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PREPARATION AND EVALUATION OF NEEM (*AZADIRACHTA INDICA*) EXTRACT MICROBEADS USING HYDROGEL SYSTEM FOR WOUND HEALING

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ABSTRACT

Introduction: Neem (*Azadirachta indica*, Meliaceae) is being used as an antimicrobial agent in traditional systems of medicines since ancient times. Neem is also applied on wounds in the form of aqueous extracts of various parts of the plant but is associated with problems of stability on long term storage. **Objective:** In the present work, the aim was to incorporate Neem (*Azardirachta indica*) extract in hydrogel system and prepare microbeads for application on wounds. **Material and methods**: The microbeads were prepared by mixing of drug and polymers to cause poly ionic complexation. The formulation was evaluated for various pharmaceutical parameters such as Solubility, Drug Release, Water Holding Capacity, % Drug Entrapped, Bead Diameter Measurement and Antimicrobial study. **Result and Discussion**: The evaluation of the optimized batch showed % drug entrapped to be 5.61 %, drug release of 65.688% in phosphate buffer pH 8 within 5 hrs and water uptake of 80% which were similar to the solutions obtained by the design expert DX7 Statease software. This suggested that the optimization model is validated. The microbeads of the optimized batch had a diameter of approximately 80 µm. **Conclusion**: Polymeric encapsulation in the form of beads allowed controlled delivery as well as enhanced stability of Azadirachtin. It provides a cost effective antimicrobial therapy.

Keywords: *Azardirachta indica*, Microbeads, Drug Release, Hydrogels, Solubility.

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INTRODUCTION

Neem (*Azadirachta indica*, Meliaceae) is being used as an anti-infective and antimicrobial agent in Ayurvedic system of medicines since ancient times. From leaves, twigs to roots and seeds almost every part of Neem is used as medicine. Neem is also applied on wounds in various forms. Neem extract being of natural origin can reduce the side effects associated with antimicrobial formulations of synthetic origin. [1] [2] Extract of Neem in the form of Neem oil is usually used in antimicrobial preparations. But oil has a tendency to get oxidized on long term storage. Neem Azal-T/S is a powdered methanolic extract from neem seed kernels so purer when compared to neem oil which is extracted by cold press technique. Also the methanolic extract is more suitable for long time stability as neem oil may get oxidized over period of time.

Incorporation of Neem extracts and formulation of hydrogels by calcium-alginate cross linking reported earlier can prolong the life of Neem formulations but they shows less entrapment of the core material due to smaller pores. This can be overcome by poly-ionic complexation that produces higher porosity, hence better entrapment. [3] [4] [5]. The present studies include the incorporation of Neem (*Azardirachta indica*) extract in hydrogel system and preparation of micro-beads for application on wounds which will not only enhance the stability of neem preparation but also shows better penetration and antimicrobial activity. Also encapsulation allows controlled delivery as well as stability of Azadirachtin. It provides a cost effective antimicrobial therapy.

MATERIAL AND METHODS

Materials

Pectin, Gelatin, Sodium Alginate, Chitosan, Sodium Carboxy Methyl Cellulose, Calcuim Chloride and other chemical and solvents were of analytical grade/IP/equivalent grade and procured from laboratory**.**

Pre-formulation Studies

The rationale behind the present project is to achieve polyionic complexation. For the same pre-formulation batches were prepared by using various positively charged polymers like chitosan and

gelatin, negatively charged polymers like sodium alginate, sodium CMC & pectin, drug, rigidizing agents like $CaCl_2$, glutaraldehyde, surfactants like Tween 80.

General method of preparation

Negatively charged polymer solution (A) of specific concentration (Table 1 and Table 2) was prepared and injected drop wise into positively charged polymer solution (B) of specific concentration under continuous stirring. The pH of the solutions was adjusted if required. Drug was homogenized into the solution A wherever required. Beads were rigidized with $CaCl_2$ or glutaraldehyde if required

Preparation of PF18

Drug (1%) was homogenized into 1% sodium alginate solution and the resulting solution was injected drop wise into a 5% solution of $CaCl_2$ under continuous stirring.

Preparation of PF21

Drug (1%) was homogenized into 1% chitosan solution (Acetic acid was added to make the chitosan solution). A 1% sodium alginate solution was prepared into which the previously prepared solution was injected drop wise under continuous stirring.

Optimization Study

Optimization of the Neem Azal – T/S microbeads comprising of a cationic polymer (chitosan), an anionic polymer (sodium alginate) and drug was done using 3^2 full factorial designs to determine the most appropriate batch of microbeads. The polymers used may affect the drug content, water holding capacity and % drug release. Hence the study was carried out considering concentration of sodium alginate and concentration of chitosan as the independent variables and drug content, swelling index and % drug release as the dependent variables, at three levels as shown in Table 3 and Table 4.

Table 1: Composition of Pre-formulation Batches 1-10

Ingredients	Batch No. & Concentration									
	PF1	PF2	PF3	PF4	PF5	PF6	PF7	PF8	PF9	PF10
Sodium alginate	2%	2%	2%	2%	2%	1%	1%	1%	-	1%
Sodium CMC	-	-	-	-	-	-	-	-	1%	1%
Gelatin	2%	4%	6%	4%	4%	-	-	-	-	-
Pectin	-	-	-	-	-	-	-	-	-	-
Chitosan	-	-	-	-	-	1%	1%	1%	1%	1%
$CaCl_2$	-	-	-	-	-	-	-	-	-	-
Glutaraldehyde	-	-	-	-	-	-	-	-	-	-
Acetic acid	-	-	-	-	-	1%	1%	1%	1%	1%
Tween 80	-	-	-	-	0.5%	-	-	-	-	-
Drug	-	-	-	1%	1%	-	1%	1%	-	-
Xanthum Gum	-	-	-	-	-	-	-	1%	-	-

Table 2: Composition of Pre-formulation Batches 11-21

Ingredients	Batch No. & Concentration										
	PF11	PF12	PF13	PF14	PF15	PF16	PF17	PF18	PF19	PF20	PF21
Sodium alginate	-	-	-	-	-	-	-	1%	1%	1%	1%
Sodium CMC	-	-	1%	1%	1%	1%	1%	-	-	-	-
Gelatin	-	1%	-	-	-	-	-	-	-	-	-
Pectin	1%	1%	1%	-	-	-	-	-	-	-	-
Chitosan	1%	-	1%	1%	1%	1%	1%	-	1%	1%	1%
$CaCl_2$	-	-	-	5%	-	-	-	5%	5%	5%	5%
Glutaraldehyde	-	-	-	-	1%	2%	3%	-	-	-	-
Acetic acid	1%	-	1%	1%	1%	1%	1%	-	1%	1%	1%
Tween 80	-	-	-	-	-	-	-	-	-	-	-
Drug	-	-	-	-	-	-	-	1%	1%	1%	1%
Xanthum Gum	-	-	-	-	-	-	-	-	-	-	-

Table 3: No. of variables and levels of the optimization model

3^2 Full Factorial Design							
No. of levels = 3			No. of independent variables = 2		No. of dependent variables =3		
-1	0	1	X1	X2	Drug	Drug release	Swelling index
1%	1.5%	2%	Conc. of sodium alginate	Conc. of sodium chitosan			

Table 4: Layout of 3^2 factorial designs with coded and corresponding actual values of independent variables

Runs	Coded Value		Actual Value	
	Factor 1 X1: Conc. of Sodium alginate	Factor 2 X2: Conc. of chitosan	Factor 1 X1: Conc. of sodium alginate	Factor 2 X2: Conc. of Chitosan
1	-1	-1	1%	1%
2	-1	0	1%	1.5%
3	-1	+1	1%	2%
4	0	-1	1.5%	1%
5	0	0	1.5%	1.5%
6	0	+1	1.5%	2%
7	+1	-1	2%	1%
8	+1	0	2%	1.5%
9	+1	+1	2%	2%

Characterization of Beads

Bead diameter measurement

Bead diameter was measured using optical microscope. A small amount of beads was taken on a clean slide and mounted on the optical microscope using glycerine. Bead diameter was measured by the following formula:

Bead diameter = Least count of eye piece * Least count of stage micrometer

Least count of stage micrometer = 0.01mm

Drug entrapped

Drug content was determined by taking 50 mg beads which were transferred into a mortar. 10 ml methanol was added to the mortar and the beads were triturated with the methanol thoroughly so that the entire drug entrapped in the beads comes out and gets dissolved in the methanol. The solution was filtered and absorbance of the filtrate was taken with the help of UV- Visible spectrophotometer with methanol as blank. Entrapment efficiency was calculated.

Water holding capacity

The water uptake of the beads was studied for all the optimization batches by accurately weighing 50 mg beads and transferring them into 9 different beakers marked as F1- F9 respectively filled with 20 ml water. The final weight of the beads was noted after 5 hrs by filtering the beads and wiping off the extra water from the surface. % Water uptake was calculated where, initial weight = 50 mg

Drug release

Drug release study was done to carried out in Franz diffusion cell using phosphate buffer pH 5.5 (physiological skin pH) and phosphate buffer pH 8 (pH of wounded skin) as the dissolution media. Magnetic beads of appropriate size were placed in two separate dissolution apparatus which were then filled with 25 ml of freshly prepared phosphate buffer (one with pH 5.5 and the other with pH 8). The mouth of the apparatus was then covered with parchment paper such that the paper gets moistened with the media underneath. Fifty milligram beads were accurately weighed and transferred onto the parchment paper placed in both the Franz diffusion cells such that the beads and the dissolution media are separated by the parchment paper. Both the apparatus were then labelled clearly and mounted onto two separate magnetic stirrers. The stirrers were then switched on and 5 ml sample was immediately withdrawn from both the media through an outlet provided in the apparatus. To maintain the sink condition 5 ml of the prepared dissolution media were introduced into both the apparatus via the same outlet port. Subsequent samples were withdrawn in the same way at regular time intervals at 30 mins, 1 hr, 2 hrs, 3 hrs, 4hrs and 5hrs. Sink condition was maintained at all the times. The absorbances of the collected samples were measured using UV-Visible spectrophotometer at 220nm. The absorbance values were then put in the standard curve equation and concentration was determined. Further the % drug release was calculated.

Validation of Optimized Batch

The optimization batch was estimated using design expert DX7 Statease software by keeping the criteria given in Table 5.

Table 5: Criteria for estimation of optimization batch

S. No.	Parameter	Criteria	
1	% Drug entrapped	Maximize	
2	Drug release after 5 hrs	Phosphate buffer pH 8	Target – 60
		Phosphate buffer pH 5.5	Minimize
3	Water holding capacity	Maximize	

The preparation method adopted was similar to the preparation of PF21. The prepared microbeads were then sieved through 45# sieve to obtain beads with uniform particle size distribution. The optimized batch was then evaluated for drug content, % water uptake and drug release. The observed results were then compared to the solution given by the design expert DX7 Statease software.

Antimicrobial Study

The antimicrobial study of the optimized batch was performed using cup plate method. [6]

Preparation of culture media

Ready to use Nutrient Agar media powder purchased from Hi-media was suspended into boiling water in required quantity as per instructions on the label. Briefly 28g of powder had to be suspended in 1000ml distilled water. Further the suspension was transferred into a conical flask and corked tightly with cotton. The flask containing suspension was then autoclaved at 121 ^{0} C, 15 psi for 20 minutes.

Preparation of nutrient agar broth

Ready to use Nutrient Agar broth powder purchased from Hi-media was suspended into boiling water in required quantity as per instructions on the label. Briefly 13g of powder had to be suspended in 1000ml distilled water. Further the solution was transferred into 3 test tubes in required quantity and corked tightly with cotton. The test tubes containing solution were then autoclaved at 121 ^{0} C, 15 psi for 20 minutes.

Preparation of inoculums

The sterilized test tubes containing nutrient agar broth was inoculated with 50 µl of standard strain of *Staphylococcus aureus* in aseptic conditions under the Laminar air flow unit. [7] The inoculated test tubes were then transferred into biological incubator and left for incubation at 37 ^{0} C ± 2^0 C for 24 hrs.

Preparation of agar plates

All the petri plates were washed properly, dried, wrapped with aluminium foil and kept for sterilization at 121 ^{0} C, 15 psi for 20 mins. The petri plates were then filled with approximately 20ml nutrient agar medium, covered with another petri plate and left undisturbed. The nutrient agar medium filled petri plates were then allowed to set in the refrigerator overnight.

Sampling of the agar plates

Three petri plates with nutrient agar medium were inoculated with 50 µl each of microbial culture from the test tube aseptically. The inoculum was spread evenly over the surface of agar medium using a spreader. Four cups were made into the nutrient media using a borer such that the plate is divided into four quadrants. The plates were marked as A, B, and C. In each plate 2 quadrants were marked for standard sample(S) and 2 for test sample (T). Plate A was filled with 25 µl of (S) and (T) in the respective cups. Similarly plate B and plate C were filled with 50 µl and 100 µl of (S) and (T) in the respective cups. The complete process was carried out under the LAF cabinet. Now the plates were covered with lid and kept in the refrigerator for 15 mins after which they were transferred into the incubator and left for incubation at 37 ^{0} C for 24 hrs. [8] [9]

(S): Drug in water

(T): Beads equivalent to the weight of drug in water

Ex- Vivo Diffusion Study

The study was conducted to examine the drug release in normal skin and in wounded skin. For the same two dissolution media were selected – phosphate buffer pH 8 equivalent to normal physiological skin pH and phosphate buffer pH 5.5 equivalent to wounded skin pH. Ex-vivo study of the optimized batch was carried out in Franz diffusion cell with goat skin as the permeable membrane in phosphate buffer pH 8 and phosphate buffer pH 5.5 for 5 hrs.

RESULT AND DISCUSSION

Pre-formulation batches were prepared as per the composition mentioned in Table 3 and Table 4 to achieve poly-ionic complexation using various combinations of cationic polymers like gelatin, chitosan and anionic polymers like sodium alginate, pectin and sodium CMC. Observations recorded in Table 6.

From all the Pre-formulation batches prepared, combinations of sodium alginate and chitosan with calcium chloride as rigidizing agent formed intact beads while all other combinations of polymers didn't form beads as depicted from Table 6. Intact beads were obtained from PF18, PF19 and PF21 batches. Therefore sodium alginate and chitosan were selected as polymers for preparation of the microbeads.

The smallest bead size was found to be that of PF21 batch as shown in Table 7. Also the PF21 beads appeared to be spherical in shape as compared to PF18 & PF19 batches. Therefore PF 21 batch containing chitosan, sodium alginate and drug rigidized with 5% $CaCl_2$ was found to be the best batch.

The highest entrapment efficiency was found to be that of PF21 beads while the lowest was that of PF18 beads as seen in Table 8. This can be attributed to the fact that poly-ionic complexation increases the pockets of drug entrapment due to more cross-linking between the polymers.

Table 6: Observations drawn from preparation of Pre-formulation batches 1-21

Batch No.	Observation
PF1	Translucent viscous mass obtained. Beads not formed
PF2	Translucent viscous mass obtained. Beads not formed
PF3	Translucent viscous mass obtained. Beads not formed
PF4	Damp mass obtained but differentiable.
PF5	Pale yellow mass obtained. Beads not formed
PF6	Beads formed but were not rigid.
PF7	Beads formed but were not rigid.
PF8	Beads formed but were not rigid.
PF9	Beads rupture easily.
PF10	Beads rupture easily.
PF11	Beads not formed
PF12	Beads not formed
PF13	Beads not formed
PF14	Beads do not get rigidized. With time the beads change shape from spherical to disc shape.
PF15	Beads do not get rigidized
PF16	Beads do not get rigidized
PF17	Beads do not get rigidized
PF18	Irregular shaped beads formed.
PF19	Irregular shaped beads obtained after drying**.**
PF20	Beads get ruptured on drying
PF21	Spherical, rigid microbeads obtained.

Table 7: Mean bead diameter of batches PF18, PF19, PF21

Batch No.	Mean Diameter (µm) ± SD (n=10)
PF18	1906.7 ± 1.212
PF19	1290.7 ± 0.850
PF21	854.3 ± 0.822

Table 8: **% Drug entrapped of batches PF18, PF19 & PF21**

S. No.	Batch No.	Mean % Drug Entrapped (%) ± SD (n=3)
1	PF18	0.769 ± 0.022
2	PF19	0.818 ± 0.014
3	PF21	1 ± 0.005

The percent drug release in phosphate buffer pH 5.5 and 8.0 for PF18, PF-19 and PF-21 were shown in Table 9 and Table 10 whereas the percentage drug release pattern in phosphate buffer pH 5.5 and pH 8.0 were presented in Figure 1 and Figure 2 respectively.

Table 9: %Drug release in Phosphate buffer pH 5.5

S. No.	Time (mins)	% DR		
		PF18	**PF19**	**PF21**
1	0	0.000	0.000	0.000
2	30	18.774 ± 0.810	13.966 ± 0.125	13.486 ± 1.02
3	60	37.673 ± 1.25	17.962 ± 0.55	18.346 ± 0.021
4	120	54.553 ± 0.71	29.168 ± 0.416	30.370 ± 0.051
5	180	78.019 ± 0.007	45.375 ± 0.060	45.856 ± 0.033
6	240	78.019 ± 1.005	58.120 ± 0.052	59.370 ± 0.001
7	300	78.019 ± 0.021	72.019 ± 0.014	72.740 ± 0.006
8	24 hrs	72.019 ± 0.021	78.019 ± 0.035	73.221 ± 0.097

Table 10: % Drug release in Phosphate buffer pH 8

S. No.	Time (mins)	% DR		
		PF18	PF19	PF21
1	0	0.000	0.000	0.000
2	30	24.543 ± 0.021	14.447 ± 0.811	14.928 ± 0.052
3	60	43.154 ± 0.115	25.269 ± 0.044	26.087 ± 0.075
4	120	64.264 ± 0.258	42.245 ± 0.014	42.486 ± 0.057
5	180	90.231 ± 0.078	52.827 ± 0.253	53.548 ± 0.059
6	240	90.231 ± 0.741	70.139 ± 0.144	77.688 ± 0.022
7	300	90.231 ± 0.103	91.298 ± 0.504	93.558 ± 0.430
8	24 hrs	90.231 ± 0.024	91.779 ± 0.061	93.798 ± 0.007

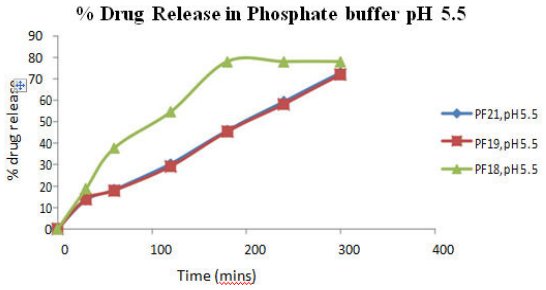

Figure 1: % drug release in phosphate buffer pH 5.5

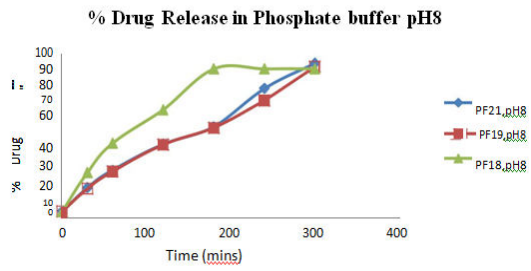

Figure 2: % drug release in phosphate buffer pH 8

The optimization was carried out using 3^2 full factorial design with the help of Design Expert software version 10 wherein entrapment efficiency, water holding capacity and drug release were compared when concentration of sodium alginate and chitosan were considered as the independent variables as shown in Table 11. % Drug Entrapped was found to be highest of batch F9 and lowest for batch F4 as given in Table 12. The entrapment efficiency of all the optimization batches was shown in Table 12.

Table 11: Summary of 3^2 full factorial designs

Batch no.	Runs	Factor 1 X_1: Conc. of Sodium alginate	Factor 2 X_2: Conc. of chitosan	Response 1 % Drug entrapped (%)	Response 2 Drug release after 5hrs (%)	Response 3 % Water uptake
F1	1	-1	-1	3.98	76.744	36
F2	2	-1	0	4.04	80.558	44
F3	3	-1	+1	2.54	88804	72
F4	4	0	-1	2.46	56.225	44
F5	5	0	0	4.35	68.435	56
F6	6	0	+1	3.56	74.422	80
F7	7	+1	-1	3.23	49.564	50
F8	8	+1	0	5.28	58.968	64
F9	9	+1	+1	5.49	68.054	86

Table 12: % Drug Entrapped of optimization batches

Batch No.	Drug Entrapped (%)
F1	3.98
F2	4.04
F3	2.54
F4	2.46
F5	4.35
F6	3.56
F7	3.23
F8	5.28
F9	5.49

Entrapment efficiency was analyzed statistically using Design Expert Statease software, version 7. Quadratic model was suggested by the software, having a p value of 0.0169. The data obtained was tabulated in Table 13.

Table 13: ANOVA for % drug entrapped

Analysis of variance table [partial sum of squares – Type III]						
Source	**Sum of squares**	**df**	**Mean square**	**F value**	**p-value Prob>F**	
Model	8.87	5	1.77	19.58	0.0169	significant
A- conc. of alginate	1.97	1	1.97	21.76	0.0186	
B- conc of chitosan	0.61	1	0.61	6.78	0.0801	
AB	3.42	1	3.42	37.76	0.0087	
A^2	0.81	1	0.81	8.94	0.0581	
B^2	2.05	1	2.05	22.66	0.0176	

The quadratic equation obtained from the software was:

% Drug Entrapped = +4.13 + 0.57*A + 0.32*B +0.93*AB +0.64A^2 - 1.01B^2

As the concentration of alginate increased an increase in the entrapment efficiency in the beads was observed. This may be because an increase in alginate concentration allowed more drug to be entrapped in the pockets of polymer complexes. However the concentration of chitosan had anomalous effect on % drug entrapped. The reduced quadratic equation would be:

% Drug Entrapped = +4.13 + 0.57*A + 0.93*AB - 1.01B^2

The contour plot shown in Figure 3 shows an increase in the entrapment efficiency with corresponding increase in the concentration of alginate as well as concentration of chitosan at the higher concentration ranges of both the polymers. Also the positive coefficient of AB suggested that there was some interaction between the two polymers which in turn suggests complexation of the two polymers which is desirable.

Water holding capacity was found by keeping 50 mg of beads in a beaker filled with 20ml water for 5 hrs and then weighing the beads after filtering it out and wiping off the extra water from the bead surface if any and the observations were tabulated in Table 14 and Table 15. Counter plot for water holding capacity was presented in Figure 4.

From the results obtained it was found that the water holding capacity of the beads was affected by the concentration of both sodium alginate and chitosan. Statistical analysis of %water uptake was done using Design Expert Statease software, version 7.

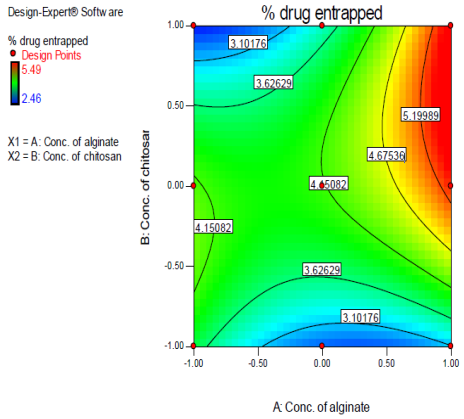

Figure 3: Contour plot for % Drug Entrapped

Table 14: Water holding capacity of optimization batches

Batch No.	Water holding capacity (%)
F1	36
F2	44
F3	72
F4	44
F5	56
F6	80
F7	50
F8	64
F9	86

Table 15: ANOVA for Water holding capacity

Analysis of variance table [partial sum of squares – Type III]						
Source	**Sum of squares**	**df**	**Mean square**	**F value**	**p-value Prob> F**	
Model	2420.44	5	484.09	116.70	0.0012	Significant
A- conc. of alginate	384.00	1	384.00	92.57	0.0024	
B- conc. of chitosan	1944.00	1	1944.00	468.64	0.0002	
AB	0.000	1	0.000	0.000	1.0000	
A^2	3.56	1	3.56	0.86	0.4228	
B^2	88.89	1	88.89	21.43	0.0190	

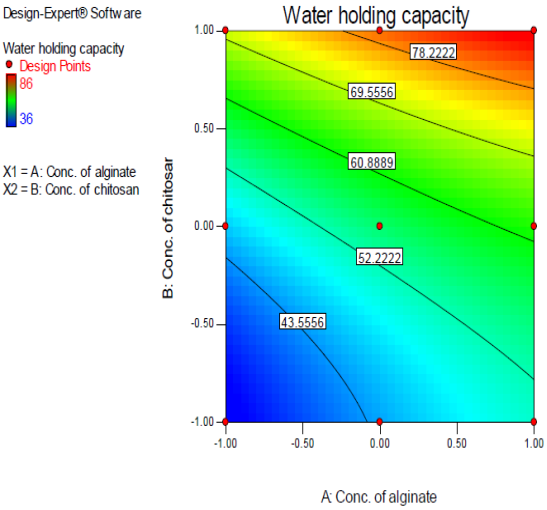

Figure 4: Contour plot for water holding capacity

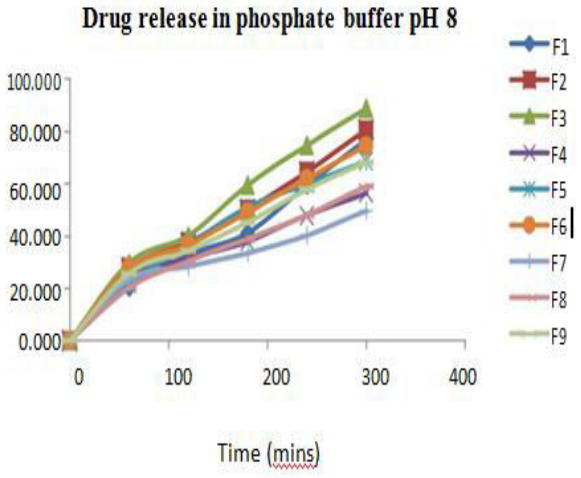

Figure 5: Comparative in vitro release study of optimization batches in phosphate buffer pH 8

The release study was carried out in Franz diffusion cell with phosphate buffer pH 8 and phosphate buffer pH 5.5 as dissolution media and the results were presented in Table 16 and Table 17 respectively. The comparative in-vitro drug release study of optimized batches in phosphate buffer pH 8 and pH 5.5 were presented in Figure 5 and Figure respectively.

Table 16: % Drug release of the optimization batches in phosphate buffer pH 8

Time (mins)	DR (%)								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
0	0	0	0	0	0	0	0	0	0
60	20.22	27.67	29.62	25.21	21.87	27.75	23.36	20.21	25.75
120	32.63	37.50	39.99	31.24	36.50	36.46	28.31	30.25	34.34
180	41.00	49.99	59.36	37.95	50.88	49.01	33.43	38.94	45.08
240	59.27	64.44	74.58	47.81	59.85	61.75	40.22	47.99	57.80
300	76.74	80.56	88.80	56.23	68.44	74.42	49.56	58.97	68.05

Table 17: % Drug release of the optimization batches in phosphate buffer pH 5.5

Time (mins)	DR (%)								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
0	0	0	0	0	0	0	0	0	0
60	15.52	16.64	20.84	23.43	17.40	22.21	17.88	15.46	16.62
120	23.31	23.01	31.06	29.46	23.95	29.64	22.65	20.59	23.64
180	33.40	34.46	42.49	35.55	32.75	37.81	28.54	26.61	30.41
240	42.94	49.37	55.89	42.33	39.47	44.57	34.56	34.84	36.35
300	56.66	59.78	64.97	48.84	46.02	51.95	41.57	42.55	44.07

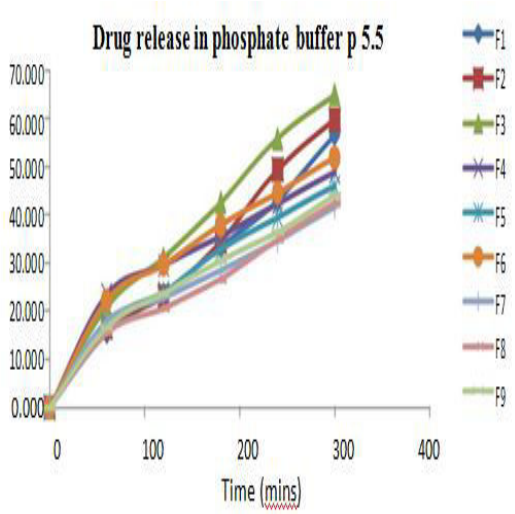

Figure 6: Comparative in vitro release study of optimization batches in phosphate buffer pH 5.5

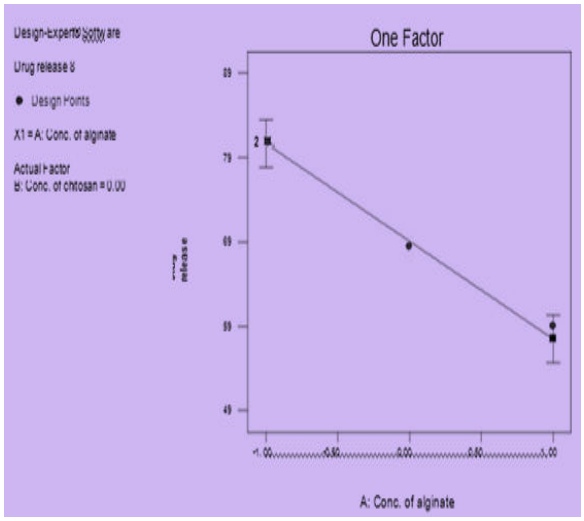

Figure 7: Contour plot of drug release in phosphate buffer pH 8 showing effect of concentration of alginate

The contour plot in Figure 8 shows an increase in the drug release in phosphate buffer pH 8 with increase in concentration of chitosan. This may be attributed to the fact that chitosan is increasing the porosity which in turn increases the release. Contour plot of drug release in phosphate buffer pH 8.0 were presented in Figure 7. ANOVA parameters for drug release in phosphate buffer pH 5.5 were recorded in Table 18.

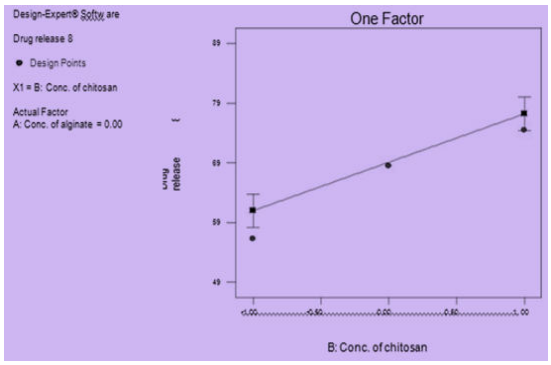

Figure 8: Counter plot of drug release in phosphate buffer pH 8 showing effect of concentration of chitosan

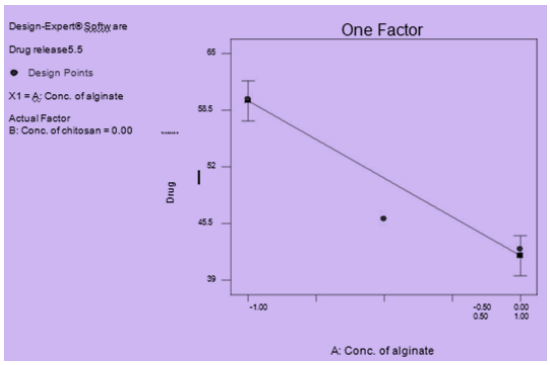

Figure 9: Contour plot of drug release in phosphate buffer pH 5.5 showing effect of concentration of alginate

Table 18: ANOVA for drug release in phosphate buffer pH 5.5

Analysis of variance table [partial sum of squares – Type III]						
Source	**Sum of squares**	**Df**	**Mean square**	**F value**	**p-value Prob> F**	
Model	505.45	2	252.72	40.17	0.0003	significant
A- conc. of alginate	472.95	1	472.95	75.17	0.0001	
B- conc. of chitosan	32.50	1	32.50	5.17	0.0634	

The quadratic equation obtained from the software was:

% Drug Release = + 50.71 – 8.88*A + 2.33*B

A p – value less than 0.05 indicated that the model is significant.

The reduced quadratic equation would be:

% Drug Release = + 50.71 – 8.88*A + 2.33*B

The negative coefficient of A (concentration of alginate) suggests that concentration of alginate is inversely proportional to drug release. While a positive coefficient of B (concentration of chitosan) suggests a directly proportional relationship of B on drug release.

The contour plot in Figure 9 shows a decrease in the drug release in phosphate buffer pH 5.5 with increase in concentration of sodium alginate similar to that observed in phosphate buffer pH 8. This may be attributed to the fact that sodium alginate is increasing the viscosity which in turn decreases the release.

The contour plot in Figure 10 shows an increase in the drug release in phosphate buffer pH 5.5 with increase in concentration of chitosan. This may be attributed to the fact that chitosan is increasing the porosity which in turn increases the release.

Optimized batch was prepared by the formula suggested in the Design Expert Statease software, version 7 when % drug entrapped, drug release and % water uptake were the parameters considered. The solution obtained in terms of coded value was to prepare a batch comprising concentration of alginate to be +1 and concentration of chitosan as 0.97 which is equivalent to 2% sodium alginate and 1.985% chitosan. The prepared batch was then evaluated for % drug entrapped; water holding capacity, drug release in phosphate pH 8 and pH 5.5, bead diameter, antimicrobial effect and permeation study by ex - vivo diffusion. The readings were recorded in Table 19 and Table 20.

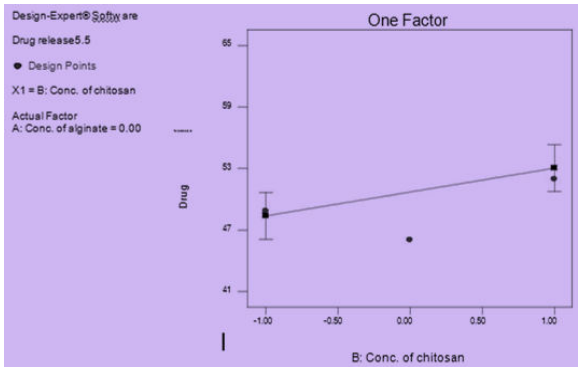

Figure 10: Contour plot of drug release in phosphate buffer pH 5.5 showing effect of concentration of chitosan

Table 19: Results of evaluation and comparison of optimized batch with Design Expert software solution

S. No.	Parameter	Software solution	Optimization batch (n=3)
1	% Drug entrapped	5.595%	5.610% ± 0.027
2	Drug Release in phosphate buffer pH 8	65.391%	65.688% ± 0.036
3	Drug Release in phosphate buffer pH 5.5	44.088%	51.715% ± 0.031
4	Water Holding Capacity	85.999%	80% ± 0.004
5	Bead Diameter	-	80.63 ± 0.755µm (n=10)

The results obtained after evaluation of the validation batch were similar to the solution suggested by the Design Expert Statease software, version 7. So it can be concluded from the above results that the prepared batch is the final, validated batch. it was confirmed that the prepared formulation showed comparable antimicrobial effect to the standard drug on the *S. aureus* strains in Figure 11. Ex-vivo study of the validated batch was performed using goat skin in Franz diffusion cell and the comparative drug release pattern at pH 8 and pH 5.5 was presented in Figure 12.

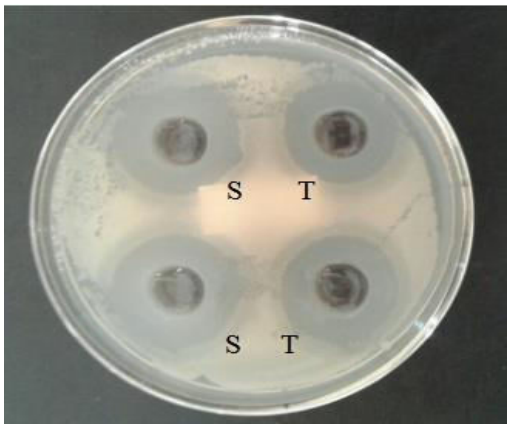

Figure 11: Petri plate showing zone of inhibition of the test and standard samples against *S. aureus* strains of bacteria.

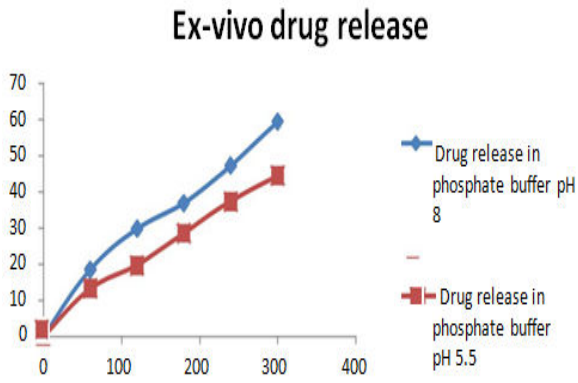

Figure 12: Comparison of drug release in phosphate buffer pH 8 and pH 5.5

Table 20: % Drug release of optimized batch in phosphate buffer pH 8 and phosphate buffer pH 5.5

S. No.	Time (mins)	DR (%)	
		Phosphate buffer pH 8	Phosphate buffer pH 5.5
1	0	0	0
2	60	18.489	13.231
3	120	29.817	19.675
4	180	36.880	28.594
5	240	47.291	37.404
6	300	59.448	44.394

CONCLUSION

Microbeads encapsulating Neem Azal -T/S were prepared by polyionic-complexation of sodium alginate with chitosan using 5% calcium chloride as rigidizing agent. The beads formed were approximately of 80 µm diameter with an entrapment efficiency of 5.61%, showing approximately 66% drug release within 5 hrs in phosphate buffer pH 8. The microbeads had good permeability through the skin of approximately 59% in 5 hrs observed from ex vivo diffusion study through goat skin membrane. The antimicrobial study showed a comparable zone of inhibition of both the formulation and pure drug. So it can be concluded that the prepared microbeads can be used for wound healing purpose successfully.

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