CHAPTER 3:

MATERIALS AND METHODS
3.0 MATERIALS AND METHODS

3.1. Paclitaxel

3.1.1. Physicochemical Properties of Paclitaxel

The physicochemical properties of paclitaxel like appearance, melting point and solubility were evaluated and recorded. The solubility of paclitaxel was studied in various solvents. The sample was qualitatively tested for its solubility in various solvents. It was determined by shaking 10 mg of drug sample in 5mL of solvent in small test tubes and the extent of solubilization was observed.

3.1.2. Identification of paclitaxel via Mass spectroscopy

The mass spectrum of paclitaxel sample was examined by mass spectroscopy using tuneable MS detector (Serial No# JAA 272; Synapt; Waters, Manchester, UK).

3.1.3. HPLC Method Development and Validation

3.1.3.1. HPLC Analytical method development

High performance liquid chromatography (Waters™ 600 Controller + 717 plus autosampler) in conjunction with ultraviolet detection (Waters™ 486) was used for the development of a sensitive assay for paclitaxel analysis for in-vitro studies.

3.1.3.1.1. Preparation of Mobile Phase

Methanol, water and tetrahydrofuran were mixed at a ratio 60:37.5:2.5 v/v, respectively. The mobile phase was then passed through 0.45µm filtered membrane and stored at 2-8°C before use.

3.1.3.1.2. Preparation of diluent

Methanol as diluent was used.

3.1.3.1.3. Preparation of blank

Diluent was used as blank.

3.1.3.1.4. Preparation of standard solution

Accurately weighed and transferred about 10 mg of paclitaxel working standard to a 10 mL volumetric flask. About 8 mL of diluent was added, sonicated to dissolve and the
volume was made up to 10 mL with diluent. Further 5 mL of drug solution was diluted up to 50 mL with diluent and mixed to make a concentration of 100 µg/mL.

3.1.3.1.5. Preparation of sample solution

Accurately weighed and transferred sample equivalent to 50 mg of paclitaxel sample preparation to a 50 mL volumetric flask. About 40 mL of diluent was added, sonicated to dissolve and the volume was made up to 50 mL with diluent. Further 5 mL of drug solution was diluted up to 50 mL with diluent and mixed to make a concentration of 100 µg/mL.

3.1.3.2. HPLC Analytical method validation

The developed method was validated for linearity, accuracy, precision, robustness and solution stability studies.

3.1.4. UPLC-MS/MS method for the determination of paclitaxel in blood plasma

For the determination of paclitaxel content in blood plasma a highly sensitive UPLC-MS/MS method was developed. UPLC was performed with a Waters Acquity™ UPLC system (Serial No# F09 UPB 920M; Model Code# UPB; Waters, MA, USA) equipped with a binary solvent delivery system, an auto-sampler, column manager and a tuneable MS detector (Serial No# JAA 272; Synapt; Waters, Manchester, UK).

3.1.4.1. Preparation of Mobile Phase

Acetonitrile-Ammonium formate buffer 2mM were mixed in a ratio 7:3 v/v, respectively. The mobile phase was then passed through 0.45µm filtered membrane and stored at 2-8°C before use. All the solvent used were of LC-MS grade.

3.1.4.2. Preparation of standard stock solution preparation

Acetonitrile was used as a diluent.

3.1.4.3. Preparation of standard stock solution (Paclitaxel): Stock A

Accurately weighed and transferred about 10 mg of paclitaxel working standard to a 100 mL volumetric flask. About 80 mL of diluent was added, sonicated to dissolve and the volume was made up to 100 mL with diluent to get a concentration of 100 µg/mL.

3.1.4.4. Preparation of internal standard stock solution (Docetaxel): Stock B
Accurately weighed approx. 10 mg docetaxel standard was dissolved in 100 mL volumetric flask. About 80 mL of diluent was added, sonicated to dissolve and the volume was made upto 100 mL with diluent to get a concentration of 100 µg/mL.

3.1.4.5. Preparation of working solution of calibration standard/ quality control sample

Working solution of calibration standard and quality control samples were prepared by serial dilutions from the standard stock solution of paclitaxel (100 µg/mL) as per the table 3.1.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Working Solution</th>
<th>Preparation</th>
<th>Concentration (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CAL-8</td>
<td>100 µL of stock A to 1000 µL</td>
<td>10000</td>
</tr>
<tr>
<td>2</td>
<td>CAL-7</td>
<td>80 µL of stock A to 1000 µL</td>
<td>8000</td>
</tr>
<tr>
<td>3</td>
<td>CAL-6</td>
<td>60 µL of stock A to 1000 µL</td>
<td>6000</td>
</tr>
<tr>
<td>4</td>
<td>CAL-5</td>
<td>500 µL of stock CAL-7 to 1000 µL</td>
<td>4000</td>
</tr>
<tr>
<td>5</td>
<td>CAL-4</td>
<td>500 µL of CAL-5 to 1000 µL</td>
<td>2000</td>
</tr>
<tr>
<td>6</td>
<td>CAL-3</td>
<td>500 µL of stock CAL-4 to 1000 µL</td>
<td>1000</td>
</tr>
<tr>
<td>7</td>
<td>CAL-2</td>
<td>500 µL of stock CAL-3 to 1000 µL</td>
<td>500</td>
</tr>
<tr>
<td>8</td>
<td>CAL-1</td>
<td>400 µL of stock CAL 2 to 1000 µL</td>
<td>200</td>
</tr>
</tbody>
</table>

Storage: All the stock and working solution were kept under refrigeration (2-8°C)

3.1.4.6. Preparation of calibration standard/QC standard in plasma

Calibration curve of paclitaxel in plasma was prepared in the range of 20 – 1000 ng/mL. Quality control samples in plasma (LQC, MQC, HQC) were prepared in the concentration 50 ng/mL, 400 ng/mL and 800 ng/mL, respectively.
Drug free plasma was thawed at room temperature and vortexed. In the 90 μL of the drug free plasma, 10 μL of respective working standard was added as per the table 3.2.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Working Solution</th>
<th>Standard</th>
<th>Conc. (ng/mL)</th>
<th>Conc. in plasma (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CAL-8</td>
<td>10000</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>CAL-7</td>
<td>8000</td>
<td>800</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>CAL-6</td>
<td>6000</td>
<td>600</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>CAL-5</td>
<td>4000</td>
<td>400</td>
<td></td>
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<tr>
<td>5</td>
<td>CAL-4</td>
<td>2000</td>
<td>200</td>
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<td>6</td>
<td>CAL-3</td>
<td>1000</td>
<td>100</td>
<td></td>
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<tr>
<td>7</td>
<td>CAL-2</td>
<td>500</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>CAL-1</td>
<td>200</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

3.1.4.7. Extraction method

Acetonitrile was selected as extraction solvent. About 1000 μL of extraction solvent was added in plasma samples and vortexed for 5 minutes followed by 5 minutes centrifugation at 10000 rpm (Eppendorf Centrifuge 5810). After centrifugation about 900 μL of supernatant was withdrawn in an Eppendorf tube and was evaporated till it completely dried in oven at 40°C under vacuum. The residue was reconstituted with 200 μL mobile phase as a diluent. The sample was vortexed for 2 minutes followed by 5 minutes sonication at room temperature, again vortexed for 2 minutes and 50 μL of the supernatant was directly injected in UPLC-MS system.

3.1.4.8. Preparation of blank plasma
About 100 µl of drug free plasma was taken and processed it as per extraction method given above.

### 3.1.4.9. Preparation of Zero sample

About 100 µl of drug free plasma was taken and 10 µl of working solution of Internal Standard was added with further processing as per extraction method given above.

### 3.1.4.10. Preparation of calibration and QC standard

Precisely 100 µl of Calibration or QC standard was taken and 10 µl of working solution of Internal Standard was added with further processing as per extraction method given above.

### 3.1.4.11. Preparation of Study Sample

Precisely 100 µl of study plasma sample was taken and 10 µl of working solution of Internal Standard was added with further processing as per extraction method given above.

### 3.1.5. Formulation Development & Evaluation: Paclitaxel

#### 3.1.5.1. Solubility Studies

Solubility studies of PCT were carried out in various oils/lipids, surfactants and co-surfactants. An excess amount of PCT was added into 1mL of each vehicle and the mixture was kept in sealed vials. A vortex mixer (Heidolph Multi Reax) was used to facilitate the solubilization. Sealed vials were stirred in a water bath (Julabo SW 23) at 37°C for 48 h. After standing for 48 h, each vial was centrifuged at 3000 rpm for 15 min using a centrifuge (Tomy Microvac). Undissolved drug was removed by filtering through a membrane filter (0.45 mm). The concentration of dissolved PCT was determined by HPLC method presented in section 3.1.3.

#### 3.1.5.2. Preparation of pseudo-ternary phase diagram

On the basis of the solubility studies, oils, surfactants and co-surfactants were selected for PCT and the ternary phase diagrams of various combinations of selected oils, surfactants and co-surfactants were established [Kommuru et al., 2001]. Ternary pre-concentrate mixtures with varying compositions of oils, surfactants and co-surfactants were prepared. The amount of oils, surfactants and co-surfactants used herein was determined on the basis of the requirements for the spontaneously emulsifying systems [Shen and Zhong,
2006]. The compositions were evaluated for the microemulsion region by diluting one part of pre-concentrate mixture with twenty parts of deionized water (1:20). At equilibrium, the time of self-microemulsification, dispersibility, appearance and flowability were evaluated and scored according to the grading system shown in Table 3.3 [Pouton, 1985]. The microemulsion regions in the diagrams were plotted, and the wider regions indicated a better self-microemulsification efficiency of the pre-concentrate mixture.

The diagram plots the relative concentration of surfactant (0-100%), the concentration of oil (0 to 100%) and the concentration of co-surfactant (0 to 100%) for the placebo system. The relative concentration of co-surfactant increases from 0% at the lower right hand margin of the diagram to 100% at the lower left corner; the relative concentration of surfactant increases from 0% at the baseline of the diagram to 100% at the apex; and the relative concentration of oil increases from 0% at the apex to 100% at the lower right hand corner of the diagram. The shaded area identifies those compositions having microemulsion region. The ratio of Surfactant to co-surfactant was held constant, while the amount of oil phase and water varied. The amount of surfactant, oil and co-surfactant folded was noted down and calculated. The pseudo-pseudo-ternary phase diagrams were mapped according to the data. The micro-emulsion regions in the diagrams were plotted and the wider region indicated the better self-micro emulsification efficiency.

3.1.5.3. Preparation of PCT loaded self-microemulsifying drug delivery system

After the identification of the microemulsification area in the ternary phase diagrams, PCT loaded SMEDDS formulations with different combinations of oils, surfactants and co-surfactants at the desired component ratios were prepared, respectively. Thirty milligrams of PCT was added to the oil phase of each of their formulations, respectively, under continuous stirring. The surfactant system (Smix) for each formulation was prepared by mixing the selected surfactant and co-surfactant at their pre-determined ratios to prepare a pre-concentrate mixture of 1.0 g. The PCT containing oil phase was added to the Smix phase under continuous stirring and vortex mixing. The stirring was continued until a homogenous mixture was formed. Finally, the mixture was kept at 25°C, then 1.0 g of the SMEDDS preconcentrate mixture (containing 30 mg PCT) were filled into hard gelatin capsules ‘size 00EL’ for dissolution testing and stability studies.
Table 3.3. Visual assessment of efficiency of self-microemulsification

<table>
<thead>
<tr>
<th>Grade</th>
<th>Dispersibility</th>
<th>Time of self-microemulsification</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Rapid forming microemulsion which is clear or slightly bluish in appearance</td>
<td>&lt; 1 min</td>
</tr>
<tr>
<td>II</td>
<td>Rapid forming, slightly less clear emulsion which has a bluish white appearance</td>
<td>&lt; 2 min</td>
</tr>
<tr>
<td>III</td>
<td>Bright white emulsion (Similar to milk in appearance)</td>
<td>&lt; 3 min</td>
</tr>
<tr>
<td>IV</td>
<td>Dull, grayish white emulsion with a slightly oily appearance that is slow to emulsify</td>
<td>&gt; 3 min</td>
</tr>
<tr>
<td>V</td>
<td>Poor or minimal emulsification with large oil droplets present on the surface.</td>
<td>&gt; 3 min</td>
</tr>
</tbody>
</table>

3.1.5.4. The effect of drug on the phase diagram

The objective of this experiment was to investigate the effects of PCT on the self-emulsifying performance of the SMEDDS. The formulation amount of (30 mg PCT) was added to the boundary formulations of the self-emulsifying formulation of the pseudo-ternary phase diagrams. The self-emulsifying performance was visually assessed after 100 times dilution with deionized water.

3.1.5.5. Determination of drug content

Drug content in the selected PCT SMEDDS formulations were determined by HPLC method presented in the section 3.1.3.

3.1.5.6. Characterization and Evaluation of the PCT SMEDDS formulations

3.1.5.6.1. Effect of dilution and pH of the aqueous phase on pseudo-ternary phase diagram of the selected system

Dilution and pH of the aqueous phase may have substantial consequences on the phase separation and stability of the self-emulsifying systems [Pouton, 1985; Kim et al., 2000;
Kawakami et al., 2000; Date & Nagarsenker, 2007]. In consideration of this, selected PCT SMEDDS formulations were diluted (20-times and 1000-times) with various diluent (i.e. deionized water, 0.1 N HCl and phosphate buffer pH 6.8). The diluted microemulsions were stored for 8 h at 25 ºC and observed by eye for phase separation or drug precipitation.

3.1.5.6.2. Droplet Size determination

SMEDDS formulations (1 mL) were subjected to droplet size determination of the resultant microemulsions after the addition of 20 mL de-ionized water by dynamic light scattering with a particle sizing apparatus (Malvern Zetasizer 3500HS, Malvern, UK).

3.1.5.6.3. Zeta potential

The emulsion stability is directly related to the magnitude of the surface charge [Pongcharoenkiat et al., 2002; Chansiri et al., 1999; Michalek and Stachurski, 1996]. The Zeta potential of the diluted SMEDDS formulation was measured using a (Malvern Zetasizer 3500HS). The SMEDDS were diluted with a ratio of 1:20 v/v with distilled water and mixed for one minute using a magnetic stirrer. Measuring the Zeta potential in simulated gastric fluid (SGF) was a challenge due to the high specific conductance of the media that restricts the maximum tolerated voltage applied through the cell. The measured zeta potential in simulated intestinal fluid (SIF) was not significantly different from the Zeta potential measured in distilled water [Belmonte and Atef, 2008]. Zeta potential of each SMEDDS was determined in triplicate.

3.1.5.6.4. Percentage Transmittance ($\lambda_{\text{max}}$ 560 nm)

A total of 1 mL of SMEDDS formulation was diluted with the 10 times and 1000 times with deionized water. Percentage transmittance were measured spectrophotometrically (Perkin Elmer Lamda 35 UV Spectrophotometer) at 560 nm using deionized water as a blank.

3.1.5.6.5. Thermodynamic stability studies

Thermodynamic stability studies were performed to evaluate the phase separation and effect of temperature variation on SMEDDS stability. All selected PCT SMEDDS formulations were diluted with deionized water (1:20) and centrifuged (Tomy Microvac) at 10,000 rpm for 20 min, and observed visually for phase separation.
Formulations that showed signs of phase separation were removed from further studies and the remaining formulations were subjected to a freeze-thaw study. Formulations were diluted with deionized water at a ratio of 1:20 and subjected to two freeze-thaw cycles between −20°C and +25°C, with storage at each temperature for not less than 4 h.

3.1.5.6.6. Transmission Electron Microscopy

A transmission electron microscope (Philips CM12 Electron Microscope, Eindhoven, The Netherlands) was used to examine droplet morphology. Selected paclitaxel SMEDDS formulation was diluted with water of a ratio of 1:100. A drop of the diluted microemulsion was directly deposited on the holey film grid to observe the morphology of formulations.

3.1.5.7. In vitro release studies

To understand the characteristics of drug release from SMEDDS, an in vitro release was carried out. When SMEDDS encountered aqueous media, the drug existed in the system in different forms including a free molecular form or mixed in the micelles or in the microemulsion droplets. The dissolution test was performed in USP type-II dissolution apparatus II (Distek) according to United State Pharmacopoeia (USP 35) dissolution procedure. PCT SMEDDS hard gelatin capsule was put into a sinker. This sinker was loaded with 900 ml of 1% Tween 80 (w/v) in PBS solution pH 7.4, 1% Tween 80 (w/v) in pH 4.5 acetate buffer and 1% Tween 80 (w/v) in simulated gastric fluid without enzymes (pH 1.2) at 37 ± 0.5°C with paddle speed of 50 rpm. Each sample (2 ml) was withdrawn at 5, 15, 30, 45 and 60 min with replacement by an equal volume of temperature-equilibrated media. The concentration of paclitaxel was determined by HPLC after appropriate dilution with methanol. The percent cumulative amount of paclitaxel released from SMEDDS was calculated as a function of time.

3.1.5.8. Ex vivo cell lines studies

3.1.5.8.1. Caco-2 Cells Uptake Study

In order to better address intestinal barrier integrity and better monitor experimental parameters, experiments were performed in vitro on selected PCT SMEDDS formulation. A well-established model to study the intestinal permeability of drugs (Caco-2 cells) [Artursson & Borchardt 1997, Balimane & Chong 2005] was chosen. Indeed, after differentiation, Caco-2 cells formed a monolayer of polarized cells, which present tight
junctions and active transporters (P-glycoprotein) [Hilgers et al. 1990; Artursson & Karlsson 1991]. The objective of the present study was to investigate the permeability of selected PCT loaded SMEDDS formulation, when co-administered with novel P-gp inhibitor (i.e. GF120918) across intestinal barrier.

3.1.5.8.1.1. Caco-2 Cell Culture

Caco-2 cells originating from a human colorectal carcinoma were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies, Gibco BRL, UK) supplemented with 20% heat-denaturated fetal calf serum (FCS, Pan Biotech GmbH, Germany), 1% (v/v) non-essential amino acids, 1.0 mM sodium pyruvate, penicillin (100 U/mL) and streptomycin (100 µg/mL) (Sigma Chemicals, St. Louis, MO). The cells were maintained at 37 ºC in an atmosphere of 95% air and 5% CO₂ at 90% relative humidity. Caco-2 cells of passage numbers 66 to 70 were used in these experiments. UV transparent 96-well Costar® Corning microplates were purchased from Corning® (Corning Life Sciences, Schiphol, The Netherlands). Transwell-COL inserts (0.4 µm pore size, 1.12 cm² growth area) and 12-well Transwell culture plates were purchased from Corning Costar, Cambridge, MA, USA.

3.1.5.8.1.2. Cell cytotoxicity study: WST-1 Assay

To ensure the Caco-2 cells integrity during the permeability study, cytotoxicity assay, i.e. WST-1 assay, was carried out. WST-1 Assay is colorimetric assay designed to be used for the non-radioactive, spectrophotometric quantification of cell proliferation, growth, viability, and chemosensitivity in cell populations using the 96-well plate format. The cytotoxic effect of paclitaxel using microemulsion formulations (formed from its SMEDDS formulation) was studied on Caco-2 Cells using WST-1 assay. Different concentration of paclitaxel (2µM and 5µM) and GF120918 (5µM and 10µM) solution were investigated to determine the suitable dose at which these drugs would not affect the cell integrity during the course time in permeability assay.

The study was carried out on different group as per the protocol mentioned below:

- Group I: Paclitaxel loaded SMEDDS with GF120918
- Group II: Paclitaxel loaded SMEDDS without GF120918
- Group III: Paclitaxel solution with GF120918
• Group IV: Paclitaxel solution alone
• Group V: GF120918 alone
• Group VI: Culture Medium alone (Positive control)
• Group VII: 20% SDS (200μg/mL) (negative control)

Collagen solution was prepared in PBS solution (1mg/ml) and loaded in 96-well plates for 1 hr. This solution was withdrawn at the predetermined time as the coating in each well was ensured. Caco-2 cells were transferred to 96-well plates at a density of $5 \times 10^4$ cells/well. As the cells reached to 80% confluence, the medium was replaced with 100 μL of medium containing varying amounts of testing groups as mentioned above. One row of the 96-well plates was used as a positive control and culture medium was added to these wells. The plates were then incubated for 4h at 37 °C. The medium was then removed and the wells were washed three times with ice-cold PBS solution (100μL).

After washing 100μL of WST-1, which was prepared by mixing 10μL of WST-1 stock solution and 90μL of incubation medium, respectively, was added to each well. Absorbance was measured using a Biotek PowerWave XS reader (Witec AG, Littau, Switzerland) at 450 nm. Cell cytotoxicity was expressed as the percent absorbance of tested group relative to absorbance measured for cells that were not exposed to any material.

### 3.1.5.8.1.3. Permeation Studies

The permeation of selected PCT SMEDDS formulation of paclitaxel was studied in the apical to basolateral direction and basolateral to apical direction in Caco-2 cells. The various groups used for the study are mentioned in table 3.4. All the SMEDDS formulations were diluted in deionized water (in the ratio of 1:20) to form respective microemulsions, which were further used to evaluate their permeability in Caco-2 cells. On the basis of cytotoxicity assay the concentrations of paclitaxel and GF120918 chosen for the studies were 5 µM and 10 µM, respectively.

**Bidirectional Permeation Assay**

The enhancement in permeation of paclitaxel using microemulsion formulation (formed from SMEDDS formulation) along with GF120918 was studied in the apical to basolateral direction and basolateral to apical direction in Caco-2 cell monolayers. For the *in vitro* permeation study, Caco-2 cells were seeded in 75 cm$^2$ flask at an initial seeding
density of $10^6$ cells/flask, harvested at 80% confluence with trypsin–EDTA and seeded onto the apical side of collagen-coated Transwell-COL inserts (0.4 μm pore size, 1.12 cm$^2$ growth area) in 12-well Transwell culture plates (Corning Costar, Cambridge, MA, USA) at an initial seeding density of $10^5$ cells/well. The culture medium was added to the apical (0.5 mL) and basolateral (1.5 mL) compartments and replaced every other day for the first week and daily thereafter. Cells were incubated for 18–21 days until the transepithelial electrical resistance (TEER) increased to between 300 Ω/cm$^2$ and 600 Ω/cm$^2$. As the cell monolayers reached the desired TEER value, they were used for further experimentations. The TEER value of monolayers was checked before and after each experiment by using a Millicell®-ER voltohmmeter equipped with chopstick electrodes (Millipore Corporation, Bedford, MA, USA).

### Table 3.4. Permeability Study Protocol

| Code  | Drug   | Formulation | Pgp-inhibitor | Direction*
|-------|--------|-------------|--------------|------
| PSME  | Paclitaxel | Microemulsion | Present | A-B  
| PSM   | Paclitaxel | Microemulsion | Absent  | A-B  
| PSE (A-B) | Paclitaxel | Solution | Present | A-B  
| PSE (B-A) | Paclitaxel | Solution | Present | B-A  
| PS    | Paclitaxel | Solution | Absent  | A-B  

Before the transport study, the culture medium (DMEM) was washed and replaced with preheated (37 °C) transport medium consisting of Hanks’ balanced salt solution (HBSS), supplemented with 10 mM HEPES (pH 7.4). After the cell monolayer was equilibrated for 30 min at 37 °C, TEER values of monolayers were determined in triplicate. For the apical to basolateral (A-B) transport study, various groups of paclitaxel were evaluated for their ability to enhance drug permeation.

For apical to basolateral (A-B) study, each test group diluted with transport medium was added to the apical side (0.5 mL) and blank media on the basolateral side (1.5 mL). After
0.5 h, 1 h, 2 h and 3 h of incubation, 0.2 mL of medium at the basolateral side was withdrawn and replaced with fresh medium. For the investigation of basolateral-to-apical (B-A) transport, each test sample was placed in the basolateral side and blank media on the other side. After 0.5 h, 1 h, 2 h and 3 h of incubation, 0.1 mL of medium at the apical side was withdrawn and replaced with fresh medium.

The concentration of paclitaxel was determined by HPLC analysis as developed earlier, and the amount of drug permeated was plotted as a function of time. The apparent permeability coefficient ($P_{app}$) was determined from the linear slope of the plot using the following equation:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{AC_0}$$

where, $P_{app}$ is the apparent permeability (cm/s), $dQ/dt$ is the steady state flux, $A$ is the surface area of the membrane (cm$^2$), $C_0$ is the initial concentration of drug at the apical (for A to B transport) or basolateral (for B to A transport) side.

### 3.1.5.8.2. Cell Uptake Studies: A549 Cell lines

Cellular uptake studies were done to investigate the potential of developed SMEDDS formulations in the presence or absence of GF120918 to enhance capture by tumor cells. A549 lung cancer cells were used for this purpose. SMEDDS formulation as evaluated earlier for paclitaxel delivery was prepared and the drug was replaced by rhodamine 123 (Rh 123). Rh 123 is a well-known P-gp substrate and was used as a model dye [Troutman and Thakker, 2003]. The resulting Rh123 loaded SMEDDS formulation was denoted as SMEDDS I. SMEDDS formulation was diluted with deionized water (in ratio of 1:20) to form microemulsion which is further incubated with A549 cells to evaluate their uptake efficiency. Various groups tested in this study were:

- Microemulsion formed from SMEDDS I along with GF120918: RSME1
- Microemulsion formed from SMEDDS I without GF120918: RSM1
- Rh 123 solution with GF120918: RSE
- Rh 123 solution alone (positive control): RS
- Culture medium alone (negative control)

### 3.1.5.8.2.1 A549 Cell Culture
A549 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were cultured in F12k medium supplemented with 10% fetal calf serum (FCS; Pan Biotech GmbH, Germany), penicillin (100 U/mL) and streptomycin (100 μg/mL) (Sigma Chemical, St. Louis, MO, USA) at 37 ºC and incubated in an atmosphere of 95% air and 5% CO2 at 90% relative humidity. The cells at an initial seeding density of 10⁶ cells/flask were allowed to grow until confluence and were trypsinized and seeded in plates for each experiment. Experiments were performed with cells of passage numbers 78-84.

3.1.5.8.2.2. Quantitative study: Cellular uptake study

A549 cells were seeded in a 24-well plate (10⁵ cells/well) and allowed to attach for 2 days. After 2 days, cells in each well were incubated with different groups as mentioned above for 4 h at 37 ºC. After 4-hour incubation, the uptake was stopped by aspirating the various groups’ supernatants and the cells were washed 3 times with ice-cold PBS. Cells were then trypsinized to detach and collected in Eppendorf tubes. Subsequently, cells were lysed with 400 μL of 0.1% Triton X-100 for 10 min at room temperature. Total protein content in each sample was estimated using the Bradford protein assay. The cells were harvested by adding 400 μL of methanol. The cell lysate was then centrifuged at 10,000 rpm for 10 min. The Rh123 content in the supernatant after centrifugation was measured at excitation and emission wavelengths of 480 nm and 520 nm, respectively, using a Biotek PowerWave XS reader (Witec AG, Littau, Switzerland). Rh123 standard curve was generated to quantify the total amount of Rh123 accumulated in each sample. Cellular accumulation of Rh123 was then normalized to the total protein content as determined by the BCA assay. Uptake was expressed as the amount of rhodamine 123 per unit weight (mg) of total cellular protein. Results were expressed as percentages against control, i.e. cellular uptake by A549 cells in the absence of the inhibitor.

3.1.5.8.2.3. Qualitative Study: Confocal Laser Scanning Microscopy (CLSM)

A549 cells were seeded in 35 mm polystyrene cell culture dishes (4 × 10⁵ cells/well) for different groups as mentioned above and allowed to attach for 48 h. After 2 days, cells in each well were incubated with different group as mentioned above for 4 h at 37 ºC. After 4-hour incubation, the uptake was stopped by aspirating all the supernatants and the cells were washed with ice-cold phosphate buffered saline (PBS). After washing, the cells were fixed with 4% formaldehyde in DPBS (Dulbecco’s Phosphate Buffer Saline) for 15 min.
Cells were then washed and stored in PBS for analysis using a Confocal Laser Scanning Microscope (Zeiss 710 2P, Zeiss, Jena, Germany) at excitation and emission wavelengths of 480 nm and 520 nm, respectively.

3.1.5.9. In vivo Study

3.1.5.9.1. Dose Selection

Dose selection of the PCT and GF120918 in Sprague-Dawley (S.D.) rats to carry out the oral single dose pharmacokinetic study was based on previous literature [Nornoo et al. 2009; Bardelmeijer et al. 2004]. PCT and GF120918 were administered safely in rats at a dose of 10 mg/kg and 20 mg/kg, respectively via oral route. The in-vivo study was done as per the protocol approved by the Institutional Animal Ethics Committee, Jamia Hamdard (approval no. 684) and their guidelines were followed.

3.1.5.9.2. Animal and Dosing Procedure/Animal Study

20 healthy female SD rats weighing 200 ± 10 gm were used in the study. The rats were divided in various groups for the study as mentioned below:

- Group I: Paclitaxel loaded SMEDDS with GF120918 solution
- Group II: Paclitaxel loaded SMEDDS alone
- Group III: Paclitaxel suspension with GF120918 solution
- Group IV: Paclitaxel suspension alone
- Group V: Control group

The rats were housed under standard conditions. All rats were dosed orally using oral feeding tube following an overnight fast; food was returned 4 hours after dosing. In the first group, GF120918 solution was administered 2 hr prior to the administration of paclitaxel loaded SMEDDS formulation. In the second group, paclitaxel loaded SMEDDS alone was administered. In the third group, GF120918 solution was administered 2 hr prior to the administration of paclitaxel suspension (0.25% CMC Na and 1% Tween 80). In the fourth group, paclitaxel suspension alone was administered. Fifth group was used as control. The dose of paclitaxel and GF120918 in each one of these formulations was adjusted to contain 10 mg/kg and 20 mg/kg body weights, respectively. Blood samples (approx. 0.5 mL) was collected from retro-orbital plexus of rat in tube containing saturated solution of di-sodium EDTA at pre-dose, and 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 12.0,
24.0, and 48.0 hrs post dose. During collection, blood sample was mixed thoroughly with di-sodium EDTA solution in order to prevent blood clotting. Plasma was separated from centrifuge the blood at 5000 rpm in cooling centrifuge for 5 min and stored frozen at – 20°C until analysis. Paclitaxel analysis was done by previously developed and validated UPLC-MS-MS method.

3.1.5.9.3. Data analysis

All values are expressed as the mean ± S.D. Statistical analysis was performed with GraphPad InStat software (version 3.00, GraphPad Software, San Diego, California, USA) using oneway ANOVA followed by Tukey–Kramer multiple comparison test. Difference with P > 0.05 was considered statistically significant.

3.1.5.10. Stability studies

Ability of a formulation to retain properties in specified limits throughout its shelf life is referred as stability. Stability of a pharmaceutical product may be defined as a capability of a particular formulation, in a specific container, to remain within its physical, chemical, microbiological, therapeutic and toxicological specifications. Stability of a formulated product on shelf becomes an important factor in successful development of dosage form. Hence, a well-designed stability-testing plan is essential for the development of SMEDDS formulations.

Hard gelatin capsules (size ‘00EL’) filled with PCT loaded preconcentrate (PCT SMEDDS) was subjected to the accelerated (40°C/75%RH) and long-term stability (25°C/60%RH) conditions for the period of 3 months. Samples were charged in stability chambers (Thermolab, Mumbai, India) with humidity and temperature control. Samples were withdrawn and analyzed for particle size and residual drug content after a period of 30, 60 and 90 days. PCT content in capsules was analysed by HPLC method presented in section 3.1.3.
3.2. Docetaxel

3.2.1. Physicochemical Properties of Docetaxel

The physicochemical properties of docetaxel like appearance, melting point and solubility were evaluated and recorded. The solubility of docetaxel was studied in various solvents. The sample was qualitatively tested for its solubility in various solvents. It was determined by shaking 10 mg of drug sample in 5mL of solvent in small test tubes and the extent of solubilization was observed.

3.2.2. Identification of Docetaxel via Mass spectroscopy

The mass spectrum of docetaxel sample was examined by mass spectroscopy using tuneable MS detector (Serial No# JAA 272; Synapt; Waters, Manchester, UK).

3.2.3. HPLC Method Development and Validation

3.2.3.1. HPLC Analytical method development

High performance liquid chromatography (Waters™ 600 Controller + 717 plus autosampler) in conjunction with ultraviolet (Waters™ 486) detection was used for the development of a sensitive assay for docetaxel analysis for in-vitro studies.

3.2.3.1.1. Preparation of Mobile Phase

Acetonitrile, 35 mM ammonium formate buffer (pH 5) and tetrahydrofuran were mixed in a ratio 50:45:5 v/v, respectively. The mobile phase was then passed through 0.45µm filtered membrane and stored at 2-8°C before use.

3.2.3.1.2. Preparation of diluent

Acetonitrile as diluent was used.

3.2.3.1.3. Preparation of blank

Diluent was used as blank.

3.2.3.1.4. Preparation of standard solution

Accurately weighed and transferred about 10 mg of docetaxel working standard to a 10 mL volumetric flask. About 8 mL of diluent was added, sonicated to dissolve and the volume was made upto 10 mL with diluent. Further 5 mL of drug solution was diluted upto 50 mL with diluent and mixed to make a concentration of 100 µg/mL.
3.2.3.1.5. Preparation of sample solution

Accurately weighed and transferred sample equivalent to 50 mg of docetaxel sample preparation to a 50 mL volumetric flask. About 40 mL of diluent was added, sonicated to dissolve and the volume was made up to 50 mL with diluent. Further 5 mL of drug solution was diluted up to 50 mL with diluent and mixed to make a concentration of 100 µg/mL.

3.2.3.2. HPLC Analytical method validation

The developed method was validated for linearity, accuracy, precision, robustness and solution stability studies.

3.2.4. UPLC-MS/MS method for the determination of docetaxel in blood plasma

For the determination of docetaxel content in blood plasma a highly sensitive UPLC-MS/MS method was developed. UPLC was performed with a Waters Acquity™ UPLC system (Serial No# F09 UPB 920M; Model Code# UPB; Waters, MA, USA) equipped with a binary solvent delivery system, an auto-sampler, column manager and a tuneable MS detector (Serial No# JAA 272; Synapt; Waters, Manchester, UK).

3.2.4.1. Preparation of Mobile Phase

Acetonitrile-Ammonium formate buffer 2mM were mixed in a ratio 7:3 v/v, respectively. The mobile phase was then passed through 0.45µm filtered membrane and stored at 2-8°C before use. All the solvent used were of LC-MS grade.

3.2.4.2. Preparation of standard stock solution preparation

Acetonitrile was used as a diluent.

3.2.4.3. Preparation of standard stock solution (Docetaxel): Stock A

Accurately weighed approx. 10 mg docetaxel standard was dissolved in 100 mL volumetric flask. About 80 mL of diluent was added, sonicated to dissolve and the volume was made up to 100 mL with diluent to get a concentration of 100 µg/mL.

3.2.4.4. Preparation of internal standard stock solution (Paclitaxel): Stock B

Accurately weighed and transferred about 10 mg of paclitaxel working standard to a 100 mL volumetric flask. About 80 mL of diluent was added, sonicated to dissolve and the volume was made up to 100 mL with diluent to get a concentration of 100 µg/mL.
3.2.4.5. Preparation of working solution of calibration standard/quality control sample

Working solution of calibration standard and quality control samples were prepared by serial dilutions from the standard stock solution of docetaxel (100 µg/mL) as per the table 3.5.

Table 3.5. Dilution for Docetaxel calibration Standard

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Working Solution</th>
<th>Preparation</th>
<th>Concentration (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CAL-8</td>
<td>50 µL of stock A to 1000 µL</td>
<td>5000</td>
</tr>
<tr>
<td>2</td>
<td>CAL-7</td>
<td>40 µL of stock A to 1000 µL</td>
<td>4000</td>
</tr>
<tr>
<td>3</td>
<td>CAL-6</td>
<td>30 µL of stock A to 1000 µL</td>
<td>3000</td>
</tr>
<tr>
<td>4</td>
<td>CAL-5</td>
<td>500 µL of stock CAL-7 to 1000 µL</td>
<td>2000</td>
</tr>
<tr>
<td>5</td>
<td>CAL-4</td>
<td>500 µL of CAL-5 to 1000 µL</td>
<td>1000</td>
</tr>
<tr>
<td>6</td>
<td>CAL-3</td>
<td>500 µL of stock CAL-4 to 1000 µL</td>
<td>500</td>
</tr>
<tr>
<td>7</td>
<td>CAL-2</td>
<td>400 µL of stock CAL-3 to 1000 µL</td>
<td>200</td>
</tr>
<tr>
<td>8</td>
<td>CAL-1</td>
<td>500 µL of stock CAL 2 to 1000 µL</td>
<td>100</td>
</tr>
</tbody>
</table>

Storage: All the stock and working solution were kept under refrigeration (2-8°C)

3.2.4.6. Preparation of calibration standard/QC standard in plasma

Calibration curve of docetaxel in plasma was prepared in the range of 10 – 500 ng/mL. Quality control samples in plasma (LQC, MQC, HQC) were prepared in the concentration 50 ng/mL, 200 ng/mL and 400 ng/mL, respectively.

Drug free plasma was thawed at room temperature and vortexed. In the 90 µL of the drug free plasma, 10 µL of respective working standard was added as per the table 3.6.
Table 3.6. Dilution for calibration standard and QC standard in plasma

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Working Solution</th>
<th>Standard</th>
<th>Conc. (ng/mL)</th>
<th>Conc. in plasma (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CAL-8</td>
<td>5000</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>CAL-7</td>
<td>4000</td>
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<td>3</td>
<td>CAL-6</td>
<td>3000</td>
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<td>4</td>
<td>CAL-5</td>
<td>2000</td>
<td>200</td>
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</tr>
<tr>
<td>5</td>
<td>CAL-4</td>
<td>1000</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>CAL-3</td>
<td>500</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>CAL-2</td>
<td>200</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>CAL-1</td>
<td>100</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

3.2.4.7. Extraction method

Acetonitrile was selected as a extraction solvent. About 1000 µL of extraction solvent was added in plasma samples and vortexed for 5 minutes followed by 5 minutes centrifugation at 10000 rpm (Eppendorf Centrifuge 5810). After centrifugation about 900 µL of supernatant was withdrawn in an eppendorf tube and was evaporated till it completely dried in oven at 40°C under vacuum. The residue was reconstituted with 200 µL mobile phase as a diluent. The sample was vortexed for 2 minutes followed by 5 minutes sonication at room temperature, again vortexed for 2 minutes and 50 µL of the supernatant was directly injected in HPLC.

3.2.4.8. Preparation of blank plasma

About 100 µl of drug free plasma was taken and processed it as per extraction method given above.

3.2.4.9. Preparation of Zero sample
About 100 µl of drug free plasma was taken and 10 µl of working solution of Internal Standard was added with further processing as per extraction method given above.

### 3.2.4.10. Preparation of calibration and QC standard

Precisely 100 µl of Calibration or QC standard was taken and 10 µl of working solution of Internal Standard was added with further processing as per extraction method given above.

### 3.2.4.11. Preparation of Study Sample

Precisely 100 µl of study plasma sample was taken and 10 µl of working solution of Internal Standard was added with further processing as per extraction method given above.

### 3.2.5. Formulation Development & Evaluation: Docetaxel

#### 3.2.5.1. Solubility Studies

Solubility studies of PCT were carried out in various oils/lipids, surfactants and co-surfactants. An excess amount of PCT was added into 1mL of each vehicle and the mixture was kept in sealed vials. A vortex mixer (Heidolph Multi Reax) was used to facilitate the solubilization. Sealed vials were stirred in a water bath (Julabo SW 23) at 37°C for 48 h. After standing for 48 h, each vial was centrifuged at 3000 rpm for 15 min using a centrifuge (Tomy Microvac). Undissolved drug was removed by filtering through a membrane filter (0.45 mm). The concentration of dissolved DCT was determined by HPLC method presented in section 3.2.3.

#### 3.2.5.2. Preparation of pseudo-ternary phase diagram

On the basis of the solubility studies, oils, surfactants and co-surfactants were selected for DCT and the ternary phase diagrams of various combinations of selected oils, surfactants and co-surfactants were established [Kommuru et al., 2001]. Ternary pre-concentrate mixtures with varying compositions of oils, surfactants and co-surfactants were prepared. The amount of oils, surfactants and co-surfactants used herein was determined on the basis of the requirements for the spontaneously emulsifying systems [Shen and Zhong, 2006]. The compositions were evaluated for the microemulsion region by diluting one part of pre-concentrate mixture with twenty parts of deionized water (1:20). At equilibrium, the time of self-microemulsification, dispersibility, appearance and
flowability were evaluated and scored according to the grading system shown in Table 3.3 [Pouton, 1985]. The microemulsion regions in the diagrams were plotted, and the wider regions indicated a better self-microemulsification efficiency of the pre-concentrate mixture.

3.2.5.3. Preparation of DCT loaded self-microemulsifying drug delivery system

After the identification of the microemulsification area in the ternary phase diagrams, DCT loaded SMEDDS formulations with different combinations of oils, surfactants and co-surfactants at the desired component ratios were prepared, respectively. Twenty milligrams of DCT was added to the oil phase of each of their formulations, respectively, under continuous stirring. The surfactant system (S$_{mix}$) for each formulation was prepared by mixing the selected surfactant and co-surfactant at their pre-determined ratios to prepare a pre-concentrate mixture of 1.0 g. The DCT containing oil phase was added to the S$_{mix}$ phase under continuous stirring and vortex mixing. The stirring was continued until a homogenous mixture was formed. Finally, the mixture was kept at 25°C, then 1.0 g of the SMEDDS preconcentrate mixture (containing 20 mg DCT) were filled into hard gelatin capsules ‘size 00EL’ for dissolution testing and stability studies.

3.2.5.4. The effect of drug on the phase diagram

The objective of this experiment was to investigate the effects of DCT on the self-emulsifying performance of the SMEDDS. The formulation amount of (20 mg DCT) was added to the boundary formulations of the self-emulsifying formulation of the pseudo-ternary phase diagrams. The self-emulsifying performance was visually assessed after 100 times dilution with deionized water.

3.2.5.5. Determination of drug content

Drug content in the selected DCT SMEDDS formulations were determined by HPLC method presented in the section 3.2.3.

3.2.5.6. Characterization and Evaluation of the DCT SMEDDS formulations

3.2.5.6.1. Effect of dilution and pH of the aqueous phase on pseudo-ternary phase diagram of the selected system

Dilution and pH of the aqueous phase may have substantial consequences on the phase separation and stability of the self-emulsifying systems [Pouton, 1985; Kim et al., 2000;
Kawakami et al., 2000; Date & Nagarsenker, 2007]. In consideration of this, selected DCT SMEDDS formulations were diluted (20-times and 1000-times) with various diluent (i.e. deionized water, 0.1 N HCl and phosphate buffer pH 6.8). The diluted microemulsions were stored for 8 h at 25 °C and observed by eye for phase separation or drug precipitation.

3.2.5.6.2. Droplet Size determination

SMEDDS formulations (1 mL) were subjected to droplet size determination of the resultant microemulsions after the addition of 20 mL de-ionized water by dynamic light scattering with a particle sizing apparatus (Malvern Zetasizer 3500HS, Malvern, UK).

3.2.5.6.3. Zeta potential

The Zeta potential of the diluted SMEDDS formulation was measured using a (Malvern Zetasizer 3500HS). The SMEDDS were diluted with a ratio of 1:20 v/v with distilled water and mixed for one minute using a magnetic stirrer. Measuring the Zeta potential in simulated gastric fluid (SGF) was a challenge due to the high specific conductance of the media that restricts the maximum tolerated voltage applied through the cell. The measured zeta potential in simulated intestinal fluid (SIF) was not significantly different from the Zeta potential measured in distilled water [Belmonte and Atef, 2008]. Zeta potential of each SMEDDS was determined in triplicate.

3.2.5.6.4. Percentage Transmittance ($\lambda_{\text{max}}$ 560 nm)

A total of 1 mL of SMEDDS formulation was diluted with the 10 times and 1000 times with deionized water. Percentage transmittance were measured spectrophotometrically (Perkin Elmer Lamda 35 UV Spectrophotometer) at 560 nm using deionized water as a blank.

3.2.5.6.5. Thermodynamic stability studies

Thermodynamic stability studies were performed to evaluate the phase separation and effect of temperature variation on SMEDDS stability. All selected DCT SMEDDS formulations were diluted with deionized water (1:20) and centrifuged (Tomy Microvac) at 10,000 rpm for 20 min, and observed visually for phase separation.

Formulations that showed signs of phase separation were removed from further studies and the remaining formulations were subjected to a freeze-thaw study. Formulations were
diluted with deionized water at a ratio of 1:20 and subjected to two freeze-thaw cycles between −20°C and +25°C, with storage at each temperature for not less than 4 h.

3.2.5.6.6. Transmission Electron Microscopy

A transmission electron microscope (Philips CM12 Electron Microscope, Eindhoven, The Netherlands) was used to examine droplet morphology. Selected docetaxel SMEDDS formulation was diluted with water of a ratio of 1:100. A drop of the diluted microemulsion was directly deposited on the holey film grid to observe the morphology of formulations.

3.2.5.7. In-vitro release studies

To understand the characteristics of docetaxel release from SMEDDS, an in-vitro release was carried out. The dissolution test was performed in USP type-II dissolution apparatus II (Distek) according to United State Pharmacopoeia (USP 35) dissolution procedure. DCT SMEDDS hard gelatin capsule was put into a sinker. This sinker was loaded with 900 ml of 1% Tween 80 (w/v) in PBS solution pH 7.4, 1% Tween 80 (w/v) in pH 4.5 acetate buffer and 1% Tween 80 (w/v) in simulated gastric fluid without enzymes (pH 1.2) at 37 ± 0.5°C with paddle speed of 50 rpm. Each sample (2 ml) was withdrawn at 5, 15, 30, 45 and 60 min with replacement by an equal volume of temperature-equilibrated media. The concentration of docetaxel was determined by HPLC after appropriate dilution with methanol. The percent cumulative amount of docetaxel released from SMEDDS was calculated as a function of time.

3.2.5.8. Ex-vivo cell lines studies

3.2.5.8.1. Caco-2 Cells Uptake Study

In order to better address intestinal barrier integrity and better monitor experimental parameters, experiments were performed in vitro on selected DCT SMEDDS formulation. The objective of the present study was to investigate the permeability of selected PCT loaded SMEDDS formulation, when co-administered with novel P-gp inhibitor (i.e. GF120918) across intestinal barrier.

3.2.5.8.1.1. Caco-2 Cell Culture

Caco-2 cells originating from a human colorectal carcinoma were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Cells were cultured in
Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies, Gibco BRL, UK) supplemented with 20% heat-denaturated fetal calf serum (FCS, Pan Biotech GmbH, Germany), 1% (v/v) non-essential amino acids, 1.0 mM sodium pyruvate, penicillin (100 U/mL) and streptomycin (100 μg/mL) (Sigma Chemicals, St. Louis, MO). The cells were maintained at 37 ºC in an atmosphere of 95% air and 5% CO₂ at 90% relative humidity. Caco-2 cells of passage numbers 66 to 70 were used in these experiments. UV transparent 96-well Costar® Corning microplates were purchased from Corning® (Corning Life Sciences, Schiphol, The Netherlands). Transwell-COL inserts (0.4 μm pore size, 1.12 cm² growth area) and 12-well Transwell culture plates were purchased from Corning Costar, Cambridge, MA, USA.

3.2.5.8.1.2. Cell cytotoxicity study: WST-1 Assay

To ensure the caco-2 cells integrity during the permeability study, cytotoxicity assay, i.e. WST-1 assay, was carried out. WST-1 Assay is colorimetric assay designed to be used for the non-radioactive, spectrophotometric quantification of cell proliferation, growth, viability, and chemosensitivity in cell populations using the 96-well plate format. The cytotoxic effect of docetaxel using microemulsion formulations (formed from its SMEDDS formulation) was studied on Caco-2 Cells using WST-1 assay. Different concentration of docetaxel (2μM and 5μM) and GF120918 (5μM and 10μM) solution were investigated to determine the suitable dose at which these drugs would not affect the cell integrity during the course time in permeability assay.

The study was carried out on different group as per the protocol mentioned below:

- Group I: Docetaxel loaded SMEDDS with GF120918
- Group II: Docetaxel loaded SMEDDS without GF120918
- Group III: Docetaxel solution with GF120918
- Group IV: Docetaxel solution alone
- Group V: GF120918 alone
- Group VI: Culture Medium alone (Positive control)
- Group VII: 20 % SDS (200μg/mL) (negative control)

Collagen solution was prepared in PBS solution (1mg/ml) and loaded in 96-well plates for 1 hr. This solution was withdrawn at the predetermined time as the coating in each
well was ensured. Caco-2 cells were transferred to 96-well plates at a density of 5×10^4 cells/well. As the cells reached to 80% confluence, the medium was replaced with 100 μL of medium containing varying amounts of testing groups as mentioned above. One row of the 96-well plates was used as a positive control and culture medium was added to these wells. The plates were then incubated for 4h at 37 °C. The medium was then removed and the wells were washed three times with ice-cold PBS solution (100μL). After washing 100μL of WST-1, which was prepared by mixing 10μL of WST-1 stock solution and 90μL of incubation medium, respectively, was added to each well. Absorbance was measured using a Biotek PowerWave XS reader (Witec AG, Littau, Switzerland) at 450 nm. Cell cytotoxicity was expressed as the percent absorbance of tested group relative to absorbance measured for cells that were not exposed to any material.

3.2.5.8.1.3. Permeation Studies

The permeation of selected DCT SMEDDS formulation of docetaxel was studied in the apical to basolateral direction and basolateral to apical direction in Caco-2 cells. The various groups used for the study are mentioned in Table 3.7. All the SMEDDS formulations were diluted in deionized water (in the ratio of 1:20) to form respective microemulsions which were further used to evaluate their permeability in caco-2 cells. On the basis of cytotoxicity assay the concentration of docetaxel and GF120918 chosen for the studies were 5 μM and 10 μM, respectively.

<table>
<thead>
<tr>
<th>Code</th>
<th>Drug</th>
<th>Formulation</th>
<th>Pgp-inhibitor</th>
<th>Direction*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSME</td>
<td>Docetaxel</td>
<td>Microemulsion</td>
<td>Present</td>
<td>A-B</td>
</tr>
<tr>
<td>DSM</td>
<td>Docetaxel</td>
<td>Microemulsion</td>
<td>Absent</td>
<td>A-B</td>
</tr>
<tr>
<td>DSE (A-B)</td>
<td>Docetaxel</td>
<td>Solution</td>
<td>Present</td>
<td>A-B</td>
</tr>
<tr>
<td>DSE (B-A)</td>
<td>Docetaxel</td>
<td>Solution</td>
<td>Present</td>
<td>B-A</td>
</tr>
<tr>
<td>DS</td>
<td>Docetaxel</td>
<td>Solution</td>
<td>Absent</td>
<td>A-B</td>
</tr>
</tbody>
</table>
Bidirectional Permeation Assay

The enhancement in permeation of docetaxel using microemulsion formulation (formed from SMEDDS formulation) along with GF120918 was studied in the apical to basolateral direction and basolateral to apical direction in Caco-2 cell monolayers. For the in vitro permeation study, Caco-2 cells were seeded in 75 cm² flask at an initial seeding density of 10⁶ cells/flask, harvested at 80% confluence with trypsin–EDTA and seeded onto the apical side of collagen-coated Transwell-COL inserts (0.4 μm pore size, 1.12 cm² growth area) in 12-well Transwell culture plates (Corning Costar, Cambridge, MA, USA) at an initial seeding density of 10⁵ cells/well. The culture medium was added to the apical (0.5 mL) and basolateral (1.5 mL) compartments and replaced every other day for the first week and daily thereafter. Cells were incubated for 18–21 days until the transepithelial electrical resistance (TEER) increased to between 300 Ω/cm² and 600 Ω/cm². As the cell monolayers reached the desired TEER value, they were used for further experimentations. The TEER value of monolayers was checked before and after each experiment by using a Millicell®-ER voltohmmeter equipped with chopstick electrodes (Millipore Corporation, Bedford, MA, USA).

Before the transport study, the culture medium (DMEM) was washed and replaced with preheated (37 °C) transport medium consisting of Hanks' balanced salt solution (HBSS), supplemented with 10 mM HEPES (pH 7.4). After the cell monolayer was equilibrated for 30 min at 37 °C, TEER values of monolayers were determined in triplicate. For the apical to basolateral (A-B) transport study, various groups of docetaxel were evaluated for their ability to enhance drug permeation.

For apical to basolateral (A-B) study, each test group diluted with transport medium was added to the apical side (0.5 mL) and blank media on the basolateral side (1.5 mL). After 0.5 h, 1 h, 2 h and 3 h of incubation, 0.2 mL of medium at the basolateral side was withdrawn and replaced with fresh medium. For the investigation of basolateral-to-apical (B-A) transport, each test sample was placed in the basolateral side and blank media on the other side. After 0.5 h, 1 h, 2 h and 3 h of incubation, 0.1 mL of medium at the apical side was withdrawn and replaced with fresh medium.

The concentration of docetaxel was determined by HPLC analysis as developed earlier, and the amount of drug permeated was plotted as a function of time. The apparent
permeability coefficient ($P_{app}$) was determined from the linear slope of the plot using the following equation:

$$P_{app} = \frac{dQ/dt \times 1}{AC_0}$$

where, $P_{app}$ is the apparent permeability (cm/s), $dQ/dt$ is the steady state flux, $A$ is the surface area of the membrane (cm$^2$), $C_0$ is the initial concentration of drug at the apical (for A to B transport) or basolateral (for B to A transport) side.

### 3.2.5.8.2. Cell Uptake Studies: A549 Cell lines

Cellular uptake studies were done to investigate the potential of developed SMEDDS formulations in the presence or absence of GF120918 to enhance capture by tumor cells. A549 lung cancer cells were used for this purpose. SMEDDS formulation as evaluated earlier for docetaxel delivery was prepared and the drug was replaced by rhodamine 123 (Rh 123). The resulting Rh123 loaded SMEDDS formulation was denoted as SMEDDS II. SMEDDS formulation was diluted with deionized water (in ratio of 1:20) to form microemulsion which is further incubated with A549 cells to evaluate their uptake efficiency. Various groups tested in this study were:

- Microemulsion formed from SMEDDS II along with GF120918: RSME2
- Microemulsion formed from SMEDDS II without GF120918: RSM2
- Rh 123 solution with GF120918: RSE
- Rh 123 solution alone (positive control): RS
- Culture medium alone (negative control)

#### 3.2.5.8.2.1. A549 Cell Culture

A549 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were cultured in F12k medium supplemented with 10% fetal calf serum (FCS; Pan Biotech GmbH, Germany), penicillin (100 U/mL) and streptomycin (100 μg/mL) (Sigma Chemical, St. Louis, MO, USA) at 37 ºC and incubated in an atmosphere of 95% air and 5% CO$_2$ at 90% relative humidity. The cells at an initial seeding density of $10^6$ cells/flask were allowed to grow until confluence and were trypsinized and seeded in plates for each experiment. Experiments were performed with cells of passage numbers 78-84.
3.2.5.8.2.2. Quantitative study: Cellular uptake study

A549 cells were seeded in a 24-well plate (10$^5$ cells/well) and allowed to attach for 2 days. After 2 days, cells in each well were incubated with different groups as mentioned above for 4 h at 37 °C. After 4-hour incubation, the uptake was stopped by aspirating the various groups’ supernatants and the cells were washed 3 times with ice-cold PBS. Cells were then trypsinized to detach and collected in Eppendorf tubes. Subsequently, cells were lysed with 400 μL of 0.1% Triton X-100 for 10 min at room temperature. Total protein content in each sample was estimated using the Bradford protein assay. The cells were harvested by adding 400 μL of methanol. The cell lysate was then centrifuged at 10,000 rpm for 10 min. The Rh123 content in the supernatant after centrifugation was measured at excitation and emission wavelengths of 480 nm and 520 nm, respectively, using a Biotek PowerWave XS reader (Witec AG, Littau, Switzerland). Rh123 standard curve was generated to quantify the total amount of Rh123 accumulated in each sample. Cellular accumulation of Rh123 was then normalized to the total protein content as determined by the BCA assay. Uptake was expressed as the amount of rhodamine 123 per unit weight (mg) of total cellular protein. Results were expressed as percentages against control, i.e. cellular uptake by A549 cells in the absence of the inhibitor.

3.2.5.8.2.3. Qualitative Study: Confocal Laser Scanning Microscopy (CLSM)

A549 cells were seeded in 35 mm polystyrene cell culture dishes (4 × 10$^5$ cells/well) for different groups as mentioned above and allowed to attach for 48 h. After 2 days, cells in each well were incubated with different group as mentioned above for 4 h at 37 °C. After 4-hour incubation, the uptake was stopped by aspirating all the supernatants and the cells were washed with ice-cold phosphate buffered saline (PBS). After washing, the cells were fixed with 4% formaldehyde in DPBS (Dulbecco’s Phosphate Buffer Saline) for 15 min. Cells were then washed and stored in PBS for analysis using a Confocal Laser Scanning Microscope (Zeiss 710 2P, Zeiss, Jena, Germany) at excitation and emission wavelengths of 480 nm and 520 nm, respectively.

3.2.5.9. In vivo Study

3.2.5.9.1. Dose Selection

Dose selection of the DCT and GF120918 in Sprague-Dawley (S.D.) rats to carry out the oral single dose pharmacokinetic study was based on previous literature [Yin et al. 2009;
Bardelmeijer et al. 2004]. DCT and GF120918 were administered safely in rats at a dose of 8 mg/kg and 20 mg/kg, respectively via oral route. The in-vivo study was done as per the protocol approved by the Institutional Animal Ethics Committee, Jamia Hamdard (approval no. 684) and their guidelines were followed.

3.1.5.9.2. Animal and Dosing Procedure/Animal Study

20 healthy female SD rats weighing 200 ± 10 gm were used in the study. The rats were divided in various groups for the study as mentioned below:

- **Group I**: Docetaxel loaded SMEDDS with GF120918 solution
- **Group II**: Docetaxel loaded SMEDDS alone
- **Group III**: Docetaxel suspension with GF120918 solution
- **Group IV**: Docetaxel suspension alone
- **Group V**: Control group

The rats were housed under standard conditions. All rats were dosed orally using oral feeding tube following an overnight fast; food was returned 4 hours after dosing. In the first group, GF120918 solution was administered 2 hr prior to the administration of Docetaxel loaded SMEDDS formulation. In the second group, Docetaxel loaded SMEDDS alone was administered. In the third group, GF120918 solution was administered 2 hr prior to the administration of Docetaxel suspension (0.25% CMC Na and 1% Tween 80). In the fourth group, Docetaxel suspension alone was administered. Fifth group was used as control. The dose of Docetaxel and GF120918 in each one of these formulations was adjusted to contain 8 mg/kg and 20 mg/kg body weights, respectively. Blood samples (approx. 0.5 mL) was collected from retro-orbital plexus of rat in tube containing saturated solution of di-sodium EDTA at pre-dose, and 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 12.0, 24.0, and 48.0 hrs post dose. During collection, blood sample was mixed thoroughly with di-sodium EDTA solution in order to prevent blood clotting. Plasma was separated from centrifuge the blood at 5000 rpm in cooling centrifuge for 5 min and stored frozen at –20°C until analysis. Docetaxel analysis was done by previously developed and validated UPLC-MS method.

3.2.5.9.3. Data analysis
All values are expressed as the mean ± S.D. Statistical analysis was performed with GraphPad InStat software (version 3.00, GraphPad Software, San Diego, California, USA) using one-way ANOVA followed by Tukey–Kramer multiple comparison test. Difference with P > 0.05 was considered statistically significant.

3.2.5.10. Stability studies

Ability of a formulation to retain properties in specified limits throughout its shelf life is referred as stability. Stability of a pharmaceutical product may be defined as a capability of a particular formulation, in a specific container, to remain within its physical, chemical, microbiological, therapeutic and toxicological specifications. Stability of a formulated product on shelf becomes an important factor in successful development of dosage form. Hence, a well-designed stability-testing plan is essential for the development of SMEDDS formulations.

Hard gelatin capsules (size ‘00EL’) filled with DCT loaded preconcentrate (DCT SMEDDS) was subjected to the accelerated (40°C/75%RH) and long term stability (25°C/60%RH) conditions for the period of 3 months. Samples were charged in stability chambers (Thermolab, Mumbai, India) with humidity and temperature control. Samples were withdrawn and analyzed for particle size and residual drug content after a period of 30, 60 and 90 days. DCT content in capsules was analysed by HPLC method presented in section 3.2.3.