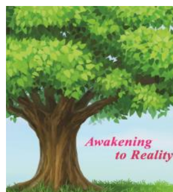


Available online at www.elixirpublishers.com (Elixir International Journal)

Pharmacy

Elixir Pharmacy 90 (2016) 37911-37920

Elixir ISSN: 2229-712X

Analysis of release kinetic models of niosomal gel of punicalagin from peels of *punica granatum*

Hanu Priya
Department of Pharmaceutics, Lovely Professional University, Phagwara, Punjab, India.

ARTICLE INFO

Article history:
Received: 14 March 2013;
Received in revised form:
20 January 2016;
Accepted: 26 January 2016;

Keywords
Punica granatum, Punicalagin,
Novel Drug Delivery System,
Niosomes, Vesicular system,
Niosomal gel,
Release kinetic models.

ABSTRACT
A very well known ancient fruit whose therapeutic qualities have rebounded and echoed throughout the millennia named as *Punica granatum* (belongs to family Punicaceae) also commonly known as Pomegranate, Anar or Dalim in North India. Plant based formulations have been used since ancient times and playing role as a remedial agent against various human and animal diseases. Therefore, in the systems of Allopathic, Ayurvedic, Homeopathic and Unani systems, much energy has been devoted to the treatment of disease and enhancement of physical and mental health. The interest in traditional medicines has increased in various parts of world. Punicalagin is chemically named as 2, 3-(S)-hexahydroxydiphenoyl-4, 6-(S,S)-gallagyl-D-glucose and belongs to a category of hydrolysable tannin. In this research, the main aim is to formulate the vesicular formulation of Punicalagin which was extracted, isolated and purified from peels of *Punica granatum*. Thus, to protect its hydrolysis, it is formulated into a nanocarrier system known as niosomes which is based on the preparation of niosomes by using a non-ionic surfactant in varying amounts and keeping the amount of cholesterol constant. The niosomal formulations were evaluated on the basis of evaluation parameters and thus optimized. The best optimized niosome formulation was then formulated as 1% w/w hydrogel and evaluated on the basis of parameters like homogeneity, rheological behaviour of hydrogel, spreadibility and gel strength, consistency, skin retention studies as well as *in vitro* and *ex vivo* drug release study. Comparative drug permeation study *in vitro*, *ex vivo* and skin retention study of niosomal gel as well as conventional gel concluded that amount of drug permeated and retained in skin from niosomal gel was much more as compared to conventional gel. The analysis of release pattern by niosomal and conventional gel was done by applying kinetic models to them and concluded that both formulations followed zero order kinetics.

© 2016 Elixir All rights reserved.

Introduction

The pomegranate (L. *Punica granatum)* is an ancient fruit which has been widely consumed in various cultures for thousands and thousands of years belongs to family Punicaceae is commonly known as Anar or Dalim in North India[1]. The Babylonians regarded pomegranate seeds as an agent of resurrection; the Persians believed the seeds conferred invincibility on the battle fields, while for the ancient Chinese, the seeds symbolized longevity and immortality[2]. Pomegranate husk is a traditional Chinese medicine used as antibacterial, anti-inflammatory and hemostasis agent which is rich in phenolic compounds. Among these polyphenols, the most abundant compound is Punicalagin. Punicalagin levels in husk depend upon the area for fruit growth, processing conditions as well as storage conditions[3]. Punicalagin is a potent antioxidant whose bioactivity can be explained by its ability to hydrolyze into Ellagic acid (EA) *in vivo* and across the mitochondrial membrane *in vitro*. Tannins are one of the most widely occurring groups of substances in different families of higher plants. They are high molecular weight plant polyphenols[4] and the secondary metabolites which are present in solution form in cell sap and also in distinct vacuoles. Chemically, tannins contain the mixture of complex organic substances in which polyphenols are present, generally with o-dihydroxy or o-trihydroxy groups on phenyl ring and they are devoid of nitrogen[5].

For the better performance of pharmaceutical formulations with respect to controlled release, bioavailability, storage stability and lesser side effects constitute the main motivation for research of novel drug delivery systems[6]. There are various novel drug delivery strategies like liposomes, niosomes, aquasomes, microsponges, microemulsions, and solid lipid nanoparticles to enhance the topical delivery of agents. Among different carriers, liposomes and niosomes are well documented for dermal drug delivery[7].Vesicles formed on hydration of mixture of cholesterol and single alkyl-chain non-ionic surfactants were first introduced by Handjani-Vila. Initially reported as a feature of cosmetic industry, they are now extensively used as drug delivery systems[6].

Niosomes may be defined as a unilamellar or multilamellar vesicles in which the aqueous solution is enclosed in highly ordered bilayers made up of non-ionic surfactants with or without cholesterol and dicetylphosphate and exhibit behaviour similar to liposomes *in-vivo*[7]. NSVs result from the self assembly of hydrated surfactant monomers. The surfactant molecules self-assemble in aqueous media in such a fashion that the hydrophobic tails face each other to minimize the high energy interactions between the solvent and tails[6]. They are

Tele:
E-mail addresses: hanupriya.yadav@gmail.com

© 2016 Elixir All rights reserved

capable of entrapping both hydrophilic and hydrophobic drugs as shown in figure 1. A number of non-ionic surfactants have been used to prepare vesicles viz. polyglycerol alkyl ethers, glucosyl dialkyl ethers, crown ethers, ester linked surfactants, polyoxyethylene alkyl ether, brij, and series of spans and tweens. They are made up of biocompatible, non-toxic, non-immunogenic, and non-carcinogenic agents. NSV's are highly resistant to hydrolytic degradation[6].

Advantages of niosomes are: It can accommodate hydrophilic, lipophilic as well as amphiphilic drug moieties; niosomes exhibit flexibility in their structural characteristics (composition, fluidity and size) and can be designed according to the desired situation; improve the therapeutic performance of the drug, protect from the biological environment, restricts its effect to target cells, thereby reducing the clearance of drug; act as depot to release the drug slowly and offer a controlled/sustained release; increase oral bioavailability of drug; increase the stability of entrapped drug; enhance the skin penetration of drugs[8,9]. They can be made to reach the site of action by oral, parenteral as well as topical routes; surfactants used are biodegradable, biocompatible and non-immunogenic; handling and storage of surfactants do not require any special conditions; the vesicle suspension being water based offers greater patient compliance over oily dosage forms[10,11].

The objective of the present study is to develop and validate a simple, precise, accurate, and economical analytical method for the estimation of Punicalagin extracted from peels of *Punica granatum*. To perform the compatibility study of drug, Punicalagin with the excipients used in formulating niosomes. To develop a vesicular system like niosomes which act as carriers and hence will help in penetration of drug through skin and provide a prolong release. The best optimized niosomal formulation was then incorporated into gel and evaluated according to evaluation parameters like homogeneity, rheological behaviour of hydrogel, spreadibility and gel strength, consistency, skin retention studies as well as *in vitro* and *ex vivo* drug release study. Several mathematical models have been developed and designed to analyze drug release mechanism and kinetics. The release kinetics of Punicalagin from niosomal and plain gel was evaluated considering five different models including zero order, first order, Higuchi's model, Hixson Crowell model and Korsmeyer's Peppas model. The obtained drug release data were analysed to determine the order of release. The selection was based on the comparisons of the relevant correlation coefficients and linearity test. The regression coefficient R^2 value closer to 1 indicates the model fitting of release mechanism.

Materials

Punicalagin was extracted from pomegranates which were purchased from local market in Bhiwani, Haryana (India). Peels were authenticated from National Institute of Science Communication and Information Resources, New Delhi, India. The common excipients like Methanol, Ethanol, Acetone, Potassium dihydrogen phosphate, Disodium hydrogen phosphate, Triethanolamine and Sodium chloride were obtained from Loba Chemie Pvt. Ltd., Mumbai. Carbopol 934, Diethyl ether, Ethyl paraben, Methyl paraben, and Propylene glycol were obtained from Central Drug House Pvt. Ltd., New Delhi. Span 60 and Cholesterol were obtained from S.D. Fine Chemicals Ltd., Mumbai, India.

Methods

Extraction, Isolation and Purification of Punicalagin from peels of Punica granatum

Extraction and Isolation of Punicalagin from peels of Punica granatum

Fruits were washed, separated from seeds and juice and cleaned to yield husks/peels. Peels of the *Punica granatum* were dried in shade and powdered them with the help of blender. Powdered husk was macerated with water and methanol for 7 days with occasional stirring. After maceration process was completed, thick husk puree was squeezed by hand and filtered through muslin cloth to yield dark brown aqueous extract. The column having Sephadex LH-20 used as stationary phase and prepared for chromatography by pre-washing in methanol and pre-equilibrated in water for 12 hr. The aqueous extract was divided into portions and adsorbed onto the Sephadex LH-20. Each column was eluted with excess amount of distilled water until sugary pale yellow elute was clear in colour. The adsorbed tannins were eluted with methanol to yield dark brown solution. The collected fractions were then passed through C18 catridges (Waters Sep-Pak® Vac 20cc) and eluted with methanol to obtain the yellowish brown solution which contained pure compound, Punicalagin. The methanol was removed by Rota-evaporator in vacuo at low temp. (37^0 C) and obtained the dark brown powder as TPT (total pomegranate tannins)[4].

Purification of Punicalagin from TPT

Sephadex LH-20 resin column was used to isolate the pure compound from TPT. TPT obtained was adsorbed onto a Sephadex LH-20 column that was pre-equilibrated with water: methanol (8:2 v/v) and eluted with increasing amount of methanol. The fraction was evaporated in vacuo and then re-chromatography was done by pre-equilibrating column with ethanol. Elution was done with increasing amounts of water and acetone then to ethanol: water: acetone (6:3:1 v/v/v) and finally with ethanol: acetone (1:1 v/v). The fraction was collected and then evaporated in vacuo to obtain yellowish brown powder as Punicalagin[4].

Preformulation Studies of Punicalagin

Organoleptic Properties: The organoleptic properties include physical state, colour and odour was done by visual inspection.

TLC Identification Test

i) Preparation of sample- The powdered drug (0.5g) was macerated in methanol for 4 days. The extract was filtered and process was repeated thrice. The filtered extract was pooled, evaporated to dryness under reduced pressure and residue was dissolved in methanol.

ii) Preparation of Solvent system- Chloroform: Ethyl acetate: Formic acid: Methanol in a ratio of 4: 5.2: 0.6: 0.2 was mixed to prepare solvent system. Spotting of the sample was done with the help of capillary on TLC plate. The plate was then placed in the solvent system till the saturation point was reached.

iii) Visualisation- TLC plate was dipped in a solution consisting of anisaldehyde (0.5ml), glacial acetic acid (9.5 ml), methanol (85ml) and conc. sulphuric acid (5ml) for a few seconds. It was then heated at 110^0 C in hot air oven till coloured band appeared and R_f value was then calculated as in equation (1).

$$\text{Rf value} = \frac{\text{Distance travelled by solute front}}{\text{Distance travelled by solvent front}}$$

Chemical identification test: To identify the drug chemically, a pinch of drug was taken in a test tube and 2 ml of ferric chloride solution was added to it. Presence of a colour shows the presence of tannin.

Characterisation of Punicalagin

UV spectroscopy- Punicalagin was dissolved in phosphate buffer saline pH 7.4 and the sample was scanned at wavelength ranging from 200-400nm by UV spectrophotometer. The absorption maxima were compared with that available in the literature[12].

IR spectroscopy- IR spectroscopy of Punicalagin was performed using FTIR 8400S (Shimadzu). KBr pellets of Punicalagin were prepared by applying a pressure of 8 tons in a hydraulic press. The pellets were scanned over a wave number range of 4000–400 cm^{-1}.The spectrum obtained was interpreted by the literature[13]. 3. Standard UV Plots

Determination of absorbance maxima (λ_{max}) - Punicalagin (10mg) was accurately weighed and transferred to a 100 ml volumetric flask. To this, pH 7.4 PBS was added to dissolve the drug. From this solution, 1 ml of solution was pipetted out in 10 ml volumetric flask and volume was made upto 10 ml with distilled water. The sample was scanned on a double beam UV-visible spectrophotometer. An absorbance maximum of Punicalagin was determined in pH 7.4 PBS.

Standard plot of Punicalagin in pH 7.4 Phosphate buffer- The standard plot of Punicalagin was prepared in pH 7.4 PBS. 10 mg of drug was weighed accurately and dissolved in 100 ml of pH 7.4 PBS. Appropriate dilutions were made with buffer to obtain test solutions ranging from 5µg/ml to 35µg/ml. The absorbance of the drug in the buffer was measured on a double beam UV-visible spectrophotometer at λ_{max} of 253.6nm against the respective blank.

Compatibility Study

Physical characterisation of drug excipient mixture- Drug and each excipient were separately passed through sieve # 20. Drug and each excipient were weighed in the ratio of 1:1 and mixed properly with 15 ml of water for injection (WFI) as shown in table 1. Different drug excipient mixtures were introduced into glass vials containing 15 ml of water covered with rubber caps which were followed by labelling. Then the vials were kept under three different conditions, one at 5 ± 3°C (refrigerated temperature), 25 ± 2°C (room temperature) and 40 ± 2°C/ 75 ± 5% RH. Observations were taken on 0th, 7th, 14th, 21st and 28th day for physical compatibility.

Chemical characterisation of drug excipient mixture- For chemical compatibility, the study was carried out by taking the different drug excipient mixtures after 28th day and analyzed spectrophotometrically by UV.

Table 1: Composition for compatibility study of Punicalagin with excipients

S.No.	Drug+ Excipient+ WFI	Ratio of drug and excipients
1.	Drug + WFI	1:1
2.	Drug+ Span 60+ WFI	1:1
3.	Drug+ Cholesterol+ WFI	1:1
4.	All excipients+ WFI	1:1
5.	Drug+ all excipients+ WFI	1:1

Preparation and Purification of Niosomes

Preparation of niosomes by ether injection method- Niosomes were prepared using Ether injection method[14]. Drug, surfactant and cholesterol were used in the ratios as indicated in Table 2. Mixture of surfactant (Span 60) and cholesterol dissolved in a mixture of diethyl ether and chloroform was slowly introduced through 20-gauge needle into warm aqueous solution of Punicalagin maintained at 20°C. Evaporation of ether leads to formation of single layered vesicles.

Purification of niosomes by centrifugation method- The prepared niosomes were separated from unentrapped drug by centrifugation method[15]. Niosome suspension was centrifuged at 40,000 rpm (1,37,088 G) for 30 minutes. Clear supernatant was removed by pipetting and remaining sediment i.e. niosomes containing only entrapped drug were obtained for further evaluation.

Table 2: Composition of niosomal formulations of Punicalagin

S.No.	Formulation	Surfactant used	Surfactant: Cholesterol: Drug
1.	F1	Span 60	1:1:1
2.	F2	Span 60	2:1:1
3.	F3	Span 60	3:1:1
4.	F4	Span 60	4:1:1
5.	F5	Span 60	5:1:1
6.	F6	Span 60	6:1:1
7.	F7	Span 60	7:1:1
8.	F8	Span 60	8:1:1
9.	F9	Span 60	9:1:1
10.	F10	Span 60	10:1:1

Evaluation of niosomes

Optical microscopy- The morphology of prepared niosomes was done by optical microscopy. The photomicrographs of the preparations were obtained with the help of photomicroscope at 1000 X.

Micromeritics studies- For the micromeritics study, the niosomes were probe sonicated and then the vesicle size and size distribution profile were determined using dynamic light scattering (DLS) method (Malvern Instruments Ltd, Worcestershire, UK). Particle size analysis was done by using particle size analyser.

Entrapment efficiency- Ultracentrifugation technique was adopted for the removal of the unentrapped drug. The centrifuged niosomes were lysed with 0.1% v/v Triton X-100 and left for 1 hour and filtered to separate drug from vesicles. Thereafter dilutions were made and solution was analyzed spectrophotometrically employing ultraviolet-visible spectrophotometer. The entrapment efficiency was calculated by using following equation:

$$\textbf{Entrapment efficiency} = \frac{\textbf{Entrapped drug}}{\textbf{Total drug}} \textbf{X 100} \quad \text{--- (2)}$$

Morphology and structure of vesicles- The prepared and optimized formulations were characterized for morphology (i.e. shape and lamellarity) employing Hitachi TEM analyzer.

Zeta potential analysis- Zeta potential of niosomal preparation is related to the stability of niosomes. Zeta potential indicates the degree of repulsion between adjacent similarly charged particles for small molecules and particles, a high value of zeta potential confers stability, i.e. the solution or dispersion will resist aggregation. Zeta potential for niosomal formulation was performed using Zeta sizer Beckman coulter instrument.

Stability studies of Punicalagin containing niosomes- Physical stability studies were carried out to investigate the leaching (leakage) of drug from niosomes (in a suspension form) during storage. The optimized niosomal formulation (F7) composed of Span 60 and cholesterol in 7:1 molar ratio were sealed in 20 ml glass vials and stored at refrigeration temperature (5 ± 3°C) for a period of 2 months. Samples were withdrawn at definite time interval of 15 days and the percentage entrapment of the drug was determined as described previously after separation from unentrapped drug.

Development of topical hydrogel

For the development of topical hydrogel, an appropriate quantity of carbopol 934 (1% by weight) was added to 100 ml distilled water. It was then left to swell and hydrate at room temperature for two to obtain a homogeneous mixture. The propylene glycol was used to dissolve ethyl and methyl paraben and this solution mixture was incorporated into the homogeneous mixture. Then, it was stirred for 60 min at 800 rpm, followed by addition of few ml of triethanolamine drop wise to neutralize the formulation. Mixing was continued until gel appeared with desired consistency. The pellets from optimized formulation (F7) equivalent to 1% w/w containing 19.5 mg drug were incorporated in carbopol gel. It was then mixed to prepare niosomal gel whose composition is given in table 3.

Table 3: Composition for optimized niosomal gel

Ingredients employed	Quantity (% w/w)
Pellets from niosomal dispersion	19.5 mg equivalent to 1%
Carbopol 934	1%
Propylene glycol	q.s.
Methyl paraben	q.s
Ethyl paraben	q.s.
Triethanolamine	q.s.
Distilled water	q.s. to 100 ml

Evaluation of gel

Rheological behavior of developed system- The gelled system was evaluated for rheological behavior using Brookfield viscometer. Viscosity of the gel was determined at various rpm and by using different types of spindles.

Homogeneity- The developed gel was tested for homogeneity by visual inspection after the gel had been set in the container. It was tested for their appearance and presence of any aggregates.

Consistency- The measurement of consistency of the prepared gel was done by dropping a cone attached to a holding rod from a fix distance of 10cm in such way that it should fall on the centre of the glass cup filled with the gel. The penetration by the cone was measured from the surface of the gel to the tip of the cone inside the gel. The distance travelled by cone was noted down after 10 seconds. This process was carried out in triplicate.

pH- The pH of the gel formulation was determined by using digital pH meter and readings were taken in triplicate.

Determination of gel strength- The method by which the properties of polymeric system may be conveniently determined is texture profile analysis. A TA-XT2 Texture analyzer with probe was used to determine the gel strength as shown in figure 2. The experiment was done by placing the gels in standard beaker below the probe. In this, an analytical probe is then immersed into the sample. The Texture Analyzer was set to the compression mode with a test-speed of 1.0 mm/s. An acquisition rate of 500 points per second, a trigger force of 5 g and a probe of P/O.5R; ½” Dia cylinder Delrin radi was used for the gel testing. The study was carried out at room temperature and readings were taken in triplicate. The force required to penetrate the gel was measured as gel strength in terms of g.

Spreadibility- Spreadibility, in simple terms, is the ease with which a spread can be applied in a thin, even layer to skin. Firmness of a gel/spread may be measured by the amount of deformation under a given force. In this process, TAXT2 texture analyser with spreadibility rig was used (as shown in figure 3) in which gel was filled in cone and extra gel was scrapped by tissue paper. The test mode was compression, the test speed was 3mm/sec and acquisition rate of 500 points per seconds were the test conditions during the process. The whole process was carried out at room temperature and readings were taken in triplicate.

In vitro drug release through cellophane membrane- To characterize the optimized formulation, the *in vitro* drug permeation studies were carried out using Franz diffusion cell. Degassing of the receptor solution was done to prevent the formation of bubbles beneath the membrane. By circulating water, the temperature of the cell was maintained at 37°C. Membrane was mounted between the donor and receptor compartments of Franz diffusion cell and excess part of the membrane was trimmed off. The prepared formulations of punicalagin i.e. Niosomal gel that contained niosomes of Punicalagin and Plain gel that contains only Punicalagin, each equivalent to 19.5 mg of Punicalagin, were applied onto the membrane in the donor compartment and covered with aluminum foil to prevent contamination and evaporation. The receptor solution was pumped by peristaltic cassette pump continuously through the receptor compartment and drained into sample collection test tubes located in the fraction collector. Aliquot (5 ml) of dissolution medium was withdrawn from the sampling port at different time interval for 24 hrs and whenever the sample was withdrawn, an equal volume of fresh dissolution medium was added to the cell to maintain a constant volume. The amount of punicalagin permeated through membrane was analyzed spectrophotometrically.

Ex vivo release studies through skin- The depilated skin from the dorsal region of the sacrificed albino wistar rat was taken for determining the permeation rate. The adipose tissue and other fatty tissues were separated from same and washed with saline and kept in PBS pH 7.4 (Dew et al., 2012; Qiu et al., 2008). Niosomal gel equivalent to 19.5 mg of Punicalagin was applied to the albino wistar rat skin in a vertical Franz diffusion cell. Other side of the skin was in contact with the dissolution medium. Franz diffusion cell was placed on a magnetic stirrer and temperature of media was maintained 37 ± 0.5°C. It was agitated at 100 rpm. The dissolution medium was 100 ml of PBS pH 7.4. Aliquot (5 ml) of dissolution medium was withdrawn from the sampling port at different time interval for 24 hrs and the amount of punicalagin permeated through membrane was analyzed spectrophotometrically. Whenever sample was withdrawn equal volume of fresh dissolution medium was added to the Franz diffusion cell to maintain a constant volume.

Skin retention studies- In this study, the skin tissue mounted on the diffusion cell was removed and washed thrice with saline solution, followed by blotting between tissue paper to remove any adhering formulation from the surface. Subsequently, the skin tissue was cut into small pieces and homogenized with 10 ml of methanol for extracting punicalagin and quantified for drug content spectrophotometrically.

Analysis of release mechanism- Several mathematical models have been developed and designed to analyze drug release mechanism and kinetics. The release kinetics of Punicalagin from niosomal and plain gel was evaluated considering five different models including zero order, first order, Higuchi’s model, Hixson Crowell model and Korsmeyer’s Peppas model. The obtained data were analysed to determine the order of release. The selection was based on the comparisons of the relevant correlation coefficients and linearity test. The regression coefficient R^2 value closer to 1 indicates the model fitting of release mechanism[16.17].

Results

Preformulation studies

Organoleptic properties

Table 4: Preformulation parameters and their observation

Preformulation property	Observations
Physical state	Solid as powder
Colour	Yellowish brown (shown in figure 4)
Odour	Pleasant smell

TLC Identification test- Purple pink colour was appeared and R_f value was observed 0.53 (whereas reported value of R_f is 0.58 in ICMR) as shown in figure 5.

Chemical identification test- On addition of ferric chloride to small amount of drug, a blue colour was appeared that indicated the presence of hydrolysable tannin.

Characterisation of Punicalagin

UV spectroscopy- The absorbance maximum (λmax) of Punicalagin in PBS pH7.4 was determined which are shown in figure 6. The λmax of Punicalagin in phosphate buffer pH 7.4 was found to be 253.6 nm.

IR spectroscopy- IR spectra of Punicalagin obtained is as shown in figure 7.

Table 5: IR spectra showing the peaks of functional groups present in Punicalagin

Wave number cm^{-1}		The peak corresponds to
Observed value	*Reference value	
1589	1560	C-O
3419 and 3720	3400-3550	O-H
1683	1680	C=O conjugated with aliphatic C=C
1261-1242	1270 -1230	-O- (Aralkyl ether)
1112 and 1350	1100 -1300	COOR
1456-1589	1450 -1600	Aromatic ring

Reported peaks in literature [13]

Standard UV plot of Punicalagin in pH 7.4 Phosphate buffer

Standard plot of punicalagin was found to be linear with R^2 = 0.9978; showing proportional increase in the absorbance with concentration which is shown in figure 8 and data is represented in table 6. Absorbance range of Punicalagin was found to be 0.154 to 0.855 nm.

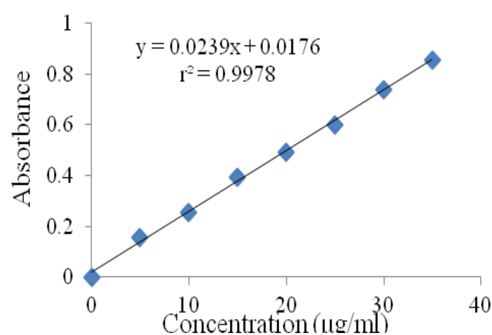


Figure 8: Standard plot data of Punicalagin in PBS pH 7.4

Compatibility Study

Physical characterisation of drug excipient mixture

The compatibility study of Punicalagin with various excipients showed that there was no colour change as well as there was no microbial growth occurred in the solution as well as in powder form as shown in table 7. This showed that drug is physically compatible with the excipients.

Table 6: Absorbance data for calibration curve of Punicalagin

Concentration (µg/ml)	Absorbance*			Average	±SD	% RSD
	Abs 1	Abs 2	Abs 3			
0	0	0	0	0	0	0
5	0.154	0.156	0.153	0.15433	0.00152	0.9897
10	0.253	0.255	0.253	0.25366	0.00115	0.4552
15	0.395	0.396	0.394	0.395	0.001	0.2531
20	0.496	0.493	0.492	0.49366	0.00208	0.4216
25	0.593	0.599	0.601	0.59766	0.00416	0.6965
30	0.736	0.743	0.74	0.73966	0.00351	0.4747
35	0.855	0.857	0.854	0.85533	0.00152	0.1785

*Each value is average of three determination, SD = Standard Deviation, %RSD = Percent Relative Standard Deviation

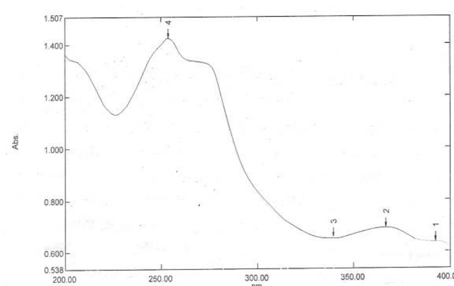
Table 7: Compatibility study data of Punicalagin with different excipients done at 0th, 7th, 14th and 28th day by physical observation

Physical compatibility study of Punicalagin with different excipients

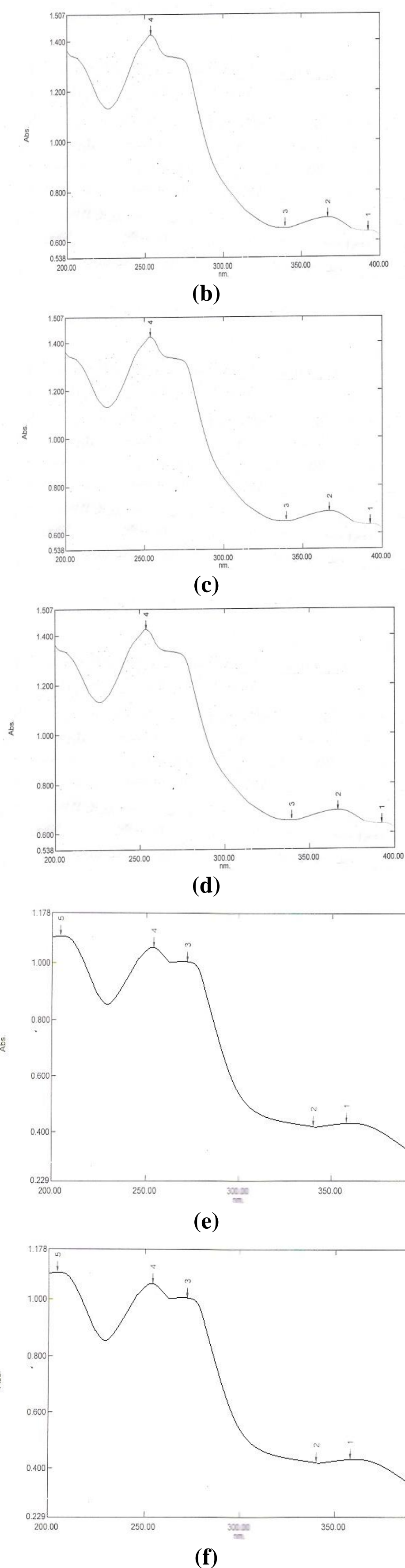
Sample (Drug+Excipients)	Ratio	Appearance at 0th day	Observation at different day and condition A (7th day); B (14th day); C (28th day)								
			5°C±3°C			25°C ± 2°C			40°C ± 2°C (75% ± 5%RH)		
			A	B	C	A	B	C	A	B	C
Drug + WFI	1:1	Yellow colour	√	√	√	√	√	√	√	√	√
Span 60	1	White colour	√	√	√	√	√	√	√	√	√
Cholesterol	1	White colour	√	√	√	√	√	√	√	√	√
Carbopol 934	1	White colour	√	√	√	√	√	√	√	√	√
Drug + Span 60 + WFI	1:1:1	Slight yellow colour	√	√	√	√	√	√	√	√	√
Drug + Cholesterol + WFI	1:1:1	Slight yellow colour	√	√	√	√	√	√	√	√	√
Drug + Carbopol 934 + WFI	1:1:1	Slight yellow colour	√	√	√	√	√	√	√	√	√
Drug+ Span 60+ Cholesterol+ Carbopol 934	11:1:1 :1	Slight yellow colour	√	√	√	√	√	√	√	√	√

Chemical characterisation of drug excipient mixture

For chemical compatibility studies, different drug-excipient mixtures were analyzed by UV spectroscopy that showed that there is no change in λmax i.e. 253.6 nm which are shown in figure 9.



(a)



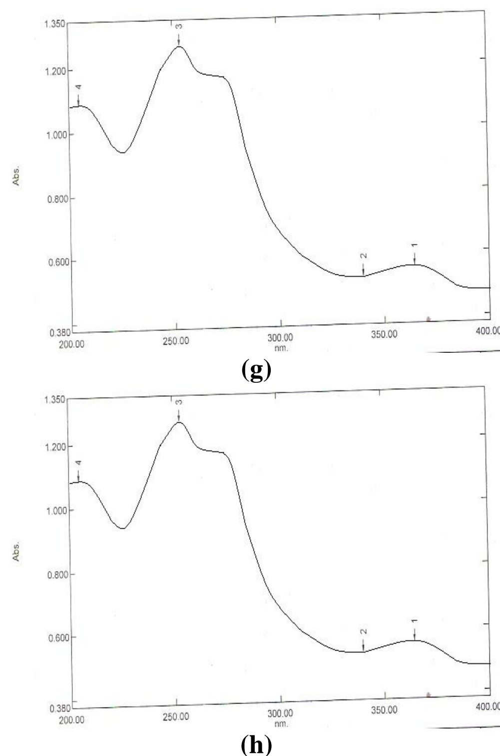
(b)

(c)

(d)

(e)

(f)



(g)

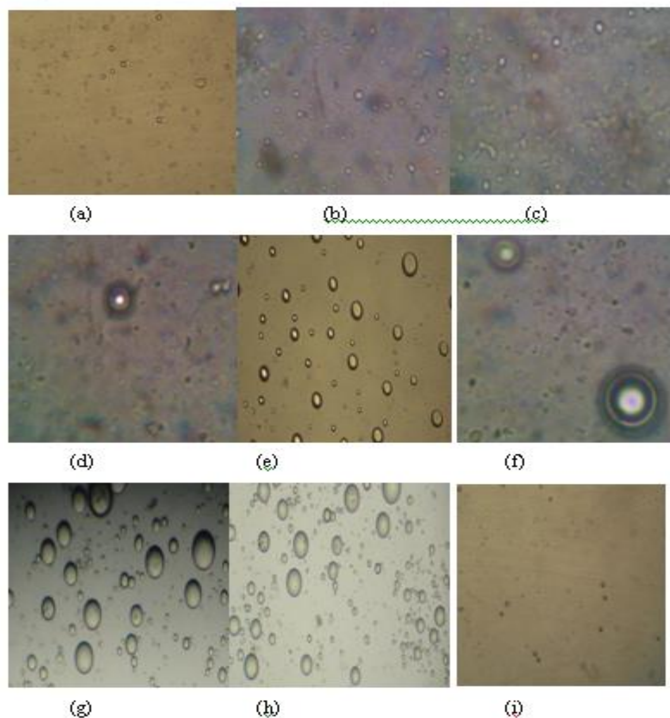
(h)

Figure 9: Compatibility study data of Punicalagin with different excipients done and by UV spectroscopy (a) Drug at 0th day (b) Drug at 28th day (c) Drug+ Span60 at 0th day (d) Drug+ Span60 at 28th day (e) Drug+ Cholesterol at 0th day (f) Drug+ Cholesterol at 28th day (g) Drug+ Mixture of all excipients at 0th day (h) Drug+ Mixture of all excipients at 28th day.

Evaluation of niosomes

Optical microscopy

The photomicrographs of prepared niosomes are shown below in figure 10. Single unilamellar niosomes were observed at 1000X by optical microscopy. The niosomes were found to be spherical in shape.



(a) (b) (c)

(d) (e) (f)

(g) (h) (i)

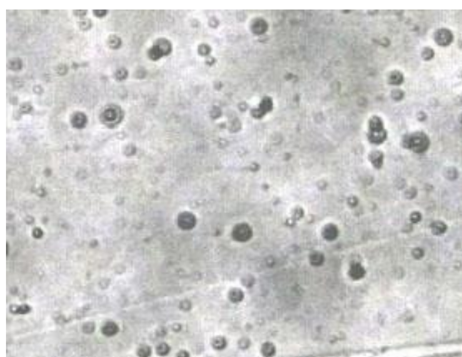


Figure 10: Photomicrographs of different formulations at 1000 X (a) F1 (b) F2 (c) F3 (d) F4 (e) F5 (f) F6 (g) F7 (h) F8 (i) F9 (j) F10

Micromeritics studies

Particle size analysis and size distribution of the sonicated niosomes was measured by Dynamic Light Scattering phenomenon using a Malvern Zeta Sizer instrument. The average particle size and PDI values are given in table 8. The average particle sizes of the sonicated niosomes were found to be in nanometer range which lies in range of 240.6nm – 625.2nm. The PDI value which characterizes the uniformity of vesicles in suspension that lies in range of 0.226 - 0.548.

Table 8: Values of Average particle size and Polydispersity index (P.D.I) for different formulations

Formulation	Particle size range (in nm)	P.D.I. range
F1	334.8	0.548
F2	279.6	0.227
F3	291.7	0.214
F4	278.7	0.236
F5	240.6	0.304
F6	336.8	0.270
F7	259.5	0.226
F8	294.1	0.324
F9	440.7	0.505
F10	625.2	0.438

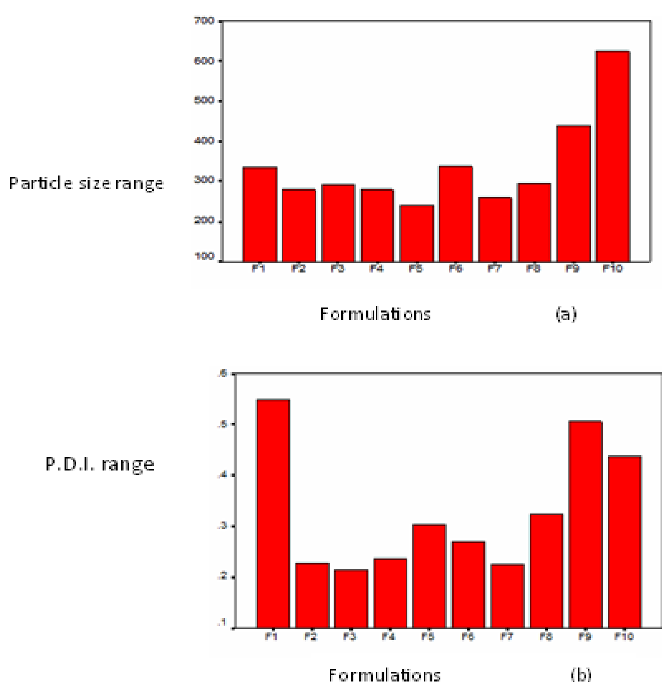


Figure 11: Graphs showing (a) Particle size (b) PDI range for all niosomal formulations

Entrapment efficiency

According to the entrapment efficiency calculated, it was found that F5, F6 and F7 has greater entrapment efficiency as compared to other formulations whereas F7 has maximum entrapment efficiency of 65.93% when compared with F5 and F6 as shown in table 9 and figure 12. Thus F7 was selected for formulating gel

Table 9: Entrapment efficiency of niosomal formulations

S. No.	Formulation name	% drug entrapment*
1	F1	28.97 ± 0.06
2	F2	32.18 ± 0.06
3	F3	35.29 ± 0.06
4	F4	44.96 ± 0.04
5	F5	56.68 ± 0.12
6	F6	59.00 ± 0.06
7	F7	65.93 ± 0.06
8	F8	55.70 ± 0.08
9	F9	46.73 ± 0.12
10	F10	42.50 ± 0.13

*Average of three determinants ± S.D.

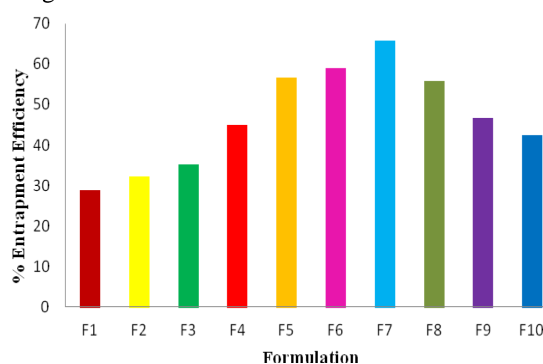


Figure 12: Comparison of entrapment efficiency of all formulations

Morphology and structure of vesicles The transmission electron micrographs of unilamellar niosomes of optimised formulation (F7) composed of span 60 and cholesterol in 7:1 molar ratio are shown in figure 13. They reveal the presence of well identified and nearly perfect spheres. In figure 13, the size of niosomes lies in range of 37.6 nm to 43.6 nm.

Zeta potential analysis- Zeta potential of the optimized formulation (F7) as shown in figure 14 which was found to be -27.2 mV depicts the stability of niosomal formulation.

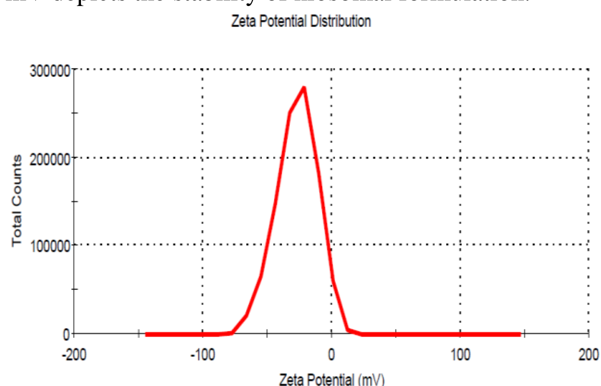


Figure 14: Zeta potential analysis for optimized niosomes (F7)

Stability studies: Drug leakage study

Physical stability studies were carried out to investigate the leaching of drug from the niosomes of optimized formulation (F7) during storage at refrigerator temperature. The percentage

of entrapment efficiency of Punicalagin after a period of 2 months got reduced to 25% as shown in table 10.

Table 10: Stability data of niosomal formulation F7

Days	5±3°C*
0th day	65.66 ± 0.14
15th day	62.66 ± 0.03
30th day	60.16 ± 0.04
45th day	52.20 ± 0.11
60st day	49.63 ± 0.10

*Average of three determinations ±S.D

Evaluation of topical gel of niosomes containing Punicalagin

Physicochemical properties of topical hydrogel

Table 11: Physicochemical characteristics of topical hydrogel formulations

Characteristics	Observation
Homogeneity	Good
Grittiness	No
Clarity	Clear
pH	7.03 ± 0.208
Consistency	6 mm

Rheological behavior of developed topical gel- The viscosity of the niosomal gel was determined at room temperature by using different types of spindle and at different speeds which is shown in table 12. The viscosity of the niosomal gel was found to be 38400 cps by spindle 63 and at 3 rpm which can be calculated by the following formula:-

$$\mathit{Dial\ reading} \times \mathit{Factor} = \mathit{Viscosity}\ (\mathit{in\ cps})$$

Table 12: Viscosity of the formulated topical gel

Speed of spindle (rpm)	% Torque reading			Viscosity (in cps)
	Spindle 61	Spindle 62	Spindle 63	
1	-	-	-	-
1.5	-	-	-	-
2	-	-	-	-
2.5	-	-	-	-
3	-	-	96%	38400

Determination of gel strength- The force required to penetrate the gel was measured as gel strength in terms of 'g' which is shown in table 13. The gel strength of niosomal gel and plain gel was carried out and the results were found to be 21.23 ± 0.99 g and 20.30 ± 0.86 g as shown in figure 15 and 16.

Table 13: Comparison of gel strength data for niosomal gel and plain gel

Sample name	Gel strength			Average	S.D.	Coefficient of variation
	1	2	3			
Niosomal gel	20.24	21.22	22.23	21.23	0.993	4.677
Plain gel	19.39	20.42	21.10	20.30	0.860	4.237

Spreadibility- Spreadibility is the ease with which a spread of a formulation can be applied in a thin, even layer to skin. According to graph as shown in figure 17 and 18, the firmness of niosomal gel and plain gel (without niosomes) in terms of force is 161.66 g and 192.55 g respectively as shown in table 14. Similarly, the work of shear which was calculated for niosomal and plain gel (without niosomes) was found to be 145.91 g.sec and 175.43 g.sec respectively as shown in table 15 and graphs are shown in figure 17 and 18. Therefore, from the above data it is concluded that niosomal gel require less force to spread as compared to plain gel.

Table 14: Firmness data for niosomal and plain gel

Sample name	Firmness in terms of force (g)			Average	S.D.	Coefficient of variation
	1	2	3			
Niosomal gel	160.43	165.70	158.83	161.66	3.5	2.2
Plain gel	199.80	191.33	186.53	192.55	6.7	3.4

Table 15: Work of shear data for niosomal and plain gel

Sample name	Work of shear (g.sec)			Average	S.D.	Coefficient of variation
	1	2	3			
Niosomal gel	144.47	150.51	142.75	145.91	4.0	2.7
Plain gel	170.45	172.81	183.05	175.43	6.7	3.8

Drug release study: The drug release study for niosomal gel and plain gel was carried out which included *in vitro* drug release through cellophane membrane and *ex vivo* study by using rat skin. The amount of drug permeated from niosomal as well as plain gel for *in vitro* and *ex vivo* study is shown in table 17 and 18 respectively. The drug release from niosomal gel is much more as compared to plain gel when compared *in vitro* and *ex vivo* study. The graphical representation showing the comparison between the amount of drug permeated from niosomal as well as plain gel for *in vitro* and *ex vivo* study is represented in figure 19 and 20 respectively.

Statistical analysis: The data obtained from *in vitro* and *ex vivo* study for niosomal gel and plain gel was statistically analysed by one way ANOVA using a software, Graph pad it was concluded that the test is significant with $p < 0.05$ that means there is a difference in the release pattern of niosomal gel when compared with plain gel by *in vitro* and *ex vivo* method. The ***p value comes out to be 0.0001 by Bonferroni's multiple comparison test.

Table 16: Comparison of the amount of drug permeated *in vitro* from niosomal and plain gel

Time in hrs	Amt permeated from plain gel (μg/cm^2)	Amt permeated from niosomal gel (μg/cm^2)
0	0	0
1	138.64	216.12
2	271.34	344.09
3	533.68	617.47
4	764.64	806.65
5	1182.91	1379.72
6	1599.99	1828.76
7	3494.84	4798.62
8	3792.20	5194.99
9	3978.33	6087.26
10	4279.31	6380.32
11	4320.64	6782.71
12	4364.20	6831.96
14	4642.45	7357.64
16	5507.34	7143.61
18	5583.27	7528.79
24	5634.41	7855.43

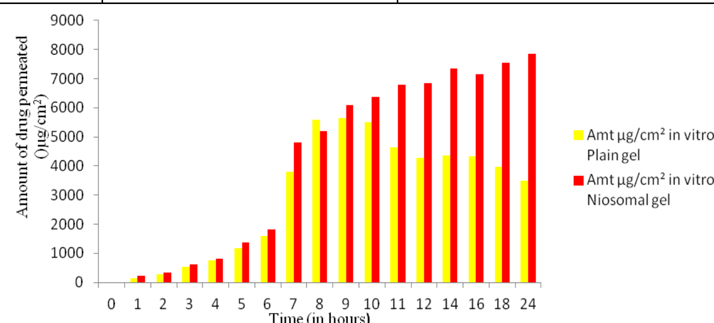


Figure 19: Comparison between the amounts of drug permeated in vitro from niosomal and plain gel in vitro

Table 17: Comparison of the amount of drug permeated *ex vivo* from niosomal and plain gel

Time (in hrs)	Amount of drug permeated from plain gel (μg/cm²)	Amount of drug permeated from niosomal gel (μg/cm²)
0	0	0
1	300.49	233.34
2	389.63	434.49
3	644.79	569.49
4	751.89	864.33
5	943.05	1252.47
6	1525.95	1720.29
7	4947.22	5541.60
8	5100.98	5754.60
9	5237.70	6262.89
10	5308.12	6650.65
11	5614.27	8667.28
12	5844.13	8601.16
14	6078.82	9282.67
16	6128.58	9154.74
18	6504.29	9626.01
24	6545.10	10038.74

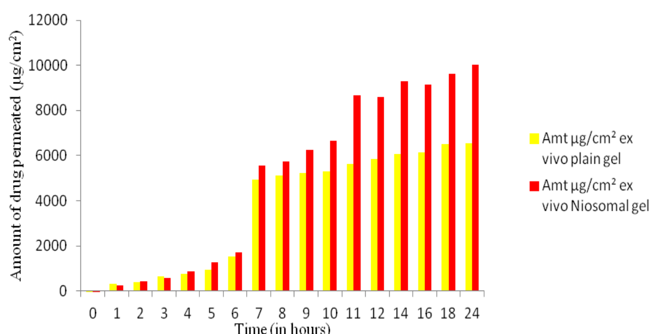


Figure 20: Comparison between the amounts of drug permeated *ex vivo* from niosomal and plain gel *ex vivo*

Skin retention studies

The amount of drug retained in skin from niosomal gel was found to be 211.27μg/cm² whereas in case of plain gel, the amount retained was 149.35 μg/cm² as shown in table 18.

Table 18: Comparison of niosomal and plain gel for skin retention study

Formulation	Absorbance	Dilution factor	Conc.(μg/ml)	D.F.*Conc.	Amount retained (μg/cm²)
Niosomal gel	0.263	50	10.26778	513.3891	211.2712
Plain gel	0.885	10	36.29289	362.9289	149.3534

Analysis of kinetic release models

To ascertain the drug release mechanism and release rate, the *ex vivo drug release* data of prepared formulations were fitted with various release models. The models selected were zero order, first order, Higuchi, Korsemeyer Peppas and Hixson Crowell model which are shown in table 19 and figure 21 - 25. R^2 value in case of zero order release was found to be higher than the other kinetic release models in niosomal gel of the optimized formulation F7 as well as in the plain gel thus suggesting that the formulation followed zero order which is best and suitable for the topical drug delivery.

Table 19: Comparison of K and R^2 values for all kinetic models

Formulation name	Zero order kinetics	First order kinetics	Higuchi model	Korsemeyer Peppas model		Hixson Crowell model
	K R^2	K R^2	K R^2	K	R^2	K R^2
Niosomal gel of F7	749.91 0.938	-0.110 0.508	2885.9 0.845	-1.42	0.434	-0.925 0.710
Plain gel	489.88 0.882	-0.099 0.459	1907.1 0.813	-1.37	0.338	-0.761 0.637

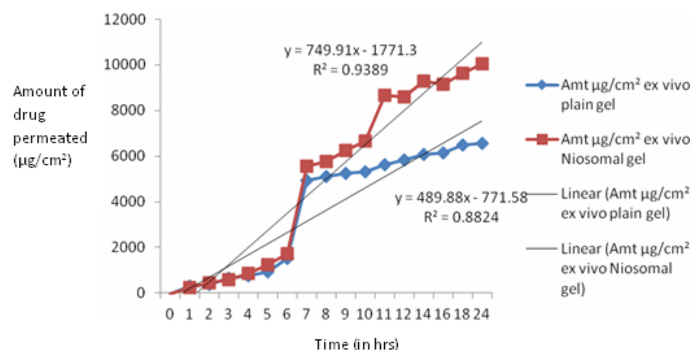


Figure 21: Analysis of release kinetics by Zero order kinetics

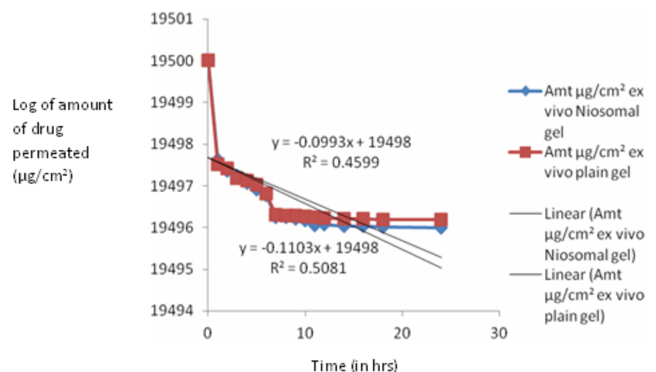


Figure 22: Analysis of release kinetics by First order kinetics

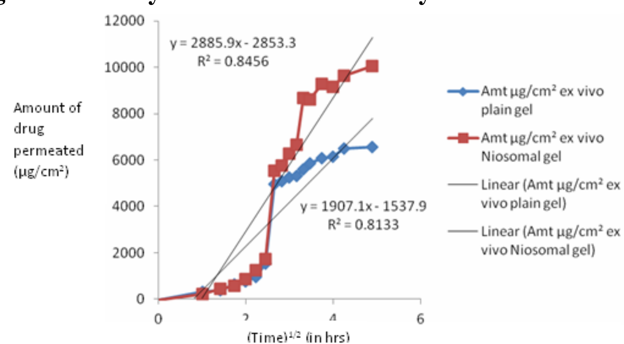


Figure 23: Analysis of release kinetics by Higuchi model

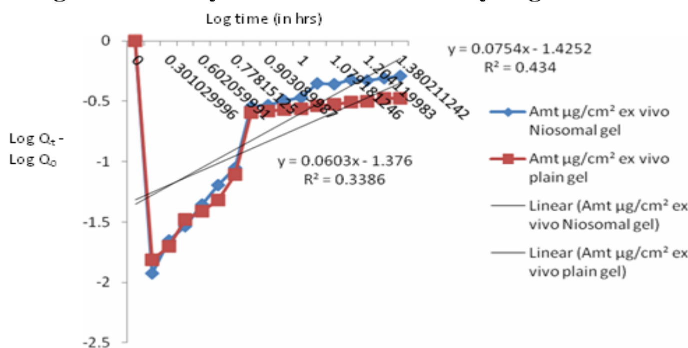


Figure 24: Analysis of release kinetics by Korsemeyer Peppas model

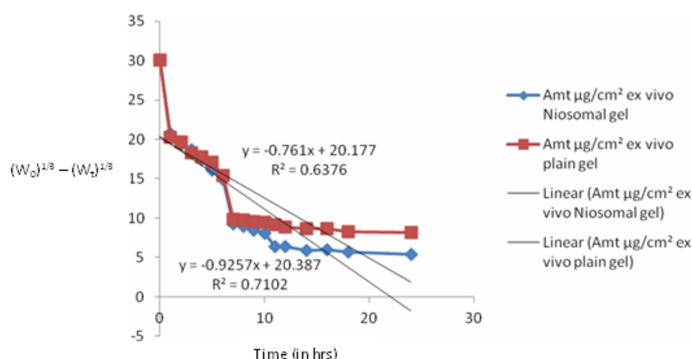


Figure 25: Analysis of release kinetics by Hixson Crowell model

Conclusion

Punicalagin was extracted, isolated and purified from the peels of the *Punica granatum* which was identified by carrying out chemical identification test, thin layer chromatography (TLC), UV spectrophotometry and FTIR. Compatibility studies of Punicalagin with the excipients used during the work were conducted for 28 days and observed that drug is compatible with the excipients physically and chemically. On the basis of the findings, it can be stated that the formulation F7 is the best formulation from all the other formulations which were prepared by different compositions of Span 60. The F7 formulation has greater entrapment efficiency as compared to other formulations, as F7 showed 65.93% whereas F5 and F6 showed 56.68% and 59% respectively. Also, F7 has particle size of lowest value as compared to others i.e. 259.5 nm as shown in figure 28. TEM, zeta potential and stability studies were conducted for the best formulation F7. TEM studies showed that niosomes in F7 are nearly perfectly spheres whereas zeta potential study showed the stability of formulation F7. The optimized formulation was formulated into a topical hydrogel and various evaluating parameters like consistency, pH, viscosity, spreadibility, gel strength, drug release study (*in vitro* and *ex vivo*) and skin retention study was done. In *in vitro* and *ex vivo* study, it was observed that amount of drug permeated from niosomal gel is more as compared to plain gel. The analysis of release pattern by niosomal and conventional gel was done by applying kinetic models to them and concluded that both formulations followed zero order kinetics as R^2 value came to be higher as compared to other models which is best kinetic model for the transdermal drug delivery system.

Acknowledgements

The Author is highly obliged to Mr. Harmanpreet Singh, guide & Assistant Professor, Department of Pharmaceutical Sciences, Lovely professional University, Phagwara, Dr. Monica Gulati, Dean, Department of Pharmaceutical Sciences, Lovely professional University, Phagwara and Dr. Uma Ranjan Lal for providing their precious and graceful support for the successful completion of work.

References

1. Agli MD, Germana, GV, Bulgari M, Basilico N, Romeo S, Bhattacharya D, Taramelli D, Bosisio E. Ellagitannins of the fruit rind of pomegranate anatagonoze in vitro the host inflammatory response mechanisms involved in the onset of malaria. Malaria Journal 2010:208:1-9.

2. Martos MV, Fernandez L, Alvarej JAP. Pomegranates and its many functional components as related to human health – A review. Comprehensive Reviews in Food Science and Food Safety 2010:9:1-20.

3. Lu J, Ding K, Yuan Q. Determination of Punicalagin isomers in pomegranate husk. Chromatographia 2008:68:303-06.

4. Seeram N, Lee R, Hardy M, Heber D. Rapid large scale purification of Ellagitannins from pomegranate husk a byproduct of the commercial juice industry. Separation and Purification Technology 2005:41:49-55.

5. Kokate CK, Purohit AP, Gokhale SB. Text book of Pharmacognosy. 33rd ed. Pune: Nirali Prakshan; 2006. p. 255-257.

6. Mehta RT, Keyhani A, Mcqueen TJ, Rosenbaum B, Rolston KV, Tarrand JJ. In vitro activities of free and liposomal drugs against Mycobacterium avium –M. Intracellulare complex and M. Tuberculosis. Antimicrobial Agents and Chemotherapy 1993:37:2584-87.

7. Shahiwala A, Misra A. Studies in topical application of niosomally entrapped Nimesulide. Journal of Pharmacy and Pharmaceutical Science 2002:5:220-25.

8. Choi MJ, Maibach HI. Liposomes and niosomes as topical drug delivery system. Skin Pharmacology and Physiology 2005:18:209-19.

9. Honeywell-Nguyen PL, Bouwstra JA. Vesicles as a tool for transdermal and dermal delivery. Drug Delivery Formulation Nanotechnology 2005:2:67-74.

10. Karim K, Mandal AS, Biswas N, Guha A, Chatterjee S, Behera M, Kuotsu K. Niosomes- A Future of Targeted Drug Delivery System. Journal of Advanced Technology and Research 2010:1:374-80.

11. Muzzalupo R, Tavano L, Trombino S, Cassano R, Picci N, Mesa CL. Niosomes from α,ώ,-trioxyethylene-bis(sodium2-dodecyloxy-propylenesulfonate): Preparation, characterization. Colloids and Surfaces B: Biointerfaces 2008:64:200–07.

12. Gil MI, Barberan FAT, Pierce BH, Holcroft DM, Kader AA. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. Journal of Agricultural and Food Chemistry 2000:48:4581-89.

13. Furniss BS, Hannaford AJ, Smith PWG, Tatchell AR, Vogel's textbook of practical organic chemistry. 5th edition. New York: Longman Scientific and Technical; 1988. p. 271-316.

14. Karki R, Mamatha GC, Subramanya G, Udupa N. Preparation, characterization and tissue disposition of niosomes containing isoniazid. Rasayan Journal of Chemistry 2008:1:224-7.

15. Hao Y, Zhao F, Li N, Yang Y. Studies on a high encapsulation of colchicine by a niosome system. International Journal of Pharmaceutics 2002:244:73-80.

16. Costa P, Lobo JMS. Modeling and comparison of dissolution profiles. European Journal of Pharmaceutical Sciences 2001:13:123-33.

17. Dash S, Murthy PN, Nath L, Chowdhary P. Kinetic modeling on drug release from controlled drug delivery systems. Acta Poloniae Pharmaceutica- Drug Research 2010:67:217-23.