Journal of Young Pharmacists, 2023; 15(3):465-477.
https://www.jyoungpharm.org

Original Article

Formulation and Optimization of Instant Film Forming Thermoreversible Chitosan Hydrogel for Wound Healing

Bharakhada Vaishali[1], Manisha Jadav[2,*], Lalit Lata Jha[3]

[1]Department of Pharmaceutics, Parul Institute of Pharmacy, Parul University, P. O. Limda, Waghodia, Gujarat, INDIA.
[2]Department of Pharmaceutical Chemistry, School of Pharmacy, Parul University, P. O. Limda, Waghodia, Gujarat, INDIA.
[3]Department of Pharmaceutics, Parul University, P.O. Limda, Waghodia, Gujarat, INDIA.

ABSTRACT

Background: Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers. The aim of the present investigation was formulation and optimization of instant film forming thermoreversible chitosan hydrogel for wound healing. **Materials and Methods:** A novel hydrogel composing of chitosan, Ethanol, Sodium α-β Glycerophosphate and combination of three drugs (Ascorbic acid, Tuftsin, Zn gluconate) were prepared. Ethanol and α-β Glycerophosphate (α-β-GP) were selected based on gelling time and film formation time. Optimization of vehicle in formulation was done by using 3^2 full factorial design using Design Expert® 8.0.7.1 software. Optimization of drug loaded thermoreversible chitosan hydrogel was done with 0.2mg, 2mg, 20mg, ratio of Zn gluconate, Tuftsin (10 µg/mL), Ascorbic acid (0.1mg/mL) by trial-and-error method. **Results and Conclusion:** The Gelling time, Film formation time, swelling ratio and Moisture retention capacity of the optimize drug loaded formulation (X_2) were 160±19.5, 390±18.4, 296±7.58, 6.8±0.76 respectively. The *in vitro* release of optimize drug loaded hydrogel formulation was carried out by Franz diffusion cell. *In vitro drug* release data of optimize batch was 86.5+1.9%, 80.66±4.52, 85.55±2.95 in 24 hr for Ascorbic acid, Tuftsin, Zn gluconate respectively. Chitosan hydrogel showed powerful antibacterial efficacy up to 100% to *Staphylococcus aureus* (ATCC 29213) and *Escherichia coli* (ATCC 87064) by hydrogel formulation with or without drug. Since the formulation contains thermosensitive drugs stability study of optimized batch was carried out as per ICH guidelines. The stability data shows that the optimized batch of thermoreversible chitosan hydrogel remains stable for 1 month-controlled room temperature (25°C±2°C/ 60%RH±5%RH) and refrigerated condition (5°C±3°C). *In vivo* studies also carried out.

Keywords: Ascorbic acid, Chitosan, Zn gluconate, Tuftsin, Wound healing.

Correspondence:
Mrs. Manisha Jadav
Department of Pharmaceutical Chemistry, School of Pharmacy, Parul University, P.O. Limda, Waghodia-391760, Gujarat, INDIA.
Email: manisha.jadav121112@paruluniversity.ac.in

Received: 27-01-2023;
Revised: 20-03-2023;
Accepted: 28-04-2023.

INTRODUCTION

A wound is defined as a defect or a break in the skin which was resulting from physical or thermal damage or as a result of the presence of an underlying medical or physiological condition. Based on the nature of the repair process, wounds can be classified in two categories acute and chronic wounds. Acute wounds are tissue injuries that heal completely usually 8–12 weeks with minimal scarring.[1] A chronic wound is defined as the loss of epithelial coverage or integrity that has failed, through an orderly and timely series of processes, to produce a durable, structural, functional, and cosmetic closure.[2] Wound healing is a biological process related to the general anomaly of growth and rebirth of tissue regeneration.[1] The objective of wound management is to heal the wound in the short period of time, with minimal pain, discomfort and scarring to the patient. At the site of wound closure, a flexible and fine scar with high tensile strength is desired. Successful management of wound patients depends on an understanding of the healing process and nutritional influences on wound healing.[3] A network of water-insoluble polymer chains known as a hydrogel can occasionally be discovered as a colloidal gel in which water serves as the dispersion medium. Although these networks have a strong affinity for water, the links that have been created between the polymer chains prevent them from dissolving.[4,5] The physical characteristics of fully expanded hydrogels are similar to those of live tissues. Also optimize drug loaded instant film forming thermoreversible chitosan hydrogel by evaluating gelling time, film formation time, swelling test, and moisture retention capacity, % *in vitro* release study and drug content. To analyses Stability study of optimized formulation. To evaluate wound healing efficacy of drug loaded thermoreversible chitosan hydrogel in rat.

DOI: 10.5530/jyp.2023.15.62

MATERIALS AND METHODS

Materials

Zinc gluconate, Ascorbic acid, Chitosan, Sodium-α-glycerophosphate, Sodium-β- glycerophosphate, Pentahydrate, Trifluroacetic acid and nutrient Agar Media were purchased from Hi-media, Mumbai. Tuftsin was obtained from Genscript, Singapor/Hongkong. Ethanol, Glacial acetic acid, Disodium Hydrogen Phosphate, Potassium Dihydrogen Phosphate, Sodium Chloride, Potassium Chloride and Acetonitrile were procured from SRL, Vadodara. Distilled Water purchased from Water Supply Specialists Pvt. Ltd., Vadodara, India.

Method of preparation

In this study first ethanol and alpha beta glycerophosphate ratio were optimize is prepared by simple mixing method. Ethanol and alpha beta glycerophosphate ratio were taken as independent variable. Gelling time, film formation time, swelling ratio, moisture retention ratio was taken as dependent variable. Based on literature survey different ratio were taken and batches were formulated and optimized by 3^2 factorial designs.

Method of preparation of instant film forming thermoreversible chitosan hydrogel (Without drug)

About 2% chitosan was dissolved in 0.25 mL 1:1 ratio of 10M acetic acid and water solution with stirring until complete dissolution.[6] Fifty percent w/v of α-β–GP (50:50, 25:75, 0:100) was prepared in distilled water. Take 0.1mL α - β -GP and add dropwise to the chitosan solution under stirring and the final chitosan α - β –GP solution was mixed with ethanol (15%, 30%, 45%) with stirring. Add sufficient amount of water up to 1 mL. 3^2 factorial designs were used for the purpose of optimization as respected to various parameters. For optimization of vehicle Gelling time, film formation time, swelling ratio, moisture retention capacity was observed.

Method of preparation instant film forming thermoreversible chitosan hydrogel (With drug)

About 2% chitosan and 0.2, 2, 20 mg Zn gluconate was dissolved in 0.4mL 1:1 ratio of 10M acetic acid and water solution with stirring until complete dissolution.[7] Fifty percent w/v of α - β –GP (50:50) was prepared in distilled water. Take 0.1mL α - β -GP was added dropwise to the chitosan solution under stirring and the final chitosan α– β –GP solution was mixed with ethanol (45%) containing 0.01 mg Tuftsin and 0.1mg Ascorbic acid, with stirring. Add sufficient amount of water up to 1mL. The formed hydrogel solution on petridish and incubate at 37°C. The hydrogel was kept at room temperature to let the water and ethanol evaporate as much as possible until the hydrogel film formed. Trial and error design were used for the purpose of optimization and various parameters were Gelling time, film formation time, swelling ratio, moisture retention capacity was observed.

Factor influence study, optimization and Characterization of instant film forming thermoreversible chitosan hydrogel (Without drug) using 3^2 factorial design

In this research work, 3^2 full factorial design was employed to evaluate the effect of independent variables i.e., Ethanol (X_1) and α-β–GP ratio (X_2) on dependent variables gelling time, film formation time, swelling ratio, moisture retention capacity.

Optimization and Characterization of instant film forming thermoreversible chitosan hydrogel (With drug) using trial and error method

Here, optimization of Drug loaded formulation was done to find out the best formulation out of all formulations. In this present work, Trial and Error method were used to optimization of Zn gluconate (0.2mg, 2mg, 22mg). Chitosan (2%), Glycerophosphate (50%), Ratio of β and α-glycerophosphate (50:50), Ethanol (45%), Tuftsin (0.01mg/mL), Ascorbic acid (0.1mg/mL), Zn gluconate (0.2, 2 and 20 mg/mL) were used for formulation of optimize batch. Gelling time, film formation time, swelling ratio, moisture retention capacity of optimize batch was measured.

In vitro diffusion experiments

For *in vitro* release phosphate buffer (pH 6.8) was used as a receptor medium. The Parchment paper was used in Franz diffusion cell.[8,15] A Parchment paper was used to apply the gel sample, which was pasted in place between the donor and receptor compartments of the diffusion cell. The receptor compartment contained 25mL phosphate buffer (pH 6.8). The temperature of diffusion medium was thermostatically controlled at 37°C± 1°C by surrounding water in jacket and the medium was stirred by magnetic stirrer. A 1mL aliquot of the receptor medium was withdrawn at time 2, 4, 6, 8, 12 and 24 hr after initiation of study for estimation of drugs and this volume were instantly replaced with a same volume of fresh buffer. Measure the absorbance of ascorbic acid in UV-visible spectrophotometer at λ_{max} 266nm and tuftsin in HPLC and Zn gluconate in to atomic absorption spectrophotometer.

Drug content

Dissolved film in 2mL phosphate buffer pH-6.8 by overnight stirring or 1mL pourable liquid form of formulation.[8,13] Such solutions were diluted up to 20mL with phosphate buffer- 6.8 by agitated stirring. These sample solutions were directly utilized for estimation of Tuftsin (HPLC), ascorbic acid (UV-visible spectrophotometer) and Zn gluconate (AAS).

Antimicrobial study

Antimicrobial test of the optimized formulation was carried out by Cup Plate method. Fresh colonies were prepared from *Escherichia coli* and *Staphylococcus aureus* which were used as test organisms.[9,12] A single loop of the bacteria was inoculated in

the test tube and kept in an air-bath shaker at 37°C for 18 hr. The organisms grown cultures of *E. coli* (ATCC 87064) and *S. aureus* (ATCC 29213) were prepared and used for the antibacterial activity. Agar plates were used to calculate the inhibition rates of the test compounds. The sample were prepared and kept the form of fluid at 38°C. The culture plates were prepared, in which 200µL solution of bacterial suspension was spread uniformly and then 150 µL fluid of the sample. All the plate were incubated at 37°C for 18 hr. Tetracycline (30µg/mL) for *S. aureus* and Cephotexime (10µg/mL) for *E. coli* were used as a standard. After incubation, the plates were taken out of the incubator, and Zone of inhibition was observed.

Stability study

As per ICH guidelines Q1A (R^2), the stability study of drug loaded hydrogel were carried out at both refrigerated temperature (5°C+3°C) and room temperature (25°C+ 2°C/60%RH+5% RH) for 1 month period of time.[10,14] The gelling time, film formation time, swelling ratio, moisture retention capacity and Drug content were evaluated.

In vivo wound healing efficacy of the formulation

Male SD rats (n=24), 8 weeks old, weighing 200-250gm were procured for the study. They were anesthetized using diethyl ether and their shaved on dorsal side. 1 cm long incision made on shaved area.[9,11] Animal were divided further in to four groups (n=6) depending upon dosing treatment. One group was normal rats, second group was testing formulation treated rats, third group was vehicle treated rats and fourth was marketed formulation treated rats. Wound of normal rats were not treated with any vehicle or formulation. Whereas optimized chitosan hydrogel film, optimized drug loaded chitosan hydrogel film and soframycin applied on wound of vehicle treated rats, test formulation treated rats, and marketed formulation treated rats respectively at one time after incisions. On 10th day after incisions, wound was morphologically analyzed for healing.

Statistical evaluation

Evaluation of all the batches (F_1 to F_9) was done by using ANOVA and Design Expert® 8.0.7.1 software to see the significant and non-significant effect of the factor selected on different responses. Contour plot and response surface plots were used to show the factor influence study graphically. The best optimized formulation was chosen using the overlay plot, and it was used for future evaluation research.

Check point method

To confirm the model validity check point batch was prepared and compared with the values obtained from the equations. Ethanol concentration of 45% and Sodium α-β-GP ratio of 50:50 (C_1 batch) was used in check point batch.

RESULTS

Analytical method of Drug (A)Tuftsin, (B)Ascorbic acid, (C) Zn gluconate

Calibration curve of tuftsin (Figures 1 and 2) was taken in range of 200-1000ng/mL (Table 3). using HPLC Retention time and area of peak was measured. Phosphate buffer pH 6.8 was taken as a solvent.

Ascorbic acid having 10 µg/mL was scanned between 200-400 nm using UV-spectrophotometer (Shimadzu 1800). Ascorbic acid exhibited UV-absorption maxima at 266.5 nm. Calibration curve of Ascorbic acid (Figure 3 and 4) was taken in the range of 2 - 10 µg/mL (Table 4). Using UV-spectrophotometer, the maximum wavelength of each solution was measured and from that the maximum wavelength of Ascorbic acid was determined

Table 1: Variables selected for present study.

Independent variables		Dependent variables
X1: Ethanol	Gelling	Time, film formation time.
X2: α - β – glycerophosphate ratio	Swelling capacity	Ratio, moisture retention.

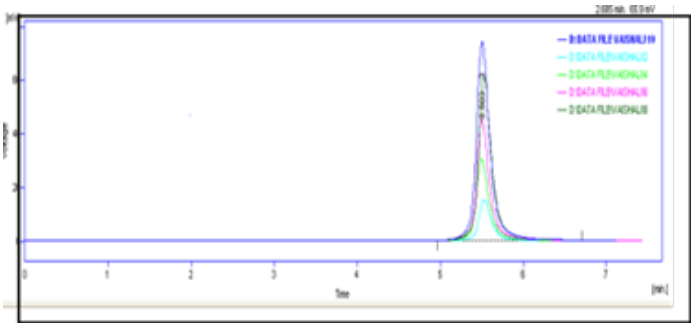

Figure 1: Overlay spectra of Tuftsin.

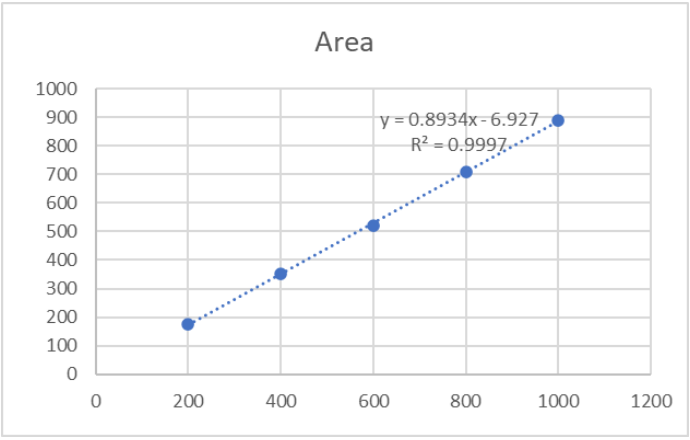

Figure 2: Calibration curve of Tuftsin.

Table 2: Coded values for factor X_1 and X_2.

Coded Value	Low (-1)	Medium (0)	High (+1)
Ethanol concentration	0.15	0.3	0.45
β-α Glycerophosphate ratio	50:50	75:25	0:100

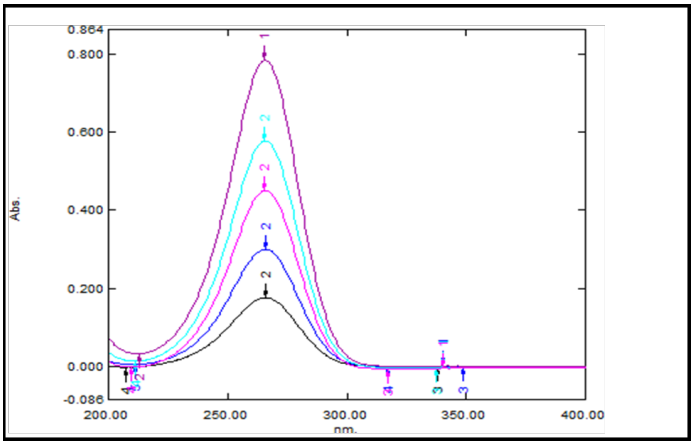

Figure 3: Overlay Spectrum of Ascorbic Acid.

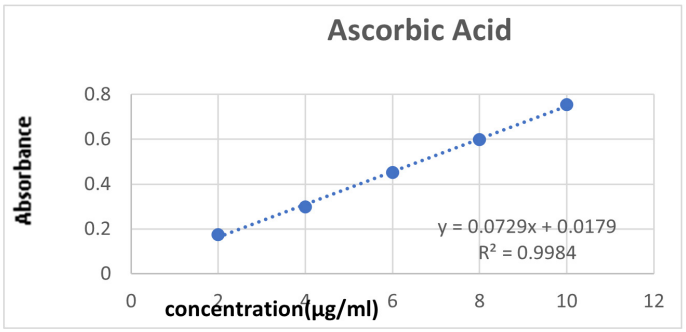

Figure 4: Calibration curve of Ascorbic Acid.

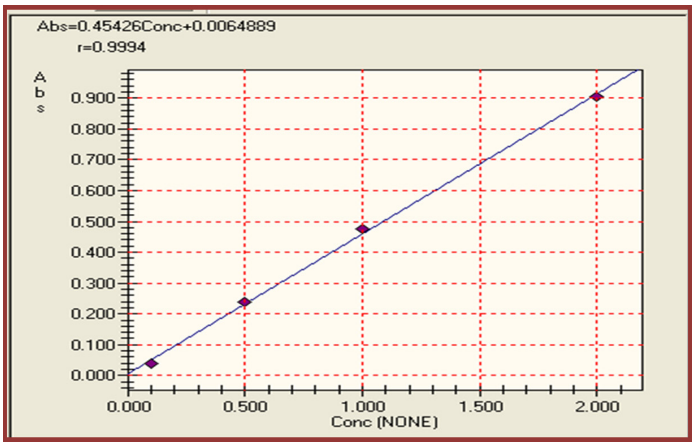

Figure 5: Calibration curve of Zn Gluconate.

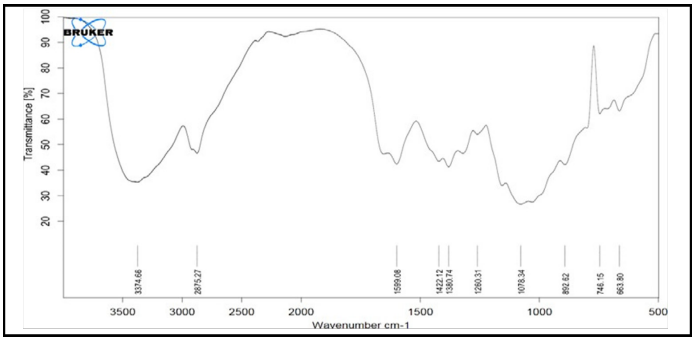

Figure 6: IR Spectra of Chitosan.

at 266.5 nm. Three different determinations of absorbance were carried out at 266.5 nm. Phosphate buffer was taken as solvent.

Calibration curve of Zn gluconate (Figure 5) were measured by atomic absorption spectrophotometer. Calibration curve of Zn gluconate was taken in the range of 0.1-2 µg/mL (Table 5). Phosphate buffer pH 6.8 was taken as solvent.

From the FTIR studies result, it showed that the peaks of Na α-Glycerophosphate (Figure 7), Na β-Glycerophosphate (Figure 9), Ascorbic acid (Figure 13), Zn Gluconate (Figure 11), Tuftsin (Figure 15) were present in the combination with Chitosan (polymer) (Figures 6, 8, 10, 12, 14, 16). It can be concluded that there is no interaction between all the above Excipient with chitosan.

In this present work, 3^2 full factorial design was employed to study the effect of independent variables (Tables 1 and 2), i.e., Ethanol (X_1) and α - β –GP ratio (X_2) on dependent variables gelling time, film formation time, swelling ratio, moisture retention capacity. All the batch contain Chitosan 2%, 10M acetic acid, Water (Table 6). Optimization of the vehicle formulation was based on the selected responses (Table 7) i.e., Gelling time, film formation time, swelling ratio, water retention capacity. Optimize study was carried out by using Design Expert ® 8.0.7.1 software.

Optimization of the formulation was done by 3^2 factorial designs by Design Expert ® 8.0.7.1 software. Contour plot (Figure 17) and response surface plot (Figure 18) of all the responses shows the curvature with change in the factor indicating Gelling time were influenced by change in both the factors like concentration of Ethanol and Ratio of Sodium α-β Glycerophosphate. Contour plot (Figure 19) and response surface plot (Figure 20) of all the responses shows the curvature with change in the factor indicating Film formation time were influenced by change in both the factors like concentration of Ethanol and Ratio of Sodium α-β Glycerophosphate. Contour plot (Figure 21) and

Table 3: Calibration Curve of Tuftsin in Phosphate buffer pH 6.8 (*n*=3).

Sl. No.	Concentration (ng/mL)	Area			Average
		I	II	III	
1	200	174.76	174.75	175.76	174.76±1.23
2	400	351.65	352.68	351.65	351.65±1.56
3	600	520.94	521.69	520.94	520.94±1.25
4	800	708.40	708.40	709.25	708.40±1.55
5	1000	889.77	890.34	889.75	889.77±1.45

Correlation coefficient = 0.999 Equation: y = 0.893x - 6.972

Table 4: Calibration Curve of Ascorbic acid in Phosphate buffer pH 6.8 (*n*=3).

Sl. No.	Concentration (µg/mL)	Absorbance			Average
		I	II	III	
1	2	0.175	0.176	0.175	0.175±1.36
2	4	0.299	0.299	0.297	0.299±2.12
3	6	0.451	0.451	0.467	0.451±1.25
4	8	0.598	0.599	0.597	0.598±1.58
5	10	0.755	0.757	0.75	0.755±1.22

Correlation coefficient = 0.998 Equation: y = 0.073x + 0.017

Table 5: Calibration Curve of Zn Gluconate in Phosphate buffer pH 6.8 (*n*=3).

Sl. No.	Concentration (µg/mL)	Absorbance			Average
		I	II	III	
1	0.1	0.02	0.022	0.036	0.02±1.36
2	0.5	0.24	0.255	0.24	0.24±1.25
3	1	0.48	0.583	0.482	0.48±1.38
4	2	0.901	0.902	0.903	0.901±1.38

Correlation coefficient = 0.9994 Equation: y = 0.45426x+0.0064

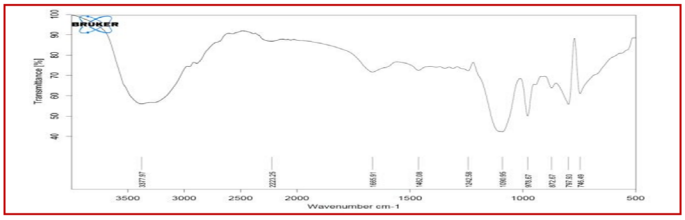

Figure 7: IR Spectra of Na α-Glycerophosphate.

response surface plot (Figure 22) of all the responses shows the curvature with change in the factor indicating Swelling ratio were influenced by change in both the factors like concentration of Ethanol and Ratio of Sodium α-β Glycerophosphate. Contour plot (Figure 23) and response surface plot (Figure 24) of all the responses shows the curvature with change in the factor indicating Water retention capacity were influenced by change in both the factors like concentration of Ethanol and Ratio of Sodium α-β Glycerophosphate. Overlay plot (Figure 25) obtained from design expert, the yellow area in which optimized formulation can be formulated. In this yellow portion, the values of both the variables i.e., Ethanol conc. and Sodium α-β Glycerophosphate ratio was selected. Here the yellow portion covered almost (+1, -1) point. So F_3 batch fully covered yellow region. It can be concluded that F_3 formulation is the optimized formulation out of all formulation. Check point batch was prepared by Ethanol concentration of 45% and Sodium α-β Glycerophosphate ratio of 50:50 (C_1 batch). The result obtained from the performed check point batch which was similar to the actual check point batch which was obtained from

Table 6: Solubility of (A) Tuftsin, (B) Ascorbic acid, and (C) Zn gluconate (D) in different medium.

Sl. No.	Ingredients	Vehicle	Solubility (mg/mL)
1	Chitosan	Water	0
2		0.1M Acetic acid	15
3		50% Ethanol (water)	5
4		50% Ethanol (0.1M Acetic acid)	10
5		1M Acetic acid	60
6		50% Ethanol (1M Acetic acid)	30
7		10M Acetic acid	100
8		50% Ethanol (10M Acetic acid)	70
9	Glycerophosphate	Water	500
10	Tuftsin	50% Ethanol	0.1 (100µg)
11	Ascorbic acid	50% Ethanol	1
12	Zinc gluconate	10M Acetic acid	200

Table 7: Composition and Responses for 3^2 Factorial design for 50% of alpha – beta Glycerophosphate ratio.

Batch	Variable level in coded form		Variable level in actual form		Response variables				
	X_1 ethanol	X_2 Beta: Alpha GP ratio	X_1 ethanol	X_2 (Beta :Alpha GP ratio)	pH	Gelling time (sec)	Film formation time (sec)	Swelling ratio% (10 min)	Water retention capacity% (24 hr)
F1	-1	-1	0.15	50:50	5.2±0.03	468.6±22.45	776±19.39	658±6.63	9.82±1.00
F2	0	-1	0.3	50:50	5.5±0.04	420.4±18.66	720±19.77	458±8.00	6.53±0.90
F3	+1	-1	0.45	50:50	5.9±0.02	181±18.50	421±18.97	272±6.63	5.8±0.82
F4	-1	0	0.15	75:25	6.3±0.04	840.6±18.98	1178±19.08	442±6.63	12.64±1.00
F5	0	0	0.3	75:25	6.2±0.03	720±18.97	890±21.45	342±6.62	9.32±1.00
F6	+1	0	0.45	75:25	6.4±0.02	482±19.08	724±19.39	225±7.24	7.52±0.92
F7	-1	+1	0.15	0:100	6.5±0.03	1248±22.45	1616±19.39	282±6.64	26.30±1.00
F8	0	+1	0.3	0:100	6.6±0.02	1180±18.97	1556±19.39	208±6.63	22.24±0.92
F9	+1	+1	0.45	0:100	6.8±0.02	1022±19.08	1510±21.45	142±6.62	16.21±0.83

the D.O.E software without any significant differences. From the result, after comparing all the parameters with F3 batch that was similar and no significant differences between performed, actual, and optimize batch. So F3 batch was selected as the optimized batch out of nine batches. The overlay plot (Figure 25) obtained from design expert.

For optimization of drug loaded vehicle in which optimize vehicle formulation (F_3 batch) was selected on which different concentration of Zn gluconate dose was incorporate and optimized the batch with trial-and-error method (Tables 8-13). The highest dose was incorporated in X_2 batch without clumping in gel. So X_2 batch was selected as a optimize batch for drug loaded vehicle. This was the final formulation of thermoreversible chitosan hydrogel. Table 14 shows the final optimized concentration of the components. Using these above components and their respective concentration the final optimized formulation was prepared. The gelling time of optimized formulation were found to be 160+19.5. It was concluded that Optimized formulation rapid converted in to gel at 37°C. The film formation time of optimized formulation was found to be 390±18.4. It was concluded that Optimized formulation rapid converted in to film at 37°C. The swelling ratio of optimized formulation was found to be 296±7.58. It was concluded that Optimized formulation has good swelling capacity at 37°C.The Moisture retention capacity of optimized formulation was found to be 6.8±0.76 (Table 15).

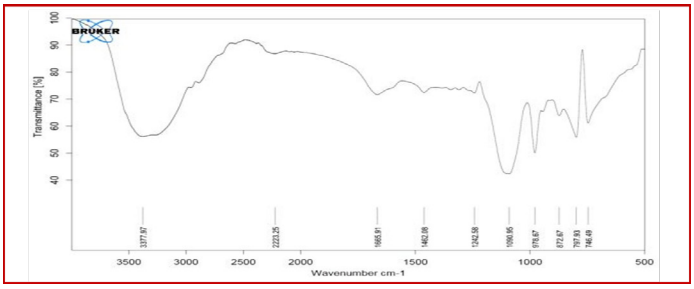

Figure 8: IR Spectra of Chitosan + Na α- Glycerophosphate.

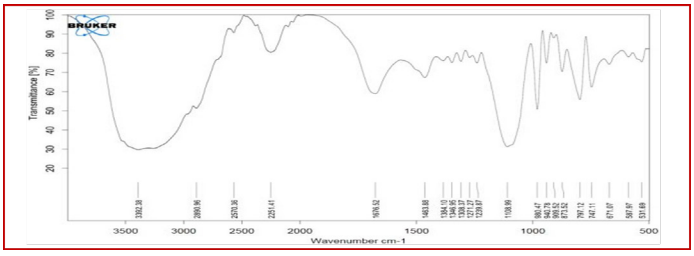

Figure 9: IR Spectra of Na β-Glycerophosphate.

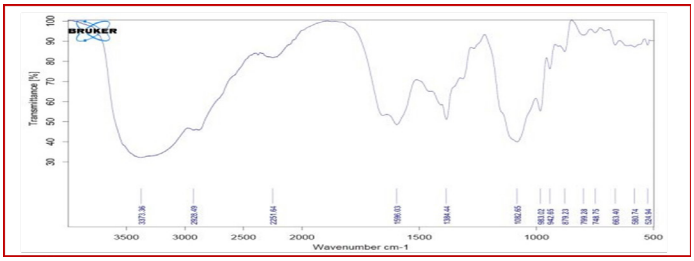

Figure 10: IR Spectra of Chitosan + Na β- Glycerophosphate.

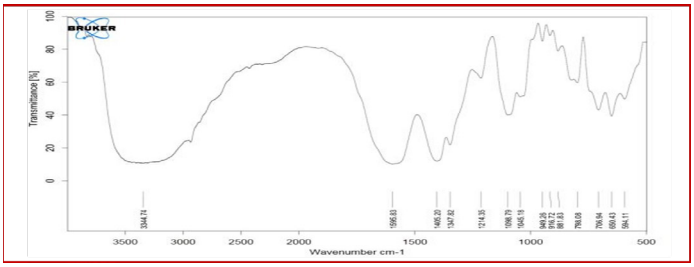

Figure 11: IR Spectra of Zn gluconate.

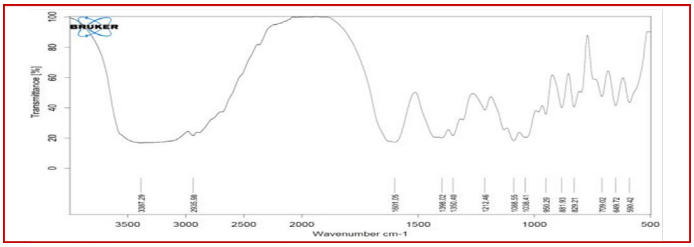

Figure 12: IR Spectra of Chitosan + Zn gluconate.

It was concluded that Optimized formulations have good Moisture retention capacity after incubation at 37°C for 24 hr. The pH of optimized formulation was found to be 6.7±0.05.

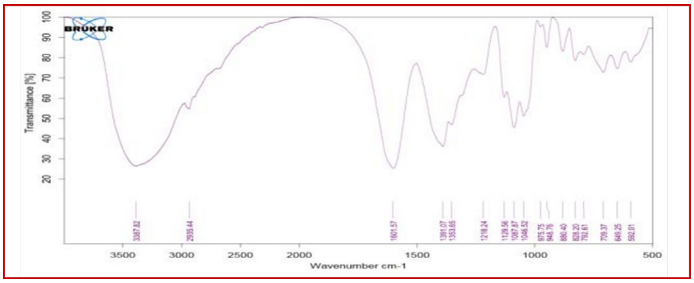

Figure 13: IR Spectra of Ascorbic acid.

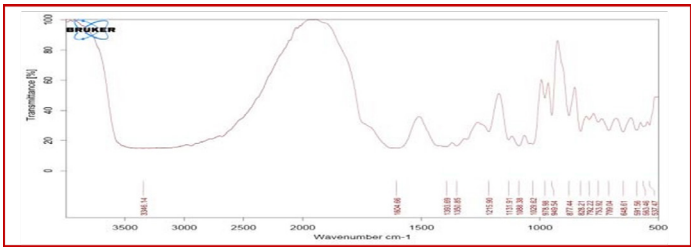

Figure 14: IR Spectra of Chitosan + Ascorbic acid.

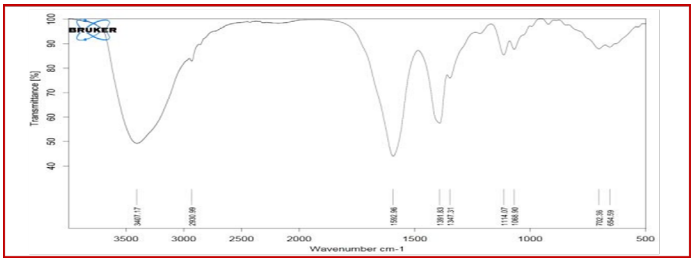

Figure 15: IR Spectra of Tuftsin.

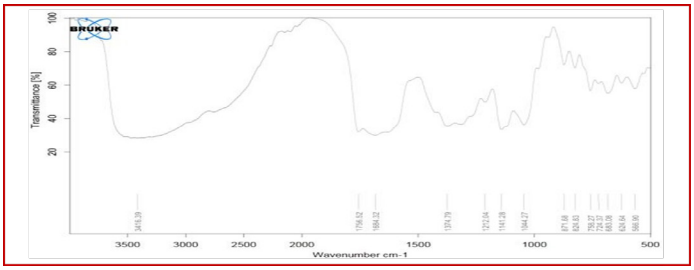

Figure 16: IR Spectra of Chitosan+Tuftsin.

It was concluded that Optimized formulations was compatible to skin pH. So, it was concluded that there was no irritation to the skin. This optimize formulation was compatible to the skin without irritation.

In vitro drug release data of optimize batch was 86.5±1.9%, 80.66±4.52, 85.55±2.95 in 24 hr by Ascorbic acid, Tuftsin, Zn gluconate respectively (Tables 16 and 17). So, this result was given sustained release action of Drug loaded thermoreversible chitosan hydrogel formulation. Drug content of optimize formulation (Table 18) in pourable liquid was found to be 93%, 90%, 95.5% and in film 90%, 90%, 92% for Tuftsin, Ascorbic acid, and Zn gluconate respectively. So, Drug content was found to be within

limits. It was concluding that % of drug was present in pourable liquid and in film form of optimize hydrogel formulation.

Antimicrobial assay was performed by cup plate method. In Figure 26 (a) showed that 100% antibacterial efficacy of *S. aureus* by hydrogel formulation with or without drug. Tetracycline (30µg/mL) was used as standard for *S. aureus* organism and it showed zone of inhibition. Figure 26 (b) Showed that 100% antibacterial efficacy of *E. coli* by hydrogel formulation with or without drug. Cephotexime (10µg/mL) was used as standard for *E. coli* organism and it showed also totally inhibition. Antimicrobial study was concluded that thermoreversible chitosan hydrogel film has good antimicrobial action against *S. aureus* and *E. coli*. So, these formulations have good antimicrobial capacity.

Optimize film forming thermoreversible Chitosan hydrogel were liquid at room temperature 25°C and gel at 37°C. The stability data (Tables 19-21) showed that the optimized batch of thermoreversible chitosan hydrogel remained stable in terms of Gelling time, Film formation time, Selling ratio, Moisture retention capacity and drug content in pourable liquid and Film at the end of 1 month. Thus, it was observed that the formulation was stable throughout 1 month.

On 10th day after incision, marketed formulation treated and normal rats showed reduction in length of wounds but remained

Table 8: Comparison of result of performed check point batch (C_1 batch) with Actual check point batch (C_2 batch) and Optimize batch (F_3 batch).

	Gelling time (Sec)	Film formation Time (Sec)	Swelling Ratio (%)	Moisture retention capacity (%)
Performed check point batch (C_1 batch)	185.18	428.36	272.36	5.6
Actual check point batch (C_2 batch)	193.16	430.06	271.54	5.4
Optimize batch (F_3 batch)	181	421	272	5.8

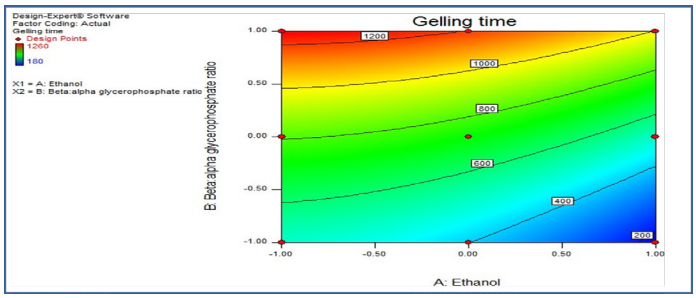

Figure 17: Contour plot of Gelling time.

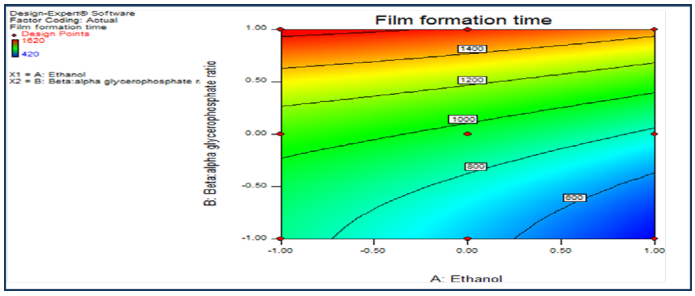

Figure 19: Contour plot of Film formation time.

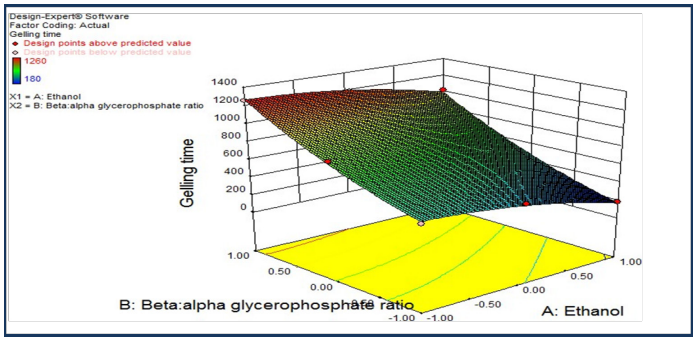

Figure 18: Response surface plot of Gelling time.

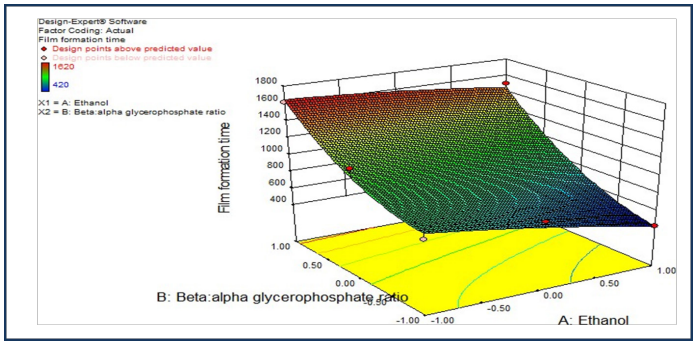

Figure 20: Response surface plot of Film formation time.

Table 9: Optimization of Drugs Loaded Vehicle.

Sl. No	Vehicle (Chitosan 2%, 50% GP, ethanol 45%)	Tuftsin (µg/mL)	Ascorbic acid (mg/mL)	Zinc gluconate (mg /mL)	pH	Gelling time (sec)	Film formation time (sec)	Swelling ratio (%10 min.)	Moisture retention capacity (% 24 hr.)
X1	Optimize vehicle formulatio n (F batch)	10	0.1	20	Precipitation in pourable liquid and clumping in gel				
X_2		10	0.1	2	6.7±0.05	160±19.5	390±18.4	296±7.58	6.8±0.76
X_3		10	0.1	0.2	6.3±0.06	190±17.35	440±20.72	282±5.65	5.6±0.74

Table 10: Formulation of optimized batch (X_2).

Formulation components	Amount
Ascorbic acid	0.1mg
Tuftsin	10 (µg/mL)
Zn gluconate	2mg
Chitosan	2%
Acetic acid: Water	(1:1) 0.4mL
Ratio of β and α-glycerophosphate	(50:50), 50%, 0.1mL
Ethanol	45% (0.45mL)
Water	Up to 1mL

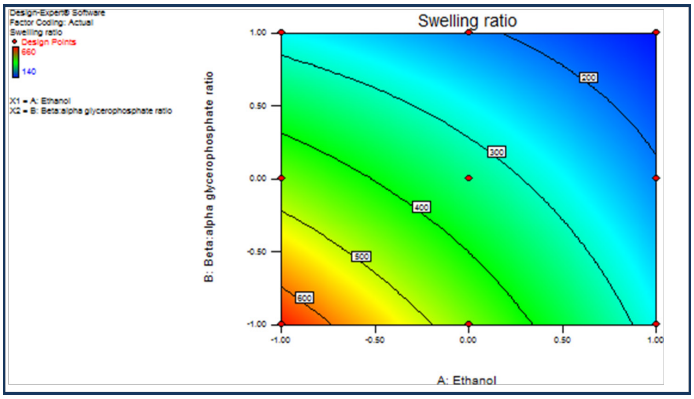

Figure 21: Contour plot of Swelling ratio.

Table 11: Results of optimized batch (*n*=3).

Gelling time (sec)	Film formation time (sec)	Swelling ratio (%)	Moisture retention capacity (%)	pH
160±19.5	390±18.4	296±7.58	6.8±0.76	6.7±0.05

Table 12: *In vitro* release profile of optimized formulation.

	Ascorbic acid	Tuftsin	Zn Gluconate
Media	Phosphate buffer pH- 6.8		
Volume	25mL		
Sample volume	2mL		
Dilution factor	No dilution	No dilution	50mL
Slope	0.073	Y=0.893X-6.972	0.4542
Label value	0.1mg	0.01mg	2mg

uncured. Whereas vehicle treated, test formulation showed (Figure 27) complete healing of wound. Apart from this, test formulation treated rats showed rapid hair growth as compare to vehicle treated rats. For the purpose of drug loaded chitosan hydrogel film promote process of hair growth and wound healing as compare to another group. Thus, drug loaded chitosan hydrogel film was effective for proper wound healing.

DISCUSSION

High Performance Liquid Chromatography, UV-visible Spectrophotometer, Atomic absorption Spectrophotometer methods used for calibration curve of Tuftsin, Ascorbic acid and Zn gluconate respectively. It was validated to be linear, precise and reproducible. Plotted calibration curve has shown the R^2 value of 0.999, 0.998, 0.999 for Tuftsin, Ascorbic acid and Zn gluconate

Table 13: *In vitro* drug release (%, mean + SD, n=3) of optimized drugs loaded film forming thermoreversible hydrogel.

Time (Hr)	Ascorbic acid	Tuftsin	Zn gluconate
2	30.42±4.23	22.8±4.23	27.36±2.89
4	41.57±7.65	33.7±7.65	36.97±2.05
6	52.63±6.45	41.4±6.45	46.87±2.76
8	63.78±2.35	49.5±5.22	54.35±2.09
10	72.98±2.94	58.4±4.39	65.67±2.98
12	79.65±2.54	69.7±5.8	76.82±3.25
24	86.5±1.9	80.66±4.52	85.55±2.95

Table 14: Drug content of optimized formulation.

Sl. No.	Formulation	Tuftsin (µg/mL) (*n*=3)			Ascorbic acid (mg/mL) (*n*=3)			Zn gluconate (mg/mL) (*n*=3)		
		Actual amount	Estimated amount	Drug content (%)	Actual amount	Estimated amount	Drug content (%)	Actual amount	Estimated amount	Drug content (%)
1	Pourable liquid form	10	9.3±2.59	93	0.1	0.09±0.002	90	2	1.91±0.22	95.5
2	Film form	10	9±3.9	90	0.1	0.09±0.004	90	2	1.84±0.38	92

Table 15: Stability study after 1 month for optimize formulation.

Temperature	pH	Gelling time (sec)	Film formation time (sec)	Swelling ratio (%10 min.)	Moisture retention capacity (% 24 hr.)
Refrigeration Temperature (5°C+ 3°C)	6.7±0.06	160±20.5	390±19.5	296±8.52	6.8±0.84
Room temperature (25°C+2°C/ 60%RH + 5% RH)	6.8±0.05	159±21.5	391±20.4	298±5.25	6.9±0.75

respectively. Coefficient of correlation (R^2) value obtained from all three methods indicated that higher linearity of the curve. Preformulation study of Tuftsin, Ascorbic acid and Zn gluconate was performed to check the solubility and drug-excipient interaction.

The result has shown that Tuftsin, Ascorbic acid and Zn gluconate has a high 50% ethanol and 10M acetic acid respectively. Chitosan have solubility in 50% ethanol in 10M acetic acid. Glycerophosphate have solubility in water. From the FTIR spectra of Chitosan (polymer) with Na α-Glycerophosphate, Na β-Glycerophosphate, Ascorbic acid, Zn gluconate and Tuftsin were remained unaffected in FTIR spectrum of drug alone and physical mixture with other components indicating no chemical interaction between drug, polymer and other component. Ethanol has been selected as a vehicle for optimization of formulation. Total nine batches were prepared by 3^2 factorial designs with different conc. of ethanol and ratio of α-β–GP and final vehicle of the formulation was optimized for the desired Gelling time, Film formation time, Swelling ratio and Moisture retention capacity. The result of all nine batches were compared and it can be concluded that the formulation F_3 having Ethanol conc. of 45% and α-β–GP ratio of 50:50(50%) shown optimum results of all the parameters. Factor influence study and optimization of the vehicle formulations were done by Design Expert® 8.0.7.1 software. From the result it has been concluded that the both the independent factor i.e., Ethanol (X_1) and α-β–GP ratio (X_2) have significant effect on dependent variables. From the overlay plot obtained from the Design Expert® 8.0.7.1, it has been clear that the formulation F_3 is the optimize formulation. From the result of gelling time and film formation time, by increasing ethanol concentration decreases both the factor and increasing Glycerophosphate ratio increase both the factor. From the result of swelling ratio, by increase ethanol concentration and Glycerophosphate ratio decreasing Swelling ratio. From the result of Moisture retention capacity, by that increase ethanol concentration decreasing Water retention capacity and increase Glycerophosphate ratio

Table 16: Drug content in optimized formulation after 1 month Refrigeration Temperature (5°C+ 3°C).

Sl. No.	Formulation	Tuftsin (µg/mL) (*n*=3)			Ascorbic acid (mg/mL) (*n*=3)			Zn gluconate (mg/mL) (*n*=3)		
		Actual amount	Estimated amount	Drug content (%)	Actual amount	Estimated amount	Drug content (%)	Actual amount	Estimated amount	Drug content (%)
1	Pourable liquid form	10	9.3±1.59	93	0.1	0.09±0.003	90	2	1.91±0.23	95.5
2	Film form	10	9±2.8	90	0.1	0.09±0.005	90	2	1.84±0.41	92

Table 17: Drug content in optimized formulation after 1 month Room temperature (25°C±2°C/ 60% RH ± 5% RH).

Sl. No.	Formulation	Tuftsin (µg/mL) (*n*=3)			Ascorbic acid (mg/mL) (*n*=3)			Zn gluconate (mg/mL) (*n*=3)		
		Actual amount	Estimated amount	Drug content (%)	Actual amount	Estimated amount	Drug content (%)	Actual amount	Estimated amount	Drug content (%)
1	Pourable liquid form	10	9.2±1.59	92	0.1	0.09±0.006	90	2	1.91±0.52	95.5
2	Film form	10	9±2.8	90	0.1	0.09±0.003	90	2	1.84±0.67	92

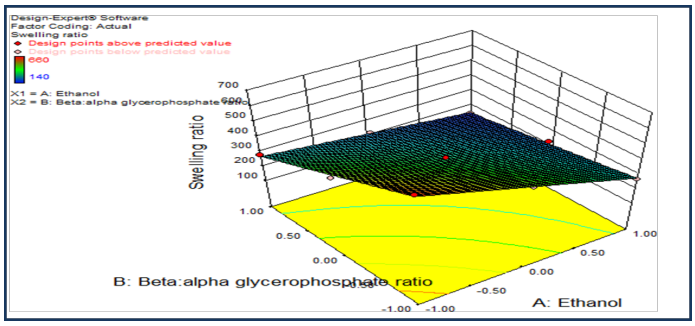

Figure 22: Response surface plot of Swelling ratio.

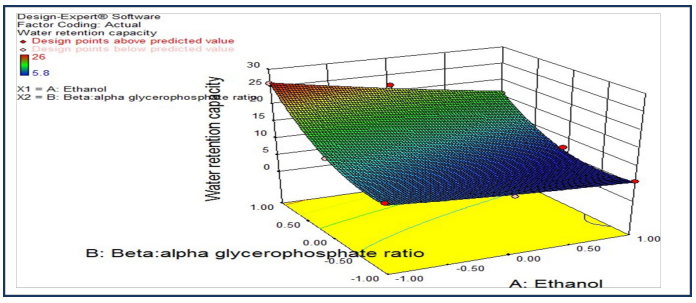

Figure 24: Response surface plot of Water retention capacity.

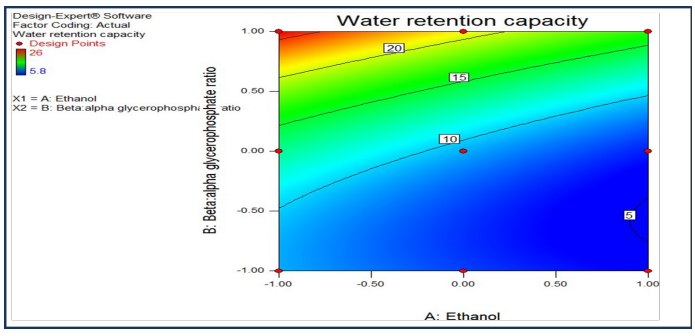

Figure 23: Contour plot of Water retention capacity.

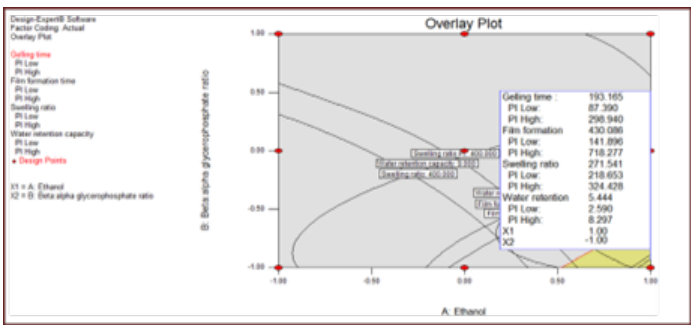

Figure 25: Overlay plot of Gelling time, Film formation time, Swelling time, Water retention capacity.

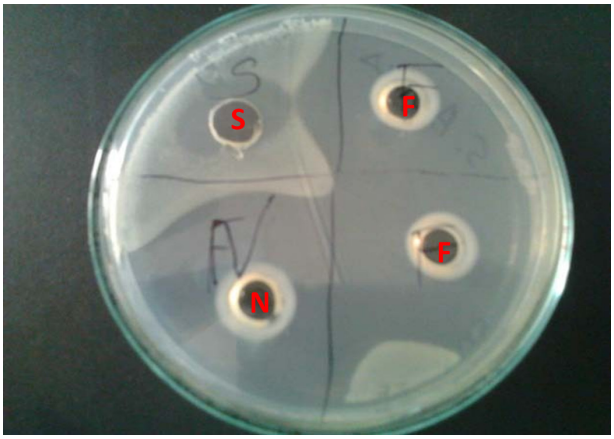

Figure 26: (a) Antimicrobial assay *S. aureus* (ATCC29213).

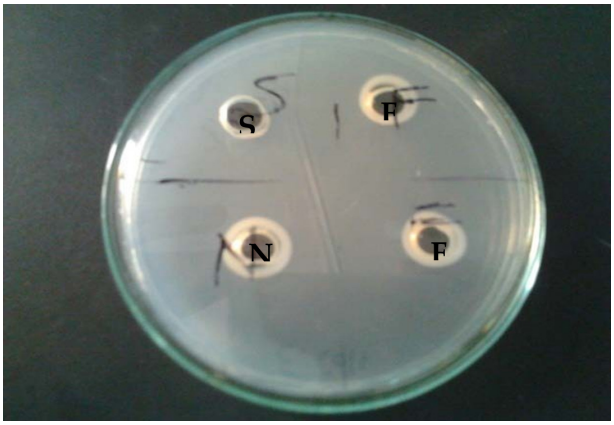

Figure 26: (b) Antimicrobial assay *E. coli* (ATCC 87064).

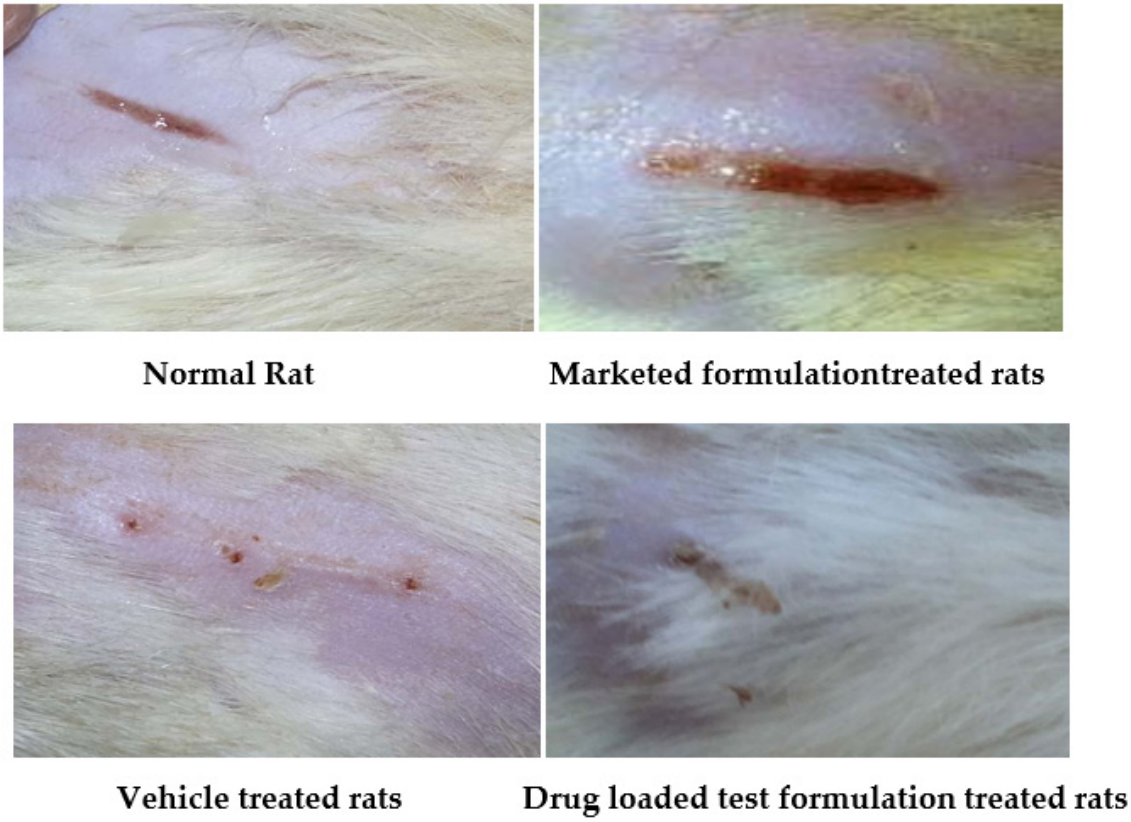

Figure 27: Morphology of wound at 10th day after incision.

increase Water retention capacity. The result of Gelling time, Film formation time, swelling ratio and Moisture retention capacity of the optimize vehicle formulation was 181±18.50, 421±18.97, 272±6.63, 5.8±0.82 respectively. Factor influence study and optimization of drug loaded thermoreversible chitosan hydrogel were done by Trial-and-Error method. Different dose of Zn gluconate was optimized in optimized vehicle formulation. 0.2mg, 2mg, 20mg, ratio of Zn gluconate was used. Tuftsin (10 µg/mL) and Ascorbic acid (0.1mg/mL) were used. Three batchesX_1, X_2, X_3 with 0.2mg, 2mg, 20mg, ratio of Zn gluconate was prepared by trial-and-error method. In the X_3 batch Precipitation in pourable liquid and clumping in gel was formed because of 20mg dose of Zn gluconate. So, the highest dose was incorporated in X_2 (2mg) batch. X_2 batch was selected as an optimized drug loaded batch of thermoreversible chitosan hydrogel. The result of Gelling time, Film formation time, swelling ratio and Moisture retention capacity of the optimize drug loaded formulation 160±19.5, 390±18.4, 296±7.58, 6.8±0.76 was respectively. pH of the optimize formulation was 6.7±0.05. The *in vitro* release of optimize drug loaded hydrogel formulation was carried out by Franz diffusion cell. *In vitro* drug release data of optimize batch was 86.5±1.9%, 80.66±4.52, 85.55±2.95 in 24 hr by Ascorbic acid, Tuftsin, Zn

gluconate respectively. From the result it can be concluded that optimized formulation was given sustained release action. Drug content of optimize formulation in pourable liquid was found to be 93%, 90%, 95.5% and in film 90%, 90%, 92% for Tuftsin, Ascorbic acid, and Zn gluconate respectively. So, Drug content was found to be within limits. It was concluding that % of drug was present in pourable liquid and in film form of optimize hydrogel formulation. Antimicrobial assay was performed by cup plate method. Chitosan hydrogel showed powerful antibacterial efficacy up to 100% to *staphylococcus aureus* (ATCC 29213) and *Escherichia coli* (ATCC 87064) by hydrogel formulation with or without drug. Tetracycline (30µg/mL) was used as standard for *S. aureus* organism and it showed zone of inhibition. Cephotexime (10µg/mL) was used as standard for *E. coli* organism and it showed 100% antibacterial efficacy. Antimicrobial study was concluded that thermoreversible chitosan hydrogel film has good antibacterial efficacy against *S. aureus* and *E. coli.* So, these formulations have good antibacterial efficacy. Stability studies of drug loaded thermoreversible chitosan hydrogel were carried out at refrigerated (5°C± 3°C) and controlled room temperature (25°C±2°C/ 60%RH ± 5% RH) for 1 month. The stability data showed that the optimized batch of Thermoreversible chitosan hydrogel remained stable in terms of Gelling time, Film formation time, Selling ratio, Moisture retention capacity and drug content in pourable liquid and Film at the end of 1 month. Thus, it was observed that the formulation was stable throughout 1 month. Finally *in vivo* wound healing study carried out in (*n*=24) rats, normal rats, marketed formulation treated rats, vehicle formulation treated rats and drug loaded thermoreversible chitosan hydrogel film treated rats. After 10th day the *in vivo* data showed that drug loaded chitosan hydrogel film promote process of hair growth and wound healing as compare to another group. Thus, drug loaded chitosan hydrogel film was effective for proper wound healing.

CONCLUSION

For the vehicle optimization different conc. of ethanol and ratio α-β–GP was selected based on Gelling time, Film formation time, Swelling ratio and Moisture retention capacity. Optimization of vehicle in formulation was done by 3^2 factorial designs by using Design Expert® 8.0.7.1 software by evaluating Gelling time, Film formation time, Swelling ratio and Moisture retention capacity. Multiple regression analysis of the data produced equations that accurately describe the impact of particular variables, such as ethanol concentration and ratio α-β-GP, on the responses under study. Antibacterial study was concluded that thermoreversible chitosan hydrogel film has good antibacterial efficacy against *S. aureus* (ATCC 29213) and *E. coli* (ATCC 87064). So, this formulation has good antibacterial efficacy. As per ICH guideline, developed formulation was stable for 1 month. Drug loaded chitosan hydrogel film promote process of hair growth and wound healing as compare to the other group. Thus, drug loaded chitosan hydrogel film formulation was effective for proper wound healing. Furthermore, preclinical and clinical studies are required to be done in order to approve marketing authorization.

ABBREVIATIONS

GP: Glycerophosphate; **RH:** Relative Humidity; **ATCC:** American Type Culture Collection; **UV:** Ultraviolet; **ANOVA:** Analysis of Variance; **ICH:** International Council for Harmonization; **Zn:** Zinc; **HPLC:** High-performance liquid chromatography; **M:** Molarity; **FTIR:** Fourier transform infrared.

CONFLICT OF INTEREST

The authors declare no conflict of interest..

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Cite this article: Vaishali B, Jadav M, Jha LL. Formulation and Optimization of Instant Film Forming Thermoreversible Chitosan Hydrogel for Wound Healing. J Young Pharm. 2023;15(3):465-77.