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Comparison of different nano biocomposites of Neomycin with marketed ointment by *in-vitro* and *in-vivo* evaluations

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Abstract

Back ground: Nano drug delivery systems have rapid onset of action with enhanced therapeutic efficacy, decreased dose of the drug and decreased toxic effects when compared to conventional drug delivery systems. Hence neomycin is formulated into nanoparticles in order to increase the therapeutic efficacy, decrease the dose of drug and to decrease the topical dose related toxic effects. Aim: Hence the present work was aimed at the preparation of zinc nanoparticles (NP1), chitosan nanoparticles (NP2), different zinc chitosan neomycin nanoparticles (NP3,NP4,NP5,NP6,NP7,NP8) by altering the concentrations of chitosan and neomycin used in the formulation in order to optimise the composition. Methods: Nanoparticles were prepared by subjecting the nanosuspension containing the specified ingredients to stirring at 40oC for 4-5 hr. The prepared nanoparticles were evaluated for particle size and surface morphology by Transmission Electron Microscopy (TEM), mean particle size and particle size distribution by zeta sizer, percentage yield, loading efficiency, in-vitro drug release by diffusion technique and agar cup plate method and invivo wound healing activity. Results: Among all the prepared zinc chitosan neomycin nanoparticles NP6 was found to possess maximum in-vitro drug release and antimicrobial activity. This may be due to the synergistic effect of all the ingredients i.e zinc, chitosan and neomycin present in the formulation. Hence zinc chitosan neomycin nanoparticles NP6 was subjected to in-vivo studies and compared with marketed neomycin ointment (nemozin). The wound healing was found to be more in group treated with ointment prepared with zinc chitosan neomycin nanoparticles compared to group treated with marketed neomycin ointment(nemozin) containing double the concentration of neomycin of NP6. Conclusion: Thus, the present work suggested that NP6 (0.2%) was found to be the best formulation of neomycin containing less than half of the concentration of neomycin of nemozin ointment(0.5%) as it shown equal *invivo* activity to nemozin ointment as this reduces the side effects, increases efficacy at low doses of drug compared to conventional formulations of neomycin.

Keywords: Chitosan, Zinc nano particles, Neomycin, Zinc chitosan neomycin, ointment, wound healing.

Introduction

Nanoparticles can be defined as sub-micron sized colloidal particles composed of synthetic or semi-synthetic polymers having size range of 1 nm to 1000 nm. [1]. They contain macromolecular materials in which the active principle (drug / biologically active agent) is dissolved or entrapped or encapsulated to which the active principle is adsorbed or attached[2]. Nanoparticles can be easily up taken by the cells and can reach every cell to produce effective delivery when compared with conventional carrier [3,4]. These enable the drug to enhance the bioavailability, decrease the toxicity[5], decreases the frequency of administration. Metallic nanoparticles can be defined as sub-nanosized colloidal particles produced from metals are called metallic nanoparticles. Advantages of metallic nanoparticles include high surface to volume ratio[6,7], uniform size distribution, better optical properties[8,9], better interaction with the biomolecules both at the surface and interior of the cell. Zinc oxide nanoparticles have anti-bacterial, anti-fungal and growth promoting activity[10,11].

Chitosan is used as a polymer because it exhibits antimicrobial activity against bacteria[12], fungi and yeast , biocompatible[13], non-toxic, biodegradable[14,15], used as wound healing accelerator[16,17] as it enhances the function of polymorphonuclear cells, macrophages and enhances fibroblastic proliferation of migration[18]. These properties render chitosan a very attractive material as a drug delivery carrier.

Nanoparticles have a relatively large (functional) surface which is able to bind, adsorb and carry other compounds such as drugs,

probes and proteins. Because of their ability to carry the drugs and proteins, zinc chitosan neomycin nanoparticles were formulated to enhance the therapeutic efficacy of neomycin to decrease the dose related toxic effects of neomycin ointment such as hypersensitivity reactions, rashes and burning sensation by decreasing the dose of the drug and enhancing its absorption. Zinc and chitosan were used because of their antibacterial activity[19]. Different zinc, chitosan, neomycin nanoparticles were evaluated and compared with the marketed neomycin ointment to optimize the composition of chitosan and neomycin in formulation.

Materials and Methods

Materials

Neomycin (Gift sample from Natco pharma pvt limited, Hyderabad), chitosan (Sigma Aldrich, Hyderabad), nanozinc oxide (Yogi dye chem pvt limited), acetic acid (Sd fine, Mumbai), agar, beef extract, sodium chloride (Himedia pvt.limited, Mumbai), methanol (Himedia pvt.limited, Mumbai), PEG 400 and PEG 4000 (Himedia pvt.limited, Mumbai) *Bacillus subtilis, Staphylococcus aureus* & two gram negative bacteria *Escherichia coli, Pseudomonas aeruginosa* obtained from NCL , Pune. All the chemicals and reagents used were of analytical and pharmaceutical grade.

Methods

Method of preparation of Nanoparticles

2% neomycin, 2% chitosan and 0.2% nano zinc oxide solutions were prepared. 50ml of zinc oxide solution was added to 40 ml each of neomycin and chitosan solutions. The above solution was stirred continuously using magnetic stirrer by heating at 40°C for 4-5 hr.. The nanosuspension i.e formed was centrifuged. The sediment was dried and the dried nanoparticles were evaluated. Different nanoparticles as shown in table 1 were prepared by following the same procedure.

Table 1: Composition of various nanoparticles

Sl.No.	Code of the formulation	Neomycin %	Chitosan %	Nano zinc oxide %
1.	NP1	-	-	0.2 %
2.	NP2	-	2 %	-
3.	NP3	2 %	2 %	0.2 %
4.	NP4	2 %	4 %	0.2 %
5.	NP5	2 %	6 %	0.2 %
6.	NP6	4 %	2 %	0.2 %
7.	NP7	4 %	4 %	0.2 %
8.	NP8	4 %	6 %	0.2 %

Characterization of nanoparticles [20,21]

Surface morphology

TEM analysis

The morphological characteristics of nanoparticles were determined by Transmission electronic microscopic (TEM) TEM1200EXJEOL, Japan. Specified quantity of the best of the nanoparticles were placed on the carbon coated copper grid making a thin film of sample on the grid and extra sample was removed using the cone of a blotting paper & kept in grid box sequentially. Then TEM microphotographs of best formulation of nanoparticles (NP6) were taken [17].

Particle size analysis

The mean particle size and particle size distribution of the drug loaded nanoparticles (NP6) and zeta potential was measured using zeta sizer (nanoparticle SZ 100, Horiba Singapore) by determining the electrophoretic mobility in a microelectrophoresis flow cell at 25° C.

Compatibility studies

The compatibility between drug , polymer and metal was determined by carrying out UV scan using UV- Vis spectrophotometer (schimadzu). The possible interaction between neomycin, chitosan and zinc was also accessed by comparing FTIR spectra of pure drug (neomycin), polymer (chitosan) and nanoparticle formulation.

Percentage Yield [22]

The formulation is centrifuged and sediments were dried. Percentage yield was calculated as follows:

$$\% \text{ Yield} = \frac{\text{Nanoparticles weight} \times 100}{\text{Total solids weight}}$$

Total solids weight = weight of nano zinc oxide + weight of neomycin + weight of chitosan

Loading efficiency

Nanosuspension with known amount of drug was centrifuged at 5000 rpm for 15 min. The supernatant solution was separated. 5 ml of supernatant was distributed with 100 ml of distilled water. Absorbance was measured using UV Spectrophotometer using distilled water as blank. The amount of drug entrapped in the supernatant was calculated from which the amount of drug entrapped and % entrapment was determined.

$$\text{Loading efficiency} = \frac{\text{Total amount of drug} - \text{Amount of unbound drug}}{\text{Weight of Nanoparticles}}$$

In vitro drug release studies

In vitro drug release by diffusion studies[21]

The *in vitro* drug diffusion of all the drug loaded formulations were carried using dialysis membrane. 5 ml of formulation was accurately placed in this assembly. The cylinder was attached to

the stand and suspended in 50 ml of dissolution medium maintained at 37± 5°C so that the membrane just touched the receptor medium surface. The dissolution medium was stirred at lower speed using magnetic stirrer. Aliquots of samples were withdrawn at regular intervals and replaced with equal volume. The samples were analyzed by UV-Visible Spectrophotometer and cumulative percentage release of all formulations were specified in the tabular form. The quantity of drug equivalent to 10 mg was taken for diffusion study.

In vitro antibacterial activity by agar cup plate method

The prepared nanoparticles were evaluated for antibacterial activity against four different strains with agar cup plate method by measuring the zone of inhibition of microorganisms. Zone of inhibition was determined for all the prepared formulations. separately by incubating for 24 hr at 37 ± 2°C. Zone of inhibition was determined in triplicate using antibiotic zone reader and average diameter was noted.

In vivo Studies

The best among the prepared zinc chitosan neomycin nanoparticles (NP6) possessing highest *in-vitro* antimicrobial activity and blank nanoparticles (NP1 and NP2) were prepared into an ointment for in vivo study of wound healing activity on albino rats by excision wound model. Pathogen free adult albino rats weighing 150-200 gm. were selected. The wound healing activity was conducted with the protocol as shown in the table 2 with each group containing 6 animals. The animal work was approved by institutional ethical committee.

Table 2 : Protocol for *in vivo* wound healing studies

Sl. No	Group number	Purpose
1.	Group 1	Control (Untreated)
2.	Group 2	Treated with zinc nanoparticles (NP1). (Blank 1)
3.	Group 3	Treated with chitosan nanoparticles (NP2) (Blank 2)
4.	Group 4	Treated with zinc chitosan neomycin nanoparticles (NP6)
5.	Group 5	Treated with the marketed neomycin ointment (nemozin).

Wound with an area of 2 cm^2 was created on the back of the anaesthetized animals on interscapular region that is 5mm away from the ears. After achieving homeostasis, the wound was blotted with sterile gauze in control group, the respective ointment was placed on the wound of animals in treatment groups. Then the following parameters were determined at specific time intervals:

Percentage of wound contraction [23,24,25]

This is measured to determine the reduction of wound area at different periods of treatment. It was measured by graphical method. Wound area was calculated on 7^{th}, 9^{th}, 11^{th}, 13^{th}, 15^{th} post wounding day by counting the number of squares of retraced wound area on graph paper. The degree of wound healing was calculated as % closure of the wound area from the original wound using the formula: % Closure = $1-[A_d/A_o] \times 100$. A_o is the wound area on day zero, A_d is the wound area on corresponding days.

Photography

The photographs of wounds from different groups were taken at regular intervals for visual comparison.

Biochemical analysis[26]

After seventh, ninth and fourteenth day of wound creation on animals, the healed skin was carefully lifted, freed of adhesions and excised along with 2 mm adjacent to normal skin. The granulation tissue was subjected for estimation of biochemical parameters.

Estimation of Hydroxyproline content

Collagen is the major extracellular protein in the granular tissue of the healing wound and there is rapid increase in the synthesis of the collagen in the wound area soon after an injury, which provides strength and integrity to tissue matrix. Measurement of hydroxyproline which comes from breakdown of collagen has been used as an index of collagen turn over. Increase in hydroxyproline content of the granular content in the wound is an indication of higher collagen content and its turnover leading to rapid healing.

Procedure

The stored tissue was dried in an oven at 60° C–70°C for 12–18 h, and the dry weight was noted. The tissues were hydrolyzed in 6 N HCl for 24 hr at 110 °C in sealed glass tubes. The hydrolysate was neutralized to pH 7.0. The sample (200 µl) was mixed with 1mL of 0.01M $CuSO_4$, 1ml of 2.5N NaOH and 1mL of 6% H_2O_2. All the tubes were incubated at 80°C for 5 min with frequent vigorous shaking. Upon cooling, 4mL of 3N H_2SO_4 was added with agitation. Finally, 2 ml of 5% para-dimethylaminobenzaldehyde was added to develop a pink color. The samples were incubated at 70°C for 16 min, cooled by placing the tubes in water at 20° C and the absorbance was measured at 540 nm using a spectrophotometer. The amount of hydroxyproline in the samples was calculated using a standard solution and its corresponding absorbance at the same time[36] by following formula:

$$\text{Concentration of hydroxyl proline in the sample} = \text{OD of the Sample} \times \frac{\text{Concentration of standard}}{\text{OD of Standard}}$$

Estimation of hexosamine content

Hexosamine and hexuronic acid are matrix molecules, which act as ground substratum for the synthesis of new extracellular matrix. These substances form a highly hydrated gel like ground substance, a provisional matrix upon which collagen substances are embedded. Hexosamine concentration increases with wound healing, ultimately increases the tensile strength.

Procedure

Granulation tissue weighing around 300mg was homogenized in 10 ml of 6N HCl in 25 ml glass ampoules and were hydrolyzed at 98° C. After hydrolysis, washed thoroughly with water and the washings were combined with the hydrolysate. The p^H of the hydrolysate was adjusted to 7, sample was diluted to 50 ml with distilled water. To 2 ml of above sample, 1 ml of 2% acetylacetone was added and heated to 96°C for 40 minutes. The mixture was cooled and 5 ml of 96% ethanol was added followed by the addition of 1 ml of ehrlich's reagent (320 mg of p-dimethyl amino benzaldehyde was dissolved in 21 ml of iso-propranol and 4 ml of con.HCl was added). The solution was thoroughly mixed and kept at room temperature for 1 hr. The absorbance of the pink colour solution was measured at 530 nm. The amount of hexosamine was determined by comparing with the standard value by following formula:

$$\text{Concentration of the sample} = \frac{\text{OD of the sample} \times \text{Concentration of standard}}{\text{OD of the standard}}$$

The results of *in-vivo* studies were compared with the results of marketed neomycin ointment (nemozin).

Statistical analysis

The results are expressed as Mean ± S.D by estimating all the above *in-vitro*, *in-vivo* parameters in triplicate. Then statistical analysis was performed by t-test, one way analysis of variance (ANOVA) for multiple comparisons using SPSS statistics software 17.0 version. Statistical significance was set accordingly at P (<0.05) level.

Results and Discussion

In the present study, zinc nanoparticles (NP1), chitosan nanoparticles (NP2) and zinc chitosan neomycin nanoparticles (NP3) were prepared by altering the concentrations of chitosan and neomycin and characterized to find out the effect of neomycin and chitosan on antibacterial activity of zinc neomycin nanoparticles. These zinc chitosan neomycin nanoparticles were formulated to get maximum loading efficiency, *invitro* drug release by diffusion studies and agar cup plate method and the best of the prepared nanoparticles were subjected to *invivo* studies using albino rats by excision wound model and compared with the marketed neomycin ointment (nemozin) along with blank nanoparticles.

Particle size and surface morphology

TEM analysis

Particle size and surface morphology of the best formulation i.e zinc neomycin nanoparticles (NP6) was determined by Transmission Electron Microscopy and its TEM microphotographs are shown in figure 1. The particle size of zinc chitosan neomycin nanoparticles(NP6) was found to be 200 nm. and are are spherical in shape with smooth surface and are in nanometric size.

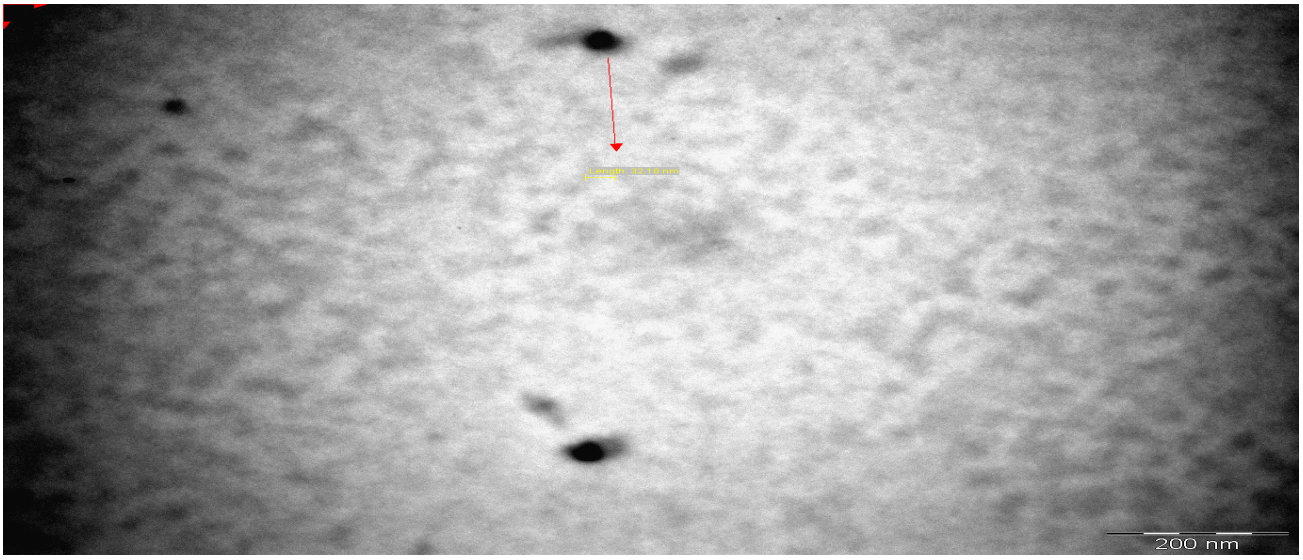
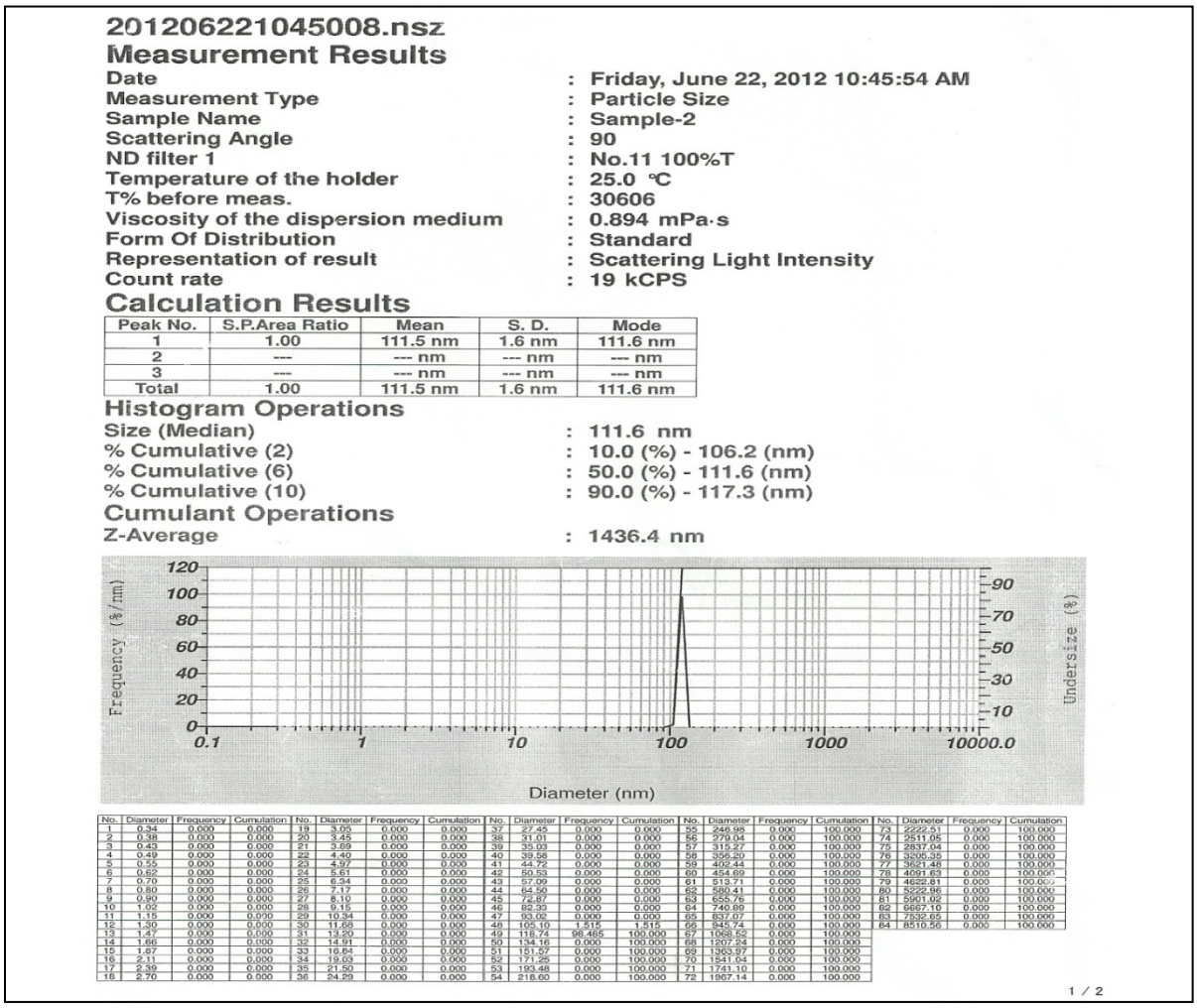


Figure 1:TEM microphotographs of zinc chitosan neomycin nanoparticles(NP6).

Zeta sizer

Mean particle size and particle size distribution of the best of the prepared nanoparticles zinc chitosan neomycin nanoparticles (NP6) was determined using zeta sizer. The mean particle size of NP6 was found to be 111.5 nm as given in figure 2. It indicated that the narrow size distribution or more uniform size distribution was found with zinc chitosan neomycin nanoparticles (NP6).



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Measurement Results

Date	: Friday, June 22, 2012 10:45:54 AM
Measurement Type	: Particle Size
Sample Name	: Sample-2
Scattering Angle	: 90
ND filter 1	: No.11 100%T
Temperature of the holder	: 25.0 ℃
T% before meas.	: 30606
Viscosity of the dispersion medium	: 0.894 mPa·s
Form Of Distribution	: Standard
Representation of result	: Scattering Light Intensity
Count rate	: 19 kCPS

Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	1.00	111.5 nm	1.6 nm	111.6 nm
2	---	--- nm	--- nm	--- nm
3	---	--- nm	--- nm	--- nm
Total	1.00	111.5 nm	1.6 nm	111.6 nm

Histogram Operations

Size (Median)	: 111.6 nm
% Cumulative (2)	: 10.0 (%) - 106.2 (nm)
% Cumulative (6)	: 50.0 (%) - 111.6 (nm)
% Cumulative (10)	: 90.0 (%) - 117.3 (nm)

Cumulant Operations

Z-Average : 1436.4 nm

1 / 2

Figure 2: Particle size determination of zinc chitosan neomycin nanoparticles (NP6)

Compatibility studies

The compatibility of neomycin with chitosan and zinc in zinc chitosan neomycin nanoparticles was determined through UV scan, FTIR analysis. The UV spectrum of pure drug solution and zinc chitosan neomycin nanoparticle formulation were identical and the characteristic absorption maxima was appeared at 273 nm. The FTIR spectra of pure neomycin, pure chitosan, zinc chitosan neomycin nanoparticles (NP6) were obtained. FTIR spectrum of pure neomycin demonstrated the characteristic absorption peaks at 3240 cm^{-1} for O-H stretching conjugated with N-H stretching and aromatic C-H stretching, at 1341 cm^{-1} for C-N stretching, at 1075 cm^{-1} for C-O stretching, at 1530 cm^{-1} for –C-C- stretching. The absorption peaks with zinc chitosan neomycin nanoparticles were almost similar to those obtained with the pure drug and polymer.

Percentage yield and Loading effieciency

The % yield of nanoparticles varied from 72.45 ± 0.12 to 83.04 ± 0.31 as given in table 3. Among all the prepared nanoparticles the % yield was found to be less for zinc nanoparticles (NP1) i.e 72.45% and highest for zinc chitosan neomycin nanoparticles (NP8) i.e 83.04%. The percentage yield was found to be increased with increased concentration of drug and polymer.

The loading efficiency of different nanoparticles varied from 75.92% to 85.44% as shown in table 3. Among all the zinc chitosan neomycin nanoparticles formulated, NP8 was found to exhibit maximum loading efficiency i.e 85.44% which may be due to higher concentration of drug and polymer used in the formulation and the ability of the polymer to form reticulated sheath which ultimately enhances the drug loading. Loading efficiency was found to be increased with increased concentration of polymer and drug.

Table 3: Percentage yield and loading efficiency of various nanoparticles

Sl. No.	Formulation code	% Yield (Mean ± S.D)	Loading efficiency (Mean ± S.D)
1.	NP1	72.45 ± 0.12	_
2.	NP2	74.94 ± 0.13	_
3.	NP3	75.13 ± 0.26	75.92 ± 0.23
4.	NP4	80.49 ± 0.28	83.21 ± 0.24
5.	NP5	81.13 ± 0.19	84.12 ± 0.11
6.	NP6	79.46 ± 0.34	81.23 ± 0.29
7.	NP7	81.67 ± 0.26	84.35 ± 0.25
8.	NP8	83.04 ± 0.31	85.44 ± 0.26

Invitro drug release

By diffusion studies

The *in-vitro* % drug release by diffusion studies was determined for all the nanoparticles for 2 hrs. At the end of 2 hrs the *in-vitro* % drug release of different nanoparticles was found to be between 74.67 to 83.36 % as shown in table 4 The *in-vitro* % drug release from zinc chitosan neomycin nanoparticles (NP3) was found to be 79.56 %. Among all the formulations, NP6 was found to possess highest *in-vitro* % drug release i.e 83.36%. The *in-vitro* % drug release was not significantly ($P<0.05$) changed with increase in concentration of polymer as shown in table 4. But the *in-vitro* % drug release was significantly increased with increase in concentration of drug ($P<0.05$). It may be due to the presence of more drug and it may also allow to assume the release by diffusion process following first order kinetics as release was increased with increase in concentration of drug which can be supported by the reports of mohammad.F et al.,2010[27] conducted up on chitosan ampicillin.

Table 4: *In-vitro %* drug release of different zinc chitosan neomycin nanoparticles with different concentrations of polymer and drug

	2% Neomycin formulations			4% Neomycin formulations		
Time (min)	NP3 (Mean ±S.D)	NP4 (Mean ±S.D)	NP5 (Mean ±S.D)	NP6 (Mean ±S.D)	NP7 (Mean ±S.D)	NP8 (Mean ±S.D)
15	73.62±0.11	72.21 ±0.32	72.05±0.41	76.96±0.23	76.51±0.43	76.11±0.15
30	73.91±0.13	74.03±0.24	72.86±0.38	78.01±0.16	78.04±0.21	77.79±0.34
45	75.23±0.24	74.98±0.49	74.01±0.16	78.96±0.33	78.80±0.33	77.95±0.51
60	75.50±0.43	75.45±0.27	75.10±0.55	79.03±0.46	78.98±0.54	78.05±0.39
75	76.21±0.36	76.07±0.18	75.96±0.44	80.91±0.55	79.90±0.19	78.89±0.11
90	77.52±0.26	77.11±0.25	76.65±0.37	82.03±0.49	80.18±0.21	79.95±0.23
105	77.83±0.19	77.91±0.19	77.79±0.29	82.98±0.33	81.15±0.43	80.02±0.14
120	79.56±0.32	79.23±0.34	79.05±0.36	83.36±0.42	81.19±0.34	80.45±0.25

Antibacterial activity by agar cup plate technique

The antibacterial activity of different nanoparticles was determined and shown in figure 3&4. The antibacterial activity of drug loaded nanoparticles was significantly more than blank nanoparticles against all selected species($P<0.05$).

Among the prepared nanoparticles, zinc nanoparticles (NP1) was found to have the least antibacterial activity and zinc chitosan neomycin nanoparticles (NP6) was found to possesses highest antibacterial activity. It may be due to the synergistic effect of all the ingredients i.e zinc, chitosan, neomycin present in the formulation (NP6)[28] and also due to increased surface area with decrease in particle size. Increase in antibacterial effect was found by increased drug loading, when compared with NP1 and NP2. However blank chitosan nanoparticles (NP2) have shown higher *in-vitro* antibacterial activity than blank zinc nanoparticles (NP1), indicated more capacity of chitosan to act against different strains of bacteria[13]. NP6 was found to possesss highest anti-bacterial activity when compared to other formulations and decreased *in-vitro* release was found with further increase in polymer concentration which may be due to retarding effect of higher concentration of polymer (NP7 and NP8). Hence it was formulated into ointment and subjected to *in-vivo* wound healing activity by excision wound model and compared with the marketed neomycin ointment (nemozin).

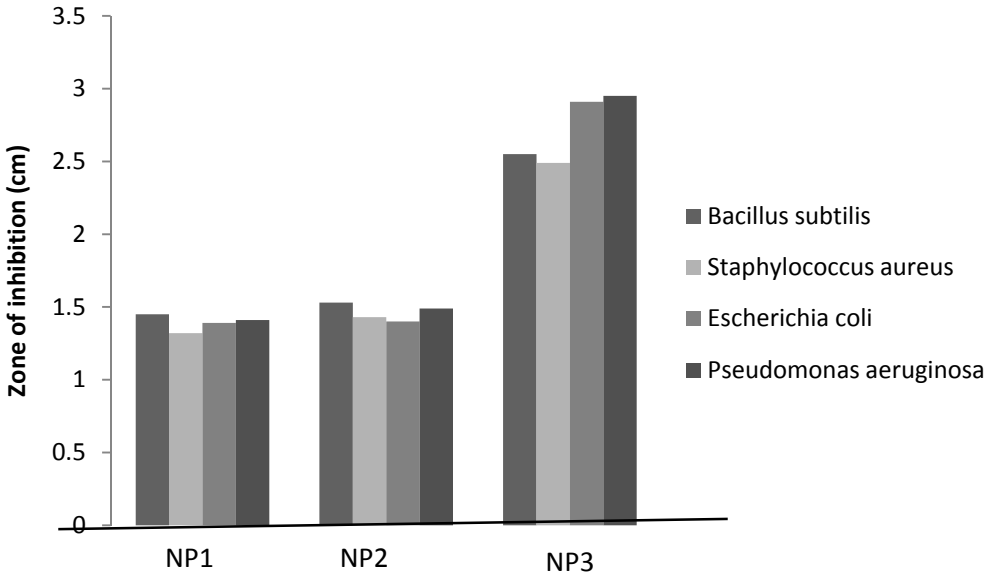


Figure 3: Zone of inhibition of NP1, NP2, NP3 (blank NP)

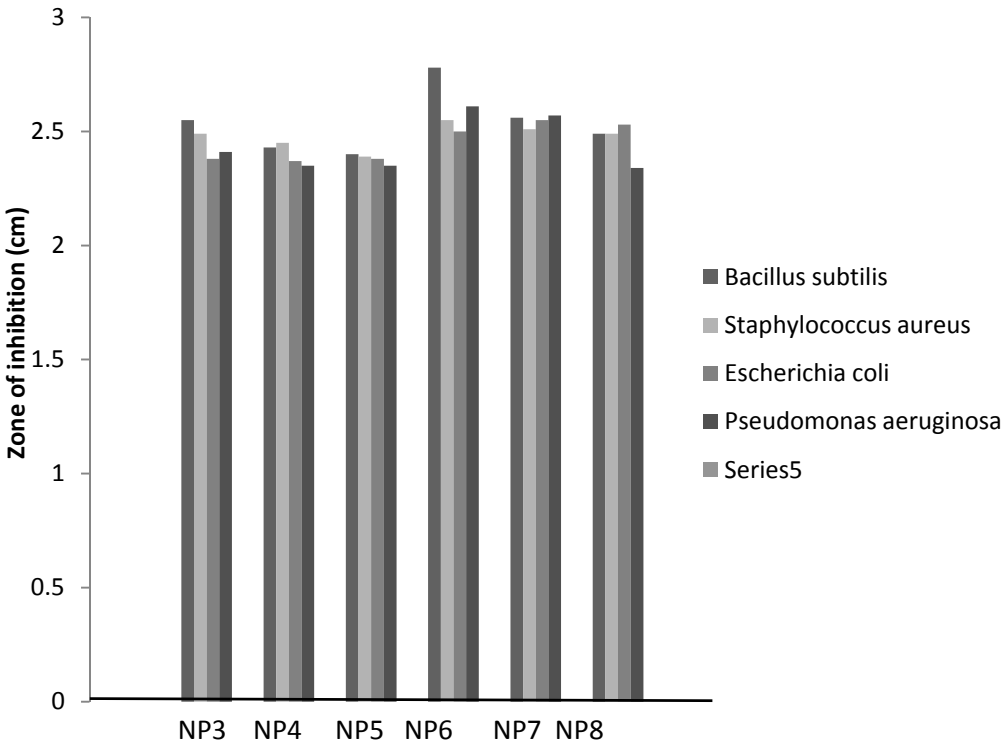


Figure 4: Zone of inhibition of different zinc chitosan neomycin nanoparticles

In-vivo studies

Percentage wound contraction

The *in-vivo* studies were conducted for ointments prepared with best zinc chitosan neomycin nanoparticles (NP6), blank nanoparticles i.e pure zinc (NP1) and pure chitosan nanoparticles (NP2) and marketed neomycin ointment (nemozin). The percentage wound contraction was measured at 7th, 9th, 11th, 13th, 15th post wounding days to estimate the reduction in wound and results are shown in table 5. 70.53% of wound was contracted in group 4 treated with NP6, whereas 69.8% wound contraction was observed with marketed neomycin ointment (nemozin) within a week. The contraction of wound at the end of 15th day for group 4 treated with NP6 and neomycin ointment (nemozin) was found to be 99.63, 98.67 respectively. This indicated that almost complete wound was healed by 15 days when compared to group 2 and 3 treated with blank zinc nanoparticles (NP1) and chitosan nanoparticles (NP2).

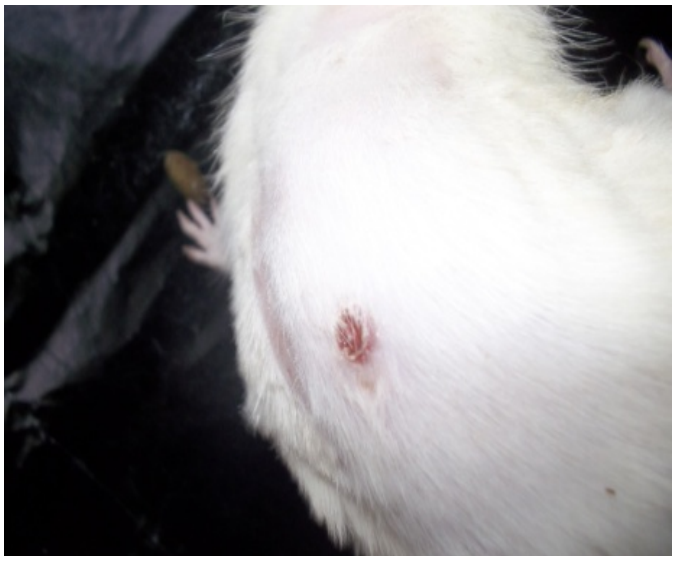
The % wound contraction was significantly increased with metallic nanobiocomposite of neomycin i.e zinc chitosan neomycin nanoparticles (NP6) when compared to zinc nanoparticles (NP1) and chitosan nanoparticles (NP2) ($P<0.05$) as per the table 5. It was found that the % wound contraction of chitosan nanoparticles (NP2) was found to be more than zinc nanoparticles (NP1), which may be due to higher antimicrobial activity of chitosan or may be due to augmentation of restoration of skin cells in wounded area[16]. It may also be due to the ability of chitosan to enhance the fibroblast proliferation , increased function of polymorphonuclear cells, macrophages, that lead to formation of new extracellular matrix by excreting collagen, which ultimately leads to wound contraction[18]. The highest % wound contraction was found with NP6 i.e zinc chitosan neomycin nanoparticles which may be due to synergistic antimicrobial effect of zinc, chitosan, neomycin[28] or due to increased surface area with decreased particle size upon incorporation of polymer. Despite of the less concentration of neomycin used in formulation of the ointment from NP6 i.e 0.2% , the prepared ointment has better wound healing activity than the marketed formulation containing 0.5% neomycin which indicated that the NP6 is the best formulation.

Table 5. Percentage wound contraction

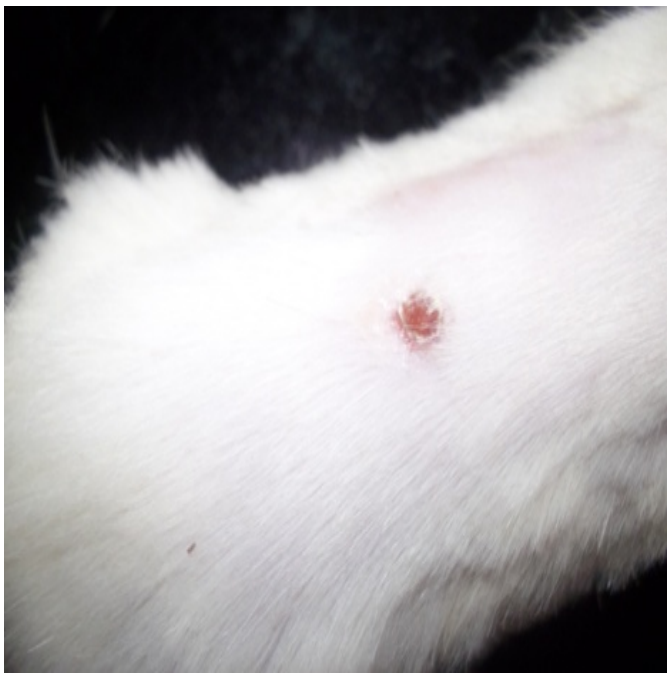
Time (days)	Group 1 (Untreated) (Mean ± S.D)	Group 2 (NP1) (Mean ± S.D)	Group 3 (NP2) (Mean ± S.D)	Group 4 (NP7) (Mean ± S.D)	Group 5 (nemozin) (Mean ± S.D)
7	29.97 ± 0.29	36.64 ± 0.47	41.96 ± 0.55	70.53 ± 0.57	69.8 ± 0.36
9	42.98 ± 0.37	45.57 ± 0.39	48.87 ± 0.47	77.76 ± 0.75	78.23 ± 0.34
11	57.78 ± 0.51	55.55 ± 0.39	59.75 ± 0.46	85.89 ± 0.45	85.86 ± 0.27
13	62.23 ± 0.32	68.56 ± 0.47	76.66 ± 0.36	96.39 ± 0.49	95.94 ± 0.66
15	76.76 ± 0.43	81.87 ± 0.49	84.87 ± 0.39	99.63 ± 0.46	98.69 ± 0.43

Photography

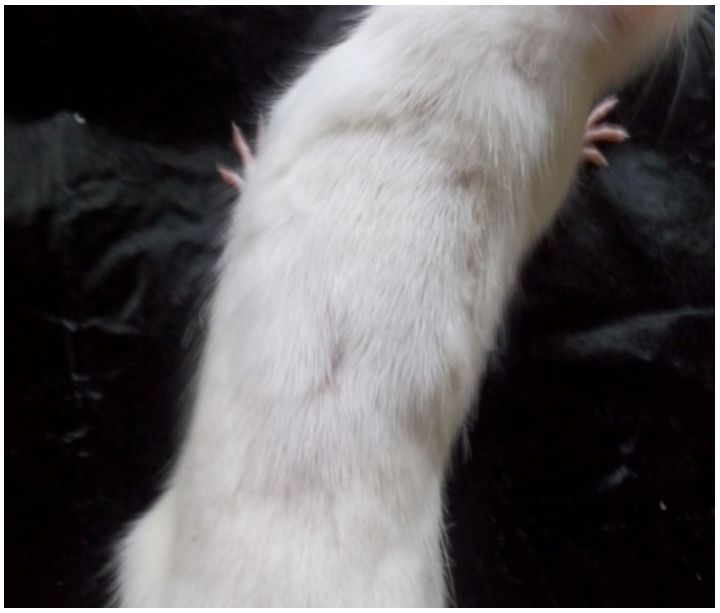
Based on the photography, the wound size was decreased in all groups by 15 days when compared to 0th day as shown in figure 5. Wound was recovered into normal skin in group 4 and 5 by 15th day. Among group 2 and 3, wound healing was found to be more in group 3, which indicated that chitosan nanoparticles (NP3) has more wound healing activity than zinc nanoparticles (NP2). The decrease in wound size was found to be more in group 4 treated with zinc chitosan neomycin ointment when compared to other groups on respective days, this indicated that loading of drug into the blank nanoparticles augmented restoration of skin cells. This may be due to broad anti-bacterial activity of neomycin which reduces infections and fastens wound healing or it may be due to the synergistic effect of all 3 components i.e zinc, chitosan, neomycin and increased surface area with decreased particle size.



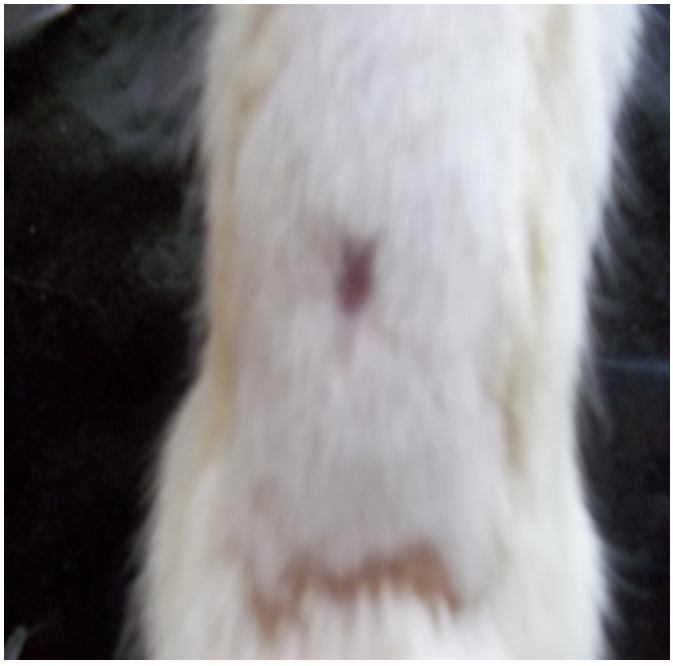
Group 1



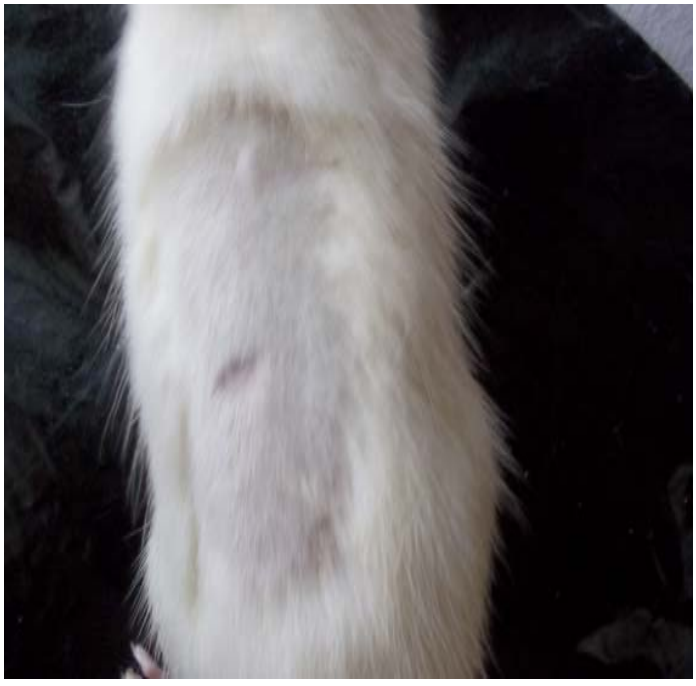
Group 2



Group 4



Group 3



Group 5

Figure 5: Comparison of photographs of wounds on 15th day in different groups

Biochemical analysis

Wound healing was also determined based upon the hydroxyproline and hexosamine levels in healed skin. Hydroxyproline was found to be increased with rapid increase in wound healing. Among all, the group 4 was found to have more hydroxyproline content, which indicated more wound healing activity as shown in table 6. Hexosamine levels were found to be highest in group 4, indicated NP6 is more effective formulation than other prepared formulations and even the marketed neomycin ointment, though the percentage of neomycin used in NP6 (0.2%) is less than the marketed formulation (0.5%). This indicates that NP6 is the best formulation for wound healing activity with less than half of the concentration of drug in marketed preparation (nemozin 0.5%).

Table 6: Hydroxy proline and Hexosamine content in different groups

Group name	Hydroxy proline content(mg/gm tissue)			Hexosamine content(mg/gm tissue)		
	7^{th} day (Mean ± S.D)	10^{th} day (Mean ± S.D)	14^{th} day (Mean ± S.D)	7^{th} day (Mean ± S.D)	10^{th} day (Mean ± S.D)	14^{th} day (Mean ± S.D)
Group 1	10.23±0.11	15.54±0.33	20.54±0.19	12.56±0.24	15.89±0.07	26.54±0.05
Group 2	16.65±0.25	18.89±0.18	25.76±0.14	14.35±0.15	18.52±0.36	29.76±0.14
Group 3	17.78±0.17	20.27±0.19	28.34±0.19	18.64±0.19	26.39±0.17	34.79±0.13
Group 4	35.73±0.29	51.36±0.22	62.23±0.21	28.45±0.37	39.93±0.13	57.75±0.22
Group 5	35.11±0.34	49.38±0.16	60.48±0.25	22.63±0.11	31.37±0.21	54.86±028

Conclusion

Of the first three (NP1,NP2,NP3) nanoparticles, zinc chitosan neomycin nanoparticles (NP3) was found to possess maximum percentage yield, loading efficiency, *in-vitro* drug release and *in-vitro* antimicrobial activity. Hence zinc chitosan neomycin nanoparticles were subjected to optimization by altering the concentration of chitosan and neomycin used in the formulation. Among all the prepared zinc chitosan neomycin nanoparticles (NP4,NP5,NP6,NP7,NP8) NP6 was found to possess maximum *in-vitro* drug release and *in-vitro* antimicrobial activity. This may be due to the synergistic effect of all the ingredients i.e zinc, chitosan and neomycin present in the formulation. Hence zinc chitosan neomycin nanoparticles NP6 was subjected to *in-vivo* studies and compared with the zinc nanoparticles (NP1), chitosan nanoparticles (NP2) by formulating into ointment and marketed neomycin ointment (nemozin). The % wound contraction, hexosamine and hydroxyproline content was found to be more in group 4 treated by ointment prepared with zinc chitosan neomycin nanoparticles (NP6). This indicates that wound healing was found to be more in group 4 treated with ointment prepared with zinc chitosan neomycin nanoparticles compared to group 5 treated with marketed neomycin ointment(nemozin) containing double the concentration of neomycin of NP6.

Thus the prepared zinc chitosan neomycin nanoparticles (NP6) can be considered as the best formulation of neomycin with less than half of the concentration of neomycin of nemozin ointment, as this reduces the side effects like hypersensitive reactions, rashes, itching, burning sensation caused by the use of conventional neomycin preparations (like ointments, creams, gels etc) with increased efficacy at low doses of drug.

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