International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Vol 13, Issue 2, 2021

Original Article

DEVELOPMENT AND OPTIMIZATION OF MANNOSYLATED NARINGENIN LOADED TRANSFERSOMES USING RESPONSE SURFACE METHODOLOGY FOR SKIN CARCINOMA

NIKITA VERMA, SWARNLATA SARAF*

University Institute of Pharmacy, Pt. Ravishankar Shukla University Raipur, Chhattisgarh, 492010 India
Email: swarnlatasaraf@gmail.com

Received: 03 Dec 2020, Revised and Accepted: 05 Feb 2021

ABSTRACT

Objective: The flavonoidal drug Naringenin offers a natural defense against free radical generation due to their antioxidant i.e. free radical scavenging property. The continuation of research work towards the invention of targeting the flavonoidal drug for skin carcinoma. Naringenin is a potent antioxidant, having remarkable reactive oxygen species scavenging potential and abundantly found in citrus fruits.

Methods: The optimization of the formulated mannosylated naringenin-loaded transfersomes (MA-NgTfs) was performed using Box–Behnken statistical design to obtain crucial variable parameters that influence vesicular size, size distribution and surface charge. Therefore keeping both the concepts in mind our objective is to design and optimize the mannosylated naringenin loaded transfersomes (MA-NgTfs) for macropahge targeting. The Box Behnken with 3D surface response design graph was employed to optimize the formulation.

Results: Phospholipids and surfactant ratio played a remarkable role to determine the mean vesicular size and the Zeta potential of the vesicles. The Zeta potential is found in the formulation having a range of-18.01±1.05 to-28.7±1.008 mV represents the good stability of the formulation. The vesicles size range was found in the range of 102.4±1.01 to 263.74±0.63 and range of Entrapment efficiency of nanovesicles was as 72.04±1.53 to 82.04±0.81. *In vitro* drug release study shows that mannosylated naringenin loaded transfersomes (MA-NgTfs), and marketed formulation dispersion was found 69.31 %, 62.03 %, 58.71 %, and 65.02 % respectively. *Ex vivo* skin permeation and deposition study shows that the marketed product and pure drug suspension optimized transfersomes through the skin of mice was of flux 6.5±3.07 and the percentage of drug retention was 0.76±1.26. The results gave us strong evidence of cellular uptake bymannose–directed transfersomes via mannose receptor-based endocytosis.

Conclusion: On the basis of findings, the study revealed that the prepared formulation has characteristic potential for targeting and the concept of ligand directed nanocarrier formulation was imparts synergistic effect against UV-induced skin carcinoma.

Keywords: Naringenin, BBM, Mannosylated Transfersomes, Macrophage targeting, Skin cancer

DOI: https://dx.doi.org/10.22159/ijap.2021v13i2.40436. Journal homepage: https://innovareacademics.in/journals/index.php/ijap

INTRODUCTION

Human skin is the largest organ and is highly exposed to environmental solar radiation and due to chronic long term exposure skin produces abnormal cell growth, which is responsible for the progression of skin cancer [1]. The solar radiation is composed of various electromagnetic radiation having UV-radiation and infrared radiation mainly. From all the three regions the UV-radiation having the range of (280-320) nm i.e. UVB is having remarkable potency to alter the biological function of the cell and causes maximum DNA damage due to free radical generation into the cells and promotes the abnormal cell growth [2]. Nowadays, various drug delivery systems are in the pharmaceutical market, to deliver the medicament at the specific site, but due to frequent wear and tear of the keratinized layer of skin, the presence of enzymes and the lipophilicity of skin membrane, and various contributing factors, site-specific drug delivery to the skin is not as easy task as it appears to be [3]. The pre-existing conventional therapy has also been failed to bring relief because they do not identify the cancerous and the normal proliferating cells. Thus the major objective of the skin cancer therapy is to develop such type of targeted drug delivery system that can able to deliver the medicaments specifically at the site of action. The macrophages are expressed into the skin keratinocyte and fibroblast due to chronic exposure of UV-radiation of the range (280-320 nm) are enhances the macrophage production, which may inhibit the p53-dependent apoptotic pathway, thereby inducing photocarcinogenesis into the skin. Hence the macrophage targeting-based therapeutics could be useful for targeting cancerous cell [4]. Mannose receptors are one of the receptors which are overexpressed on the surface of the macrophage (MMR), while the other MR+cells (i.e. dendritic and endothelial cells) and other different lectins with having mannose-binding activity have been subsequently identified. The glycotargeting exploits highly significant interactions of endogenous lectins with the moiety of carbohydrates (often with more than one carbohydrate) [5]. Functionalization of nanovesicles through the conjugation of ligands that are specifically recognized by surface receptors on target cells may favor the stimulation of the immune system. Targeting of mannose receptors can be achieved by the technique of mannosylation, which is an effective strategy to design and develop nano-systems that could be able to target mannose receptors, which are highly expressed in cells of the immune system and can play crucial role in preventing UV radiation caused skin carcinoma in which the immunity is hampered directly [6]. The flavonoids drug naringenin offers a natural defense against free radical generation due to their antioxidant i.e. free radical scavenging property. The continuation of research work towards the invention of targeting drug delivery systems for skin cancer. Hence we proposed the macrophage targeting for skin cancer which may be useful for future perspective and effectively used as a site-specific drug delivery of Naringenin to the cancerous cells and also decrease the adverse effect related to the drug and treat skin carcinoma synergistically [7]. Mannose receptor is the most suited receptor to formulate the drug delivery system for herbal bioactive and other small and macromolecules. The Mannose ligands and their associated delivery system are cost-effective and easily affordable to use and for future perspective. On the basis of previous findings, a formulation based on targeted therapy of natural bioactive for skin cancer is yet present in the market; therefore, our objective of the study was to conceptualize and formulate the optimized mannosylated naringenin loaded transfersomes based formulation for targeting macrophages of cancerous cells via Mannose ligand.

MATERIALS AND METHODS

Materials

Naringenin (Ng) was purchased from Sigma Aldrich, USA; D-Mannosamine HCl was purchased from Himedia Laboratories and Soya Phospholipids, Rhodamine, was purchased from Himedia Mumbai India. Phosphatidyl-Choline from soy lecithin containing

not less than 98% Phosphatidyl Choline, DMSO. Phosphate-buffered saline (PBS) (pH 7.4 and pH 5.5) was used for the determination of drug release. All other consumables used throughout the experiment were of analytical grade.

Chemicals for cell culture

The different solvents that were used in the process were of HPLC grade. Millipore super Q water system was used for purification of water. The National Center for Cell Science, Pune in India provided the two cell lines. The 3-(4, 5-Dimethylthiazol-2-yr)-2, 5-diphenyltetrazolium bromide for (MTT) assay was obtained from Sigma Aldrich. HaCaT cells were cultured in Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM (DMEM F-12 HAM)with 2 mmol L-glutamine supplemented with 10% fetal bovine serum (FBS), 45 IU/ml penicillin, and 45 IU/ml streptomycin (HaCaT media), at 37 °C in 5% CO_2. Preparations of mannosylated naringenin loaded transfersomes (MA-NgTfs) were formed by slight modification with the previously reported method [8]. The total lipid content and Tween 80 was mixed in a small amount of chloroform the plant active naringenin is about 20 mg and 10 mg of D-mannosamine HCl was dissolved in ethanol and the whole solution was subsequently added into lipid and Tween-80 mixture solution the solvent was evaporated at rotary evaporator (Buchi rota vapor R-3000 with 14000 water bath, Switzerland) until a dry layer of a mixture solution is formed and hydrated with phosphate buffer pH (5.5) at the temperature of 45 °C by rotation speed of 70 rpm and the nanovesicles forms were allowed to absorb and swell at room temperature for 3 hr. The soaked and hydrated prepared transfersome formulation was sonicated for 25 min in a probe sonicator to uniform and reduce the vesicles size. The developed MA-NgTfs were stored in a tightly closed container for performing further experiments.

Design of experiment statistics

The Box Behnken Response Surface methodology has been used to characteristically identify the influence of three critical and suitable formulation variables on vesicular size, % Entrapment efficiency, and Zeta potential of the developed (MA-NgTfs). The formulation details of the optimization were dictated in table 1. The limit and range of every factor were selected according to preliminary experiments results and the feasibility of formulating the transfersomes at the optimum values [8]. The soya phospholipid: surfactant ratio (L: S) % W/W in mg, Rotating Speed (RS) in rpm, and sonication time (ST) in min are taken as independent variables, whereas the vesicular size (VS) in nm, % Entrapment efficiency (%EE), Zeta potential (ZP) in mV, were chosen as dependent variables the amount of bioactive Naringenin and ligand (D mannosamine HCl) was taken 20 mg and 10 mg respectively and kept in constant amount throughout the experiment. The batch code and value of all variables coded and actual are shown in table 1. The selection of optimized formulation was decided accordingly the solution originated by the software.

Characterization of optimized mannosylated naringenin laded transfersomes (O-MA-NgTfs)

Vesicular size, PDI and zeta potential analysis

The vesicular diameter of mannosylated transfersomes was measured by the help of using Zetasizer instruments (Zetasizer, Malvern UK). The unimodal Size distribution was measured on the basis of the polydispersity index (PDI) [9]. A small range of PDI is considered as homogenous population, and a wider the range indicates higher heterogeneity. Zeta Potential indicates the charge in the form of electric ions on the surface of the vesicles that indicates the physical stability of the vesicular delivery system, was determined by using the Malvern Zetasizer 3000 HAS (Malvern Instruments, UK) [10]. The vesicular delivery system was diluted by ultrapure water just before the experiment. The results of size and zeta potential are shown in the.

Surface morphology study

The surface morphology of prepared optimized vesicles of mannosylated naringenin-loaded transfersomes (O-MA-NgTfs) was studied by transmission electron microscope (TEM), (Hitachi J500, H7500 Japan) [11]. In the carbon-coated copper grids, the sample of MA-NgTfs was placed and staining was done by aq solution of phosphor-tungstic acid (1 %) and observed under the microscope in an accelerated voltage of 100 kV

X-Ray diffraction study

X-ray diffraction study was performed for the analysis of the nature of (Crystalline or Amorphous) of mannosylated Naringenin loaded transfersomes [12]. Naringenin Soya Lecithin (Phospholipid), D-Mannosamine HCl physical mixture and transfersomes were carried out by using powder X-ray diffractometer (PAN analytical 3KWX' Pert Powder, Cambridge U. K.) followed by the sample of the formulation was kept and sputtered out in sample stage and scanned for the range of 2Θ to 60Θ with an accelerating voltage of about 40 kV and with load current of 30 mA. A Cu-Ka radiation source and scanning rate (2 h/min) was 5 °C/min.

FTIR

FTIR spectrum is used for the detection of the compatibility aspect of active pharmaceutical ingredients (API) and the excipients. FTIR spectra of Naringenin, Phospholipids, and D-Mannosamine HCl were determined by using (Shimadzu Bruker Alpha-II FTIR) by pellet method. All the Excipients and Naringenin was mixed with KBr and pellet was formed individually and samples were examined over wave number 4000-400 cm^{-1} range [13]. The characteristics peaks that shows the significant absorption peaks of the functional group of the drug.

Differential scanning colorimetry study (DSC) study

The study of DSC for the pure drug (Ng), soya phospholipid, D-mannosamine and formulated optimized formulation (MA-NgTfs) were performed using DSC instrument (Perkin Elmer, SOPS, Rajiv Gandhi Proudyogiki Vishwavidyalaya, Bhopal India) for each drug ligand and excipient the samples amount about 5 mg work kept in aluminum pans (Al-crucibles, 40 Al) and sealed. The nitrogen atmosphere was created and the props get heated from the range 50 to 400 °C in a rate of 10 °C.

Measurement of entrapment efficiency

The entrapment efficiency of prepared mannosylated transfersomes is evaluated by the direct method previously described by [14]. The amount of Ng encapsulated in mannosylated transfersomes in each formulation is detected by using Eppendorf tubes at low temperature. The frozen samples of formulations were centrifuged at rpm of 14000 by the help of a centrifuge (Remi Centrifuge model 5430 R) for a duration of 30 min. after that the supernatant solution was investigated for drug loading spectrophotometrically at λ_{max} of 289 nm by (UV-Spectrophotometer Shimadzu Model UV-1800, Tokyo Japan) followed by dilution with phosphate buffer pH 5.5. Therefore the % EE and % DL was calculated by the formula

$$EE\ \% = [NgT - NgS / NgT] \times 100$$

$$DL\ \% = [NgT - NgS / NgL] \times 100$$

Where NgT is the amount of total drug which is added into transfersomes system, NgS is the amount of drug detected in supernatant and NgL total amount of lipid used in the formulation.

Evaluation of storage stability

The stability study was determined for the thermodynamic stability of the system for determination of the ability of formulated formulation to withstand against environmental stress. The changes occur in vesicular size and physical visualization of the mannosylated Naringenin-loaded transfersomes. 10 ml of the formulation was kept at normal 25±2 °C, 60±5 % RH and temperature of 40±2 °C, 75±5 % RH. At a certain period of time interval i.e. 1, 3 and 6 mo the formulation was examined by vesicular Size, % Entrapment Efficiency and Zeta potential [15].

***In vitro* release study of drug**

The *in vitro* release study of MA-NgTfs, NgTfs, pure drug suspension, and the marketed formulation was carried out by modified Franz

diffusion cell (Mol. Wt. cut off 6000-8000, HI Media Ltd, Mumbai, India) separately. The diffusion cell was validated and designed prior to while performing the study. The mounted cellophane membrane on cell assembly of Franz diffusion with having an area of diffusion 2.5 cm^2 and the receptor compartment consist about 22.5 ml of PBS pH 5.5 the receptor fluid stirred 100 rpm with the temperature at 37±0.5 °C throughout the experiment. The MA-NgTfs was taken at donor compartment membrane [15] and about 2 ml of samples aliquot was withdrawn in suitable intervals of time and again filled immediately with the equal volume of freshly prepared diffusion medium. The release studies have been done in triplicate pattern and percentage drug release of the drug has been calculated and the graph against % drug release versus time was plotted.

Cellular uptake studies

The HaCaT macrophages cell line was procured from the (National centre for cell science, pune) was cultured and maintained as a supportive culture media in 96 well-cultured plates having EMEM (Eagle's Minimum Essential medium, low glucose with glutamine)supplemented with 10 % FBS and with 1 % antibiotics**.** After incubating overnight in a humidified atmosphere (5 % CO_2) in at 37±1 ºC the cells were separated and mechanically adjusted with a required concentration of the viable cells ($1x10^5$/well), the counting was made by the help of a haemocytometer. For the quantitative cellular uptake estimation, the cells were treated with the free drug and arrange in suspension dissolved in (0.5 % DMSO), NgTfs and MA-NgTfs (5 µ/ml, Ng/well) and incubated for 37 ºC in the atmosphere for 30 min, for the time in hrs 1, 3,6,12 and for 24 h. the medium was removed at each time point and PBS 5.5 was used to wash the cells in three-time, and collected by trypsinization and at 3000 rpm centrifuged for 3 min decanted supernatant re-suspended with the periods and 1 ml of ethanol forming vertex for 5 minute for extraction of Ng in ethanolic fraction and the resulting lysate found was centrifuged in at 5000 rpm in 5 min and the absorbance were recorded of Supernatant at 283 nm using UV visible spectrophotometer [16], from the ethanol extract of Naringenin was used for the calibration curve. The graph of calibration of ethanol Ng 283 nm, the Ng amount which is uptaken by cells was determined. The cellular uptake of Ng was denoted as micrograms of $Ng/10^5$ cells. For the confirmation of the mannose receptor-mediated uptake of MA-NgTfs, the mannose receptor found on the surface of cancerous macrophages cells were blocked by the help of using incubation of the cells within a sufficient amount of free mannose (0.05 M) for the period of time 1 hr just prior to incubation with the free Ng, NgTfs and MA-NgTfs in an individual experiment and the cellular uptake was determined after 24 h treatment. In this experiment, the amount of Ng 10 µM was used.

***Ex-vivo* permeation study**

The fully thicker skin of hairless (Swiss albino) mice was taken for conducting the Ex vivo skin permeation study of naringenin from nanovesicles by the use of fabricated Franz diffusion cell with enhanced permeation area having with 2.5 cm^2. Freshly prepared phosphate buffer of pH 5.5 was filled in the receptor chamber of the diffusion cell at 37 ºC temperature; the diffusion cell was maintained and by magnetic stirrer with a hot plate (Magnetic Stirrer Remie equipment) and the receptor chamber was agitated constantly with the rpm of 300. In the donor chamber, the nanovesicular formulation (MA-NgTfs), consisting of drug naringenin was placed gently and at 1, 2, 3, 4, 5, 6, 12, 18 and 24 h and about 5.0 ml of a solution containing drug were removed and the concentration of drug determined by UV-Spectrophotometer (UV Spectrophotometer SHIMADZU, 1800) and was replaced quickly with the same volume of freshly prepared PBS having pH 5.5. All experiments have been done in triplicate.

Skin deposition study

The studies of skin retention have been done to determine how much the content of bioactive i.e. Naringenin diffuses from the skin after 24 h. The skin-bearing diffusion cell was separated carefully and the surface of the skin rinsed carefully around 5 times with the help of 45 ºC receptor chamber medium for the determination of the amount of Naringenin in the skin. The small pieces of skin were cut from cleaned skin and mashed entirely in a beaker. In this mass 10 ml of 50% ethanol was added with the help of a water shaker bath. The complete extraction of Naringenin has been done by using a water shaker bath in 37 ºC for 2 hr in a mechanically shaken beaker. The appropriate dilutions have been made for the determination of Naringenin content in the resulting solution by UV spectroscopy.

Statistical analysis

The data and the findings obtained by the optimization were expressed in the form of mean±SD (Standard deviation). The results found by the analysis were represented as an analysis of variance p-value less than 0.05, ($p<0.05$) considered as significant. The Stat - Ease, Design Expert trial version 12.0.1.0 was used for the statistical data analysis and 3D plotted graph.

Table 1: Box behnken model of actual experimental design and obtained actual responses

F-code	Coded value			Actual value			Vesicular size (VS) (nm)=Y_1	Entrapment efficiency (EE%)=Y_2	Zeta potential ZP (-mV)=Y_3	PDI	Drug loading (DL in %)
	X_1=(L: S) (%W/W)	X_2=(RS) (rpm)	X_3=(ST) min	X_1= (L: S) (%W/W)	X_2= (RS) (rpm)	X_3= (ST) Min					
Tfs-1	0	0	0	92:8	60	20	156.2±0.68	79.03±0.96	22.8±0.99	0.229±0.102	9.017±0.019
Tfs-2	1	0	-1	88:12	60	25	263.74±0.63	74.09±0.63	18.8±0.91	0.385±0.076	7.018±0.009
Tfs-3	0	1	-1	92:8	70	15	209.81±1.57	77.8±1.15	18.04±1.09	0.135±0.108	3.019±0.028
Tfs-4	-1	0	-1	96:4	60	15	225.07±1.08	74.81±1.37	18.01±1.05	0.210±0.298	2.321±0.089
Tfs-5	1	0	1	88:12	60	25	112.3±0.66	79.02±0.99	24.2±0.63	0.543±0.086	8.43±0.19
Tfs-6	0	1	1	92:8	70	25	102.4±1.01	82.04±0.61	28.7±1.008	0.477±0.017	9.01±0.076
Tfs-7	0	0	0	92:8	60	20	149.7±1.18	79.8±1.41	25.02±1.19	0.169±0.065	7.026±0.49
Tfs-8	0	0	0	92:8	60	20	133.8±2.11	81.03±0.60	24.8±2.8	0.229±0.033	6.047±0.048
Tfs-9	0	0	0	92:8	60	20	165.04±0.58	78.2±2.09	23.09±1.63	0.579±0.210	5.069±0.035
Tfs-10	1	1	0	88:12	60	20	154.6±1.30	76.4±2.270	23.02±1.77	0.170±0.057	6.047±0.78
Tfs-11	0	0	0	92:8	60	20	174.8±1.40	78.6±1.43	21.09±1.21	0.212±0.201	7.043±0.056
Tfs-12	0	-1	-1	92:8	50	15	188.2±0.71	75.2±1.09	19.6±1.50	0.190±0.091	4.046±0.067
Tfs-13	-1	0	1	96:4	60	25	119.7±1.37	72.04±1.53	25.8±1.93	0.199±0.013	7.01±0.036
Tfs-14	-1	-1	0	96:4	50	20	198±1.09	73.04±1.45	20.03±1.19	0.389±0.200	6.09±0.082
Tfs-15	-1	1	0	96:4	70	20	191.7±1.35	74.8±1.58	22.03±1.19	0.140±0.027	5.023±0.098
Tfs-16	1	-1	0	88:12	50	20	173.02±1.50	73.83±1.48	20.21±1.45	0.212±0.070	6.046±0.019
Tfs-17	0	-1	1	92:8	50	25	109.8±1.42	79.08±1.85	24.03±1.53	0.280±0.047	8.016±0,02

*mean±SEM (n = 6).

RESULTS AND DISCUSSION

Statistical analysis

The Box-Behnken Model (BBM) with response surface quadratic model has been used for the maximum variables at different three levels low (-1), medium (0), and high (+1) along with the respective limited number of runs. The present research work aimed at the investigation of variables viz. Dependent and independent were analyzed at 3 levels of factorial design and three responses were studied. The 3 different values were found in ranging from for VS,

for % EE and ZP. On the basis of data and results, the quadratic model was suitable for these responses viz. Mean Vesicular Size (VS), Entrapment efficiency (% EE) and zeta potential (ZP).

The Quadratic equations generated by the software were mentioned below:

(VS), Y_1=155.91-3.85X_1-1.31X_2-55.33X_3-3.03X_1X_2-11.52X_1 X_3-7.25X_2 X_3+25.54X_1^2-2.11X_2^2-1.24X_3^2

(%EE), Y_2=79.33+1.08 X_1+1.24 X_2+1.29 X_3+0.2025 X_1X_2+1.92 X_1 X_3+0.0900 X_2 X_3-4.18 X_1^2-0.6373 X_2^2-0.1647 X_3^2

(ZP), Y_3=23.26+0.0450 X_1+0.9900 X_2+3.54 X_3+0.2025 X_1X_2-0.5975 X_1 X_3+1.56 X_2 X_3-1.46 X_1^2-0.5738 X_2^2-0.1937 X_3^2

Where X_1, X_2, and X_3 denoted the coded value of the Lipid: Surfactant ratio (L: S), X_2=Rotating Speed (RS), X_3=Sonication time (ST). In the equation, all the positive signs represent the synergistic effect on the responses, whereas the value of lack of fit is not significant in most of cases. The remaining parameters existing with a significant value with p-value ≤0.05. The experimental v alue as performed at the average of 3 batches (n=3) of transfersome formulation and the optimized range closer to the predicted value and on the basis of the difference between the predicted value and observed value, the low percentage bias were found, which indicates in the table 2 the optimization was reasonable and consistent [17].

Response surface plot

The BBM was employed to find surface responses. The 17 experimental runs were obtained exclusively for 3 level low (-1), medium (0), and high (+1) and three factors. The findings of optimization were shown in the table 3 along with both the variables independent as well as dependent. Other surface responses were plotted. The 3D response surface graph was generated by the help of software Design Expert trial version 12.0.1.0 and the value of separated other statistical parameter was obtained like the coefficient of variation, p-value, standard deviation and predicted F-value was summarized in with their significant value which was shown in the table 3. The graph of 3D surface response Have been used for the detection of Interaction pattern the 3D response surface plots imparts the information that soya phospholipids: surfactant ratio rotating speed and sanitation time significantly exhibited at the mean vesicle size, Entrapment efficiency (% EE) and Zeta potential (ZP) with the help of software Design expert The independent variable viz the ratio of Soya Phospholipids: Surfactant plays a vital role in the determination of the size of vesicles (VS) and Entrapment efficiency (% EE) the limit of soya phospholipids: surfactant for Entrapment efficiency of transfersomes was found in the range of 92:8 the justification for Entrapment efficiency (% EE) is that when surfactant ratio is in medium concentration were added the Entrapment efficiency was found to be optimum in range but slightly increasing the ratio of surfactant the Entrapment efficiency (% EE) suddenly decreased due to pore formation in the surface and leaking of drug. The rotating speed of 70 rpm was considered as good but not affected remarkably. The sonication time (ST) is about 25 minute was for small vascular size and Entrapment efficiency; the Zeta potential of all formulations was in the range of with good stability table 3 indicates the suitable polynomial equation with interaction factors which was selected for the estimation of several parameters statistics such as the adjusted multiple correlations adjusted R square multiple correlation Coefficient R square and predicted residual sum of squares generated by software design expert 12.0.1.0.

Table 2: Comparison of predicted and observed values in the prepared MA-NgTfs under predicted optimum condition

Response	Prediction	Observation	% Bias
Y_1 = Vesicle Size (nm)	102.4±1.01	104.2±0.64	1.75
Y_2 = % Entrapment Efficiency	82.04±0.61	80.07±0.96	2.40
Y_3 = Zeta Potential (-mV)	-28.7±1.008	-27.06±3.02	5.75

% Bias = [(Predicted value–Observed value)/Predicted value] X 100, *mean±SEM (n = 6).

Table 3: Summary of results found in regression analysis for response and analysis of variance for vesicular size, % entrapment efficiency and zeta potential

Statistical parameters	DF	SS	MS	F-value	p-value	R^2	SD	C. V %
Mean Vesicular Size								
Model	9	28147.40	3127.49	7.61	0.0070Significant	0.9073	20.27	12.19
Residual	7	2876.54	410.93					
Core total	16	31023.94						
% Entrapment Efficiency								
Model	9	127.17	14.13	8.59	0.0049Significant	0.9170	1.28	1.67
Residual	7	11.51	1.64					
Core total	16	138.68						
Zeta Potential								
Model	9	130.33	14.48	8.69	0.0047Significant	0.9178	1.29	5.79
Residual	7	11.67	1.67					
Core total	16	142.00						

DF: Degree of freedom, SS: Sum of Square, R^2: Correlation Coefficients, CV: coefficient of variation, SD: Standard Deviation

Effect of phospholipids and surfactant ratio on the particle size, PDI and zeta potential

The phospholipids and surfactant ratio played a remarkable role to determine the mean vesicular size and the Zeta potential of the vesicles which required for the stability of the formulation. The formulation was designed by using the range of lipid surfactant ratio at the range 96:4 %-88:12 % W/W. The drastic size increment was made when the lipid surfactant ratio is in increasing order. The Zeta potential is found in the formulation having a range of-18.01±1.05 to-28.7±1.008 mV represents the good stability of the formulation. Zeta potential represents the surface charge of the vesicles and required for the determination of the stability and L: S ratio play a remarkable role in imparting the stability of the nanovesicular system. The vesicles size range was found in the range of 102.4±1.01 to 263.74±0.63 table 1 and the range of Entrapment efficiency of nanovesicles was as 72.04±1.53 to 82.04±0.81. The Entrapment efficiency of transfersomes was increased significantly ($p<0.05$) by increasing the ratio of surfactant concentration ranging from 4 to 8 % W/W for formulated transfersomes prepared using Tween 80 as the surfactant.

Size analysis

The vesicular size analysis values for optimized transfersomes determined by Malvern Zeta sizer showed size ranging from

102.4±1.01 to 263.74±0.63 nm table 1. The effect of less lipid ratio of phospholipids and surfactant concentrate on the particle size can be seen from the particle size of sample Tfs–5 and Tfs–6 (112.3 nm and 102.4 nm), respectively. The effect of sonication and rotation speed was not significant on decreasing the size of the particle separately but together, they showed decreases in particle size. Sonication time also led to an alteration in the zeta potential of the formulation. On increasing the sonication time, zeta potential changes from 18.01 mV to 28.7 mV. Additionally, there was no significant effect of rotation speed on PDI.

Surface morphological study

The surface morphology of optimized mannosylated naringenin-loaded transfersomes (O-MA-NgTfs) is depicted. The tables shows that the prepared Mannosylated nanovesicles are nanosized with having the single lamella the surface of O-MA-NgTfs are displayed as retention and sealed vesicular structure and also indicates the uniform homogeneous size distribution.

X-ray diffraction

Powder X-ray diffraction patterns of pure Naringenin (Ng), soy lecithin, an optimized lyophilized sample of mannosylated naringenin-loaded Transfersomes were recorded. The Ng showed distinct characteristic sharp and intense peaks at 2h values of, and in the diffractogram. These peaks indicate that the Ng was crystalline nature. These peaks of Ng were absent in Mannosylated Naringenin-loaded Transfersomes (MA-NgTfs) diffractogram, which indicates that Ng was present in the amorphous form within the transfersomes. If Ng was present outside the transfersome, it would occur crystallization because its poor aqueous solubility and might be affected the diffraction patterns of the Ng-loaded transfersome. But no changes occur in diffractogram, which indicates that Ng was successfully encapsulated into the transfersome. The X-ray diffraction patterns of empty and Ng-loaded transfersome were quite similar, indicating that the presence of Ng did not affect the nature of transfersome. In case of bulk soya lecithin diffractogram peaks were obtained between 2 h values of 14.67–26.56. These peaks were also obtained in empty and Ng-loaded transfersome diffractogram but having lower intensity, which suggests a less ordered structure of soya lecithin in transfersome.

Differential scanning colorimetric (DSC) study

At the 251 ºC, pure drug naringenin in a sharp melting transition point was observed at 77.52 to and 222.13 ºC soya phosphatidylcholine chlorine and Tween 80. At 218.19° C, a peak has shown by DSC thermogram of optimized formulation it is indicated by new sharp melting endothermic peaks of prepared transfersomes formulation which is absent initially due to crystalline phase of ingredient and the bioactive. The characteristic peak of naringenin was completely absent in the case of MA-NgTfs, which indicated the incorporation of drug Ng in the lipid system. Therefore it is stated that the drug present in the nanocarrier is in the amorphous state and due to this, the drug does not show its endothermic peak in the optimized formulation.

FTIR study

The Fourier transform infrared spectroscopic study of plant bioactive Naringenin (Ng) shows the significant peaks recorded by the scanning pellets with the range of 500-4000 cm^{-1}. The spectra of Ng characteristics bands and peaks at 3326.66 cm^{-1}(Alkenyl C-H stretching) 3175.58 cm^{-1}(Alkynyl C-H stretching), 1645.17 cm^{-1}(carbonyl groups stretching) and 1400-1500 cm^{-1}(Aromatic ring C=C bending). The confirmation of conjugation between lipid and the D-mannosamine HCl shows the identifying peak at 1600 $cm^{-1}$and the availability of strong sharp peak between 1620 and 1640 cm^{-1}. The IR spectrum of Ng ring modes which corresponds to the localization of benzene part of the Naringenin, but it is disappeared in the case of MA-NgTfs transfersomes formulation. Whereas the new and characteristics peaks are recorded at 1550 cm^{-1}C-C stretches and 1620 cm^{-1} for aromatic C-H bending. Rest of the peaks is either remaining same or slightly shifted in the IR spectrum of the formulation. This corroborating the entrapment of drugs and conjugation of mannose in the lipid.

***In vitro* drug release study**

Phosphate buffer saline (pH 7.4) was used as dissolution media and the dialysis bag was submerged in 100 ml of media with 100 rpm stirring at 37 ºC temperature. Subsequently, 1 ml samples were withdrawn at definite time-points and the same amount of fresh media was added to maintain sink condition. Percentage release of samples was estimated at 289 nm using UV spectrophotometer. The experiment was performed in triplicate. No drug precipitation was observed during release study, as confirmed by the physical observation of the dialysis bag. The agenda of Mannosylated naringenin-loaded transfersomes to control burst release phenomenon by Tfs as well as maintain the sustained duration of release. Mannosylated naringenin-loaded transfersomes, and marketed formulation dispersion was found 69.31 %, 62.03 %, 58.71 %, and 65.02 % respectively as shown in fig. 1. The release of hydrophobic drug form naringenin is dependent on the diffusion ability from the oil core into the surfactant layer and finally to continuous phase. The dynamic layer of Mannosylated creates additional partition through continuous phase. This leads to slow release of drug from the oil core and it could be predicted that it will maintain the therapeutic window at the site of action for a longer period of time. Therefore, it is expected to obtain a prolonged release of incorporated naringenin at the site of application to maintain the supply of the therapeutic agent towards effective healing of wound. The result is represented in table 4.

Table 4: *In vitro* drug release profile of optimized mannosylated naringenin loaded transfersomes (O-MA-NgTfs), plain naringenin loaded tansfersomes (NgTfs), pure drug suspension (PDS) and marketed formulation (MF)

S. No.	Time intervals	% Cumulative drug release from various formulation			
		O-MA-NgTfs	NgTfs	Marketed formulation (MF)	Pure drug suspension (PDS)
1	0	0	0	0	0
2	2	9.83±2.14	8.49±1.23	13.52±2.41	14.02±0.32
3	4	20.17±1.12	18.13±0.41	25.11±1.06	29.43±0.51
4	6	32.81±1.37	30.02±2.13	37.57±2.03	44.06±2.41
5	12	48.07±2.71	45.13±1.51	53.31±0.82	60.21±0.83
6	24	62.03±0.42	58.71±2.53	65.02±0.37	69.31±2.31

*mean±SEM (n = 6)

***Ex vivo* skin permeation and skin deposition and study**

The ex vivo permeation study revealed that the cumulative concentration of naringenin permeated in Swiss albino mice skin through vesicular formulation was significantly higher ($p \leq 0.05$) than the marketed product and pure drug suspension optimized transfersomes through the skin of Mice was of flux 6.5±3.07 and the percentage of drug retention was 0.76±1.26 whereas the non-mannosylated Naringenin loaded transfersomes (NgTfs), marketed product and pure drug suspension was significantly minimized transdermal flux and percent drug retention as shown in table 5. The nanosize vesicle range was achieved by the concentration of surfactants and the surfactant plays a vital role and act as a penetration enhance due to surfactant dilated the layer of skin in the

stratum corneum and facilitate to penetrate the vesicles into the intact skin.

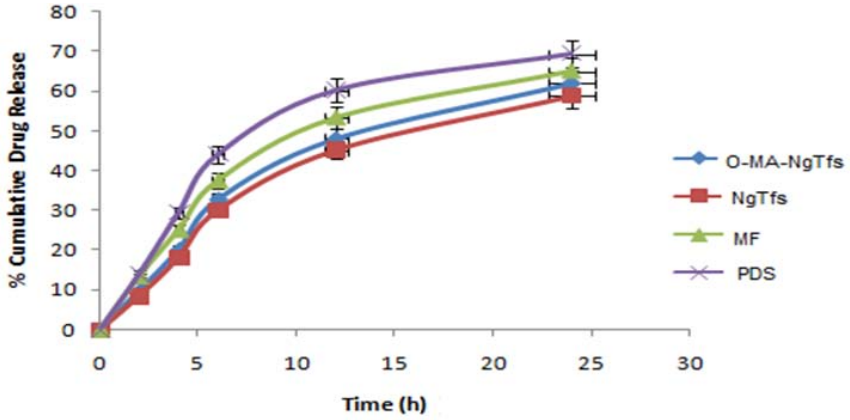

Fig. 1: *In vitro* drug release profile of optimized mannosylated naringenin loaded transfersomes (O-MA-NgTfs)

Table 5: Represented the permeation and % drug retention data for PDS, MF, NgTfs, O-MA-NgTfs across thick skin of (Swiss albino mice)

Formulation code	Jss (μg/ch^{-2}/h)	P (Ch/h)	LT (h)	D^d (Ch^2/h)	% drug retention after 24 h
PDS	5.47±1.51	0.256±2.39	2.01	5.3	.47±0.35
MF	5.8±2.05	0.243±1.53	1.5	6.7	.56±2.51
NgTfs	6.03±1.36	0.223±3.47	1.63	7.4	.62±0.57
O-MA-NgTfs	6.5±3.07	0.221±1.33	1.9	8.2	.76±1.26

Jss: transdermal flux, P: permeability coefficient, LT: Lag Time, D^d: Diffusion Cofficient and all the values represented as mean±SD (n=3), *mean±SEM (n = 6).

Cellular uptake study

The estimation of the targeting potential of formulated ligand directed delivery system of naringenin could be connected on the basis of their targeting potential in macrophages. Therefore the cellular uptake of formulated MA-NgTfs was studied in HaCaT macrophages cell line [18]. The uptake studies of transferesomes have been done in the formulation and plain drug suspension (PDS) where one is plain optimized NgTfs and another is optimized mannosylated naringenin loaded transfersomes (MA-NgTfs) and results revealed that the prominent difference in Naringenin levels. The highest amount of drug concentration was observed in 3 h post-treatment and the level of drug concentration in NgTfs and MA-NgTfs treated cells 1.51±0.71 μg/10^5 cells and 2.93±0.48/10^5μg/10^5 cells respectively after 3 h where is the highest concentration of drug 2.41±0.51 μg/10^5 cells was achieved just after 30 min in naringenin plain suspension solution. The results threw light the concept act that the free naringenin suspension containing 0.5 % DMSO rapidly uptaken by HaCaT cells in the 30 min initially after that the concentration of the drug decreases rapidly over time and level of drug concentration was reduced at 0.01 μg/10^5 cells after the 12 h incubation. In contrast of NgTfs or MA-NgTfs the concentration of drug moderately increased in the initial stage (30 min 3 hr) and during the subsequent time of incubation (3-6 h) was reduced. All the concentration of drug in uptake level of MA-NgTfs was almost higher than the NgTfs at every time Point ($p \leq 0.05$). The outline of results on the basis of finding the reorganization mode of MA-NgTfs specific binding to the mannose-specific receptor of macrophages which eventually might have uptake their higher level. Another parallel experiment was performed for corroboration of the experiment previously reported. The mannose receptor blocking experiment has conducted the level of the drug from MA-NgTfs was found significantly in less amount in the blocked cells 0.81±0.31μg/10^5 cells as comparatively to unblocked cells. 2.1±0.63 μg/10^5 cells. Hence the drastic reduction in drug uptake was found in blocked cells. The results gave us strong evidence of cellular uptake bymannose–directed transfersomes via mannose receptor-based endocytosis [19, 20].

Stability studies

There was a minor increase in the size of the vesicles from nm to 106.57±0.5 nm to 106.02±0.16 during the condition of storage (40 °C and 25 °C) for 6 mo and in the stability studies, the optimized transfersomes in the initial percent Entrapment efficiency was found to be 80.07±0.96 percent and after storing it 6 mo at 40 °C and 25 °C it was found to be 76.01±0.53 percent and 76.74±3.36 respectively and vice versa for six months at 4 and 25 °C there was no significant change in the percent me during the storage of formulation after restoring 6 mo Zeta potential at 4 and 25 °C there was a slight reduction found for 6 mo at 40 °C and 25 °C temperatures optimized formulation was found stable in table 6 data are represented.

Table 6: Result summary of stability of O-MA-NgTfs

Time period	Parameters					
	Y_1 = VS (nm)		Y_2 =%EE		Y_3= ZP (-mV)	
	40±2 °C,75±5 %RH)	(25±2 °C, 60±5 %RH)	40±2 °C,75±5 %RH)	(25±2 °C, 60±5%RH)	40±2 °C,75±5 %RH)	(25±2 °C, 60±5%R H)
0 Mo	104.2±0.64	104.2±0.64	80.07±0.96	80.07±0.96	27.06±3.02	27.06±3.02
1 Mo	104.43±1.92*	104.03±0.45*	79.75±0.48	79.83±2.49**	26.01±0.23	26.81±1.71
3 Mo	105.05±0.54	105.54±1.28*	77.02±1.58	77.82±0.89**	24.07±1.69	23.87±1.67
6 Mo	106.57±0.5	106.02±0.16	76.01±0.53	76.74±3.36	23.05±0.76	23.86±0.85

*mean±SEM (n = 6). Significant at **p<0.01

CONCLUSION

The Designing and optimization of mannose conjugated transfersomes have been successfully performed and formulated optimized mannosylated Naringenin loaded transfersomes was evaluated and the results revealed that the formulated mannose directed nano transfersomes has a remarkable capacity for targeting the macrophages and the data obtained from the BBM and surface response curve was found to be significant. The medium amount of lipid and surfactant ratio shows highest entrapment efficiency and the highest sonication time i.e. 25 min was required for smaller size vesicles which imparts the higher Zeta potential of the formulation it provide the thermodynamical stability of vesicular delivery system. The FTIR result of optimized MA-NgTfs shows the attachment of D-mannosamine HCl moiety in the transfersomes. The formulated delivery system for skin cancer has the ability to penetrate the skin and deposition of naringenin was found to be high concentration than the marketed formulation. Therefore the mannose-directed transfersomes also improve the dermal delivery of lipophilic bioactive. So that the results of our study demonstrated that the successful entrapment of bioactive in developed system and their targeting affinity towards macrophages. For the authentication of macrophage targeting many more studies are still required.

ACKNOWLEDGEMENT

I pay my warm gratitude to the head of Phyto-formulation Laboratory, University Institute of Pharmacy, Pt. Ravishankar Shukla University, Raipur, Chhattiisgarh for their untiring efforts to make this manuscript.

FUNDING

Nil

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

The authors declare no conflict of interest related to the submission of this manuscript and the manuscript is approved for publication by all authors.

REFERENCES

1. D Orazio A, Jarrett S, Amaro Ortiz A, Scott T. UV radiation and the skin. Int J Mol Sci 2013;14:1222-48.
2. Saraf S, Gupta A, Kaur CD, Jangde M. Dermatological consequences of photosensitization with an approach to treat them naturally. Pak J Biol Sci 2014;17:167-72.
3. Gupta A, Kaur CD, Saraf S, Saraf S. Formulation, characterization and evaluation of ligand-conjugated biodegradable quercetin nanoparticles for active targeting. Artif Cells Nanomed Biotechnol 2016;44:960-70.
4. Tavana O, Benjamin CL, Puebla Osorio N, Sang S, Ullrich SE, Ananthaswamy H, *et al.* Absence of p53-dependent apoptosis leads to UV radiation hypersensitivity, enhanced immunosuppression and cellular senescence. Cell Cycle 2010;9:3348-56.
5. Chen P, Zhang X, Jia L, Prud'homme RK, Szekely Z, Sinko PJ. Optimal structural design of mannosylated nano carriers for macrophage targeting. J Controlled Release 2014;28:341-9.
6. Gupta A, Kaur CD, Saraf S. Comparative evaluation of two different novel formulations of quercetin against non melanoma skin cancer in human subjects. J Clin Exp Dermatol Res 2016;7:34-9.
7. Martinez RM, Pinho Ribeiro FA, Steffen VS, Silva TC, Caviglione CV, Bottura C, *et al.* Topical formulation containing naringenin: efficacy against ultraviolet B irradiation-induced skin inflammation and oxidative stress in mice. PloS One 2016;11:e0146296.
8. Pukanud P, Peungvicha P, Sarisuta N. Development of mannosylated liposomes for bioadhesive oral drug delivery via M cells of Peyer'spatches. Drug Delivery 2009;16:289-94.
9. Ahad A, Aqil M, Kohli K, Sultana Y, Mujeeb M, Ali A. Formulation and optimization of nanotransfersomes using experimental design technique for accentuated transdermal delivery of valsartan. Nanomed Nanotech Biol Med 2012;8:237-49.
10. Verma DD, Verma S, Blume G, Fahr A. Particle size of liposomes influences dermal delivery of substances into the skin. Int J Pharma 2003;258:141-51.
11. Tsai MJ, Huang YB, Fang JW, Fu YS, Wu PC. Preparation and characterization of naringenin-loaded elastic liposomes for topical application. PloS One 2015;10:1013-26.
12. Yang S, Liu C, Liu W, Yu H, Zheng H, Zhou W, *et al.* Preparation and characterization of nanoliposomes entrapping medium-chain fatty acids and vitamin C by lyophilisation. Int J Mol Sci 2013;14:19763-73.
13. Jangdey MS, Kaur CD, Saraf S. Efficacy of concanavalin-a conjugated nanotransfersomal gel of apigenin for enhanced targeted delivery of UV induced skin malignant melanoma. Art Cel Nanomed Biotech 2019;47:904-16.
14. Shinde G, Patel M, Mehta M, Kesarla R, Bangale G. Formulation, optimization, and characterization of repaglinide loaded nanocrystal for diabetes therapy. Adv Pharma 2015. https://doi.org/10.1155/2015/363061
15. Sayyad MK, Zaky AA, Samy AM. Fabrication and characterization of sildenafil citrate loaded transfersomes as a carrier for transdermal drug delivery. Pharm Pharmacol Int J 2017;5:100-13.
16. Sumathi R, Tamizharasi S, Sivakumar T. Formulation and evaluation of polymeric nanosuspension of naringenin. Int J Appl Pharm 2017;9:60 70.
17. Jangdey MS, Gupta A, Saraf S, Saraf S. Development and optimization of apigenin-loaded transfersomal system for skin cancer delivery: *in vitro* evaluation. Artif Cells Nanomed Biotechnol 2017;45:1452-62.
18. Chaubey P, Patel RR, Mishra B. Development and optimization of curcumin-loaded mannosylated chitosan nanoparticles using response surface methodology in the treatment of visceral leishmaniasis. Exp Opin Drug Delivery 2014;1:1163-81.
19. Qushawy M, Nasr A, Abd-Alhaseeb M, Swidan S. Design, optimization and characterization of a transfersomal gel using miconazole nitrate for the treatment of candida skin infections. Pharmaceutice 2018;10:26-32.
20. Upasana Y, Nuzhat H, Qamar R. Formulation of nanoparticles of E prosartan mesylate for better drug delivery by improving solubility. Asian J Pharm Clin Res 2018;11:260-3.