Experimental

Novel Delivery Systems for the Treatment of Vitiligo

Chapter 4
### 4.1. Materials

Table 4.1 gives the details of the materials used in the present research work.

**Table 4.1: List of chemicals and excipients**

<table>
<thead>
<tr>
<th>Materials</th>
<th>Manufacturer/Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-gingerol</td>
<td>Sami Labs Ltd., Bangalore, India</td>
</tr>
<tr>
<td>Psoralen</td>
<td>Natural Remedies., Bangalore, India</td>
</tr>
<tr>
<td>Carbopol 934 P</td>
<td>Dow Pharmaceuticals, Michigan, USA</td>
</tr>
<tr>
<td>Sodium Starch Glycolate</td>
<td>Dow Pharmaceuticals, Michigan, USA</td>
</tr>
<tr>
<td>Aerosil</td>
<td>FMC Biopolymer, Philadelphia, USA</td>
</tr>
<tr>
<td>Magnesium stearate</td>
<td>Mallinkrodt, Hazelwood, MO, USA</td>
</tr>
<tr>
<td>Avicel® PH 102 (Microcrystalline cellulose)</td>
<td>FMC Biopolymer, Philadelphia, USA</td>
</tr>
<tr>
<td>Isopropyl alcohol</td>
<td>Qualigens Fine Chemicals, Mumbai, India</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>Qualigens Fine Chemicals, Mumbai, India</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>Qualigens Fine Chemicals, Mumbai, India</td>
</tr>
<tr>
<td>Potassium dihydrogen orthophosphate</td>
<td>Qualigens Fine Chemicals, Mumbai, India</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>Qualigens Fine Chemicals, Mumbai, India</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>Merck, Mumbai, India</td>
</tr>
<tr>
<td>Triethanolamine</td>
<td>S D Fine-Chem Ltd., Mumbai, India</td>
</tr>
<tr>
<td>Orthophosphoric acid</td>
<td>Qualigens Fine Chemicals, Mumbai, India</td>
</tr>
<tr>
<td>Lauroglycol 90</td>
<td>Gattefössse, Saint Priest, Cedex, France</td>
</tr>
<tr>
<td>Tween 80</td>
<td>S D Fine-Chem Ltd., Mumbai, India</td>
</tr>
<tr>
<td>Ethanol (99.9%)</td>
<td>Jiangsu Huax Co. Ltd., Jiangsu, China</td>
</tr>
</tbody>
</table>

All the other chemicals and reagents used were of analytical grade.
4.2. Equipments

Table 4.2 gives the details of the equipments used for carrying out the present research work.

Table 4.2: List of equipments

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Model/Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tablet compression machine</td>
<td>CIP Machinery Co. Pvt. Ltd., Ahmedabad, India</td>
</tr>
<tr>
<td>Mechanical stirrer</td>
<td>Universal motors Ltd., Mumbai, India</td>
</tr>
<tr>
<td>Electronic weighing balance</td>
<td>Mettler Toledo Inc., OH, USA</td>
</tr>
<tr>
<td>Hot air oven</td>
<td>Scientific Equipment, Delhi, India</td>
</tr>
<tr>
<td>Micropipettes</td>
<td>Eppendorf, Hamburg, Germany</td>
</tr>
<tr>
<td>Water bath shaker</td>
<td>Nirmal international, New Delhi, India</td>
</tr>
<tr>
<td>Dissolution apparatus</td>
<td>Veego Progressive Instruments, Mumbai, India</td>
</tr>
<tr>
<td>UV spectrometer</td>
<td>Shimadzu-UV-1601, Kyoto, Japan</td>
</tr>
<tr>
<td>Differential scanning calorimeter</td>
<td>Perkin Elmer, Massachusetts, USA</td>
</tr>
<tr>
<td>pH meter</td>
<td>Microprocessor pH System, Punjab, India</td>
</tr>
<tr>
<td>Zeta potential and Particles size analyzer</td>
<td>Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, United Kingdom</td>
</tr>
<tr>
<td>Transmission Electron Microscope</td>
<td>Morgagni 268(D), FEI Company, Oregon, USA</td>
</tr>
<tr>
<td>Syringe filters (0.22 and 0.45 μ)</td>
<td>Millipore (Nylon, PVDF and PTFE), MA, USA</td>
</tr>
<tr>
<td>Hot air oven</td>
<td>Widsons Scientific Work, New Delhi, India</td>
</tr>
<tr>
<td>Scanning electron microscope</td>
<td>LEO VP, Tokyo, Japan</td>
</tr>
<tr>
<td>Stability chamber</td>
<td>Labtop Instruments, Mumbai, India</td>
</tr>
<tr>
<td>Sonicator</td>
<td>US - 250 W, Altrasonics, Mumbai, India</td>
</tr>
<tr>
<td>Friability test apparatus</td>
<td>Scientific instruments, Mumbai, India</td>
</tr>
<tr>
<td>Hardness tester (Pfizer type)</td>
<td>Jagsons Scientific, New Delhi, India</td>
</tr>
<tr>
<td>HPLC</td>
<td>Waters HPLC solutions, MA, USA</td>
</tr>
<tr>
<td>Research centrifuge</td>
<td>Remi Labs, Mumbai, India</td>
</tr>
<tr>
<td>USP I Tapped density tester</td>
<td>Electrolab, New Mumbai, India</td>
</tr>
</tbody>
</table>
4.3. Methods

4.3.1. Physicochemical Characterization and Drug Identification

The organoleptic properties, partition coefficient and pH were determined to characterize psoralen and 6-gingerol. It was absolutely necessary to confirm the identity of the drugs procured before proceeding to its formulation into sustained release tablets and nanoemulsion gel.

4.3.1.1. Physical description / Organoleptic properties: The organoleptic properties refer to the appearance, color, odor, and taste of the substance. Characterization of these properties is the primary step in the study and helps with the primary identification of the drug substance and also helps in determining the likelihood of patient acceptability to the odor, taste and color of the raw material and its possible inclusion in the final dosage form. Sometimes changes in the stability of a formulation can also be easily recognized by changes (under similar conditions) in the color and odor of the raw material in the formulation (Wells, 2002).

4.3.1.2. Partition coefficient: The partition coefficient of the drug was determined by shake flask method (D'Cruz and Uckun, 2006; Krishna et al., 2001). Equal quantities of n-octanol (previously saturated with water) and distilled water was taken in a conical flask and drug was added. The mixture was shaken for 24 h at 25°C in a shaker water bath. The two phases were separated carefully and drug content was analyzed. The experiment was carried out in triplicate.

4.3.1.3. pH determination of the drugs: A 5 % w/v solution of the drug in demineralized (DM) water was prepared and evaluated for its pH by using calibrated pH meter (Microprocessor pH System, Punjab, India) and compared with the value given in the certificate of analysis (COA).

4.3.1.4. Differential scanning calorimetry: The DSC thermogram of drugs were obtained using DSC - 821 (Perkin Elmer, Massachusetts, USA) equipped with Star ® Software. Nitrogen gas flow was 60 mL min⁻¹. Sample was weighed about 5 - 10 mg,
sealed in an aluminum pan of 40 µL capacity and equilibrated at 25°C, was subjected to the DSC run over the temperature range of 25 to 350°C at a heating rate of 10°C min⁻¹. The temperature was calibrated using pure indium with a melting point of 156.60°C. An empty pan was used as a reference.

4.3.1.5. Thin-Layer Chromatography: A thin mark was made at the bottom of the ready made plate (stationary phase) with a pencil to apply the sample spots. Then samples solutions were applied on the spots marked on the line at equal distances. The mobile phase was poured into the TLC chamber to a level few centimeters above the chamber bottom. A filter paper moistened in mobile phase was placed on the inner wall of the chamber to saturate the mobile phase and thereby avoid edge effect. Then the plate prepared with sample spotting was placed in TLC chamber such that the side of the plate with sample line is towards the mobile phase. Then the chamber was closed with a lid. The plate was immersed such that sample spots are well above the level of mobile phase but not immersed in the solvent for development. After development of spots, the plates were removed and allowed to dry. The sample spots were visualized in UV light chamber as recommended for the said samples.

4.3.1.6. UV spectral analysis: The drug (5 mg) was dissolved in 500 ml methanol in a volumetric flask and the solution was scanned for the determination of λ_max (absorption maxima) in the spectral range of 200-800 nm of UV-visible region.

4.3.2. Pre-formulation:
4.3.2.1. Loss on drying (LOD): About 1 g of the drug was accurately weighed and evenly spread in an aluminum tray and placed in the LOD assembly (Mettler Toledo, IL, USA) for the determination of its water and volatile substance content under the pre-set conditions of 105°C for 10 min and then the observed LOD was compared with standard range of LOD given in the COA (NMT 2 %).
4.3.2.2. Solubility of the drug: Psoralen and 6-gingerol in various components (oils, surfactants and cosurfactants) and various physiological pH media (1.2 to 6.8) were required in the process of developing nanoemulsion and sustained release tablets
respectively for solubility studies. The solubility of the drugs was determined by adding an excess amount of drug in 2 mL of selected vehicle in 5 mL capacity stoppered vials, and mixing using a vortex mixer (Nirmal International, Delhi, India). These vials were then kept at 25 ± 1 °C in an isothermal shaker (Nirmal International, Delhi, India) for 72 h to reach equilibrium. The equilibrated samples were removed from shaker and centrifuged at 3000 rpm for 15 min. The supernatant was taken and filtered through a 0.22 μm filter and the concentration of drug was determined using HPLC at 245 nm as described in section 4.3.3.

4.3.2.3. Flow and consolidation properties: Flow and consolidation properties of 6-gingerol and psoralen were determined by measuring the following parameters:

i) Angle of Repose: The angle of repose is a simple practical measurement for indicating the flow of powders. It is the tangent of angle that is formed between the free standing surface of a powder heap and the horizontal plane (Marshall and Rhodes, 1979) and is given by the following (Eqn. 4.1)

\[ \tan \theta = \frac{h}{r} \]

(4.1)

where, \( \tan \theta \) = tangent of angle, \( r \) = radius of base of the heap (cm) and \( h \) = height of the heap (cm). The angle of repose is independent of the mass of the powder. The angle of repose was determined by setting up the assembly (Staniforth and Aulton, 2002). The cone was kept in an upright position on a piece of white printing paper and filled with the drug (10 g). Then the filled cone was smoothly and gently lifted allowing the flow of powder onto the paper. The flowed powder that collected on the paper had a cone shape and the radius and height of the heap of powder was measured with a calibrated scale and the angle of repose was calculated using the equation \( \theta = \tan^{-1} \frac{h}{r} \), where \( h \) is the height and \( r \) is the radius of the base of heap (USP 30). According to the Carr’s Index, the theoretical lower limit of the angle of repose for powders that are suitable for direct compression is 30°. Powders that have lower values of angles of repose show better flow. As the cohesive force among particles of powder gives rise to resistance to particle flow, the cohesive force can be correlated with the angle of repose. As the angle of repose
increases, the flow of particles decreases due to increase in the cohesive forces among the particles (Carstensen, 1993).

ii) Density Studies: Density is another property that provides characterization of the compressibility of powders.

a) Bulk Density: Bulk density was determined by accurately weighing 10 g of the drug and transferring it to a 50 ml graduated cylinder. The unsettled volume occupied by the drug was measured and the bulk density was calculated by the following formula (Eqn. 4.2):

\[
\text{Bulk Density} = \frac{M}{V_o} \tag{4.2}
\]

Where, \(M\) = Weight of test sample; and \(V_o\) = Apparent unsettled volume.

b) Tapped Density: Tapped density was determined by accurately weighing 10 g of the drug and transferring it to a 50 ml graduated cylinder. Cylinder was tapped mechanically (USP I Tap Density Tester) by raising the cylinder and allowing it to drop under its own weight that provides a fixed drop of 14 ± 2 mm at a normal rate of 300 drops per minute. The cylinder was tapped 500, 750 and 1250 times initially and the tapped volumes were measured until the difference between the two volumes was less than 2% (USP 30). Tapped density was calculated using the following formula (Eqn. 4.3):

\[
\text{Tapped Density} = \frac{M}{V_f} \tag{4.3}
\]

Where, \(M\) = Weight of the test sample; and \(V_f\) = Final tapped volume.

iii) Powder Compressibility: The Compressibility Index and Hausner Ratio are measures of the porosity of a powder to be compressed. They measure the relative importance of interparticulate interactions. For poor flowing materials, there are frequently greater interparticulate interactions and a greater difference between the bulk and tapped densities. These differences are reflected in the Compressibility index and Hausner Ratio. They are calculated by the following equation (Eqn. 4.4 & 4.5).

- Carr’s Compressibility Index = \(100 \frac{(V_o - V_d)}{V_o}\) \(\tag{4.4}\)

- Hausner Ratio = \(\frac{V_o}{V_f}\) \(\tag{4.5}\)
were, $V_u=$Unsettled apparent volume; and $V_f=$Final tapped volume

4.3.3. Analytical Method Development

To quantify psoralen and 6-gingerol at various stages and different samples of the studies, efforts have been made towards the development and validation of analytical method.

4.3.3.1. HPLC Simultaneous Estimation for Analysis of 6-Gingerol and Psoralen

High-performance liquid chromatography (HPLC) was developed for analysis of psoralen and 6-gingerol simultaneously. The samples were separated with an $C_{18}$ column (250×4.6 mm) by linear gradient elution technique using orthophosphoric acid buffer adjusted to pH 3 (A) and Acetonitrile (B) as mobile phase (Table: 4.3) at a flow rate of 1.0 ml/min and detected at 245 nm using UV Vis detector.

Table 4.3: Gradient elution program for psoralen and 6-gingerol

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% of Mobile phase A (Acetonitrile)</th>
<th>% of Mobile phase B (water adjusted to pH 3 with orthophosphoric acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
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</tr>
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<td>40</td>
</tr>
<tr>
<td>25</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>30</td>
<td>10</td>
<td>90</td>
</tr>
</tbody>
</table>

4.3.3.2. Preparation of Standard Plots/Calibration Curves in different media: For the purpose of quantification of psoralen and 6-gingerol, development and validation of a HPLC method in methanol was carried out as follows. The method was validated according to ICH guidelines, Q2 (R1) (ICH, 2005) with respect to linearity and range, precision, accuracy, detection limit (DL) and quantitation limit (QL). The method was also evaluated for its application as a stability indicative method using forced degradation studies. For the purpose of quantification of psoralen and 6-gingerol in the aqueous
phases during the various \textit{in vitro} evaluation of the developed sustained release tablet and nanoemulsion gel, standard plots were prepared (n=3) in phosphate buffer saline (pH 7.4) with 20% v/v methanol, phosphate buffer (pH 7.4) with 20% v/v methanol, 0.1N HCl (pH 1.2), Acetate Buffer (pH 4.5) and Phosphate Buffer (pH 6.8).

4.3.3.3. Preparation of reagents and buffers

To determine the \textit{in vitro} release of psoralen and 6-gingerol from the sustained release tablets, following buffer solutions were prepared.

\textit{i) Method for Preparation of 0.1N HCl (pH 1.2):} Two grams of sodium chloride and 7 g of concentrated hydrochloric acid were taken and added to make up 1000 mL of distilled water. The salts were mixed thoroughly with the aid of magnetic stirrer. The pH of the buffer was checked using a calibrated pH meter and adjusted if found necessary (USP XXIV).

\textit{ii) Method for preparation of acetate buffer (pH 4.5):} An accurately weighed 2.99g of sodium acetate trihydrate and 14 mL of 2N acetic acid were dissolved and made up to 1000 mL with demineralized water. If necessary, the pH shall adjusted to 4.5±0.05 with sodium hydroxide or 2N acetic acid).

\textit{iii) Method for preparation of phosphate buffer (pH 6.8):}

71.64 g of 0.2M dibasic sodium phosphate was dissolved and made up to 1000 mL in water (Solution 1) and 27.6 g of 0.2M monobasic sodium phosphate was dissolved in water and made up volume to 1000 mL (Solution 2). Mix 245 mL of solution 1 with 255 mL of solution 2 and made volume up to 1000 mL with distilled water to form 0.1 M phosphate buffer of pH 6.8. The pH of the buffer was checked using a calibrated pH meter and adjusted if found necessary.

4.3.3.4. Preparation of calibration curves in different media

\textit{i) 6-gingerol:} 6-gingerol (50 mg) was weighed accurately and was dissolved in minimum amount of medium and the volume was made to 100 ml in a volumetric flask. This gives us a master stock solution with a concentration of 500 \( \mu \)g/ml which were further diluted to 200, 100, 50, 10, 5 and 1 \( \mu \)g/ml with the respective physiological media. The chromatogram of all concentrations were measured at 245 nm (n=3) and regression
coefficient was calculated to establish linearity. Sharp and identical peaks of 6-gingerol was separated at 16.3 min (Gupta., 2010).

ii) Psoralen: Psoralen (10 mg) was weighed accurately and was dissolved in minimum amount of medium and the volume was made to 100 ml in a volumetric flask. This gives us a master stock solution with a concentration of 100 µg/ml which were further diluted to 50, 20, 10, 5, 1 and 0.1 µg/ml with the respective physiological media. The chromatogram of all concentrations were measured at 245 nm (n=3) and regression coefficient was calculated to establish linearity. Sharp and identical peaks of 6-gingerol was separated at 14.7 min.

To determine the *in vitro* and *ex vivo* release of psoralen and 6-gingerol from the nanoemulsion gels, phosphate buffer (pH 7.4) and phosphate buffer saline (PBS) (pH 7.4) were prepared.

4.3.3.5. Phosphate buffer (pH 7.4) with 20% *v/v* methanol

To determine the *in vitro* release of 6-gingerol and psoralen from the nanoemulsion gels, a calibration plot of 6-gingerol and psoralen were obtained in phosphate buffer pH 7.4 with 20% *v/v* methanol which was used as the dissolution medium.

a) 6-gingerol:

50 mg of 6-gingerol was dissolved in 20 ml of methanol and volume was made to 100 ml by adding PB (pH7.4) to form stock solution of 500 µg/ml. For HPLC analysis, a series of 6-gingerol solutions (1–500 µg/ml) was prepared from the stock solution by dilution with methanol: PB (20:80) vehicle. A calibration curve was plotted between concentration (µg/ml) and peak area.

b) Psoralen:

10 mg of psoralen was dissolved in 20 ml of methanol and volume was made to 100 ml by adding PB (pH7.4) to form stock solution of 100 µg/ml. For HPLC analysis, a series of psoralen solutions (0.1–100 µg/ml) was prepared from the stock solution by dilution with methanol: PB (20:80) vehicle. A calibration curve was plotted between concentration (µg/ml) and peak area.
4.3.3.6. Phosphate buffered saline (PBS) (pH 7.4) with 20% v/v methanol

To determine 6-gingerol and psoralen during *ex vivo* skin permeation studies of the nanoemulsion gels, a calibration plot of psoralen and 6-gingerol were obtained in phosphate buffered saline (PBS) (pH 7.4) containing 20% v/v methanol which was used as the receptor phase. The calibration curve was plotted using the concentration range of 0.1 - 50 \( \mu \text{g mL}^{-1} \) for psoralen and 1 - 200 \( \mu \text{g mL}^{-1} \) for 6-gingerol. A calibration curve was constructed by plotting peak area vs. concentration of standard solution and the regression equation was determined. The experiment was carried out in triplicate.

4.3.3.7. HPLC method validation:

i) Linearity: Various concentrations from 0.01 to 500 \( \mu \text{g/ml} \) for psoralen and 0.1 to 500 \( \mu \text{g/ml} \) for 6-gingerol were prepared and areas under peak were calculated. The graph was plotted between concentration and area under peak for linearity.

ii) Accuracy as recovery: Accuracy was determined by standard addition method. The preanalyzed samples of psoralen (10 \( \mu \text{g/ml} \)) were spiked with the extra 0, 80, 100 and 120 % of the standard psoralen and the mixtures were analyzed by the proposed method. The experiment was performed in triplicate. The % recovery of samples, % RSD and standard error of mean (SEM) were calculated at each concentration level.

iii) Precision: Precision was considered at two levels of ICH suggestions i.e., repeatability and intermediate precision. Repeatability of sample application was determined as intraday variation whereas intermediate precision was determined by carrying out inter-day variation for the determination of psoralen and 6-gingerol at three different concentration levels of 100, 200 and 300 \( \mu \text{g/ml} \) in triplicates.

iv) Reproducibility: Reproducibility of the method was investigated by obtaining precision on a different instrument, which was analyzed by another person in different laboratory. Both intraday and interday precision was calculated at three different concentration level i.e., 100 \( \mu \text{g/ml} \), 200 \( \mu \text{g/ml} \) and 300 \( \mu \text{g/ml} \). This was done in triplicates.
v) Detection (LOD) and quantification (LOQ) limits: LOD and LOQ were determined by standard deviation ($S_{yn}$) method. For the determination of LOD and LOQ, blank samples were injected in triplicate to the chromatograph, and then peak area of this blank was calculated. The LOD and LOQ were determined using the slope of the calibration curve and $S_{yn}$ of the blank sample by following formulae:

$$\text{LOD} = 3.3 \times \frac{S_{yn}}{S}$$

$$\text{LOQ} = 10 \times \frac{S_{yn}}{S}$$

Where $S_{yn}$ is the standard deviation of the blank response and $S$ is the slope of the calibration curve.

vi) Robustness: Robustness was carried out to evaluate the influence of small but deliberate variation in the chromatographic conditions for the determination of psoralen and 6-gingerol. Robustness of the method was determined by changing the flow rate (0.8 & 1.2 ml/min) of mobile phase.

4.3.3.8. Preparation of forced degradation samples (Hamrapurkar et al., 2011)

In order to establish the stability of the analytical methods, forced degradation studies of psoralen and 6-gingerol were performed. It was focused towards a suitable method of quantification of active compounds during accelerated stability studies of the sustained release tablets and nanoemulsion gel. Simultaneous estimation method by HPLC was tried for the development of a stability indicating assay method.

**Acid induced degradation:** Acid-induced forced degradation was performed by adding 10 mL stock solution (2 mg mL$^{-1}$) of psoralen and 6-gingerol in combination (1:1) to 10 mL each of methanol and 0.1 M HCl, and refluxing the mixture at 60°C for approximately six hours. The solution was then left to reach ambient temperature, neutralized to pH 7 by addition of 0.1 M NaOH, and then diluted to 100 mL, with methanol, so as to get a concentration of 100 μg mL$^{-1}$ each.

**Base induced degradation:** Base-induced forced degradation was performed by adding 10 mL stock solution (2 mg mL$^{-1}$) of psoralen and 6-gingerol in combination (1:1) to 10 mL each of methanol and 0.1 M NaOH and refluxing the mixture at 60°C for approximately six hours. The solution was then left to reach ambient temperature,
neutralized to pH 7 by addition of 0.1 M HCl, and then diluted to 100 mL with methanol, so as to get a concentration of 100 µg mL\(^{-1}\) each.

**Oxidative degradation:** To study the effect of the oxidizing conditions, 10 mL stock solution (1 mg mL\(^{-1}\)) of psoralen and 6-gingerol in combination (1:1) was added to 10 mL of 30% H\(_2\)O\(_2\) solution and the mixture was refluxed at 60°C for approximately six hours. The solution was left to reach ambient temperature and then diluted to 100 mL with methanol, so as to get a concentration of 100 µg mL\(^{-1}\) each.

**Thermal degradation:** To study the effect of temperature, accurately weighed about 100 mg of psoralen and 6-gingerol in combination (1:1) was stored at 80°C for 48 hours. Then it was dissolved in methanol and the volume was adjusted to 50 mL to give a solution of final concentration equivalent to 1000 µg mL\(^{-1}\) of psoralen and 6-gingerol each.

**Photolytic degradation:** To study the effect of UV light, accurately weighed about 100 mg of psoralen and 6-gingerol in combination (1:1) was exposed to short and long wavelength UV light (254 and 366 nm, respectively) for 48 h. Then it was dissolved in methanol and the volume was adjusted to 50 mL to give a solution of final concentration equivalent to 1000 µg mL\(^{-1}\) of psoralen and 6-gingerol each.

Availability of a suitable HPLC method for the estimation of psoralen and 6-gingerol will always be advantageous as the method is simple and rapid. Till date no studies have been reported as a stability indicating assay method for the estimation of psoralen and 6-gingerol.

The following sections explain in detail the methods employed for the successful formulation development and preparation of psoralen with 6-gingerol for the treatment of vitiligo.

### 4.3.4. Development and Evaluation of Sustained Release Tablets

#### 4.3.4.1. Drug-Excipient compatibility studies:

Drugs were weighed accurately and mixed with the selected excipients in the ratio of 1:1. Seven sets of each physical mixture and control were prepared and placed in glass vials which were then hermetically sealed stored at 25°C, 4°C and 40°C/75% RH for 4 weeks (Banker, 1992) after which the vials were opened and observed for caking, liquefaction, discoloration and odor or gas formation.
4.3.4.2. Dose Determination Studies:

The anti-emetic activity of 6-gingerol was investigated against cisplatin-induced delay in gastric emptying in Wistar albino rats. The protocol for animal studies was approved (Proposal No. 758, dated 23.07.10) by the Institutional Animal Ethics Committee (IAEC) of Jamia Hamdard, New Delhi (173/CPCSEA, 28th Jan 2000).

Species: Adult Wistar Albino Rats

Weight: 150-200 g

Gender: Either sex

Table 4.4: Treatment plan for the pharmacodynamic evaluation of 6-gingerol in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Treatment</th>
<th>Dose of 6-gingerol/cisplatin (mg kg⁻¹ body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>Control (cisplatin)</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>Standard (ondansetron) + cisplatin</td>
<td>3 + 10</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>6-gingerol + cisplatin</td>
<td>2 + 10</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>6-gingerol + cisplatin</td>
<td>4 + 10</td>
</tr>
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<td>5</td>
<td>5</td>
<td>6-gingerol + cisplatin</td>
<td>8 + 10</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>6-gingerol + cisplatin</td>
<td>12 + 10</td>
</tr>
</tbody>
</table>

Procedure of the study

The animals were kept under standard laboratory conditions of temperature at 25 ± 2°C and relative humidity of 55 ± 5%. The animals were housed in polypropylene cages, five animals per cage with free access to standard laboratory diet (Lipton feed, Mumbai, India; providing 3630 kcal gm⁻¹ energy and containing 22.10% crude protein, 4.10% crude oil, 4.05% crude fiber, 10.05% ash, 0.75% sand silica) and water *ad libitum*. The animals were deprived of food for 24 h prior to the experiment but allowed free access to water until 2 h before the experiment. A dose of cisplatin (10 mg/kg, i.p.), 30 min before meal administration produces a significant decrease in gastric emptying in rats i.e. 28.31 ± 91.65% compared to control 80.21 ± 92.12% (Sharma and Gupta, 1998). 6-gingerol were investigated for their effect on delay in gastric emptying induced by cisplatin 10 mg/kg, i.p. 6-gingerol was administered orally (p.o.) through intragastric tube 30 min before cisplatin administration. Four doses of 6-gingerol 2, 4, 8 and 12 mg/kg were tested. In each group 5 rats were used. The effects of 6-gingerol were compared with that of ondansetron, administered in the dose of 3 mg/kg, p.o., 30 min before cisplatin administration.
administration. After drug treatments, rats were administered test meal; 30 min after the test meal administration, rats were sacrificed for the measurement of gastric emptying.

Preparation of drugs:
6-gingerol were suspended in 1% gum acacia. Cisplatin was dissolved in normal saline at 50°C and cooled to 37°C before administration. Ondansetron was dissolved in saline.

Measurement of gastric emptying:
Gastric emptying of non-nutrient solution was determined according to the method described previously (Scarpignato et al., 1980). Briefly, a test meal (0.05% phenol red in a 1.5% aqueous methyl cellulose solution) in a volume of 1.5 ml/rat was given by gastric tube. Thirty minutes after the meal administration, the stomach was removed and homogenized in 100 ml of 0.1 N sodium hydroxide. Proteins (in 5 ml of homogenate) were precipitated with 0.5 ml of trichloro acetic acid (20% w/v), centrifuged and separated out. The supernatant was mixed with 4 ml of 0.5 N sodium hydroxide and the absorbance of the sample was read at wavelength of 560 nm. Phenol red recovered from stomach of rats sacrificed immediately after administration of methyl cellulose meal served as standard stomach. The percentage gastric emptying was calculated from the following formula: Gastric emptying (%) =1-[(concentration of phenol red in test stomach)/ (concentration of phenol red in standard stomach)] X100.

4.3.4.3. Dose calculation:
Human dose was calculated taking into consideration ratio of surface area of a rat to that of a human being. The dose to be given to human on the basis of surface area ratio was determined by dividing the dose of 200 g rat by a factor of 0.018 (Ghosh, 2005; Freireich, et al., 1996).
- 8 mg/kg is the determined dose of 6-gingerol for rat.
- Therefore, for 200 g rat, 6-gingerol dose will be 1.6 mg.
- 6-gingerol dose for humans was calculated to be 1.6/0.018 =88.88 (90) mg

Dose of psoralen was determined by dividing the dose (30 mg) of 200 mg rat by a factor of 0.018.

4.3.4.4. Selection of punches and dyes: The dies and punches were of standard round concave designed with dimensions of 10 mm in diameter.
fabricated in such a way to satisfy patient acceptability; accuracy and uniformity of drug content and reproducibility.

4.3.4.5. Preparation of SR tablets using dry granulation method (study 1):

Table 4.5: Psoralen with 6-gingerol SR Tablets (study 1)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Ingredients</th>
<th>Required quantity (mg/tablet)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T1</td>
</tr>
<tr>
<td>1</td>
<td>Psoralen</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>6-gingerol</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>Microcrystalline cellulose</td>
<td>54</td>
</tr>
<tr>
<td>4</td>
<td>Carbopol 934-P</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>Aerosil</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>Magnesium stearate</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Sustained release tablets were prepared by direct compression method using the ingredients given in the formula (Table 4.5).

A. Ingredients 1-4 were accurately weighed, mixed and passed through sieve # 40.

B. Mixed, A uniformly using poly bag.

C. Ingredients 5 and 6 were accurately weighed and passed through sieve # 60.

D. Mixed ingredient 5 uniformly with B.

E. Lubricated D with ingredient 6 using poly bag thoroughly.

F. Prepared blend (E) was processed for compression after blend analysis using 10 mm biconcave standard round punches and dies in single-punch tablet compression machine (Cadmach, Ahmedabad, India). Each tablet was punched with the total weight of 200 mg.

4.3.4.6. Preparation of SR tablets using wet granulation method (study 2):

Sustained release matrix tablets were prepared by wet granulation technique method using the ingredients given in the formula (Table: 4.6).

A. Ingredients 1, 3 and 4 were accurately weighed and passed through sieve # 40.

B. Mixed, A uniformly using poly bag.

C. Solublized ingredient 2 completely in required amount of ethanol using a mechanical stirrer.
D. Blended B with C thoroughly and were air dried at 30°C for 30 mins. Obtained granules were passed through sieve # 16.

Table 4.6: Psoralen with 6-gingerol SR Tablets (study 2)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Ingredients</th>
<th>Required quantity (mg/tablet)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T5</td>
</tr>
<tr>
<td>1</td>
<td>Psoralen</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>6-gingerol</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>Microcrystalline cellulose</td>
<td>54</td>
</tr>
<tr>
<td>4</td>
<td>Carbopol 934-P</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>Aerosil</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>Magnesium stearate</td>
<td>0.5</td>
</tr>
</tbody>
</table>

E. Air dried D further until the moisture content reached between 1 - 1.5%.

F. Passed ingredient 5 through sieve # 60 and were mixed homogenously with (E).

G. Lubricated ingredient 6 (60 mesh passed) with F using poly bag thoroughly.

H. Prepared blend (G) was processed for compression after blend analysis using 9 mm biconcave standard round punches and dies in single-punch tablet compression machine (Cadmach, Ah-medabad, India). Each tablet was punched with the total weight of 200 mg.

4.3.4.7. Preparation of SR tablets using wet granulation method (study 3):

Table 4.7: Psoralen with 6-gingerol SR Tablets (study 3)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Ingredients</th>
<th>Required quantity (mg/tablet)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T9</td>
</tr>
<tr>
<td>1</td>
<td>Psoralen</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>6-gingerol</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>Microcrystalline cellulose</td>
<td>46</td>
</tr>
<tr>
<td>4</td>
<td>Sodium Starch Glycolate</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>Carbopol 934-P</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>Aerosil</td>
<td>0.5</td>
</tr>
<tr>
<td>7</td>
<td>Magnesium stearate</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Sustained release tablets were prepared by wet granulation technique method using the ingredients given in the formula (Table: 4.7).

A. Ingredients 1, 3, 4 and 5 were accurately weighed and passed through sieve # 40.
B. Mixed, A uniformly using poly bag.
C. Solublized ingredient 2 completely in required amount of ethanol using a mechanical stirrer.
D. Blended B with C thoroughly and were air dried at 30°C for 30 mins. The obtained granules were passed through sieve # 16.
E. Air dried D further until the moisture content reached between 1–1.5%.
F. Passed ingredient 6 through sieve # 60 and mixed homogenously with (E).
G. Lubricated ingredient 7 (60 mesh passed) with F using poly bag thoroughly.
H. Prepared blend (G) was processed for compression after blend analysis using 9 mm biconcave standard round punches and dies in single-punch tablet compression machine (Cadmach, Ah-medabad, India). Each tablet was punched with the total weight of 200 mg.

4.3.4.8. Physical parameters of compressed tablets
i) Weight variation: Twenty tablets were weighed individually and average weight was calculated, and the individual weight was compared with average weight. The difference in weight was determined and % variation was calculated.

ii) Dimensional variation: Thickness of the tablets (n=20) were measured with a calibrated vernier caliper.

iii) Hardness test: Hardness of tablets (n=10) was measured by Pfizer type hardness tester. Tablet was placed between the jaw of the tester and pressure was applied until it broke. Hardness was noted from the dial.

iv) Friability test: Since the weight of the tablet was less than 650 mg, a sample of tablets corresponding to as near as possible to 6.5 g was taken for friability measurement in a Roche’s friabilator. Tablets were accurately weighed and were placed in the drum which was made to rotate at 25 RPM for 4 min. The tablets were removed, de-dusted and were weighed again and the percentage friability was calculated.

4.3.4.9. Assay: 6-gingerol and psoralen content were determined by weighing the powder of crushed tablets equivalent to the total weight of the tablet. The samples were
dispersed in 10 ml of methanol by vortex mixing followed by centrifugation at 10,000 rpm for 5 min on a Minispin, Eppendorf (PCI, Mumbai, India) centrifuge. The supernatant was filtered through 0.45 μm Millipore membrane filter, and the filtered solutions were suitably diluted and analyzed at 245 nm using HPLC.

4.3.4.10. Dissolution studies: Dissolution study is an approach to evaluate drug release characteristics of a product (tablets/capsules) in vitro.

- Method: USP II method is said to be the most suitable method for tablet dosage form analysis in vitro.
- RPM: 50

Table 4.8: Dissolution Medium for SR Tablets

<table>
<thead>
<tr>
<th>Medium</th>
<th>pH</th>
<th>Time point (h)</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 N HCl</td>
<td>1.2</td>
<td>0-2</td>
<td>900</td>
</tr>
<tr>
<td>Acetate Buffer</td>
<td>4.5</td>
<td>2-4</td>
<td>900</td>
</tr>
<tr>
<td>Phosphate Buffer</td>
<td>6.8</td>
<td>4-24</td>
<td>900</td>
</tr>
</tbody>
</table>

- Sampling: 5 ml
- Replacement of medium: Yes
- Method of analysis: HPLC
- Wavelength: 245 nm
- Temperature: 37°C
- Sampling Time points: 1, 2, 4, 6, 8, 12, 16, 20 and 24 h. The selection of the sampling time point was based on the time by which complete drug release.

The in vitro analysis of all the test batches was carried out with the above conditions for the potential batches depicting satisfactory release.

4.3.4.11. Determination of mechanism of in vitro drug release: To describe the kinetics of the psoralen and 6-gingerol release from the formulations, mathematical models namely zero-order, first-order, Higuchi’s model, Hixson-Crowell cube root law and Peppas-Korsemeyer equation were used. The data obtained from the in vitro drug release studies were plotted in various kinetic models: Zero order (Eqn. 4.6) as cumulative percentage drug released versus time and describes the systems where the drug release rate is independent of its concentration. The First order (Eqn. 4.7) describes
the release from system where release rate is concentration dependent (Bourne, 2002) and plotted as log cumulative percentage of drug remaining versus time, and Higuchi’s model (Eqn. 4.8) as cumulative percentage of drug released versus square root of time (Merchant et al., 2006) and described the release of drugs from insoluble matrix as a square root of time dependent process based on Fickian diffusion. The Hixson-Crowell cube root law (Eqn. 4.9) describes the release from systems where there is a change in surface area and diameter of particles or tablets (Hixson and Crowell, 1931). Korsmeyer et al (1983) derived a simple relationship which described drug release from a polymeric system (Eqn. 4.10) and plotted as log cumulative % drug release vs. log time. The criterion for selecting the most appropriate model for the release kinetics of the SR tablets was the goodness-of-fit test using correlation coefficient.

\[ C = K_0 t \]  (4.6)

where \( C \) is the concentration of drug, \( K_0 \) is the zero-order rate constant expressed in units of concentration/time and \( t \) is the time in hours. The slope of a graph of concentration versus time would yield a straight line with a slope equal to \( K_0 \).

\[ \log C = \log C_0 - k t / 2.303 \]  (4.7)

where \( C_0 \) is the initial concentration of drug, \( k \) is the first order constant, and \( t \) is the time.

\[ Q = K t^{1/2} \]  (4.8)

where \( K \) is the constant reflecting the design variables of the system and \( t \) is the time in hours. \( Q \) is the drug release.

\[ Q_0^{1/3} - Q_t^{1/3} = K_{HC} t \]  (4.9)

Where, \( Q_t \) is the amount of drug released in time \( t \), \( Q_0 \) is the initial amount of the drug in tablet and \( K_{HC} \) is the rate constant for Hixson-Crowell rate equation.

\[ M_t / M_w = K t^n \]  (4.10)

Where \( M_t / M_w \) is fraction of drug released at time \( t \), \( k \) is the rate constant and \( n \) is the release exponent.

4.3.4.12. Accelerated stability studies of SR Tablets

The accelerated stability study was carried out according to both International Conference on Harmonisation (ICH) guidelines and Arrhenius method.
i) Accelerated stability testing according to ICH guidelines

i.i) Controlled temperature and humidity condition

The accelerated stability study under controlled temperature and humidity conditions was carried out according to International Conference on Harmonisation (ICH) Q1A(R2) guidelines. The sustained release formulation was proposed to be stored at room temperature. The stability studies were carried out to determine the effect of the presence of formulation additives on the stability of drug and also to determine the physical stability of the prepared formulation under conditions of storage temperature and relative humidity. Three batches of the optimized formulations were prepared and filled into amber coloured bottles. The sealed bottles were packed in cartons. The packed tablets were placed in stability chamber maintained at 40±2°C and 75±5% RH. The cartons containing the tablets were placed in a humidity chamber maintained at a relative humidity of 75%. The humidity chambers were created by placing a beaker with saturated aqueous sodium chloride solution in desiccators (Kopleman et al., 2001; Uhumwangho and Okor, 2005). The humidity chambers were placed in a hot air oven maintained at 40°C. The samples were withdrawn from the humidity chambers at time intervals of 0, 1, 2, 3 and 6 months. The tablets were evaluated for in vitro drug release and drug content. Different humidity chambers were used for the different sampling intervals such that the samples were not in contact with the external environment during the storage interval in the chambers (Uhumwangho and Okor, 2005). Zero time samples were used as controls. The shelf life of the SR tablets, based on change in percentage drug content, was determined using SigmaPlot® 12 (Cranes Software International Limited, Bangalore, India) software. Percentage label claim (% drug remaining) was plotted against time in months to determine the shelf life using SigmaPlot® 12 software. The shelf life was determined as the time at which the 95% one-sided confidence limit for the mean curve intersects the acceptance criterion of 90% of drug content (percentage label claim).

i.ii) Photostability testing

The accelerated photostability study was carried out according to International Conference on Harmonisation (ICH) Q1B guidelines (ICH, 1996). Three batches of the optimized SR tablets were prepared and filled into amber coloured bottles. The sealed
bottles were packed in cartons. Photostability was evaluated by exposing the packed samples of optimized SR tablets to 200 watt hours/square meter in the UV range of 320 to 400 nm and 1.2 million lux hours in the VIS range (400-700 nm) using a cool white fluorescent lamp and a near UV fluorescent lamp respectively. The samples were evaluated for % drug content (assay). Samples were exposed side-by-side with a validated quinine actinometric system to ensure the specified light exposure is obtained. Aqueous solution of quinine monohydrochloride dihydrate (2% w/v) was prepared and filled in a 1 cm quartz cell and was used as the sample. Separately filled another 1 cm quartz cell, wrapped in aluminum foil to protect completely from light, and was used this as the control. The sample and control quinine solutions were exposed to the light sources during the photostability testing along with the tablets. After exposure the absorbances of the sample (AT) and the control (Ao) at 400 nm were determined (Pradhan et al., 2011). The change in absorbance, ΔA = AT - Ao was calculated. The length of exposure was confirmed to be sufficient to ensure a change in absorbance as indicated by its value of 0.58 (a value greater than 0.5).

iiii) Accelerated stability study of SR tablets by conventional method using Arrhenius equation

For the determination of shelf life by conventional method, the optimized SR tablets were stored at 30 ± 2 °C; 40 ± 2 °C and 50 ± 2 °C for three months. Samples were withdrawn after predetermined time intervals (0, 30, 60, and 90 days) and the percentage drug content was determined using the developed and validated stability indicating HPLC method as described in section 4.3.3. Zero time samples were used as controls. Logarithm of percent drug remaining versus time (in days) was plotted. The degradation rate constant ‘k’ was determined from the slope of the plots at each elevated temperature using the following equation (Eqn. 4.11).

\[
\text{Slope} = \frac{-k}{2.303}
\]

(4.11)

Plot of the logarithm of k values at various elevated temperatures against the reciprocal of absolute temperature was drawn (Arrhenius plot). From the plot, k value at 25 °C was determined and was used to calculate shelf life by substituting in the following equation (Eqn. 4.12).

\[
t_{0.9} = \frac{0.1052}{k_{25}}
\]

(4.12)
Where, $t_{0.5}$ is the time required for 10% degradation of the drug and is referred to as shelf life.

4.3.4.13. *In vivo* study for estimating the pharmacokinetic parameters in optimized SR Tablets:

i) Protocol of study: Animal used in study: Rats

* Species: Albino Wistar
* Weight: 200 – 250 g
* Gender: Either sex
* Numbers of animals in each group: Three

ii) Dose calculation for rats
This was done as described in section 4.3.4.3.

iii) Procedure of the study
The animals were kept under standard laboratory conditions, temperature at 25 ± 2°C and relative humidity of 55 ± 5%. The animals were housed in polypropylene cages, three per cage with free access to standard laboratory diet (Lipton feed, Mumbai, India; providing 3630 kcal/g energy and containing 22.10% crude protein, 4.10% crude oil, 4.05% crude fiber, 10.05% ash, 0.75% sand silica) and water *ad libitum*. Average weight of tablets providing 30 mg dose of psoralen was found to be 200 mg. So, for a 200 mg rat requiring 0.54 mg of drug, the required weight of tablet to be used was 3.6 mg. Hence, SR Tablet was crushed, mixed and powder equivalent to dose of drug was taken and administered to animal for *in vivo* study. Further after ingestion of tablet, study was made for analysis of both drugs.

a) Psoralen:
The rats were anaesthetized using diethyl ether and blood samples were withdrawn from the tail vein at 0 (pre-dose), 1, 2, 4, 6, 8, 10, 12 and 24th h in microcentrifuge tubes in which 8 mg of EDTA was added as an anticoagulant. The collected blood was mixed...
properly with the anticoagulant and centrifuged at 5000 rpm for 20 min. The plasma was separated and stored at -21 °C until drug analysis was carried out using HPLC. A simple, specific and sensitive HPLC method was developed and validated for the determination of psoralen in rat plasma (Wang and Jiang, 2006). Separation was carried out on a reverse-phase C18 column (250×4.6mm, 5μm particle size), using a mixture of acetonitrile and water in the ratio of 30:70 (% v/v) as mobile phase. The chromatographic analysis was performed at ambient temperature at a flow rate of 1 ml/min and the UV detector wavelength was set at 245 nm.

b) 6 gingerol:
The rats were anaesthetized using diethyl ether and blood samples were withdrawn from the tail vein at 0 (pre-dose), 1, 2, 4, 6, 8, 10, 12 and 24th h in microcentrifuge tubes in which 8 mg of EDTA was added as an anticoagulant. The collected blood was mixed properly with the anticoagulant and centrifuged at 5000 rpm for 20 min. The plasma was separated and stored at -21 °C until drug analysis was carried out using HPLC. Reverse-phase HPLC was used to quantify 6 gingerol in plasma. Chromatographic separation was accomplished with the use of a reverse-phase C18 column (250×4.6mm, 5μm particle size). The mobile phase was acetonitrile and water 45:55, v/v with a flow-rate of 1.0 mL/min. The eluate from the HPLC column was monitored by the spectrophotometric detector at 280 nm. The injection volume was 20 μL. 6 gingerol was identified based on its retention time compared with the reference standard (Suzhanna et al., 2008).

The pharmacokinetic parameters AUC, T_{max}, C_{max}, and K_{e} were calculated using Win Lin software (Cole-Parmer Instrument Co., Chicago, IL, USA). All the parameters were calculated individually for each animal and the values expressed as mean ± S.D.

4.3.4.14. Validation of bio analytical method:

i) Precision and Accuracy: Precision was considered at two levels of ICH suggestions i.e., repeatability and intermediate precision. Repeatability of sample application was determined as intraday variation whereas intermediate precision was determined by
carrying out inter-day variation for the determination of psoralen and 6-gingerol at three
different concentration levels of 3, 10 and 17 ng/mL in triplicates. Accuracy was
determined by standard addition method. The blank plasma samples were spiked with the
concentration of 3, 10 and 17 ng/mL of the standard drug solutions and the mixtures were
analyzed by the proposed method. The experiment was performed in triplicate. The %
recovery of samples, % RSD and standard error of mean (SEM) were calculated at each
concentration level.

**ii. Extraction recovery:** The recovery of 6-gingerol and psoralen was determined at the
three concentrations 3, 10 and 17 ng/mL. Three replicates of each sample were treated as
mentioned in the sample preparation previously and injected into HPLC system. The
extraction recovery was calculated by the following equation:

\[ \text{Recovery} = \left( \frac{\text{peak area after extraction}}{\text{peak area after direct injection}} \right) \times 100 \]

4.3.4.15. Pharmacokinetic and statistical analysis

Pharmacokinetic parameters were calculated using PK Functions for Microsoft Excel
software (designed by Joel I. Usanky, Atul Desai and Diane Tang-Liu, Allergan, CA,
U.S.A.). Following pharmacokinetic functions were calculated using Microsoft Excel
worksheets: \( C_{\text{max}}, t_{\text{max}}, \text{AUC}_{0\rightarrow24h}, \) and \( \text{AUMC}_{0\rightarrow24h} \). Mean residence time is the average
amount of time a drug remains in a compartment or system and was computed as
\( \text{MRT}_{0\rightarrow24h} = \frac{\text{AUMC}_{0\rightarrow24h}}{\text{AUC}_{0\rightarrow24h}} \). The pharmacokinetic data among different
formulations were compared for statistical significance by one way analysis of variance
(ANOVA) followed by Tukey-Kramer multiple comparison test using GraphPad Instat
software (GraphPad Software Inc., CA, USA).

4.3.4.16. In vitro-in vivo correlation (IVIVC): The in vitro — in vivo correlation
(IVIVC) for a pharmaceutical product is a mathematical relationship between an in vitro
property of the product and its in vivo performance. The in vitro release data of the active
substance normally serve as characteristic of the in vitro property, while the in vivo
performance is represented by the time course of the plasma concentration of the active
substance. These data are then treated mathematically to determine whether a correlation
exists; a correlation can usually be expected when drug release from the product is the
step governing the subsequent absorption kinetics. This is an essential design element for a modified-release dosage form like the one in the present work. For oral dosage forms, the in vitro drug release is routinely measured and characterized as dissolution rate. The relationship between the in vitro and in vivo characteristics is expressed mathematically by a linear or nonlinear correlation. However, the plasma concentration profiles cannot be related directly to the in vitro release rate; they have to be converted first to the underlying in vivo release or absorption data. The latter is usually accomplished mathematically by using the deconvolution/convolution method (Sangalli et al., 2001; Emami, 2006; Rettig and Mysicka, 2008). In vitro drug release profiles of optimized formulation were compared with the in vivo profile by plotting the percentage drug released in vivo against the percentage drug released in vitro to establish correlation.

4.3.5. Development and Evaluation of Nanoemulsion Gel

4.3.5.1. Dose determination of 6-gingerol against psoralen induced irritation:

Skin irritation tests were conducted in albino Wistar rats to determine the effect of 6-gingerol against redness of the skin induced after application of psoralen. Fifteen rats of either sex (weighing 200–250 g) with removal of hair on the back 24 h prior to administration were used in this experiment. 1 mL (0.2 mg) of prepared 0.02% methanolic solution of psoralen was applied uniformly on the dorsal skin regions to each rat and they were randomly divided into five groups (3X5) as control, 1 mL (0.2 mg), 2 mL (0.4 mg), 3 mL (0.6 mg) and 4 mL (0.8 mg) of 0.02% of 6-gingerol solutions respectively. These preparations were applied on the same targeted site and were exposed under sun for 20 min. Administrated sites were assessed for signs of skin irritation (erythema and edema), and this test procedure was repeated for another 3 days. After withdrawal, observation was continued for 3 days. The irritation scores of the test area were obtained by judging the extent of erythema and edema according to the criteria proposed (Paolino et al., 2002). Erythema and edema were graded as follows: 0 for no visible reaction, 1 for just present reaction, 2 for slight reaction, 3 for moderate reaction, and 4 for severe reaction. Eventually, the total scores for irritation test in each condition were calculated using the following equation.

\[
\text{Average irritation scores} = \frac{\text{Erythema reaction scores} + \text{Dropsy reaction scores}}{\text{Amount of animals}}
\]
4.3.5.2. Construction of pseudo ternary phase diagrams: Solubility studies of the drugs were performed in various oils like olive oil, jojoba oil, castor oil, carbitol, lauroglycol FCC, lauroglycol 90, peccol, labrafil M and Maisine 35. On the basis of solubility studies Lauroglycol 90 was selected as the oily phase. Surfactant (tween 80) and cosurfactant (ethanol) were selected in different volume ratios (5:1, 4:1, 3:1, 2:1, 1:1 and 1:2). Oil phase and surfactant mix \( S_{\text{mix}} \) prepared were mixed well in different ratios ranging from 1:9 to 9:1. Existence zone of nanoemulsion was determined using water-titration method by visual observations. All ratios were made so that maximum ratios were covered in order to define the boundaries of phases specifically formed in each phase diagram. Pseudo ternary phase diagrams were developed using aqueous titration method by adding drop by drop external aqueous phase under gentle vortexing to each oil and \( S_{\text{mix}} \) ratio (spontaneous emulsification technique). Visual observation was carried out for transparency and easily flowability of o/w nanoemulsions. The phase conversion of nanoemulsion from w/o to o/w was checked on the basis of conductance measurement. The physical state of the nanoemulsion was marked on a pseudo-three-component phase diagram with one diagonal of triangle representing aqueous phase, the other one representing the oily phase and the third representing a mixture of surfactant and cosurfactants \( S_{\text{mix}} \) ratio. The nanoemulsion area in the each phase diagram was plotted and the wider region indicated the better self nanoemulsifying efficiency.

4.3.5.3. Thermodynamic stability studies: Various thermodynamic stability tests like centrifugation study, heating cooling cycle and freeze-thaw cycle were performed as described below.

4.3.5.4. Centrifugation study: The prepared formulations were centrifuged (REMI International, Mumbai, India) at 5000 rpm for 30 min. and observed for phase separation, creaming or cracking. The formulations which did not show any instability
(creaming, cracking, and/or phase separation) were selected and subjected to heating-cooling cycle (Shafiq et al., 2007).

4.3.5.5. Heating cooling cycle: It is used to observe the stress effect of variations in temperature on the stability of nanoemulsion. Six cycles between refrigerator temperature (4 °C) and 40 °C with storage at each temperature for not less than 48 h were performed. The formulations that were found to be stable at these temperatures were subjected to Freeze thaw stress test (Shafiq et al., 2007).

4.3.5.6. Freeze thaw cycle (Accelerated ageing): This test was performed for the accelerated stability testing of the nanoemulsion formulations. In this study formulations were subjected to three freeze thaw cycles at the temperatures between -21 and +25 °C with storage at each temperature for not less than 48 h.

4.3.5.7. Refractive Index: Refractive index of selected formulations was determined using a refractometer. The results obtained were analyzed by one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test using GraphPad Instat software (GraphPad Software Inc., CA, USA).

4.3.5.8. Droplet size analysis (Particle size distribution): Droplet size of the prepared nanoemulsion was determined using Dynamic light scattering, which analyzed the fluctuations in the intensity of light scattering due to Brownian movement of the particles (Attwood et al., 1992). The formulation (0.1 mL) was dispersed in 50 mL (500 times dilution) of double distilled water and gently mixed by inverting the flask. Dynamic light scattering was performed at 25 °C, using He-Ne laser having wavelength 632.8 nm at an angle of 90° on a digital correlator (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, United Kingdom). The results obtained were analyzed by one way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test using GraphPad Instat software (GraphPad Software Inc., CA, USA).

4.3.5.9. Determination of zeta potential: The formulation (0.1 mL) was diluted 100 times using double distilled water and analysed using Zeta potential measuring instrument (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, United Kingdom).

4.3.5.10. Transmission electron microscopic (TEM) analysis: Transmission electron microscopic (TEM) analysis was done to determine the morphology of the dispersed
A drop of diluted (100 times) nanoemulsion was applied to a 300 mesh copper grid and was left for 1 min. After this the grid was kept inverted and a drop of phosphotungastic acid (PTA) was applied to the grid for 10 sec. Excess of PTA was removed by absorbing on a filter paper and the grid was analyzed using Morgagni 268D (FEI Company, OR, USA) operated at 60-80 KV at 1550X magnification.

4.3.5.11. Preparation of Nanoemulsion Gel: 1 g of psoralen and 3 g of 6-gingerol were weighed and sieved together through mesh # 40 and were used for nanoemulsion formulation by spontaneous emulsification method. Mixed drugs were dissolved in 16.5 % w/w of lauroglycol 90 (oil phase). Then 13 % w/w of Tween-80 and 26 % w/w of ethanol were added slowly in the oil phase and kept aside as drug solution. Nanoemulsions gel (NG1) was optimized by comparing physical parameters of the product by varying concentration of the polymer (i.e., 0.5, 0.75 and 1%). Three formulations were prepared simultaneously by dispersing required quantity (Table 4.9) of Carbopol-940, 0.2% w/w of methyl paraben and 0.02% w/w of propyl paraben in sufficient quantity of distilled water. After complete swelling of Carbopol-940 in distilled water. Prepared drug solution was added slowly to Carbopol-940 dispersion. Then remaining quantity of distilled water was added to get the final formulation 100% w/w.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>F4</th>
<th>NG1</th>
<th>NG2</th>
<th>NG3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psoralen (%w/w)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6-gingerol (% w/w)</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Carbopol-940 (%w/w)</td>
<td>-</td>
<td>0.5</td>
<td>0.75</td>
<td>1.0</td>
</tr>
<tr>
<td>Tween 80 (%w/w)</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Ethanol (%w/w)</td>
<td>26</td>
<td>26</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Lauroglycol 90 (%w/w)</td>
<td>16.5</td>
<td>16.5</td>
<td>16.5</td>
<td>16.5</td>
</tr>
<tr>
<td>Methyl paraben (%w/w)</td>
<td>-</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Propyl paraben (%w/w)</td>
<td>-</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Sandal DC (%w/w)</td>
<td>-</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Distilled water (%w/w) *</td>
<td>Qs</td>
<td>Qs</td>
<td>Qs</td>
<td>Qs</td>
</tr>
</tbody>
</table>

* Quantity sufficient to make up to 100 g

The 0.2 % w/w of triethanolamine (TEA) was added in the above mixture to neutralize Carbopol-940. Finally, 0.2% of sandal DC was mixed homogenously to add desired fragrance to the formulation. The composition of nanoemulsion (F4) and nanoemulsion gel (NG1, NG2 and NG3) are given in Table 4.9.
4.3.5.12. Determination of pH: The 2.5 g gel was accurately weighed and dispersed in 25 mL of purified water. The pH of the dispersion was measured using pH meter, which was calibrated before each use with buffered solution at 4.0, 7.0 and 9.0.

4.3.5.13. Drug content: The 1.0 g of each gel formulations was dissolved in methanol, filtered, and the volume was made to 100 mL. The resultant solution was suitably diluted with methanol and absorbance was measured at 245 nm. From this the drug content was determined using calibration curve for psoralen and 6 gingerol.

4.3.5.14. Viscosity determination: Viscosity of different formulations was determined using Brookfield viscometer with spindle no. 6 at 10 rpm at temperature 37±0.5°C.

4.3.5.15. Spreadability: The spreadability of the gel formulation was determined, by measuring diameter of 1 g gel between horizontal plates (20X20 cm²) after 1 min. The standardized weight tied on the upper plate was 125 g. The spreadability was calculated by using the following formula.

\[
\text{Spreadability} = \frac{\text{Weight} \times \text{Length}}{\text{Time}}
\]

4.3.5.16. Extrudability: Measuring the quantity of gel extruded from collapsible tube on application of constant weight. A closed collapsible tube containing 20 g of gel was pressed by applying constant load of 1 kg at the crimped end. When the cap was removed, gel extruded until pressure dissipated. The extruded gel was collected and weighed (Kamboj et al., 2012).

4.3.5.17. In vitro drug release studies

The paddle over disc apparatus (USP Apparatus 5; type II apparatus (Hanson Research SR8PLUS dissolution test station, Hanson Research Corporation, CA, USA), with the addition of a small stainless steel disk assembly designed for holding the gel at the bottom of the vessel) was used for studying the in vitro drug release from the nanoemulsion gels (Joshi and Patravale, 2008; USP, 2009). The temperature was maintained at 32±0.5°C (Arora and Mukherjee, 2002; Joshi and Patravale, 2008; USP, 2009). Phosphate buffer solution (pH 7.4) with 20% v/v methanol was used as the dissolution medium and 900 mL of this medium was placed in the dissolution vessel and maintained at 32±0.5°C (Arora and Mukherjee, 2002; Joshi and Patravale, 2008). One gram of the nanoemulsion gel was applied on the disk assembly, assuring that the release
surface was as flat as possible. The disk assembly was gently inserted at the bottom of the dissolution vessel. The vessel was covered during the test to minimize evaporation of the medium. The speed of rotation of the paddle was maintained 25 rpm (Joshi and Patravale, 2008). The drug was protected from light by use of amber colored dissolution vessels to prevent any possible photodegradation. Samples of 5 mL were withdrawn at predetermined time intervals of 0, 0.5, 1, 2, 3, 4, 5 and 6h and were replaced with same volume of fresh medium. The samples were analyzed using HPLC at 245 nm as given in section 4.3.3. All the experiments were performed in triplicate. The amount of drug release at different intervals was determined from the standard plot prepared in the dissolution medium. A graph of cumulative percentage drug release against time (h) was plotted. To describe the kinetics of the psoralen and 6-gingerol release from the formulations, mathematical models namely zero-order, first-order, Higuchi’s model, Hixson-Crowell cube root law and Peppas-Korsemeyer equation were used. The data obtained from the in vitro drug release studies were plotted in various kinetic models: Zero order (Eqn. 4.13) as cumulative percentage drug released versus time, first order (Eqn. 4.14) as log cumulative percentage of drug remaining versus time and Higuchi’s model (Eqn. 4.15) as cumulative percentage of drug released versus square root of time (Merchant et al., 2006). Korsmeyer et al (1983) derived a simple relationship which described drug release from a polymeric system (Eqn. 4.16) and plotted as log cumulative % drug release vs. log time. The criterion for selecting the most appropriate model for the release kinetics of the SR tablets was the goodness-of-fit test using correlation coefficient.

\[ C = K_0 t \] \hspace{1cm} (4.13)

where \( C \) is the concentration of drug, \( K_0 \) is the zero-order rate constant expressed in units of concentration/time and \( t \) is the time in hours. The slope of a graph of concentration versus time would yield a straight line with a slope equal to \( K_0 \).

\[ \log C = \log C_0 - k t / 2.303 \] \hspace{1cm} (4.14)

where \( C_0 \) is the initial concentration of drug, \( k \) is the first order constant, and \( t \) is the time.

\[ Q = K t^{1/2} \] \hspace{1cm} (4.15)

where \( K \) is the constant reflecting the design variables of the system and \( t \) is the time in hours. \( Q \) is the drug release.
\[
\frac{M_t}{M_\infty} = K t^n \hspace{1cm} (4.16)
\]

Where \( \frac{M_t}{M_\infty} \) is fraction of drug released at time \( t \), \( k \) is the rate constant and \( n \) is the release exponent.

4.3.5.18. Ex vivo skin permeation studies

Ex vivo skin permeation experiments were carried out using vertical Franz-type diffusion cells having a receptor compartment capacity of 11.0 mL. Phosphate buffered saline (PBS) (pH 7.4) containing 20% v/v methanol was used as the receptor phase. The area for diffusion was 2.404 cm\(^2\). The abdominal skin of rat was excised, the hair of which had been previously removed with an electric clipper. The subcutaneous tissues were surgically removed and dermis side was wiped with isopropyl alcohol to remove residual adhering fat. The skin was washed with phosphate buffer saline (PBS) (pH 7.4), wrapped in aluminium foil and stored in a deep freezer at -20°C till further use. The excised skin was equilibrated in the receptor phase for 1 h before being mounted on the diffusion cell.

On the donor side accurately weighed quantities of the nanoemulsion gels equivalent to 5 mg of psoralen and 15 mg of 6-gingerol was applied uniformly over the diffusion area. The receptor phase was maintained at 32±2°C by circulating water jacket and was stirred at 500 rpm using a magnetic stirrer (Remi instruments Ltd., Mumbai, India) (Arellano et al., 1996; Arora and Mukherjee, 2002; Joshi and Patravale, 2008). At predetermined time intervals of 0, 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 h, 0.5 mL samples were taken from the receiver compartment and replaced by the same volume of fresh receptor phase. All the experiments were performed in triplicate. The amount of drug released into the receptor phase from the nanoemulsion gels was determined by HPLC after suitable dilution at 245 nm as given in section 4.3.3. By determining the amount of drugs permeated at various time intervals, the cumulative amount of drug permeated (\( \mu \text{g} \cdot \text{cm}^{-2} \)) versus time (h) graph was plotted. The linearity of the steady state in the plot was checked using statistical testing of linear regression. After the linearity is confirmed, the mean flux (\( \mu \text{g} \cdot \text{cm}^{-2} \cdot \text{h}^{-1} \)) was determined by calculating the slope at the steady state of the plot (Joshi and Patravale, 2008). From the value of flux, mean permeability coefficient (cm h\(^{-1} \)) was calculated using the drug load (Arellano et al., 1996; Mutalik and Udupa 2003; Nokhodchi et al., 2007; Zhao and Singh, 1998).
4.3.5.19. Histopathological Studies
Approval to carry out these studies was obtained from the Animal Ethics Committee of Jamia Hamdard, New Delhi, India. Histopathological studies were carried out for elucidation of skin irritation potential of the investigated formulation. In this study abdominal skin of wistar rat was treated separately with the prepared nano emulsion gel. After 24 h, rat was sacrificed and the skin samples from treated and untreated (control) area were taken. Each specimen was stored in 10% formalin solution in phosphate buffer saline (pH 7.4). The specimen was cut into sections vertically. Each section was dehydrated using ethanol, embedded in paraffin for fixing, and stained with hematoxylin and eosin. These samples were then observed under light microscope (Motic, Japan) and compared with control sample.

4.3.5.20. Pharmacodynamic evaluation of the nanoemulsion gels
The anti-inflammatory activity of the developed nanoemulsion gels was evaluated on Wistar albino rats by carrageenan induced rat paw edema test. The protocol for animal studies was approved (Proposal No. 658, dated 23.07.10) by the Institutional Animal Ethics Committee (IAEC) of Jamia Hamdard, New Delhi (173/CPCSEA, 28th Jan 2000).

- **Species**: Adult Wistar Albino Rats
- **Weight**: 150-200 g
- **Gender**: Either sex

**Procedure of the study**
The animals were kept under standard laboratory conditions of temperature at 25±2°C and relative humidity of 55±5%. The animals were housed in polypropylene cages, five animals per cage with free access to standard laboratory diet (Lipton feed, Mumbai, India; providing 3630 kcal gm⁻¹ energy and containing 22.10% crude protein, 4.10% crude oil, 4.05% crude fiber, 10.05% ash, 0.75% sand silica) and water *ad libitum*.
Table 4.10: Treatment plan for the pharmacodynamic evaluation of the NEGs in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Treatment</th>
<th>Dose of diclofenac/6-gingerol (mg kg⁻¹ body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>Control (Placebo gel)</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>Standard (Diclofenac gel)</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>NG1</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>NG2</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>NG3</td>
<td>30</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>6-gingerol</td>
<td>30</td>
</tr>
</tbody>
</table>

Adult Wistar albino rats of either sex weighing between 150 and 200 g were randomly divided into six groups of five rats (Table 4.10) and were weighed, numbered and marked. Experiments were performed with placebo gel, marketed diclofenac emulgel, NG1, NG2, NG3 and pure 6-gingerol. The NEGs and 6-gingerol were applied to the rats at 6-gingerol dose of 30 mg kg⁻¹ and marketed diclofenac emulgel at a dose of 10 mg kg⁻¹ (Patil et al., 2009; Reuter et al., 1994). Animals of each group were pretreated with topical application of gels and 6-gingerol by rubbing with index finger (Gupta and Gaud, 2006) half an hour before injection of carrageenan. Carrageenan (0.1 ml) in 0.9% saline was injected into the planter aponeurosis of the right hind paw of each rat. Volume of the foot was measured using a digital plethysmometer (Nautica International, Uttaranchal, India) at predetermined time intervals of 0, 1, 2, 3, 6, 9 and 12h after carrageenan administration. The percentage edema (percentage paw volume increase) and percentage inhibition of edema in each group were calculated using following equations (Eqn. 4.17 and 4.18) (Arora and Mukherjee, 2002; Joshi and Patravale, 2008).

\[
\% \text{ Edema} = \left( \frac{V_t - V_o}{V_o} \right) \times 100 \quad \text{(4.17)}
\]

\[
\% \text{ Inhibition of edema} = \left( \frac{E_c - E_t}{E_c} \right) \times 100 \quad \text{(4.18)}
\]

where \( V_o \) is the mean paw volume before the carrageenan injection, \( V_t \) the mean paw volume after the carrageenan injection at time ‘t’, \( E_c \) the % edema of the placebo group and \( E_t \) is the % edema of the treated group at time ‘t’. The percentage inhibition of rat paw edema by the diclofenac emulgel, nanoemulsion gels and 6-gingerol was determined with respect to that of placebo gel. The pharmacodynamic data of different formulations were compared for statistical significance by one way analysis of variance (ANOVA) followed by Turkey-Kramer multiple comparison test using GraphPad Instat software (GraphPad Software Inc., CA, USA). A column chart of percentage inhibition obtained...
for different experimental groups which is normalized against placebo group versus time in hours was prepared.

4.3.5.21. Accelerated stability testing of nanoemulsion gels

The accelerated stability study was carried out according to both International Conference on Harmonisation (ICH) guidelines (ICH, 2003; 1996) and Arrhenius method.

i) Accelerated stability testing according to ICH guidelines

i.i) Controlled temperature and humidity condition

The accelerated stability study under controlled temperature and humidity conditions was carried out according to International Conference on Harmonisation (ICH) Q1A (R2) guidelines. The prepared nanoemulsion gels were proposed to be stored at room temperature. The stability studies were carried out to determine the effect of the presence of formulation additives on the stability of drug and also to determine the physical stability of the prepared formulation under conditions of storage temperature and relative humidity. Three batches of the nanoemulsion gel formulations were prepared and filled in high density polyethylene (HDPE) tubes for stability studies under controlled temperature and humidity condition. A quantity of 5 g gel was filled in the tubes and the tubes were sealed using an electric sealer. The filled and sealed tubes were finally packed in cartons. The packed samples (in cartons) of the freshly prepared nanoemulsion gels were placed in stability chamber maintained at 40±2°C and 75±5% RH. The cartons containing the nanoemulsion gels were placed in a humidity chamber maintained at a relative humidity of 75%. The humidity chambers were created by placing a beaker with saturated aqueous sodium chloride solution in desiccators (Kopleman et al., 2001; Uhumwangedo and Okor, 2005). The humidity chambers were placed in a hot air oven maintained at 40°C. The samples were withdrawn from the humidity chambers at time intervals of 0, 1, 2, 3 and 6 months. The nanoemulsion gels were evaluated for pH, spreadability, extrudability, in vitro drug release and drug content. Different humidity chambers were used for the different sampling intervals such that the samples were not in contact with the external environment during the storage interval in the chambers (Uhumwangedo and Okor, 2005).
The pH, spreadability and extrudability of the samples were determined. These evaluation parameters at different sampling intervals were tested for statistical significance by one way analysis of variance (ANOVA) followed by Turkey-Kramer multiple comparison test using GraphPad Instat software (GraphPad Software Inc., CA, USA). Zero time samples were used as control. For stability testing carried for the estimation of psoralen and 6-gingerol content in the nanoemulsion gels, the developed and validated stability indicating HPLC method was used (Section 4.3.3). The shelf life of the nanoemulsion gels, based on change in percentage drug content, was determined using SigmaPlot® 12 (Cranes Software International Limited, Bangalore, India) software. Percentage label claim (% drug remaining) was plotted against time in months to determine the shelf life using SigmaPlot® 12 software. The shelf life was determined as the time at which the 95% one-sided confidence limit for the mean curve intersects the acceptance criterion of 90% of drug content (percentage label claim).

i.ii) Photostability testing
The accelerated photostability study was carried out according to International Conference on Harmonisation (ICH) Q1B guidelines (ICH, 1996). Three batches of the nanoemulsion gels were prepared and filled in low density polyethylene (LDPE) tubes. The tubes were sealed using an electric sealer. The filled and sealed tubes were finally packed in cartons. Photostability was evaluated by exposing the packed samples of nanoemulsion gels to 200 watt hours/square meter in the UV range of 320 to 400 nm and 1.2 million lux hours in the VIS range (400-700 nm) using a cool white fluorescent lamp and a near UV fluorescent lamp respectively. The samples were evaluated for % drug content (assay). Samples were exposed side-by-side with a validated quinine actinometric system to ensure the specified light exposure is obtained. Aqueous solution of quinine monohydrochloride dihydrate (2% w/v) was prepared and filled in a 1 cm quartz cell and was used as the sample. Separately filled another 1 cm quartz cell, wrapped in aluminum foil to protect completely from light, and was used this as the control. The sample and control quinine solutions were exposed to the light sources during the photostability testing along with the nanoemulsion gels. After exposure the absorbances of the sample (AT) and the control (Ao) at 400 nm were determined. The change in absorbance, $\Delta A =$
AT - Ao was calculated. The length of exposure was confirmed to be sufficient to ensure a change in absorbance as indicated by its value of 0.58 (a value greater than 0.5).

i.iii) Accelerated stability study of nanoemulsion gels by conventional method using Arrhenius equation

For the determination of shelf life by conventional method, the nanoemulsion gels were stored at 30 ± 2 °C; 40 ± 2 °C and 50 ± 2 °C for three months. Samples were withdrawn after predetermined time intervals (0, 30, 60, and 90 days) and the percentage drug content was determined using the developed and validated stability indicating HPLC method as described in section 4.5.4. Zero time samples were used as controls.

Logarithm of percent drug remaining versus time (in-days) was plotted. The degradation rate constant 'k' was determined from the slope of the plots at each elevated temperature using the following equation (Eqn. 4.19).

\[ \text{Slope} = -\frac{k}{2.303} \]  

\[ \text{Eqn. 4.19} \]

Plot of the logarithm of k values at various elevated temperatures against the reciprocal of absolute temperature was drawn (Arrhenius plot). From the plot, k value at 25 °C was determined and was used to calculate shelf life by substituting in the following equation (Eqn. 4.20).

\[ t_{0.9} = \frac{0.1052}{k_{25}} \]  

\[ \text{Eqn. 4.20} \]

Where, \( t_{0.9} \) is the time required for 10 % degradation of the drug and is referred to as shelf life.