### 6. MATERIALS AND METHODS

#### 6.1. Materials Used

##### 6.1.1. List of materials used

<table>
<thead>
<tr>
<th>S.No</th>
<th>Chemicals/Materials</th>
<th>Manufacturers/Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Curcumin</td>
<td>Sigma, Aldrich, Mumbai</td>
</tr>
<tr>
<td>2</td>
<td>Quercetin</td>
<td>S.D. Fine Chem Ltd, Mumbai</td>
</tr>
<tr>
<td>3</td>
<td>Piperine</td>
<td>S.D. Fine Chem Ltd, Mumbai</td>
</tr>
<tr>
<td>4</td>
<td>Rutin</td>
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<td>5</td>
<td>Black pepper – <em>Piper nigrum</em></td>
<td>Natural remedies, Bangalore</td>
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<td>6</td>
<td><em>Curcuma</em> extract – <em>Curcuma longa</em></td>
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<td>Green Tea – <em>Camellia sinensis</em></td>
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<td>Orange peel - <em>Citrus sinensis</em></td>
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<td>Liquorice extract - <em>Glycyrrhiza glabra</em></td>
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<td>Polyvinyl pyrrolidine</td>
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<td>31</td>
<td>Tween 80 LR</td>
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### 6.1.2. List of equipments used

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<td>RP-UFLC</td>
<td>Shimadzu LC-20 AD, SPD-M201 230V, Japan</td>
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<td>2</td>
<td>UV/Visible spectrophotometer</td>
<td>UV-1700 series, Shimadzu</td>
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<td>3</td>
<td>FTIR spectrophotometer</td>
<td>FTIR-8400S, Shimadzu, Japan</td>
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<td>Research centrifuge</td>
<td>Remi Instruments, Mumbai, India</td>
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<td>Digital pH meter</td>
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<td>Freeze dryer</td>
<td>Christ Alpha1-2 LD plus, Germany</td>
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<td>Magnetic stirrer</td>
<td>Remi Equipments, Mumbai, India</td>
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<td>Malvern ZS 90, UK</td>
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<td>Differential scanning calorimeter</td>
<td>TA Instrument, USA</td>
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<td>Tablet punching machine</td>
<td>Rimek, Ahmedabad, India</td>
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<td>17</td>
<td>Dissolution apparatus</td>
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<td>Friability apparatus</td>
<td>Electro lab, Mumbai, India</td>
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<td>19</td>
<td>Tray dryer</td>
<td>Cadmach, Ahmedabad, India</td>
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6.2. Methods

6.2.1. Analytical method development and validation

A. Analytical method

Development of the analytical method depends upon the nature of the compounds such as the molecular weight, solubility and ionic strength. For the analysis of the analytes in high performance liquid chromatography (HPLC) with ultra violet/photodiode array detector (UV/ PDA) detector, it’s important that the compound should have the chromo pores. In the present study, reverse phase ultrafast liquid chromatography (RP-UFLC) method was considered for the analysis of the analytes. Analytical method for the selected phytoconstituents was carried out by following parameters such as,

- Selection of detection wavelength
- Selection of stationary phase
- Selection of mobile phase

I. Determination of detection wavelength

The sensitivity of the method depends upon the proper selection of the wavelength for detection of the phytoconstituents. The idea wavelength of the select phytoconstituents is one that gives a good response. A stock solution of curcumin, quercetin and piperine was prepared by dissolving 10 mg of each phytoconstituents in 10 ml of acetonitrile. Similarly rutin solution was prepared by dissolving 10 mg of rutin in 10 ml of DMSO. The stock solutions were stored in refrigerator at –20 ± 2°C until analysis. From the stock solution, suitable dilutions were prepared using the same solvent to produce 100 μg/ml solutions. \( \lambda_{\text{max}} \) of the drugs was determined by scanning the prepared solution between 800 to 200 nm using a UV-visible spectrophotometer.

II. Selection of stationary phase

Selection of the stationary phase was carried out by the nature of the polarity of the compounds. In this method development, different reverse phase columns (C₈, C₁₈ and ODS) were used for the separation of the phytoconstituents. Depending on the response, retention time and peak shape, Phenomenex C₁₈ (250 x 4.6 mm i.d., 5μ) column was used.
III. Selection of mobile phase
Selection of the mobile phase was done based on the solubility and stability of the phytoconstituents. Various mobile phases were tried in the RP-UFLC to achieve a good peak response and peak separation. Initially different methanol: buffers ratios were carried out for the separation of the phytoconstituents. Followed by different ratio of water: buffers, water: methanol and acetonitrile: buffers were carried out. The different chromatography condition like pH of the buffer, flow rate, different in organic phase ratios and peak modified were studied.

IV. Buffer preparation
A weighed quantity of 0.9635 gm of ammonium acetate was taken in a 500 ml beaker 300 ml of milli Q water was added and kept for sonication for about 10 min. The resulting solution was made up to 500 ml with milli Q water and the pH was adjusted to 3 with ortho phosphoric acid and prepared solution degassed on a sonicator.

V. Chromatographic conditions
Stationary phase : Phenomenex C_{18} (250 x 4.6 mm i.d., 5μ)
Mobile Phase : Acetonitrile: ammonium acetate buffer
Mobile phase ratio : 80:20
Flow rate : 1.0 ml/min
Sample volume : 20 μl using Rheodyne 7725i injector
Detection : 380 nm
Detector : Photo Diode Array (PDA)
Data station : LC Solutions
Retention time for rutin : 2.2 min
Retention time for quercetin : 2.8 min
Retention time for curcumin : 3.8 min
Retention time for piperine : 4.5 min
Buffer strength : 25 mM
pH : 3
B. Validation of the developed method

Validation is a process which involves confirmation or establishment by laboratory studies that a method / procedure / system / analyst can give the required accuracy, precision, sensitivity, ruggedness, etc. In the most basic form, validation of an analytical procedure demonstrates that the procedure developed is suitable for its intended purpose. Validation of the method was carried out after the development of the HPLC methods. Validation parameters tested such as, selectivity/ specificity, sensitivity, linearity, precision, accuracy, ruggedness & robustness were carried out as per the ICH Q2R1 guidelines [100].

B.1. Solution preparation

I. Preparation of standard solution

A stock solution of curcumin, quercetin and piperine was prepared by dissolving 10 mg of each phytoconstituents in 10 ml of acetonitrile. Similarly rutin solution was prepared by dissolving 10 mg of rutin in 10 ml of DMSO. The stock solutions were stored in refrigerator at –20 ± 2°C until analysis.

II. Preparation of sample solution

Each extract was accurately weighed (100 mg) and dissolved in 70 ml of methanol and then sonicated for 30 min. The final volume of solution was adjusted to 100ml with methanol and filtered through a membrane filter (0.22 μm). Then it was subjected to RP-UFLC analysis.

B.2. Validation

I. Specificity

A developed method is said to be specific when it produces a response only for a single analyte. Method selectivity is the ability of the method to produce a response for the analyte in the presence of other interferences. In order to prove that the method chosen was specific and selective the standard peak of the phytoconstituents and the sample peak of extracts were compared with the retention time.
II. Sensitivity

It is expressed as limit of quantitation. It is the lowest amount of analyte in a sample matrix that can be determined. Three injections of the lowest concentration were injected and the spectra were determined.

III. Precision

The precision of the method was determined by analyzing two batches each consisting of six injection at three different concentration of rutin (20, 60, 100 μg/ml), quercetin (20, 60, 100 μg/ml), curcumin (20, 60, 100 μg/ml) and piperine (20, 60, 100 μg/ml). Precision is expressed as the percentage coefficient of variation (% CV) which was calculated as per the following expression

\[
% \text{ CV} = \frac{\text{Standard Deviation}}{\text{Mean}} \times 100
\]

Intra-run precision

Intra-run precision was determined by calculating the percentage coefficient of variation (% CV) of the results obtained in the same run.

Intra-day precision

Intra-day precision was determined by calculating the percentage coefficient of variation (% CV) of the results obtained in the same day.

Inter-day precision

Inter-day precision was determined by calculating the percentage coefficient of variation (% CV) of the results obtained over at least two days.

IV. Accuracy

Accuracy of the developed method was determined by recovery studies. Recovery of the method was carried out by adding the known concentration of the standard to the sample extract and analyzed. The results of the solution were compared with the true results. This accuracy experiment was carried out by analyzing six replicates (n=6) at three different concentration of quality control levels. The mean, standard deviation (SD) and percentage relative standard deviation (% RSD) were calculated.
Accuracy was reported as % nominal of the analyzed concentration which was calculated as

\[
\% \text{ Nominal} = \frac{\text{Measured Concentration}}{\text{Actual Concentration}} \times 100
\]

V. Linearity

Linearity and range of the methods were analyzed by preparing calibration curves using different concentrations range of the standard phytoconstituents solutions. Linearity for rutin, quercetin, curcumin and piperine were established over the range of 10-100 µg/ml using the weighted least square regression analysis. From the prepared stock solutions (1000 µg/ml), aliquots of 0.1, 0.2, 0.4, 0.6, 0.8 and 1 ml were pipette out separately into a 10ml volumetric flask and made up the volume to 10 ml with acetonitrile solution to produce the concentration of 10-100 µg/ml. This solution was then analyzed in the RP-UFLC method at 380 nm. The calibration curve was plotted using mean peak area on X axis and concentration of the standard phytoconstituents solutions on y axis. From the calibration curve intercept, slope and regression equation were calculated.

VI. Ruggedness

Ruggedness of the method was studied by changing the experimental conditions such as operators, instruments, source of reagents, solvents and column of similar type. Chromatographic parameters such as retention time, asymmetric factor, capacity factor and selectivity factor were evaluated.

VII. Robustness

Robustness of the method was studied by injecting the standard solutions with slight variations in the optimized conditions namely, ± 1% in the ratio of acetonitrile in the mobile phase, ± 0.1 in the pH value and ± 0.1 ml of the flow rate.

VIII. Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantitation (LOQ) were reported as 3 and 10 times the noise level obtained from three replicate injections of samples respectively.
6.2.2. Standardization of herbal extract

A. Quantification of herbal extract

About 100 mg of the each herbal extracts as shown in the table 1 was accurately weighed in a 100 ml of volumetric flask separately to this 60-70 ml of methanol was added and sonicated for 45-60 min and the final volume was made up with methanol. This solution was then filtered through 0.45 \( \mu \) membrane filter and diluted with acetonitrile solution. The diluted solutions were injected in to the RP-UFLC system for analysis and the amount of phytoconstituents was estimated.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Herbal extracts</th>
<th>Phytoconstituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Black pepper – <em>Piper nigrum</em></td>
<td>Piperine</td>
</tr>
<tr>
<td>2</td>
<td>Curcuma extract – <em>Curcuma longa</em></td>
<td>Curcumin</td>
</tr>
<tr>
<td>3</td>
<td>Green Tea – <em>Camellia sinensis</em></td>
<td>Rutin, Quercetin</td>
</tr>
<tr>
<td>4</td>
<td>Orange peel - <em>Citrus aurantium</em></td>
<td>Rutin, Quercetin</td>
</tr>
<tr>
<td>5</td>
<td>Long pepper - <em>Piper longum</em></td>
<td>Piperine</td>
</tr>
<tr>
<td>6</td>
<td>Thyme extract - <em>Thymus vulgaris</em></td>
<td>Rutin, Quercetin</td>
</tr>
<tr>
<td>7</td>
<td>Liquorice extract - <em>Glycyrrhiza glabra</em></td>
<td>Rutin, Quercetin</td>
</tr>
</tbody>
</table>

B. Forced degradation studies of phytoconstituents

Forced degradation studies was determined for the selected phytoconstituents as per ICH Q1A (R2) guidelines [101], parameters such as acid, alkali, oxidative and thermal degradation were carried out. Sample solutions were prepared individually for different degradation analysis were shown in the table 2.

I. Hydrolytic studies

*Acid hydrolysis study for quercetin, curcumin and piperine*

About 10 mg of quercetin, curcumin and piperine was weighed in a 100ml volumetric flask to that 60-70 ml of methanol was added and kept for sonication for 5-10 min to complete dissolve. 1ml of 0.1N HCl solution was added and made up the volume to 100ml with methanol. Samples were withdrawn at regular time intervals of 1, 2, 18
and 24 h and filtered. The filtered solutions were analyzed in the optimized RP-UFLC method.

**Acid hydrolysis study for rutin**
About 10 mg of rutin was weighed in a 100 ml volumetric flask to that 50 ml of DMSO was added and kept for sonication for 5-10 min to complete dissolve. 1ml of 0.1N HCl solution was added and made up the volume to 100 ml with methanol. Samples were withdrawn at regular time intervals of 1, 2, 18 and 24 h and filtered. The filtered solutions were analyzed in the optimized RP-UFLC method.

**Alkaline hydrolysis study for quercetin, curcumin and piperine**
About 10 mg of quercetin, curcumin and piperine was weighed in a 100ml volumetric flask to that 60-70 ml of methanol was added and kept for sonication for 5-10 min to complete dissolve. 1ml of 0.1N NaoH solution was added and made up the volume to 100 ml with methanol. Samples were withdrawn at regular time intervals of 1, 2, 18 and 24 h and filtered. The filtered solutions were analyzed in the optimized RP-UFLC method.

**Alkaline hydrolysis study for rutin**
About 10 mg of rutin was weighed in a 100ml volumetric flask to that 50 ml of DMSO was added and kept for sonication for 5-10 min to complete dissolve. 1ml of 0.1N NaoH solution was added and made up the volume to 100 ml with methanol. Samples were withdrawn at regular time intervals of 1, 2, 18 and 24 h and filtered. The filtered solutions were analyzed in the optimized RP-UFLC method.

**II. Oxidative study**

**Oxidative study for quercetin, curcumin and piperine**
About 10 mg of quercetin, curcumin and piperine was weighed in a 100ml volumetric flask to that 60-70 ml of methanol was added and kept for sonication for 5-10 min to complete dissolve. 1ml of 40 % of hydrogen peroxide was added and made up the volume to 100ml with methanol. Samples were withdrawn at regular time intervals of 1, 2, 18 and 24 h and filtered. The filtered solutions were analyzed in the optimized RP-UFLC method.
Oxidative study for rutin

About 10 mg of rutin was weighed in a 100ml volumetric flask to that 50 ml of DMSO was added and kept for sonication for 5-10 min to complete dissolve. 1 ml of 40% hydrogen peroxide, was added and made up the volume to 100ml with methanol. Samples were withdrawn at regular time intervals of 1, 2, 18 and 24 h and filtered. The filtered solutions were analyzed in the optimized RP-UFLC method.

III. Thermolytic study

Thermolytic studies for quercetin, curcumin and piperine

Sample solutions of phytoconstituents (quercetin, curcumin and piperine) were prepared by taking 10 mg phytoconstituents in a 100ml of volumetric flask, to this 60-70 ml of methanol was added and kept for sonicator for 5-10 min for complete dissolve and the volume was adjusted. The above solution was heated at 70°C for 30 min and the volume was adjusted for 100ml. Samples were withdrawn at regular time intervals of 1, 2, 18, and 24 h and filtered. The filtered solutions were analyzed in the optimized RP-UFLC method (as shown in table 2).

Thermolytic studies for rutin

Sample solution of phytoconstituents (rutin) was prepared by taking 10 mg phytoconstituents in a 100 ml of volumetric flask, to this 50 ml of DMSO was added and kept for sonicator for 5-10 min for complete dissolve and the volume was adjusted with methanol. The above solution was heated at 70°C for 30 min and the volume was adjusted for 100 ml with methanol. Samples were withdrawn at regular time intervals of 1, 2, 18, and 24 h and filtered. The filtered solutions were analyzed in the optimized RP-UFLC method.
### Table 2. Conditions for forced degradation studies for phytoconstituents

<table>
<thead>
<tr>
<th>S.No</th>
<th>Phytoconstituents</th>
<th>Amount added (mg)</th>
<th>Acid degradation (0.1N HCl)</th>
<th>Alkali degradation (0.1N NaOH)</th>
<th>Peroxide degradation (40% v/v)</th>
<th>Thermal degradation</th>
<th>Conditions</th>
<th>Total volume (ml)</th>
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<tbody>
<tr>
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<td>Rutin</td>
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</table>
6.2.3. Biopharmaceutical classification system for herbal extracts and the phytoconstituents

Biopharmaceutical classification system for the herbal extract and the phytoconstituents were carried out based on the solubility and permeability studies.

A. Solubility studies (Shake flask method)

The pH solubility profile of herbal extracts and phytoconstituents was determined by shake flask method [102]. Selected herbal extracts, curcuma (Curcuma longa), green tea (Camellia sinensis), black pepper (Piper nigrum), long pepper (Piper longum), orange peel (Citrus sinensis), thyme (Thymus vulgaris) and liquorice (Glycyrrhiza glabra) were weighed as per the maximum dose number of the phytoconstituents such as rutin 500 mg, quercetin 500 mg, curcumin 500 mg and piperine 10 mg in a separate 500 ml conical flask. To the above conical flask, about 250 ml of various pH buffer solutions like 1.2, 4.5, 6.8 and 7.4 were added. These solutions were kept inside orbital shaker as shown in the fig 3 and maintained at 100 rpm for 72 h at 37 ± 1°C. Minimum of three replicate determinations of solubility in each pH (1.2, 4.5, 6.8 and 7.4) condition were carried out. Standard buffer solutions described in the USP are considered appropriate for use in solubility studies. After equilibrium the solutions were filtered and then diluted to measure the concentration of the phytoconstituents and herbal extracts by a RP-UFLC method and the dose number was calculated as follows

**Dose number (D₀)**

Dose number of the herbal extract and phytoconstituents were calculated by the relationship between maximum dose strength and solubility.

$$D_0 = \frac{M_0}{V_0} \times C_S$$

Where $M_0$ – Highest dose strength (mg); $V_0$ – Volume of water taken with dose (250 ml); $C_S$ – minimum physiologic solubility at pH 1.2, 4.5 and 6.8 at 37°C (mg/ml).

In general compounds with $D_0$ value as less than 1 are considered as highly soluble.
B. Permeability studies

Design of the Permeability apparatus (Gowshan)

The permeability apparatus was fabricated and patented (4590/CHE/2013 dated on 10-10-13) consisted of two parts of disposable syringes, both the two disposable syringes were tied in glass rod at a distance of 6 cm separately at the top position of the syringe, at the bottom position of the syringe was tied with the syringe piston at the distance of 5 cm in order to prevent the damage of the intestinal. The inner diameter of the syringe consists of 1 cm and at the end of the attachment position of the syringe and intestinal consists of 0.4 cm. The assembly allows the adjustment to tie the intestinal segment at both the end. This apparatus allow mounting in dissolution apparatus for further permeability studies as shown in fig 4. The design model was validated with different BCS class of drugs such as caffeine (high permeable) and paracetamol (low permeable).
Fig 4. Permeability apparatus model (Gowshan)

Everted Intestine model
Male white Leghorn chicks weighing between 500 and 600 gm were bought from the local market. The Krebs–Ringer solution was prepared by combining 6.3 gm NaCl, 0.35 gm KCl, 0.14 gm CaCl₂, 0.16 gm KH₂PO₄, 0.15 gm MgSO₄·7H₂O, 2.1 gm NaHCO₃ and 5 gm glucose in one litre of distilled water. For isolation of everted intestine, the chick was slaughtered, a median incision of the abdomen was performed, and the small intestine was freed (Fig 5 A). The lumen was carefully cleared from mucus by rinsing with a pH 7.4 buffer solutions (Krebs–Ringer solution). An intestinal segment of the first 6-cm length was removed and transferred to oxygenated Krebs–Ringer solution (Fig 5 B). It was washed thoroughly with warm Krebs–Ringer solution and the fat layer adjoining the intestine was removed carefully (Fig 5 C). The proximal extremity of the intestine was turned back and checked for any damage (Fig 5 D, E) and ligated on a specially design glass rod to form an everted bag (Fig 5 F, G). Permeability of each extract, curcuma (Curcuma longa), green tea (Camellia sinensis), black pepper (Piper nigrum), long pepper (Piper longum), orange peel (Citrus aurantium), thyme (Thymus vulgaris) and liquorice (Glycyrrhiza glabra); and the phytoconstituents such as rutin, quercetin, curcumin and piperine were determined separately by placing weighed quantity as per the dose number of the phytoconstituents in a specially design model (Gowshan Fig 5 G) which was placed in the dissolution vessel (Fig 5 H, I). This vessel was then filled with the SGF media, supplied with the oxygen. Permeability studies were performed
on six vessels. About 5 ml of samples were withdrawn at 0.0, 15, 30, 45, 60, 90 and 120 min time intervals. Equal quantity of the SGF medium was replaced to the dissolution jar after each sampling as shown in the (Fig 5 I). The amount of the permeably released was estimated by optimized and validated RP-UFLC methods.

Fig 5. Permeability studies by everted chicken intestine model

C. Classification of herbal extracts and phytoconstituents into BCS
Based on the dose number and permeability values of the respective phytoconstituents and herbal extracts were fitted into the biopharmaceutical classification system (BCS).
Class I – High soluble and high permeable
Class II – Low soluble and high permeable
Class III – High soluble and low permeable
Class IV – Low soluble and low permeable
6.2.4. Physical modification approach for enhancing solubility and permeability
In the present study quercetin, curcumin and piperine were selected for the further physical modification to obtain poly herbal nano preparation by omitting rutin due to the difficulty in accumulate four phytoconstituents at a time in a single process. The possible reason is that the selected phytoconstituents number was more (> 4) could not be encapsulated properly with lipids. The amount of lipids was low when compared with that of phytoconstituents. In connection with the percentage recovery rutin showed low recovery when compared with that of other phytoconstituents. Hence, due to the practical difficulties rutin was rejected.

A. Preparation of poly herbal nano crystals
I. Optimization of poly herbal nano crystals
Poly herbal nano crystals (NC) were prepared by solvent / antisolvent precipitation technique [16, 103]. 35mg of quercetin, 35 mg of curcumin and 1.4 mg of piperine were dissolved in 100 ml ethanol. The obtained phytoconstituents solution then added in drop wise to the different surfactant concentration as shown in the table 3 which was dissolved in 20 ml of water. The reaction was maintained in a magnetic stirrer at 2000 rpm for 20 min. Precipitation of solid drug particles occurred immediately upon mixing. This suspension then was subjected to sonication with the help of probe sonicator for 5 min. This suspension was subjected to centrifugation at 11000 rpm for 15 min. The upper clear liquid was removed and the remaining residue was separated. Lyophilization of the NC dispersion was carried out by using 2% w/w of mannitol as cryoprotectant. The poly herbal NC dispersions were frozen in aqueous cryoprotectant solution at -20°C for about 24 h and then the samples were transferred to the freeze-dryer (Christ, Alpha 2-4 LD plus, Germany) operated at -40°C and pressure of 0.001bar for 72 h to obtained the NC free flowing powder for further experiments. The influence of other stabilizers such as triethyl amine (TEA), phronic F 68 and tween 80 was also studied in the above said procedure.
Table 3. Composition of surfactant concentration used

<table>
<thead>
<tr>
<th></th>
<th>FC*</th>
<th>Drug (mg)</th>
<th>TEA</th>
<th>Pluronic F 68 (mg)</th>
<th>Tween 80</th>
<th>Speed (rpm)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC-1</td>
<td>71.4</td>
<td>0.2%</td>
<td>250</td>
<td>-</td>
<td>-</td>
<td>2000</td>
<td>20</td>
</tr>
<tr>
<td>NC-2</td>
<td>71.4</td>
<td>0.5%</td>
<td>500</td>
<td>-</td>
<td>-</td>
<td>2000</td>
<td>20</td>
</tr>
<tr>
<td>NC-3</td>
<td>71.4</td>
<td>-</td>
<td>250</td>
<td>0.2%</td>
<td>-</td>
<td>2000</td>
<td>20</td>
</tr>
<tr>
<td>NC-4</td>
<td>71.4</td>
<td>-</td>
<td>500</td>
<td>0.5%</td>
<td>-</td>
<td>2000</td>
<td>20</td>
</tr>
</tbody>
</table>

*Formulation code

II. Evaluation of poly herbal nano crystals

**Particle size and zeta potential**

Particle size and zeta potential of the poly herbal NC were measured by photon correlation spectroscopy using a Malvern Zetasizer Nano ZS 90 (Malvern Instruments, Worcestershire, UK), which works on the Mie theory. All size and zeta potential measurements were carried out at 25°C using disposable polystyrene cells and disposable plain folded capillary zeta cells, respectively, after appropriate dilution with original dispersion preparation medium.

**Polydispersity Index**

Polydispersity was determined according to the equation,

\[
\text{Polydispersity} = \frac{D(0.9) - D(0.1)}{D(0.5)} \times 100
\]

Where,

- \( D(0.9) \) corresponds to particle size immediately above 90% of the sample.
- \( D(0.5) \) corresponds to particle size immediately above 50% of the sample.
- \( D(0.1) \) corresponds to particle size immediately above 10% of the sample.

**External Morphological Study (Scanning electron microscopy)**

External surface morphology of NC was determined using Scanning Electron Microscopy (SEM). Samples were weighed about 9-10 mg which was then mounted on an aluminum stub with double side adhesive tape. The tape was firmly attached to the stub and lyophilized sample was scattered carefully over its surface carefully over its surface. The stub with the sample was then sputter coated with a thin gold layer (JFC 1200 fine coater, Japan) to ensure the sample conductive. This processed sample
was subject for the SEM analysis for external surface morphology determination. The samples were spread on a sample holder and dried using vacuum.

**B. Preparation of poly herbal solid lipid nano particles (SLN)**

**I. Compatiblility studies**

Infrared spectral matching approach was employed to detect any possible chemical interaction between quercetin, curcumin, piperine, lipid, soya lecithin and pluronic F 68. Physical mixtures of the herbal phytoconstituents and the excipients (1:1) were mixed with 400 mg of potassium bromide (IR grade). About 100 mg of the mixture was taken and compressed to form a transparent pellet in a hydraulic press at 15 ton pressure. The samples were scanned from 4000 to 400 cm\(^{-1}\) in a Shimadzu FT-IR spectrophotometer. Similarly, the IR spectra of quercetin, curcumin, piperine and the excipients were also recorded. Physical appearance of the samples and appearance / disappearance of peaks in the spectra were observed to assess any possible physical and chemical interactions.

**II. Lipid solubility studies**

Lipid solubility of the selected phytoconstituents was determined in different lipids viz. Glyceryl mono stearate (GMS), tween 80, tween 20, stearic acid and tristearin. 10mg of phytoconstituents was weighed accurately in a separate beaker and it was dispersed in a mixture of melted lipid (1gm) and shaken for 30min in a water bath shaker maintained at 10°C above the melting point of lipid. This lipid solution was centrifugation at 10000 rpm for 5 min. The clear supernatant obtained was suitably diluted with organic solvent and content was determined in RP-UFLC.

**III. Optimization of method**

*Optimization of lipid quantity*

Solid lipid nanoparticles for phytoconstituents were prepared by micro-emulsion technique. Lipid optimization for the GMS was carried out by keeping the phytoconstituents concentration constant and varying the amount of the lipid. Six different concentration batches were prepared by varying GMS lipid such as 1:2, 1:4, 1:6, 1:8, 1:10 and 1:15. The particle size, zeta potential and PDI were evaluated for the optimization of lipid concentration batches.
**Optimization of stirring time**

Optimization of the stirring time was carried out by keeping the phytoconstituents concentration, lipid concentration, surfactant concentration and stirring speed constant and varying the stirring time of 1, 2, 3 and 4 h.

**Optimization of stirring speed**

Optimization of the stirring time was carried out by keeping the phytoconstituents concentration, lipid concentration, surfactant concentration and stirring time constant and varying the stirring speed of 1000, 1500 and 2000 rpm respectively.

**Optimization of surfactant concentration**

Optimization of the stirring time was carried out by keeping the phytoconstituents concentration, lipid concentration, stirring speed and stirring time constant and varying the surfactant concentration (Pluronic F 68) of 1%, 1.5% and 2% w/v respectively.

**IV. Preparation of poly herbal solid lipid nanoparticles by micro emulsion method**

Solid lipid nanoparticles (SLN) were prepared by micro emulsion technique and the Schematic representation of preparation of poly herbal SLN was shown in the fig 6 [104, 105]. In which O/W micro emulsion was prepared and the oil phase was glycerol mono stearate. Soy lecithin was used as a lipophilic surfactant and Pluronic F 68 (hydrophilic surfactant) solution was used as continuous phase. The chosen lipid (720 mg) and soy lecithin (1%) were melted at 60°C and 35 mg of curcumin, 35 mg of quercetin and 2 mg of piperine were added under stirring. 10 ml of aqueous surfactant solution containing Pluronic F 68 (1.5%) heated at same temperature (60°C) was added to the melted lipid with mechanical stirring for 15 min. A clear micro emulsion was obtained under stirring at a temperature close to the melting point of the lipid used. SLN were obtained by dispersing the warm o/w micro emulsion drop wise into ice cold water in a beaker under continuous stirring speed of 2000 rpm. Poly herbal SLN dispersion was further stirred after complete addition of micro emulsion. After completion of stirring for 3 h, the poly herbal SLN dispersion was subjected to sonication using probe sonicator for 2 min (table 4). Then, the solution was centrifuged at 11,000 rpm for 15 min and the Supernatant liquid was
Materials and methods

Lyophilization of the SLN dispersion was carried out by using 2% w/w of mannitol as cryoprotectant. SLN dispersions were frozen in aqueous cryoprotectant solution at -20°C for about 24 h and then the samples were transferred to the freeze-dryer (Christ, Alpha 2-4 LD plus, Germany) operated at ~40°C and pressure of 0.001 bar for 72 h to obtained the SLN powders for further experiments.

**Fig 6. Schematic representation of preparation of solid lipid nanoparticles**
Table 4. Optimized parameters for solid lipid nanoparticles

<table>
<thead>
<tr>
<th>S.No</th>
<th>Parameter</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lipid</td>
<td>GMS</td>
</tr>
<tr>
<td>2</td>
<td>Amount of lipid</td>
<td>720 mg</td>
</tr>
<tr>
<td>3</td>
<td>Drug</td>
<td>72 mg</td>
</tr>
<tr>
<td>4</td>
<td>Soy lecithin</td>
<td>1% w/w</td>
</tr>
<tr>
<td>5</td>
<td>Surfactant (pluronic F 68)</td>
<td>1.5% w/w</td>
</tr>
<tr>
<td>6</td>
<td>Stirring time</td>
<td>3 h</td>
</tr>
<tr>
<td>7</td>
<td>Stirring speed</td>
<td>2000 rpm</td>
</tr>
</tbody>
</table>

Soy lecithin (% w/w w.r.t. lipid)

V. Evaluation for solid lipid nanoparticles

**Percent recovery of nanoparticles**

Recovery of the prepared poly herbal SLN was calculated by using the following formula,

\[
\% \text{ Recovery} = \frac{\text{Total mass of the nano particle recovered}}{\text{Mass of the drug, mass of the polymer and other excipients used}} \times 100
\]

**Particle size and zeta potential**

Particle size and zeta potential of the prepared SLN were measured by photon correlation spectroscopy using a Malvern Zetasizer Nano ZS 90 (Malvern Instruments, Worcestershire, UK), which works on the Mie theory. All size and zeta potential measurements were carried out at 25°C using disposable polystyrene cells and disposable plain folded capillary zeta cells respectively, after appropriate dilution with original dispersion preparation medium.

**Poly dispersity Index**

Polydispersity was determined according to the equation

\[
\text{Polydispersity} = \frac{D(0.9) - D(0.1)}{D(0.5)} \times 100
\]

Where,

D (0.9) corresponds to particle size immediately above 90% of the sample.
D (0.5) corresponds to particle size immediately above 50% of the sample.
D (0.1) corresponds to particle size immediately above 10% of the sample

**Encapsulation efficiency and drug loading**

Entrapment efficiency and drug loading of prepared poly herbal SLN were determined according to the procedure described as follows. About 10 mg of poly herbal SLN were weighed accurately in a 100 ml beaker and 20 ml of methanol was added and kept under water bath at 70°C for 30 min and then cooled to room temperature to dissolve the drug. Supernatant liquid was separated after centrifuged at 4000 rpm for 15 min. The resulting solution was determined by RP-UFLC method.

\[
\text{Entrapment efficiency} = \frac{\text{Particle weight of the drug in SLN}}{\text{Theoretical weight of the drug in the SLN formulation}} \times 100
\]

\[
\text{Drug entrapment efficiency (\%)} = \frac{\text{Analyzed weight of drug in SLN}}{\text{Theoretical weight of drug loaded in SLN}} \times 100
\]

\[
\text{Drug loading (\%)} = \frac{\text{Analyzed weight of drug in SLN}}{\text{Analyzed weight of SLN}} \times 100
\]

\[
\text{Entrapment efficiency} = \frac{1 - \text{Free drug}}{\text{Theoretical drug loaded}} \times 100
\]

**External Morphological Study (Scanning electron microscopy)**

External surface morphology of poly herbal SLN was determined using Scanning Electron Microscopy (SEM). Samples were weighed about 9-10 mg which was then mounted on an aluminum stub with double side adhesive tape. The tape was firmly attached to the stub and lyophilized sample was scattered carefully over its surface carefully over its surface. The stub with the sample was then sputter coated with a thin gold layer (JFC 1200 fine coater, Japan) to ensure the sample conductive. This processed sample was subject for the SEM analysis for external surface morphology determination. The samples were spread on a sample holder and dried using vacuum.
6.2.5. Physically modified poly herbal NC/SLN towards the BCS

A. Solubility (Shake flask method)

The pH solubility profile of the prepared poly herbal nano crystals and poly herbal solid lipid nano particles were determined by shake flask method. The developed poly herbal NC and poly herbal SLN were weighed as per the maximum dose number of the phytoconstituents such as rutin 500 mg, quercetin 500 mg, curcumin 500 mg and piperine 10 mg in a separate 500 ml conical flask. To the above conical flask, about 250 ml of various pH buffer solutions like 1.2, 4.5, 6.8 and 7.4 were added. These solutions were kept inside orbital shaker and maintained at 100 rpm for 72 h at 37 ± 1°C. Minimum of three replicate determinations of solubility in each pH (1.2, 4.5, 6.8 and 7.4) condition were carried out. Standard buffer solutions described in the USP are considered appropriate for use in solubility studies. After equilibrium the solutions were filtered and then diluted to measure the concentration of the phytoconstituents by a RP-UFLC method and the dose number was calculated as follows,

Dose number \((D_0)\)

Dose number of the phytoconstituents was calculated by the relationship between maximum dose strength and solubility.

\[ D_0 = \frac{M_0}{V_0} \times C_s \]

Where \(M_0\) – Highest dose strength (mg); \(V_0\) – Volume of water taken with dose (250 ml); \(C_s\) – Minimum physiologic solubility at pH 1.2, 4.5 and 6.8 at 37°C (mg/ml). In general compounds with \(D_0\) value as less than 1 are considered as highly soluble.

B. Permeability studies (Everted Intestine model)

Male white leghorn chicks weighing between 500 and 600 gm were bought from the local market. The krebs–ringer solution was prepared by combining 6.3 gm NaCl, 0.35 gm KCl, 0.14 gm CaCl₂, 0.16 gm KH₂PO₄, 0.15 gm MgSO₄·7H₂O, 2.1 gm NaHCO₃ and 5 gm glucose in 1 litre of distilled water. For isolation of everted intestine, the chick was slaughtered, a median incision of the abdomen was performed, and the small intestine was freed. The lumen was carefully cleared from mucus by rinsing with a pH 7.4 buffer solutions (krebs–ringer solution). An intestinal segment of the first 6 cm length was removed and transferred to oxygenated krebs–ringer solution. It was washed thoroughly with warm krebs–ringer solution. The
proximal extremity of the intestine was turned back and ligated on a specially design glass rod to form an everted bag. Permeability study of developed poly herbal nano crystals and poly herbal solid lipid nano particles were determined separately by placing weighed quantity as per the dose number of the phytoconstituents in a specially design model (Gowshan Fig 4) which was placed in the dissolution vessel. This vessel was then filled with the SGF media, supplied with the oxygen. Permeability studies were performed in six vessels. 5 ml of the samples were withdrawn at 0.0, 15, 30, 45, 60, 90 and 120 min time intervals. Equal quantity of the SGF medium was replaced to the dissolution jar after each sampling. The amount of the permeably released was estimated by optimized and validated RP-UFLC methods.

C. Categorization of physically modified poly herbal NC and SLN into biopharmaceutical classification system

Based on the aqueous solubility with the dose number and permeability values of the respective prepared poly herbal nano particles were fitted into one of the categories of biopharmaceutical classification system (BCS) as a special interest.

6.2.6. Formulation of poly herbal immediate releasetablets

- Poly herbal immediate release (IR) tablets consisting of quercetin, curcumin and piperinewere prepared by wet granulation method.
- Poly herbal nano crystals immediate release (NCIR) tablets consisting of physically modified nano crystals particles of quercetin, curcumin and piperinewere prepared by wet granulation method.

A. Compatibility studies (IR/NCIR)

Infrared spectral matching approach was employed to detect any possible chemical interaction between quercetin, curcumin, piperine and excipients. Physical mixtures of the herbal phytoconstituents and the excipients (1:1) were mixed with 400 mg of potassium bromide (IR grade). About 100 mg of the mixture was taken and compressed to form a transparent pellet in a hydraulic press at 15 ton pressure. The samples were scanned from 4000 to 400 cm⁻¹ in Shimadzu FTIR spectrophotometer. Similarly, the IR spectra of quercetin, curcumin, piperine and the excipients were also recorded. Physical appearance of the samples and appearance / disappearance of
peaks in the spectra were observed to assess any possible physical and chemical interactions.

**Differential scanning calorimeter (DSC)**

Differential scanning calorimetric analysis was used to characterize the thermal behavior of the phytoconstituents, excipients, and their physical mixtures. Sample was prepared by crimped in standard aluminum pans and heated from 20 to 400°C at a heating rate of 10°C/min under constant purging of dry nitrogen at 30 ml/min. An empty pan, sealed in the same way as the sample, was used as a reference. DSC thermograms were obtained using an automatic thermal analyzer system. Temperature calibration was performed using indium as calibration reference standard.

**B. Preparation of granules (IR/NCIR)**

Poly herbal immediate release tablets and poly herbal nano crystals immediate release tablets containing quercetin, curcumin and piperine were prepared separately by wet granulation method. Three batches of immediate release (IR-1 to 4) and nano crystals (NCIR-1 to 4) were prepared. All the compositions, with the excipients were shown in the table 5 were thoroughly mixed in a tumbling mixer for 5 min and wetted in a mortar with isopropyl alcohol. The wet mass was sieved (16 mesh) and granules were dried at 60°C for 2 h. The dried granules were sieved (22 mesh) and these granules were lubricated with a mixture of magnesium stearate and talc (2:1).
### Table 5. Composition for immediate release tablets

<table>
<thead>
<tr>
<th>S.No</th>
<th>Formulation code</th>
<th>Drug content for quercetin (% w/w)</th>
<th>Drug content for curcumin (% w/w)</th>
<th>Drug content for piperine (% w/w)</th>
<th>MCC* (% w/w)</th>
<th>PVP-K-30* (% w/w)</th>
<th>Magnesium Stearate* (% w/w)</th>
<th>Talc* (% w/w)</th>
<th>Total weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IR-1</td>
<td>17.5</td>
<td>17.5</td>
<td>0.7</td>
<td>58.8</td>
<td>2.5</td>
<td>2</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>IR-2</td>
<td>17.5</td>
<td>17.5</td>
<td>0.7</td>
<td>56.3</td>
<td>5.0</td>
<td>2</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>IR-3</td>
<td>17.5</td>
<td>17.5</td>
<td>0.7</td>
<td>53.8</td>
<td>7.5</td>
<td>2</td>
<td>1</td>
<td>100</td>
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<tr>
<td>4</td>
<td>IR-4</td>
<td>17.5</td>
<td>17.5</td>
<td>0.7</td>
<td>51.3</td>
<td>10.0</td>
<td>2</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>NCIR-1</td>
<td>17.5</td>
<td>17.5</td>
<td>0.7</td>
<td>58.8</td>
<td>2.5</td>
<td>2</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>NCIR-2</td>
<td>17.5</td>
<td>17.5</td>
<td>0.7</td>
<td>56.3</td>
<td>5.0</td>
<td>2</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>NCIR-3</td>
<td>17.5</td>
<td>17.5</td>
<td>0.7</td>
<td>53.8</td>
<td>7.5</td>
<td>2</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>NCIR-1</td>
<td>17.5</td>
<td>17.5</td>
<td>0.7</td>
<td>58.8</td>
<td>2.5</td>
<td>2</td>
<td>1</td>
<td>100</td>
</tr>
</tbody>
</table>

*MCC (microcrystalline cellulose) – diluents, PVP-K-30 – binder, magnesium stearate – lubricant and talc - glidant and lubricant*
C. Evaluation of granulations (IR/NCIR)

Evaluation of granulations parameters such as flow properties, granular densities and percentage of fines were determined for the prepared granules as per the standard IP 2010 procedure and the limit was calculated [106].

The following parameters were used for the characterization of prepared granules.

1. Percentage of fines
2. Flow properties
3. Granular densities

I. Determination of percentage of fines (IR/NCIR)

Percentage of fines was determined by passing the granules through sieves 22 superimposed on sieve 40. The particles which pass through #40 were considered as fines.

II. Flow Properties (IR/NCIR)

The flow properties are critical for an efficient tableting operation. A good flow of the powder or granulation is necessary to assure efficient mixing and acceptable weight uniformity for the compressed tablets. When a heap of powder is allowed to stand with only the gravitational force acting on it, the angle between the free surface of the static heap and the horizontal plane can achieve a certain maximum value for a given powder. This angle is defined as the static angle of repose and is a common way of explaining flow characteristics of powder granulation. The prepared granules were allowed to flow through the funnel fixed to a stand at definite height of 2.5 cm. The angle of repose was then calculated by measuring the height and radius of the heap of granules formed as follows.

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

Where \( h \) and \( r \) are the height and radius of the powder cone and \( \theta = \tan^{-1} \frac{h}{r} \).

III. Bulk Density

Both loose bulk density (LBD) and tapped bulk density (TBD) were determined by tapped density apparatus. A known amount of granules from each formula, previously lightly shaken to break any agglomerates formed was introduced into a graduated
measuring cylinder. After the initial volume was observed, the cylinder was allowed to fall under its own height onto a hard surface from the height of 2.5 cm at 2 sec intervals. The tapping was continued until no further change in the volume was noted. LBD and TBD were calculated using the following formulas.

\[
LBD = \frac{\text{Weight of the powder}}{\text{Volume of the packing}} \times 100
\]

\[
TBD = \frac{\text{Weight of the powder}}{\text{Trapped volume of the packing}} \times 100
\]

**IV. Compressibility Index IR and NCIR**

The compressibility index of the granules was determined by Carr’s compressibility index formula

\[
\text{Carr’s index (\%)} = \frac{(\text{TBD} - \text{LBD})}{\text{TBD}} \times 100
\]

Where,

TBD is Tapped bulk density and LBD is Loose bulk density.

**V. Hausner’s Factor (IR/NCIR)**

Hausner found that the ratio \(D_F/D_O\) was related to inter particle friction and, as such, could be used to predict powder flow properties. It was calculated by using the following formula

\[
\text{Hausner’s factor} = \frac{D_F}{D_O}
\]

Where,

\(D_F\) - Tapped bulk density and \(D_O\) - Loose bulk density.

**D. Compression of immediate release tablets (IR/NCIR)**

Poly herbal immediate release tablets and poly herbal nano crystal immediate release tablets containing quercetin, curcumin and piperine were prepared by wet granulation method. Compression of granules was performed using a 12 station Rimek RSB-4 mini press–I tablet punching machine (Ahmadabad, India) using a compression force of a 12 KN equipped with a 6 mm concave punch as shown in fig 7. A batch of 50
tablets was considered in the preliminary work. Four batches of both poly herbal immediate release and nano crystal immediate release formulations were prepared and evaluated for hardness, friability, weight variation and drug content as per Indian pharmacopoeia [110] and the limits were calculated.

![Image of tablet compression](image)

**Fig 7. Compression of IR tablet by Rimek tablet punching machine**

**E. Evaluation of prepared poly herbal IR/NCIR tablets**

**I. Weight variation**
Randomly selected 20 tablets were weighed using electronic balance. The deviation from the average weight was calculated and the deviation from the average weight of tablets should be ± 7.5 % w/w [107].

**II. Thickness**
Thickness of the prepared formulations was determined by using digital Vernier callipers. The result of the thickness was expressed in mm.

**III. Hardness**
Monsanto hardness tester was used to determine the hardness of the tablets. It consisted of a barrel containing a compressible spring held between two plungers. The tablet was placed in contact with lower plunger and zero reading was noted. The plunger was then forced against a spring by turning the thread bolt until the tablet fractured. The force required to fracture the tablet was recorded and expressed in kg/cm² [107].
VI. Friability
Friability of the tablets was determined by using roche friabilator. Ten tablets from each batch were accurately weighed, placed in the friabilator and rotated at 25 rpm for a period of 4 min. The tablets were removed from friability apparatus and weighed again. The friability was determined using the following formula [107].

\[
\text{Friability (\%)} = \frac{(\text{Initial weight} - \text{Final weight})}{\text{Initial weight}} \times 100
\]

V. Disintegration
The disintegration of the formulated tablets was determined in the disintegration apparatus and in 1000 ml purified water maintained at 37 ± 2°C. One tablet was placed in each of the six tubes and the apparatus was operated. The time required for the tablet to disintegrate and pass through the mesh was noted [107].

6.2.7. Development of dissolution method
A. Selection of dissolution medium
I. Selection of dissolution medium for quercetin
Saturation solubility profile studies for quercetin was carried out in different media such as 0.5 and 1.5% (w/v) of SLS in water and phosphate buffer pH 6.8, 0.5 and 1.5% (w/v) of tween 80 in water and phosphate buffer pH 6.8, 0.5 and 1.5% (w/v) of DMSO in water and phosphate buffer pH 6.8, 1% (w/v) of SLS and 1.0% (w/v) of DMSO in water and phosphate buffer pH 6.8, 1.0% (w/v) of PEG 6000 in water and phosphate buffer pH 6.8, 1.0% (w/v) of PEG 6000 and 1.0% (w/v) of DMSO in water and phosphate buffer pH 6.8, 1.0% (w/v) of DMSO in water, 1.0% (w/v) of ethanol and phosphate buffer pH 6.8, 30, 40 and 50% (w/v) of DMSO and phosphate buffer pH 6.8 respectively at 37°C. As per the biopharmaceutical classification guidelines for solubility studies, highest dose strength of quercetin was added to 250 ml of above mention media separately in a 500 ml conical flask and shaken continuously at 37°C for 48 h in an orbital shaker. Sink conditions of the phytoconstituents was calculated when the amount of phytoconstituents that can be dissolved in the dissolution medium is three times greater than the amount of phytoconstituents to be dissolved. The ratio of saturation solubility of the phytoconstituents concentration (dose) is expressed as Cs/Cd which represents to the closeness to sink conditions. The solutions were then filtered through a membrane filter (0.22 μm) and analyzed.
through RP-UFLC. The study was performed in three replicates and the mean data was recorded with the sink condition data. Dissolution media was selected based on the highest value of sink condition.

II. Selection of dissolution medium for curcumin and piperine

The saturation solubility profile studies for curcumin and piperine was carried out by using following media: 0.5 and 1.5 % (w/v) of SLS in water and phosphate buffer pH 6.8, 0.5 and 1.5 % (w/v) of tween 80 in water and phosphate buffer pH 6.8, 0.5 and 1.5 % (w/v) of DMSO in water and phosphate buffer pH 6.8, 1 % (w/v) of SLS and 1.0 % (w/v) of DMSO in water and phosphate buffer pH 6.8, 20 % (w/v) of ethanol and phosphate buffer pH 6.8, 10, 20 and 20 % (w/v) of methanol and phosphate buffer pH 6.8 respectively at 37°C. As per the biopharmaceutical classification guidelines for solubility studies, highest dose strength of quercetin 500 mg was added to 250 ml of above mention media separately in a 500ml conical flask and shaken continuously at 37°C for 48 h in an orbital shaker. The solutions were then filtered through a membrane filter (0.22 µm) and analyzed through RP-UFLC. The study was repeated three times and the mean data was recorded with the sink condition data. Dissolution medium were selection based on the highest value of sink condition. Sink conditions of the phytoconstituents were calculated when the amount of phytoconstituents that can be dissolved in the dissolution medium is three times greater than the amount of phytoconstituents to be dissolved. The ratio of saturation solubility of the phytoconstituents concentration (dose) is expressed as Cs/Cd which represents to the closeness to sink conditions. A low Cs/Cd value shows non-sink conditions in this the rate of phytoconstituents dissolution will be slowed by the limited solubility of the phytoconstituents in that medium.

B. Determination of dissolution media stability

Dissolution media stability was carried out for the above selected ideal solution by adding the known concentration of phytoconstituents to the solution and analyzed over 48 hat room temperature. Aliquots (1 ml) were collected initially at 0 and 24 h intervals for 2 days and analyzed. The drug concentrations observed at 0, 24 and 48 h samples were analyzed and percentage degradation were calculated.
C. *In vitro* release studies for the developed poly herbal formulations (IR/NCIR).

The release characteristics of poly herbal immediate release tablets and physically modified poly herbal nano crystals immediate release tablets consisting of quercetin, curcumin and piperine were determined using USP XXIII dissolution apparatus (type II, paddle) at 50 rpm. The dissolution medium (900 ml) used for quercetin was phosphate buffer pH 6.8 containing (40 % of DMSO) and the dissolution media (900ml) used for curcumin and piperine were phosphate buffer pH 6.8 containing (1.5 % of tween 80) for 120 min maintained at 37°C ± 0.5°C. Dissolution tests were performed on six tablets. Five ml of samples were withdrawn at 0.0, 15, 30, 45, 60, 90 and 120 min time intervals. Equal quantity of the dissolution medium was replaced to the dissolution jar after each sampling. The amount of the drug released was estimated by optimized and validated RP-UFLC methods. Percentage drug release and cumulative release at various time intervals were calculated and compared with the developed formulations.

6.2.8. Bio-analytical method development and validation

A. Bio-analytical method development

Bio-analytical method development was done for the quercetin, curcumin and piperine by RP-UFLC method. Development of the bio-analytical method depends upon the nature of the compounds such as the molecular weight, solubility and ionic strength. For the analysis of the analytes in RP-UFLC with UV/ PDA detector it’s important that the compound should have the chromopores. In the present study reverse phase ultra-fast liquid chromatography (RP-UFLC) method has been considered for the analysis of analytes. Bioanalytical method for the selected phytoconstituents was carried out by following parameters such as

- Selection of detection wavelength
- Selection of stationary phase
- Selection of mobile phase

I. Determination of detection wavelength

The sensitivity of the method depends upon the proper selection of the wavelength for detection of the phytoconstituents. The idea wavelength of the select phytoconstituents is one that gives a good response. A stock solution of curcumin,
quercetin and piperine was prepared by dissolving 10 mg of each phytoconstituents in 10 ml of acetonitrile. The stock solutions were stored in refrigerator at −20 ± 2°C until analysis. From the stock solution, suitable dilutions were prepared using the same solvent to produce 100 μg/ml solutions. λmax of the drugs was determined by scanning the prepared solution between 800 to 200 nm using a UV-visible spectrophotometer.

II. Selection of stationary phase
Selection of the stationary phase was carried out by the nature of the polarity of the compounds. In this method development, different reverse phase columns (C8, C18 and ODS) were used for the separation of the phytoconstituents. Depending on the response, retention time and peak shape, Phenomenex C18 (250 x 4.6 mm i.d., 5μ) column was used.

III. Selection of mobile phase
Selection of the mobile phase was done based on the solubility and stability of the phytoconstituents. Various mobile phases were tried in the RP-UFLC to achieve a good peak response and peak separation. Initially different methanol: buffers ratios (pH 3-6.5) were carried out for the separation of the phytoconstituents. Followed by different ratio of water: buffers, water: methanol and acetonitrile: buffers were carried out. The different chromatography condition like pH of the buffer, flow rate, different in organic phase ratios and peak modified were studied.

IV. Chromatographic conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stationary phase</td>
<td>Phenomenex C18 (250 x 4.6 mm i.d., 5μ)</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>Acetonitrile: water</td>
</tr>
<tr>
<td>Mobile phase ratio</td>
<td>54:46</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.0 ml/min</td>
</tr>
<tr>
<td>Sample volume</td>
<td>20 μl using Rheodyne 7725i injector</td>
</tr>
<tr>
<td>Detection</td>
<td>380 nm</td>
</tr>
<tr>
<td>Detector</td>
<td>PDA</td>
</tr>
<tr>
<td>Data station</td>
<td>LC Solutions</td>
</tr>
<tr>
<td>Retention time for quercetin</td>
<td>5.2 min</td>
</tr>
<tr>
<td>Retention time for curcumin</td>
<td>9.2 min</td>
</tr>
</tbody>
</table>
Retention time for piperine : 10.1 min
Retention time for rutin : 14.5 min

**B. Validation of the developed method**

Validation is a process which involves confirmation or establishment by laboratory studies that a method / procedure / system / analyst can give the required accuracy, precision, sensitivity, ruggedness, etc. In the most basic form, validation of an analytical procedure demonstrates that the procedure developed is suitable for its intended purpose. Validation of the method was carried out after the development of the HPLC methods. Validation parameters tested such as, selectivity/ specificity, sensitivity, linearity, precision, accuracy, ruggedness and robustness were carried out as per the US-FDA guidelines.

**I. Solution preparation**

*Preparation of standard solution*

A stock solution of curcumin, quercetin and piperine was prepared by dissolving 10 mg of each phytoconstituents in 10 ml of acetonitrile. The stock solution was stored in refrigerator at $-20 \pm 2^\circ C$ until analysis.

*Preparation of internal standard solution*

A stock solution of internal standard was prepared by dissolving 10 mg of Isradipine in 10 ml of acetonitrile. The stock solution was stored in refrigerator at $-20 \pm 2^\circ C$ until analysis.

*Preparation of sample solution*

About 0.5 ml of rabbit plasma, 0.5 ml of phytoconstituents (quercetin, curcumin and piperine), 0.5 ml of internal standard (Isradipine) and 0.5 ml of precipitating agent (30 % perchloric acid) were taken in a 2 ml eppendorf tube and vortexed for 2 min and this solution was centrifuged at 4000 rpm for about 5 min. Supernatant was separated carefully and analysed in RP-UFLC method.

**II. Specificity**

A developed method is said to be specific when it produces a response only for a single analyte. Method selectivity is the ability of the method to produce a response
for the analyte in the presence of other interferences. In order to prove that the method chosen was specific and selective the following two sets of samples were processed and injected into the RP-UFLC using the extraction procedure.

- Blank samples from six different lots of biological matrix (plasma containing sodium citrate as anticoagulant).
- Samples from the same six lots of biological matrix mentioned in step 1 spiked with the analyte at the lower limit of quantification (LLOQ) of the calibration curve and with the internal standard at the concentration level in the study.

To calculate % interference, the response obtained for each sample in step 1 was compared with the response obtained for each corresponding sample in step 2.

\[
\text{% Interference} = \frac{\text{Peak area response of blank}}{\text{Peak area response of LLOQ}} \times 100
\]

**III. Sensitivity**

It is expressed as limit of quantitation. It is the lowest amount of analyte in a sample matrix that can be determined. Three injection of the lowest concentration were injected and the spectra were determined.

**IV. Precision**

The precision of the method was determined by analyzing two batches each consisting of six injection at three different concentration of quercetin (50, 500, 1000 ng/ml), curcumin (100, 800, 2000 ng/ml) and piperine (20, 200, 400 ng/ml) of low (QCL), middle (QCM) and high (QCH).

Precision is expressed as the percentage coefficient of variation (%CV) which was calculated as per the following expression

\[
\text{% CV} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100
\]

**Intra-run precision**

Intra-run precision was determined by calculating the percentage coefficient of variation (% CV) of the results obtained in the same run.
**Intra-day precision**

Intra-day precision was determined by calculating the percentage coefficient of variation (\% CV) of the results obtained in the same day.

**Inter-day precision**

Inter-day precision was determined by calculating the percentage coefficient of variation (\% CV) of the results obtained over at least two days.

**V. Accuracy**

Accuracy of the developed method was determined by \% recovery studies. \% recovery of the method was carried out by addition of known concentration standard solution to the plasma sample and analyzed. Results obtained were compared with the true results. Accuracy study was performed by analyzing 6 replicates (n=6) at three different concentration levels and mean, standard deviation (SD) and \% relative SD were calculated. Accuracy was reported as \% nominal of the analyzed concentration which was calculated as

\[
\text{\% Nominal} = \frac{\text{Measured Concentration}}{\text{Actual Concentration}} \times 100
\]

**VI. Linearity**

Linearity and range of the methods were analyzed by preparing calibration curves using different concentrations of the standard phytoconstituents solution containing the internal standard. Linearity was established for quercetin, curcumin and piperine over the range of (50-1000 ng/ml), (100-2000 ng/ml), (20-400 ng/ml) using the weighted least square regression analysis. The calibration curve was plotted using response factor (peak area standard phytoconstituents / peak area of internal standard) vs concentration of the standard phytoconstituents solutions. A calibration curve consisted of

- Aqueous standard at middle concentration level to check retention time of analyte and internal standard
- Blank sample (matrix sample processed without internal standard and analyte)
- Zero sample (matrix sample processed with internal standard but without analyte)
• Eight non-zero standards covering the expected range. The lowest and highest standards were prepared in duplicates.

VII. Ruggedness
Ruggedness of the method was studied by changing the experimental conditions such as operators, instruments, source of reagents, solvents and column of similar type. Chromatographic parameters such as retention time, asymmetric factor, capacity factor and selectivity factor were evaluated.

VIII. Robustness
Robustness of the method was studied by injecting the standard solutions with slight variations in the optimized conditions namely, ±1% in the ratio of acetonitrile in the mobile phase and ±0.1 ml of the flow rate.

XI. Limit of detection and limit of quantification
Limit of detection (LOD) and limit of quantitation (LOQ) were reported as 3 and 10 times the noise level obtained from three replicate injections of samples respectively.

6.2.9. Pharmacokinetic studies
The protocol of the animal study was submitted to the institutional animal ethical committee. All animal experiments were approved by Institutional Animal Ethical Committee, JSS College of Pharmacy, Udhagamandalam (Proposal no:JSSCP/IAEC/Ph.D/Ph.eutics/02/2012-13). Bioavailability studies were carried out in the albino white healthy rabbits weighing between 1.5-2 kg. Pharmacokinetic studies were carried between the developed poly herbal IR tablets, poly herbal NCIR tablets, SLN and pure drug suspension, to prove the bioavailability of the developed formulations. All the received animals were kept overnight fasting before the experiments but had free access to water. Healthy overnight fasted animals were used for the experiments. Zero hour fasting blood samples were withdrawn early in the morning. The dosing for the rabbits was selected based on the surface area ratio of rabbit and man. The animals were then divided in to 5 groups as follows,

Groupings: Animals were divided into 5 groups (n=3)
Group 1 - Rabbits received poly herbal immediate release tablets
Group 2 - Rabbits received poly herbal nano crystals immediate release tablets
Group 3 - Rabbits received solid lipid nanoparticles
Group 4 - Rabbits received pure drug suspensions (curcumin/piperine/quercetin)
Group 5 - Rabbits was kept as control (0.9% saline solution)
Immediately after administration the animals were given 5ml of water. In case of group 4 animals, pure drugs were suspended into 0.3 % of sodium carboxy methyl cellulose and administered orally. Blood samples (0.5 ml) were withdrawn from the marginal ear vein at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 18 and 24 h period using a sterilized syringe as shown in fig 8. The blood samples collected in 8ia vials containing the anti-coagulant (100μl of 11% sodium citrate) and centrifuged at 4000 rpm for 4 min. The supernatant layer (plasma samples) were separated and stored at -20°C. The plasma samples were mixed with IS solution and deproteinized by mixing the samples with equal volume of perchloric acid and the contents were vortexes for 2 min. It was then centrifuged at 4000 rpm for 4 min and the supernatant liquid was separated and analyzed. Estimation of plasma sample by RP-UFLC was carried out using optimized chromatographic conditions.

Fig 8. Collection of blood sample form rabbit marginal ear vein
A. Method of analysis

The processed standards and samples were analysed using optimised chromatographic conditions mentioned earlier and the chromatograms were recorded. The quantification of the chromatogram was performed using peak area ratios (response factor) of the drug to internal standard. The calibration curves were constructed routinely during the process of pre-study validation and in-study validation.

Analytical batch organization:

Samples were injected in the following order:

i) Aqueous standard
ii) Plasma blank
iii) Zero sample
iv) Calibration curve samples
v) Quality control samples
vi) Subject samples

B. In vivo data analysis

The pharmacokinetic parameters $C_{\text{max}}$, $t_{\text{max}}$, AUC$_{0-t}$, AUC$_{0-\infty}$, $t_{1/2}$ and $k_{\text{el}}$ were determined using PK1 and PK2 solution (software) for individual drug treatments from the observed plasma concentration-time data. The measured plasma concentrations were used to calculate the area under the plasma concentration-time profile from time zero to the last concentration time point (AUC$_{0,\infty}$). The AUC$_{0,t}$ was determined by the trapezoidal method. AUC$_{0,\infty}$ was determined by the following equation

$$\text{AUC}_{(0-\infty)} = \text{AUC}_{(0-t)} + C_0/k_{\text{el}}$$

$k_{\text{el}}$ was estimated by fitting the logarithm of the concentrations versus time to a straight line over the observed exponential decline.