RESEARCH PAPER

PEGylated Liposomes of Opuntiol: Formulation Optimization, Characterization and in-vitro Anticancer Activity

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Abstract

Breast cancer is the most frequently diagnosed cancer in women worldwide. Opuntiol is the potent phytoactive of *Opuntia elatior* Mill. (Cactaceae), and exhibited potent anticancer activity against MCF7 breast cancer cells. The liposomes have successfully improved the delivery of many phytoactives having anticancer activity. Therefore, the present study aimed to develop and optimize PEGylated Opuntiol liposomes using a three-factor, three-level Box–Behnken design for breast cancer activity against MCF7 cells. The mean particle sizes were observed in the range of 122.13 ± 38.17 to 214.08 ± 38.46 nm, PDI values <0.3, and zeta potentials -7.5 ± 0.4 to -28.8 ± 0.8 mV. The entrapment efficiency and drug loading capacity of the formulations ranged from 80.18 ± 0.87% to 97.54 ± 1.10% and 33.25 ± 0.07% to 39.48 ± 0.11% respectively. The percent cumulative drug release of Opuntiol from prepared liposomes OPN-DSPE-PEG-2000 ranges from 75.23 ± 1.1% to 97.56 ± 1.32 %, after 12 h. The MTT assay on breast cancer MCF7 cells demonstrates cytotoxic effect of the standard 5-FU (IC50= 25.30 µg/mL), OPN (IC50= 55.75 µg/mL), and OPN-DSPE-PEG-2000 (IC50= 44.68 µg/mL), suggesting that PEGylation enhanced cellular uptake and therapeutic efficiency. The present study therefore concluded that PEGylated liposomes serve as an efficient nanocarrier system for Opuntiol by enhancing its pharmaceutical properties, including stability, controlled drug release, and dispersion in the biological environment, ultimately resulting in enhanced in vitro anticancer activity against MCF7 breast cancer cells.

Keywords:

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Introduction

The *Opuntia elatior* Mill. (Cactaceae), often known as the "nagphani or prickly pear cactus", is a medicinal plant extensively distributed in semi-arid and arid environments.[1] It has been historically used in herbal therapy for the treatment of several disorders, including wounds, inflammation, diabetes, oxidative stress and gastrointestinal issues.[2] The therapeutic effects of Opuntia elatior are due to the presence of many bioactive phytochemicals, among which Opuntiol is regarded as one of the key active ingredients.

Chemically Opuntiol is a bioactive flavonol (6-hydroxymethyl-4-methoxy-2H-pyran-2-one).[3] Due to the wide range of physiological actions, Opuntiol has developed significant scientific attention. Experimental evidence have reported the pharmacological activities of Opuntiol such as anti-inflammatory, gastroprotective, hepatoprotective, potent antioxidant, and anticancer. These bioactivities are mostly attributed due to its capability to regulate inflammatory mediators, to scavenge free radicals and protection of cell from oxidative damage.[4]

Recently, the Opuntiol has gaining the interest on detailed mechanism of action and pharmacological potential. Preclinical investigations suggest that it may play a promising role in the prevention and management of chronic disorders related with oxidative stress, inflammation and cancer. However, further clinical trials are needed to prove its safety, efficacy, and therapeutic applications in people.[5] Kandan PV, and co-workers, 2019; and Ashfaque A, and colleagues 2021; have demonstrated Opuntiol exhibits anticancer activity by suppressing proliferation of oral carcinoma (KB) cells ($IC_{50} \approx 30$ µM), inducing ROS generation, causing mitochondrial dysfunction, triggering apoptosis, and increasing active caspase-3 expression in glioblastoma cells.[6-7]

Since liposomes have successfully improved the delivery of many natural anticancer compounds, developing an Opuntiol-loaded liposomal formulation is a logical research strategy, although direct experimental evidence for this specific formulation is still lacking.

Therefore, an attempt was made to develop PEGylated liposomes encapsulating Opuntiol as a potential nanocarrier for breast cancer therapy. In the present investigation, PEGylated liposomes were expected to offer several advantages over plain Opuntiol, including improved dispersion in biological fluids, enhanced colloidal stability, and protection of the encapsulated drug from premature enzymatic and chemical degradation, thereby prolonging its biological activity and improving formulation stability.[8-9] The incorporation of DSPE-PEG into the liposomal bilayer forms a hydrated PEG corona around the vesicles, which provides steric stabilization, minimizes vesicle aggregation, and contributes to sustained drug release. Such prolonged drug release is expected to maintain therapeutic drug concentrations for an extended duration while reducing premature drug leakage. Furthermore, PEGylated liposomes have been reported to facilitate cellular internalization through endocytic pathways, thereby enhancing intracellular drug delivery and improving the antiproliferative activity of encapsulated anticancer agents. Improved intracellular drug retention may also contribute to enhanced induction of apoptosis in cancer cells.[10–12]

Although Opuntiol has demonstrated promising anticancer activity in several experimental models, its pharmaceutical application is still limited by its poor aqueous solubility and inadequate stability in biological systems. To the best of our knowledge, no systematic investigation has optimized PEGylated liposomes for the delivery of Opuntiol using a Quality-by-Design (QbD)

approach. Therefore, the present study aimed to formulate and optimize PEGylated Opuntiol liposomes using a three-factor, three-level Box–Behnken design and to evaluate their physicochemical characteristics, entrapment efficiency, in vitro drug release, stability, and anticancer activity against MCF7 breast cancer cells.

2. Materials and methods

2.1 Materials

2.1.1 Plant Selection, Collection, and authentication

The fully matured, dark red colored fruits of Opuntia elatior Mill (Cactaceae), commonly known as "nagphani," were collected from the local areas of Mahakal, Wardha, Maharashtra, India, in the months of June-July and November-December. Fruits were identified and authenticated by an expert Botanist, with a specimen voucher number 08/Botany/2021-22. The fruits were washed thoroughly and cut into small pieces and used for extraction.

2.2 Chemicals and Reagents

1,2-distearoyl-sn-glycero-3-phospho-ethanolamine-N-[amino (polyethylene glycol)-2000] (DSPE-PEG-2000) was purchased from Topclass Enterprises LLP, New Delhi, India. Hydrogenated soya phosphatidylcholine (HSPC), and cholesterol were purchased from Loba Chemie Pvt. Ltd. Mumbai, India. Opuntiol (OPN) was separated indigenously from the fruits of Opuntia elatior Mill, (Cactaceae). Analysis and characterization of Opuntiol was accepted in Jordan Journal of Pharmaceutical Sciences. All the solvents and chemicals were of AR and HPLC grade.

2.3 Preparation of PEGylated Opuntiol liposomes by Box Behnken Design

The thin lipid film hydration method was modified to prepare PEGylated liposomes encapsulating OPN. A mixture of HSPC, CHL, DSPE-PEG-2000, solubilized in chloroform:methanol (2 : 1 v/v) and OPN were separately dissolved in chloroform: methanol (2 : 1 v/v) and added to the lipid phase, thoroughly mixed, evaporated to dryness under reduced pressure at 40°C by using a rotating vacuum evaporator. A thin film was coated on the inside wall of a 50 mL round-bottom glass flask. The dried lipid film was dispersed gently in tris buffer along with 5% dextrose solution at room temperature. The resulting liposome dispersion was sonicated for 5 min with gentle shaking. Prepared liposomes were filtered using a PES hydrophilic sterile membrane filter [0.22µm, 25mm diameter, HiMedia (Cellbio), India] to remove large liposomes. The final liposomes in the Tris buffer solution were preserved at 4°C until use.[13]

2.4 Characterization of PEGylated Opuntiol liposomes (OPN-DSPE-PEG-2000)

2.4.1 Determination of particle size, polydispersity index and zeta potential

The average particle size, polydispersity index, and zeta potential of formulated liposomes (B1 to B15) were determined by the dynamic light scattering (DLS) technique (Litesizer DLS 500, version 4.6.0, Anton Paar) at 25°C with an optimal detection angle of 173°. The size of the liposomes was measured immediately after diluting 1mL of liposomes with 100mL of filtered PBS (pH 7.4) using a PES hydrophilic sterile membrane filter. The results (mean ± S.D.) given are for three consecutive measurements.[14]

2.4.2 Determination of drug encapsulation efficiency (% EE)

The EE of B1 to B15 was calculated indirectly by separating the free drug using a cooling centrifuge. The OPN-loaded liposomes were centrifuged at 4 °C, 20 min at 10000 rpm. Following centrifugation, the liposomes encapsulating the entrapped drug precipitated at the bottom, whilst the unbound drug remained in the supernatant. The unencapsulated drug from the supernatant was quantified using UV spectrophotometry at 280 nm. The entrapment efficiency was calculated using the subsequent formula.[15]

% EE = (Total drug-Free drug/ Total drug) x 100

2.4.3 Determination of drug loading capacity (% DL)

Drug loading capacity (B1 to B15) was assessed for the drug encapsulated within the liposomes. The quantity of free drug was ascertained using the indirect method for OPN-loaded liposomes. The OPN-loaded liposomes were centrifuged at 10000 rpm for 15 minutes at 4 °C. Following centrifugation, the liposomes encapsulating the entrapped drug precipitated at the bottom, whereas the unbound drug persisted in the supernatant. The quantity of unentrapped drug was used to calculate the drug loading efficiency of OPN-loaded liposomes employing the subsequent formula.

% DL= (Actual amount of drug /weight of liposomes) x100

2.4.4 Morphological Characterization

(A) Field emission scanning electron microscopy (FESEM)

The surface morphological features of OPN-DSPE-PEG-2000 liposomes were assessed using field-emission scanning electron microscopy. Liposome samples were coated with platinum for 5 minutes at 20mA using an ion sputter (JSM-IT810, JEOL Ltd., Tokyo, Japan). The observation was accomplished with the accelerating voltage of 20 kV and the working distance of 11.9 mm. The SEM images were recorded at 10,000× and 20,000×, respectively.

(B) Transmission electron microscopy (TEM)

A drop of OPN-DSPE-PEG-2000 liposomal solution was placed on carbon film coated on a copper grid for TEM. Observation was performed at 80 kV in a JEM-2000 FXII. The TEM images were taken at 50,000x and 100,000x magnification.

Transmission electron microscopy (HT7800, Series 120kV, Hitachi, Japan) was employed to analyze the surface morphological features of liposomes. The liposomes was diluted with deionized water, stained and then fixed to the mesh grids. Samples were examined at 120 kV subsequent to the removal of residual solution.[16]

2.5 In vitro release study

The in vitro drug release study of OPN, and OPN-DSPE-PEG-2000 liposomes was evaluated for the percent cumulative drug release by using diffusion cell apparatus (LEDC/06) having Hanson type-Franz cell (Donor & Receptor), 15 mm diameter and 7.5 mL capacity (Milton Enterprises, India). The dialysis membrane with a MWCO of 12,000 Da was immersed in a hydroalcoholic solution overnight to activate the membrane for further testing. The OPN and its PEGylated liposomes were placed in the donor compartment and samples of the dissolution medium were obtained from the receiver compartment. The diffusion cell was fitted to diffusion

cell apparatus at 100 RPM and a temperature of 37 ± 0.5 °C. At pre-determined time points, 0.5 mL sample was extracted and the same volume of PBS 7.4 was automatically replaced to maintain the sink condition. The samples were taken at time intervals of 0.5, 1, 2, 3, 4, 5, 6, 8, 10 and 12 h. After dilution, the samples were spectroscopically examined for absorbance at 280 nm and the percentage cumulative release was calculated. The in-vitro release data were further analyzed by fitting the cumulative percentage drug release data to different mathematical kinetic models, including zero-order, first-order, Higuchi, and Korsmeyer–Peppas models, to elucidate the drug release mechanism. The coefficient of determination (R^2) was used to determine the goodness of fit for each model, and the model exhibiting the highest R^2 value was considered to best describe the release kinetics. The release exponent (n) obtained from the Korsmeyer–Peppas model was used to predict the mechanism of drug release from the PEGylated liposomes.[17]

2.6 Optimization of formulation variables using the desirability function

A three-factor, three-level Box–Behnken design was employed to investigate the effects of formulation variables on the characteristics of Opuntiol-loaded PEGylated liposomes. The molar proportions of HSPC, cholesterol, and DSPE-PEG-2000 were selected as the independent variables, while particle size and entrapment efficiency were selected as the dependent responses. The experimental responses were fitted to a second-order polynomial model, and the significance of the linear, quadratic, and interaction effects was evaluated by analysis of variance.

Numerical optimization was performed using the desirability function. The optimization goals were set to minimize particle size and maximize entrapment efficiency, with equal weight and importance assigned to both responses.

2.7 In-vitro cytotoxicity by MTT assay

The in-vitro cytotoxicity of Opuntiol and its PEGylated liposomes e on MCF7 breast cancer cells was examined via MTT [3-(4, 5-Dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide] assay (HI Media, India). The 96-well tissue culture plates were seeded at (1×105 cells/ml) cells per well in 100 µl (Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum) of culture medium. 100 µl of the OPN, OPN-DSPE-PEG-2000 and 5-fluorouracil (positive control) at concentrations of 10 to 200µg/mL were tested. After incubation (24 h), 20 µl of MTT solution (5 mg/mL) was added to each well and kept it further for incubation (4h). The formation of formazan crystals, indicating viable cells, was observed under a microscope. The developed formazan crystals by metabolically active cells were dissolved in DMSO (0.2%), and absorbance was recorded at 570 nm. The experiment was conducted in triplicate to ensure reproducibility, by an ELISA microplate reader (Athenese-Dx-ALTA-ELISA reader, India).[18-20]

3. Results and Discussion

3.1 Plant Selection, collection and authentication

The fully matured, dark red colored fruits of Opuntia elatior Mill. (Cactaceae), commonly known as "nagphani" were collected from the local areas of Mahakal, Wardha, Maharashtra, India, in the month of June-July and November-December. Fruits were identified and authenticated by an expert Botanist, with a specimen voucher number 08/Botany/2021-22. The fruits were washed thoroughly and cut into small pieces and used for extraction.

3.2 Preparation of PEGylated Opuntiol liposomes by Box Behnken Design

PEGylated Opuntiol liposomes were successfully prepared by the thin-film hydration method using HSPC, cholesterol, and DSPE-PEG-NH2. Hydrogenated soybean phosphatidylcholine (HSPC) was selected as the principal phospholipid because of its high phase transition temperature and predominantly saturated acyl chains, which facilitate the formation of a rigid and well-organized lipid bilayer. The increased membrane rigidity imparted by HSPC is expected to reduce drug leakage, improve entrapment efficiency, and enhance the physical stability of the liposomal formulation during storage. Furthermore, HSPC has been widely employed in long-circulating PEGylated liposomal formulations owing to its compatibility with PEGylated phospholipids and its ability to maintain bilayer integrity, thereby contributing to sustained drug release and improved formulation stability.[2]

Table 1: Experimental design and response variables of Opuntiol-loaded PEGylated liposomes prepared according to the Box–Behnken design (mean ± SD, n = 3).

Batch No.	HSPC (Molar Parts)	CHL (Molar Parts)	DSPE-PEG-2000 (Molar Parts)	OPN-DSPE-PEG-2000 Liposomes characterization					
				Particle size (nm)	PDI	Zeta potential (mV)	%EE	%DL capacity	% Cumulative drug release at 12 h
1	10	10	3	133.68 ± 85.42	0.218 ± 0.72	-13.8 ± 0.5	82.32 ± 1.32	34.88 ± 0.08	96.15 ± 1.38
2	15	15	1	140.30 ±102.34	0.267 ± 1.31	-7.5 ± 0.4	83.81 ± 1.	35.11 ± 0.02	94.23 ± 1.90

							83		
3	15	10	2	150.02 ± 49.04	0.228 ± 1.52	-10.5 ± 0.8	85.86 ± 0.94	39.48 ± 0.11	91.82 ± 1.58
4	15	15	3	157.46 ± 24.53	0.215 ± 0.88	-11.4 ± 0.9	89.14 ± 1.21	37.59 ± 0.02	88.90 ± 0.69
5	10	15	2	122.13 ± 38.17	0.273 ± 1.45	-25.6 ± 0.7	80.18 ± 0.87	36.87 ± 0.07	97.56 ± 1.32
6	15	5	1	158.20 ± 57.24	0.214 ± 1.42	-28.8 ± 0.8	90.17 ± 0.66	36.17 ± 0.03	87.81 ± 1.46
7	15	5	3	161.93 ± 54.80	0.229 ±1.34	-20.1 ± 0.2	90.80 ± 0.57	37.48 ± 0.14	85.68 ± 1.19
8	15	10	2	152.16 ± 64.12	0.203 ± 1.21	-9.6 ± 0.1	88.13 ± 1.54	38.59 ± 0.06	89.57 ± 0.48
9	10	10	1	128.09 ± 24.87	0.209 ± 0.98	-8.7 ± 0.6	80.62 ± 1.	33.48 ± 0.14	97.23 ± 1.20
							72		
10	10	5	2	138.76 ± 23.52	0.308 ± 0.87	-27.7 ± 0.4	82.55 ± 1.09	36.59 ± 0.12	95.61 ± 1.80
11	20	5	2	214.08 ± 38.46	0.235± 1.02	-16.5 ± 0.3	97.54 ± 1.10	33.25 ± 0.07	75.23 ± 1.11
12	20	10	1	180.3 ± 102.34	0.268± 1.46	-22.7 ± 0.7	93.57 ± 0.91	37.17 ± 0.08	83.04 ± 1.31
13	15	10	2	151.54 ± 20.78	0.247± 1.23	-19.4 ± 0.9	88.59 ± 0.84	34.48 ± 0.24	90.24 ± 0.91
14	20	10	3	204.5 ± 57.63	0.216± 0.68	-21.2 ± 0.4	96.03 ± 1.43	36.52 ± 0.08	78.35 ± 1.51
15	20	15	2	164.35 ± 48.38	0.221± 0.97	-15.6 ± 0.6	92.15 ± 1.50	36.78 ± 0.12	84.51 ± 0.71

The OPN loaded liposomes particle sizes, PDIs, and zeta potentials were shown in Table 1. The incorporation of DSPE-PEG-2000 into the liposomal bilayer resulted in an increase in particle size. The optimized OPN-DSPE-PEG-2000 liposomal formulation exhibited mean particle sizes in the range of 122.13 ± 38.17 to 214.08 ± 38.46 nm. This increase may be attributed to the presence of hydrated polyethylene glycol chains extending outward from the liposomal surface, forming a hydrophilic steric barrier commonly referred to as a PEG corona. Such a hydrated PEG shell increases the hydrodynamic diameter as measured by dynamic light scattering and enhances colloidal stability. This observation is supported by the relatively narrow PDI values (<0.3) obtained for OPN-

3.4 Characterization of PEGylated Opuntiol liposomes

3.4.1 Particle size, polydispersity index and zeta potential

Figure 1: (A) Particle size (nm) and (B) zeta potential of OPN-DSPE-PEG-2000 (mV)

DSPE-PEG-2000 liposomes, indicating a homogeneous particle population.[22]

The negative zeta potentials observed for OPN-DSPE-PEG-2000 (-27.7 ± 0.4 mV) further contributed to colloidal stability by generating electrostatic repulsion between vesicles. From a biological perspective, the moderately negative surface charge is advantageous because highly positive nanoparticles tend to interact strongly with plasma proteins and cellular membranes, leading to rapid opsonization and clearance.[23]

3.4.2 Entrapment efficiency and drug loading capacity

Table 1 presents the entrapment efficiency of the fifteen OPN-DSPE-PEG-2000 liposomal formulations prepared with varying molar ratios of HSPC, cholesterol, and DSPE-PEG-2000. The entrapment efficiency of the experimental formulations ranged from 80.18 ± 0.87% to 97.54 ± 1.10%, indicating efficient incorporation of Opuntiol within the PEGylated liposomal bilayer. The observed improvement in entrapment efficiency with increasing HSPC concentration may be attributed to the formation of a more rigid and tightly packed phospholipid bilayer, which minimizes drug diffusion from the vesicles and enhances retention of Opuntiol within the liposomal membrane. Similar observations have been reported for HSPC-based PEGylated liposomes, where the saturated phospholipid contributes to enhance membrane stability and sustained drug release.[24] Also, the improvement in drug encapsulation can be attributed to the structural modifications introduced by PEGylation which enhanced the stability of the lipid bilayer and reduced premature drug loss.

The drug loading capacity of the prepared PEGylated liposomal formulations ranged from 33.25 ± 0.07% to 39.48 ± 0.11% (Table 1), indicating efficient incorporation of Opuntiol within the lipid bilayer. The observed variations in drug loading were primarily influenced by the phospholipid composition of the formulations. A slight increase in drug loading was observed with increasing HSPC concentration, which may be attributed to the availability of a larger hydrophobic lipid domain for accommodation of the lipophilic Opuntiol molecules. Moreover, the predominantly saturated acyl chains of HSPC facilitate the formation of a compact and rigid lipid bilayer, thereby improving drug retention within the vesicles. Conversely, increasing cholesterol concentration beyond the optimum level produced only marginal improvement or a slight reduction in drug loading. This may be explained by the intercalation of cholesterol between phospholipid molecules, resulting in competition with the drug for space within the hydrophobic region of the bilayer and consequently reducing the amount of drug that can be accommodated. Similar observations have been reported in HSPC-based PEGylated liposomal systems, where phospholipid composition and cholesterol content significantly influence the drug loading capacity by modulating bilayer organization and membrane packing.[25]

3.4.3 Morphological Characterization

The OPN encapsulated OPN-DSPE-PEG-2000 liposomes prepared from the aforementioned optimal conditions were approximately 125 nm in size and exhibited a comparatively spherical shape with a smooth surface in the SEM observation (Figure 3). These results were in close agreement with those of the TEM observation (Figure 4) and DLS analyzer measurements. These results demonstrate that this method was successful in the preparation of PEGylated liposomes containing OPN. The liposomes exhibited a good morphology and a particle size of approximately 125 nm.

Figure 3: (A) FESEM image at 10,000x, and (B) TEM image (5 nm) of OPN-DSPE-PEG-2000 liposomes

3.5 In vitro release study.

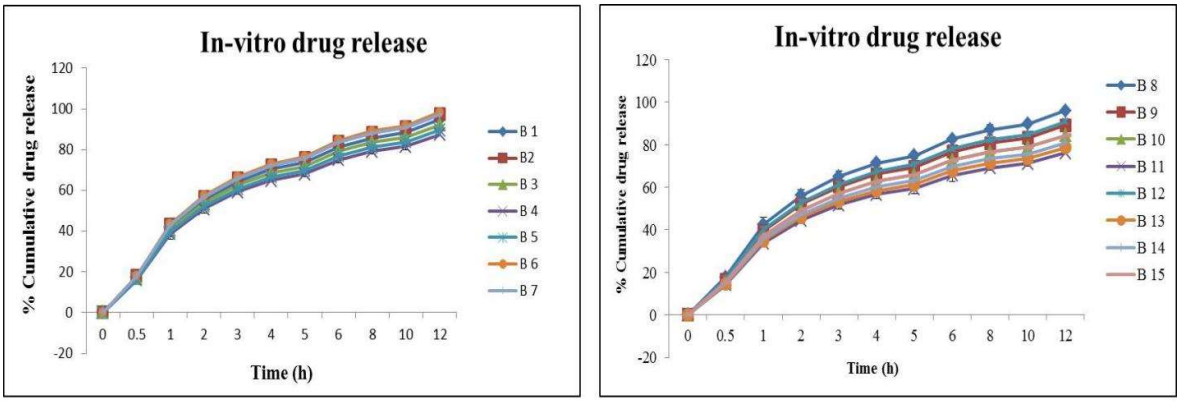

Figure 4: In-vitro release profile of OPN-DSPE-PEG-2000 (A-Batch 1 to 7 and B-Batch 8 to 15) in phosphate buffer pH 7.4 (n=3)

The release characteristics of OPN from OPN-DSPE-PEG-2000 liposome in phosphate buffer pH 7.4 media are shown in Table 1 and Figure 5. The percent cumulative drug release of OPN from prepared liposomes OPN-DSPE-PEG-2000 (batch 1 to batch 15) ranges from 75.23 ± 1.1% to 97.56 ± 1.32 % was released from the dialysis membrane after 12 h.

R2 value was maximum across all formulations for Higuchi equation (>0.96) while n-value was less than 0.5 for all the formulations when treated with Korsmeyer–Peppas equation. This indicates that drug release occurred by fickian diffusion mechanism from all the formulations. The controlled release of Opuntiol was observed from all prepared OPN-DSPE-PEG-2000 liposomes batches. This outcome is probably attributable to the incorporation of PEGylated phospholipid into OPN-DSPE-PEG-2000 liposomes. [13,26]

3.6 Effect of formulation variables

3.6.1. Effect of formulation variables on particle size

The quadratic polynomial equation in coded terms is:

Particle size (nm) = $151.00 + 30.13A - 11.21B + 6.26C + 7.96A^2 + 0.73B^2 + 2.49C^2 - 8.31AB + 4.80AC + 3.27BC$

The coefficients and their statistical significance are reported in the regression output.

The particle size of PEGylated Opuntiol liposomes was significantly affected by the lipid bilayer composition. The quadratic model demonstrated statistical significance ($p = 0.002$) and accounted for 97.30% of the variability in particle size, indicating a robust association between the formulation variables and vesicle size. The adjusted R^2 value of 92.44% further confirmed the model's adequacy. However, the predicted R^2 was lower at 57.08%, and the lack of fit was significant ($p = 0.012$). Consequently, while the model effectively describes the experimental data, its predictive accuracy beyond the studied conditions should be interpreted with caution.

Among the variables examined, HSPC demonstrated the most substantial effect on particle size, as evidenced by its large positive coefficient (+30.13) and highly significant p-value ($p < 0.001$). An increased proportion of HSPC led to a pronounced rise in vesicle size. This outcome is likely due to the greater availability of phospholipid molecules for bilayer formation, which results in thicker or more extensively organized lipid bilayers and larger vesicles. HSPC is primarily composed of saturated phospholipids that form highly ordered and rigid bilayers; at elevated concentrations, increased lipid packing and bilayer material may promote the formation of larger vesicular structures.[27]

Conversely, cholesterol exhibited a significant negative linear effect on particle size, with a coefficient of −11.21 ($p = 0.007$). Within the studied formulation range, increasing cholesterol content resulted in smaller vesicle sizes. Cholesterol intercalates between phospholipid molecules and enhances molecular packing within the bilayer. At optimal concentrations, this condensing effect reduces bilayer defects and yields more compact vesicular structures, which likely accounts for the observed decrease in particle size.[28]

DSPE-PEG-2000 displayed a positive coefficient (+6.26), suggesting a trend toward increased particle size with higher PEG-lipid concentrations. The linear effect approached statistical significance ($p = 0.056$). Incorporation of DSPE-PEG-2000 introduces hydrophilic PEG chains onto the liposomal surface. The hydrated PEG corona increases the hydrodynamic diameter as measured by dynamic light scattering, which may contribute to the observed increase in apparent particle size.

Among the quadratic terms, A^2 ($HSPC^2$) exhibited the most pronounced curvature, with a positive coefficient of +7.96 and a near-significant p-value ($p = 0.086$). This positive coefficient indicates that the increase in particle size becomes more substantial at higher HSPC levels, deviating from a strictly linear relationship. In contrast, the B^2 and C^2 terms were relatively small and not statistically significant, indicating minimal curvature effects for cholesterol and DSPE-PEG-2000 within the tested ranges.[23]

The interaction between HSPC and cholesterol (AB) yielded a negative coefficient (−8.31) and approached statistical significance ($p = 0.068$). This finding is notable as it suggests that cholesterol may partially offset the particle-size-increasing effect of HSPC. While HSPC increases the amount and rigidity of the bilayer, cholesterol may enhance packing between saturated phospholipid chains, leading to a more condensed membrane structure and limiting excessive vesicle enlargement.[29]

The AC and BC interactions were positive but nonsignificant. The AC and BC interaction terms were

positive but not statistically significant, indicating comparatively weaker combined effects of HSPC with DSPE-PEG-2000 and cholesterol with DSPE-PEG-2000 on particle size dominantly:

HSPC > cholesterol > DSPE-PEG-2000

HSPC most strongly increased particle size, cholesterol tended to reduce it, and PEGylation resulted in a comparatively modest increase in hydrodynamic size.

Figure 5: (A) Surface plot and (B) contour plot of particle size Vs cholesterol and HSPD of OPN-DSPE-PEG-2000 liposomes.

3.6.2. Effect of formulation variables on entrapment efficiency

The quadratic polynomial equation in coded terms is:

Entrapment efficiency (%) = $87.467 + 6.688A - 1.988B + 1.275C + 0.104A^2 + 0.454B^2 + 0.529C^2 - 0.775AB + 0.200AC + 1.150BC$

The quadratic model describing entrapment efficiency demonstrated high statistical significance ($p < 0.001$) and strong goodness of fit, as indicated by an R^2 of 98.88%, adjusted R^2 of 96.87%, and predicted R^2 of 96.10%. The close alignment between adjusted and predicted R^2 values supports the model's predictive reliability. Furthermore, the nonsignificant lack of fit ($p = 0.973$) confirms that the model accurately represented the experimental data within the investigated formulation space.

HSPC was identified as the most influential factor affecting entrapment efficiency, with a strong positive coefficient (+6.688) and a highly significant effect ($p < 0.001$). Increasing HSPC concentration substantially increased Opuntiol entrapment.

This result is likely due to the formation of a more extensive and structurally ordered lipid bilayer at higher HSPC concentrations. As Opuntiol is incorporated within or associated with the liposomal lipid domain, increasing phospholipid content provides greater bilayer volume and additional accommodation sites for the drug. The saturated acyl chains of HSPC also produce a rigid and tightly packed membrane, which restricts diffusion of the entrapped drug from the vesicles and reduces leakage during preparation and processing. These combined effects explain the observed increase in entrapment efficiency with higher HSPC concentrations.[30]

Cholesterol exerted a significant negative linear effect on entrapment efficiency (coefficient −1.988; $p = 0.002$). While cholesterol typically enhances bilayer rigidity and reduces membrane permeability, excessive cholesterol can compete with lipophilic or amphiphilic drug molecules for space within the hydrophobic region of the phospholipid bilayer. Increasing cholesterol within the studied range may have reduced the available bilayer volume for Opuntiol incorporation, resulting in a modest reduction in entrapment efficiency.[31]

DSPE-PEG-NH_2 demonstrated a significant positive effect on entrapment efficiency, with a coefficient of +1.275 ($p = 0.014$). Incorporation of PEGylated phospholipid may improve structural organization and steric stabilization of the vesicles, reducing aggregation, fusion, and drug leakage during formulation processing. The hydrophobic DSPE anchor integrates into the lipid bilayer, while the PEG chains extend into the aqueous environment to form a hydrated steric barrier. This stabilization likely contributes to improved retention of Opuntiol within the liposomal system.[32]

The quadratic terms A^2, B^2, and C^2 were all statistically nonsignificant, suggesting that the effects of the individual formulation variables on entrapment efficiency were predominantly linear within the selected experimental ranges.

Among the interaction terms, the cholesterol × DSPE-PEG-NH_2 interaction (BC) showed a positive coefficient (+1.150) and approached statistical significance ($p = 0.063$). This suggests a potentially beneficial combined effect of cholesterol and PEG-lipid on drug retention. Cholesterol can improve bilayer packing and rigidity, while DSPE-PEG-NH_2 provides steric stabilization; together, at suitable proportions, these components may produce a more stable bilayer capable of retaining Opuntiol more effectively.

Figure 6: (A) Surface plot and (B) contour plot of entrapment efficiency (%) Vs cholesterol and HSPC of OPN-DSPE-PEG-2000 liposomes.

3.7 Optimization by desirability

The desirability function was used in the numerical optimization process to arrive at several feasible formulations that met the established optimization criteria. Of these, the one consisting of 14.27 molar parts of HSPC, 15 molar parts of cholesterol, and 2.53 molar parts of DSPE-PEG-2000 was chosen as the optimized formulation since it offered a desirable balance between particle size and entrapment efficiency. The software anticipated a particle size of 142.91 nm and an entrapment efficiency of 86.50%. The optimized formulation was prepared in the laboratory, and the measured responses were compared with the predicted values in order to verify the optimization model. The experimentally obtained particle size and entrapment efficiency were found to be in close agreement with the predicted values, thus confirming the validity and reliability of the optimization model.

3.8 Cytotoxicity study by MTT assay on MCF7 breast cell line

Figure 8: In-vitro cytotoxicity assay of OPN and OPN-DSPE-PEG-2000, against MCF7 breast cancer cell line.

The MTT assay on breast Cancer MCF7 cells demonstrates a clear concentration-dependent increase in growth inhibition for OPN and its liposomal formulations (20–100 µg/mL), indicating enhanced cytotoxic activity with increasing dose. The standard 5-FU showed the highest cytotoxic effect (IC50= 25.30 µg/mL) at all concentrations, confirming strong efficacy against MCF7 cells. Among the test samples, OPN exhibited the lowest

cytotoxic activity, having IC50= 55.75 µg/mL concentrations. The OPN-DSPE-PEG-2000 formulation demonstrated improved anticancer activity compared to free OPN, with IC50= 44.68 µg/mL, suggesting that PEGylation enhanced cellular uptake and therapeutic efficiency.

The findings of the present study therefore suggest that PEGylated liposomes serve as an efficient nanocarrier system for Opuntiol by improving its pharmaceutical properties, including stability, controlled drug release, and dispersion in the biological environment, ultimately resulting in enhanced in vitro anticancer activity against MCF7 breast cancer cells.

The in vitro cytotoxicity study showed that PEGylated Opuntiol liposomes had much stronger antiproliferative effects on MCF7 breast cancer cells than plain Opuntiol. This stronger effect likely comes from the improved physical and chemical properties of the PEGylated liposomes, not just the drug itself. Encapsulating Opuntiol in PEGylated liposomes helps it mix better in water, making more of the drug available in the cell culture. Because Opuntiol does not dissolve well in water, putting it into the liposomal lipid bilayer allows for even drug distribution and longer contact with cancer cells.

Adding DSPE-PEG-2000 to the liposomal membrane makes the formulation more stable by creating a water-based barrier around the vesicles. This barrier helps prevent the vesicles from clumping together or merging during the experiment, so the liposomes stay small throughout the study. The stable mixture keeps the drug available in the surrounding medium for longer, which means cancer cells are exposed to Opuntiol for an extended time. This longer exposure likely increases the overall cytotoxic effect compared to the quick spread and possible breakdown of the free drug.

The rigid lipid bilayer made from HSPC and cholesterol helps control how the drug is released by stopping Opuntiol from leaking out of the liposomes too soon. In this study, the drug was released slowly over time, which kept its concentration steady in the cell environment during incubation. This longer-lasting availability of the drug may explain why PEGylated liposomes reduced cell viability more than plain Opuntiol.

The small size of the PEGylated liposomes, about 120 to 160 nanometers, and their uniform size give them a larger surface area to interact with cell membranes. While this study did not measure how much of the drug enters the cells, the better mixing, less clumping, and slow release of the PEGylated liposomes should help the drug carrier stay in contact with cancer cells longer. This is expected to improve the overall effectiveness of Opuntiol.[33]

3.9 Stability Study

The optimized OPN-DSPE-PEG-2000 liposomal formulation was evaluated for physical stability by monitoring particle size, polydispersity index, zeta potential, and entrapment efficiency during storage at 2–8°C for six months. The formulation remained physically stable throughout the storage period, with no significant changes in particle size, PDI, or zeta potential, indicating the maintenance of colloidal stability. A slight reduction in entrapment efficiency was observed during storage, which may be attributed to gradual diffusion of a small fraction of the entrapped Opuntiol from the lipid bilayer rather than extensive vesicle disruption. Nevertheless, the decrease was minimal and did not significantly affect the overall integrity of the formulation.

The excellent stability of the PEGylated liposomes can be attributed to the combined effects of HSPC, cholesterol, and DSPE-PEG-2000. The saturated phospholipid HSPC forms a rigid and highly ordered lipid bilayer, while cholesterol enhances membrane packing and reduces bilayer permeability, thereby minimizing drug leakage during storage. In addition, the hydrated PEG corona provided by DSPE-PEG-2000 imparts steric stabilization, preventing vesicle aggregation and fusion, which contributes to the preservation of nanosized vesicles and sustained drug retention. No visible changes in the physical appearance of the formulation, such as aggregation, phase separation, or drug precipitation, were observed throughout the storage period, confirming the excellent physicochemical stability of the PEGylated liposomal formulation. These findings demonstrate that the formulation remained stable for at least six months under refrigerated conditions and is suitable for further pharmaceutical development.

4. Conclusion

This study developed and optimized PEGylated Opuntiol-loaded liposomes using a three-factor, three-level Box–Behnken design. The formulation variables had a significant impact on the liposomes' physicochemical properties. Numerical optimization with the desirability function produced a formulation with nanosized vesicles, high entrapment efficiency, and good drug loading. The optimized PEGylated liposomes showed sustained drug release in vitro and stayed physically stable for six months when stored in the refrigerator, maintaining good colloidal stability and drug retention. The PEGylated liposomal formulation also showed much stronger in vitro anticancer activity against MCF7 breast cancer cells than plain Opuntiol. This improvement may be due to better dispersion in water, greater stability, sustained drug release, and longer exposure of cancer cells to the drug. In summary, the PEGylated liposomal system developed here is a promising nanocarrier for enhancing the pharmaceutical performance and anticancer effects of Opuntiol. More in vivo studies on pharmacokinetics, biodistribution, and therapeutic effects are needed to confirm its potential for breast cancer treatment.

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