DEVELOPMENT AND EVALUATION OF ALGINATE FILM CONTAINING OKRA MUCILAGE FOR WOUND HEALING.

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CERTIFICATE

This is to certify that **VIVEK SAHU** (Class Roll No.: 002111402028 and Reg. No.: 160256 of 2021-2023), has carried out the research work on the subject entitled "***DEVELOPMENT AND EVALUATION OF ALGINATE FILM CONTAINING OKRA MUCILAGE FOR WOUND HEALING***" under my supervision in the Pharmaceutics Research Laboratory in the Department of Pharmaceutical Technology of this university. He has incorporated his findings into this thesis of the same title, being submitted by him, in partial fulfilment of the requirements for the degree of **Master of Pharmacy** of Jadavpur University. He has carried out this research work independently and with proper care and attention to my entire satisfaction.

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DECLARATION OF ORIGINALITY AND COMPLIANCE OF ACADEMIC ETHICS

I hereby declare that this thesis contains literature survey and original research work by the undersigned candidate, as part of her Master of Pharmaceutical Technology studies. All information in this document have been obtained and presented in accordance with academic rules and ethical conduct. I also declare that as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

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LIST OF ABBREVATION

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1	3D	Three dimensional	1
2	PBS	Phosphate buffer saline	23
3	TS	Tensile strength	24
4	AG	Alginate	39
5	OK	Okra	39
6	AGOK	Alginate-Okra	39

CONTENTS

CHAPTER-I

INTRODUCTION

1. INTRODUCTION

Biodegradable films based on natural origin polymers have been widely investigated for several biomedical applications, including tissue engineering [1], drug delivery [2] and wound healing [3], due to its biocompatibility, biodegradability and properties similar to the human tissues [4,5]. Hydrogels 3D hydrophilic polymer networks that can swell in aqueous solutions without dissolution, maintaining its structure [6,8]. Natural hydrogels are attractive materials for the treatment of different types of wounds, due to its properties such as smoothness, high water content, elasticity, malleability and ability to provide a moist environment protecting the wound from desiccating [8,9]. The healing of a wound is a complex, dynamic and continuous process aiming at the repairing of damaged tissue. The efficient treatment system of a wound is very important to improve the healing process, in terms of both quality and time, as well to reduce the costs associated with the treatment. Currently, there is a great variety of wound-care products, available in the market, including creams, solutions, dressings or skin tissue engineering substitutes [10,11]. Among these products, polymeric dressings represent an effective method for wound treatment, presenting a good relationship between clinical efficacy and manufacturing cost. However, for some types of wounds, such as infected wounds, the isolated use of polymeric dressings cannot be sufficient to promote the healing process, as many of these materials do not present therapeutic activity (e.g., antibacterial and antiseptic characteristics). In order to solve this limitation, some dressings, based on natural polymers, were developed incorporating different synthetic drugs to reduce the growth of microorganisms in wounds [12]. The continuous administration of synthetic drugs in infected wounds, though associated with the development and spread of antibiotic-resistant strains of bacteria, present satisfactory clinical results.

1.1 Classification of Hydrogels

Based on source:

They can be of natural or synthetic origins.

- Natural polymers including proteins such as collagen and gelatin and polysaccharides such as alginate, starch, cellulose, glucomannan, pectin, hemicellulose, gums, and agarose forming hydrogels.
- Synthetic polymers including polyvinyl alcohol (PVA), polyethylene glycol (PEG), polyacrylic acid (PAA), polyacrylamide (PAM) that form hydrogels are conventionally synthesized using chemical polymerization methods [13].

Based on polymeric composition or synthesis techniques:

- Homopolymeric hydrogels have basic structural and functional unit comprising of a single type of monomer in the polymeric network. They can have cross-linked skeletal depending on the polymerization technique as well as on the type of monomer [14].
- Copolymeric hydrogels are derived from a variety of monomeric units with at least one hydrophilic component. The polymeric network chains can be arranged in a random, block or alternating configuration [15].
- Multipolymer interpenetrating polymeric network (IPN) can be synthesized using two independent cross-linked natural or synthetic polymer component, confined in a network form. In case of Semi-interpenetrating hydrogel, one polymer is a cross- linked and other polymeric component is a non-cross-linked [16]. They interact without any chemical bonding between them, one straight polymeric chain penetrates into another crosslinked network [17].

Based on physical structure and chemical composition:

- Non-crystalline (Amorphous)
- Semicrystalline, a composite of amorphous and crystalline phases.
- Crystalline.

Based on cross-linked networks:

- Chemical cross-linking: Chemically cross-linked networks have permanent bonding involving covalent interaction.
- Physical cross-linking: Physical networks have transient junctions involving entanglements of polymeric chain involving hydrogen bonds, polar or ionic, hydrophobic type of physical interactions [2].

Based on electrical charge:

- Neutral (non-ionic),
- Ionic (including anionic or cationic),
- Amphoteric having both acidic and basic groups,
- Zwitterionic (polybetaines) possess both cationic and anionic functionality in each repeating unit [2].

1.2 ALGINATE

Alginate is an anionic polysaccharide, which is capable to form hydrogels under very mild conditions, at room temperature and in the absence of toxic solvents [21]. Alginate has been widely used and investigated due to its biocompatibility, biodegradability, relative low cost, low toxicity and gelling properties [18,21,22]. Commercially available alginates are typically extracted from brown algae (Phaeophyceae), including Laminaria hyperborea, Laminaria digitata, Laminaria japonica, Ascophyllum nodosum, and Macrocystis pyrifera. Alginates can also be obtained by bacterial biosynthesis from Azotobacter and Pseudomonas, exhibiting a more defined chemical structure and physical properties, when compared with the seaweed-derived alginates [21]. Its chemical structure contains blocks of (1,4)-linked d-mannonate (M) and l-guluronate (G) residues that can be arranged in the form of homopolymeric sequences (MMM or GGG) or alternating sequences (MGMG) along the polymeric chain [23]. The content of M and G blocks, their distribution in the polymeric chain and the length of each block strongly determine the alginate physical and chemical properties and its gelling properties [23,24]. Alginate hydrogels are mostly prepared by external gelation, using calcium ions as cross-linking agents. The cation interacts and binds with guluronate blocks of alginate chains, forming the gel network [21,23]. Due to its high-water content, elasticity, permeability and ability to create a moist environment in the wound bed, alginate gels have been widely used for the treatment of severe kinds of wounds [25–26]. In particular, calcium alginate hydrogels were investigated and used for wound healing applications, due to both the hemostatic properties of the calcium ion and the ability of the gel to serve as a matrix for the aggregation of platelets and erythrocytes [20,21,27,28]. Additionally, the porosity of the hydrogels allows the entrapment/immobilization of therapeutic agents (drugs, growth factors, etc.), which are then released in the wound [21,23]. This approach can be used, for example, in the treatment of wound infections, which are frequently present in the early stages of the wound healing process. The healing of a wound is a very complex and dynamic process that involves four main phases, which are distinct, sequential and overlapping [19]: hemostasis, inflammation, proliferation and tissue remodeling. A great variety of processes occur during these phases, including vasoconstriction, thrombogenesis, angiogenesis, synthesis of collagen, extracellular matrix (ECM) formation and ECM remodeling [19,28,29]. An ideal wound dressing should present adequate properties to create a favorable environment for the healing process, such as flexibility, durability, permeability to water vapor, adequate mechanical properties and adherence to the tissue. In addition, the dressing should have the ability to hydrate/dehydrate the wound, maintain a moist environment, protect the wound from infections and avoid maceration [19,25,27]. The incorporation of therapeutic agents into wound dressings and their topical release

in the wound is an attractive approach to control the inflammatory reaction, prevent/eliminate infections and, simultaneously, to promote tissue regeneration. In the last years, topical administration of therapeutic agents into the wounds gained importance due to the limited efficacy of systemic treatment. This is related with the inadequate wound perfusion, which restricts the delivery of therapeutic agents to the wound [28,30] Antibiotics, such as gentamycin, ofloxacin and minocycline, are commonly used in the treatment of wound infections, which are generally characterized by the presence of bacteria (e.g., Staphylococcus aureus, Pseudomonas aeruginosa) that can compromise the wound healing [18,27–30]. However, the constant administration of these drugs can increase the resistance of the microorganisms to them, which represents a great challenge for the scientific community [31].

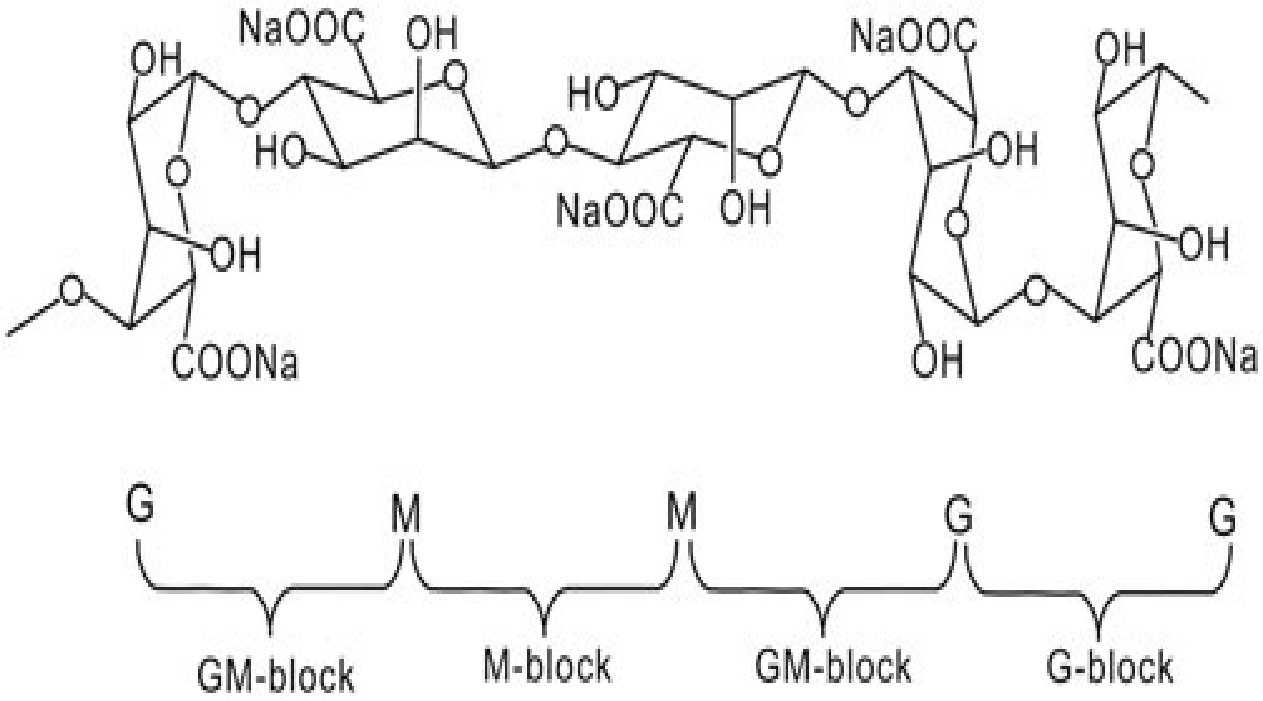

Fig: 1.1 *Structure of Sodium Alginate*

1.3 OKRA

Abelmoschus esculentus (L.) Moech is a fruit-producing species popularly known as "okra", and is widely appreciated in Brazilian cuisine. Antidiabetic, anti-oxidant, and anti-cholesterol activities have been reported for okra, as well as the presence of minerals, carbohydrates, proteins and vitamins [32,33]. In addition, the presence of mucilage is also reported in okra. Okra mucilage consists of random coil polysaccharides of galactose, rhamnose, and galacturonic acid [34] and

must have others essential constituents. The use of mucilage presents advantages over synthetic polymers due to the lower procurement cost and its biodegradability [35]. Mucilage is used as a thickener, stabilizing emulsion, suspending agent and binder in the food and pharmaceutical industries [36]. In this case, its use also offers biocompatibility and non-toxicity advantages [37]. Okra mucilage is mainly constituted by pectin, which is a polysaccharide formed by galacturonic acid units and their methyl esters [38,39]. Studies on the chemical, physicochemical and rheological characterization of mucilage and okra gum have been reported [40,41], as well as on its structural modifications [42], pharmaceutical applications and as bio flocculants [43,44]. Okra mucilage films with carboxymethylcellulose were studied to evaluate the physical mechanics parameters and antibacterial activity [45]. Other studies have related okra mucilage films to verify thermoplastic properties [46] and other applications have been reported. In contrast, starch-based edible films are widely used because these materials have high hydrophilicity, which gives the films considerable solubility in water and hence weaken the barrier to water vapor [47,48]. To address this challenge, the potential use of biodegradable films is being investigated. The aim of this study is to investigate some relevant properties of alginate and okra film to apply in wound dressing, including the thickness, transparency, fluid uptake capacity and invitro degradation properties for biomedical applications.

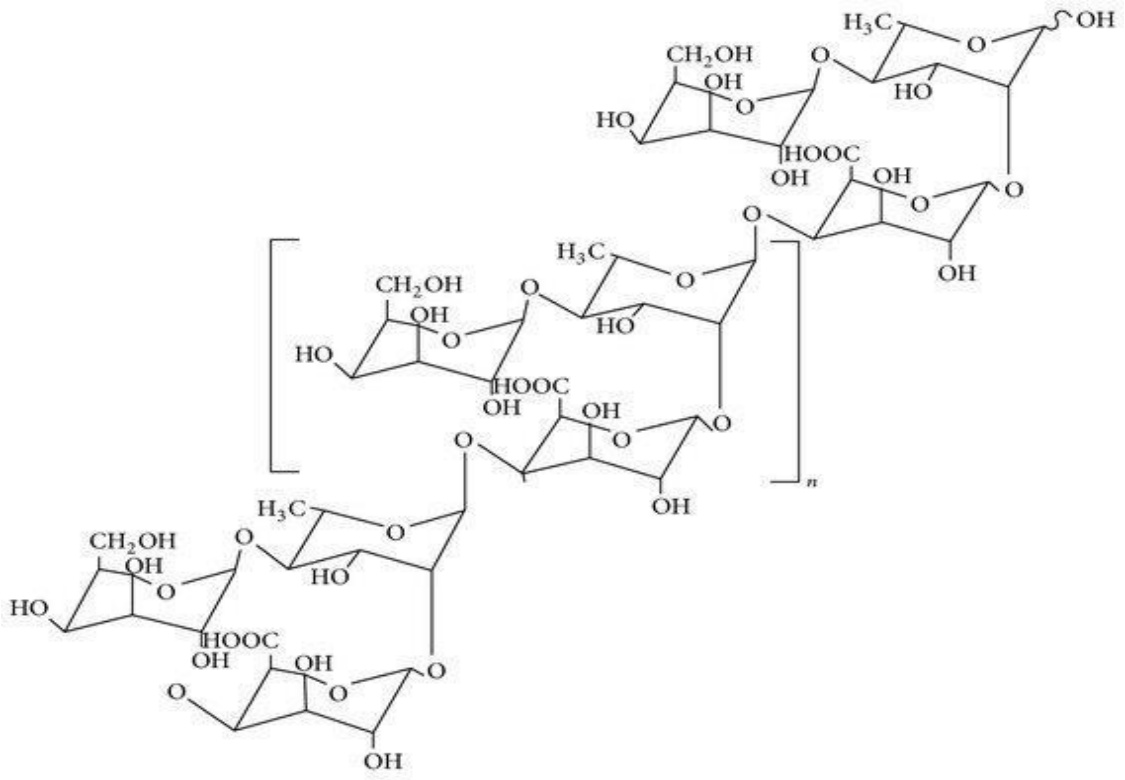

Fig: 1.2 *Structure of Okra mucilage*

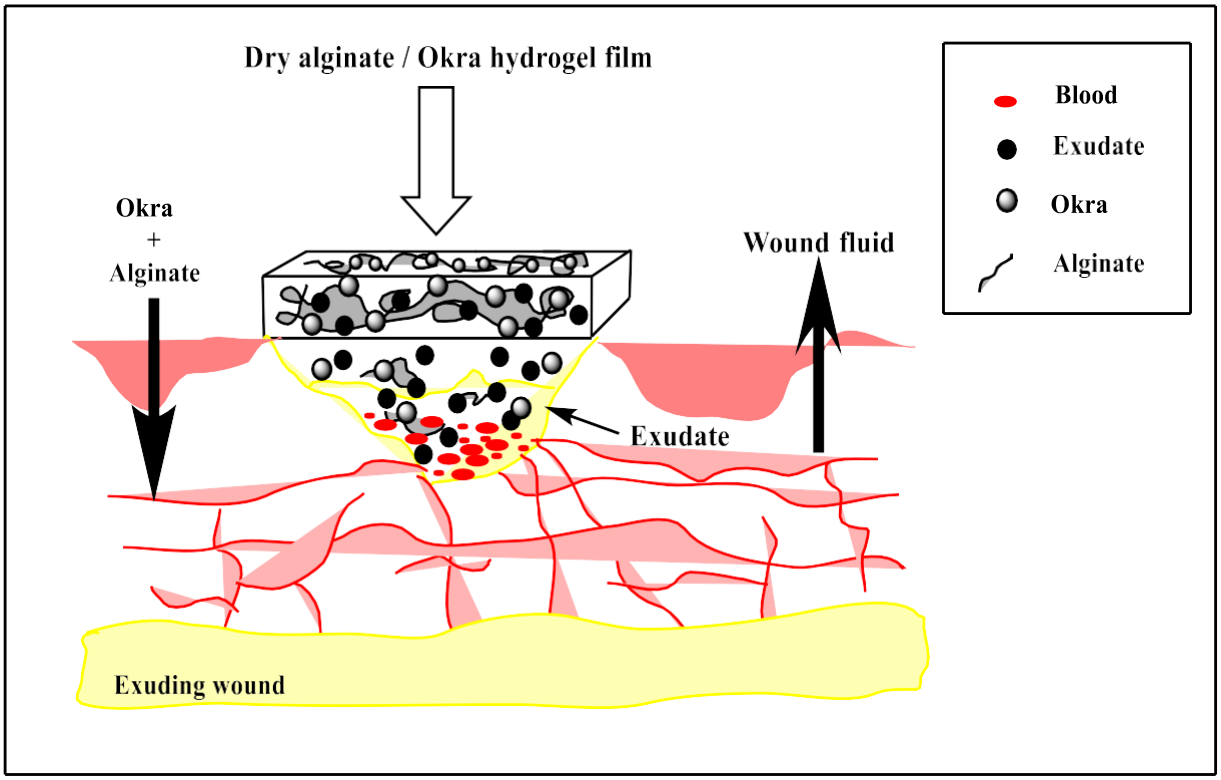

Fig: 1.3. *Application of the alginate/ okra film into exuding wound.*

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CHAPTER-II

LITERATURE REVIEW

2.1. LITERATURE REVIEW

Pereira et al. [1] describes a methodology to produce hydrogel films, composed of alginate and *Aloe vera*, for wound healing and drug delivery applications. The films were prepared through the solvent-casting method and subsequently submitted to an additional cross-linking step to improve their properties. Alginate films with different *Aloe vera* contents (5, 15 and 25%) were prepared and its properties evaluated in terms of thickness, transparency, swelling behavior and *in vitro* degradation. Results show a positive influence of *Aloe vera* on the transparency of the films, in both dry and wet state. Films were immersed in acetate buffer at pH 5.5 simulating the value of the skin, to evaluate water absorption capacity. It was found that water absorption increases as the *Aloe vera* content increases, suggesting that *Aloe vera* enhances the hydrophilic properties of the films. The *in vitro* degradation tests were performed through the incubation of the films, for 10 weeks, in a simulated body fluid at 37 °C. After this period, films kept its structural integrity exhibiting a weight loss in the range of 14.1-18.6%.

Rúben Pereira et al. [2] states that Alginate and *Aloe vera* are natural materials widely investigated and used in the biomedical field. In this research work, thin hydrogel films composed by alginate and *Aloe vera* gel in different proportions (95:5, 85:15 and 75:25, v/v) were prepared and characterized. The films were evaluated regarding the light transmission behavior, contact angle measurements, and chemical, thermal and mechanical properties. These thin hydrogel films, prepared by crosslinking reaction using 5% calcium chloride solution, were also investigated relatively to their water solubility and swelling behavior. Results showed that *Aloe vera* improved the transparency of the films, as well their thermal stability. The developed films present adequate mechanical properties for skin applications, while the solubility studies demonstrated the insolubility of the films after 24 h of immersion in distilled water. The water absorption and swelling behavior of these films were greatly improved by the increase in *Aloe vera* proportion.

Mariana et al. [3] manufactured the composite biofilms of alginate and LM-pectin crosslinked with calcium ions requires a two-step contact with Ca^{2+}: initially a low-structured pre-film is formatted which is further crosslinked in a second contact with a more concentrated Ca^{2+} solution containing plasticizer. This research evaluated the influence of the plasticizer (glycerol) concentration (1–15% w/v) in this finishing reticulation step on final films characteristics. The results indicated that the extent of the simultaneous Ca^{2+} crosslinking and plasticization with glycerol was determined by the level of structural organization obtained in

the pre-reticulation. Increasing the glycerol concentration of the crosslinking solution increased film solubility in water, moisture content, volumetric swelling and flexibility and decreased the resistance to tensile stress. Transparent alginate and pectin composite films with acceptable mechanical properties, low solubility and limited degree of swelling were obtained with 10% glycerol in the second contact solution.

Birgit et al [4] suggested that hydrogels are interesting as wound dressing for burn wounds to maintain a moist environment. Especially gelatin and alginate based wound dressings show strong potential. Both polymers are modified by introducing photocrosslinkable functionalities and combined to hydrogel films (gel-MA/alg-MA). In one protocol gel-MA films are incubated in alg-MA solutions and crosslinked afterward into double networks. Another protocol involves blending both and subsequent photo crosslinking. The introduction of alginate into the gelatin matrix results in phase separation with polysaccharide microdomains in a protein matrix. Addition of alg(-MA) to gel-MA leads to an increased swelling comparedto 100% gelatin and similar to the commercial Aquacel Ag dressing. In vitro tests show bettercell adhesion for films which have a lower alginate content and also have superior mechanical properties. The hydrogel dressings exhibit good biocompatibility with adaptable cell attachment properties. An adequate gelatin-alginate ratio should allow application of the materials as wound dressings for several days without tissue ingrowth.

Ganzhe et al. [5] mixed the solution of sodium alginate (SA) and arginine (Arg) and dried into a film and then crosslinked with zinc ion to form sodium alginate-arginine-zinc ion (SA- Arg-Zn^{2+}) hydrogel for skin wound dressings. SA-Arg-Zn^{2+} hydrogel had higher swelling ability, which was beneficial to absorbing wound exudate. Moreover, it exhibited antioxidant activity and strong inhibition against *E. coli* and *S. aureus*, and had no obvious cytotoxicity to NIH 3T3 fibroblasts. Compared with other dressings utilized in rat skin wound, SA-Arg-Zn^{2+} hydrogel showed better wound healing efficacy and the wound closure ratio reached to 100 % on the 14th day. The result of Elisa test indicated that SA-Arg-Zn^{2+} hydrogel down-regulated the expression of inflammatory factors (TNF-α and IL-6) and promoted the growth factor levels (VEGF and TGF-β_1). Furthermore, *H&E* staining results confirmed that SA-Arg-Zn^{2+} hydrogel could reduce wound inflammation and accelerate re-epithelialization, angiogenesis and wound healing. Therefore, SA-Arg-Zn^{2+} hydrogel is an effective and

innovative wound dressing, moreover, the preparation technique is simple and feasible for industrial application.

Antonio et al. [6] developed okra mucilage/corn starch films to apply in food were developed by casting and then characterized to know its main requirements for packing material. The film was submitted to an acute toxicity analysis in rats by ingesting the filmogenic solution. An okra mucilage material obtained by precipitation was analyzed by thermal analysis, Fourier Transformed Infrared Spectroscopy and Scanning Electron Microscopy with Energy Dispersive Spectroscopy. Okra mucilage and corn starch films presented compact and uniform structure, low water vapor permeability (1.32–2.84 g/m s Pa),low solubility (around 15%), good thermal and mechanical properties, swelling capacity (about 95%), and no toxicological responses. The precipitated material presented similar characteristics to those of polysaccharides. Thus, okra mucilage differed in view of its improvement in film properties because of high quality intermolecular bonds. The film obtained with okra mucilage has excellent potential to be applied in food packaging.

Yinghui et al [7] investigated that self-healing conductive hydrogels have attracted widespread attention as a new generation of smart wearable devices and human motion monitoring sensors. To improve the biocompatibility and degradability of such strain sensors, we report a sensor with a sandwich structure based on a biomucopolysaccharide hydrogel. The sensor was constructed with a stretchable self-healing hydrogel composed of polyvinyl alcohol (PVA), okra polysaccharide (OP), borax, and a conductive layer of silver nanowires. The obtained OP/PVA/borax hydrogel exhibited excellent stretchability (~1073.7%) and self-healing ability (93.6% within 5 min), and the resultant hydrogel-based strain sensor demonstrated high sensitivity (gauge factor = 6.34), short response time (~20 ms), and good working stability. This study provides innovative ideas for the development of biopolysaccharide hydrogels for applications in the field of sensors.

Rishikesh et al. [8] prepared a novel green hydrogel (PGCO) of Okra (*Abelmoschus esculentus*) mucilage-reinforced poly-vinyl alcohol-guar gum (PG) cross-linked by citric acid containing nanocurcumin (NC) as a model drug is reported. The citric acid (CA) cross-linked hydrogel (PGC) without okra is also prepared. The hydrogels are characterized using FTIR, XRD, FE-SEM, and TGA techniques. Okra reinforced green hydrogel (PGCO) provided comparable swelling behavior with better mechanical and thermal properties compared to the neat PGC hydrogel. Network parameters of PGC and PGCO hydrogels are estimated

using Flory-Rehner equation and strong correlation between the cross-link density and swelling behavior is established. 45.68 % NC loading in the PGCO hydrogel is achieved. Release study in phosphate buffer (PB) of pH 7.4 provided sustained release of NC over aperiod of 100 h. The release study of NC followed primarily the Korsmeyer-Peppas modelwith less-Fickian diffusional character ($n < 0.5$). The average diffusion coefficients_of NCand curcumin are found to be 3.52×10^{-5} cm^2 s^{-1}, and 3.43×10^{-5} cm^2 s^{-1} respectively demonstrating the quick release of NC in early time, which is a pre-requisite in drug delivery.The study provides initial evidence of the usefulness of okra mucilage in green hydrogel development and drug delivery applications.

Chokboribal et al. [9] discovered the main component of mucilage found in the fruit of okra (Abelmoschus esculentus) is okra gum consisting of pectic polysaccharides reported to exhibit, inter alia, antibacterial and antioxidant characteristics. It was used in the pharmaceutical industry,e.g., as a stabilizer or a modifier drug release. In this work, various formulations of xanthan gum (XG)/κ-carrageenan (CN)-based hydrogel with okra mucilage (OM) powder were examined to achieve the goal of developing OM-hydrogel pads that make good use of the advantages of OM polysaccharides relevant to drug delivery and/or stability. A straightforward cosmeceutical application of the OM-hydrogels is as under-eye masks since the OM itself is reported to contain diffusible small compounds with proven skincare benefits. OM from blended fresh okra pods without seeds was precipitated with ethanol. Preliminary studies showed that hydrogel pads prepared with XG, CN, glycerol, and NaCl at 0.5, 0.5, 13, and 4 wt%, respectively, possess a suitable hardness while exhibit the highest adhesive strength among other formulations tested. When OM powder was added at 0.02, 0.05, 0.06, 0.1, 0.2, 0.4, or 0.6 wt%, the OM-hydrogels' hardnesses substantially drop. Increases in hardnesses and adhesive strengths with increasing amounts of OM powder were observed. The upward trend of adhesive strengths continues onlyup to 0.2 wt% where the adhesive strength begins to drop. In OM-hydrogels containing 0.2 wt% OM powder, normalized rates of moisture loss are the lowest while water contents and water holding capacities are the highest. The formulation with 0.2 wt% OM powder is of highest potential for the fabrication of XG/CN-based OM-hydrogel pads for topical administration of active compounds.

Sheng et al. [10] prepared a hydrogel film with antibacterial activity and excellent stability in enzymes as potential wound dressing. Using hyaluronic acid, carboxylated chitosan and gentamicin, antibacterial film HA-CC-GS was prepared through intermolecular covalent bonding. Surface morphology of film was evaluated by scanning electron microscope images. The film exhibited good water absorption capacity and appropriate water vapor permeation rate (WVPR), outstanding mechanical properties and remarkable resistance to enzymatic hydrolysis. The biocompatibility of the film was tested by CCK-8 assay, and the results showed that the viability of NIH3T3 cell was not affected by coculture with hydrogel film. In vitro antibacterial test indicated that the synthesized hydrogel film had obvious antibacterial properties. In addition, in vivo studies showed that antibacterial hydrogel film could protect wound from infection and promote wound healing. These results indicated that the prepared antimicrobial hydrogel films by cross-linking had great potential application in skin/tissue repair, suggesting as a hopeful wound dressing.

Dibya et al. [11] investigated the property of Gum Ghatti (GG) on topical application accelerates wound healing similarly pectin is widely used in wound dressing materials. However, development of composite hydrogel using synergistic interaction of both gum ghatti and pectin and research characterizing their properties is scare. Synergistic advantages of both natural polymers were utilized in designing new antibiotic eluting biodegradable hydrogel film without using synthetic polymer for slow release of ciprofloxacin hydrochloride_and the engineering aspects of the film were emphasized. Initially gum ghatti (GG) was modified to sodium carboxymethyl gum ghatti (NaCMGG) and then NaCMGG- pectin film was prepared by solvent casting method followed by crosslinking in AlCl 3 solution (18% to 22% w/v) for 2 to 20mins. Swelling indices of low crosslinked film (LCL) and high crosslinked film (HCL) were 67% and 53% respectively that prevent accumulation of exudates in wound. The water vapour transmission rate of HCL and LCL were 511.36 gm/m^2/day and 568.18 gm/m^2/day respectively, maintaining moist environment over the

wound. Molecular evidence of probable healing mechanism could be found in in-vivo model.

Diana et al. [12] found that Xyloglucan is a polysaccharide isolated from chia seed gum (*Salvia hispanica* L.) and can act as a soluble fiber. In this investigation, several porous hydrogels were prepared from mixtures of chitosan and xyloglucan. To characterize these biomaterials, their mechanical, hydrophilic, structural, and morphological properties were measured, as well as their biodegradability and antimicrobial activity. The pore sizes of the porous hydrogels were 32.8–101.6 μm, and their water retention capacity is proportional to the added amount of xyloglucan. Dynamic degradation of the porous hydrogels with lysozymes showed progressive weight loss during the 14 days of testing. The mechanical properties improved slightly after the addition of xyloglucan. All of these results indicate that the incorporation of vegetable-derived polymers such as xyloglucan improves the properties of chitosan without affecting its antimicrobial capacity. Thus, biomaterials based on chitosan and xyloglucan are a promising option for the design of hydrogel wound dressings for medical applications.

Satish et al. [13] prepared Lupeol entrapped chitosan-gelatin hydrogel (LCGH) films by solution cast method by blending chitosan and gelatin solution using glycerol as plasticizer, followed by crosslinking with glutaraldehyde. LCGH films were characterized by scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), equilibrium water content (EWC), Water vapor transmission rate (WVTR) and *in vitro* release studies. SEM confirmed presence of the uniform porous network of both blank and LCGH films. The incorporation of lupeol in hydrogel was confirmed FTIR and DSC. The LCGH film was smooth, flexible, non-brittle and showed excellent swelling ability. EWC (85.40%) and WVTR (2228 ± 31.8) met the condition of ideal wound dressing. The biological activity of lupeol was assessed by antioxidant and antibacterial assay. Antioxidant assay confirmed that lupeol and LCGH film have excellent antioxidant properties by scavenging both radicals at steady increasing rate which increases with time due to steady release of lupeol. Antibacterial activity of lupeol in LCGH film was found to be retained as assessed by disc diffusion method. Cell viability was evaluated by MTT assay with NIH/3T3 fibroblast cells. The MTT assay showed that the CGH film evidently offered acceptable cell viability and non-toxicity. These observations depicted that chitosan/gelatin hydrogel film can be an ideal delivery system for sustained released of lupeoland LCGH film for enhanced wound healing.

Kelly et al [14] prepared chemically-crosslinked glycosaminoglycan (GAG) hydrogel films and evaluated as bio-interactive wound dressings. Hyaluronan (HA) and chondroitin sulfate (CS) were first converted to the adipic dihydrazide derivatives and then crosslinked with poly (ethyleneglycol) propiondialdehyde to give a polymer network. The crosslinking occurred at neutral pH in minutes at room temperature to give clear, soft hydrogels. After gelation, a solvent-castingmethod was used to obtain a GAG hydrogel film. A mouse model was used to evaluate the efficacy of these GAG films in facilitating wound healing. Full-thickness wounds were created onthe dorsal side of Balb/c mice and were dressed with a GAG film plus Tegadermt or Tegadermt alone. A significant increase in re-epithelialization was observed on day 5 (po0:001) and day 7 (po0:05) for wounds treated with a GAG film plus Tegadermt versus those treated with Tegadermt alone. While no significant differences in wound contraction or inflammatory response were found, wounds treated with either HA or CS films showed more fibro-vascular tissue by day 10. The GAG hydrogel films provide a highly hydrated, peri-cellular environment in which assembly of other matrix components, presentation of growth and differentiation factors, and cell migration can readily occur.

2.2. OBJECTIVE OF THE WORK

The objective of this project work is to prepare and evaluate the okra-based film for wound healing and its biomedical application. The aim is to prepare film by using natural polymer over synthetic polymer to enhance the biocompatibility with skin.

- Preparation of pH 7.4 phosphate buffer saline solution
- Preparation of standard curve in pH 7.4 phosphate buffer saline solution
- Extraction and purification of mucilage from Okra fruit
- Preparation of film for wound healing
- Characterization of film thickness and transparency
- Performing fluid absorptivity test of film
- Performing water vapor transmission rate
- In-vitro degradation test of film
- Performing mechanical strength study of film
- Performing XRD study
- Performing FT-IR study

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CHAPTER-III

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Materials

Ofloxacin supplied as a gift sample (Crest Life science Pvt. Ltd, Himachal Pradesh, India), Sodium alginate (purchased from SD Fine - chem limited), Okra (from local market), Ethanol and Glycerol were purchased commercially fromLoba Chemie Pvt. Ltd, Mumbai, India. All reagents used were of analytical grade. Double distilledwater was used throughout the study.

3.2. Methods

3.2.1. Preparation of pH 7.4 phosphate buffer saline solution

1litre of a phosphate buffer saline solution was prepared by taking 8 gm of sodium chloride, 0.2 gm of potassium chloride, 1.44 gm of disodium hydrogen phosphate, 0.245 gm of potassium dihydrogen phosphate in 800 ml distilled water. The pH was then adjusted to 7.4 using dilute HCl. According to the Indian Pharmacopoeia (IP), the volume was made up to 1litre with distilled water and the pH was finally measured using pH meter (Toshniwal Instruments Mfg. Pvt. Ltd., Ajmer, India).

3.2.2. Preparation of standard curve in pH 7.4 buffer saline solution

25 mg of drug (Ofloxacin) was accurately weighed and transferred into a 250 ml volumetric flask and the volume was made up with pH 7.4 buffer saline solution (primary stock solution) (100µg/ml). Then the flask was shaken in order to dissolve the drug. From the primary solution, further dilutions were made with pH 7.4 buffer saline solution to obtain concentrations of 2, 4, 6, 8, 10 µg/ml. Absorbance were noted at 287 nm using UV-Visible Spectrophotometer (UV- 2450, Shimadzu, Japan) against blank to get the calibration curve.

3.2.3. Extraction and purification of okra mucilage

Okra fruits were purchased from a local market. The seeds did not contain any mucilage and were removed prior to extraction. The okra was sliced and homogenized with water (okra: water = 1:3) and later centrifuged at 4000 rpm for 15 min and the clear, viscous solution was decanted. The solution was heated at 70°C for 5 min and recentrifuged after cooling [1]. The polysaccharide was precipitated with three volumes of ethanol (1:3) and washed with more ethanol followed by acetone. The greenish-brown colored polysaccharide was dried under oven at 50° C for 7 hrs. and gave a yield of 22 g polysaccharide/kg okra [2].

3.2.4. Preparation of film for wound healing

Solution of alginate (4% w/v) was initially prepared by dissolving it in distilled water. During the preparation of the alginate solution, glycerol was added at percentages of 15%, 20% and 30% (w/w, based on the mass of the alginate), in order to increase the flexibility of the films. Subsequently, okra gum was added in varying amounts (Table 3.1) to obtain the final alginate/ okra films. Afterwards, 40 mL of each mixture was casted into petri dishes (ø=8.8 cm) and left to dry at room temperature (25ºC) and for controlled humidity (50%) [3].

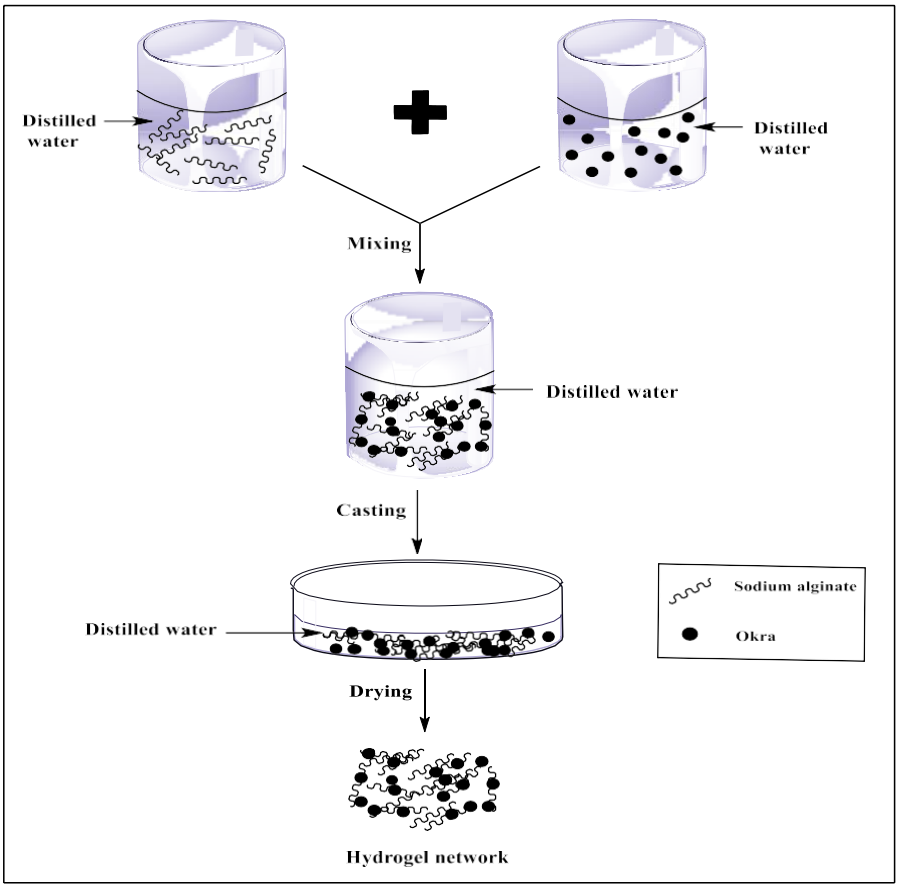

Fig: 3.1 *Preparation of alginate/ okra film.*

Table 3.1: Composition of films

Formulation Code	Glycerin (%)	Okra mucilage (mg)	Alginate (mg)	Alginate: Okra	Water (ml)
F1	15	-	1600	-	40
F2	20	-	1600	-	40
F3	30	-	1600	-	40
F4	20	120	1480	3.7:0.3	40
F5	20	200	1400	3.5:0.5	40
F6	20	280	1320	3.3:0.7	40

3.2.5. Characterization of film

3.2.5.1. Film thickness and transparency

The thickness of the films was determined using a digital vernier calipers (CD-6"CS, Mitutoyo, Japan). The transparency of the films was evaluated by spectrophotometry. Film samples of 10 x 30 mm were placed on a spectrophotometer cell (UV- 2450, Shimadzu, Japan) and analyzed at a range of 200-800 nm. Tests were performed in triplicate [4]. The transparency was determined by the following equation:

$$\% \text{ transparency} = 1/\text{ absorbance} \times 100$$

3.2.5.2. Fluid absorptivity

Fluid absorptivity was measured by measuring the swelling index of the film. 50 mg of the films were cut and dipped into PBS solution at pH 7.4. After a fixed interval of time films weretaken out and blotted with tissue paper to remove excess buffer on the surface of the film. The weight of the hydrated films was measured after removing the excess fluid with the help of blottingpaper [5]. The swelling index was calculated using the formula:

$$\mathbf{S.I\ (\%) = (W_s - W_d) / W_d \times 100}$$

where W_s and W_d are weights of the swollen film and dry film.

3.2.5.3. Mechanical properties

Stress-strain measurements were performed with a custom-built tensile stretcher composed of two clamps, holding the film. Weights are placed on a platform attached at the bottom of thefilm (figure 1). The alginate and okra films were cut into circular (diameter= 2 cm) shapes. Both

the ends were attached with the hanging clamps. Six samples were tested for each composition to obtain a set of statistically significant results. The ratio of the force to the initial cross-sectional area of the sample gauge section was designated as stress [6]. Stress and strain measurements at the fracture point provided TS according to the following formula:

TS= Maximum force at break (N) / Initial cross-sectional area of film (mm^2)

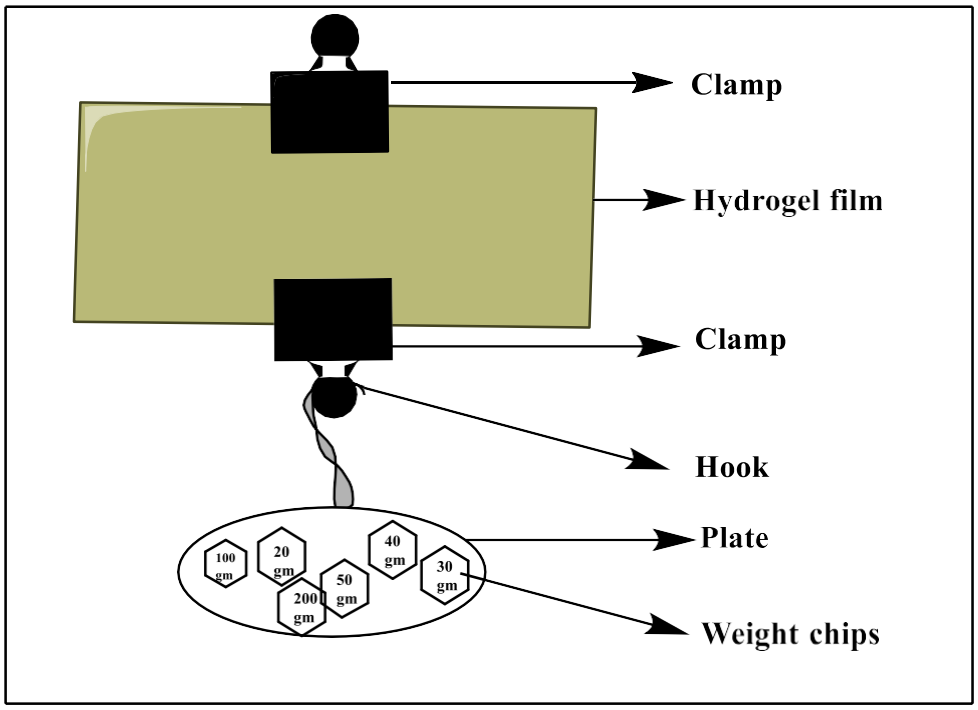

Fig: 3.2. Schematic diagram of mechanical strength analyzer.

3.2.5.4. In vitro degradation tests

The in vitro degradation tests were performed by the immersion of dry film samples (30 x 10) mm^2 in 10mL of PBS solution (pH 7.4) at 37ºC, for 7 days. At pre-determined time periods, the samples were removed from the degradation medium. They were then placed in an oven and dried at 37ºC until a constant mass was achieved, to evaluate the weight loss. The degradation medium was replaced every 2 days, and six samples were used for each condition [7]. The degradation was accessed by determining the weight loss (WL), as follows:

$$(\%)\ WL = (W_i - W_f) \times 100 / W_i$$

Where, W_i is the initial weight of film at time 0, W_f is the weight of film at time t after immersion in the test medium

3.2.5.5. Water vapor permeation rate (WVPR)

The WVPR of film was investigated according to the American Society for Testing and Materials (ASTM) standard. Each kind of film samples (diameter = 1 cm) with approximate thickness was used to cover the top of a tube containing 10 mL deionized water and sealed tightly with Teflon tape. The tube was placed into a desiccator having a relative humidity of 75%. The relative humidity was maintained by using a mixture of 10.23 gm of water and 20 gm of NaCl and placing it at the base of the desiccator [8]. After 24 h, films were removed and the weight gain per unit area was determined. Each sample was tested three times, and the results were averaged. WVPR of wound dressings was calculated using the following equation [9]:

Weight gain = Final weight – initial weight

WVPR (g. m^2. day $^{-1}$) = Weight gain (g) / Exposed surface area of film (m^2)

3.2.5.6. Fourier transform infrared (FT–IR) analysis

FT-IR spectra of okra, alginate and film containing okra and alginate were recorded usinga FT-IR spectrophotometer (Alpha-E, Bruker, USA). The samples were first mixed with KBr andthen they were converted into pellets with the help of a hydraulic press. The spectra were recordedin the wavenumber region of 4000–400 cm^{-1} [10-13].

3.2.5.7. X-ray diffraction (XRD) study

The X-ray diffraction studies of alginate, okra and film containing okra and alginate wereperformed using an X-ray diffractometer (Bruker axs, D8 advance) supported by LynxEye superspeed detector and a Ni-filtered Cu-Kα radiation (λ= 1.54 Å) radiation generated at 40 kV voltage and 40 mA current. The diffractometer was operated with a scan speed of 0.5 s for steps of 0.01º in 2θ [14-15].

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CHAPTER-IV

RESULTS, DISCUSSION & CONCLUSION

4. RESULTS AND DISCUSSION

4.1 Development of standard curve of Ofloxacin in pH 7.4 buffer saline solution

Standard curve of ofloxacin in pH 7.4 buffer saline solution was prepared by taking concentration in X-axis and absorbance in Y-axis. The slope of the standard curve is 0.0683 and R^2 value is 0.9996. Table 4.1 represents the different absorbance value at different concentrations using pH 7.4 buffer saline solutions. Figure 4.1 shows the graphical representation of absorbance versus concentration using pH 7.4 buffer saline solutions.

Table 4.1. Absorbance values in different concentrations of ofloxacin in pH 7.4 buffer saline solutions for the preparation of standard curve

Concentration (µg/ml)	Abs 1	Abs 2	Abs 3	Mean	SD (n=3)
0	0	0	0	0	0
2	0.139	0.186	0.142	0.155	0.026
4	0.273	0.265	0.289	0.275	0.012
6	0.399	0.39	0.421	0.403	0.015
8	0.535	0.555	0.553	0.547	0.011
10	0.678	0.663	0.705	0.682	0.021

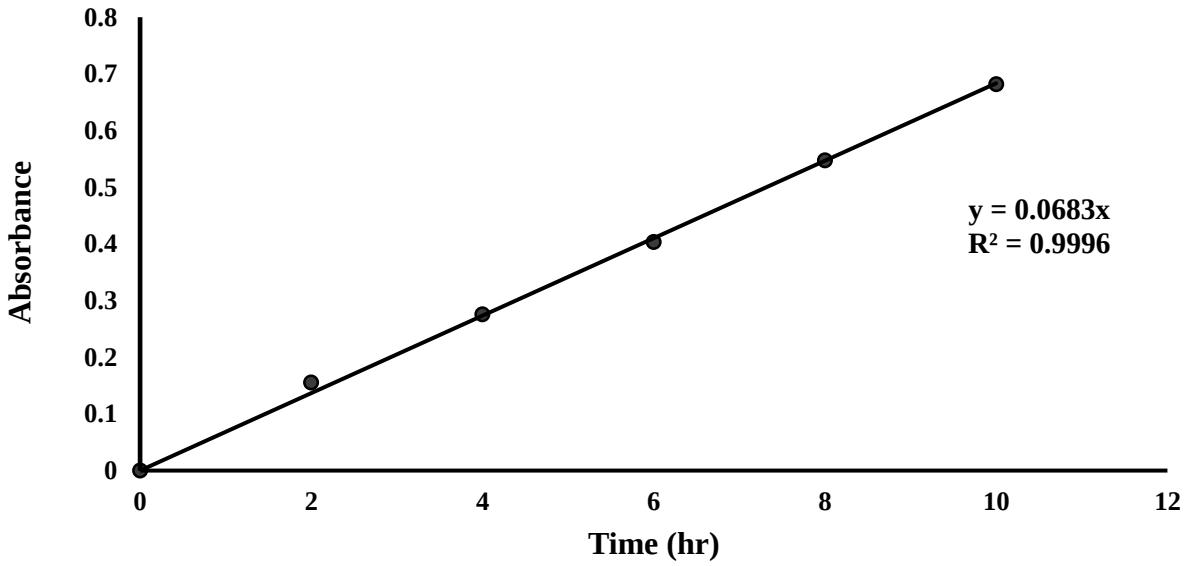

Figure 4.1. Preparation of standard curve of ofloxacin in pH 7.4 buffer saline solution

4.2. Characterization of films

4.2.1. Film thickness and transparency

The thickness of the films was evaluated as illustrated in and Table 4.2 and Fig. 4.2. The film thickness values lied between 0.47 mm and 0.48 mm. Various amounts of glycerol, sodium alginate and Okra mucilage having different concentrations were added. Results show that with an increase in theglycerol concentration, the thickness of the film decreases. This is because the glycerol molecules are able to occupy the spaces surrounding the hydroxyl group of alginates and can form crosslinks between the alginate molecules thus restricting the number of contacts made between alginate and water molecules [1]. For wound dressing, a thickness of the dressing lower than that of human skin is desirable. The dermis thickness depends upon the age, genderand places in the body and varies from 0.5 mm to 2.0 mm [2]. Since UV rays (200-400 nm) areharmful and cause further degradation of the wound, the prepared films must show less transmittance in the UV range for protection of the wound whereas, the transmittance mustbe more in the visible range. Table 4.2 shows that as glycerin concentration increases, film thickness decreases and Table 4.3 shows % Transmittance of the films also decreases. Films with 15 % glycerin show more transmittance and have more thickness as compared to 20 % and 30 % glycerin. Films with 30% glycerol show more absorbance and less transmittance which indicates more protection from UV radiation but high glycerol content decreases the film thickness. Films containing 20% glycerol shows optimum thickness and significantly havenot much difference with 30% glycerol films. Therefore, formulation containing 20% glycerin is considered as the desirable formulation [1]. Similarly, films were prepared with different ratios of alginate: okra (3.7:0.3, 3.5:0.5, 3.3:0.7). Films with alginate: okra (3.7:0.3) show minimum thickness with higher transmittance in the UV region. Although the film with alginate: okra (3.3:0.7) shows less transmittance, it is not considered as the optimum formulation because it did not have the desirable thickness of ≤ 0.5mm. The film containing

alginate: okra (3.5:0.5) has the desirable thickness with transmittance. Hence it is considered as the optimum formulation [1].

Table 4.2. Thickness of films.

Code	Ingredient	Thickness 1(mm)	Thickness 2(mm)	Thickness 3 (mm)	Avg Thickness (mm)	SD
F1	15% Glycerin	0.54	0.56	0.55	0.55	0.01
F2	20% Glycerin	0.48	0.47	0.47	0.47	0.005773503
F3	30% Glycerin	0.44	0.42	0.44	0.43	0.011547005
F4	0.3 Okra + 20% Glycerin	0.42	0.4	0.43	0.41	0.015275252
F5	0.5 Okra +20% Glycerin	0.49	0.48	0.5	0.49	0.01
F6	0.7 Okra + 20% Glycerin	0.52	0.53	0.52	0.52	0.005773503

Table 4.3. Transmittance of films.

Wavelength	F1	F2	F3	F4	F5	F6
0	0	0	0	0	0	0
200 nm	5	5	5	5	5	5
300 nm	40.81	27.77	23.77	5	5	5
400 nm	172.41	95.23	92.59	102.04	56.11	50.5
500 nm	440.52	203.25	210.52	146.62	138.31	91.24
600 nm	657.89	289.01	321.54	225.73	209.64	136.98
700 nm	819.67	362.31	425.53	322.58	306.74	200
800 nm	990.09	421.94	518.13	436.68	427.35	280.11

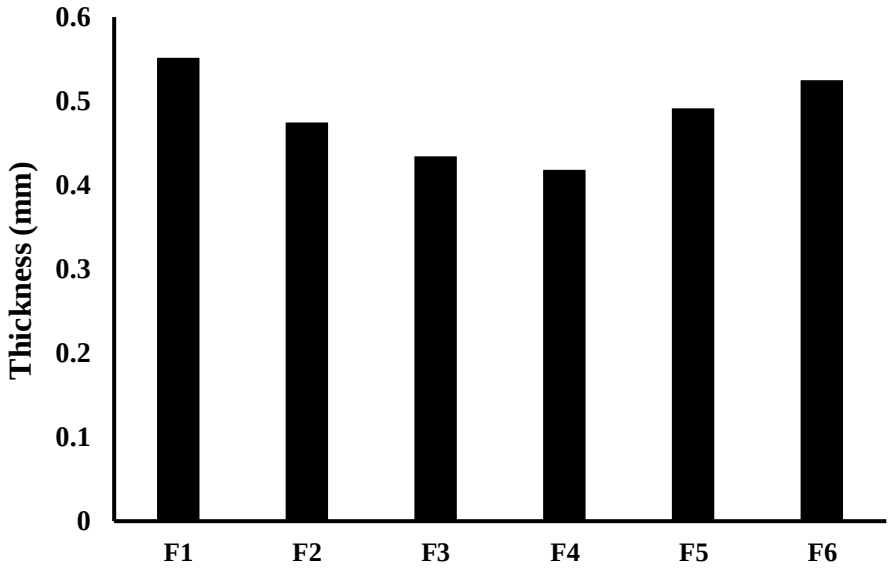

Fig 4.2: Thickness of the films.

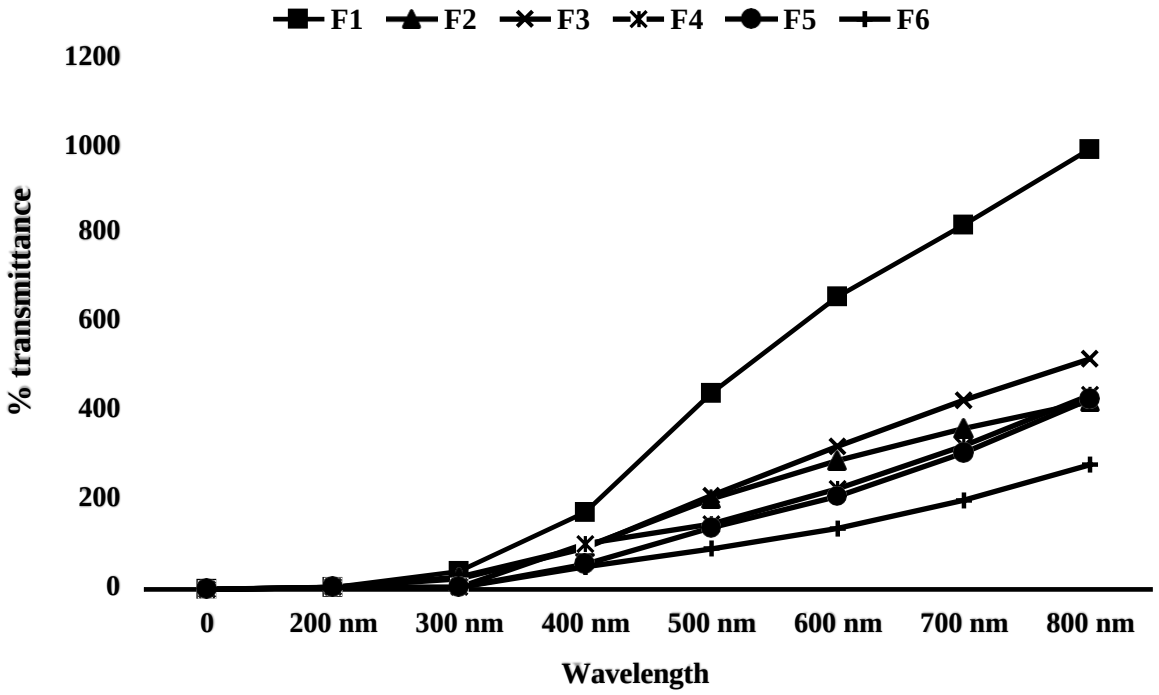

Fig: 4.3. Transmittance of films.

4.2.3. Fluid absorptivity

The fluid uptake capacity of the film was observed by measuring its percentage of swelling in PBS solution. The fluid uptake values of 15%, 20% and 30% glycerin were studied and presented in Fig. 4.4. It showed that as alginate concentration decreased simultaneously with an increase in glycerin concentration, the swelling percentage decreased. The films swelled

rapidly for 20 mins and gradually reached equilibrium in between 40-60 mins as was seen in the report of Sinha et al [3]. The fluid uptake capacity reached a saturation point after 60 mins. Initial rapid swelling of 1148% (15% glycerin), 1052% (20% glycerin) and 856% (30% glycerin) occurred within 20 min and then gradually decreased after 60 mins. Slight decreasein water uptake was probably due to some degradation [3].

When hydrophilic okra mucilage was added to the formulation, it caused an increase in the viscosity of the films. Due to an increase in the number of hydroxyl groups, the swelling of the films increases leading to an increased volume and thickness of the films. This canbe explained by possible interaction between the mucilage and alginate granules with glycerinacting as the plasticizing agent. The results were similar to those found in the work of Rahmaniet al. Table (Fig. 4.4) shows the fluid absorptivity behavior of the different formulation. [4].

Table 4.4. Fluid absorptivity of films containing alginate and okra.

Time	F1	F2	F3	F4	F5	F6
0	0	0	0	0	0	0
20	1148	1052	856	1252	1464	1608
40	1184	1090	914	1464	1672	1852
60	1236	1144	948	1610	1874	2004
80	1234	1154	964	1624	1706	1896
100	1176	1134	958	1560	1580	1786
120	1120	1092	950	1492	1522	1720

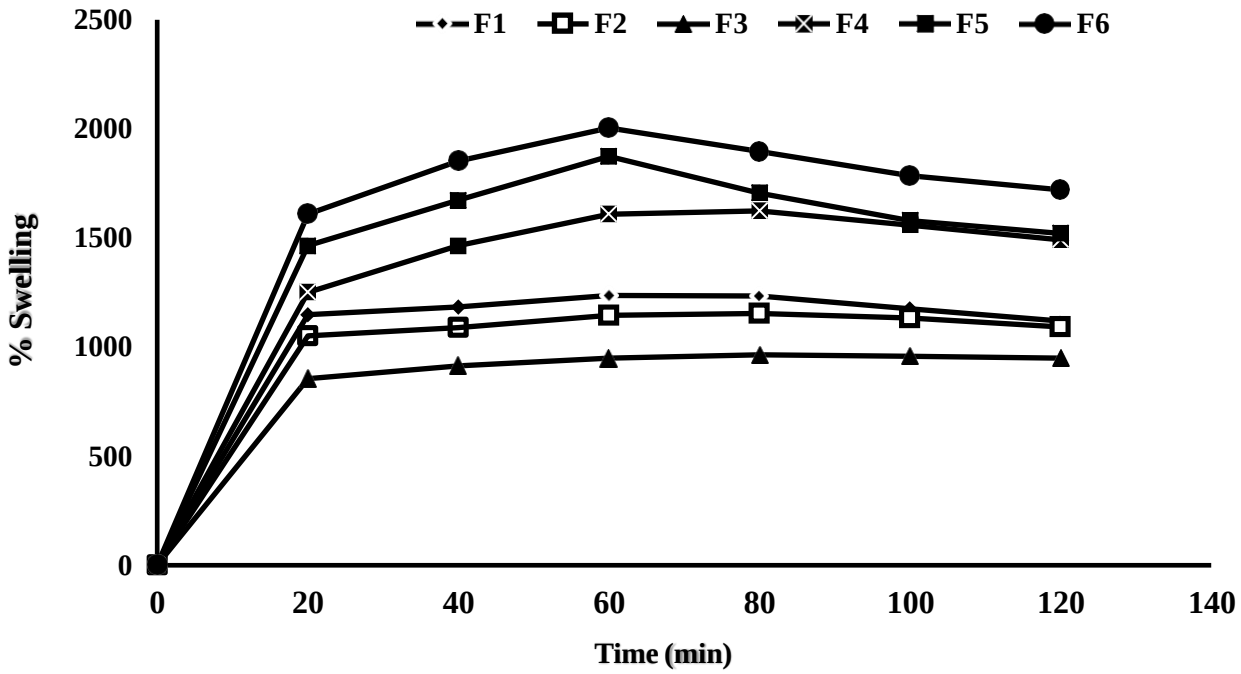

Fig. 4.4. Fluid absorptivity of films containing alginate and okra.

4.2.4. Water vapor transmission rate

Ideal wound dressing should provide an optimum rate of moisture transmission to prevent excessive dehydration and build-up of exudates. A high-water vapor transmission rate causes rapid drying of wounds, whereas wound exudates are accumulated at a low water vapor transmission rate which retards the healing process and increases bacterial growth. Water vapor transmission rate for normal skin is 204 g/m^2 /24 h whereas, for injured skin, it can range from 279 to 5138 g/m^2 /24 hr. as reported by Sinha et al [3]. The water vapor transmission rate for film containing 15% glycerin is lower than that of normal skin, hence it cannot be considered whereas the films containing 20% and 30% glycerin have no significant difference in water vapor transmission rate, ranging from 280-331 g/m^2/24 hr as shown in Table 4.5. Film containing 20% glycerin is taken as the optimized formulation since it has the optimum thickness ($\leq$ normal skin thickness of 0.5mm) and can maintain a moist environment on the wound area. Film having alginate: okra = 3.7: 0.3 exhibits the desirable range for water vapor transmission rate but due to its decreased thickness, it is not considered. On the other hand, films having alginate: okra = 3.5: 0.5 and 3.3: 0.7 show no significant difference in water vapor transmission rate. Hence alginate: okra = 3.5: 0.5 having thickness equivalent to normalskin, is considered as the optimized formulation [3].

Table 4.5. Water vapor transmission rate of films

Composition	WVTR (gm/sq.mt/24hr)
F1	190.98
F2	280.11
F3	331.04
F4	292.84
F5	343.77
F6	356.50

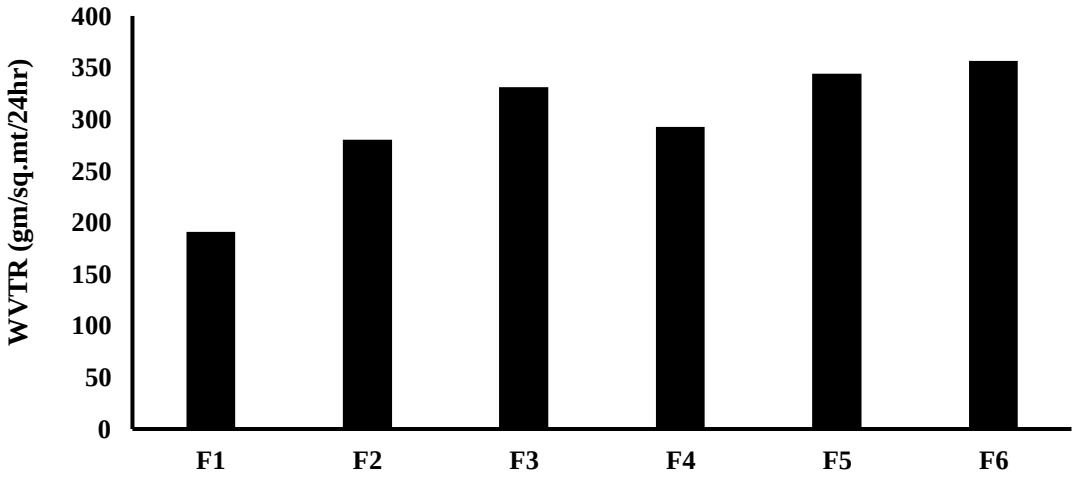

Fig. 4.5. Water vapor transmission rate of films.

4.2.5. In-vitro degradation

The in vitro degradation tests were performed to evaluate the influence of glycerin and okra on the degradation behavior of the films, as well to investigate the durability of the films when immersed in PBS solution. Fig. 4.6. displays the weight loss profiles of the films throughout the degradation period. Films with 15% glycerol and alginate presented a quick gain in weight during the first day of degradation due to more concentration of alginate and less concentration of glycerin as compared to other formulations. This was followed by a slower and gradual loss of weight during the following days which was possibly due to gradual leaching of the plasticizer over the period of time. Similarly films with 20% and 30% glycerol and alginate, showed less gain in weight during the first day of degradation as compared to 15 % glycerin due to less concentration of alginate. This was followed by a slower and gradual loss of weight during the following days. This can be related to the leaching of the plasticizer agent (glycerol).

Hence, Table 4.6 shows that F1 is not completely degraded even on the 7th day and F3 is completely degraded after the 4th day, whereas F2 exhibits complete degradation after the 6th day and is therefore considered as the optimum formulation. Subsequently F4-F6 contain varying amounts of alginate: okra as mention earlier. The films having alginate: okra = 3.5: 0.5 (F5) exhibit optimum degradation with time as compared to F4 which did not degrade completely even on the 7th day and F6 which degraded completely after the 4th day. Hence F5 film was used as the optimized formulation [5].

Table 4.6. In vitro degradation of films containing alginate and okra.

No of days	F1	F2	F3	F4	F5	F6
1	150	120	105	170	185	210
2	135	102	85	150	165	150
3	126	95	55	125	141	93
4	115	75	25	92	110	48
5	101	48	0	78	62	0
6	90	29	0	54	0	0
7	78	0	0	30	0	0

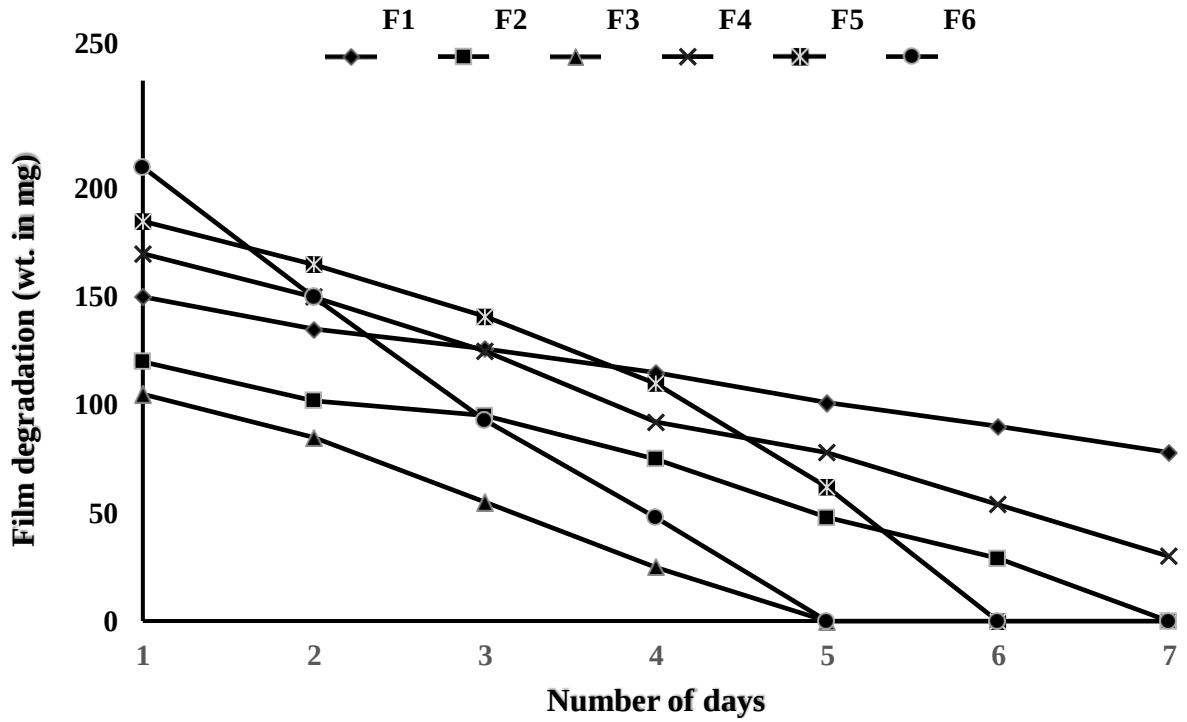

Fig. 4.6. In vitro degradation of films containing alginate and okra.

4.2.6. Mechanical properties

An ideal wound dressing should present good mechanical properties and maintain their integrity during use. Additionally, the films should present adequate resistance to the mechanical abrasion and be flexible to follow the skin movements. The value of the tensile strength of the skin is usually in the range of 2.5-16 MPa as reported by Pereira et al [6]. The mechanical properties of the films were evaluated regarding its tensile strength as shown in Fig. 4.7. The inclusion of glycerol within the alginate film has a significant influence in its mechanical properties, causing a significant reduction in the rigidity and decreasing the tensile strength. An increase in the amount of plasticizer (glycerol) from F1 to F3 (15%, 20%, 30%) and a simultaneous decrease in the alginate concentration leads to a decrease in the tensile strength as shown in Table 4.7. The behavior of the films prepared with alginate andglycerin can be explained by the plasticizing effect of the glycerol, which improves the free volume between the polymeric chains, thereby reducing the polymer-polymer interactions and increasing the mobility of the polymeric chains Hence, F2 (20% glycerin) is considered as the optimized formulation as it exhibits similar tensile strength as reported by Pereira et al. Similarly, formulations F4 to F6 that contain alginate: okra in the ratio of 3.7: 0.3, 3.5: 0.5 and

3.3: 0.3 have a slight increase in the thickness of the films leading to an increase in the tensile strength of the films. Alginate increases the rigidity of the film while okra increases the thickness of the film [6].

Table 4.7. Mechanical property of films

Composition	Tensile Strength (MPa)
F1	21.06
F2	17.16
F3	12.48
F4	15.60
F5	17.94
F6	23.40

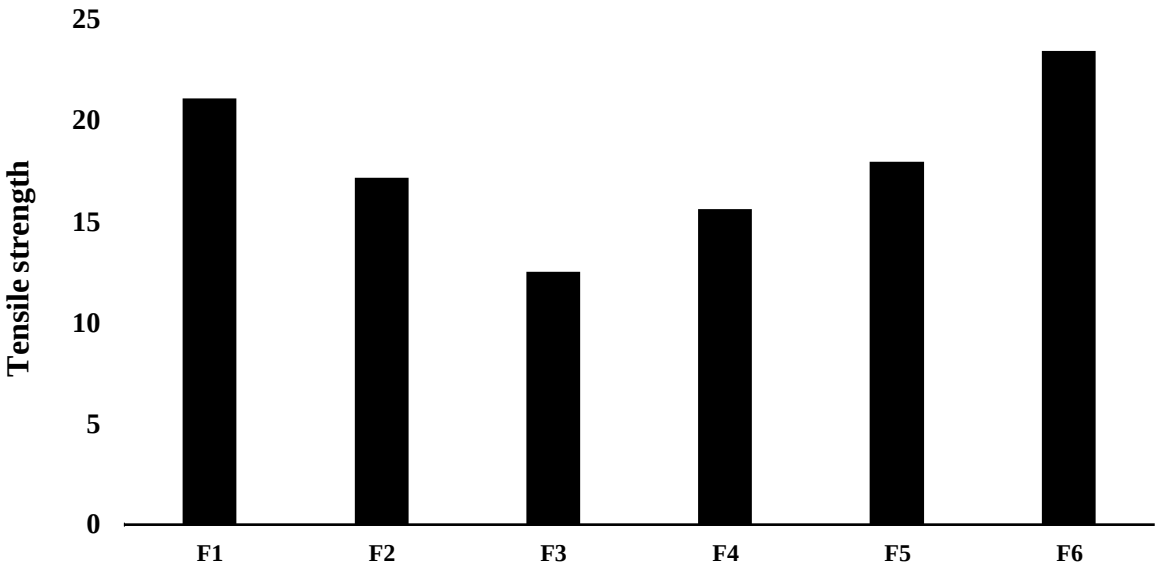

Fig. 4.7. Mechanical property of films

4.2.7. X-ray diffraction (XRD) study

XRD diffractogram of alginate and okra extract are shown in Figure 4.8. XRD of alginate sample [Fig. 4.8 (A)] showed a characteristic semi-crystalline nature with a peak at 2θ equals to 20.87° - 23.54º [7]. The okra extract appeared to form a semi-crystalline body, as indicated

2θ values 18.29° - 23.75° [Fig. 4.8 (B)] [8]. The broad peak shows the optimized formulation having amorphous structure ranging from 20.95° - 23.68° [Fig. 4.8 (C)]. The presence of the internal strain might be the reason for the fracturing of the grains into sub grains which led to a decrease in the semi-crystalline size to amorphous.

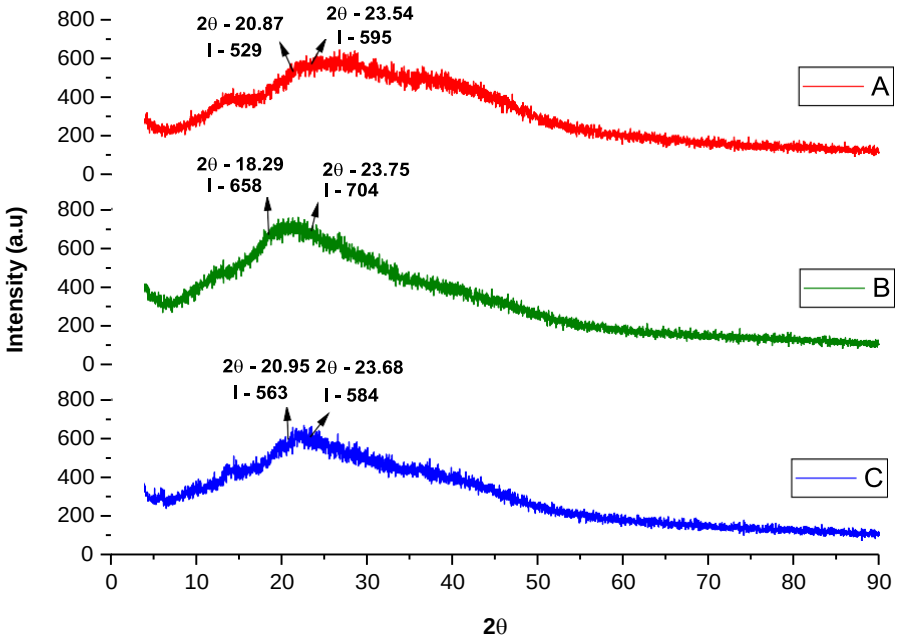

Fig. 4.8. XRD analysis of films

4.2.8. Fourier transform infrared (FT–IR) analysis

The absorption bands obtained from the FTIR spectra have been demonstrated in the Table 4.3. Figure 4.9 shows the FTIR spectra of pure alginate (AG.), pure okra (OK) and film (AGOK) respectively. The optimized Alg.-Okra film (obtained at alginate: okra =3.5: 0.5, drying temperature=50°C, glycerin concentration=20%) was used for FTIR analysis as shownin table 4.9. In case of pure Alg., asymmetric stretching vibrations of -COO- were observed at1420 cm^{-1} [6] and -CH stretching vibrations are observed at 2962 cm^{-1} while -C-OH- stretchingvibrations were present at 1105 cm^{-1}. This is because -C-O stretch of alginate gets converted to -C-OH group when it gets dissolved in water due to hydrogen bonding and water is not completely removed during drying [9]. In case of pure okra, asymmetric stretching vibrations of -COO- were observed at 1417 cm^{-1}, -CH stretching vibrations are observed at 2962 cm^{-1}, - C-OH stretching vibrations observed at 1262 cm^{-1} [4], while -C=O stretching vibration at 1660cm^{-1} [10] and -N-H bending vibration at 1580 cm^{-1}. The film thus showed asymmetricstretching vibrations of -COO- at 1420 cm^{-1} and -CH stretching vibrations at 2962 cm^{-1} and - C-OH-stretching vibrations at 1105 cm^{-1} thereby indicating the presence of unchanged alginate

in the film. The presence of -C=O stretching vibration at 1660 cm^{-1} and -N-H bending vibration at 1580 cm^{-1} indicated the presence of okra in an unchanged form in the film. A common peak arises in all the formulations at 2360 cm^{-1} which can be attributed to -O=C=O and is possibly due to the presence of carbon dioxide in the instrument.

Table: 4.8 Various absorption bands obtained from FTIR spectra

Functional Group	Pure Alginate	Pure Okra	Film
-COO (Asym. Str.)	1420	1417	1420
-C-OH (Str.)	1105	--------	1105
O=C=O (Str.)	2360	2360	2360
-C-H (Str.)	2962	2962	2962
-C-OH (Str.) alcohol	----------	1262	---------
-C=O (Str.)	----------	1660	1660
-N-H (Bending)	----------	1580	1580

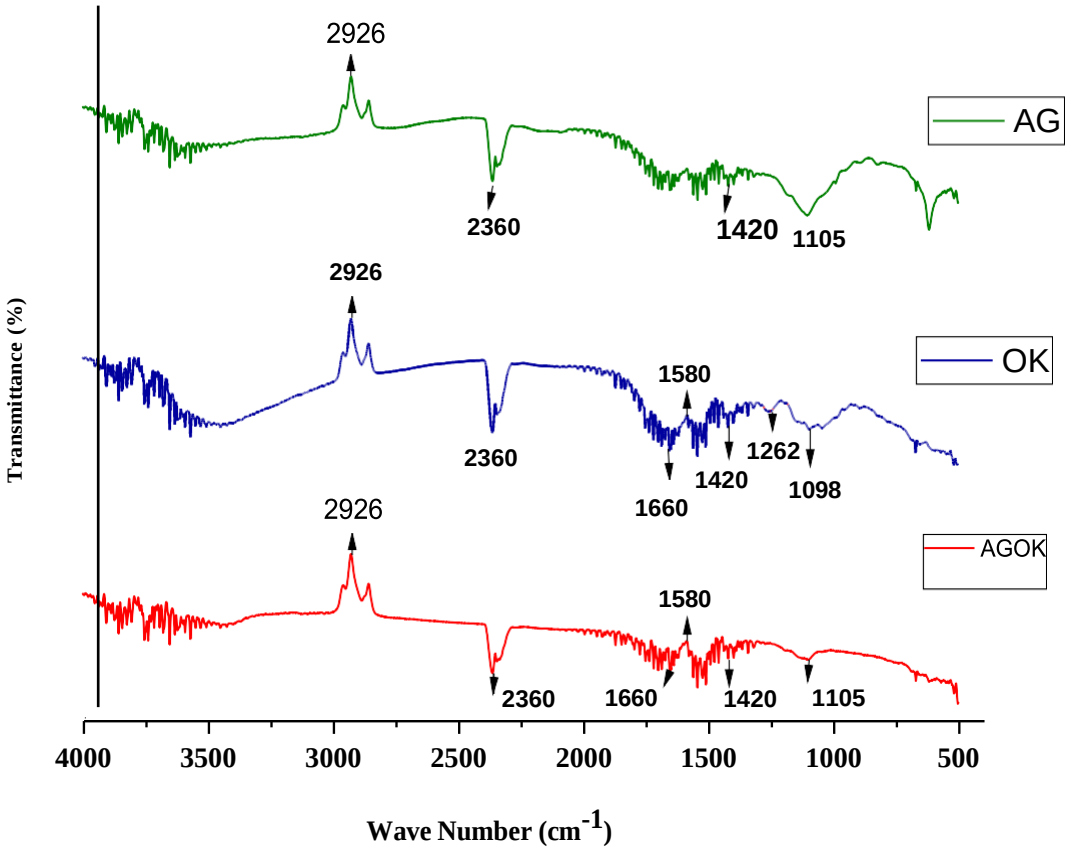

Fig. 4.9. FT-IR analysis of film

4.3. Conclusion

Films, composed of alginate and okra polysaccharide were prepared for wound healing applications. The films were developed by combining the hemostatic property of alginate and therapeutic property of okra. Films with desirable transparency and thickness, water vapor transmission rate, fluid uptake capacity, in vitro degradation and mechanical property can therefore be obtained by changing the concentration of glycerin and okra in the film. High absorbance of harmful UV radiation is a valuable property for wound healing. Filmscontaining optimum thickness and transparency equivalent to that of normal skin (≤ 5 mm) were prepared. Water vapor transmission rate of the films were found to be in the appropriaterange for wound healing. The fluid uptake capacity of the films which increases initially for 60mins and then gradually decreases is considered to be useful for the rapid removal of exudatesfrom the injured skin. In vitro degradation of the films containing alginate and glycerin werefound to occur from 5^{th} to 7^{th} days. As glycerin concentration increased, degradation was morerapid whereas in case of alginate with okra-based film, degradation was observed from 4^{th} to 7^{th} days. In this case, as okra concentration increased, degradation was more rapid due to more hydrophilic nature leading to bond breaking by hydrolytic cleavage. From all theseobservations, F5 was concluded to be the optimized formulation. An increase in mechanical strength and decrease in fracture strain due to increase in okra concentration was also observedin this formulation. The FTIR study confirmed the presence of alginate and okra in their original form in film, whereas XRD study demonstrated the amorphous nature of thefilm. All these results suggest that alginate/okra films can be potentially explored forwound healing application.

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