CHAPTER 4: EXPERIMENTAL (MATERIALS AND METHODS)

4.1 Materials and Equipment

4.1.1 Chemicals and excipients

Various chemicals used in the present work are enlisted in Table 4.1.

Table 4.1: List of chemicals and excipients

Chemical	Manufacturing Company
2,2-diphenyl- 1-picrylhydrazyl (DPPH)	Sigma Aldrich, USA
Absolute alcohol	Merck Ltd., India
Acetic acid	Merck Ltd., India
Acetonitrile (HPLC)	Merck Ltd., India
Ascorbic acid	Merck Ltd., India
Carbopol (Carbomer) 934 P, 974 P	Sigma Aldrich, USA
Castor oil	CDH, India
Chloroform	Merck Ltd., India
Coconut oil	Persona, Amway India
Coumarin 6 (C6)	Sigma Aldrich, USA
Dialysis Membrane	Himedia Pvt. Ltd
Disodium Hydrogen Phosphate	Himedia Pvt. Ltd
ELISA kits for assay cytokines	Krishgen Biosystem Ltd., Mumbai, India
Ellman's reagent	Himedia Pvt. Ltd

Eosin and Haematoxylin	Himedia Pvt. Ltd
Ethyl oleate	Himedia Pvt. Ltd
Ethylenediaminetetraacetic acid (EDTA)	Himedia Pvt. Ltd
Formalin	CDH, India
Glycerine	Himedia Pvt. Ltd
Hair remover Cream (Anne French)	Wyeth Limited, India
Hydrogen peroxide	SDFCL, India
Imiquimod (IMQ)	Imiquad, Glenmark Pharmaceuticals 5% w/w
Methanol	Merck Ltd., India
Methanol (HPLC Grade)	SDFCL, India
NaOH	CDH, India
n-Butanol	Merck Ltd., India
n-Octanol	CDH, India
Olive oil	Leonardo, India
Paraffin wax	CDH, India
PEG 400	Himedia Pvt. Ltd
Phosphoric acid	Merck Ltd., India
Poloxamer F127	Sigma Aldrich, USA
Poloxamer P123	Sigma Aldrich, USA

Potassium Bromide	CDH, India
Propylene glycol	Merck Ltd., India
Resveratrol*	Sami Labs, Bangalore, India
Sodium chloride	CDH, India
Sodium Dihydrogen Phosphate	Himedia Pvt. Ltd
Transcutol P	Himedia Pvt. Ltd
Triethanolamine	Himedia Pvt. Ltd
Tween 20	Himedia Pvt. Ltd
Tween 80	Himedia Pvt. Ltd
Vitamin E	Himedia Pvt. Ltd

Gift samples supplied *ex gratis

4.1.2 Equipment

Various types of equipment employed in the present study are enlisted in Table 4.2. Table 4.3 enlists various softwares used.

Table 4.2: List of different instruments

S. No.	Instrument	Model number	Manufacturer
1.	Brookfield viscometer	R/S plus	Brook-field Engineering Laboratories, Inc, Middleboro, MA
2.	Cell crusher	Lark	Lark, USA
3.	Centrifuge machine	R-24C	Remi Motors, India

4.	Confocal microscope	LSM 510 META,	Zeiss, Germany
5.	Franz Diffusion cell	4G- 01- 00-09-05	Prefit, India
6.	FTIR	380	Thermo Scientific, USA
7.	High-Performance Liquid Chromatography	515	Waters, USA
8.	Homogenizer		Heidolph, Germany
9.	Hot air oven	PMTC-3040	PERFIT, India
10.	Incubator	1005RE	Daihan, Korea
11.	Magnetic mixer	122	Remi work, India
12.	Melting point apparatus	31354	Decibels, India
13.	Micropipette	(100-1000 µl)	Tarsons, India
14.	Microtome	HM 325	Thermo Scientific, USA
15.	Motic digital microscope	BA 310	Motic Incorporation, Hong Kong
16.	pH meter	ME 963-P	Remi, India
17.	Plate reader	680	BIO-RAD, USA
18.	Rota evaporator	4011	Heidolph, Germany
19.	Sonicator	40050	Lark, USA
20.	Spinner	3020	Tarsons, India
21.	Stability chamber	400G	Thermolab, India

22.	Tissue homogenizer	WiseMix HG-15D	Dathan Scientific, New Delhi, India
23.	Transmission electron microscope	H-7000	Hitachi Ltd., Japan
24.	Ultracentrifuge	3K- 30	Sigma, USA
25.	UV-VIS Spectrophotometer	UV-1700	Shimadzu, Japan
26.	Vacuum pump	65	Welch, USA
27.	Water Purifier	Milli-Q	Millipore, USA
28.	Weighing balance	AB265	Mettler Toledo, UK
29.	Zeta-sizer	Nano 4 C	Beckman Coulter, USA

Table 4.3: List of various computer software used in the present research

Software	Manufacturer	Use
Design-Expert 11	Stat-Ease Inc. Minneapolis, USA	For optimization of formulation
Minitab 18	Minitab Inc., Philadelphia, USA	For risk assessment analysis (Development of Fishbone Ishikawa's Diagram)
Graph Pad Instat 3	GraphPad Software Inc., USA	Statistical Analysis
Mendeley Desktop	Elsevier, UK	Reference management and generate bibliographies
MS-Office 2007	Microsoft Corp., USA	Thesis Writing

4.2 Preformulation studies of Bioactive (Resveratrol)

Preformulation studies are "indispensable elements of the drug development process. Preformulation entails the application of biopharmaceutical concepts to the physicochemical parameters of a drug to design a foremost drug delivery system".

Preformulation studies are vital for evaluating and confirming the physicochemical properties as well as the identity and purity of the raw material to be used in formulation development, especially the active pharmaceutical ingredient.

Preformulation studies of Resveratrol were carried out to confirm its identity and purity as well as to establish the most suitable analytical technique for determining its concentration in various formulations. The studies helped in selecting the appropriate formulation ingredients, solvent system process variables, etc to develop a novel, patient compliant, high quality and safe formulation."The main intention of preformulation studies is to develop innovative, stable, safe, cost-effective dosage forms capable of delivering the drug at the intended site" (Kharia & Singhai 2013). Figure 4.1 is representing various studies conducted during the preformulation of Resveratrol.

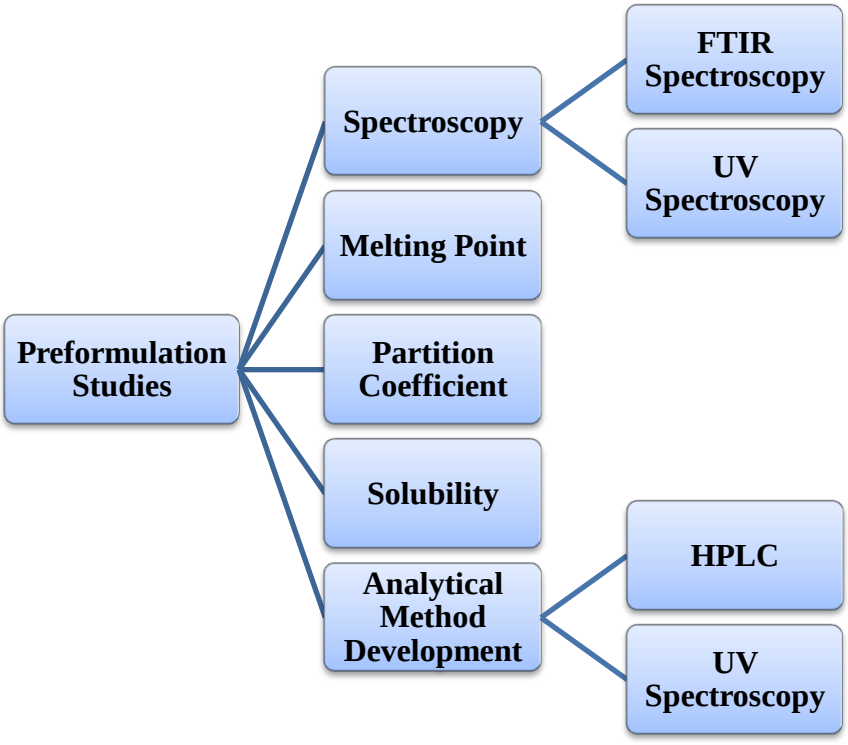

Figure 4.1: Preformulation studies of Resveratrol

4.2.1 Identification of Resveratrol

Identification was done by evaluating its organoleptic properties, ultraviolet spectroscopy, IR spectroscopy, and melting point.

4.2.1.1 Organoleptic properties

The substantial appearance of Resveratrol was observed and noted visually.

4.2.1.2 UV Spectroscopy for determining λ_{max}

The following procedure was employed to determine the λ_{max} of Resveratrol as shown in Figure 4.2.

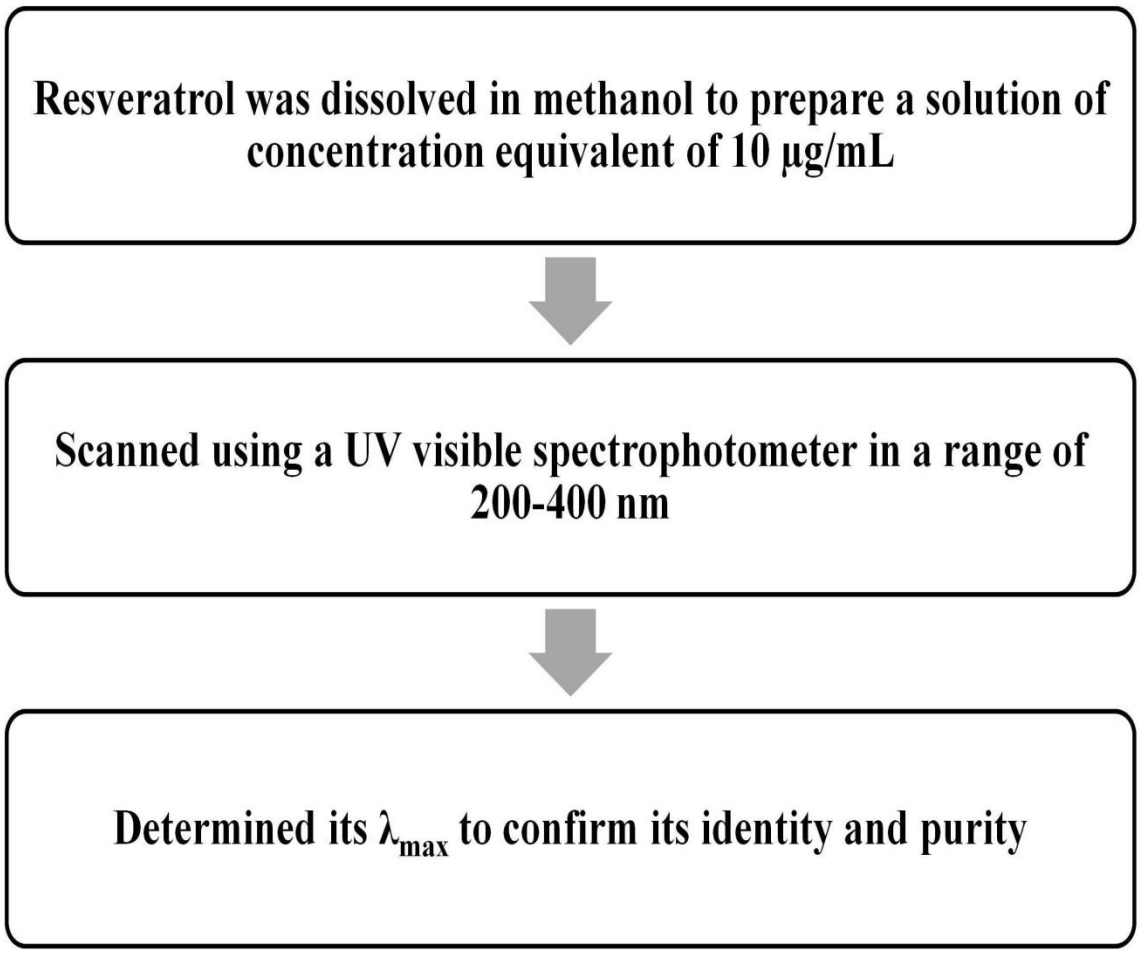

Figure 4.2: Procedure for performing UV Spectroscopy

4.2.1.3 FTIR spectroscopy

FTIR spectroscopy studies of Resveratrol were done using the following procedure as mentioned in Figure 4.3.

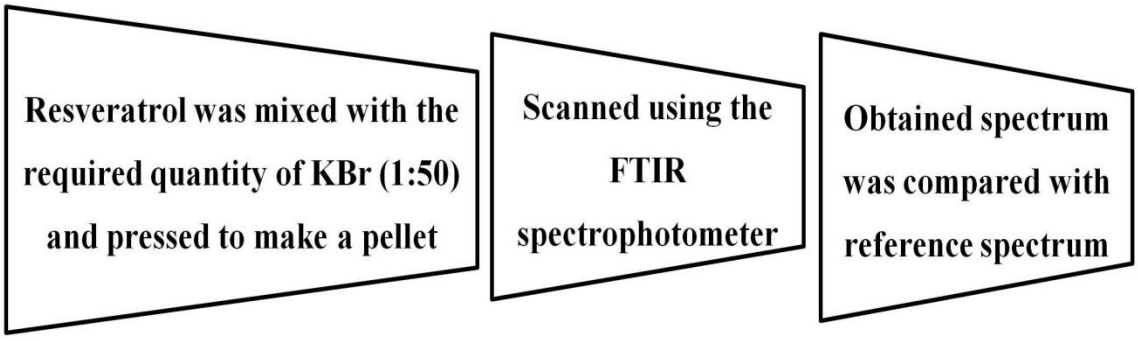

Figure 4.3: Procedure for performing FTIR Spectroscopy

4.2.1.4 Determination of melting point

The following procedure as depicted in Figure 4.4 was employed for determining the melting point of Resveratrol.

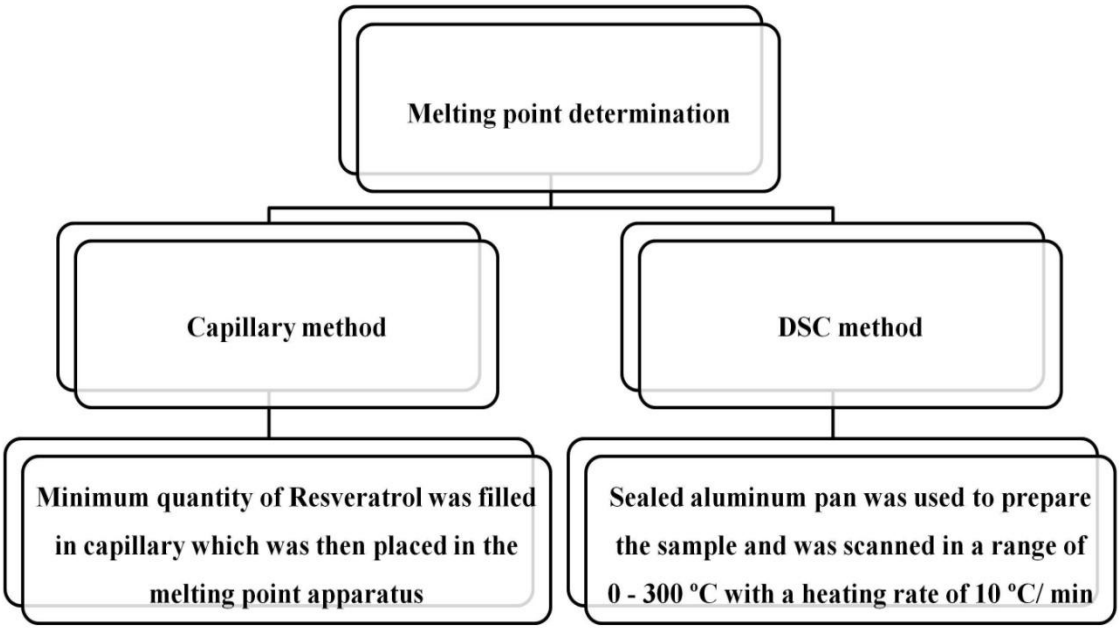

Figure 4.4: Procedure for determining the melting point of Resveratrol

4.2.2 Development of the analytical methods

4.2.2.1 UV-Visible spectroscopy studies

The standard plot of Resveratrol was developed in methanol at observed λ_{max} i.e. 306 nm. Figure 4.5 represents the schematic representation of the procedure employed. Similarly, it was also plotted in a mixture of PBS pH 7.4 and ethanol (7:3 v/v).

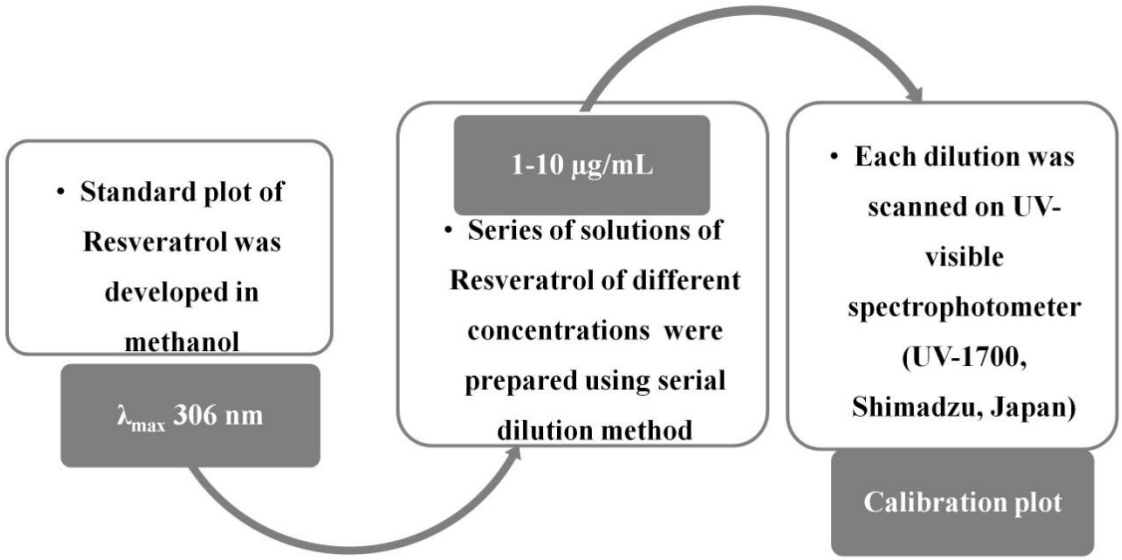

Figure 4.5: Schematic representation for plotting a standard curve of Resveratrol in methanol

4.2.2.2 HPLC method for Resveratrol determination

Instrumentation: The HPLC system (Waters, USA) consists of a "LiChrospher® C18 reversed-phase column (150 mm× 4.6 mm i.d., 5 µm particle size; Merck, Mumbai, India)" was used.

Parameters and detection: A mobile phase of methanol, water, and acetic acid (69:30:1, v/v/v). "The flow rate and UV wavelength were set at 1 mL/min and 306 nm, respectively. The detection was done at room temperature. All of the samples were filtered through a 0.20 µm pore-size nylon membrane filter and injected using a Rheodyne injector fitted with a 20 µl loop". The detailed procedure employed is depicted in the following Figure 4.6.

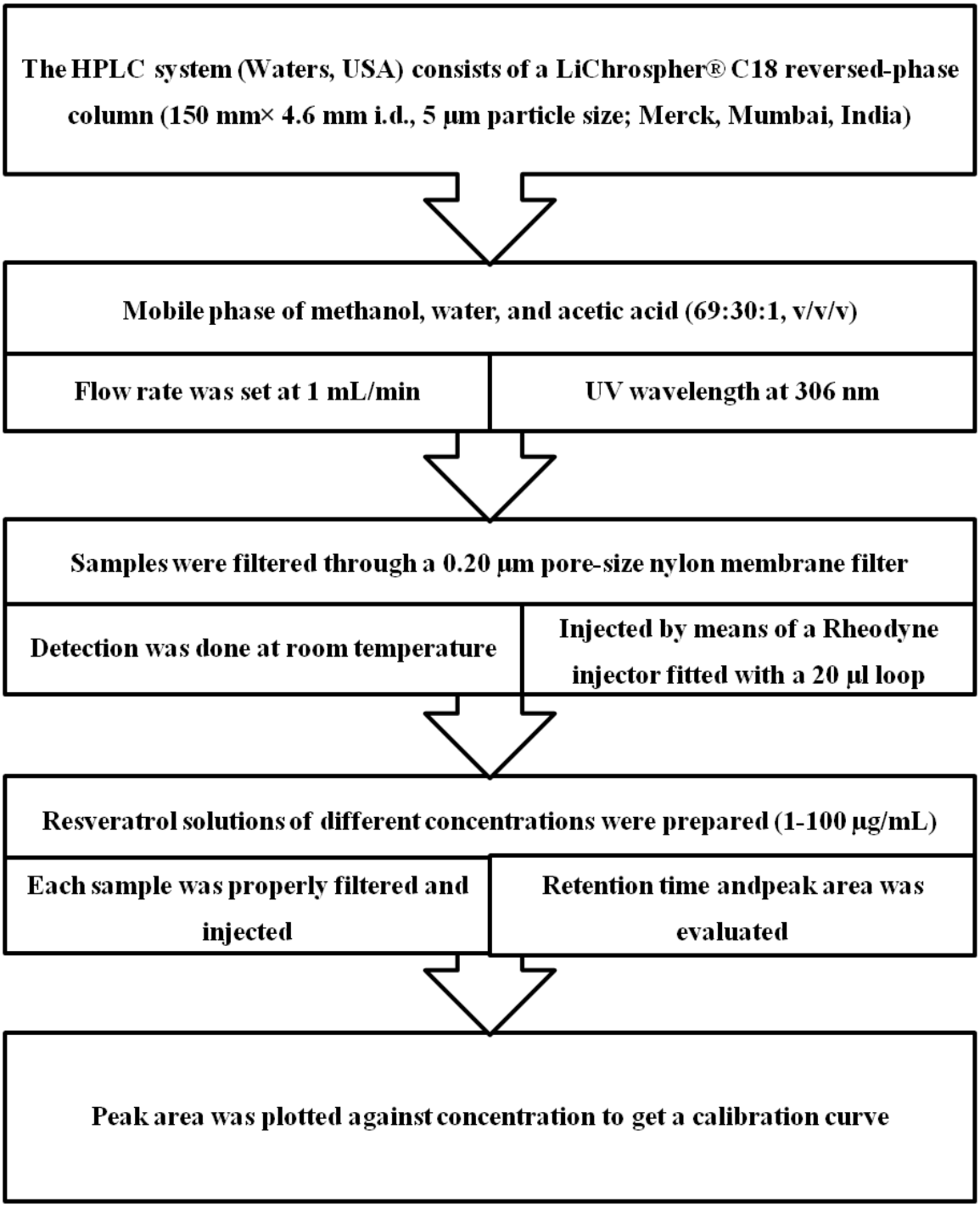

Figure 4.6: Schematic representation for developing an analytical method by HPLC method

4.2.3 Determination of partition coefficient

Figure 4.7 depicts the procedure used for evaluating the partition coefficient of Resveratrol. Partition coefficient was determined using the following equation (Eqn. 4.1):

$$\log P = \log \frac{\text{concentration of Resveratrol in } n \text{ octanol}}{\text{concentration of Resveratrol in water}} \qquad \text{-eqn. 4.1}$$

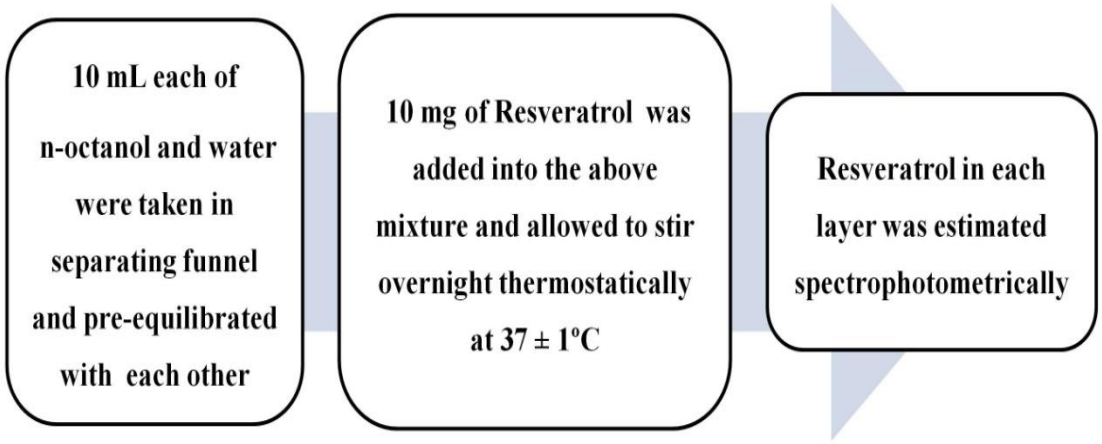

Figure 4.7: Schematic representation of the procedure for determining the partition coefficient of Resveratrol

4.2.4 Determination of solubility

Figure 4.8 represents the schematic layout for determining the solubility of Resveratrol.

4.3 Formulation Development

In present studies, two types of nanocarriers were developed for successful topical delivery of Resveratrol. The first one is polymer-based polymeric micelles and the second is a lipid-based delivery system i.e. vitamin-E oil-based nanoemulsion. Both of these systems were then incorporated into the carbomer based hydrogels.

The development of such complex nanocarriers involves the combination of several diverse material and process variables. To achieve the desired product profile of target formulation and to achieve the prerequisite quality characteristics of the developed drug product, the formulation scientist needs to optimize all the associated variables

systematically as suggested by ICH Q8 (R2) guideline for product development. Traditionally one variable at a time (OVAT) approach of optimizing drug delivery does not solve the purpose completely, while a more systematic multivariate response surface methodology is preferred nowadays as per regulatory requirements also.

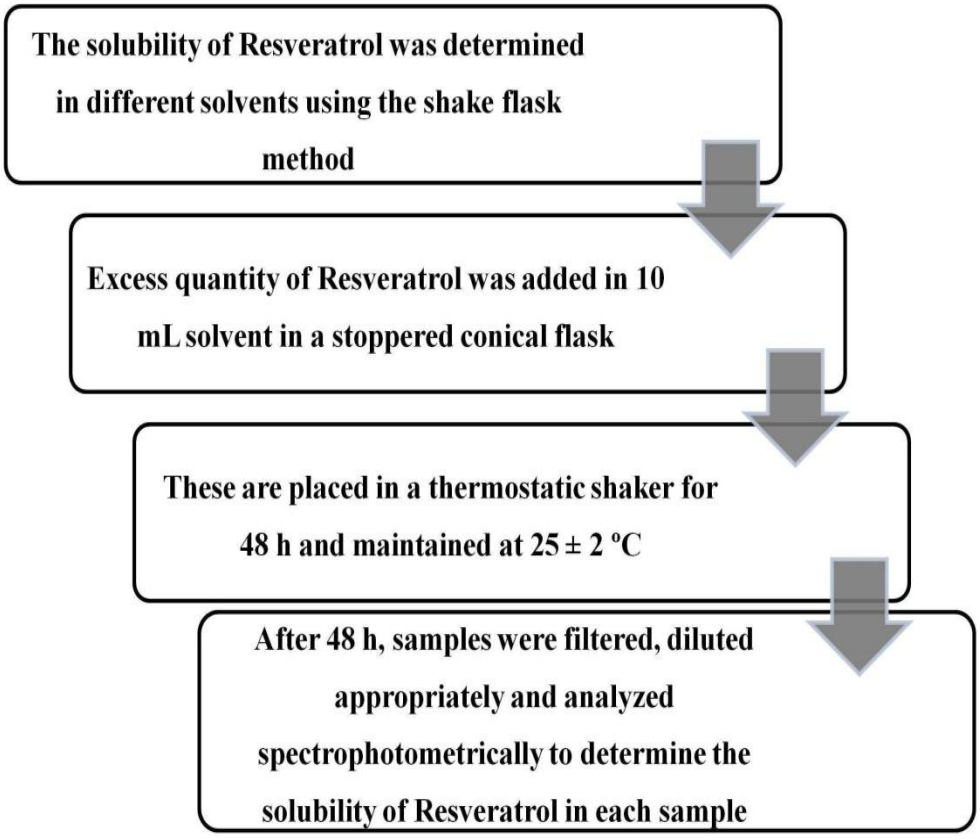

Figure 4.8: Schematic layout for determining the solubility of Resveratrol

Thus, the Quality by Design (QbD) approach has a pivotal role in formulation optimization and development which states that it is "a systematic approach of development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management" (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use 2009; Saydam & Takka 2018).

The basic elements and tools of QbD approach such as identifying Quality Target Product Profile (QTPP), Critical Quality Attributes (CQAs), Critical Material Attributes

(CMAs), and Critical Process Parameters (CPPs) were employed along with the application of DoE to establish the relationship between CQAs and CMAs.

4.3.1 Formulation of Resveratrol loaded polymeric micelles: QbD based optimization

Resveratrol loaded Polymeric Micellar formulation was formulated constituted of a mixture of two block copolymers i.e. Pluronic P123 and Pluronic F127.

4.3.1.1 Defining QTPP and Identifying CQAs for applying QbD approach

The first step of QbD based development is to define the quality target product profile (QTPP) which endows an eventual outline regarding the quality characteristics of a drug product to be developed (Simões et al. 2018; Gurumukhi & Bari 2020). The intended target is better skin permeation and enhanced therapeutic activity of loaded bioactive cargos in the polymeric micellar formulation.

Hence, it could be the basis for designing the formulation emphasizing the quality, safety, and efficacy of the formulation considering the dosage type, route of administration, stability, product characteristics in terms of skin permeation, etc (Prasad et al. 2016; Sousa et al. 2019). Based on defined QTPP, its subset i.e. indispensable CQAs were recognized which constitutes formulation and physical attributes. Most crucial attributes were screened among the identified and defined characteristics including particle size, micellar incorporation efficiency, and extent of Resveratrol deposition potential in the skin. These were selected based on the literature survey, previous skill, and knowledge with their apt justifications (Torregrosa et al. 2020).

4.3.1.2 Risk assessment

Risk assessment was done to screen influential formulation and process variables/ parameters affecting selected CQAs. An Ishikawa fish-bone diagram was created using Minitab 19 software (M/s Minitab Inc., Philadelphia, USA) indicating a cause and effect relationship between all possible material and process variables on selected CQAs of polymeric micelles (Arora & Nanda 2019). Failure mode and effect analysis (FMEA) was executed to screen out the crucial parameters affecting the quality, safety, and

efficacy of the drug product. All associated materials and process attributes were scored to strategically rank associated chances of failure during the production of micelles. The score to the material and process parameters attributes were assigned ranging between a value of 1-10, based on their severity (S), occurrence (O), and detectability (D).

$$RPN = Severity\ (S) * Occurence\ (O) * Detectability\ (D) \quad \text{-eqn. 4.2}$$

The product of all three values was designated as risk priority number (RPN) as indicated in equation 4.2 which was used to rank failure modes and identify potential critical process parameters (Herneisey et al. 2019). The attributes having high RPN values were selected for further screening studies and taken for principal component analysis for computing their qualitative and quantitative effect on CQAs.

4.3.1.3 Preparation of Resveratrol loaded polymeric micelles (PM)

Resveratrol loaded polymeric micelles were developed by film hydration method with slight modifications as per laboratory setup (Fares et al. 2018). In brief, the mixture of Polaxomer F127 and P123 (200 mg, in different ratios) and Resveratrol in different quantities as per optimization design were dissolved in a mixture of methanol: chloroform (1:2, v/v) in the round bottom flask.

Then rotary evaporator with vacuum and reduced pressure was employed to remove the organic solvents to form the thin film of polymer on the inner lines of the round bottom flask. The vacuum was further applied for an additional 4 hours to remove traces of solvents. The dried deposited polymeric film was rehydrated with a solution of 4 mL phosphate buffer pH 7.4 for 1 h and kept under refrigeration to attain sufficient swelling. Probe sonication was employed to disperse the final formulation evenly using Probe sonicator, Lark, USA for 3 cycles of 5 minutes each (15 sec on/off cycle) as shown in Figure 4.9. All other process variables were kept constant.

The free or unentrapped bioactive and unstructured polymer was then separated using the ultracentrifugation method from the final formulation which was then stored in the refrigerator till further characterization. Also, blank formulations of the same composition without bioactive were formulated.

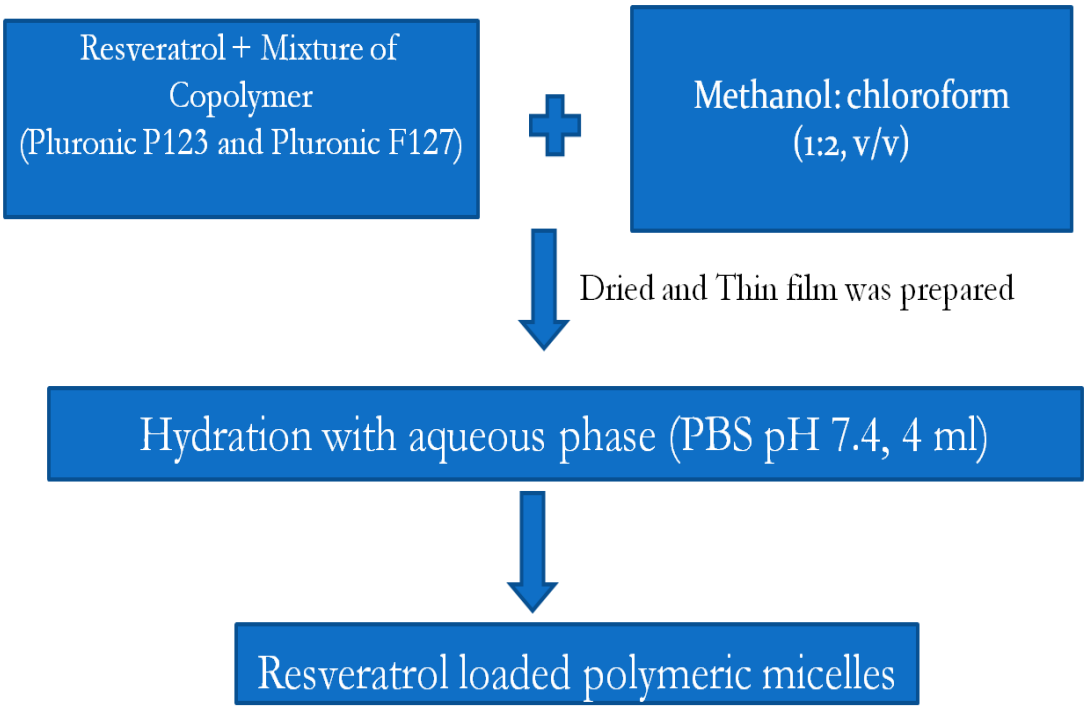

Figure 4.9: Flowchart for preparation of Resveratrol loaded polymeric micelles

4.3.1.4 DoE (Central Composite Design) based optimization of Resveratrol loaded polymeric micelles

After selecting and identifying CMAs and CPPs through risk assessment studies, the next tool of QbD is to apply response surface methodology using DoE to earmark and find out the optimum levels of selected CMAs and/or CPPs. These were varied at different levels to evaluate their main and interaction effect on selected CQAs. The Face Centered Central Composite Design (FCCD) was employed to optimize Resveratrol loaded polymeric micelles. Selected CMAs (independent attributes) was the percentage of Pluronic P123 in the mixture of two pluronic (P123 and F127) (X1) and amount of Resveratrol (X2), which were varied at three different levels i.e. low, medium, and high represented by coded levels (-1, 0 and +1) and actual values (10, 50 and 90 % for X1 and 50, 75 and 100 mg for X2). The identified CQAs (dependant attribute) were micellar incorporation efficiency (Y1), particle size (Y2), and extent of skin deposition (Y3) as shown in Table 4.4.

Table 4.4: Variables (CQAs and CMAs) used in Central Composite Design with coded and actual values for developing polymeric micelles

CMAs	**Coded and Actual Levels**		
	Low (-1)	Medium (0)	High (+1)
Ratio of P123, % (X1)	10	50	90
Amount of Resveratrol, mg (X2)	50	75	100
CQAs	**Target**		
Micellar Incorporation efficiency (MIE, %) (Y1)	Maximum		
Particle size (PS, nm) (Y2)	In range (100-200 nm)		
Extent of Skin deposition (SD, %) (Y3)	Maximum		

To employ statistical optimization, Design-Expert software (Design Expert 11.0.4, Stat-Ease, Minneapolis, MN) was employed to create a design matrix of formulations that suggested a total of thirteen batches including four additional center points per block as shown in Table 4.5. Thus the formulations comprising the suggested combination of CMAs were developed in triplicate and analyzed for respective CQA. The obtained results were statistically evaluated by ANOVA employing Design-Expert software to check the significance of model terms generated. Multiple Linear Regression Analysis (MLRA) was employed to generate the polynomial equations and ascertain the interaction effects of CMAs on CQAs. Along with this, 2D Contour Plots and 3D Response Surface Plots were exploited to establish interaction relationships. The target/constraint values for CQAs were defined as per QTPP. Both numerical and graphical optimization were carried out using the desirability approach and checkpoint analysis was done to get the final optimized micellar formulation (Negi et al. 2016; Beg et al. 2018).

Table 4.5: Different experimental runs for the development of polymeric micelles

Batch No	Variable levels in coded form		Variable levels in Actual form	
	X1	X2	Ratio of P123, %	Amount of Resveratrol, mg
PM 1	-1	-1	10	50
PM 2	-1	0	10	75
PM 3	-1	+1	10	100
PM 4	0	-1	50	50
PM 5	0	0	50	75
PM 6	0	+1	50	100
PM 7	+1	-1	90	50
PM 8	+1	0	90	75
PM 9	+1	+1	90	100
PM 10*	0	0	50	75
PM 11*	0	0	50	75
PM 12*	0	0	50	75
PM 13*	0	0	50	75

[* indicates additional center points, All other parameters were kept constant]

4.3.1.5 Characterization of developed colloidal micellar formulation

4.3.1.5.1 Size, polydispersity index, and zeta potential

DLS technique utilizing Zetasizer (Nano 4 C, Beckman Coulter, USA) was utilized to evaluate the particle size of polymeric micellar dispersion. PDI and surface charge were also estimated using zetasizer. The standard protocol of diluting the samples before all measurements were followed (Arora et al. 2011; Pahal et al. 2011; B. Khurana et al. 2013).

4.3.1.5.2 Morphological evaluation

The developed colloidal system was evaluated for its morphological characteristics and shape using TEM. The sample was placed on a carbon-coated copper grid and stained with phosphotungstic acid, air-dried and photographs were captured using a microscope.

4.3.1.5.3 Micellar incorporation efficiency

The encapsulation efficiency of colloidal carriers was determined by evaluating the amount of Resveratrol entrapped into them after separating free/unentrapped Resveratrol from them by the ultracentrifugation method (Arora & Nanda 2019). The supernatant was diluted appropriately with the required quantity of methanol and Resveratrol was quantified spectrophotometrically using the previously developed method at 306 nm (Arora & Nanda 2019; Sousa et al. 2019). Entrapment efficiency was calculated using the following formula (equation 4.3):

$$\%\ Micellar\ Incorporation\ Efficiency = \frac{(Amount\ of\ Resveratrol\ entrapped)}{(Amount\ of\ Resveratrol\ added)} * 100$$

- eqn. 4.3

4.3.1.5.4 Skin permeation and skin deposition studies

The extent of Resveratrol deposition in the skin was evaluated using the *ex vivo* skin permeation method. Franz diffusion cell and porcine ear skin were used in this study. A detailed procedure is mentioned later in section 4.4.5.

4.3.1.6 Formulation of carbomer based hydrogel embedded with colloidal micelles (PMG)

The micellar dispersion has poor skin applicability due to less viscosity, thus the colloidal dispersions were transformed into the carbomer-based hydrogel. Carbomer was selected due to its ideal characteristics (i.e. gelling property at a lower concentration and ease of preparation) and its wide use in a topical formulation.

Colloidal dispersion was homogenized with the appropriate quantity of glycerine which was used as humectants and carbomer which was added as a gelling agent. 1 g carbopol was added into a mixture of required quantities of PM (carrying 1 g resveratrol), 6 g glycerine, and purified water (quantity sufficient to make 100 g) and the mixture was constantly stirred at 1000 rpm for 60 min. The properly homogenized mixture was then neutralized by adding 0.2 mL of triethanolamine dropwise with continuous stirring which induced the gelling. Different types of hydrogel were formulated i.e. Polymeric micelles hydrogel (PMG) and conventional hydrogel (CG) having a strength of 1% w/w. The conventional hydrogel was formulated by using distilled water instead of micellar dispersion and the equivalent quantity of Resveratrol. It is developed as the control for comparative purposes. The physical and chemical properties of the developed gel were then examined to ensure their stability (Arora & Nanda 2019).

4.3.2 Formulation of Resveratrol loaded Vitamin E based nanoemulsion

Preliminary screening studies: Solubility studies of resveratrol in several excipients

Preliminarily, various excipients such as oil (castor oil, ethyl oleate, olive oil, coconut oil, vitamin E and their combinations); surfactants (tween 20 and tween 80); and co-surfactants (propylene glycol, PEG 400, and transcutol P) were screened based on the solubility of resveratrol in them. For determining the solubility of resveratrol, an excess amount of resveratrol was added in 2 mL of each solvent in amber glass stopper vials having 5 mL capacity. The mixtures were then shaken using a water bath shaker at room temperature for 48 h. The saturated solution was separated by centrifuging the mixtures at 4000 rpm for 15 mins (Pangeni et al. 2014; Salem et al. 2019). The supernatants obtained were diluted with methanol appropriately and the quantity of resveratrol dissolved in each

solvent was evaluated spectrophotometrically using a UV spectrophotometer (Shimadzu, UV-1800 PC, Japan) at 306 nm. All the studies were conducted in triplicates.

4.3.2.1 Pseudo ternary phase diagram

The different combinations of the Smix ratio constituted of tween 80 as the surfactant, transcutol P as the co-surfactant; oil (vitamin E: ethyl oleate; 1:1) and water were utilized to scrutinize the phase behavior using the titration method and pseudo ternary phase diagrams were constructed. The surfactant and co-surfactant (Smix) were mixed in altered volume ratios (1:1, 1:2, 2:1, 3:1). to construct a total of four ternary phase diagrams using Tri-plot software (A product of Todd Thompson Software, version 4.1.2). For every phase diagram, altered volume ratios of oil and Smix, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1 were prepared and titrated with water (dropwise) with continuous stirring till one phase showing clear solution was observed (El-Leithy et al. 2018). The content of excipients (oil, Smix, and water) in each batch was noted and the nanoemulsion area of the ternary system was observed as triangular phase diagrams using tri-plot computer software. The Smix ratio resulting maximum nanoemulsion region was further selected for formulation and optimization studies.

4.3.2.2 Formulation of Resveratrol loaded vitamin E based nanoemulsion (NE)

Resveratrol loaded nanoemulsion was developed by reported high energy emulsification method as with appropriate modifications (Sharma et al. 2019). Briefly, Resveratrol was firstly dissolved in a mixture of selected oil (ethyl oleate and vitamin E, 1:1) with continuous stirring using a high-speed magnetic stirrer. A varying quantity of selected Smix (tween 80 and transcutol P) was added to the mixture of oil containing resveratrol and was then mixed with continuous stirring. Then the required quantity of aqueous phase i.e. distilled water was added to this mixture under continuous vortexing to get primary o/w emulsion which was then subjected to homogenization for 15 mins (Heidolph, Germany) at 25 ± 2^{o}C. The evenly dispersed nanoemulsion globules were formed. The formulation was then stored in the refrigerator for further characterization. The concentration of resveratrol in the final formulation was 1% w/v; all other process parameters were kept constant in all batches as shown in Figure 4.10.

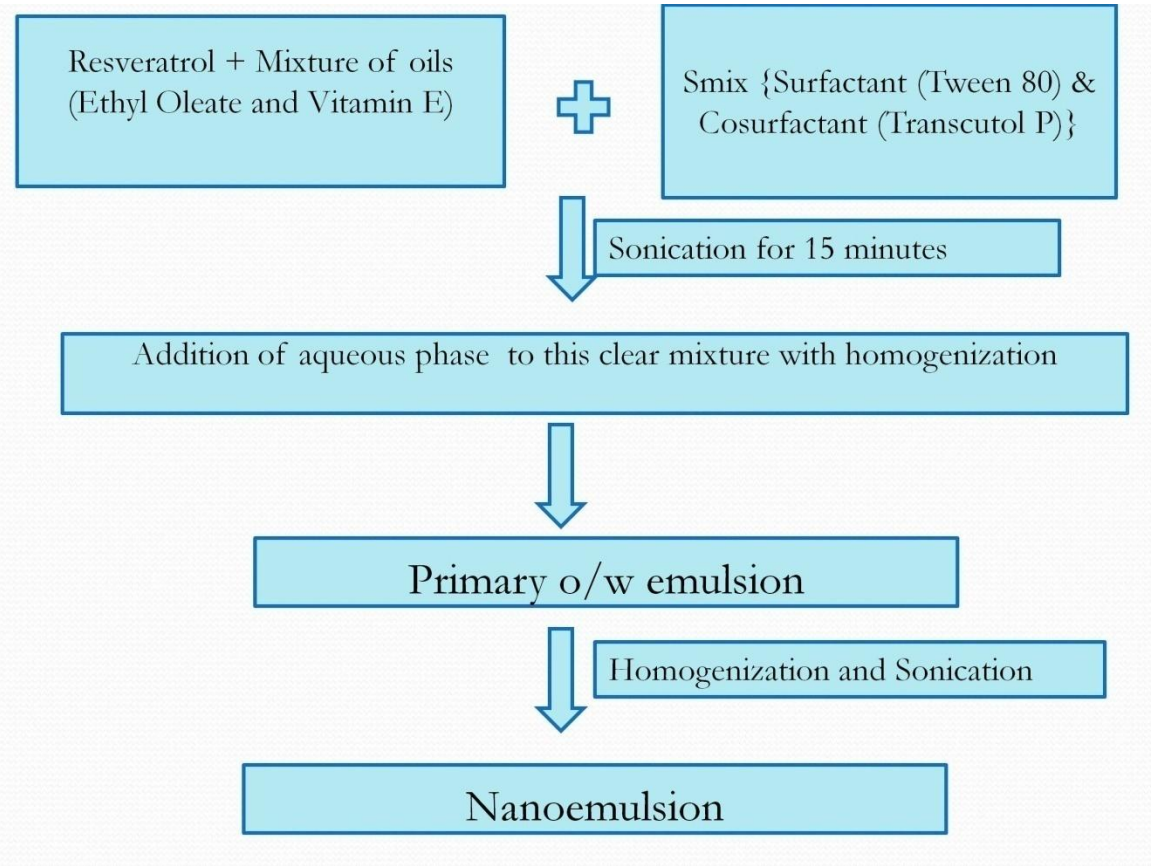

Figure 4.10: Flowchart for the formulation of Resveratrol loaded vitamin E based nanoemulsion

4.3.2.3 Factorial design and Response surface methodology employed optimization of resveratrol loaded nanoemulsion

The effect of different formulation variables on selected CQAs was studied by employing a 3^2 full factorial design consisting of two factors which were varied at three levels i.e. high, intermediate, and low. The different levels of independent variables (CMAs) i.e. X1 = concentration of oil (% v/v) and X2 = concentration of Smix (% v/v) were selected as low, medium, and high. Dependant variables or response variables (CQAs) were selected as Y1 [globule size (nm)], Y2 [% cumulative drug permeation], Y3 [permeation flux (J) (µg hr^{-1} cm^{-2})] and Y4 [skin deposition (µg cm^{-2})] as shown in Table 4.6. Smaller globule size would facilitate superior permeation and retention of the bioactive within the dermal layers; therefore it was selected as a highly critical quality attribute. Another critical parameter selected was % cumulative drug permeation as this is extremely

important for attaining meaningful pharmacodynamic effects. Since the primary objective of the present study is to enhance the drug deposition in the dermal layer of the drug, at the same time, permeation flux has to be controlled. Thus these both parameters were also selected as critical response variables in optimization studies.

A total of 13 batches including four additional center points were prepared as shown in Table 4.7. The response data obtained after analyzing the formulations were fitted into Design-Expert software (Design-Expert 11.0.4, Stat-Ease, Minneapolis, MN). Further software-based correlations between dependent and independent variables and their interactions were analyzed by constructing contour plots and (3D) response surface plots and generation of multiple linear regression models were done to understand their interaction effects (Sailor et al. 2015). Validation of the design suggested by the software was done by applying the analysis of variance (ANOVA). The range of targets was set as constraint values for CQAs and checkpoint analysis combined with the desirability approach and numerical optimization procedure was used to find the optimum formulation composition using Design-Expert software (Arora et al. 2016; Arora & Nanda 2019).

Table 4.6: Variables (CQAs and CMAs) used for employing Factorial Design with coded and actual values for developing nanoemulsion

CMAs	**Coded and Actual Levels**		
	Low (-1)	**Medium (0)**	**High (+1)**
Concentration of Oil (X1)	5 %	10 %	15 %
Concentration of Smix (X2)	2.5 %	5 %	7.5 %
CQAs	**Target**		
Globule size (Y1)	Minimum		
Percent Cumulative Permeation (Y2)	Maximum		
Permeation flux J (µg hr^{-1} cm^{-2}) (Y3)	Enhanced but in range		
Skin deposition SD (µg cm^{-2}) (Y4)	Maximum		

Table 4.7: Different experimental runs for development of nanoemulsion

Batch No	Coded factors		Actual factors	
	X1	**X2**	Concentration of Oil (%v/v)	Concentration of Smix (%v/v)
NE 1	-1	-1	5	2.5
NE 2	0	-1	10	2.5
NE 3	+1	-1	15	2.5
NE 4	-1	0	5	5
NE 5	0	0	10	5
NE 6	+1	0	15	5
NE 7	-1	+1	5	7.5
NE 8	0	+1	10	7.5
NE 9	+1	+1	15	7.5
NE 10*	0	0	10	5
NE 11*	0	0	10	5
NE 12*	0	0	10	5
NE 13*	0	0	10	5

[* indicates additional center points, All other parameters were kept constant]

4.3.2.4 Formulation of carbomer based nanoemulsion hydrogel (Nanoemulgel, NEG)

To enhance the skin applicability and to improve the consistency of formulated nanoemulsion, it was converted into carbomer-based nanoemulgel. Colloidal dispersion was homogenized with the appropriate quantity of glycerine which was used as humectants and carbomer which was added as a gelling agent. 1 g carbopol was added into a mixture of required quantities of NE (carrying 1 g resveratrol), 6 g glycerine, and purified water (quantity sufficient to make 100 g) and the mixture was constantly stirred at 1000 rpm for 60 min. The properly homogenized mixture was then neutralized by adding 0.2 mL of triethanolamine dropwise with continuous stirring which induced the gelling.

Different types of hydrogel were formulated i.e. Polymeric micelles hydrogel (NEG) and conventional hydrogel (CG) having a strength of 1% w/w. The gel was evaluated visually for checking its color and physical stability as well as for size, PDI, and zeta potential.

4.3.2.5 Characterization of developed nanoemulsion and nanoemulgel

4.3.2.5.1 Globule size, zeta potential, and PDI

The globule size and polydispersity index were determined by the dynamic light scattering technique (Zetasizer Nano 4C, Beckman Coulter, USA). All the formulations were properly diluted with deionized distilled water, followed by vigorous shaking to minimize multiple scattering effects before each measurement (Khurana et al. 2010). An average of three measurements was taken for size determination at temperature 25 ºC. The surface charge of dispersion and nanoemulgel was also evaluated after diluting the formulations (Arora et al. 2011).

4.3.2.5.2 Surface morphology via transmission electron microscopy

The transmission electron microscopy (TEM) (H 7500 Hitachi, Japan) was used to study the surface morphology of globules. Prepared formulations were stained negatively with 1 % w/v phosphotungstic acid solution after diluting appropriately with triple distilled water and (Pahal et al. 2011).

4.3.2.5.3 Resveratrol content in nanoemulgel

The weighed quantity of hydrogel was taken and diluted appropriately with methanol to extract the resveratrol completely. The amount of Resveratrol was quantified accurately using validated UV spectrophotometric method at λ_{max} 306 nm in triplicate (Arora & Nanda 2019).

4.3.2.5.4 Skin permeation and skin deposition studies

The permeation flux and the extent of Resveratrol deposition in the skin were evaluated using the *ex vivo* skin permeation method. Franz diffusion cell and porcine ear skin were used in this study. A detailed procedure is mentioned later in section 4.4.5.

4.4 Some other Important Evaluations of Formulated Micellar Hydrogel and Nanoemulgel

4.4.1 Determination of pH

1 g of the formulated gel was diluted in 50 mL distilled water and mixed well for half an hour using magnetic stirring at room temperature. The pH of the gel formulations (n=3) was measured using a calibrated pH meter (Dhawan & Nanda 2018).

4.4.2 Spreadability studies

To assess the ease of application on the skin, topical formulations were tested for spreadability analysis using the plate method (Nayak et al. 2018). The plate method was used to assess the spreadability of the hydrogel. A small quantity of formulation was kept at the center between two acrylic plates. A weight of 500 g was put for 5 minutes on the upper plate to apply some pressure. The initial and final diameter of the circles in which gel was spread was evaluated. The increase in diameter was determined which was correlated with the spreadability of the gel.

4.4.3 Rheological behavior and viscosity

Brookfield R/S plus viscometer (Brook-field Engineering Laboratories, Inc, Middleboro, MA) equipped R3- C75 spindle as shown in Figure 4.11 adjusted at 100 rpm was used for

evaluating viscosity and flow behavior of the PMG and NEG. A small amount of the formulation was applied at the base of the viscometer at a temperature of 25±2°C. A shear rate ranging from 1-100 sec^{-1} was applied. The viscosity was determined from the flow curve obtained at different values of shear rate (Yadav et al. 2016).

Figure 4.11: Pictorial representation of Brookfield R/S plus viscometer

4.4.4 *In vitro* Resveratrol release

The dialysis bag method was employed to determine the *in vitro* release profile of Resveratrol from formulated colloidal dispersions as well as hydrogels. The dissolution medium used was 500 mL of 3:7 (v/v) ethanol-pH 7.4 phosphate buffer which was maintained at 32 ± 0.5 °C and 50 rpm. A dialysis bag of MWCO 12,000

g/ mole containing different formulation (Micellar dispersion, PMG, CG, and drug solution) equivalent to 10 mg Resveratrol was placed in the dissolution medium. At periodic intervals i.e. 0.5, 1, 1.5, 2, 4, 6, and 12 h; 1 mL of aliquots from dissolution medium was withdrawn which was replaced with an equal volume of fresh medium. The sample withdrawn was analyzed for Resveratrol content spectrophotometrically at 306 nm.

4.4.5 Skin permeation and deposition profile

The ability of the formulated delivery system to permeate through the upper skin barrier and to deposit the loaded cargos at the target site was evaluated using *ex vivo* skin penetration studies. The skin was excised from pig ear (pinna) which was procured from the slaughterhouse and defeated adequately as per the standard protocol. Franz - diffusion cell having a cross-sectional area of 3.14 cm^2 and a capacity of 30 mL was employed on to which skin was mounted as represented in Figure 4.12. The receptor compartment was filled with 3:7 (v/v) ethanol-pH 7.4 buffer, the temperature was maintained at 32 ± 0.5 °C at kept stirred at 100 rpm. In donor compartment formulation equivalent to 1.5 mg of Resveratrol was applied. At predetermined intervals, aliquots were taken from the receptor compartment and the concentration of Resveratrol was analyzed using the validated HPLC method as described earlier (Arora et al. 2020).

Data Analysis: The cumulative amount of resveratrol permeated through the skin (Qn) was calculated using the following equation (equation 4.4):

$$Qn = Cn * Vo \sum_{i=1}^{n=1} Ci * Vi \qquad \text{- eqn. 4.4}$$

where *Cn* represents the concentration of resveratrol in the receptor compartment at any sampling time, V_0 and *Vi* represent the volumes of the receptor compartment (30 mL) and volume of aliquot respectively, and *Ci* is the resveratrol concentration of the *i*th sample. The calculated cumulative amount of Resveratrol permeated through unit area of the skin was plotted on the y-axis against time on the x-axis known as permeation curve from

which permeation rate (flux; J) was calculated (S. Khurana et al. 2013). While the following equation was used to calculate the permeability coefficient (Kp) (equation 4.5):

$$Kp = \frac{Jss}{C_0} \qquad \text{- eqn. 4.5}$$

Where Jss is the steady-state flux (μg/cm^2/h) and Co symbolizes the initial concentration of resveratrol in the donor compartment. The flux of control formulation was compared with vesicular formulation to calculate the enhancement ratio (Er) (S. Khurana et al. 2013; Negi et al. 2016).

After 24 h of permeation studies, the skin tissue was taken from the diffusion cell. It was properly rinsed to remove adhered formulation and the heat was applied to separate the dermis and epidermis layer of skin. The dermis tissue samples were homogenized using a tissue homogenizer at 8000 rpm for 10 minutes (in 2 mL of ethanol) (S. Khurana et al. 2013; Negi et al. 2016). The complete extraction of Resveratrol from the dermis layer of skin tissue was carried out by further sonicating the homogenate for 30 minutes. The supernatant obtained after centrifuging the above mixture for 10 minutes at 4,000 rpm was membrane filtered using 0.45 μM nylon membrane filters. The filtrate was analyzed for the amount of Resveratrol deposited per unit area of the dermis portion of the skin using a previously developed HPLC method.

4.4.6 Skin targeting studies: Histopathology by CLSM

Targeting of active payloads into the dermal layer of skin was confirmed by histopathological studies using confocal laser scanning microscopy technique. For this, coumarin 6 (C6) loaded PMG and NEG were formulated following the same optimized procedure as for the Resveratrol loaded hydrogel. C6 containing conventional carbomer hydrogel (CG) was also formulated. Similar to permeation studies, porcine ear skin was taken and processed as previously. The C6 containing formulations were then applied to it and stored for 6 h. Then skin tissue was washed properly with phosphate buffer saline (pH 7.4) and cryo-sectioned using cryostat microtome (Thermo scientific instruments, Microme, Germany). The cut sections were placed on the glass slides and imaged using CLSM (LSM 510 META, Zeiss, Germany) (Nayak et al. 2018; Arora & Nanda 2019).

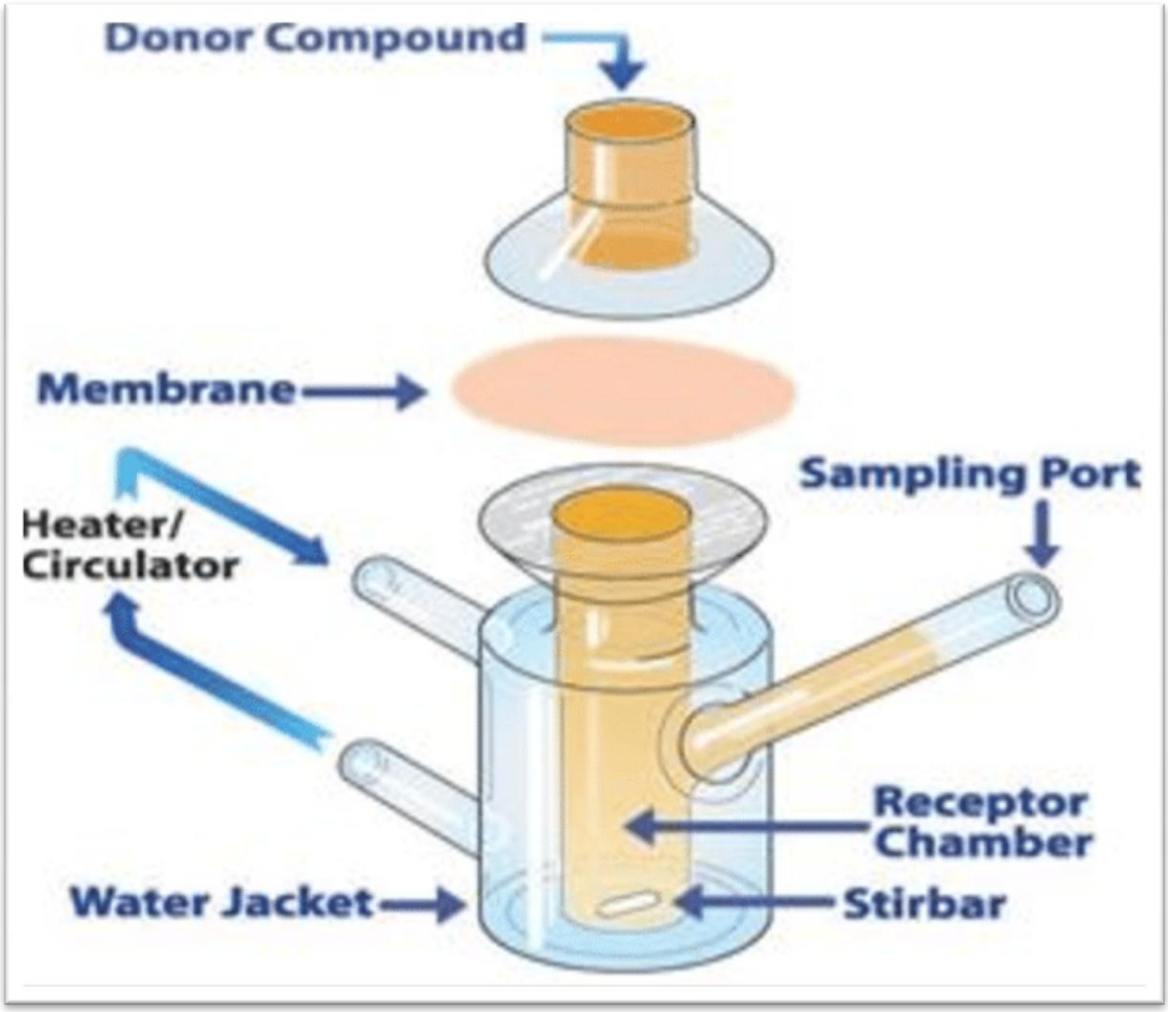

Figure 4.12: Franz Diffusion cell for skin permeation studies

4.4.7 *In vitro* antioxidant activity using DPPH assay

Free radical scavenging and antioxidant property of the developed formulation were evaluated by conducting the DPPH assay method (Hangun-balkir & Mckenney 2012; Arora et al. 2020). Ascorbic acid was used as standard and its antioxidant potential is compared with the Resveratrol suspension and the developed hydrogel formulations. The hydrogel formulations, Resveratrol, and ascorbic acid were diluted in a serial dilution from 1–10 µg/mL using methanol. One ml of each sample was added to one ml of 0.004% w/v DPPH solution in methanol. The absorbance was measured after 30 min at 517 nm using methanol as the blank (without the drug). The percentage of inhibition was calculated using the following formula (equation 4.6):

$$\% \, Radical \; scavenging \; ability = \frac{Absorbance \; of \; control \; (A_O) - Absorbance \; of \; sample \; (A_S)}{Absorbance \; of \; sample \; (A_S)} \times 100 \quad \text{- eqn. 4.6}$$

4.5 *In vivo* efficacy studies (antipsoriatic activity) using IMQ-induced psoriatic-like plaque model in Swiss Albino Mice

Animal Protocol was duly permitted by the Institutional Animal Ethics Committee, ISFCP, Moga [ISFCP/IAEC/CPCSEA/Meeting No. 23/2018/ Protocol No. 391]. Studies were carried out according to the CPCSEA guidelines. 2-3 months old Swiss Albino mice weighing 20-25 g were utilized and kept in an adequate environmental condition (25 ± 2 °C with 45% relative humidity and 12 h light/dark cycle) and provided *ad libitum* food and water. Before initiating the studies, they were acclimatized for 1 week. The experimental protocol consists of 5 groups each containing six animals.

Table 4.8: Different animal groups used in antipsoriatic studies

S. No.	**Groups**	**Number of animals**
I	Control (No disease and no treatment)	6
II	Negative control (Disease induced)	6
III	Disease + Conventional Resveratrol gel formulation (CG)	6
IV	Disease + Test drug (Resveratrol loaded polymeric micelles gel) (PMG)	6
V	Disease + Test drug (Resveratrol Loaded Nanoemulsion gel) (NEG)	6

Group 1 was taken as control (placebo), group 2 was the diseased group (psoriasis induced), groups 3, 4, and 5 were diseased animals treated with CG, PMG, and NEG respectively as shown in Table 4.8.

Animals were shaved on their back using an animal hair clipper. Imiquimod (IMQ) cream (brand name: Imiquad, Glenmark Pharmaceuticals 5% w/w) was employed for inducing psoriasis in group 2-5 by applying it on shaved back of animals. Accurately weighed cream (62.5 mg of cream equivalent to 3.125 mg of IMQ) was applied daily morning for seven days on the shaved skin of each animal. The signs of induction of plaque-like psoriatic skin lesions were examined after every application.

After disease induction, groups 3, 4, and 5 were treated topically with CG, PMG, and NEG respectively for consecutive 7 days. After the completion of the treatment period, the antipsoriatic efficacy of the formulations was determined based on the following parameters.

4.5.1 Psoriasis area and severity index (PASI) score

A scoring system was used to score the severity of inflammation in animals in which a clinical PASI score was assigned based on erythema, scaling and thickening of the skin from 0 to 4 where 0 = none; 1 = slight; 2 = moderate; 3 = marked; 4 = very marked. The scoring was done on the 7^{th} and 15^{th} days after starting the protocol (Sharma et al. 2020).

4.5.2 Cytokines level in serum and spleen dimension & weight

On the 15^{th} day of the experiment, the animals were sacrificed and spleen, blood sample, and skin biopsy were collected. Levels of pro-inflammatory cytokines (i.e. IL-17, IL-23 & TNF-α) were estimated. Blood was collected from the mice and it was centrifuged at 2500 rpm for 5 min at 25 °C to collect serum from it. The levels of different pro-inflammatory cytokines i.e. TNF-α, IL-17, and IL-23 were estimated by the following methods as specified in respective ELISA kits. The isolated spleen was also measured and weighed to determine its size and shape (Sahu et al. 2018).

4.5.3 Histology of skin

Histopathological alterations in skin samples were analyzed by histological evaluation of skin samples. The isolated skin biopsy was stored in a 10 % neutral formalin buffer. The section of size 5µm was cut and stained with hematoxylin and eosin (H&E) as per standard protocol and then examined using the light optical microscope for histopathological features (Sahu et al. 2018).

4.6 Stability Studies

Formulated polymeric micellar hydrogel and nanoemulgel were subjected to stability studies to evaluate the effect of different storage conditions. The study was conducted by keeping the formulations at "refrigerated conditions (5 ± 3 ºC)" and "room temperature (25 ± 2 ºC/ 60 ± 5 % RH)" for 6 months. The formulations were evaluated for physical as well as chemical stability at consecutive time intervals of "0, 1, 3, and 6 months". Physical stability was studied by analyzing surface charge, micrometric, and pH of the dispersion. The hydrogel was analyzed for physical appearance, pH, consistency, and phase separation. For chemical stability tests, NEG was evaluated for residual drug content in the formulation while micellar incorporation efficiency of PMG was investigated at both temperatures for up to 6 months (Negi et al. 2016). At periodic time intervals, evaluations were done by following the same procedure as reported in the previous sections.

4.7 Statistical Analysis

The results were evaluated statistically using Graph Pad Prism software. The responses are presented as mean ± SD. One-way analysis of variance (ANOVA) was deployed for multiple comparisons followed by post hoc analysis using the Tukey's test and Dunnett test.