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DEVELOPMENT OF *PTEROCARPUS SANTALINUS* NANOLIPOSOME GEL: PHYSICAL CHARACTERIZATION AND *IN-VITRO* ANTI-INFLAMMATORY ACTIVITY

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ABSTRACT

To develop nano-liposomal gel of methanolic extract of *Pterocarpus santalinus* for its anti-inflammatory action. Formulation of various excipient combination of liposomes were formulated by using the thin film hydration method. Nine formulations (F1-F9) were designed and prepared by varying the concentrations of cholesterol. The formulation was further tested for their physical characterization like particle size, entrapment efficiency, % drug release and SEM. Stability study of optimized formulation was performed at refrigerated condition. Gels were prepared by incorporating Carbopol 940 as gelling agent and eucalyptus oil as permeation enhancer. The prepared gel formulations were assessed for various properties such as particle size, pH, spreadability, extrudability, viscosity, zeta potential, anti-inflammatory activity. Formulation of liposomes F5 was found to be optimal which was based on the entrapment efficiency in range 92.7 ± 2.08% with a desired mean particle size distribution 495.9 nm. Developed liposomal gel of *Pterocarpus santalinus* could have great effect for the anti-inflammatory treatment.

Keywords: *Pterocarpus santalinus*, liposomes, anti-inflammatory activity, gel

INTRODUCTION

Human skin plays a vital role for the target site of topical application of drugs and is the outer covering of the body for protecting the body against pathogens and

unnecessary water loss besides many of vital functions. It acts to regulate the body homeostasis, to defend the body from external pathogens and chemicals. It is composed of 3 layers mainly: the epidermis is the outer most layer, the thinnest layer of the skin and provides the most significant barrier function, the dermis is second layer which lies beneath the epidermis to provide mechanical support to the skin and the hypoderm is the third layer of subcutaneous fat to attach the skin to underlying bone, muscle, blood vessels and nerves **[1]**. Permeation of drugs across the skin is the base route of transdermal drug delivery system (TDDS) and topical delivery. Many factors like physicochemical properties of the drug, vehicle, and formulation components have influenced on drug permeation through different skin layers. TDDS has certain advantages like drug release in a controlled manner, preventing the first pass metabolism, constant drug delivery, and faciliting drug localization at target site **[2]**.

The inflammation is a sequence of actions occur in response to either toxic stimuli, infection, trauma, or injury in the living tissues. A cascade of events including enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown, and repair processes initiated the inflammation. It releases white blood cells (WBCs) as a protective measure against injury. WBCs release several biomolecules after injury leading to swelling and redness. Prostaglandins (PG) are one of the important biomolecules of inflammation playing a key role in the inflammatory response induction as their biosynthesis is considerably increased. These inflammatory responses are produced as a defense mechanism by an organism or tissues. Though, continued inflammation can lead to undesired effect as a consequence of interplay of various biomolecules that are secreted during the process of inflammation in several diseases e.g. cancer. The agents that contain or block inflammation may play significant role in treating pathologies associated with inflammatory reactions **[3]**. As a result of inherent problems associated with the current non-steroidal anti-inflammatory agents (NSAIDs) as well as steroidal anti-inflammatory agents (SAIDs), there is a continuous search for alternative agents, especially from natural sources. For centuries, a large numbers of natural products have been involved in the treatment of inflammation by natural healers.

Pterocarpus santalinus Linn (*Family:* Leguminosae*)* is known as a Rakta-chandan /red sandal wood has been reported to possess anti-diabetic **[4]**, anticancer **[5]**, antibacterial **[6]**, hepatoproactive, anti-ulcer, wound healing

activity. Heartwood contains pterocarpol, santalin A, B, pterocarptriol, ispterocarpolone, pterocarpo-diolones with *β*-eudeslol and cryptomeridol. In addition, Auron glycosides viz., 6-OH-1-Methyl-3', 4', 5' trimethoxyaurone-4-*O*-rhamnoside and 6, 4'-dihydroxyaurone-4-*O*neohesperidoside, and isoflavone glycoside 4', 5-dihydroxy7-*O*-methyl isoflavone 3'-*O*-beta-*D*-glucoside are also present. Heart wood is known to possess isoflavones glycosides, savinin, calocedrin and triterpene. From the ancient time *P. santalinus* wood having anti-inflammatory activity via decreasing the burning sensation, arresting bleeding, alleviating edema and ameliorating various skin disorders also **[7]**.

Liposome is an aqueous medium and surrounded by single or multilayer of phospholipids for the reason liposome known as microparticulate colloidal vesicle. Due to physico-chemical property of liposome most of hydrophilic and lipophilic medicaments can be incorporated in multilayered matrix of phospholipids. It provides controlled release of drug at targeted site so it helps in enhancing therapeutic efficacy with minimal dosing rate. Its size varies from very small (0.025 μm) to large (2.5 μm) vesicles having either one or bilayer membranes. The circulation half-life of liposomes depends on the vesicle size as an acute parameter. The amount of drug encapsulation in the liposomes determines on size and number of bilayers. Two groups of liposomes are as: (1) Multilamellar Vesicles (MLV) in which vesicles have an onion structure and (2) unilamellar vesicles has a single phospholipid bilayer sphere enclosing the aqueous solution **[8]**.

MATERIALS AND METHODS

Authentication and Extraction of *Pterocarpus santalinus*

Preparation of *Pterocarpus santalinus* Linn. heartwood extract: *Pterocarpus santalinus* Linn. 500 gm heartwood powder was obtained along with petroleum ether and ethanolic extract was collect by soxhlet apparatus. The percentage yield of methanol extract was found to be 15% w/w. Methanolic extract of *Pterocarpus santalinus* was labeled as PSME. It was procured and authenticated from YUCCA Enterprises Mumbai-400037.

Chemicals

Soya lecithin, methanol, cholesterol, chloroform, Carbopol 940, eucalyptus oil, methyl paraben were purchased from RL Fine Chem Pvt. Ltd. Mumbai India.

Solubility study

Different solvents distilled water, methanol, acetone, HCl were tested to check the solubility of PSME. One ml of various solvents were taken in the centrifuge tube and maximum amount of PSME were added. The mixture was vortexed for 72 hr

at 25 ^{0}C temperature by using Remi vortex mixer for 10 mints at 1008 rcf then followed for 72 hrs. The super tent 10 ml of above mixture was removed and transferred to fresh tube and volume was made with the ethanol. Then the mixture was filtered with syringe filter (0.22 micrometer). The filtered mixture was then vortexed. We prepared correct dilution with each solvent to for UV absorbance at the 247 nm, then concentration for each solvent was determined by calibration curve method.

Preformulation study

Organoleptic properties

Organoleptic tests were carried out such as colour, odour and physical appearance for *Pterocarpus santalinus* Methanolic Extract (PSME) and compared with standards available in monographic literature.

UV visible spectral analysis of PSME

Suitable dilutions (10µg/ml) of PSME were prepared in the methanol. The dilution was scanned in the UV visible range 200-800 nm by using UV spectrophotometer (Jasco V-630, Japan). For the baseline correction methanol was used in both cuvette and reference cuvette at the time of sample analysis.

FTIR spectroscopy of PSME

FTIR spectroscopy has studied the interacti on of PSME. The drug was observed under IR spectrophotometer (Jasco FT/IR-4600) and the spectrum obtained was interpreted. The scanning was performed at scanning range 4000-400 cm-1.

Preparation of liposome:

Full factorial design was used concerning three variables at two levels for formulations. Nine different formulations with low and high values of cholesterol (410 and 820 mg) and vortex time (5 and 15 minutes) were used to prepare liposomal formulations. Liposomes were prepared by thin film method. Moreover, various concentrations of cholesterol **(Table 1)** were dissolved in the chloroform-methanol (1:1) and 100 mg of *Pterocarpus santalinus* was added to the solution, then the mixture was evaporated in a rotary evaporator at on water bath 60^{0}C, 30 rpm vacuum until thin film was formed in the round-bottoms flask, it was hydrated with phosphate buffer (pH 7.4). The suspension was agitated by vortex for 30 minutes and then sonicated for one hour (20-24).

Full factorial design

A 3^2 full factorial design was to examine the effect of two variables of formulation, each at 2 levels, and the probable nine combinations of *Pterocarpus santalinus* liposomes. As independent variables, the low and high values of cholesterol and vortex time have been taken. The particle size and drug release are considered dependent variables.

Table 1: A 3^2 factorial design formulation of liposomes
Formulation Batch F1 to F9

Batches	*Pterocarpus santalinus* (mg)	Soya lecithin (mg)	Cholesterol (mg)	Vortex time (min)
F1	100	400	820	5
F2	100	400	410	10
F3	100	400	615	10
F4	100	400	410	5
F5	100	400	410	15
F6	100	400	615	5
F7	100	400	615	15
F8	100	400	820	10
F9	100	400	820	15

Incorporation of prepared liposomes into carbopol gel

Nine batches of liposomal gel were prepared by using carbopol940 (1% w/v) was soaked in a small amount of double distilled water for 1 hour. The soaked carbopol 940 mixture was mixed until swelled mass get completely dissolved in the water. Then 5 ml Liposome suspension of *Pterocarpus santalinus* extract was added and the mixture was stirred at 1200 rpm by using homogenizer at controlled temperature 30 ºC, after observing clear solution, pH was adjusted to 7.4 by 0.1 N NaOH. In additional to enhance rate of penetration Eucalyptus oil was added in liposomal gel and methyl paraben was added as preservative. The liposomal gel was left equilibrating for 24h at room temperature (25 ± 1º C).

Evaluation test of liposomes

Particle size measurement

The particle sizes of the formulation were measured using Zeta Sizer 1000 HS_A, (Malvern Instrument, UK) which is based on the basic principle of photon correlation spectroscopy.

Scanning electron microscopy

Scanning Electron Microscopy (SEM) was used to determine the surface morphology of the formulation. This study was performed using scanning electron microscope SEM.

Entrapment efficiency

The amount of drug entrapped is calculated by deducting the amount of un-entrapped drug. Entrapment efficacy of *Pterocarpus santalinus* liposomal mixture was determined by ultracentrifuge (Remi) equipped with a TLA-45 rotor at 15,000 rpm at 4 ºC for 3 h. After separation of *Pterocarpus santalinus* entrapped liposome vesicles, by using UV-Visible spectroscopy the concentration of un-entrapped molecules was determined at 247 nm. On the basis of concentration the amount of drug entrapped was calculated and by using below formula % entrapment efficacy was determined.

$$\textbf{Entrapment efficiency}(\%) = \frac{\textbf{amount of free drug}}{\textbf{total amount of drug}} \times 100$$

***In-vitro* drug release study**

To study the *in-vitro* release of all batches F1 to F9 Franz diffusion cells were used.

Receiver compartment volume of 10 ml and effective diffusion area of 2.84 cm^2 were used to determine release of *Pterocarpus santalinus* liposome. The dialysis membrane 0.65 micrometer was used. The receptor compartment was filled with 60 % w/w ethanol and temperature was maintained at 33^0c. One g *Pterocarpus santalinus* liposome of formulation was placed in the donor compartment and 1ml of sample from receiver compartment was withdrawn at the appropriate time. The same amount of fresh sample was added and volume was kept constant. The samples were analyzed on UV-Spectrophotometer (Systronics AU 2701) at the wavelength of 247 nm and the concentration of was determined of each batch from the standard curve. For standard curve working standard of *Pterocarpus santalinus* with percentage purity 99.9 % were used.

Evaluation test of liposomal gel:

pH

Digital pH meter (Lab India) was used to determine pH of formulation. pH was determined by dissolving 1 gm of gel in 100 ml double distilled water and allowed mixture to stabilize at 4 ºC for 2 hours. The pH reading was validated for 3 times for each sample.

Spreadability

In this study, spreadability was calculated by taking two glass slides having 7.8 cm length and then the prepared liposomal gel were sandwiched in-between them. Then a weight of 50 g was placed over the upper slide of glass for uniform spreading of the liposomal gel and the takeoff that applied weight of 50 mg, and then the spreadability of the gel was measured with respect to a known applied weight of 20 g with the help of a pulley, and the time taken to roll down the glass slide was noted. Subsequently, the procedure was repeated thrice to take an average of the total spreadability achieved by placing 20 g of weight over it.

Further, spreadability was calculated by the following formula:

$$S = \frac{M \times L}{T}$$

Where, S is the spreadability of the liposomal gel, M is the weight tied on the upper slide (20 g), L is the length of the slide (7.8 cm) and t is the time taken by the upper slide to roll down.

Viscosity

For determination of gel viscosity (cps), Brookfield Rotational Digital Viscometer DV II RVTDV-II was used. The spindle No.63 was rotated at 200 rpm. The determination test was performed in controlled temperature at (25 ± 1°C) and samples of gels was allowed to settled down for 30 min.

Extrudability test

In extrudability study, the amount in percentage of gel and gel extruded from lacquered aluminum collapsible tube on application of weight in grams required to extrude at least 0.5 cm strip of gel in 10

seconds. Higher quantity of extruded was directly proportional to extrudability. The value of extrudability was validated by taken average of triplicate. The calculation of extrudability was done by using following formula,

$$\text{Extrudability} = \frac{\text{Applied weight to extrude gel from tube (in g)}}{\text{Area in (cm2)}}$$

Anti-inflammatory activity of *Pterocarpus santalinus* liposomal gel

***In-vitro* anti-inflammatory activity by protein denaturation method**

The test mixture was prepared by adding egg albumin 0.4 ml (from fresh hen's egg), 4 ml of synthetic derivatives of liposomal mixtures (1000, 800, 600. 400, 200 µg/ml) and total 10 ml volume makeup by using Phosphate buffer saline (pH 6.4). Control sample was prepared by above procedure by using double distilled water at the place of liposomal mixture. After that mixtures were stored in incubation chamber at (37 ºC ± 2) for 15 min. after 15 min. of incubation time, mixtures were heated at 70 ºC for 5 min.. Then mixtures were placed aside for cooling and after cooling, the absorbance was measured at 660nm and water was selected as blank. The reference medicament, Diclofenac sodium was taken at concentration 1000, 800, 600, 400, 200 µg/ml) and equivalent amount was selected for determination of absorbance. The percentage inhibition of protein denaturation was calculated by using the following formula,

$$\%\ \text{inhibition} = \frac{(\text{absorbance of control} - \text{absorbance of test})}{\text{absorbance of control}} \times 100$$

Stability study

The ICH guidelines were referred to perform stability study. As per guidelines formulated gel was poured in collapsible tubes and stored in humidity and temperature controlled chamber. Mainly,

1. 25°C ± 2°C/60% RH ± 5%RH
2. 30°C ± 2°C/65% RH ± 5%RH
3. 40°C ± 2°C/75% RH ± 5%RH

The evaluation test was performed every after 3 months under various evaluation criteria. The stability tests were evaluated by pH, drug content uniformity, physical appearance, poly-dispensability, extrudability and spreadability.

RESULTS AND DISCUSSION

Preformulation study of PSME

Organoleptic properties

The colour of PSME was found to be reddish and amorphous.

Determination of drug solubility

From the results obtained it was clear that PSME were highly soluble in methanol with highest solubility of 32 mg/ml and soluble in the distilled water, acetone and HCl with solubility 22, 10 and 12 mg/ml respectively **(Table 1)**.

UV visible spectral analysis of PSME

From the spectra analysis it was concluded that the absorption maxima (λ max) for

PSME was 247nm in the ultraviolet range of UV visible spectra **(Figure 1)**.

Calibration curve determination

Calibration of curve of PSME was carried out as per the standard procedure by preparing the 10 µg/ ml concentration and the absorbance was taken at different concentrations shows straight line which passes from the origin. The ranges found in the calibration were obeyed Beer's lamberts law **(Figure 2)**.

The regression coefficient of above equation was found to be 0.996.

FTIR spectroscopy of PSME

In the PSME, FTIR spectrum strong absorption peaks at 3615.88 cm^{-1} indicates OH stretching in hydroxyl group. Peaks at 2854.13 cm^{-1}indicates C-CH stretching in methyl and methylene groups. Absorption peaks at 1618.3 cm^{-1}indicates C=O stretching in flavones and 1536.99 cm^{-1} indicates aromatic skeletal stretching. Absorption peaks at 1419.35 cm^{-1} aromatic skeletal combined with C-H in plane deforming and stretching and 1457.92 cm^{-1} CH_2 deformation stretching in lignan and xylan. Absorption peaks at 1155.15 cm^{-1} C-O-C indicates stretching in pyranose rings, C=O stretching in aliphatic groups. Absorption peaks 842.74 cm^{-1}indicates character of cellulose βchains, C-H stretching out of aromatic ring **(Figure 4)**.

Particle size analysis of liposomal gel

All batches of formulation were measured for particle size analysis and out of all batches, the particle size of fifth formulation shown in **Figure 5**.

***In-vitro* drug release study**

Calibration curve determination

The regression coefficient of above equation was found to be 0.996.

y = mx +c

y = Absorbance

x = (concentration)

m= Slope

c = Intercept

Factorial Design

Central composite design was applied to examine the combined effect of two formulation variables, each at 3 levels and the possible 9 combinations of liposomes. The concentration of cholesterol (X1) and vortex time (X2) were taken as independent variables. The particle size (Y1), % drug release (Y2) were taken as dependent variables. The data obtained by experiment work were analyzed statistically using Design-Expert 11 software (Stat-Ease Inc., USA). The F5 batch showed less particle size and maximum drug release **(Table 3, 4)**.

Two dimensional counter plots are very helpful for studying the factor's interaction effect on the responses. These kinds of plots are useful for concurrently studying the impacts of two variables on the response. Counter plot as indicates regions

where cholesterol & vortex time in the liposome formulation influence particle size of liposomes. It was determined from counter plot that particle size of liposomes less with decreasing concentration of cholesterol& increasing vortex time **(Figure 6)**.

The linear surface model obtained from the regression analysis was used to build up 3-D graphs in which the responses were represented by curvature surface as a function of independent variables. The relationship between the response and independent variables can be directly visualized from the response surface plots. 3D Response surface plot as indicates regions were the concentration of cholesterol and vortex time in liposome formulation influences the particle size of liposomes. It showed that particle size of liposomes was found to be less with decreasing concentration of cholesterol and increasing vortex time **(Figure 7)**.

Two dimensional counter plots are very useful to study the interaction effect of the factor on the responses. These types of plots are useful in study of the effects of two factors on the response simultaneously. Counter plot as indicates regions were the cholesterol and vortex time in liposome formulation influences the % drug release. It was observed that as decreasing concentration of cholesterol and decreasing vortex time **(Figure 8)**.

Response surface plot as indicates regions were the cholesterol and vortex time in liposome formulation influences the. It % drug release was observed that as the less concentration of Cholesterol and vortex time increases then % drug release increases **(Figure 9)**.

The bar graph indicates the overall desirability of the responses .The optimal region values has the overall desirability value of 0.991 indicating central composite design incorporated with desirability functions could be effectively used to optimize the liposome. Desirability function is used and the estimated response is transformed into a scale free value (di) called desirability ranging from 0 to 1 and completely dependent on closeness to the lower and upper limits. If the desirability value shows 1 it represents the ideal case; 0 indicates the one or more responses are being outside their acceptable limits. The bar graph indicates the overall desirability of the responses. The optimal region values have the overall desirability value of 0.991 indicating closeness to target response **(Figure 10)**.

Graphical optimization was done on the basis of overlay plot generated by the design expert software version 11. This gave the over lay plot comprising of two regions viz, yellow region describing an area of design space with feasible response

values and grey region describing an area where response did not fit the quality product criteria. Criteria for optimization of liposome formulation. The goals for optimization in this study were to particle size (nm) and % drug release within 24 hours **(Figure 11)**.

Table 2: Solubility of *Pterocarpus santalinus* extracts

Solvents	Solubility in mg/ml
Methanol	32 mg/ml
HCl	12 mg/ml
Acetone	10 mg/ml
Water	22 mg /ml

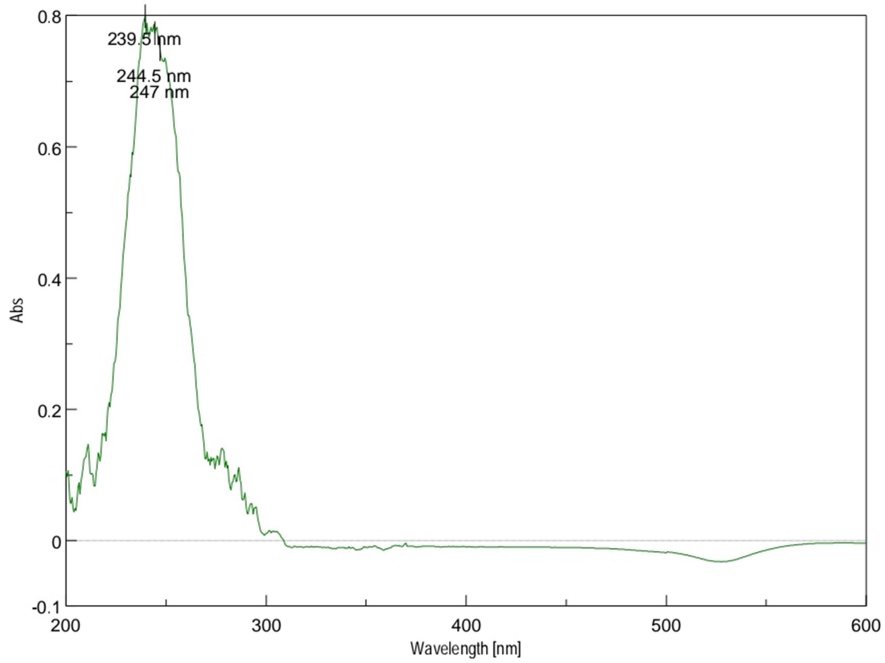

Figure 1: ƛ max of PSME

$$y = mx + c$$

y = Absorbance
x = (concentration)
m= Slope
c = Intercept

Concentration (µg/ml)	Absorbance
10	0.006
20	0.070
30	0.120
40	0.340
50	0.594

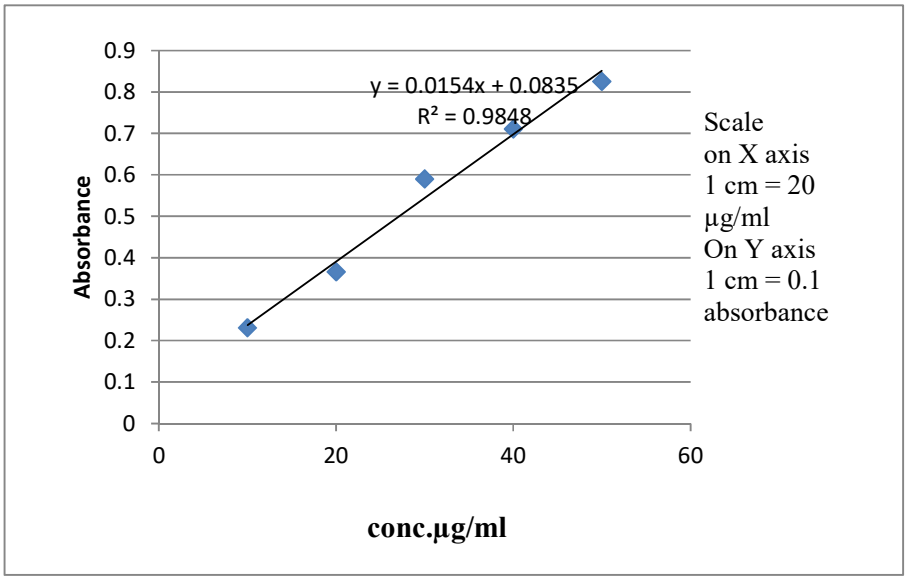

Figure 3: Calibration curve of *P. santalinus*

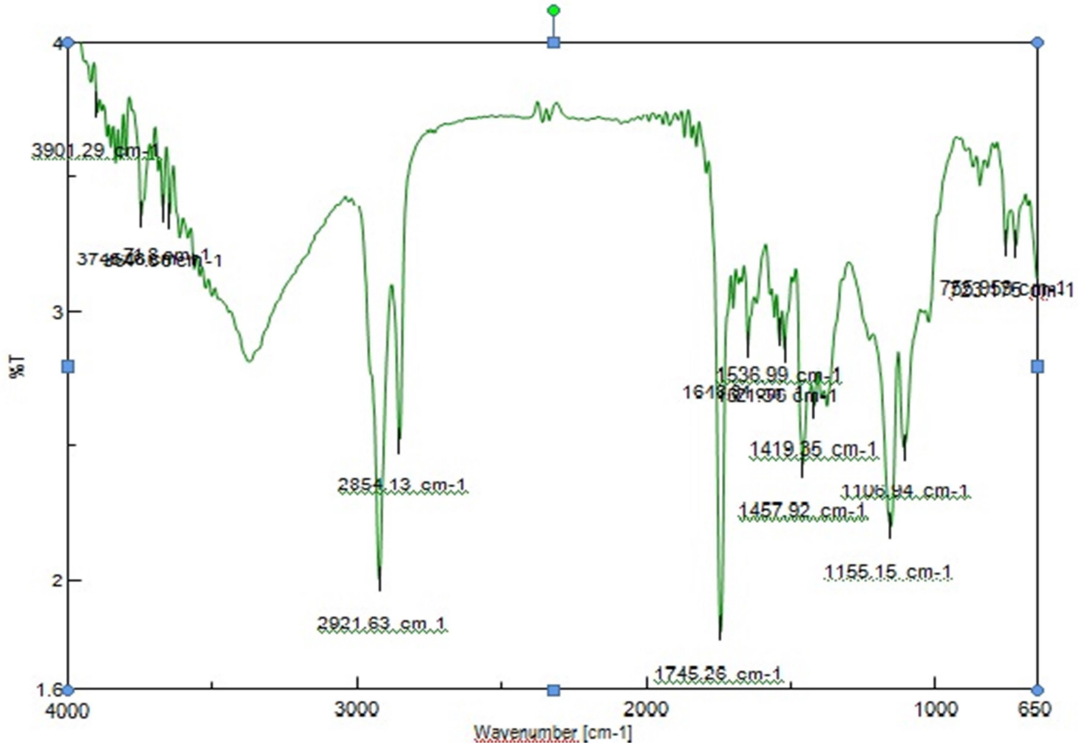

Figure 4: FTIR spectroscopy of PSME

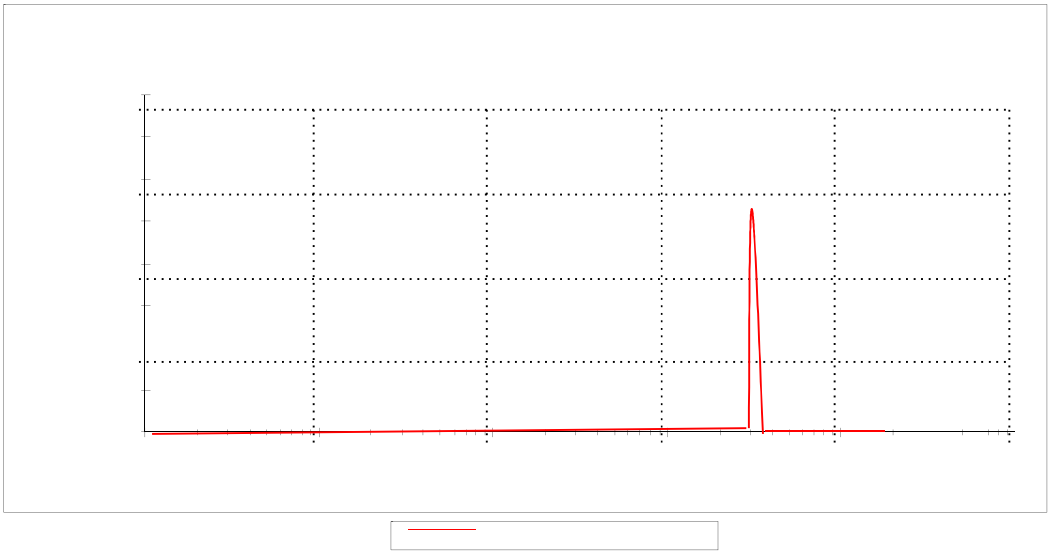

Figure 5: Particle size analysis of liposomal gel batch f1 (Z average = 820 nm)

Table 3: Comparative of all batches F1 to F9

Liposome parameters	F1	F2	F3	F4	F5	F6	F7	F8	F9
Colour	Red	Red	Red	Red	Red	Red	Red	Red	Red
Odour	Pleasant odour	Pleasant odour	Pleasant odour	Pleasant odour	Pleasant odour	Pleasant odour	Pleasant odour	Pleasant odour	Pleasant odour
Appearance	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Particle size(nm)	820	573	689	620	495.9	715	635	780	740
% drug release	76.12 %	91.65 %	83.46 %	88.2 %	94.2%	82.5 %	87.32 %	78.29 %	80.92 %
Drug entrapment efficiency	70.91±1.2	81.34±2.3	83.7±1.46	75.5±3.09	92.7±2.08	80.6±2.6	77.8±0.87	65.78±3.31	61.9±1.67
Polydispersity index	0.902	0.83	0.958	0.947	0.1	0.969	0.865	0.982	0.948
pH	6.5±0.05	7.5±0.04	6.4±0.09	7±0.05	5.5±0.05	6.8±0.05	6.5±0.06	6.2±0.04	5.7±0.04
Viscosity	4520± 0.75	5685± 0.47	6225± 0.098	5148± 0..89	6728± 0.59	6857± 0.45	5982± 0.84	6287± 0.77	3364± 0.95
Spreadability (gcm/sec)	10.12±0.23	32.1±0.87	11.8±0.36	13.3±0.58	14.3±0.46	12.6±0.52	12.0±0.98	12.5±0.34	13.1±0.21
Anti-inflammatory activity % Inhibition (1 mg/ml)	56.92%	53.38%	49.53%	48.76%	90 %	49%	50.69%	61.69%	61.92%

Table 4: Comparative percentage drug release at 10 min, 20 min and 30 min

Batch	Percentage drug release at 10 min (%)	Percentage drug release at 20 min (%)	Percentage drug release at 30 min (%)
F1	45.67 %	52.12 %	76.12 %
F2	74.12%	80.71%	91.65%
F3	68.61%	70.21%	83.46%
F4	50.16 %	69.61 %	88.2%
F5	65.16 %	80.91 %	94.2 %
F6	53.21 %	66.12 %	82.5 %
F7	57.21%	70.13%	87.32%
F8	59.71%	67.61%	78.29%
F9	62.21 %	73.12 %	80.92 %

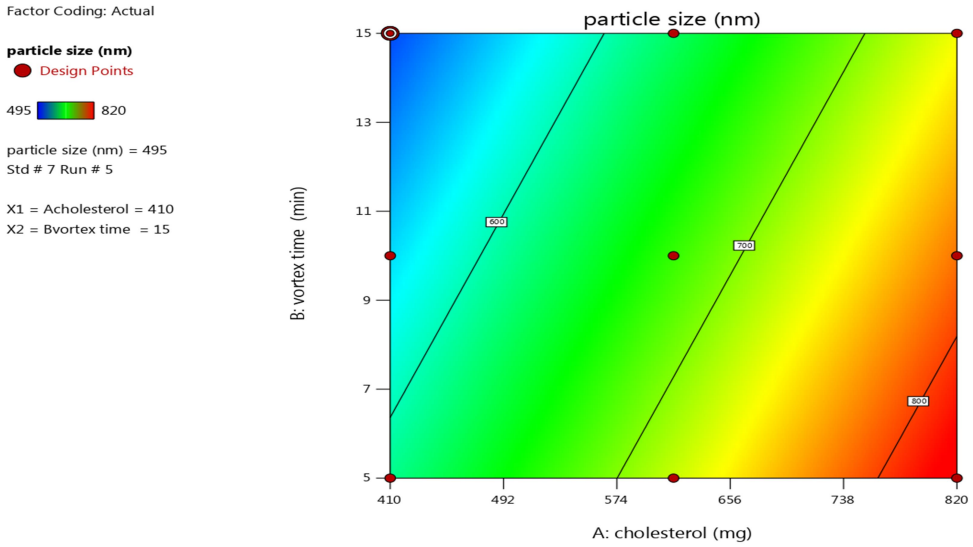

Figure 6: Counter plot showing the effect of X1 and X2 on particle size (nm)

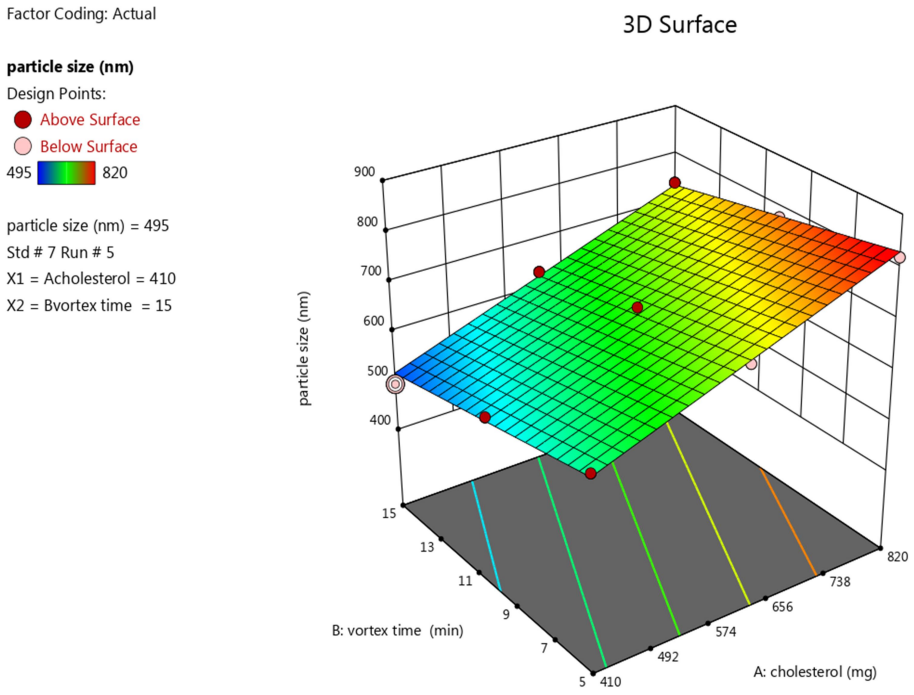

Figure 7: D Response surface plot showing the effect of X_1 and X_2 on particle size

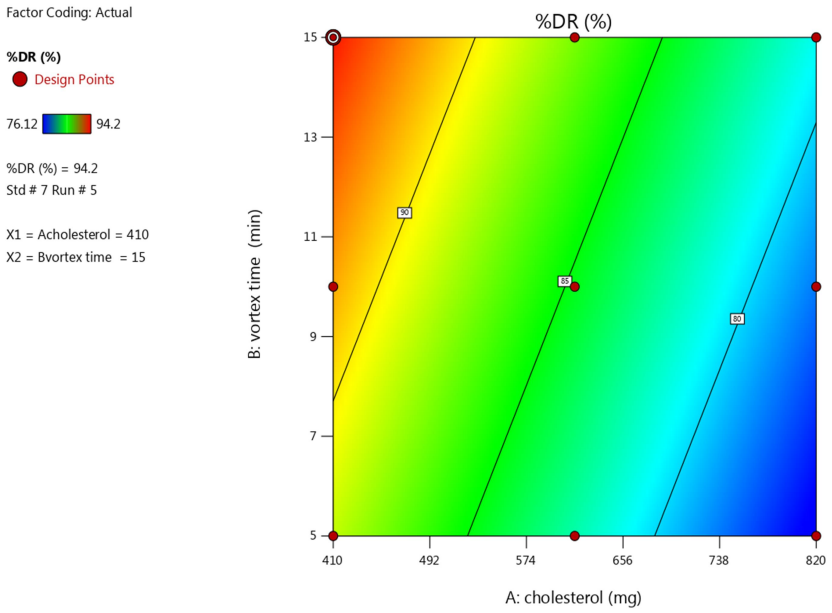

Figure 8: Counter plot showing the effect of X_1 and X_2 on % drug release

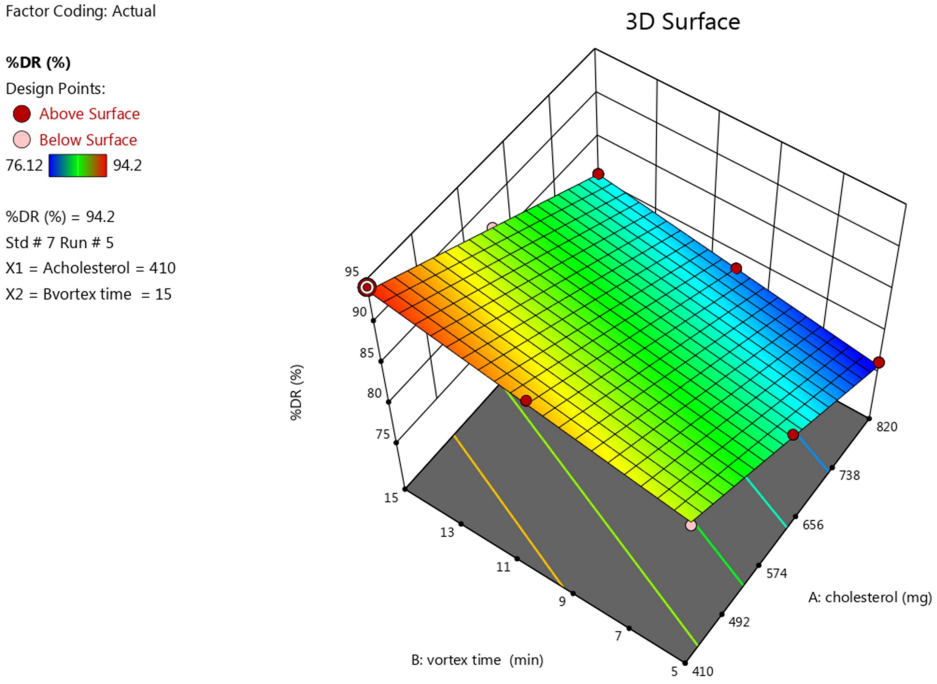

Figure 9: D Response surface plot showing the effect of X_1 and X_2 on% drug release

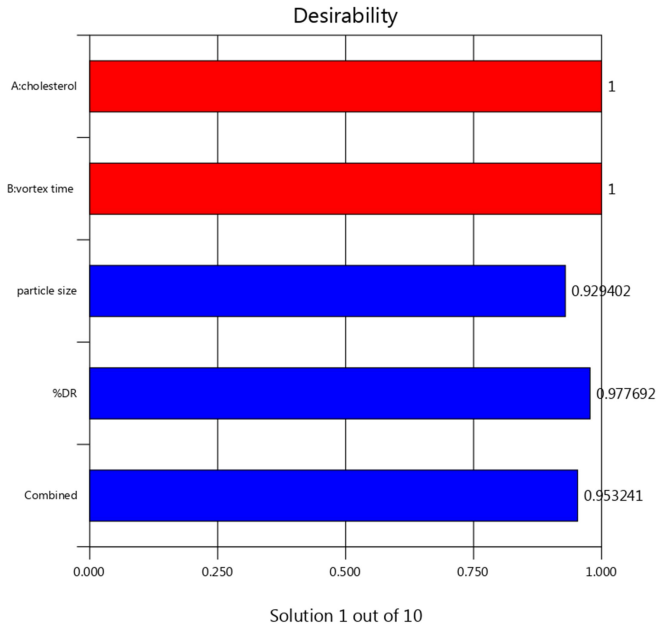

Figure 10: Bar graph indicates desirability for each parameter and response the best optimal conditions

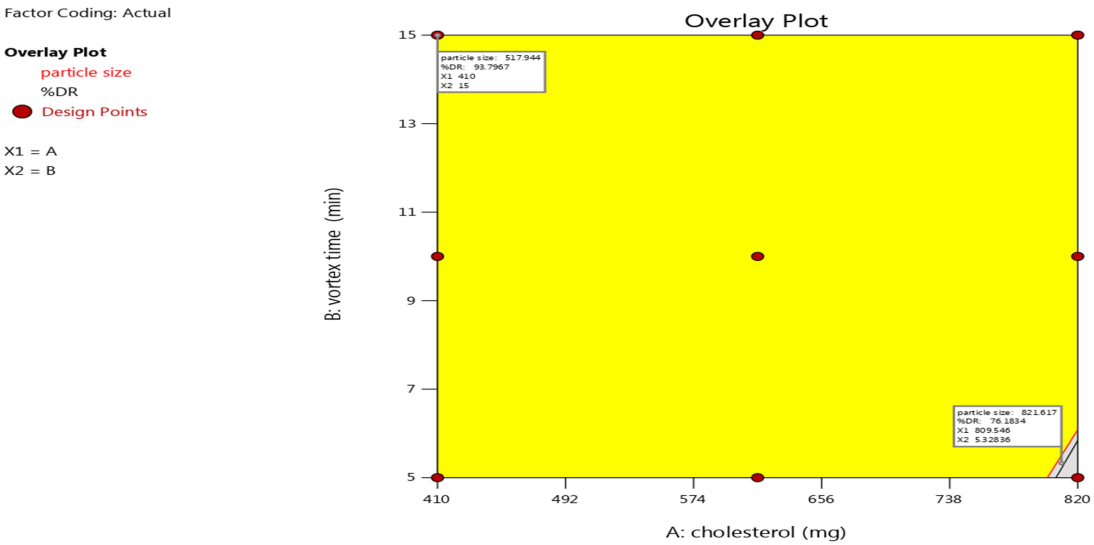

Figure 11: Over lay plot showing optimized batch

CONCLUSION

The developed liposomal gel of *Pterocarpus santalinus* methanolic extract could have great effect for the anti-inflammatory treatment.

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