. Annual growth in penicillin-resistant pneumococci is dangerous [130].

.Fig No.:16 Gram Positive Bacteria(https://images.app.goo.gl/rKhWiwLYUHNJCzek9)



1.6.1 Pathogenicity of gram-positive bacteria:

There are six gram-positive human-injuring species, two being Streptococcus and Staphylococcus, spherical cocated, and the others are baseless, or rod-shaped animals, that are divided depending on their capacity to generate diseases: bezylus and clostridium anarbis, forms. The spore-producing bacteria are also differentiated depending on their behavior: Clostridium is an ignorant, while the basilus is a faculty anarob [131]. Bacteria cause harmful and, sometimes deadly diseases in infants.

Example: Staphylococcus, Enterococcus, Streptococcus pyogenes, Corynebacterium .

1.7 Gram Negative Bacteria

Crylet dyelet dye planned in gram blurred technology of bacteria discrimination is not conducted by gram-negative bacteria, unlike gram-positive bacteria. Their cell envelope consists of a thin peptidoglycan cell wall located between an outer membrane and an internal (cytoplasmic) membrane. All environment that supports life on Earth porters these microorganisms. Gram-negative bacteria (GNB) are one of the largest public health concerns in the world due to their important antibiotic resistance.

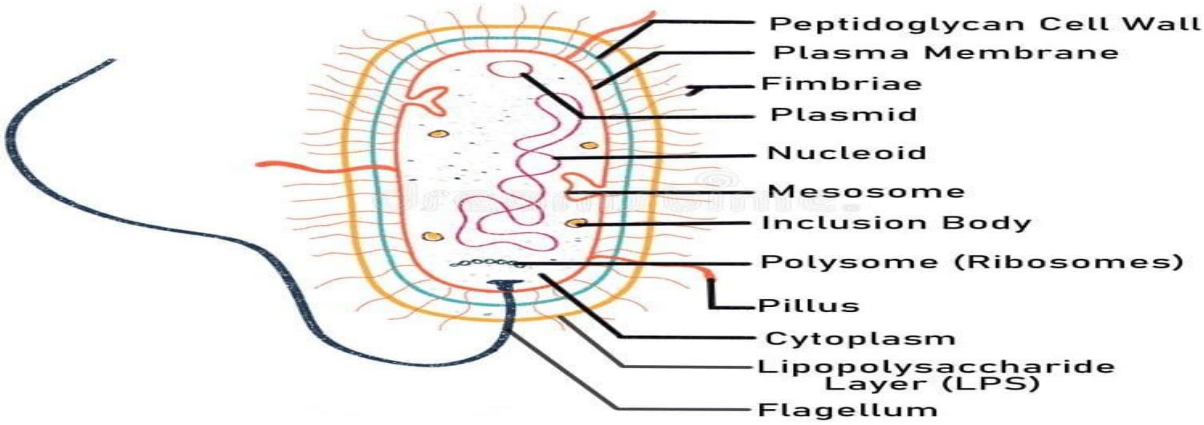


Fig No.:17 Gram Negative Bacteria(https://images.app.goo.gl/dQo9VWoWfkUyTdwR8)

1.7.1 Pathogenicity of gram-negative bacteria:

Gram-negative bacteria are particularly fatal because they are capable of bringing many diseases and antibiotic-resistant. Their outer membranes have lipopolescarides (LPS), which are able to trigger a strong immune response, and a range of viral factors that enable them to enter and destroy host organs. Their growing antibiotic resistance, which is often caused by genetic changes or mutations, is a leading resistance to genes, is another major treatment problem. Bacteria are the most important diseases that affect warmwatter fish held in captivity.

Example: Pseudomonas, Klebsiella, Proteus, Salmonella, [132]

1.8 CALCIUM CHLORIDE

Calcium chloride is an inorganic salt composed of calcium and chlorine ions. It exists as a white, crystalline solid at room temperature and is very soluble in water to form a clear solution. The compound is exceedingly disparate, i.e., it readily picks up the point of dissolving dampness from the air, which defines many of its expansive uses.

Chemically $cacl_2$, is an ionic compound, which exists in the form of calcium salt and hydrochloric acid. It may be produced in many ways, typically as a by-product of the solve process (sodium carbonate manufacturing) or by reacting with hydrochloric acid to hydrochloric acid .[133]

The versatile application of calcium chloride is a key factor due to its distinctive physical and chemical attributes. Its capacity to drastically inhibit the cold point of water confers to it an efficient function as a de-hijing agent on roads and pavements, where there is cold weather. Moreover, utilization of its hygroscopic quality is for dust control on roads and as a desiccant in some industrial and domestic processes, including the air decimidifier.

Beyond these general uses, calcium plays an important role in a wide range of chloride industries, including food processing (as a firming agent and preservative), construction (as a solid accelerator), and including various chemical processes.

1.8.1 Properties of calcium chloride:

Calcium chloride is typically available as colorless crystalline solid. It can be in the form of bunches, pellets, corpuscles, or powders.

- Odour- Calcium chloride is odourless. That is to say that it has no smell which could be easily known.
- Solubility in water- It is very soluble in water. A solution resulting from dissolving is clear and translucent. It has a high solubility with increasing temperatures.
- Melting points: Unemjal calcium chloride has a relatively high melting point, about 772 ° C (1422 ° F).

- Boiling points: Its boiling point is also very high, around 1935 ° C (3515 ° F).
- Density: The density of the waterless form is about 2.15 grams per cubic centimeter. Hydrated forms, which contain water molecules within their crystal structure, have low density.[134]

1.8.2 USES

- D-icing and dust control: Take advantage of your ability to reduce the cold point of water and absorb moisture, Cacl2 is widely used to melt ice on roads and pavements. It effectively controls dust on unexpected roads by tying fine particles.
- Food Industry: In food, it acts as a firming agent in canned vegetables (such as tomatoes and pickles) and tofu, maintains texture. It is also used as cheesymaking, brooing (to accommodate water mineral content) and an electrolyte in sports drinks.
- Construction: cacl2 as a concrete accelerator , concrete setting and tightening, especially beneficial in cold conditions.
- Desiccant: Its strong hygroscopic nature makes it an excellent desiccant to remove moisture from air, gases and fluids in industrial processes and domestic dehumidifiers.[135]

PLAN OF WORK

1. Preformulation Studies of Tetracycline Hydrochloride
    - ♦ Melting Point
    - ♦ Solubility
2. Mucilage Extraction from Abelmoschus Esculentus (Ladies Finger)
    - Collection Of Ladies Finger
    - Boiling Of Ladies Finger
    - Extraction Of Mucilage
    - Drying
3. Microsphere Preparation
    - Preparation Of Mixture Of Sodium Alginate & Mucilage
    - Preparation Of $CaCl_2$ Solution
    - Preparation of Microsphere
    - Drying
4. Antimicrobial Studies
    - Preparation Of Culture Media
    - Staining
    - Incubation
    - Microbial Studies
    - Zone Of Inhibiton

METHODOLOGY

1. Preformulation Studies of Tetracycline Hydrochloride

A. Solubility

i. *Preparation:*
   a. Weigh excess TCH.
   b. Add to fixed volumes of each solvent in separate containers.

ii. *Agitation:*
   a. Stir solutions for 24 hours at controlled temperature (usually 25°C or 37°C).

iii. *Filtration:*
   a. Filter the solutions to remove undissolved drug.

iv. *Analysis:*
   a. Measure concentration of TCH in the filtrate using UV spectrophotometry or HPLC.

B. Melting Point of Tetracycline Hydrochloride

- **Reported Melting Point:**

Approximately **220°C to 230°C (with decomposition)**.

Procedure:

Sample Preparation

↓

Finely powder Tetracycline Hydrochloride (Dry if needed to remove moisture)

↓

Fill capillary tube (2-3 mm of sample)

↓

Seal Capillary Tube (optional if apparatus requires)

↓

Insert Into Melting Point Apparatus

↓

Set initial temperature (20-30°C below expected MP)

↓

Heat Slowly (1-2°C per minute near expected MP)

↓

Observe Melting Range

(Start of melting to completely liquefied)

↓

Note Melting Point

↓

Repeat For Accuracy (optional)

↓

Clean Apparatus After Use

2. Mucilage Extraction from <u>Abelmoschus Esculentus</u> (Ladies Finger)

- *Collection*: Fresh, healthy ladies finger fruits are selected.
- *Boiling*: Helps to release mucilage into water.
- *Filtration & Extraction*: Separates mucilage from plant material.
- *Drying*: Removes moisture for storage and further use.

Procedure:

Collection Of Ladies Finger (From Dubrajpur Market 100gm)

↓

Wash thoroughly to remove dirt & impurities

↓

Cut into small pieces

↓

Boiling Of Ladies Finger

↓

Boil in distilled water (60-80°C for 30-60 min)

↓

Cool the boiled mixture

↓

Filtration

↓

Filter through muslin cloth to separate mucilage

↓

Extraction Of Mucilage

↓

Add suitable solvent (ethanol) to precipitate mucilage

↓

Collect Precipitated Mucilage

↓

Drying

↓

Dry at room temperature or in hot air oven (40-50°C)

↓

Collect & Store Dried Mucilage

3. Microsphere Preparation

PREPARATION OF MIXTURE OF SODIUM ALGINATE & MUCILAGE

↓

Weigh required amounts of sodium alginate & extracted mucilage

↓

Dissolve both in distilled water under stirring (magnetic stirrer) to form uniform solution

↓

Remove air bubbles (allow to stand or use sonication)

↓

PREPARATION OF $CaCl_2$ SOLUTION

↓

Prepare calcium chloride solution (e.g., 2-5% w/v) in distilled water

↓

Stir until completely dissolved

↓

PREPARATION OF MICROSPHERES

↓

Drop sodium alginate-mucilage mixture into $CaCl_2$ solution using syringe or pipette (dropwise)

↓

Microspheres form instantly due to ionic crosslinking (gelation)

↓

Allow microspheres to cure in $CaCl_2$ solution (15-30 min)

↓

Collect microspheres by filtration

↓

WASH microspheres with distilled water to remove excess $CaCl_2$

↓

DRYING

↓

Dry microspheres at room temperature or in hot air oven (40-50°C)

↓

COLLECT & STORE DRIED MICROSPHERES

Batch No.	Sodium Alginate (% w/v)	Mucilage (% w/v)	Total Volume (mL)	Calcium Chloride (% w/v)
1	1.0%	0.5%	100	2%
2	1.5%	0.5%	100	2%
3	1.0%	1.0%	100	2%
4	2.0%	1.0%	100	2%
5	2.0%	1.5%	100	2%

Table No.:2 Various Formulation of Microsphere Preparation

Among the various formulations prepared, **Batch No. 3 (1% Sodium Alginate and 1% Mucilage)** was found to be the most suitable for microsphere preparation. This batch provided:

- Good microsphere formation with uniform spherical shape.
- Optimum viscosity of the polymer solution, allowing easy drop formation.

- Smooth surface and good mechanical strength of microspheres.
- Stable cross-linking with calcium chloride, ensuring proper gelation.

Thus, the balanced combination of Sodium Alginate and Mucilage at 1% each resulted in microspheres with desirable characteristics suitable for further pharmaceutical applications.

4. Antimicrobial Study

Procedure:

PREPARATION OF CULTURE MEDIA
↓
Weigh and dissolve required amount of Nutrient Agar or Mueller-Hinton Agar in distilled water
↓
Sterilize media by autoclaving (121°C, 15 psi, 15-20 min)
↓
Pour sterile media into petri plates and allow to solidify
↓
STAINING (for microbial identification if needed)
↓
Prepare smear of microbial culture on slide
↓
Stain using Gram staining or other suitable method
↓
Observe under microscope for organism confirmation
↓
INOCULATION
↓
Inoculate the solidified media with test microorganism (e.g. *E. coli*, *S. aureus*)
↓
Apply test sample (microspheres or drug) on the inoculated media (disc or well diffusion method)
↓
INCUBATION
↓
Incubate plates at 35-37°C for 24-48 hours
↓
MICROBIAL STUDIES
↓
Observe microbial growth and compare with control
↓
ZONE OF INHIBITION
↓
Measure the clear zone (no growth area) around the sample
↓
Record zone diameter in mm
↓
ANALYZE RESULTS

RESULT

1. Preformulation Studies of Tetracycline Hydrochloride

A. Solubility

SOLVENT	SOLUBILITY	REMARKS
Water	Highly Soluble	Approx. 50 mg/ml at room temperature forms a yellow solution
Hot water	Very Soluble	Solubility increases with temperature
Ethanol	Slightly Soluble	Limited solubility

Methanol	Slightly Soluble	Slightly more soluble than ethanol
Dilute Acid	Soluble	Stable and soluble in acidic medium

Table No.:3 Solubility Of TCH

From the above table we conclude that

- Tetracycline hydrochloride is highly soluble in water and acidic pH.
- Its solubility decreases as pH increases (due to its weakly basic nature).
- Organic solvents show poor solubility.
- These results help in selecting suitable solvents and pH for drug formulation.

C. MELTING POINT

NUMBER OF ATTEMPTS	MELTING POINT
1st	180°C
2nd	185°C
3rd	180°C
4th	185°C
5th	177°C
6th	190°C
7th	183°C

Table No. 4: Melting point of tetracycline hydrochloride

Average:

(180+185+180+185+177+190+183/7) = 182.85°C

2. **Mucilage Extraction from Abelmoschus Esculentus (Ladies Finger)**

Parameter	**Observation / Result**
Plant Material Used	*Abelmoschus esculentus* pods (Ladies Finger)
Weight of Fresh Pods Taken	100 g
Volume of Water Used	200 mL
Extraction Method	Hot water extraction followed by filtration and drying
Dry Mucilage Yield	3.5 g (from 100 g fresh pods)
Percentage Yield	3.5%
Colour of Mucilage	Pale yellow to off-white
Texture	Sticky, viscous
Solubility	Swells in water; insoluble in organic solvents
pH of 1% Mucilage Solution	6.2 (near neutral)
Other Observations	No Odor, forms a gel-like mass when hydrated

Table No. 5 : Result of Mucilage Extraction from Abelmoschus Esculentus

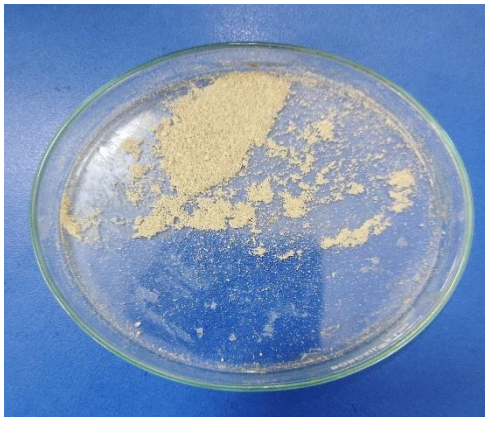


Fig No.18: Mucilage Extraction from Abelmoschus Esculentus

3. **Microsphere Preparation**

Microspheres of the drug were successfully prepared using the cross linking method, showing **good yield, high entrapment efficiency, and desirable sustained-release behaviour**. The prepared microspheres are suitable candidates for **controlled drug delivery** applications.

Fig No.19 : Microsphere Without Drug

Fig No .20: Microsphere with drug (TCH)

5. Antimicrobial Study

Antimicrobial activity of tetracycline hydrochloride and chloramphenicol loaded microspheres was tested against four disease-causing microorganisms : escherichia coli, bacillus subtilis, staphylococcus aureus, and candida albicans by the agar well diffusion technique. The zone of inhibition distance was determined in millimeters at a uniform concentration of 5 mg/ml for all formulations.

The results indicated that the microspheres exhibit wide-range antimicrobial activity, with differences in effectiveness based on the individual microorganism. Of all the tested strains, staphylococcus aureus was the most sensitive towards the tetracycline-loaded microspheres with 4.2 mm zone of inhibition, followed by candida albicans (3.0 mm), and both e. Coli and b. Subtilis (2.5 mm for each). Chloramphenicol was more inhibitory for e. Coli (3.2 mm) and b. Subtilis (2.3 mm) but were relatively less effective against staphylococcus aureus (2.5 mm) and candida albicans (1.2 mm).

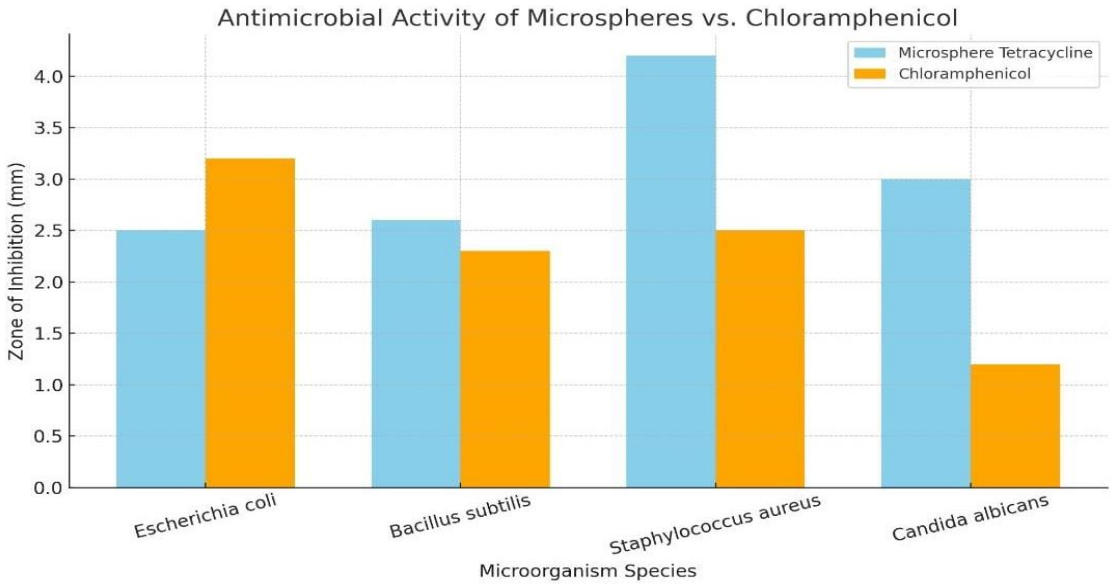
Zone of Inhibition

Post-incubation, the diameter of the zone of inhibition surrounding each well was measured in millimeters (mm) using a transparent ruler or Vernier caliper. All tests were performed in triplicate to ensure reproducibility, and the mean values were recorded.

SL NO	Microorganism species	Concentration (mg/mL)	Microsphere Tetracycline hydrochloride (mm)	Chloramphenicol (mm)
1	Escherichia coli	5	2.5	3.2

2	Bacillus subtilis	5	2.5	2.3
3	Staphylococcus aureus	5	4.2	2.5
4	Candida albicans	5	3.0	1.2

Table No 6: Antimicrobial activity of microsphere containing tetra cyclin hydrochloride and Chloramphenicol against Pathogenic microorganism.



Graph 1: Antimicrobial Activity of Microspheres vs. Chloramphenicol

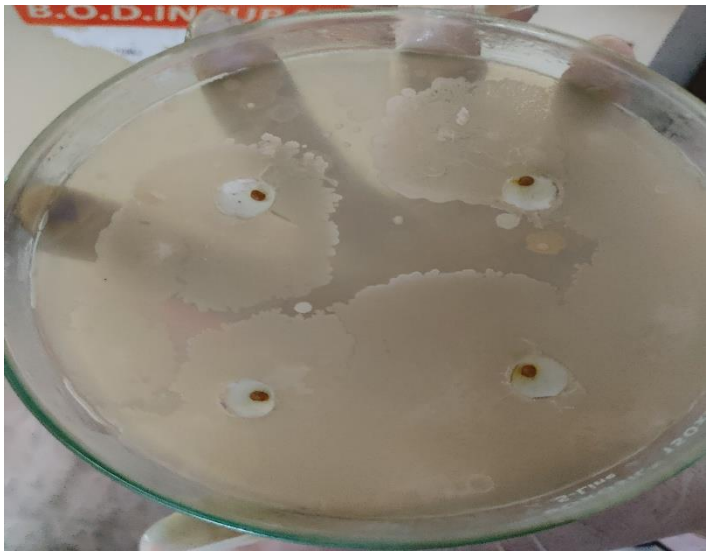


Fig No.21: Antimicrobial Study

DISCUSSION

The present study evaluated the antimicrobial efficacy of tetracycline hydrochloride-loaded microspheres using okra mucilage against common pathogenic microorganisms. The zones of inhibition were assessed and compared to standard chloramphenicol, providing insight into the formulation's effectiveness.

The microsphere formulation showed significant antimicrobial activity, particularly against Staphylococcus aureus (4.2 mm), which surpassed the inhibition zone of chloramphenicol (2.5 mm) for the same organism. This suggests that the microspheres facilitated enhanced localized drug concentration and release at the infection site, likely due to the sustained release properties of the okra mucilage and sodium alginate polymeric matrix. This is promising for treating gram-positive infections, especially in wound or tissue regeneration contexts.

Escherichia coli, a gram-negative bacterium, was also effectively inhibited (2.5 mm), although slightly less than the standard (3.2 mm), indicating that while the microspheres are effective, chloramphenicol maintains a broader spectrum against certain gram-negative strains. The moderate activity against Bacillus subtilis (2.6 mm vs. 2.3 mm) further validates the formulation's broad-spectrum potential.

The lowest activity was observed against Candida albicans, a fungal strain, where the microsphere zone (3.0 mm) was significantly greater than chloramphenicol (1.2 mm), suggesting that tetracycline-loaded microspheres may provide some antifungal effects, though tetracyclines are not traditionally fungicidal. This could be attributed to the physical barrier or pH-modifying microenvironment created by the polymer matrix.

From a formulation standpoint, the use of natural polymers like okra mucilage offers numerous advantages: biocompatibility, biodegradability, sustained release potential, and low toxicity. The successful incorporation of tetracycline hydrochloride into a microsphere system supports its suitability as a drug delivery vehicle for chronic infections and wound healing applications.

Overall, this study highlights the potential of okra-based microsphere systems as a viable alternative to conventional antibiotic delivery, with potential to reduce dosing frequency, improve patient compliance, and minimize side effects due to targeted and controlled release.

ACKNOWLEDGMENT:

The successful completion of this research work titled "Preparation and Anti-Microbial Studies of Tetracycline Hydrochloride Loaded Microsphere Using Mucilage Extracted from Abelmoschus esculentus for Tissue Regeneration Applications" would not have been possible without the invaluable support and contributions of several individuals and institutions.

First and foremost, I express my deepest gratitude to my research supervisor, Mr. Sourav Mandal, for their continuous guidance, insightful suggestions, and unwavering encouragement throughout the course of this study. Their expertise and constructive feedback were instrumental in shaping the direction and outcome of this work.

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