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PREPARATION AND EVALUATION OF WOUND HEALING ACTIVITY OF URSOLIC ACID NANOEMULGEL FORMULATIONS IN RATS

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ABSTRACT
In this study, we employed a mixture of the surfactants poloxamer 407 with tween 80 to produce a nanoemulgel containing ursolic acid (UA) aimed to study the effect of each independent variable on dependent variables using Stat-Ease Design Expert software (DX10). The effects of the three factors (Water, oil and S: CoS) on the globule size, drug content, PDI, spreadability, gel strength were tested. Through preliminary screening the water, oleic acid and surfactant/Co-surfactant ratio were identified as the most significant variables within the range of 58-60g, 10-12g and 18-20g, respectively. These results clearly indicate that all the dependent variables are strongly dependent on the selected independent variables as they show a wide variation among the 14 batches.

Keywords: Ursolic Acid, Nanoemulgel**,** Design Expert software, wound healing activity

INTRODUCTION
Ursolic acid, is a constituent of Lantana camara (Verbenaceae) and possesses high medicinal value. It is used in several herbal medicines marketed in Asia and worldwide for inflammatory conditions [1–3]. Chemically, it is a pentacyclic triterpenoids ensuring chemical name (3ß-hydroxy-urs-12-en-28oic acid) and used for numerous pharmacological activities such as anti-inflammatory activity [4,5], trypanocidal, antiviral, antioxidant, and anti tumour activities [6]. It possesses strong anti-inflammatory activity by inhibiting the activity of COX-2, also inhibiting the TNF-induced activation of NF-κB in Jurkat cells as well as NF-κB transcription in human T lymphocytes [4,7]. The therapeutic efficacy of UA is limited due to its poor oral bioavailability. The poor oral bioavailability of UA has been attributed to its poor aqueous solubility and extensive first pass metabolism [8]. Therefore, for effective treatment of arthritis there is a need to develop a formulation to enhance the bioavailability and therapeutic efficacy. Regrettably, oral formulations have a number of limitations such as extensive first pass metabolism and gastrointestinal irritation. For these purposes, advanced localized and transdermal delivery has extended a lot of importance these days [9,10].

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Emulgels are emulsions, either of the oil-in water or waterin- oil, which are filled by mixing with a gelling agent. In fact, the presence of a gelling agent in the water phase converts a classical emulsion into an emulgel [11,12]. Generally, direct (oil-in-water) system is used to entrap lipophilic drugs whereas hydrophilic drugs are encapsulated in the reverse (water-in oil) system.

In this study, we have expand a drug delivery system called nanoemulgel through a novel formulation strategy, which utilizes the ‘‘Multi Absorption Mechanism’’ (MAM) concept and has a broad applicability. Nanoemulgel contains of two varieties of matrices; A & B. Matrix A comprises the nanoemulsion whilst matrix B includes the nanomicelles. The premise of the present study is that every nano drug delivery system is distinctive and its rate, extent and mechanism of absorption depend on the size, charge and composition of the nano drug delivery system. So, when a combination of entirely different drug delivery systems is used for the delivery of a drug, the absorption of the combined system would be healthie than either of the individual drug delivery systems due to the utilization of the maximum favorable paths of absorption available for that particular drug (Figure. 1).

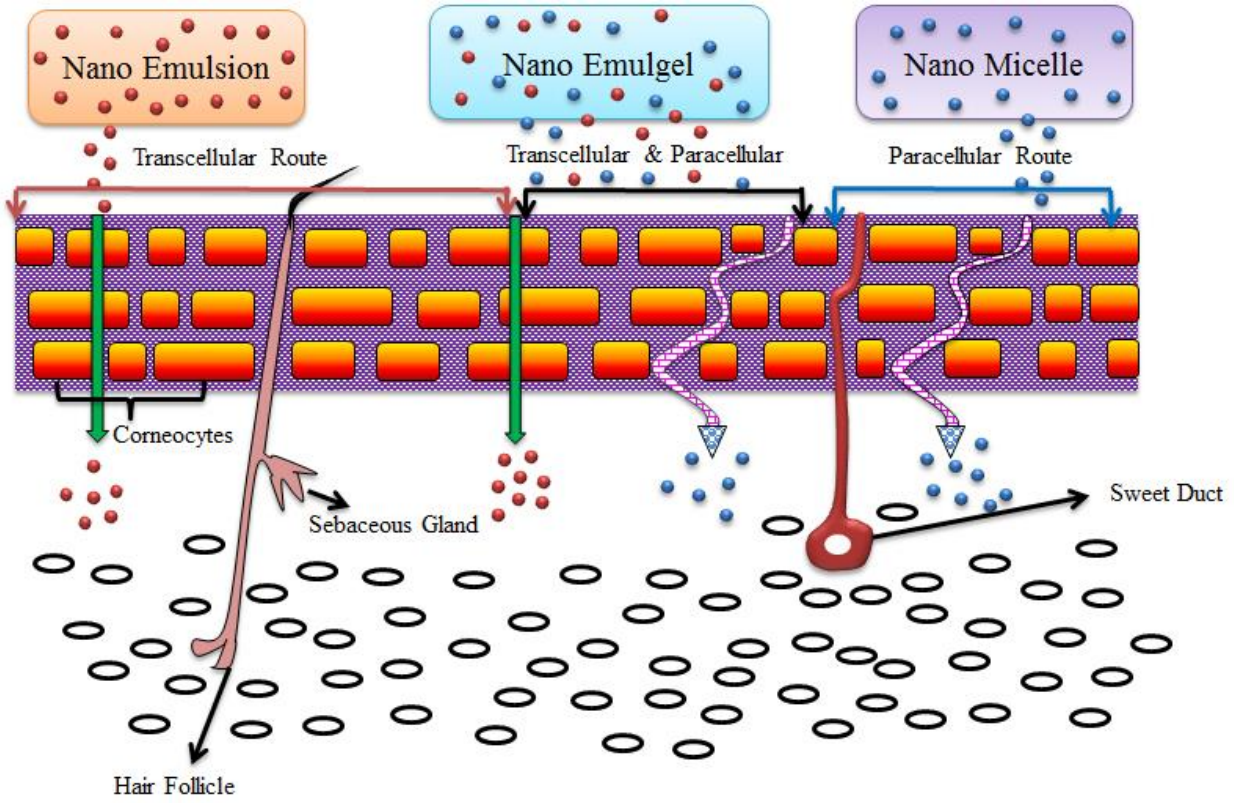
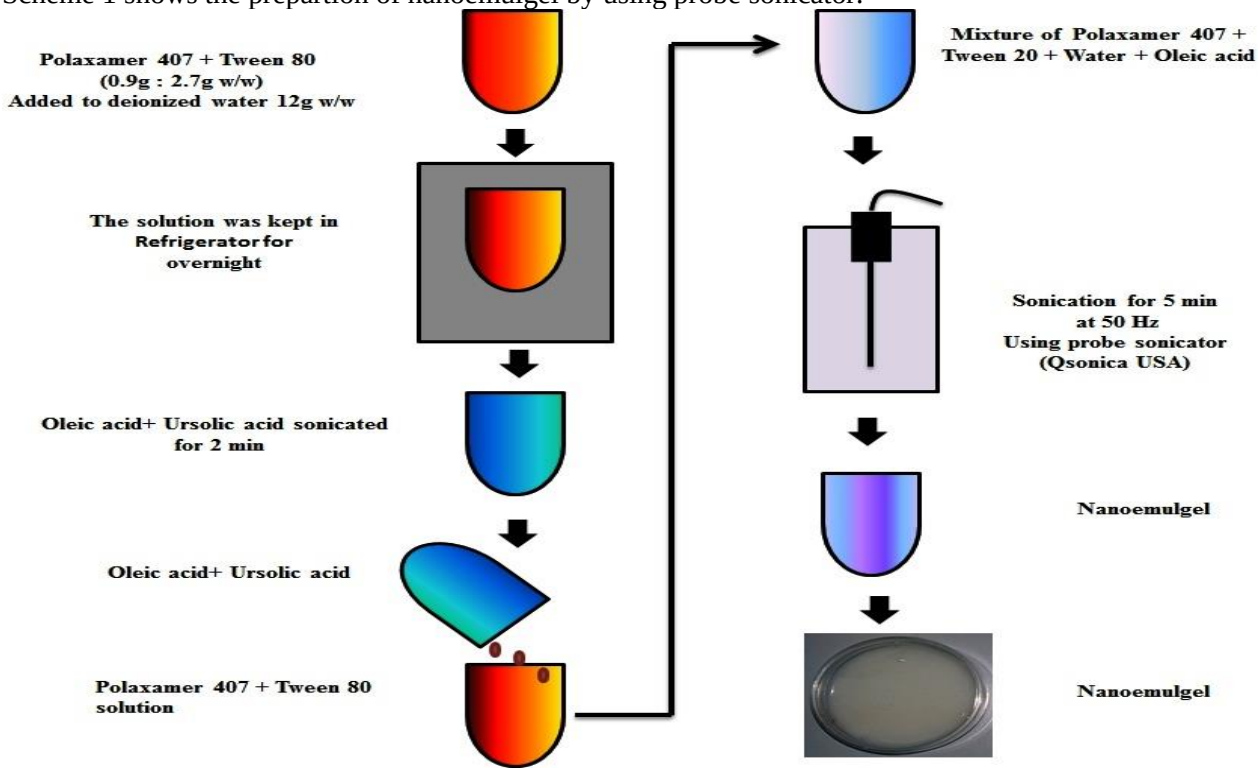


Figure -1: Nanoemulgel and its hypothetical paths of entry into the skin

METHOD OF PREPARATION

Scheme 1 shows the prepartion of nanoemulgel by using probe sonicator.



Scheme 1. Schematic representation of urosolic acid nanoemulgel preparation

MATERIAL AND METHODS

Materials:

Urosolic acid (pure) obtained from Sigma-Aldrich Co., Tween 20 was purchased from R&M Chemical., Poloxamer 188 was purchased from Merck KGaA (Darmstadt, Germany). All water used in the formulation was of Milli-pore grade. HPLC grade of acetonitrile, water and ethanol were purchasedfrom Sigma Aldrich Co (St Louis, MO).

Nanoemulgel glouble size:

The mean droplet size and polydispersity index of the nano-emulsions were determined by dynamic light scattering (DLS). A Malvern 4700 photon correlation spectrometer (Malvern Instruments, Malvern, UK).

Drug content:

Determination of drug content in the samples was conducted using a high performance liquid chromatography (HPLC) method previously developed and validated at our laboratory [13]. The method employed a Shimadzu liquid chromatographic system equipped with a LC-20AD solvent delivery system (pump), SPD-20A photo diode array detector, and SIL-20ACHT injector with 50µL loop volume. The LC solution version 1.25 was used for data collecting and processing (Shimadzu, Japan). The HPLC was carried out at a flow rate of 1.0 ml/min using a mobile that is phase constituted of acetonitrile, 20mm potassium dihydrogen phosphate buffer (40:60, v/v), and detection was made at 215 nm. The mobile phase was prepared daily, filtered through a 0.45µm membrane filter (Millipore) and sonicated before use. A Thermo C18 column (25cm × 4.6mm i.d., 5µ) was used for the separation. The drug content was calculated from the calibration curve and expressed as drug content**.**

Rheological studies:

A rheological property is significant to evaluate and control the flow properties of semisolid pharmaceutical products to ensure quality and effectiveness of the formulation. Rheological analysis of nanoemulgels to test the oscillation stress sweep was performed using Anton paar MCR rheometer (Malaysia) with cone- plate geometry sensor with the diameter of the cone being 40 mm and 1° cone angle, operating in the oscillation and static mode. The sample of nanoemulgel to be studied was placed on the plate and left to equilibrate at a controlled temperature (25 ± 0.1°C) for 3 min before bringing the cone down. This was done to ensure the thermal as well as the structural equilibration of all samples [14].

Spreadability:

A small portion of the nanoemulgel was applied on a glass slide and was compressed to uniform thickness by placing 50 g for 5 minutes [15]. The time in which the upper glass slide move over the lower slide was taken as measurement of spreadablility (S)

S= ML/T

Where, M= weight tide to upper slide (g) L= length moved on the glass tide (cm) T= time taken (sec)

Gel Strength:

A sample of 50gm of nanoemulgel was placed in a 100 ml graduated. The apparatus for measuring gel strength (weighing 27 gm) was allowed to penetrate in gel. The gel strength, which means the viscosity of the nanogels was determined by the time (seconds), the apparatus took to sink 5cm down through the prepared gel [16].

Drug diffusion studies:

Franz diffusion cell method was applied using phosphate buffer (pH 5.8) at room temperature for in-vitro drug release studies. A cellophane membrane (dialysis membrane) was used to carry out the study and soaked overnight in phosphate buffer at room temperature to be prepared. The membrane was then placed between donor and receptor compartment of diffusion cell with an exposed membrane surface area of 2.97 cm^2 to the receptor compartment. The receptor compartment was filled with 16.4 ml of freshly prepared ethanolic phosphate buffer (pH 7.4) maintained at 35 ± 0.5°C with constant stirring using a teflon coated magnetic stir bead. One g of proniosomal gel formulation was placed on the membrane and the top of the diffusion cell was covered with paraffin paper. At appropriate time intervals (1, 2, 3, 4, 5, 6, 8, 10, 12 and 24 h), 2 ml aliquots of the receptor medium were withdrawn and immediately replaced by an equal volume of fresh receptor solution to maintain sink conditions. The amount of drug released from was determined by HPLC as mentioned above [17].

FT-IR spectroscopy:

FT-IR spectrum of urosolic acid (pure), poloxamer 407 and physical mixture of urosolic acid & poloxamer 407 were recorded on a Perkin Elmer, Germany. FT-IR spectrometer with 3 scans in the scan range of 10000–370 cm−1.

Animals:

Healthy adult Sprague Dawley rats (160-200 g) of male gender were used for the experiment. The animals were obtained from the Animal House, AIMST University, Malaysia and allowed to adapt to the laboratory conditions. All the experimental

animals were housed at a temperature of 25 ± 2 °C and at a humidity of 30-50% in a 12:12 ± 1 h light-dark cycle. The rats were fed with standard rat pellets and water *ad libitum*. The study was approved by the AIMST University Human and Animal Ethics Committee he study was conducted according to the Animal Research Review Panel guidelines.

Wound healing activity of UA nanoemulgel:

Wound healing activity of formulation was studied using excision wound model. The rats were anesthetized with diethyl ether. Under anesthesia the dorsal skin is shaved and an approximately 3 cm diameter wound was inflicted by cutting and removing the skin. The wound was left undressed for 2 days, exposed to the environment. From the third day after excision, the standard and investigational formulations were applied once a day till the wound healed completely. The wound area was determined as the percentage reduction in the wound area. The progressive changes in the wound area were measured planimetrically by tracing the wound margin at weekly interval. The percentage reduction was calculated using the formula: [Healed area/ Initial area] x 100 [18, 19].

Statistical analysis:

The values are expressed as mean ± standard error of the mean. Statistical difference between normal to control and control to drug treatments were analyzed by One-way analysis of variance followed by Bonferroni test by using Graph pad prism. A $P < 0.05$ was considered statistically significant.

OPTIMIZATION OF PROCESS VARIABLES FOR THE UA NANOEMULGEL

The effects of the three factors (Water, oil and S: CoS) on the globule size, drug content, PDI, spreadability, gel strength were tested. Through preliminary screening the water, oleic acid and surfactant/Co-surfactant ratio were identified as the most significant variables within the range of 58-60g, 10-12g and 18-20g, respectively. On the basis of the preliminary trials a simplex-lattice mixture design was employed to study the effect of each independent variable on dependent variables. The response surfaces of the variables inside the experimental domain were analyzed using Stat-Ease Design Expert software (DX9).

In the UA nanoemulgel development, a three-level 14 full factorial experimental design was used to identify and estimate the main and interaction effects of three different formulation factors water phase (A), oil phase (B), and surfactant: Co-surfactant ratio (1:3) (C) on critical quality attributes of the developed nanoemulgel. Based on the experimental design, the factor combinations yielded different responses as presented in Table 1. These results clearly indicate that all the dependent variables are strongly dependent on the selected independent variables as they show a wide variation among the 14 batches. Mathematical relationship generated in terms of L. pseudo components analysis for the studied variables are expressed.

Following equation expresses mathematical relationship generated using U Pseudo Components for the studied response variables

Globule size *= +163.72* A +164.32* B +184.24* C -12.20* AB -0.64* AC -3.18* BC +104.88 * ABC*

Drug content *= +93.98*A+93.15* B+85.07* C+2.85*AB+1.97* AC+5.10*BC+29.29* A^2BC-7.25*AB^2C-271.22* ABC^2*

PDI *= +0.051* A+0.061* B+0.081* C+3.292E-003* AB+0.19* AC+0.25* BC+9.87* ^2BC+18.51*AB^2C-11.25*ABC^2*

Spreadability *= +2.78* A+2.36* B+0.43* C-1.72*AB-0.98*AC-0.33*BC-16.81*A^2BC+106.13* AB^2C-92.48* ABC^2*

Gel strength *= +71.80*A+74.80* B+39.30* C-4.78* AB-33.35* AC-35.35* BC-295.61* A2BC-655.61* AB^2C+438.49* ABC^2*

The independent variables A, B, C and the quadratic term of A, B and C have significant effects on the globule size, since the Model F-value of 144.80 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, AB, ABC is significant model terms. It was found that all the variables are having interactive effects for the response. Normal % probability plot of the externally studentized residuals plot, Residuals vs. Predicted plot, Residuals vs. Run plot, Actual Response vs. Predicted plot, Box-Cox plot, Residuals vs. A; water, 2D contour plot, 2D real contour plot, 3D response surface plot of the globule size were shown in Figure (1a, b, c, e, f, g, h and i) to depict the interactive effects of independent variables on globule size. Photography 3&4 were shown physical appearance and surface morphology of nanoemulgel formulation.

Table-1: Factorial design of UA nanoemulgel formulations

Run	A:water % (w/w)	B:oil % w/w	C:S:CoS 1:3 % (w/w)	Globule size (nm)	Drug content (%)	PDI	Spreadability gm.cm/sec.	Gel strength (s)
1	60	10	20	164.31	93.86	0.05	2.38	75
2	59.66	10.66	19.66	168.02	91.37	0.33	2.99	53
3	58	12	20	163.03	94.01	0.04	2.55	70
4	58	12	20	164.52	94.06	0.06	2.99	74
5	58.66	11.66	19.66	167.62	91.86	0.24	2.01	55
6	60	12	18	185.04	85.14	0.07	0.43	39
7	60	10	20	164.33	92.56	0.07	2.31	75
8	59.33	11.33	19.33	172.06	89.77	0.31	1.24	51
9	59	11	20	160.44	94.52	0.05	2.01	71
10	60	11	19	173.11	90.61	0.13	1.26	49
11	59	11	20	161.61	94.26	0.06	2.22	74
12	60	12	18	183.06	85.11	0.09	0.41	40
13	59	12	19	173.55	90.24	0.11	1.31	48
14	59.66	11.66	18.66	180.07	84.81	0.08	0.25	43

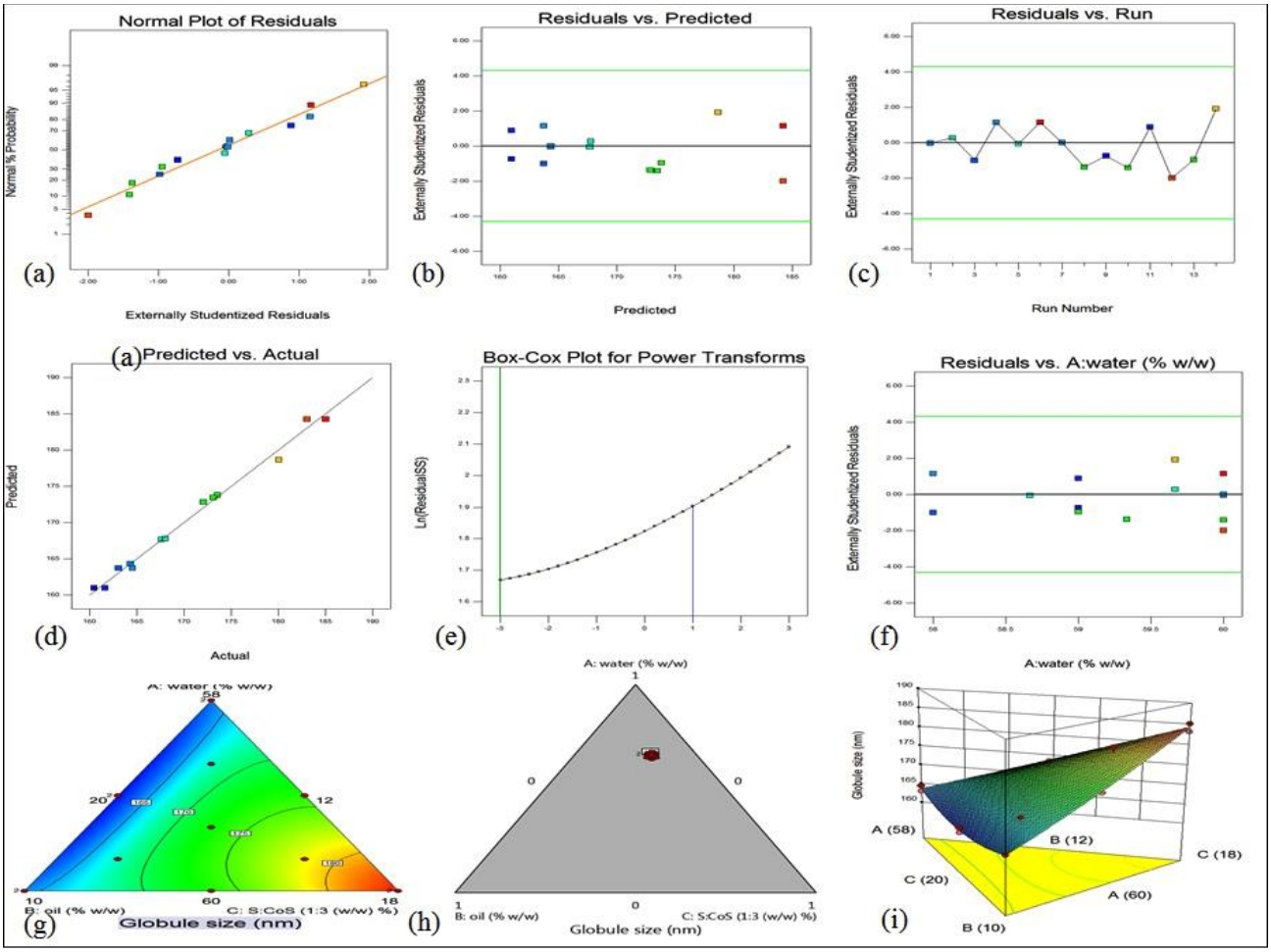


Figure-1: (a) Normal % probability plot, (b) Residuals vs. Predicted Plot, (c) Residuals vs. Run Plot, (d) Actual Response vs. Predicted Plot, (e) Box-Cox Plot, (f) Residuals vs. A; water, (g) 2D contour plot, (h) 2D real contour plot , (i) 3D response surface plot for globule size

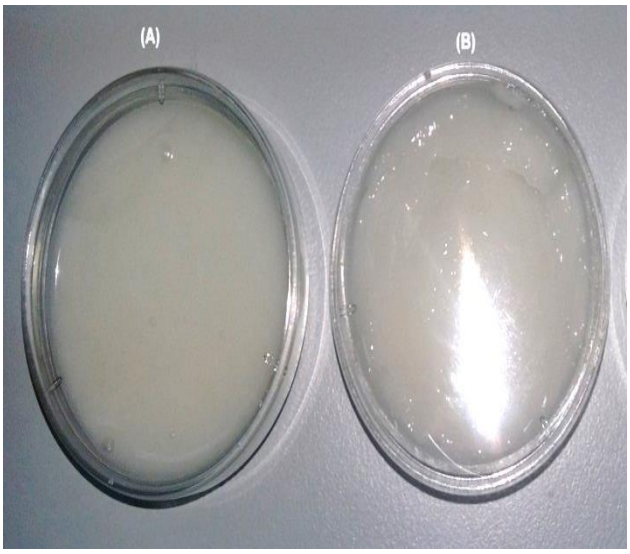


Figure-3: A&B photography shows appearance of nanoemulgels

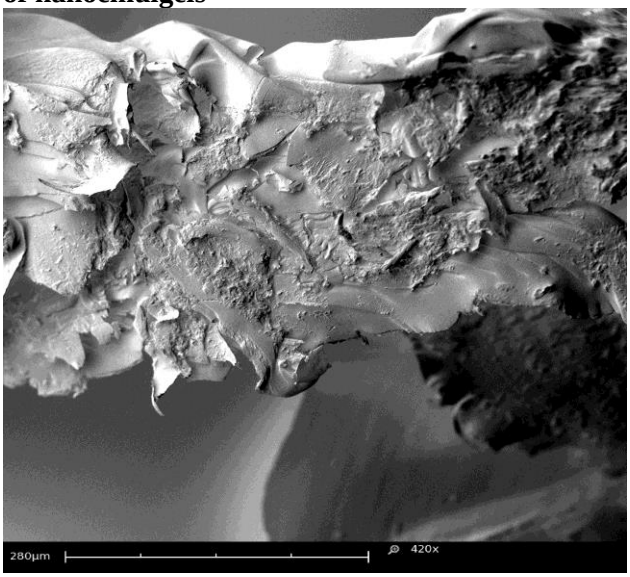


Figure-4: SEM image of nanoemulgel

The PDI values of the formulations prepared as the experimental design are shown in Table 2. The "Pred R-Squared" value for PDI is 0.4630 not as close to the "Adj R-Squared" of 0.9753 as one might normally expect; i.e. the difference is more than 0.2. This may indicate a large block effect or a possible problem with our model and/or data. The influence of the main and interactive effects of independent variables on the particle size was further elucidated using the plots as shown in Figure (5a to 5i).

The two main prerequisites of an gelling system are viscosity and gelling capacity. To instill easily at the affected site the formulation must possess optimum viscosity. Hence, the viscosity of UA nanoemulgel formulations was determined at various shear rates and it was found to be shear thinning systems as shown in Figure 6.

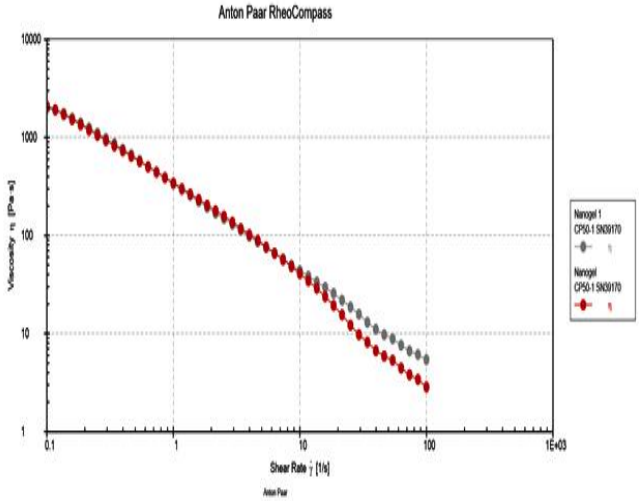


Figure-6: Viscosity vs Shear Rate

The mathematical model generated for % drug content was found to be significant with F-value of 29.08 ($p < 0.0001$) and R2 value of 0.9885. Results of the equation indicate that the effects of A, B, C, ABC^2 are more significant model. Here, the "Pred R Squared" of 0.9790 is in reasonable agreement with the Adj R-Squared of 0.9453. The % drug content range (84.81to 94.52) of nanoemulgel formulations was found to be a ideal in the all runs. The interactive effects of independent variables on % drug content as shown in Figure (7a to 7i).

Gel strength of UA nanoemulgel was found to be in the range of 39–75 seconds as shown in Table 1.The factorial equation for particle size exhibited a good correlation coefficient (1.000) and the Model F value of 36.18 which implies the model is significant. Values of "Prob> F" less than 0.0500 indicate model terms are significant. In this case A, B, C, BC are significant model terms. Gel strength is important because strong gels will support a much higher pressure than weak gels before they are washed out of the targeted site. The Normal % probability plot of the externally studentized residuals plot, Residuals vs. Predicted plot, Residuals vs. Run plot, Actual Response vs. Predicted plot, Box-Cox plot, Residuals vs. A; water, 2D contour plot, 2D real contour plot, 3D response surface plot of the gel strength were shown in Figure (6a to 6i) to depict the interactive effects of independent variables on gel strength.

The values of spreadability indicate that the gel is easily spreadable by small amount of shear. The spreadability of UA nanoemulgel was found to be in

the range of (0.25 to 2.99 g.cm/sec.). At low levels of A, spreadability increased from 2.55 to 2.99 seconds. Similarly at high levels of A, spreadability increased from 0.41 to 2.38 seconds. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Our ratio of 14.738 indicates an adequate signal. This model can be used to navigate the design space. The relationship between the dependent and independent variables was further elucidated using externally studentized residuals plot, Residuals vs. Predicted plot, Residuals vs. Run plot, Actual Response vs. Predicted plot, Box-Cox plot, Residuals vs. A; water, 2D contour plot, 2D real contour plot, 3D response surface plot are shown in figure (7a to 7i).

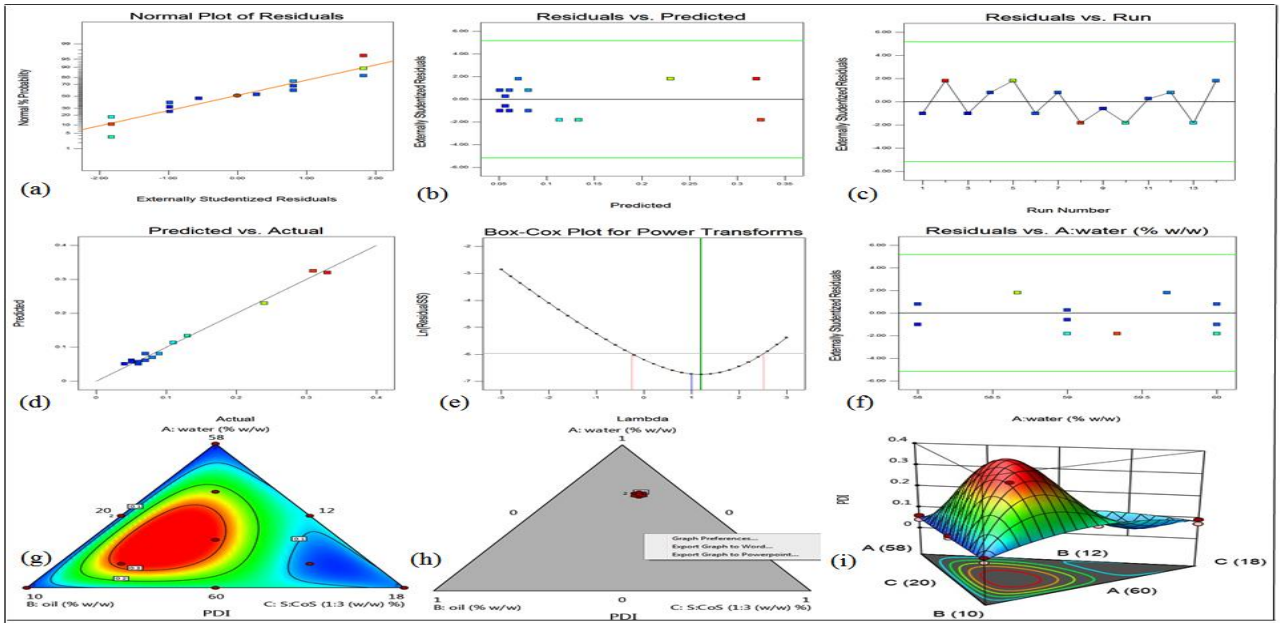


Figure-5: (a) Normal % probability plot, (b) Residuals vs. Predicted Plot, (c) Residuals vs. Run Plot, (d) Actual Response vs. Predicted Plot, (e) Box-Cox Plot, (f) Residuals vs. A; water, (g) 2D contour plot, (h) 2D real contour plot , (i) 3D response surface plot for PDI

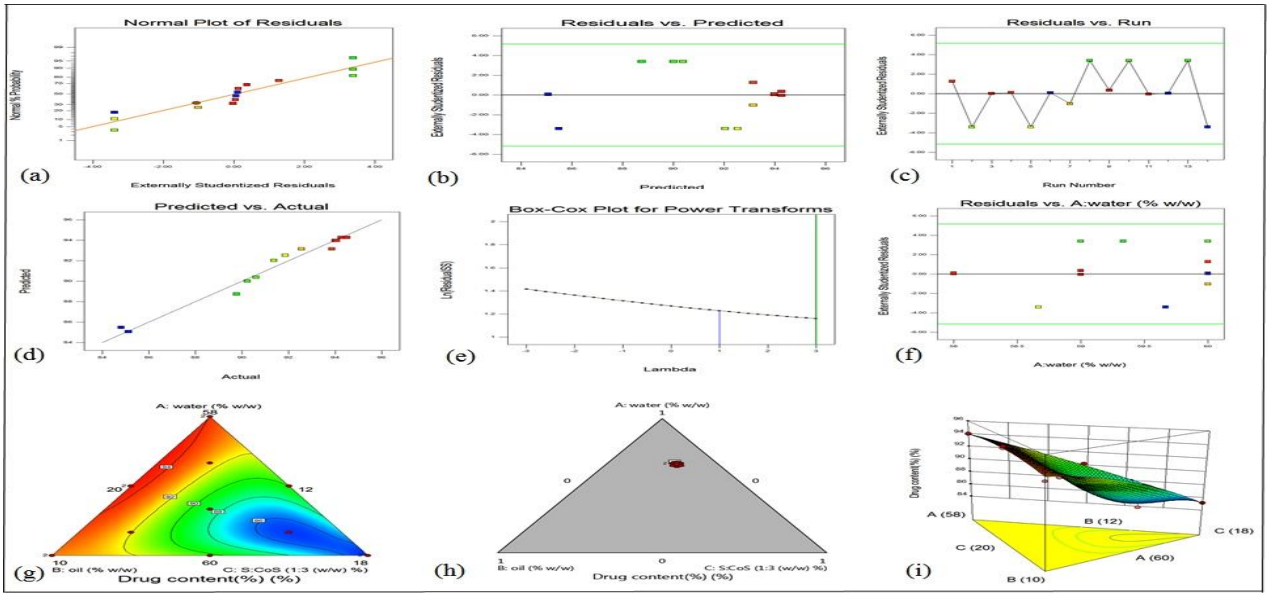


Figure-7: (a) Normal % probability plot, (b) Residuals vs. Predicted Plot, (c) Residuals vs. Run Plot, (d) Actual Response vs. Predicted Plot, (e) Box-Cox Plot, (f) Residuals vs. A; water, (g) 2D contour plot, (h) 2D real contour plot , (i) 3D response surface plot for drug content

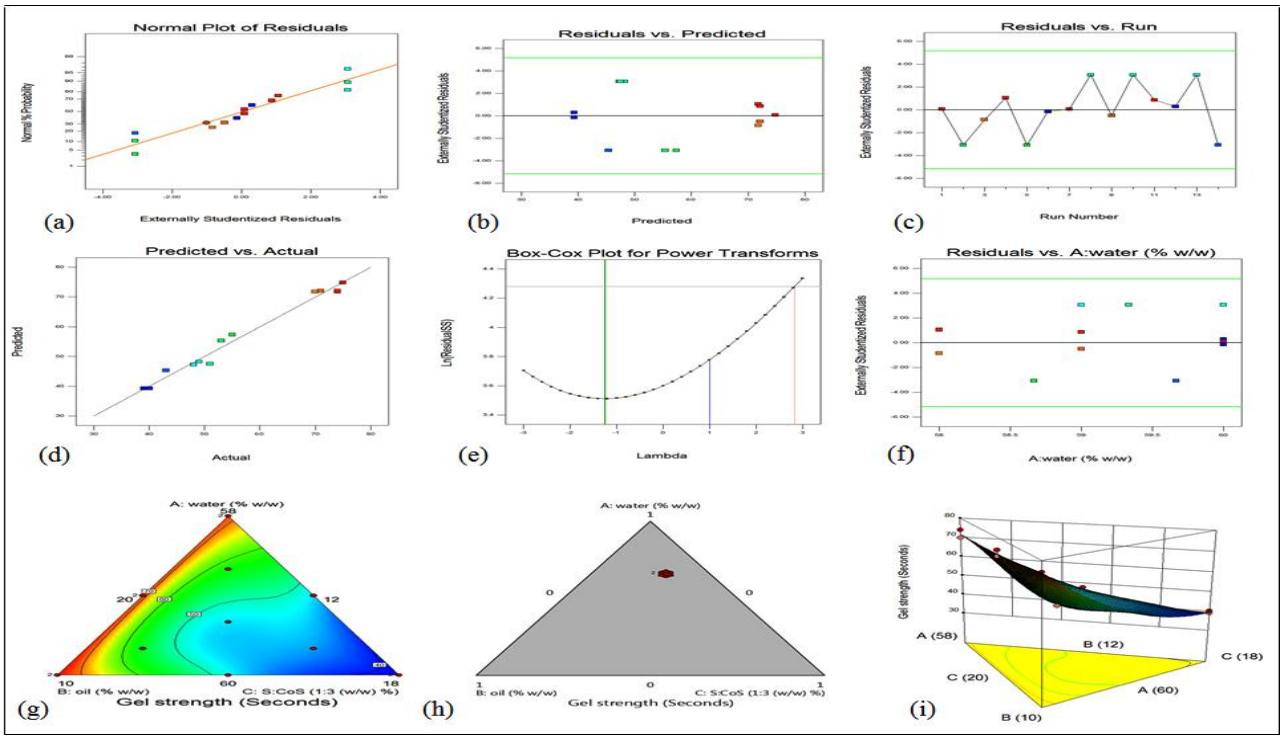


Figure-6: (a) Normal % probability plot, (b) Residuals vs. Predicted Plot, (c) Residuals vs. Run Plot, (d) Actual Response vs. Predicted Plot, (e) Box-Cox Plot, (f) Residuals vs. A; water, (g) 2D contour plot, (h) 2D real contour plot , (i) 3D response surface plot for gel strength

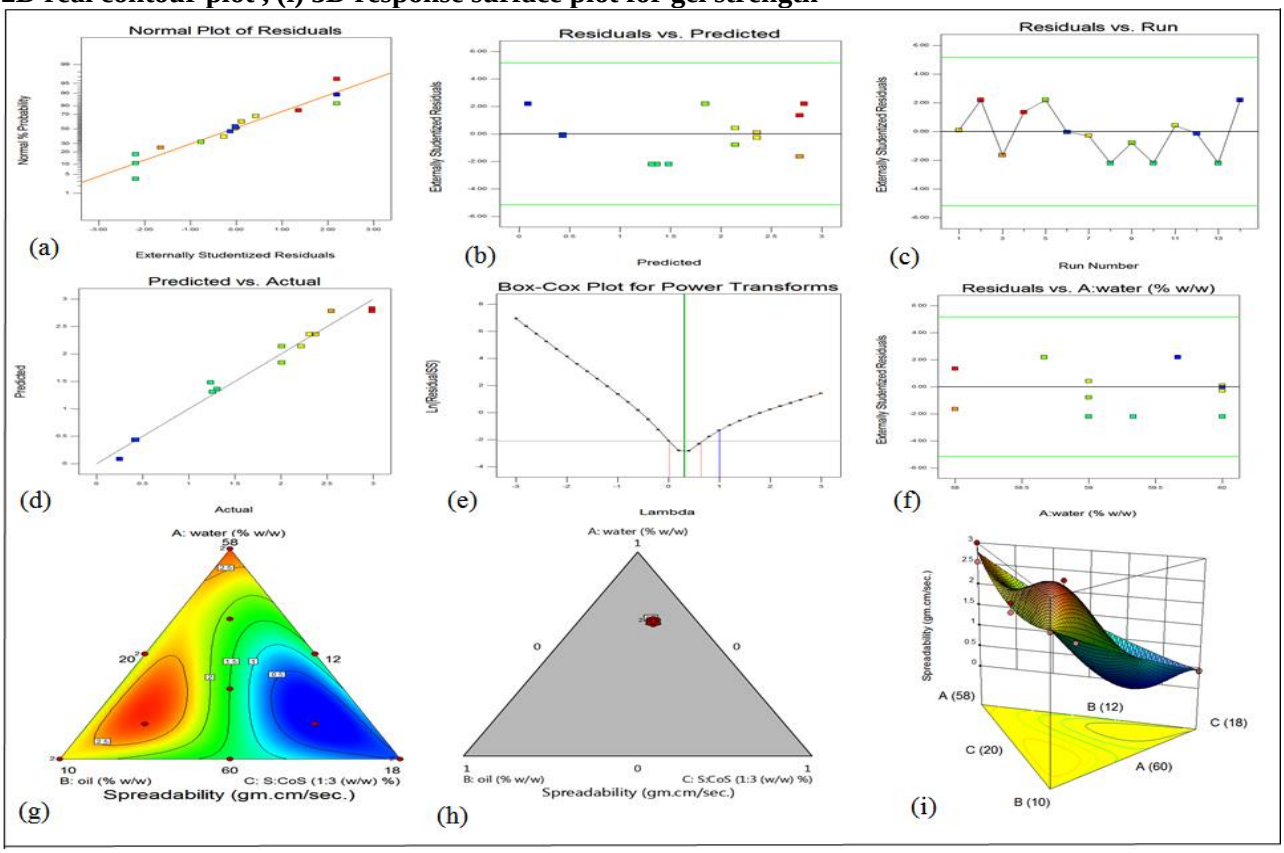


Figure-7: (a) Normal % probability plot, (b) Residuals vs. Predicted Plot, (c) Residuals vs. Run Plot, (d) Actual Response vs. Predicted Plot, (e) Box-Cox Plot, (f) Residuals vs. A; water, (g) 2D contour plot, (h) 2D real contour plot , (i) 3D response surface plot for spreadability

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Percentage cumulative drug release after 24 h from the UA nanoemulgel was found to be 75.2% and 78.4% of rutin release from formulation Run 8 and Run 10, respectively. When cumulative percentage of drug released was plotted against time, the release profile showed a linear relationship with time for 10 h after which release becomes constant as shown in Figure 8.

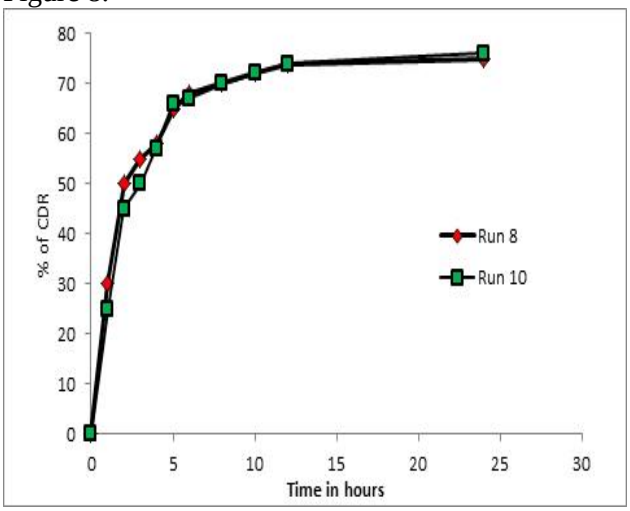

Figure-8: Showing the cumulative % of drug release

FTIR study was carried out to confirm the compatibility between the poloxamer 407 and drug are presented in Figure 9. The spectra obtained from the I.R. studies are from 10000 cm^{-1} to 370 cm^{-1}.

It was confirmed that the peak of pure drug, polymer and drug combination retain same, there are no major shifting as well as no loss of functional peaks between the spectra of drug, polymer and drug.

In wound healing study (Table 30), wound contraction progresses in wound treated with formulation and control group. In animals group treated with nanoemulgel 67.73% healing was observed up to 14th day. While untreated group I (control) animals showed 33.86% healing of wounds on 14th day. On 21st day control animal showed 65.50% of wound contraction, whereas PVP Iodine gel and UA nanoemulgel showed 92.91% and 90.51% of wound contraction, respectively.

Batch 8 and batch 10 of nanoemulgel were prepared according to these optimized levels. Observed responses were in close agreement with the predicted values of the optimized process was shown in Table 8, thereby demonstrating the viability.

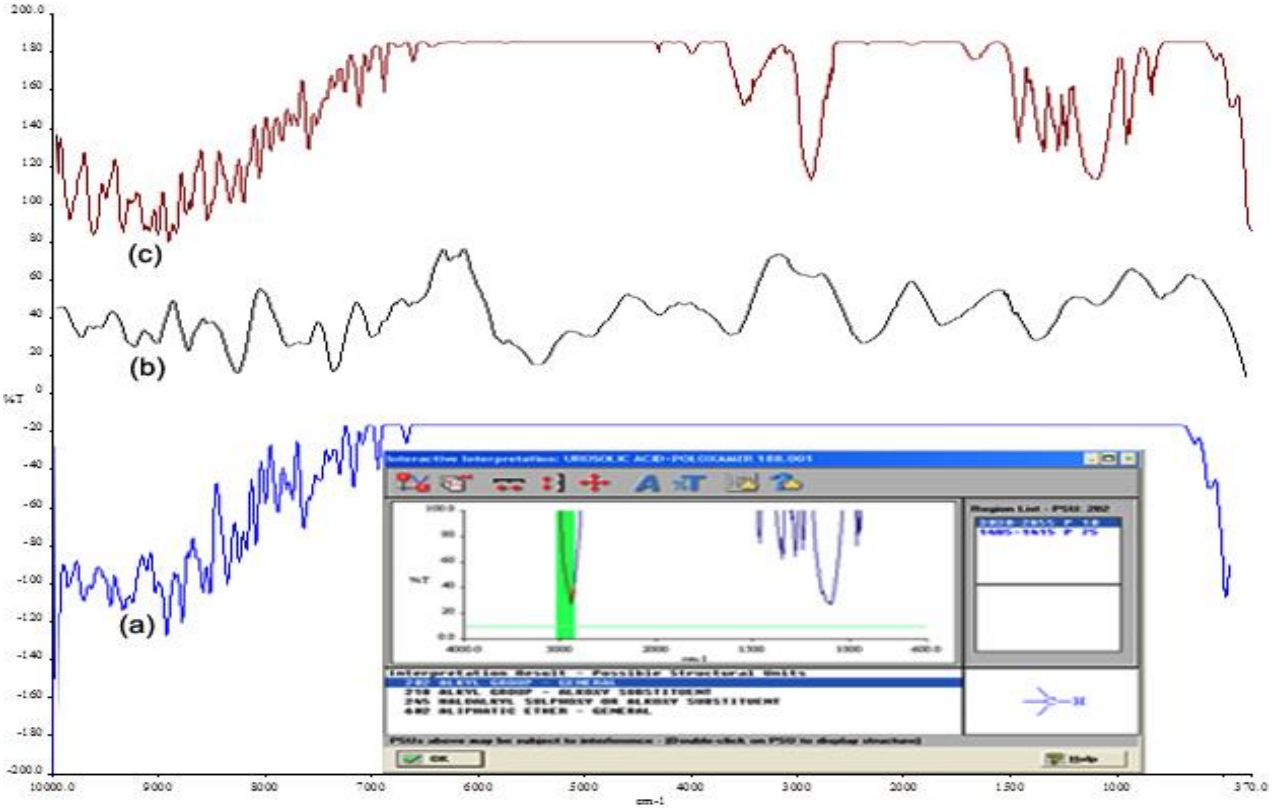

Figure-9: (a) showing the FTIR spectra of pure drug, (b) showing the FTIR spectra of P407, showing the FTIR spectra of pure drug and P407

Table-2: Effect of UA nanoemulgel on wound healing

Days after infliction of wound	Wound area (mm^2) (Percentage of wound contraction)		
	Control	PVP Iodine gel	UA nanoemulgel
1	492.20 ± 6.68 (0)	493.40 ± 9.01 (0)	499.80 ± 9.21 (0)
7	431.60 ± 14.42 (12.13 ± 3.94)	377.40 ± 24.34 (23.35 ± 5.48)	394.20 ± 9.90 (21.04 ± 2.35)
14	325.20 ± 11.99 (33.86 ± 2.68)	140.20 ± 15.83*** (71.60 ± 3.14)	161.60 ± 12.83*** (67.73 ± 2.25)
21	169.80 ± 12.17 (65.50 ± 2.43)	35.20 ± 5.74*** (92.91 ± 1.06)	46.80 ± 8.67*** (90.51 ± 1.84)

All the values are mean ± SEM (n=5). **P<0.001 compare with control (One-way ANOVA followed by Bonferroni post-hoc test)

Table-8: Optimized values obtained by the constraints

Response	**Predicted**	**Observed Batch 8**	**Observed Batch 10**
Globule size	172.8	172.4	172.4
Drug content (%)	88.7	88.5	88.5
PDI	0.3	0.3	0.3
Spreadability	1.4	1.5	1.5
Gel strength	47.4	48.0	48.0

CONCLUSION

This study has focused on the role of each independent variable on dependent variables. From the present study, it has made clear that nanoemulgel loaded with ursolic acid has shown promising results during in-vitro as well as animal studies with desirable physicochemical parameters thus holds a good future potential.

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