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Fabrication and Characterization of Metformin, Gentamicin and Curcumin Loaded Hydrogel for Wound Healing

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Abstract:

Chitosan is a sugar derived naturally and used in medicine for its different uses, like wound dressings, drug delivery systems, medical implants, and hydrogels. This study fabricated a chitosan hydrogel loaded with curcumin, gentamicin, and metformin. The physical properties included swelling index, tensile strength, elongation profile, and drug release. The chitosan hydrogel is used to treat burn wounds and boost the healing process in Wistar rats. The chitosan hydrogel showed bonding with acetate, and after the formation of chitosan acetate, the pH was raised using sodium hydroxide to achieve cross-linking using formaldehyde and tween 80. The hydrogel characterization showed all the peaks and parameters that ensured the fabrication of drug-loaded chitosan hydrogel. The physical properties revealed increased tensile strength and elongation percentage with increased chitosan concentration. At the same time, there was a negative effect on the porosity and water adsorption. The best tensile strength and elongation percent were shown at 1.2 grams of chitosan, 0.32 Mpa, and 62 percent, respectively. Scanning electron microscopy showed that the pore sizes were 50±2 µm. The surface showed drug loading, which is the reason the drug was released initially as a burst and then uniform. When compared to the control group, the in vivo study found that the drug-loaded chitosan and chitosan hydrogel improved wound healing. Compared to the control group, there was an increased formation of collagen, rete ridges, epidermal bed length, and scar formation in the chitosan and drug-loaded chitosan groups. The best wound closure was seen in the drug-loaded chitosan group, which was 12.5% wound size by the end of the 3rd week. Using chitosan alone can accelerate burn wound healing, and the lower wound PH in the respective group also showed this. However, certain drugs could boost wound healing even more, as showcased in this study. Drug-loaded hydrogel wound dressings could revolutionize the wound-healing process.

Keywords: SEM; HPLC; FTIR; Physical properties of hydrogels; Swelling index; Elongation percentage; pH; Wound dressing.

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1. Introduction

Chitin is the substance from which chitosan is derived, and it is used in medicine for different purposes. It is naturally abundant and is taken from sources like insect exoskeletons, cell walls of fungi, and exoshells of crustaceans [1]. Chitin is deacetylated to get chitosan, which is a distinctive polysaccharide [a (1→4) 2-amino-2-deoxy-bD-glucan] [2]. It has been used as a biomedical material due to its sturdy properties like biocompatibility, low toxicity, mucoadhesive nature, biodegradability, hemostasis, antibacterial activity, wound healing, and high-speed granulation [1-3]. Hydrogels, with their precise and perfectly arranged structure, have given an

eye to their use in the biomedical sciences for many purposes. A chitosan-based hydrogel's proper activity and modulation depend upon the formation mechanisms [4]. Modifying chitosan's physical or chemical properties leads to the formation of new and unique products [5]. Hydrogels, for the first time, were used by Wichterle and Lim in the 1960s in the biomedical field [6]. The hydrogels have different sizes ranging from nanometers to centimeters, so they can easily be deformable and acquire any space in which they are made [7]. Hydrogels are used in medicine for tissue engineering, skin grafts, drug delivery systems, and cell encapsulation. Chitosan-based hydrogels have been used recently for wound-healing purposes.

Curcumin, also called diferuloylmethane, is found in the Curcuma longa rhizomes. It has three compounds, of which 77% is curcumin, and the remaining two are bis-demethoxycurcumin and demethoxycurcumin [8]. Curcumin is best known for its epigenetic modulation, which helps treat cancer [9]. Studies have shown that curcumin has wound-healing effects; it helps to activate pathways and mechanisms like increased epithelization, fibroblast cells, neutrophils, and macrophages [10].

Gentamicin is a well-known antibiotic; it belongs to the aminoglycoside class [11]. Gentamicin has some side effects that can be reduced when used in conjugation with other antibiotics [12]. Studies have shown that Gentamicin is used to treat skin infections and has activity in wound healing to some extent [13, 14].

Metformin has an herbal lineage from Galega officinalis, also known as professor weed or goat's rue. It belongs to the biguanide class of glucose-lowering drugs [15]. A study has shown that metformin can increase circulating endothelial precursor cells for better wound healing [16]. Other studies have shown that metformin can re-epithelialize the wound, help restore connective tissue, and enhance transforming growth factor expression [17].

Several studies have been conducted to reduce the wound healing time; different hydrogels and nanoparticles that behave as drug delivery systems have been introduced to aid in the cause [5, 18-20]. The hydrogels have similar properties, composition, and mechanical properties to the cellular matrix; hence, they could serve both the functions of tissue support, cell generation, and drug availability [6, 18, 21]. A study has shown that chitosan can assist in wound healing due to its activity in activating fibroblasts, type IV collagen synthesis stimulation, giant cell migration, and cytokine production [5]. Recent studies have also shown that chitosan hydrogel treats burn wounds in rats [22, 23]. Many drugs are used to treat wounds, among which studies have shown that metformin increases the activity of wound healing by activating activated protein kinase signaling pathways [24].

In this present study, for the first time, three different drugs were incorporated into the chitosan hydrogel to enhance burn wound healing by the combined effects of all the drugs infused in the hydrogel.

2.Materials & Methods

2.1. Materials

Low molecular weight chitosan powder provided by the Gluchi Bio-Industry®, Acetic acid from Merck Emsure®, Curcumin drug standard powder from Genacol Aminolock®, Metformin standard powder from Merk Serono®, Gentamicin sulfate powder from Intracin, Tween 80 or polysorbate 80 from Thermo Scientific®, and other chemicals were also procured needed for the study.

2.2. Preparation of chemical solutions

All the chemical powders were dissolved in the respective dissolving solutions; for example, deionized water was used to dissolve gentamicin sulfate powder, and dimethyl sulfoxide was used to dissolve curcumin powder. The dissolving solutions were identified using different studies and online sources.

2.3. Preparation of Chitosan Hydrogel

Chitosan powder was dissolved in the 1% acetic acid solution; this was achieved by stirring the solution on the magnetic stirrer for 4 hours at a moderate temperature to ensure complete powder mixing. The chitosan acetate solution is low in pH, and the pH was adjusted using 1M sodium hydroxide solution, which was added drop by drop to prevent clumping of the chitosan acetate.

After a pH of 7.6, drug solutions of curcumin, metformin, and gentamicin were added dropwise. Then, the chitosan hydrogel was cross-linked using formaldehyde and tween 80. The solution was stirred with the dropwise addition of the cross-linkers until a thick gel was obtained.

2.4. Lyophilization of Chitosan Acetate

After the initial characterization of the hydrogel, the chitosan acetate initially made with drug loading prior to

cross-linking was lyophilized with the lyophilizer (Ilshin BioBase, Europe) for further processing. The lyophilization was carried out per the lab protocols and steps. The solution was loaded in the vials before loading; the lyophilizer was set to the required settings. The solution was pre-frozen at -40°C overnight and then lyophilized by freeze-drying at -50°C.

2.5. HPLC of chitosan hydrogel

High-performance liquid chromatography evaluated the prepared chitosan hydrogel's cross-linking efficiency and drug-loading capacity (HPLC, Shimadzu LC-20A). This evaluation was done with the C18 reverse-phase column, which was optimally suited for separating compounds that possess both hydrophilic and hydrophobic regions, such as the components in the hydrogel and the drugs. The flow rate was kept at 1.0 ml/min, and the mobile phase used was procured liquid chromatography (HPLC) grade water to provide smooth flow and proper separation of peaks in the chromatogram. Hydrogel solution was passed through the HPLC instrument after cross-linking, and certain peaks were found in the chromatogram associated with the O-H and N-H regions. These peaks indicated the appropriate cross-link formation in the hydrogel matrix, which is crucial for stabilizing and controlling the release of active agents.

Apart from cross-link analysis, encapsulation and retention of therapeutic agents by the hydrogel were also assessed. The drug-loaded hydrogel was analyzed under the same HPLC conditions, and curcumin, gentamicin, and metformin retention times were determined. The retention peaks mimicked each drug's unique profiles, verifying that they had been incorporated into the hydrogel matrix. In addition, the intensity of every peak was proportional to the concentration of each drug in the formulation. Therefore, this ability to encapsulate multiple drugs with the structural integrity of the hydrogel facilitates its potential application in controlled drug release in burn wound healing. Results Section: The chromatographic data and their interpretations will be presented in the results section.

2.6. FTIR of Chitosan hydrogel

For the confirmation of the formation of the non-cross-linked chitosan acetate, cross-linked unloaded chitosan, and drug-loaded chitosan, Fourier transform infrared spectroscopy (FTIR) (Perkin Elmer, USA). The process was done with all the solutions separately; 1 mg of the respective sample powder was mixed with 200 mg of KBr to obtain the pallet. Home protocols performed FTIR. The settings were set to 400–4000 cm1 at a resolution of 4 cm1. All the bonds and their peaks are mentioned in the results section.

2.6 Scanning electron microscopy of chitosan hydrogel:

After the initial characterization of the chitosan hydrogel, it was further processed to evaluate the surface, 3-D structure, and size of the hydrogel formed. Scanning electron microscopy (JEOL Japan, JSM-6490A) was used to evaluate all these parameters. The photographs were taken at ten KV, and all the photos have the appropriate resolution and magnification to be studied.

2.7. Physical Properties

The physical properties studies of the chitosan hydrogel, like swelling index, tensile strength, elongation percentage, and drug release index, were carried out. Swelling index studies were determined using phosphate-buffered saline and the lyophilized chitosan acetate solution [25]. The universal testing machine determined the elongation percentage and the tensile strength (Shimadzu Japan, AGX-Plus). The drug release index studies were carried out using the phosphate buffer saline as a medium for the drug release. They were calculated with the help of a spectrophotometer at certain intervals.

2.8. Wistar rats processing

Three groups of Wistar rats were taken and processed under the Animal Research Act, and the Internal Review Board approved the use of rats in research for Animal Testing at the National Institutes of Health in Islamabad (Ethical Approval Code: NIH-AWC-ISB-2023/01). All Wistar rat operations followed the NIH Guidelines for the Care and Use of Laboratory Animals [26]. The care and handling of the Wistar rats adhered strictly to the ethical guidelines for laboratory animals.

The Wistar rats used in this investigation were kept in typical laboratory settings, which included a 12-hour light/dark cycle and a regulated temperature (20–24°C). The rats had unrestricted access to food and water in hygienic, well-ventilated cages. They were fed a nutritionally balanced rat chow designed to satisfy their

requirements. Environmental enrichment was offered, including nesting materials and items for exploration to encourage natural behaviors and lessen stress. The animals were kept in the best possible health during the trial thanks to routine health examinations. The mean age of the rats at the time of initializing the study was about 7–10 weeks, and the mean weight was around 200–220 grams. All the mice tested were males. We used two rats for each of the three groups. A total of six rats were used in this study.

For the study, three groups were created: the first group had chitosan hydrogel only, the second group was drug-loaded chitosan hydrogel, and the last group was the control group with normal saline or PBS. Two rats were tested for each group. A total of six rats were used in this study.

To give the wounds to the rats, they were anesthetized with ketamine 40 mg/kg [26]. After this, the hair of a particular site was trimmed and shaved and then disinfected with an alcohol swab. The rats were given a burn wound by heating a stamp and placing it at the site of the shaved area. After the burn wound, initial skin tissue/biopsy was taken for the histopathological studies, and the rats were processed as per their respective groups. After applying the bandages, the rats were put in separate cages and fed as per the requirements of the animal department. They were kept in favorable conditions and were under constant monitoring. The weight gains and healthy body indicated that they were kept in a suitable environment. The rats were processed for skin biopsy 7, 14, and 21 days after the initial histopathological study.

2.9. Histopathological studies

Rat wound biopsies were processed on days 1st, 7th, 14th, and 21st. The samples were preserved in 10% formalin, and the process of tissue processing was carried out as follows: Initially, the samples were gross sectioned and put in the tissue-processing unit for tissue fixation. After that, the samples were removed and put into the paraffin blocks on the cryo-plates of the paraffin block embedding. The paraffin-embedded blocks were processed on a microtome to get small 20nm sections of the embedded sample. After the sectioning, the sample sections were transferred into the water bath and, afterward, on a slide on a hotplate. The slides were then stained with eosin hematoxylin (H/E) stains. The prepared slides were then seen under a microscope to evaluate the selected parameters: wound closure, rete ridge formation, and collagen deposition at the wound site.

2.10. Wound site studies

On the first day of the wound bearing, the rats were photographed by the DSLR camera, and the wound size, appearance, and gross looks were captured.

2.11. Statistical Analysis

Comparison was done using graphs, T-tests, and one-way ANOVA to compare p values ($p<0.05$) for different parameters. The tests were performed on IBM SPSS Statistics 20.

3. Results & Discussion

3.1. Hydrogel Properties and Morphology

Before drug loading, the color of the hydrogel was yellowish-white, and it was processed on the FTIR and HPLC to confirm chitosan acetate formation. After the drug was loaded, the color of the hydrogel became dark yellow because the curcumin had an orange-to-yellow solid color. The surface and three-dimensional structure of the hydrogel were revealed with a scanning electron microscope that showed high porosity and a pore size of 50±2µm. Some studies showed that different polysaccharides could be used in medicine [18, 22]. However, chitosan is the most favorable for wound dressings and drug delivery as it has better biodegradability, biocompatibility, and cost-effectiveness. That is why chitosan was chosen for the hydrogel preparation in this study. The hydrogel made in this study was cross-linked using formaldehyde and tween 80 [27].

3.2. Fourier transform infrared spectroscopy

FTIR (Perkin Elmer, USA) was processed and recorded in the 4000-400 cm^{-1} at the resolution of 4 cm^{-1}. The 3700-2950 cm-1 peak corresponds to the series of O-H and NH_2 bonds. The peak band around 3000-3550 cm^{-1} shows the vibration of amine N-H and the H bond in the O-H. The carbohydrate rings of vibrations of asymmetric and symmetric CH_2 of the chitosan are shown at the peak

2850–2980 cm^{-1}. The absorption peak of the chitosan corresponds to the peak 1750 cm^{-1} and its corresponding regions, covering three-amide bond interactions and vibrations. The first one is C=O in the first amide vibrations, the second at peak 1645 cm1 of the CH_2 bending of the second amide, and the last at peak 1550 cm^{-1}, corresponding to the N-H vibrations of the third amide. The peak at 1230 cm^{-1} corresponds to the asymmetric C=O bond, and the peak at around 950–1200 cm^{-1} corresponds to symmetric C-O-C stretching. It can be confirmed that there was an interaction between chitosan and acetic acid, resulting in the formation of chitosan acetate (Figure 1). The peak at 1550 cm^{-1} confirms the bond between the acetic acid's carboxylate salt and the chitosan amine group. This confirms the natural electrostatic interaction between the chitosan and the acetate ions.

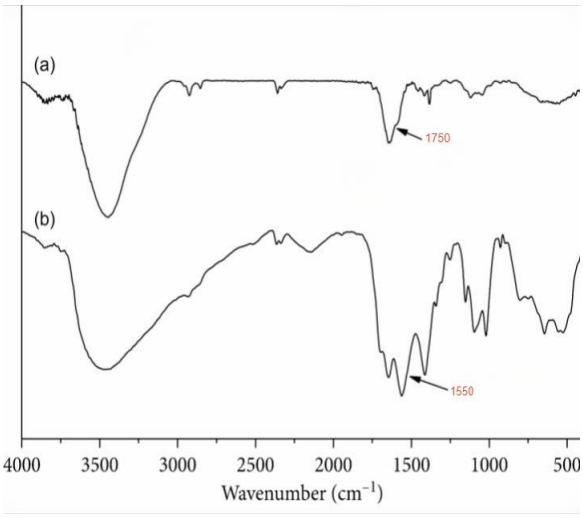

Figure 1. FTIR of (a) Chitosan and (b) Chitosan-Acetate.

After the characterization and confirmation of the chitosan acetate interaction, the chitosan loaded with drugs (curcumin, gentamicin, and metformin) was processed on the FTIR under the same protocols and settings. As indicated in the above sections, the peaks and the bond and interaction confirmation between the chitosan acetate and the drugs were discovered. In Figure 2, it can be seen that the formation of the bond between chitosan and the drugs (curcumin, gentamicin, and metformin) was shown at the peak of 1750 cm^{-1}, thus confirming the formation of the chitosan hydrogel loaded with drugs. Studies showed the FTIR peaks parallel to our study [5, 24, 25, 27].

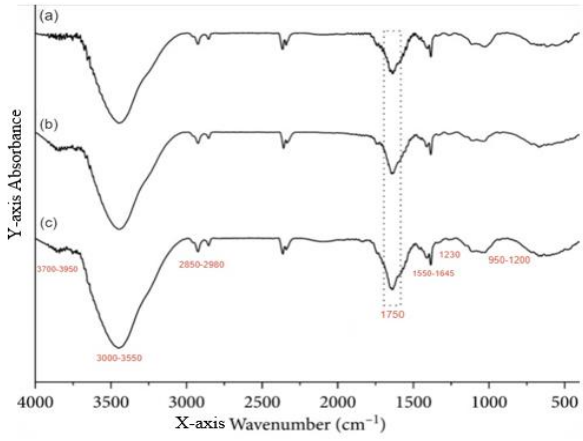

Figure 2. FTIR of drug-loaded chitosan hydrogel (a) Curcumin-loaded CH, (b) Metformin-loaded CH, and (c) Gentamicin-loaded CH.

3.3. High-pressure liquid chromatography

The high-pressure liquid chromatography HPLC (Shimadzu LC-20A) of the chitosan and the chitosan acetate showed an interaction between the chitosan and the acetic acid naturally, called the electrostatic interaction. The mobile phase was chosen according to the previous studies, while HPLC-grade water was used at 1.0 ml/min. The retention time of chitosan was 22 minutes; in contrast, the retention time peaked at around 2 min for the acetic acid overall. Furthermore, the HPLC confirmation of the drugs was also done, for which curcumin's retention time peak was 8.7 mins, gentamicin retention time peaked at 14.8 mins, and metformin at around 3 min. All the peaks are given in Figures 3-5.

3.4. Scanning electron microscopy

The SEM (JEOL Japan, JSM-6490A) of different samples was explicitly performed as chitosan, cross-linked chitosan, and drug-loaded chitosan hydrogel. The results clearly showed that the chitosan had initial porosity, as shown in Figure 6. (a) these pores indicated a potential for the interlinking of different chemicals and drugs. These pores were big, and the structure of the chitosan represented a sponge-like appearance, whereas the size of the pores was 50±2µm. Later, when the chitosan acetate was cross-linked with the formaldehyde and tween 80, it showed that the size of the pores was significantly reduced.

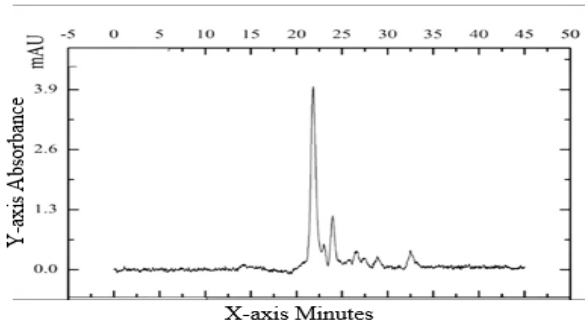

Figure 3. HPLC of Chitosan.

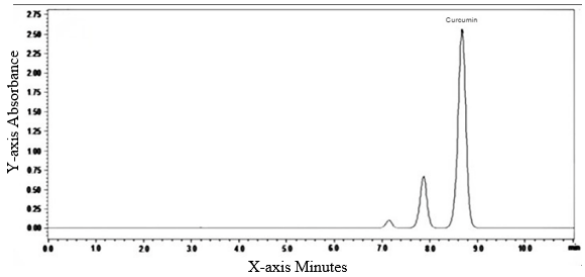

Figure 4. HPLC peak of curcumin standard.

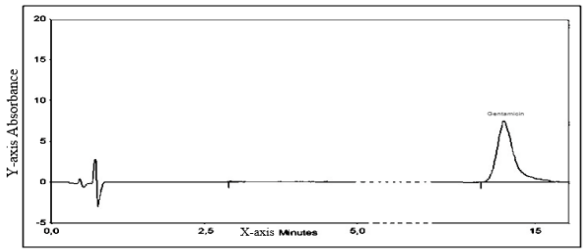

Figure 5. HPLC of Gentamicin sulfate standard.

The hydrogel showed a composite structure with more integrity and less sponginess, as shown in Figure 6 (b), in which chitosan loaded with drugs was processed in the final segment. The structure unexpectedly showed vivid drug loading on the surface, indicating that the drug is mostly bound to the surface of the chitosan hydrogel. Furthermore, in the 3-D spaces of the hydrogel shown in Figure 6. (c), the drug release studies also confirm the presence of drugs on the surface more than the inside. In a study, the SEM analysis showed the pore sizes to be 50–150 µm and a decrease in porosity as the chitosan concentration was increased [3]. However, in our study, the SEM analysis showed the pore size to be 50±2µm, and along with this confirmation, it determined that there was an increased amount of drug on the surface of the hydrogel compared to the inside. Some studies have also revealed that the low chitosan concentration causes irregular pore sizes, whereas our study does not have such a finding.

3.5. Swelling index

The swelling index of the chitosan hydrogel is shown in Figure 7. The swelling of the hydrogel is attributed to the presence of pores in the structure, which is due to the degree of cross-linking [28]. The lesser the cross-linking, the more the pores numbers and the greater the affinity to absorbance. This study, as well as a previous study [29], also displayed that the swelling of the hydrogel was directly proportional to the time and the size of the pores in the structure. The results were based on previous studies [28, 29]; hence, the hydrogel was approved for drug release and wound dressing studies. It was also noted that after reaching equilibrium after 24 hours, the structure and integrity of the hydrogel were unaffected, and it was not entirely soft but not brittle. In a study, the swelling index determined in the water and PBS showed that the swelling was greater in the water compared to PBS, with the values setting as high as 980 percent in the case of water and 770 percent in the case of PBS [25]. The swelling index in our study using a single concentration of the chitosan in triplicates in PBS for 24 hours showed that the initial swelling of the chitosan ranges from 350 percent to 900 percent; after that, it becomes stagnant and in a state of equilibrium.

3.6. Tensile Strength of Chitosan Hydrogel

As the hydrogel is to be used for biomedical purposes, specifically wound dressing, the hydrogel needs an appropriate tensile strength with proper stress/strain index. Four different concentrations of chitosan were used for this study (Figure 8); it was indicated that the increase in the chitosan concentration increased the tensile strength. It might be because more chitosan means less porosity and more composite structure, giving it more stress/strain indices [30]. These results showed that the chitosan was not strong enough to make implants, but it was in the range of use for medical dressings, which in our case was burn wound dressing. In a study, the increased chitosan concentration could increase the hydrogel's tensile strength and physical integrity [3].

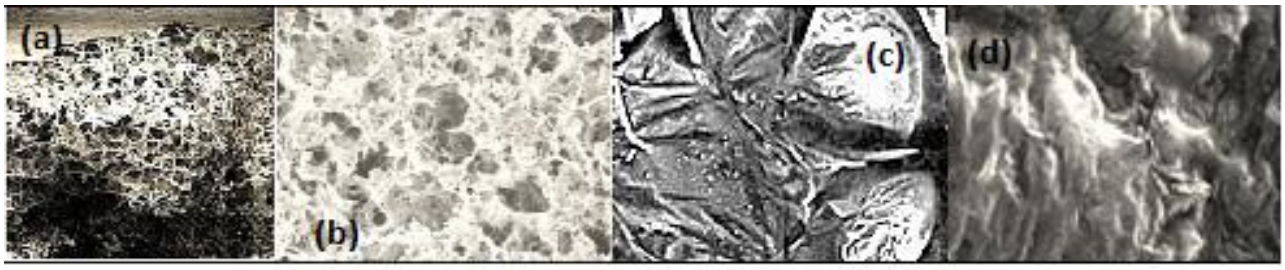

Figure 6. SEM of (a) Non-cross-linked chitosan, (b) Cross-linked Chitosan with Formaldehyde and Tween 80, (c) Non-Drug loaded Chitosan, (d) Drug loaded Chitosan with Curcumin, gentamicin, and metformin.

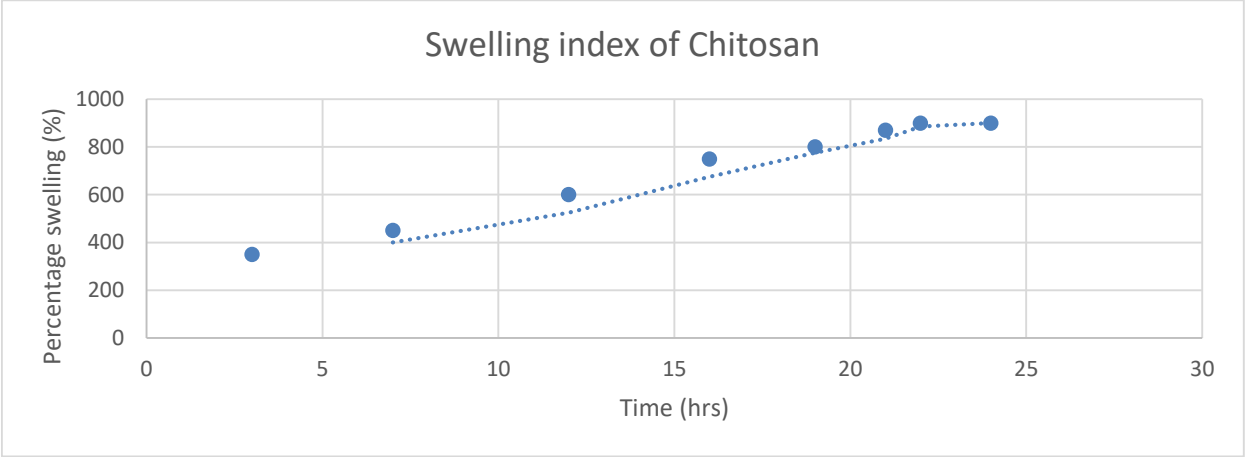

Figure 7. Swelling index of chitosan hydrogel, the dot trend line represents the increasing swelling of the chitosan hydrogel in the phosphate buffer saline over a period of 24 hours.

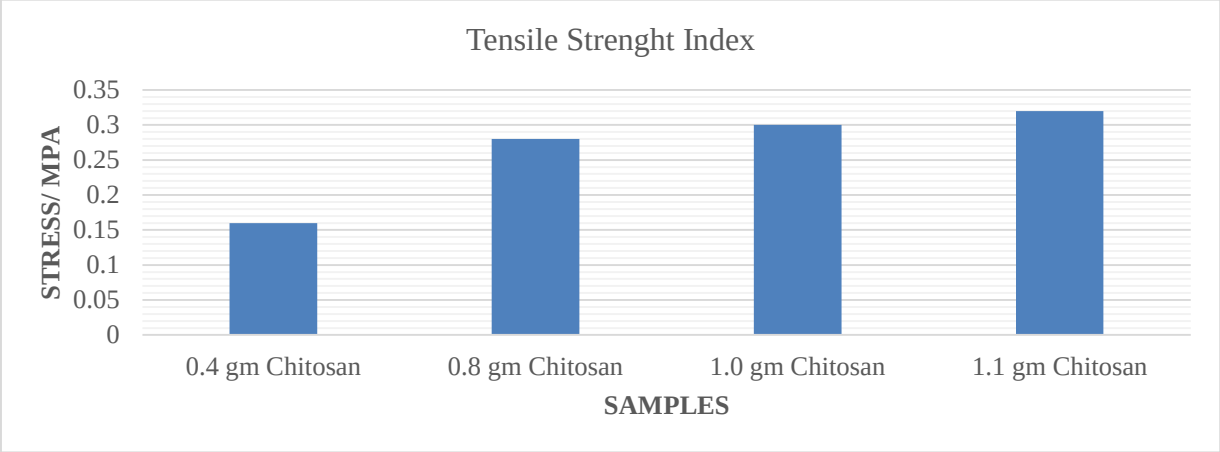

Figure 8. Tensile Strength index of chitosan hydrogels, different samples are tested where the concentration of chitosan is increased from 0.4 gm to 1.1 gm in four different hydrogels. The tensile strength is increased with the increase in the concentration of the chitosan.

However, it reduced the hydrogel's water adsorption and porosity of the hydrogel, similar to the results in our study. In another study, the chitosan's tensile strength was as high as 0.35 MPa, and the minimum recorded was 0.03 MPa [25].

Our study also noticed that the increase of the chitosan from 0.4 g to 1.2 g showed an increase in the tensile strength, i.e., 0.16 MPa to 0.32 MPa.

3.7. Elongation percentage index of chitosan hydrogel

Hydrogel should have appropriate elongation strength because it is used in biomedical applications. With low elongation or tensile strength, the hydrogel can be damaged during applications. The elongation percentage was determined using the method and formula given in the methodology. Four different chitosan concentrations were taken to evaluate the role of chitosan concentration in elongations. From the results in Figure 9, it can be shown that with the increase in the concentration of chitosan, there is an increase in the elongation percentage, as in the case of tensile strength. These results showed that the hydrogel could be used in biomedical applications like wound dressings. In a study, the elongation percentage was 20 to 60 percent [1]. In our study, the elongation percentage was 30 percent to 62 percent.

3.8. Drug release index of chitosan hydrogel

The drug release index, as shown in Figure 10, displayed that there was an initial burst release of the drugs from the hydrogel and a later slow release.

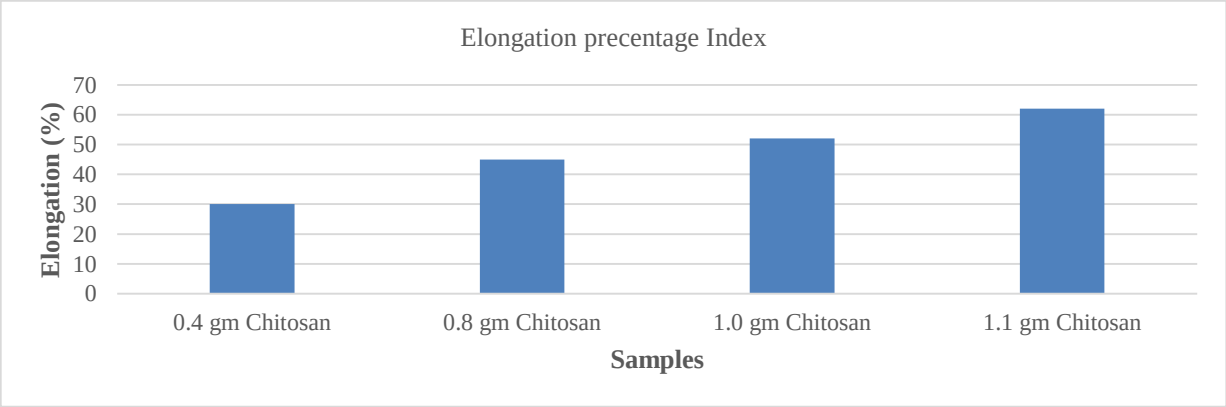

Figure 9. Elongation percentage index of Chitosan hydrogels, the concentration of chitosan is increased in the four different hydrogels and the figure shows that the higher the concentration of the hydrogel the better elongation percentage index.

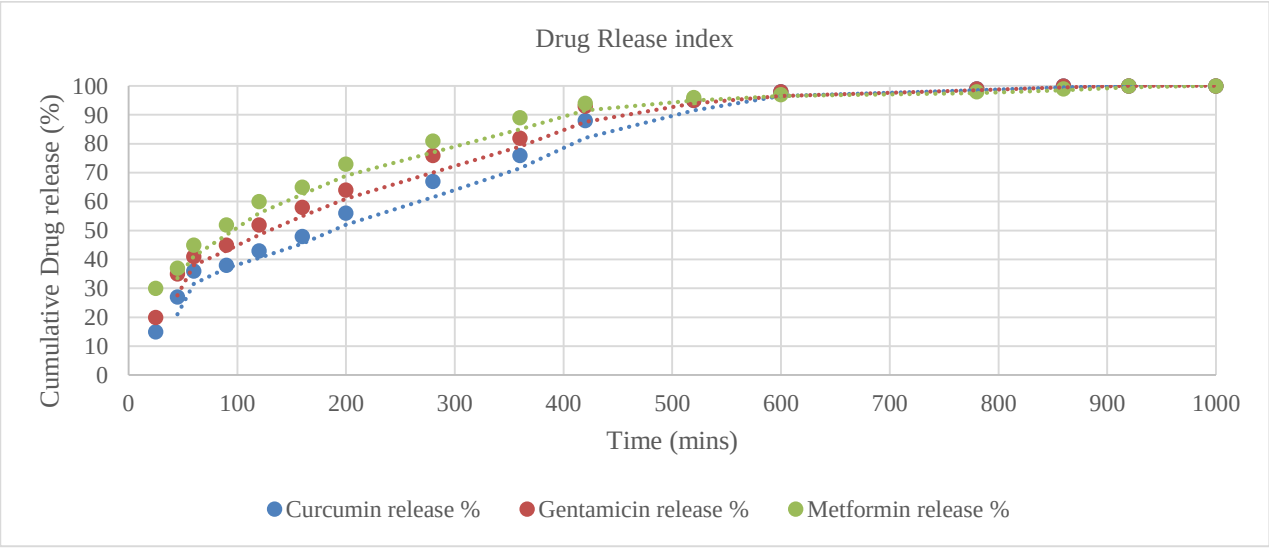

Figure 10. Drug release index, the blue dot trend line represents the curcumin release percentage after certain amount of times, the orange dot trend line represents gentamicin release percentage and the gray dot trend line represents metformin release percentage.

The burst release might be due to drugs on the surface of the hydrogel [7], as shown in the SEM analysis. The burst initial release for the curcumin was 32% in the first hour. The burst release for gentamicin sulfate was 41% in the first hour, which might be due to the more solubility of gentamicin in the water than the curcumin. Whereas the initial burst, the release of the metformin standard was 45% in the first hour, also because of its solubility in the water. After about eight hours, all the drugs were released from the hydrogel. The results were from the previous studies of other hydrogel drug releases. The trend lines show that the drugs are released with time progression, and at some point, all the drug content escapes the hydrogel in Figure 10. The blue dots represent curcumin, red represents gentamicin, and green represents metformin, loaded in the chitosan hydrogel. The results were tested statistically on SPSS, with the mean indicating the drug release was higher in the metformin than in the other two drugs.

3.9. Macroscopic evaluation of wound site

In vivo studies showed that the hydrogel was easy to apply to the wound area; it did not adhere to the skin on the wound site, and the application and removal were very smooth. This result has been supported by previous studies [31]. Initially, the wound site was observed macroscopically and with the naked eye. The initial studies showed that the wound sites in the chitosan and drug-loaded chitosan groups were shrinking in size faster than the control group. The wound was thick, and there was a scab formation on the wound site in the chitosan and drug-loaded chitosan groups by the end of the first week, whereas in the control group, the scab appeared on the 14th day. In addition, it was clear that the scab formed in the drug-loaded chitosan group was firm and dry, with no significant oozing compared to the other groups. The wound sites healed more quickly in the chitosan group than in the control group, as shown in Figure 11.

The wound size was measured using a ruler; the ruler was aligned on the periphery of the wound area, and the sizes were taken on days 1, 7, 14, and 21 for each respective group. The calculations were done by the method mentioned above. It showed that there was a quick decrease in the size of the wound in the drug-loaded chitosan group as compared to the chitosan and control groups. The values are shown in Figure 12. A study showed an increased wound closure when the control group to chitosan group values were compared in the third week [26]; this was the case in our

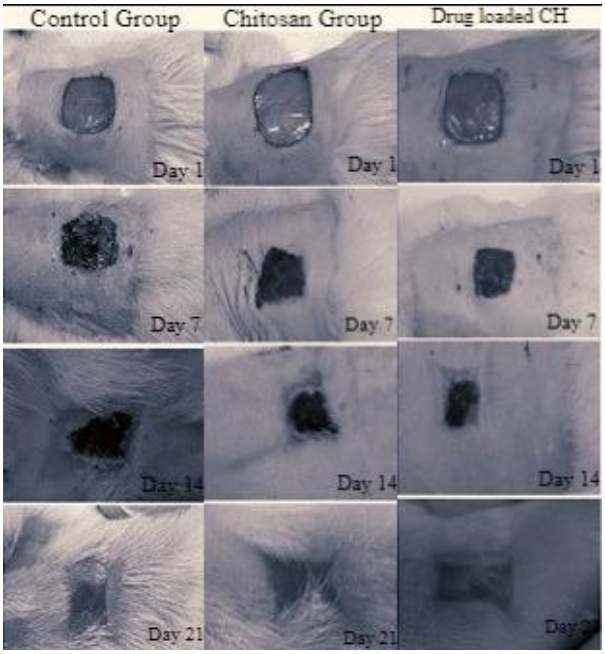

Figure 11. Wound site of all the groups.

study. The wound size observed on days 1st, 7th, 14th, and 21st was evaluated statistically with a one-way ANOVA test and paired sample T-test that showed the results to be significant ($P<0.05$) for all the groups compared to the untreated control group.

3.10. Histopathological studies

The biopsies were taken on days 7, 14, and 21 for the respective group. It was noted that an increased epithelial layer formation, known as epithelization, was present in the chitosan loaded with drugs group compared to the chitosan and control groups. It was also noted that the dermal-epidermal layer was thicker in the case of the chitosan-loaded with drugs group as compared to others. When studied, this healing pattern was progressive from day seven onward. Under light microscopy, keratinocyte deposition was increased in the drug-loaded chitosan group, which progressively increased as the weeks passed. As in the chitosan group, keratinocytes can also be seen at the end of week 1, but fewer were shown in Figure 13. In the case of the control group, the wounds had fewer keratinocytes even at the end of week 2 but significantly increased by week 3 compared to weeks 1 and 2 (Figure 13). The dermal-epidermal layer was thicker and faster in the drug-loaded chitosan group than the others, as supported by the previous study [26].

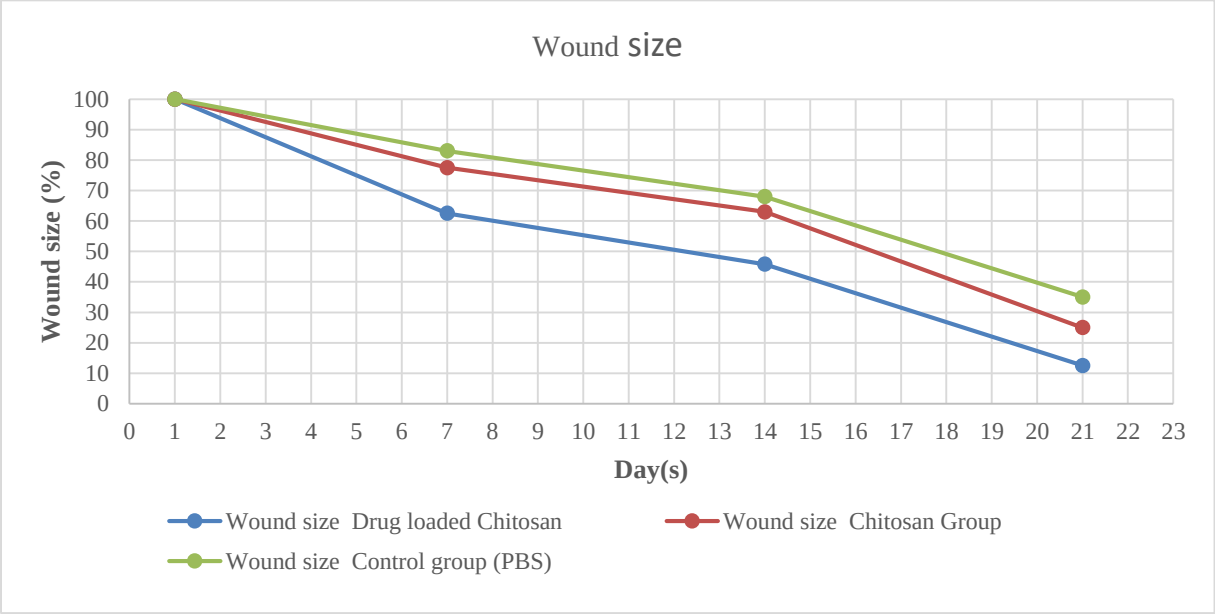

Figure 12. Wound size percentage evaluation, the blue trend line represents the wound size reduction in drug loaded chitosan group from 100% to the corresponding value on a day 1st, 7th, 14th, and 21st, the orange trend line represents chitosan group and the gray one represents control group.

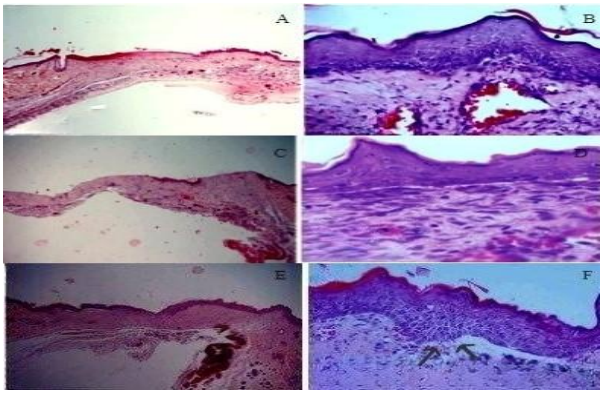

Figure 13. Hematoxylin-eosin H/E staining Slide of Wound sites.

In addition to the attention paid, it can be seen that there was an increased number of rete ridges, as shown by the arrows in Figure 13. (f) in the drug-loaded chitosan group earlier in the studies, closer to the end of week 1, and increasing in number progressively. In the case of the chitosan group, the number is smaller, and the formation is late, whereas the control group showed inferior and late formation of rete ridges in the wound. There was little to no inflammation or granulomatous mass formation under the light microscope in the case of drug-loaded chitosan ($P<0.05$) and chitosan hydrogel group ($P<0.05$) as compared to the control group that had small oozing macroscopically and some mass and inflammation patterns under the light microscope [37].

3.11. pH studies of the wound site

The wound inflicted upon the rats was evaluated for the pH changes on the 1st, 7th, 14th, and 21st days. The initial wound sites were tested almost neutral, with the pH at ±0.20 and 7.45 for all the groups. The drug-loaded hydrogel group showed a progressive pH lowering when measured on the respective days. The mean pH of this group was determined to be around 0.25±6.56. The chitosan hydrogel group and the control also had progressive pH lowering but not as low as the drug-loaded chitosan hydrogel group ($P<0.05$). The mean pH of the chitosan hydrogel group was 0.20±6.70, and the control group was 0.20±6.97. The low pH of the wound site shows better wound healing. The chitosan group loaded with drugs showed better wound healing, which was also confirmed by the microscopic studies and the wound size evaluation of the wound site. As shown in **Figure 14**, the drug-loaded chitosan group showed a significant reduction in the wound pH as the day progressed compared to the other groups. The pH reduces from 7.45 at day 1 to 6.34 at day 7, then to 6.24 at day 14, and 6.2 at day 21. The results were tested on SPSS with a one-way ANOVA test, which showed that the test results were significant ($P<0.05$) when the drug-loaded chitosan and the chitosan groups were compared with the control group.

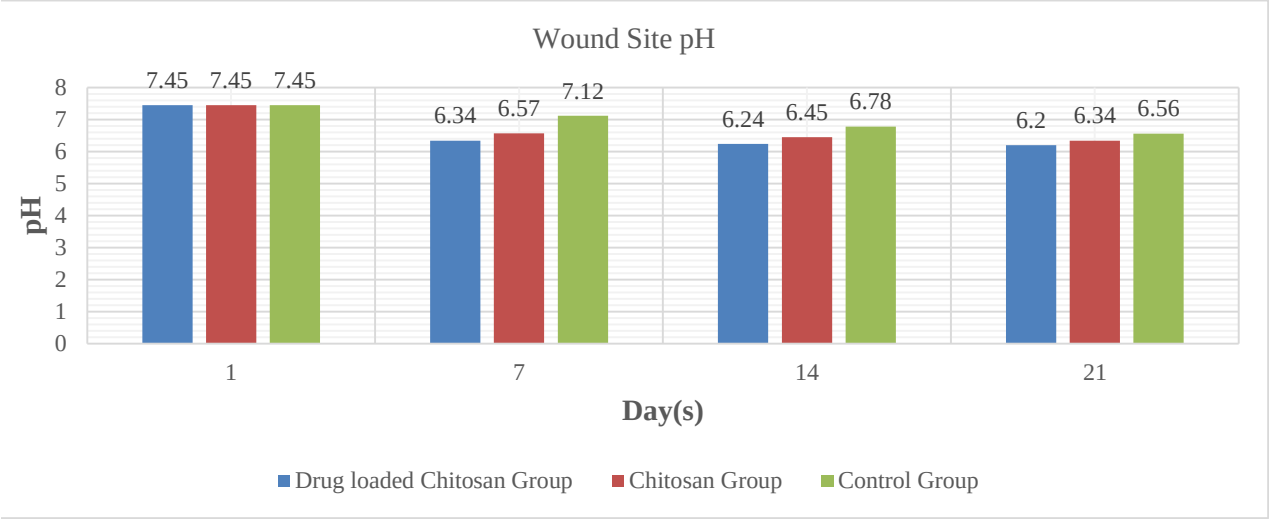

Figure 14. Wound site PH, Blue bars represents drug loaded chitosan hydrogel, orange bars represent chitosan group and the gray bars represents control group.

The healing process was significantly affected by the pH at the wound site; lower pH could enhance healing better. The most pronounced change in pH was seen in the group with drug-loaded chitosan hydrogel, which was shifted from 7.45 to 6.20, consistent with better healing results. There was a drop in pH levels for both the chitosan hydrogel and control groups, but this was less significant, meaning lower pH levels are associated with enhanced wound healing.

Different types of hydrogels are used in the medical field for different purposes. There are different hydrogel types, including pH-sensitive, temperature-sensitive [33], and ionic strength-sensitive, drug delivery hydrogels, medical implants made from hydrogels, and wound dressings designed by hydrogels. A study showed that different polysaccharides could be used in medicine [33, 34]. However, chitosan is the most favorable for wound dressings and drug delivery as it has better biodegradability, biocompatibility, and cost-effectiveness.

In this study, the wound dressing type of hydrogel was fabricated and characterized for its various physical and chemical properties. Traditionally, normal bandages and wound dressings were used for burn wounds that would need extra care, medications, and check and balance regarding the moistness and dryness of the wound site. All these problems can be overcome using this chitosan-based wound dressing hydrogel; the hydrogel made in this study had curcumin, gentamicin, and metformin. Secondly, the hydrogel provides a perfect wound environment suitable for wound healing in case of burns.

Some studies have shown that gentamicin, curcumin, and metformin are used harmoniously with chitosan to treat burn wounds [35-39]. A study that worked on the chitosan hydrogel in harmony with the fucoidan for the burn wound healing activities of chitosan alone and then with the conjugation of fucoidan [35]. It was noted that chitosan alone has burn wound healing activities. In our study, the chitosan hydrogel group exhibited burn wound healing activity compared to the control groups. Our study used glacial acetic acid to make chitosan acetate, which was later cross-linked. In this study, chitosan hydrogel loaded with drugs showed results like in the case of fucoidan-chitosan hydrogel [35]. The addition of drugs in the chitosan group improved the wound healing activity of the hydrogel. The hydrogel made in this study was cross-linked using formaldehyde and tween 80, as was done [36]. Here, chitosan hydrogel was made infused with silver nanoparticles. In our study, the hydrogel was infused with different drugs. However, both studies prove that using these additives with the generic chitosan hydrogel can tremendously improve the burn wound healing activity. The increase in the chitosan concentration can increase the hydrogel's tensile strength and physical integrity. However, it reduces the hydrogel's water adsorption and porosity [37]. In a study, the chitosan's tensile strength was as high as 0.35Mpa, and the minimum recorded was 0.03Mpa [38]. In another study, the elongation percentage was 20 to 60 percent [39]. Our study also noticed that the increase of the chitosan from 0.4gms to 1.2gms showed an increase in the tensile strength, i.e., 0.16MPa to 0.32Mpa, and an

increase in the elongation percentage from 30 percent to 62 percent.

In our study, only one batch of chitosan concentration of 1.2 grams was used to calculate the swelling index percentage. The swelling index, drug release profile, tensile strength, and elongation profile are also determined. In a study, the swelling index determined in the water and PBS showed that the swelling is more in the water than in PBS, with the values setting as high as 980 percent in the case of water and 770 percent in the case of PBS [38]. The swelling index in our study used a single concentration of the chitosan in triplicates in PBS for 24 hours, showing that the initial swelling of the chitosan was 350 to 900 percent; after that, it became stagnant and in a state of equilibrium. The release of drugs from the hydrogel varies depending on the type of the drug and the method of loading in the hydrogel. In our study, the initial release of all the drugs was maximum, which explained that there was more drug on the surface of the hydrogel as compared to the inside network.

The presence of drugs on the surface of the hydrogel is confirmed through scanning electron microscopy. The total pattern of release of the drugs from the hydrogel is around eight hours; during this time, all the drugs from the hydrogel are released. Other studies that used different drugs showed periods similar to this as well. The results and the values of the hydrogel characterization presented with the FTIR peaks corresponding to the peaks in our study [40-42]. The peaks of the chitosan and acetic acid are identical, showing that a successful bond has been formed between the chitosan and acetate. The peaks corresponding to the drug's bond formation also confirmed an interaction between the chitosan hydrogel and the drugs used in our study. The FTIR results confirmed the chitosan hydrogel's formation and the successful loading of the drugs on it. Different peaks in the HPLC also confirmed the presence of chitosan, gentamicin, and curcumin in the solution. In a study, the SEM analysis showed the pore sizes to be 50-150 µm and a decrease in porosity as the chitosan concentration was increased [37]. However, in our study, the SEM analysis showed the pore size to be 50±2µm, and along with this confirmation, it determined that there was an increased amount of drug on the surface of the hydrogel compared to the inside. Some studies also revealed that the low chitosan concentration causes irregular pore sizes, whereas our study did not find such a finding.

In our study, curcumin, gentamicin, and metformin were loaded on the chitosan, and a group called drug-loaded chitosan; secondly, a group without drugs called the chitosan group, and lastly, a control group was created. In a study, the wound size evaluation showed increased wound healing when chitosan was applied topically on the wound site compared to the control group [43]. The wound closure percentage was 100% by the end of the third week. Initially, the wound size was 100%; by the end of the first week, the wound size was 77%; in the second week, it was almost 31%. In the control group, by the end of the first week, the wound size was 92%; by the second week, 38%; and by the end of the last week, that was, third, the wound size was shown to be 15%. Our study has shown increased wound healing ability when chitosan was used, which was increased when drugs were used in conjugation with the hydrogel. By the end of the first week, the chitosan group showed 77.5%, the second week 63%, and by the end of the third week, it was 25%. In the drug-loaded chitosan group, the wound size by the end of the first week in our study was 62.5%, the second week was 45.8%, and finally, by the end of the third week, 12.5%.

Meanwhile, in the control group, the wound size was 83% by the end of the first week, 68% by the end of the second week, and 35% by the end of the last week. The wound-closing ability of the control group in our study was lower than the control group in the other studies. In contrast, the addition of chitosan and drugs to the wound increases the ability accordingly.

The histopathological studies of the wound are necessary to get information and confirm whether wound healing is accelerated in different groups. In addition, histopathology determines the presence of neutrophils, different cytokines, granulomatous masses, rete ridges, inflammation, and wound bed length [43]. Histological studies were carried out on days 7, 14, and 21 after applying the initial dressing. The study revealed an increase in the epithelial layer generation in the case of the chitosan group compared to the control group. The epithelial layer formation increased by the end of the first week, progressively increasing and then decreasing, showing wound healing. Our histological studies also revealed increased epithelial bed length in the loaded chitosan and chitosan groups in the initial days compared to the control group. The formation of the epithelial layer in the case of drug-loaded chitosan was better than that of the other two groups. In another study, the control group exhibited necrotic development, low

epithelization, low number of rete ridges, edema, and inflammation [42]. The groups with chitosan showed better epithelization and lesser inflammation than the control group. By the end of the 12th day, there was an improved collagen formation and capillary bed formation in the chitosan group compared to the control group. However, by the end of week 3 (the 21st day), the chitosan and other groups exhibited epidermal covering and scar formation, indicating wound healing. However, compared to the data, the chitosan group showed better wound healing than the other groups. Our study showed that by the end of the first week, there was an increased epithelial layer formation. The formation of collagen in the chitosan and drug-loaded chitosan group confirmed the accelerated wound healing by such days, and there was no collagen formation in the control group. The capillary beds formed at the end of the first week; this showed an increased wound-healing activity in the chitosan group compared to the control group. In the histological slides, it can be confirmed that there was an increased formation of rete ridges in the drug-loaded chitosan group. Our study also noted little to no inflammation in the chitosan groups compared to the control group. By the end of the last week, scars had formed in all groups, and the length of the epidermal wound bed had been reduced, but the best results were shown by the drugs loaded in the chitosan group.

In the initial formation of the chitosan hydrogel, adding sodium hydroxide was necessary because cross-linking with formaldehyde requires basic pH; otherwise, the solution becomes brittle and breaks upon touch. This was confirmed when the chitosan acetate was initially cross-linked in the acidic pH. The color of the chitosan hydrogel changes from whitish to yellowish upon the addition of drugs due to the strong yellow color of curcumin. The porous structure of the chitosan helps in the infusion of different chemicals and drugs in the network; in this study, the porosity of the hydrogel exhibits perfect drug loading. The chitosan's porous structure also explains the chitosan hydrogel's swelling. Further studies need to be done to use more drugs or a single drug that can achieve the same or better results. This hydrogel wound dressing can also be converted to a skin graft with drugs loaded in it that will be released and the skin graft left on the spot for a better overall experience in burn wound healing. Further studies need to be conducted to pave the way for achieving this.

4. Conclusion

The development of chitosan hydrogel loaded with the drugs could help wound healing in case of burn wounds. The cross-linking of chitosan acetate with formaldehyde requires basic pH, and it causes brittleness and non-uniform gel. The porosity of the chitosan hydrogel is inversely proportional to the concentration of the chitosan. At the same time, physical strength, such as elongation and tensile profiles, increases with increasing chitosan concentration. In order to have a uniform and controlled drug release, the method used in this study should not be used, as there was an initial burst release of drugs here. The uniformity of release can be achieved by making thermosensitive hydrogels. The presence of chitosan and acetic acid on the wound site increases the wound healing capability and effectiveness. Adding drugs, i.e., curcumin, gentamicin, and metformin, increases the effectiveness, and the wounds are healed quicker. Hydrogel was better because of its biodegradability, biocompatibility, and low cost. Using chitosan also prevents dryness at the wound site and keeps the wound area favorable for better wound healing. Chitosan hydrogel infusion with different chemicals and drugs can increase the wound healing of burn wounds.

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Conflict of interest

The authors of this article announce that we have no conflict of interest.

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