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A study of chitosan nanofibers containing neomycin sulphate for wound healing activity

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ABSTRACT

The rationale behind this project work was to prepare neomycin sulphate loaded chitosan nanofibers via electrospinning methods so as to enhance wound healing property. Natural polymers are used as main compounds for design of therapeutic drug delivery systems for treatment of different ailments. Chitosan and Neomycin sulphate have proven wound healing properties individually. The combination of these two, polymers and incorporation of drugs into the composite nanofibers show improvement in wound healing property. The drug loaded chitosan nanofibers were prepared with increasing concentrations of polymer and cross linker (glutaraldehyde). The characterization of these nanofibers is done by FT-IR, DSC and SEM studies. These prepared nanofibers were evaluated for fibers diameter, tensile strength, drug content, fluid uptake, moisture vapor transmission rate, antibacterial activity, in vitro drug release by diffusion studies and in vivo studies by excision wound model. The fibers diameter, tensile strength, drug content, fluid uptake, moisture vapor transmission rate, antibacterial activity for optimized formulation were observed to be 512± 60nm, 6.14± 0.63MPa, 98.58± 0.76%, 200.24± 0.31%, 2430.52±0.45, 2.46±0.081cm (S.aureus), 2.33±0.06cm (E.coli) and 59.49±0.52% respectively. The drug loaded nanofibers shown significant difference in antibacterial activity when compared to neomycin cream. Percentage of wound contraction was more for wounds treated with neomycin sulphate loaded chitosan nanofibers than the blank nanofibers and neomycin cream. With the above results, neomycin sulphate loaded chitosan combination had shown better results when compared to chitosan nanofibers and neomycin cream alone in wound healing activity.

Keywords: Nanofibers, electrospinning, wound healing, anti-bacterial activity, *in vivo* studies

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INTRODUCTION

The use of neomycin sulphate as an antimicrobial agent and antibacterial agent has a long history.[1-5] use of combination of neomycin sulphate and chitosan for wound healing activity show synergistic activity, they show epidermal proliferation and possessed antibacterial properties against *Staphylococcus aureus, Pseudomonas aeruginosa and Escherichia coli*.[6] Neomycin sulphate can be effective against a wide range of microorganisms, including both aerobic and anaerobic bacteria, fungi and viruses. The antimicrobial activity neomycin sulphate involves several mechanisms: it interferes with the respiratory chain in the cytochromes of micro bacteria: inhibit the protein synthesis of bacterial cell wall: it interferes with components of the microbial electron transport system:[7] and it binds DNA and inhibits DNA replication.

Recently there has been a rapid increase in the number of commercially available dressing material such as $AgNO_3$, Silver sulfadiazine, neomycin film, gentamicin and nanocrystalline silver. It is possible to produce pure neomycin particles at a nanoscale (nanofibers) with advanced nanotechnology. When cells or tissues are exposed to neomycin

nanofibers, their active surface is significantly larger than that of other neomycin formulations used for wound healing activity. As a result, neomycin sulphate in combination with chitosan are able to exhibit unusual physicochemical properties with remarkable biological activity. Ideal antimicrobial wound dressings should have more controlled and prolonged release of neomycin sulphate (nanofibers) compared with neomycin cream formulations during their entire period of usage. This will result in less frequent dressing changes, thereby reducing the risk of nosocomial infection, cost of care, further tissue damage and patient discomfort. Many factors affect the clinical performance of a dressing, such as the drug content, chemical and physical form of neomycin sulphate, distribution of neomycin sulphate and its affinity for moisture. Chitosan (CH) has been found to have potential in the area of biomedical science and engineering due to its distinctive biological properties, including biocompatibility and oxygen and water vapour permeability, biodegradability and minimally induced inflammatory responses *in vivo*.[8-12] It was reported that chitosan loaded neomycin sulphate nanofibers could be useful for the culture of fibroblasts and keratinocytes, because it could enhance adhesion, growth and differentiation of cells with benefits similar to those extra cellular matrices. In addition, nanofibrous scaffolds of biocompatible CH have great potential as dressing for wounds when combined with neomycin sulphate because they have a high specific surface area and nanoporous structure, and show good adhesion to damaged skin.

In this study, CH nanofibers containing neomycin sulphate were prepared as antimicrobial wound dressings. Re-epithelization and wound contraction, the two important components in process of wound healing, are mediated by keratinocytes and fibroblasts, respectively. The effect of CH nanofibers containing neomycin sulphate on wound healing was compared with neomycin cream in an animal wound model.

MATERIALS AND METHODS

Materials:
Neomycin sulphate was purchased from Bmr Pharmaceuticals (Mumbai), chitosan was purchased from Sigma-Aldrich (Germany), trichloro acetic acid purchased from Sigma- Aldrich (USA) and glutaraldehyde was obtained from Loba chem (Mumbai).

Preparation of electrospun CH nanofibers containing neomycin sulphate[13-15]:
Chitosan was dissolved in the trichloro acetic acid (TCA) to form different concentration of chitosan w/v. This base solution was stored for 24 hrs at room temperature for protonation process. Then blend was loaded with 2% neomycin sulphate solution and stirred at 80° C for another 5 hrs in the magnetic stirrer (Remi equipments, India). Electrospinning (Chungpa EMT-CPS, Korea)was performed as follow under room temperature. The solutions were filled into a 5 ml plastic syringe with the blunt-ended needle (ID = 0.21mm). The syringe was located in a syringe pump and dispensed at a rate of 0.8ml/h. A voltage of 15 kV using a high voltage power supply was applied across the needle and ground collector, which was placed at a distance of 12-15 cm. The collector plate is covered by aluminum foils and nanofibers are obtained.

Cross- linking[16-18]:
The CH nanofibers containing neomycin sulphate were cross-linked in glutaraldehyde vapour at room temperature for several hours, and heated at 80° C for another 4 hours to remove any residual glutaraldehyde.

Physicochemical characterization of prepared nanofibers:
Ultraviolet- visible spectroscopy: The UV spectra of neomycin sulphate were recorded using spectrophotometer (Shimadzu-1800, Japan) at pH 5.5 acetate buffer. The scanning wave were ranged from 200-800 nm.

Fourier transform infrared spectroscopy: FT-IR analysis was conducted to verify the occurrence of chemical bonds between drug and polymer. FT-IR spectra were obtained by powder diffuse reflectance on a FT-IR spectrophotometer type 8400 S Shimadzu. FT-IR spectrum of NS was compared with FT-IR spectra of CH-NS optimized formulation. Disappearance of NS peaks or shifting of peak in any of the spectra was studied.

Differential scanning calorimetry (DSC): Differential scanning calorimetry was performed on pure drug and formulations using Shimadzu, DSC 60 apparatus. Calorimetric measurements were made with empty cell (high purity alpha alumina discs) as the reference. The instrument was calibrated using high purity indium metal as standard. The dynamic scans were taken in nitrogen atmosphere at a heating rate of 10 °C min^{-1}. The range of temperature is 100-300 °C.

Scanning electron microscopy [19-21]: The morphology and diameter of the nanofibrous mats were determined via SEM (Joel SEM, Model JSM 840A, Japan). The samples were fixed on SEM sample holder with a double sided adhesive tape and coated with a layer of gold of 150 Å for 2 min using sputter coated in a vacuum of 3x10-1atm of argon gas. The sample was then examined using a scanning electron microscope (JSM-840 A scanning microscopy, Tokyo, Japan).

Mechanical property measurements[22-25]: The tensile strength of the electrospun nanofibers was tested using tensile tester machine (Simpletech, India). Test specimens (10mm wide x 20mm long) were tested at a crosshead speed of 10 mm/ min and length of 25mm under ambient conditions.

Drug content: A weighed amount of nanofibers was taken in a 100 ml volumetric flask, methanol was added to make up the volume. Drug concentration was determined by measuring the absorbance of solution at 315nm using UV spectrophotometer (Shimadzu 1800, Japan)

Drug content = Concentration from graph x dilution factor/1000

Determination of fluid uptake[26-28]: The fluid uptake of drug-loaded fiber mats was measured after the samples were submerged in acetate buffer solution (pH 5.5) at 37°C for 24 hours, according to the given equations

% fluid uptake = (W1-W2)/W2*100

Moisture vapour transmission rate[27-29]: Moisture vapour transmission Rate (MVTR) is an important criterion for a wound dressing material. The liquid formed inside the wound layer first changes to vapour state and then transported to atmosphere. The CH-NS nanofibers were tested for MVTR. A test sample of 40 mm diameter is taken and fixed over a container of 35.7 mm inner diameter, containing 20 mL of distilled ^{water}. The test sample container is weighed (W1) before the start of the test. Then the container is kept inside an incubator for 24 h (conditions maintained inside the incubator) Temperature: 37 ± 5^0C and RH 20%). After 24 h the container is taken out and again weighed (W2). MVTR is calculated based on the formula:

X = (W1 –W2) x 1000 x 24/T

Where, X is MVTR (g/m2/24 h)

Drug diffusion Studies: The CS-NS nanofibers were electrospun and (approximately 25 mg) were immersed in a 50 mL of acetate buffer (pH 5.5). The samples were incubated at 37°C and stirred at 100 rpm. Sample (1 mL) were taken from the release medium at 60 minutes intervals and diluted to 5 mL with fresh buffer solution to assess the quantity of drug released at various time interval for 6 hrs. The sample were analyzed by UV- Spectroscopy.

Determination of antibacterial activity[30-32]: Microbial assay measures the activity of antibiotics (Extent of ability to inhibit the growth of microorganism) whereas chemical assays of such substances estimate only their potency i.e concentration or amount. Antibiotics can be assayed by both turbidimetry and diffusion assay methods. The sterile inoculating loop was inserted into the stock culture of *S. aureus* and *E.coli* to remove small amount of bacteria. This inoculum was transferred to inoculated slant. The necks of the tube were close with the respective caps. Heat the inoculating loop after inoculation to red hot. Slants were incubated for 24 hrs at 30-37°C.

***In vivo* studies to assess wound healing activity [33-36]:** Healthy adult wistar rats weighing 150-200 g were selected. The rats were housed in polypropylene cages under standard laboratory conditions with 12-hour light dark cycle. The rats were fed with standard laboratory chow and water. The wound healing activity was conducted with the protocol as shown in Table 1.

Table 1: Protocol for *In Vivo* wound healing studies

SL No.	Group	No. of animals	Treatment	Evaluation
1.	Control	06	Excision the skin flap with sterile scissors and forceps	Excision wound model for wound healing activity
2.	Standard	06	Neomycin cream	
3.	Blank	06	Chitosan nanofibers	
4.	Test	06	Optimized formulation	
	Total no. of animals	24		

RESULTS AND DISCUSSION

The present study was carried out to formulate and evaluate the CH-NS loaded nanofibers by electrospinning method for wound healing activity.

From the standard solution, a solution was prepared to give a concentration of 10µg/ml in methanol and UV scan was taken between the wavelengths of 200-800 nm. The wavelength, spectrum and overlain is reported in the figure1. The absorbance maxima of NS at 315 nm was selected and utilized for farther studies.

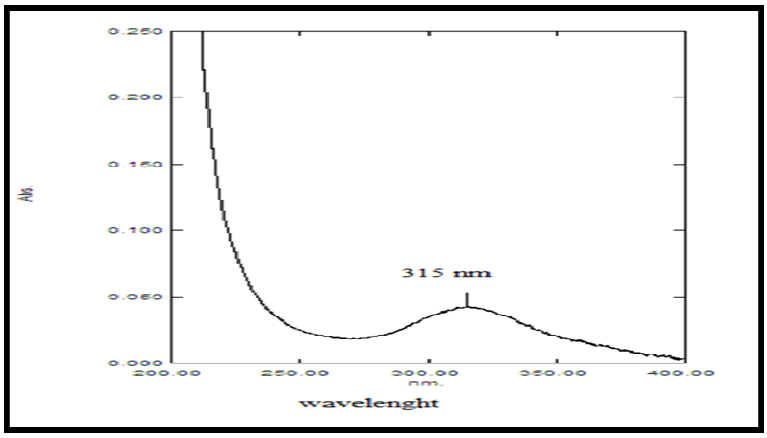


Figure. 1:UV spectrum of neomycin sulphate in acetate buffer pH 5.5 λ_{max} 315nm

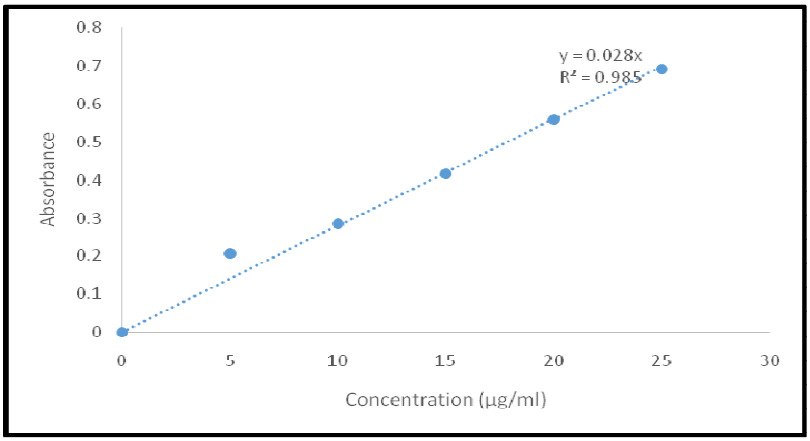


Figure. 2: standard plot of neomycin sulphate in acetate buffer pH 5.5

Drug polymer compatibility studies

Fourier Transform infrared (FT-IR) Spectroscopy:

The infrared spectrum of NS confirms the presence of the relevant functional groups (as the important peaks are listed in the table given below) and is compared with the literature findings. This study is done to find the

information regarding chemical bonding and molecular structure of a material and intermolecular interaction in solid material. FT-IR spectrum of pure NS and CH-NS loaded nanofibers were compared. The CH-NH loaded nanofibers showed the characteristics peaks as shown in the table given below.

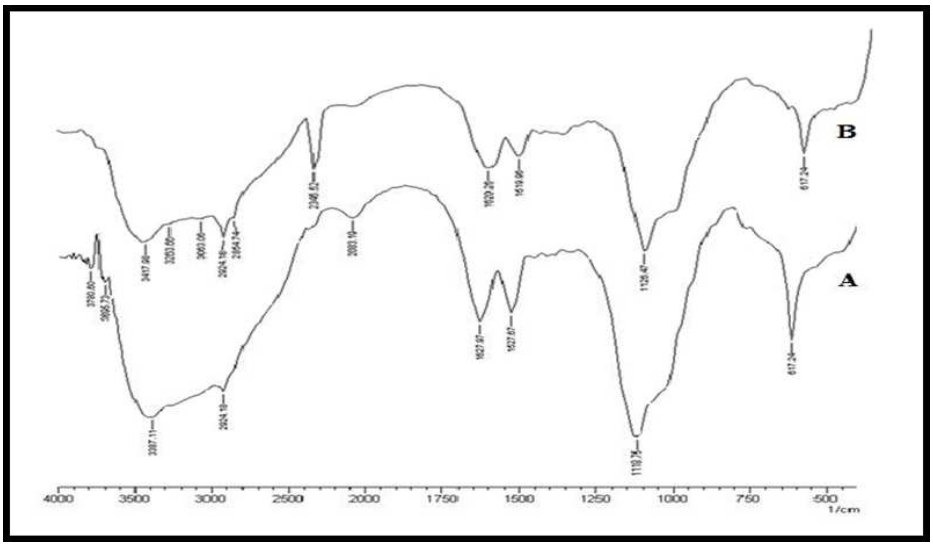


Figure. 3: FT-IR spectra for (A) Neomycin sulphate (B) optimized formulation (F4)

Table 2: FT-IR Spectra peak values

Functional groups	IR Absorption Band (cm^{-1}) (literature)	IR absorption band (cm^{-1}) (Pure drug)	IR Absorption Band (cm^{-1}) (F4)
NH stretching	3300-3500	3387.11	3263.66
OH stretching	3500-3700	3780.60	3417.98
C-H stretching (Aromatic)	2900-3100	2924.18	2924.18
C=C stretching	1620-1680	1527.67	1519.96
C-H stretching (Alicyclic)	3300- 3970	3895.75	3063.06
C-O stretching	1000-1300	1118.75	1126.47
C=O stretching	1600-1820	1627.97	1620.26

The spectra of CH-NS loaded nanofibers do not show any changes in peak position from pure NS spectra. These result revealed that there is an absence of chemical interaction between Neomycin sulphate and other excipients.

Differential scanning colorimetry (DSC):

In order to study possible interaction between drug and polymer, DSC studies was carried out for pure drug (NS) and CH-NS loaded nanofibers (F4) showed peak at 187-270°C. The DSC thermograms obtained are reported in the below figure. From the thermograms it was observed that, NS show a single peak at 263.78°C corresponding to its melting point and formulation (F4) showed peak at 261.18°C. This shows that NS which is loaded in CH-NS nanofibers is completely encapsulated without any traces of drug on the surface of the nanofibers and also determines that they are stable without any degradation.

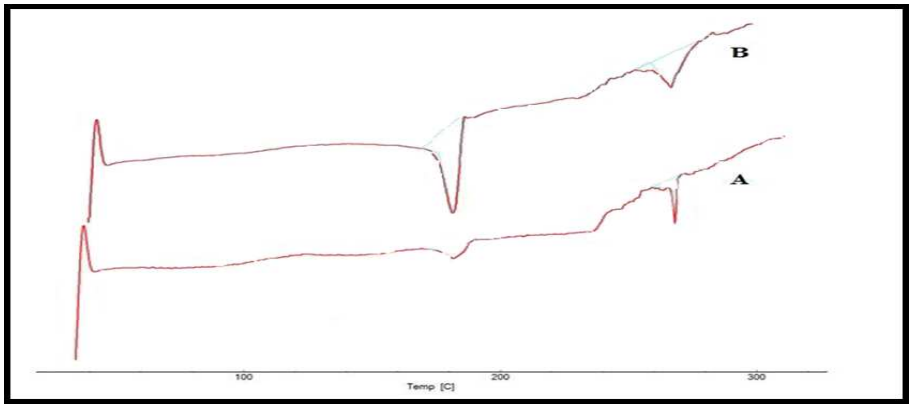


Figure. 4: DSC thermogram peak of (A) NS (B) optimized formulation (F4)

Table 3: DSC data of NS and optimized formulation (F4)

SL no.	Sample	T^0 (₀C)	T^m(₀C)	T^c(₀C)	Melting range (°C)
1.	NS	263.78	262.34	265.26	1.48
2.	F4	255.73	261.18	270.08	14.35

Determination of fibers diameter and surface morphology by SEM: Surface morphology of prepared CH-NS loaded nanofibers were carried out using SEM (JSM-840 A scanning microscopy, Tokyo, Japan). Samples analyzed under SEM were found to have a nano-pores structure, elongated surface with smooth texture and nano size diameter ranging from 256 ± 30nm to 1214 ± 321nm given in below table 4 and SEM images are reported in figure 4.

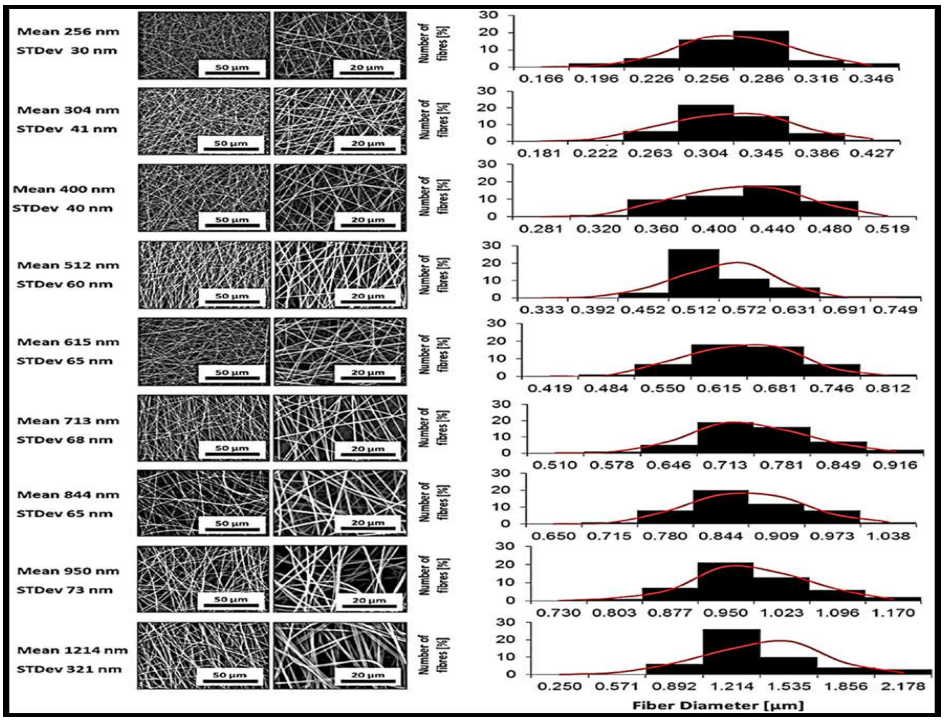


Figure 4. SEM showing surface morphology and fibers diameter of CH-NS loaded nanofibers by electrospinning method

Table 4: Fibers diameter of prepared CH-NS nanofibers

SL No.	Formulation	Fiber diameter (nm)
1	F1	256 ± 30
2	F2	304 ± 41
3	F3	400 ± 49
4	**F4**	**512 ± 60**
5	F5	615 ± 65
6	F6	713 ± 68
7	F7	844 ± 65
8	F8	950 ± 73
9	F9	1214 ± 321

Determination of tensile strength: The tensile strength of the cross-linked Chitosan loaded neomycin sulphate fibers membranes increased from 1.2±0.26 MPa to 13.21±0.23 MPa with the increased content of chitosan. The elongation at break of the cross-linked nanofibrous membranes showed an increased trend with the increasing proportion of chitosan. These results suggested that the addition of chitosan was beneficial for enhancing the mechanical properties of CH-NS nanofibers. This indicate that the mechanical properties of the CH-NS nanofiber membranes are good for handling during the fabrications process. The tensile strength of prepared CH-NS nanofibers were measured and data is represented graphically. The tensile strength of optimized formulation was found to be 6.14 ± 0.63 MPa, which is ideal for wound dressing purpose.

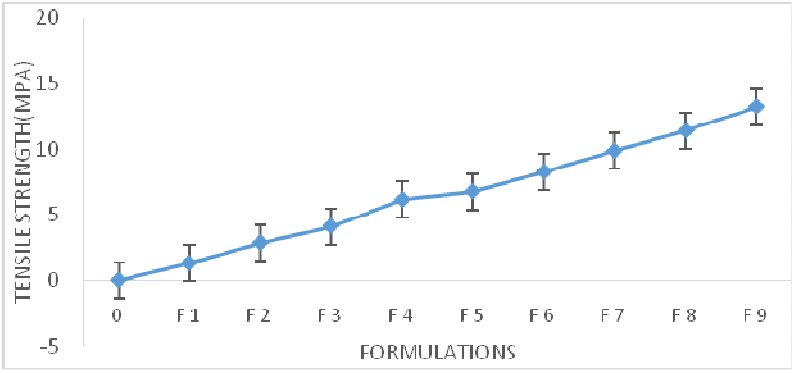


Figure. 5: Graph showing tensile strength of prepared CH-NS nanofibers formulations

Drug content: The prepared formulations were analyzed for drug content and the data is reported in below table. It was observed that the drug content in the prepared nanofibers was satisfactory and the drug was uniformly distributed in all the formulations. The percentage drug content is highest for optimized formulation that is 98.58 ± 0.76% respectively.

Table 4: Drug content of prepared CH-NS nanofibers

SL No.	Formulation	Drug content (%)
1	F1	97.76±0.19
2	F2	98.21±0.10
3	F3	98.00±0.23
4	**F4**	**98.58±0.76**
5	F5	97.81±1.05
6	F6	98.10±0.60
7	F7	97.84±0.11
8	F8	97.68±0.83
9	F9	97.74±0.63

**Standard deviation, N= 3*

Determination of fluid uptake: The prepared CH-NS nanofibers were analyzed for percentage fluid uptake, the percentage of fluid uptake for the optimized formulations is found to be 200.24±0.31% and represented in figure 5.

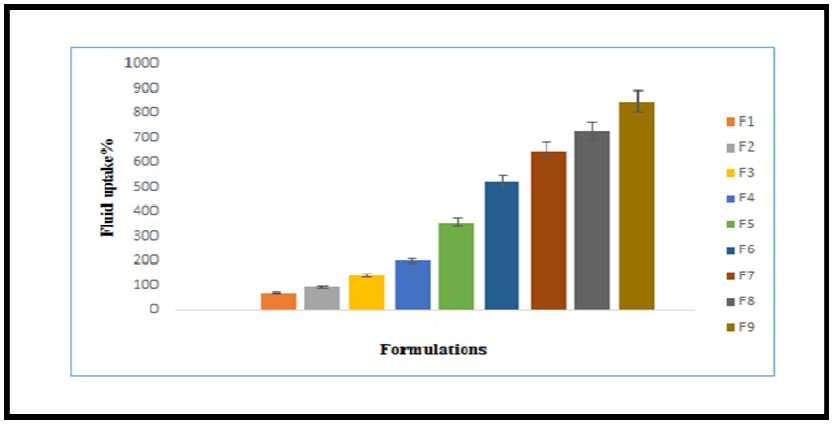


Figure. 7: Column graph showing fluid uptake for CH-NS nanofibers

Determination of moisture vapour transmission rate: The MVTR of CH-NS nanofibers were determined, it was reported that with increase in chitosan in the blend, MVTR values tend to increases, which is ideal dressing should have MVTR value of 2000 to 2500 g/m²/ h. The MVTR value of optimized formulation was found to be 2430.52 ± 0.45 g/m²/ h, which is suitable for wound dressing for healing activity.

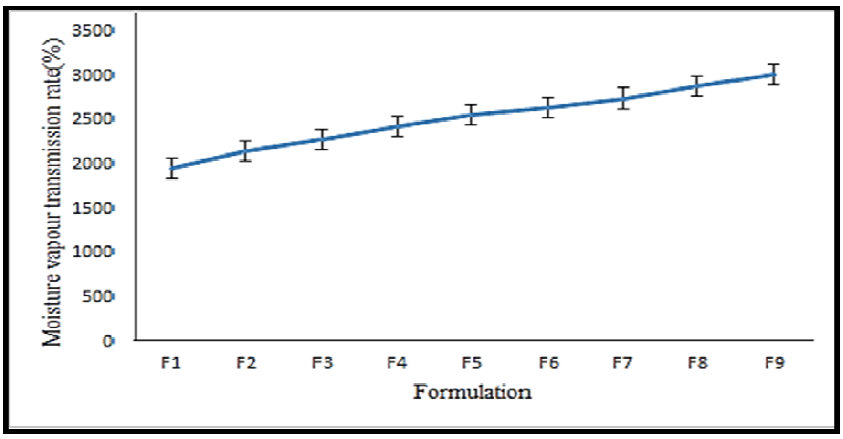


Figure.8: Graph showing MVTR of CH-NS nanofibers

Drug diffusion studies:

The diffusion studies of prepared CH-NS nanofibers were carried out for 6 hours in pH 5.5 acetate buffer. The % drug release of prepared formulations is reported in graphically, the result reveal that there is control release of drug after 6 hours. The % cumulative drug release of optimized formulation was found to be 59.49±0.52 %.

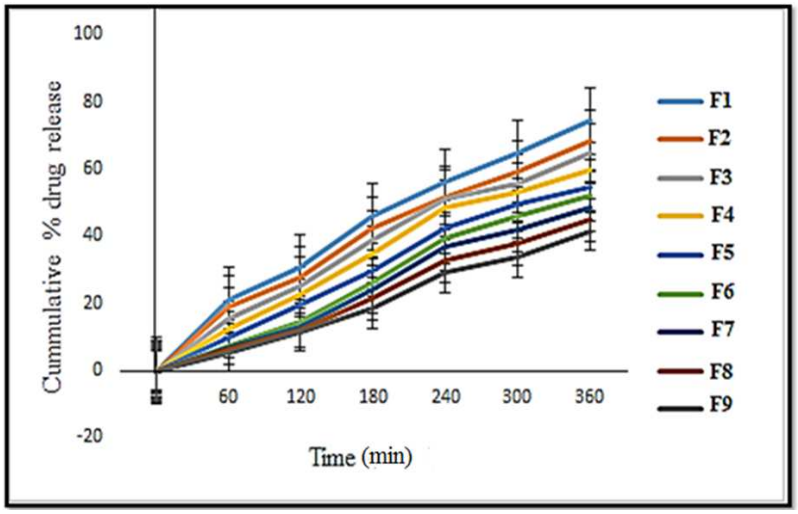


Figure. 9: Drug diffusion studies of prepared CH-NS nanofibers

Determination of antibacterial activity:
Antibacterial activity of optimized formulation was determined by using agar plate diffusion method. The bactericidal effect of F4 formulation were determined against gram positive bacteria (*S. aureus*) and gram negative bacteria (*E. coli*). After incubation, inhibition of growth can be seen as a clear zone around each petri dish. The diameter of this is proportional to the log concentration of antibiotic. The zone of inhibition is examined and measured with the help of scale. The results are represented below in figure 10 and table 5.

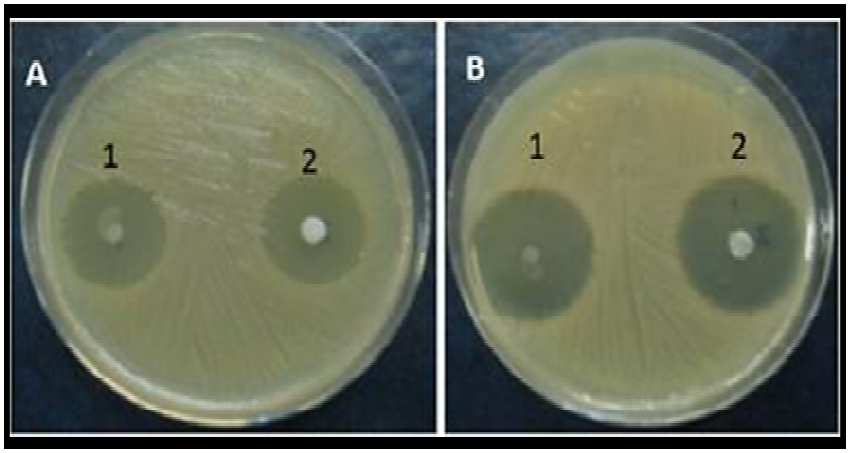


Figure. 10: Zone of inhibition of NS cream (A) and optimized formulation (B)

Table 5: Zone of inhibition data for optimized formulation

Formulation	Diameter of zone of inhibition (cm)	
	S. aureus	***E. coli***
NS cream	2.22±0.060	2.12±0.08
F4	2.46±0.081	2.33±0.06

***In vivo* studies to assess wound healing activity:**
Percentage of wound contraction: The wound contraction on day 0^{th} was considered as 100%, which was considered to compare the wound area on days 7^{th}, 14^{th} and 21^{st}. Table 6, shows percentage wound contraction of different groups at different time intervals. There was significant difference in percentage of wound contraction between untreated group and treated group. 100% of wound contraction was observed in groups treated with selected drug loaded nanofibers and blank nanofibers within 21days. Though there was increased wound contraction

with drug loaded nanofibers, but no significant difference was observed between drugs loaded nanofibers and NS cream. Restoration and recovery of cells was observed in wounds treated with blank nanofibers, optimized formulation and NS cream when compared to untreated wounds in 21st days. Decrease in wound size was observed in wounds treated with CH-NS loaded nanofibers on 21st days when compare to other groups. This suggested that CH-NS loaded nanofibers may have capacity for fast recovery and rapid epithelization of skin than in the untreated and wounds treated with NS cream.

On 7th, 14th and 21st days more decrease in wound size was observed in group treated with CH-NS loaded nanofibers when compare to groups treated with blank nanofibers and NS cream alone. It indicate that the combination of CH-NS showed better wound healing property than NS and chitosan alone. This may be due to broad antibacterial activity of NS and CH which reduces infections and thus fastens the healing of wound.

Table 6: Percentage of wound contraction at different time intervals in different groups

Group	Wound contraction (%)		
	7th day	14th day	21st day
Control	21.31 ± 4.20	59.80 ± 2.95	75.91 ± 2.10
Standard	54.33 ± 4.12	78.74 ± 1.33	95.74 ± 0.69
Blank	50.32±3.21	75.87±1.74	91.05±1.40
Test (optimized formulation)	60.36 ± 2.42	83.66 ± 2.44	98.84 ± 0.08

Wound healing time:

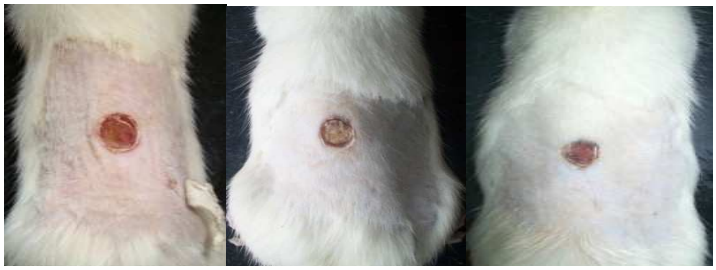
The mean wound healing time of control, standard, blank and test was reported in table 7, compare with control group there was significant decrease in wound healing time of optimized formulation group. Whereas, when compared to NS treated group and blank group there was no significant difference in wound healing time (figure 10).

Table 7: Wound healing time at 21st days

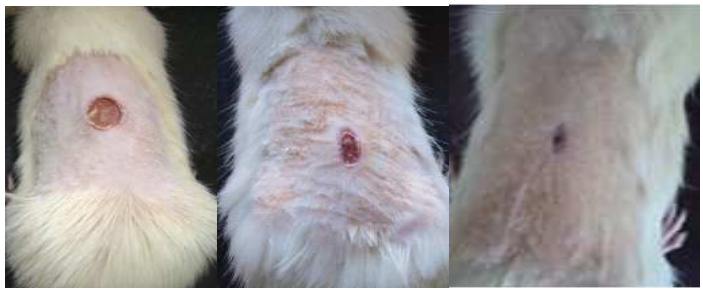
Group	Wound healing time % (21st days)
Control	20.50 ± 0.50
Standard	16.84 ± 0.23
Blank	16.20 ± 0.48
Test (optimized formulation)	16.50 ± 0.31

Wound healing in control group

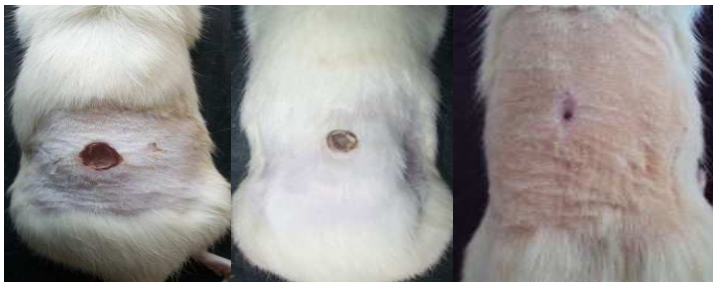
7th day **14th day** **21st day**



Wound healing in standard group treated with NS cream



Wound healing in blank group treated with chitosan nanofibers



Wound healing in test group treated with optimized formulation

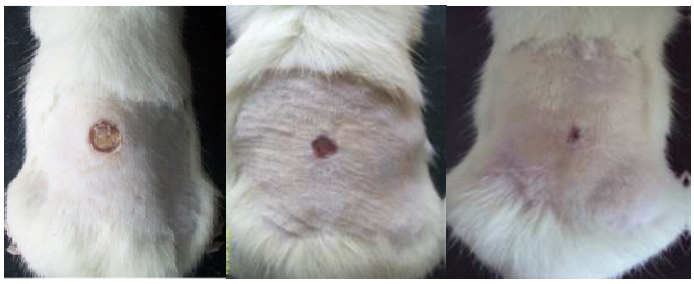


Figure. 11: Wound appearances treated with control, standard and test at days 7th, 14th, and 21stdays

CONCLUSION

In the present study, chitosan loaded neomycin sulphate nanofibers were successfully prepared using an electrospinning method. The presence of CH-NS nanofibers was confirmed via scanning electron microscopy, Fourier transform infrared spectroscopy, and differential scanning calorimetry. The CH-NS nanofibers mats were in the nanometer range, were nontoxic and biocompatible, and displayed sustained-release characteristics, demonstrating excellent antimicrobial activity against Gram-positive *S. aureus* and Gram-negative *E. coli*. Moreover, the nanofiber mats accelerated the early stages of wound healing compared with the chitosan nanofiber mats and neomycin cream, suggesting that these materials have great potential for use as wound dressings.

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