Chapter 4

Materials and Methods

4.1 List of Materials used in the Present Investigation

Table 4.1 Materials used in the present investigation

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Chemicals</th>
<th>Manufacturers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Petroleum Ether (60-80{\degree} C)</td>
<td>Ranchem Ltd. India.</td>
</tr>
<tr>
<td>2</td>
<td>Chloroform</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Methanol</td>
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</tr>
<tr>
<td>4</td>
<td>Chromic acid</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Ethanol</td>
<td>Baroda Chemicals Ltd</td>
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<tr>
<td>6</td>
<td>Oleic acid</td>
<td>Seva Fine Chemical Ltd. Mumbai</td>
</tr>
<tr>
<td>7</td>
<td>Linoleic acid</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Peppermint oil</td>
<td>Central Drug House, India</td>
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<tr>
<td>9</td>
<td>0.1 M Sodium citrate buffer</td>
<td>Seva Fine Chemical Ltd. Mumbai</td>
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<td>10</td>
<td>Streptozotocin</td>
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<td>11</td>
<td>Citric acid</td>
<td>Sigma Aldrich (USA)</td>
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<tr>
<td>12</td>
<td>Sodium hydroxide</td>
<td></td>
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<tr>
<td>13</td>
<td>Sodium citrate</td>
<td>Seva Fine Chemical Ltd. Mumbai</td>
</tr>
<tr>
<td>14</td>
<td>Potassium dihydrogen phosphate</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Hydrochloric acid</td>
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</tr>
</tbody>
</table>
4.2 List of Equipments used in the Present Investigation

Table 4.2 Equipments used in the Present Investigation

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Equipments</th>
<th>Manufacturers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Digital Balance</td>
<td>Shimadzu Corporation, Japan</td>
</tr>
<tr>
<td>2</td>
<td>Soxhlet Apparatus</td>
<td>JSWC</td>
</tr>
<tr>
<td>3</td>
<td>Dessicator</td>
<td>JSWC</td>
</tr>
<tr>
<td>4</td>
<td>Oven</td>
<td>Tempo instruments and equipment</td>
</tr>
<tr>
<td>5</td>
<td>FT-IR</td>
<td>Shimadzu, Japan.</td>
</tr>
<tr>
<td>6</td>
<td>UV-VIS</td>
<td>Shimadzu, Japan.</td>
</tr>
</tbody>
</table>

4.3 Collection and preparation of plant material

The *Gymnema sylvestre* (GS) leaves were procured locally Udupi district, Karnataka state, cleaned, shade-dried, powdered coarsely and passed through 40mesh and kept in, labeled, air tight vessel for further use.

4.3.1 Preliminary pharmacognostic studies of the leaves of *gymnema sylvestre* (Leon Lachman & Lieberman 1991; Khandelwal 2009; Ayurvedic pharmacopoeia of India 2008)

The powdered GS leaf was evaluated for the following pharmacognostic parameters.

4.3.1.1 Loss on Drying

The GS leaf powder was weighed accurately to 5 g and was kept in petri dish for drying in hot-air oven at 110°C for 4 hours. It was kept in dessicator after cooling and the weight loss was documented and process was done in repetitive manner till constant weight was achieved.

\[ \text{Loss on Drying} = \frac{\text{Weight loss}}{W} \times 100 \]

Where, \( W \) = Powdered drug’s weight in grams
4.3.1.2 Ash Value

4.3.1.2.1 Procedure for Total Ash value determination

The powder of *Gymnema sylvestre* leaves was weighed precisely about 5 gm in a previously ignited tarred silica crucible. Burned progressively by elevating the temperature (not more than dull red heat) till it become carbon free. Reweighed them after cooling. The percentage of total ash was computed with respect to dry powder of GS leaves.

4.3.1.2.2 Procedure for determination of Acid Insoluble Ash

Total ash was added to 25 ml of dilute HCl and kept for heating for 10 min. The insoluble matter was gathered at the bottom which was taken in an earlier ignited and weighed silica crucible. The percentage of acid-insoluble ash was computed with respect to dry powder of GS leaves.

4.3.1.2.3 Procedure for determination of Water Soluble Ash

Total ash was added to the 25 ml of water and boiled them for 5 min. The insoluble matter was gathered at the bottom which was taken in an earlier ignited and weighed silica crucible. It was washed using hot water. Then it was burned and reweighed. The percentage of water soluble ash was computed with respect to dry powder of GS leaves.

4.4 Exhaustive extraction of plant material

Dry powder of *Gymnema sylvestre* was subjected to continuous and exhaustive Soxhlet ion, done sequentially with petroleum ether, chloroform and methanol. The final methanolic extract (known henceforth as GSE for *Gymnema sylvestre* methanolic extracts respectively) was concentrated *in vacuo* and dried under reduced pressure.

4.4.1 Extractive value

Percentage of alcohol soluble extractive value was computed with respect to dry powder material.
4.5 Preliminary Phytochemical studies of the *Gymnema sylvestre* Extract

4.5.1 Qualitative analysis (Khandelwal 2009)

Preliminary phytochemical screening was done to show the existence of alkaloids, steroids, flavonoids, terpenes, saponins, volatile oil, tannins, carbohydrates, phenolic substances and mucilage.

1. Detection of Carbohydrates

5ml of distilled water was taken and extract was added. Made the extract soluble then it was filtered. The filtrate was collected and used to perform the following tests which show the existence of carbohydrates.

a. Molisch’s Test

In test tube, 2 drops of alcoholic α-naphthol solution was added to the filtrate. 2 ml of concentrated H$_2$SO$_4$ was poured at the test tube’s side wall very carefully. The violet colored ring formed at the junction indicated that carbohydrates were present.

b. Benedict’s Test

Benedict’s reagent was added to the filtrate and kept for heating on water bath till an orange red precipitate were formed which specify the existence of reducing sugars.

c. Fehling’s Test

Both the solutions Fehling A & B were mixed equally 1-1 ml and kept for boiling for 1 min. To this solution mixture add equal volume of aqueous fraction of extract. It was heated for 5-10 min in water bath. First of all yellow and then brick red precipitate observed which specify the presence of carbohydrate.

2. Detection of Alkaloids

Dilute HCl was taken and extract was added in test tube and then it was filtered. Made the extract soluble then it was filtered. The filtrate was collected. Using filtrates the following tests were performed with several alkaloid reagents.
a. **Mayer’s Test**

To the filtrate, potassium mercuric iodide solution known as Mayer’s reagent was incorporated and yellow cream colored precipitates were observed which indicated that alkaloids were present.

b. **Wagner’s Test**

Iodine in potassium iodide was known as Wagner’s reagent which was added to the filtrate and a brown/reddish brown colored precipitates were formed which specify the existence of alkaloids.

c. **Dragendorff’s test**

To the filtrate, potassium bismuth iodide solution known as Dragendorff’s reagent was added and red precipitates were formed which specify the existence of alkaloids.

3. **Detection of Glycosides**

Using dilute HCl the extract was hydrolyzed and the obtained hydrosylate was exposed to following glycosides tests.

a. **Legal’s Test**

The hydrosylate was taken in the test tube and the pyridine was added followed by addition of sodium nitroprusside and made alkaline. Pink to red coloration shows the existence of cardiac glycoside.

b. **Killer Killani Test**

0.5 gm of dried extract was taken then 2 ml of glacial CH\textsubscript{3}COOH was added followed by addition of 1 drop of FeCl\textsubscript{3} solution. After solubilizing the extract, it was under laid with 1 ml of conc. H\textsubscript{2}SO\textsubscript{4}. A brown colored ring was formed which shows the existence of cardenolides.
4. Detection of Flavonoid
   a. With Lead Acetate
      To the extract, little amount of lead acetate solution was incorporated. Yellow colored precipitates specified that flavonoids were present.

   b. With Sodium Hydroxide
      The ethanolic extract showed yellow coloration by gradually adding sodium hydroxide and after the addition of acid it was decolorized.

5. Detection of Tannins and Phenolic compounds
   a. Gelatin Test
      The extract was treated with 1 % gelatin solution having sodium chloride. The white precipitates offered the existence of tannins.

   b. Vanillin hydrochloride Test
      To the extract, few drops of vanillin hydrochloride was added. The red color showed the presence of tannins.

   c. FeCl₃ Test
      To the extract, little amount of 5 % Fecl₃ alcoholic solution was added. The deep blue-black color showed the presence of phenolic compound.

   d. Matchsticks Test
      The matchsticks was placed into the extract and dried for 2 min. After it was again placed into conc. sulphuric acid and gently heated for 2 min and the red color showed the presence of tannins.
6. Detection of Saponins
   a. Foam Test
      Little amount of extract was taken in the test tube then minor quantity of NaHCO₃ and water were added and shaken vigorously. Characteristic and stable honeycomb like froth was observed which indicates the presence of saponins.

7. Detection of Sterols
   a. Salkowski Test
      2 ml of chloroform was added to the little amount of extract. 2 ml of concentrated H₂SO₄ was poured at the test tube’s side wall. Then it was shaken for some time. The red coloration in the chloroform layer indicated that sterols were present.
   b. Libermann-Burchard Reaction
      To the little amount of extract, chloroform was added. Made the extract soluble 2 ml of acetic anhydride was incorporated to the solution. 2 drops of concentrated H₂SO₄ was poured at the test tube’s side wall. The greenish transient coloration showed the existence of sterols.

8. Detection of Steroids and Terpenoids
   Chloroform was taken to dissolve 10mg of the extract and into that few drops of acetic anhydride was added. 1 ml of concentrated H₂SO₄ was incorporated. Change is blue color of chloroform layer to green indicates the existence of steroids, whereas pink color indicates the existence of terpenoids.

9. Detection of Quinone
   Little amount of extract in alcohol was reacted with H₂SO₄. The development of color showed the existence of Quinone.
4.6 Anti-Diabetic Activity

4.6.1 Ethical approval
All the experimental protocols were conducted in Pinnacle Biomedical Research Institute (PBRI) Bhopal and approved by their Institutional Animal Ethics (IAEC) (Reg No. 1283/PO/c/09/CPCSEA).

4.6.2 Animals
4.6.2.1 Selection of Animals
Albino Wistar rats (200±25gm) were used in the present investigation.

4.6.2.2 Housing
Animals were kept in a group of three in separate cages at room temperature (22±2 °C), relative humidity (60±5%) and at 12:12, light: dark cycle.

4.6.2.3 Diet
Standard diet and water were given to all animals (Golden Feed, New Delhi).

4.6.3 Experimental design and treatment schedule
In the experiment, 30 No. of rats were used which were divided. In one group six animals were taken, so five groups of animals were made.

**Group 1:** Rats were given 0.1 M citrate buffer hence known as Normal Control.
**Group 2:** Streptozotocin (60 mg/kg) was administrated to the rats intra-peritoneally (i.p.) in 0.1 M Citrate buffer having pH 4.5 hence known as Diabetic control.
**Group 3:** Standard drug Glibenclamide (0.6 mg/kg) was intended orally to the diabetic rats.
**Group 4:** Diabetic rats were given the test sample HF01 (GSE-300 mg/kg) orally.
**Group 5:** Diabetic rats were given the test sample HF02 (GSE-500 mg/kg) orally.
4.6.4 Chemicals and reagents

Analytically graded chemicals were used in the study. Streptozotocin was procured from Sigma Aldrich (USA).

4.6.4.1 Chemicals

- 0.1 M citrate buffer
- Streptozotocin
- Citric acid
- Sodium citrate
- Sodium hydroxide

4.6.4.2 Instruments

- Glucometer
- Glucometer strip

4.6.4.3 Preparation of 0.1 M Citrate Buffer

- Citric acid – 10.5 gm
- Sodium citrate – 14.7 gm
- Distilled water – 1000 ml

Adjust the pH by Sodium hydroxide solution.

4.6.5 Induction of diabetes (Pandit et al. 2010; Erejuwa et al. 2010)

Streptozotocin (60 mg/kg) was administered to rats intra-peritoneally (i.p.) to induce diabetes. STZ was prepared by dissolving them in ice cold 0.1 M citrate buffer. The animals were allowed to drink 5% glucose solution overnight to overcome STZ induced hyperglycemia. Normal control rats were given citrate buffer only which act as placebo. If the glucose level in blood increased above 240 mg/dl they were considered as diabetic. The treatment schedule was began on 4th day after diabetic induction and it was counted as 1st day of treatment. It was continued till 21 days. Body weight and level of glucose in blood were observed on 0, 7, 14 and 21 day of post treatment.
4.6.6 Estimation of biochemical parameters (Pandit et al. 2010; Erejuwa et al. 2010)

The retro-orbital plexus of the rats from where the blood samples were collected after induction of diabetes in animals and after 21st day of the treatment with GSE. The serum was separated, and the biochemical estimations of alkaline phosphatase (ALP), aspartate aminotransferase (SGOT), alanine aminotransferase (SGPT) and total bilirubin were done. Decreased blood glucose level was measured. Lipid profile, high density lipoprotein (HDL), low density lipoprotein (LDL) and Total cholesterol (TC) in all groups were measured.

4.6.7 Histology study (Shidhaye et al. 2008)

On the day of 21st, by using mild ether anesthesia all animals were sacrificed, whole pancreas was removed and placed in 10% formalin solution. Then tissue was treated instantly by the paraffin technique. 5 μ thick sections were cut and stained by haematoxylin and eosin (H & E) to examine histology of pancreas.

4.7 Preformulation Studies

Preformulation studies were performed for the determination of the compatibility of initial excipients i.e. polymers with the active substance for a physicochemical, biopharmaceutical, and analytical investigation in support of promising experimental formulations. Successful preformulation studies take into account the change in its physicochemical properties due to drug’s interactions with other ingredients and their interactions with each other during preformulation studies to produce a safe, stable, beneficial, and marketable product. In the current research work solubility determination of $\lambda_{\text{max}}$, compatibility study of drug with other excipients using FT-IR was performed. The results for the same are discussed below:
4.7.1 Solubility of extract

Solubility forms the prerequisite of preformulation studies since poorly soluble compounds can intensely decrease production in drug discovery and development. The saturation solubility of *Gymnema sylvestre* extract was determined in different solvents viz. chloroform, methanol, diethyl ether, phosphate buffer (PBS) pH 7.4, 6.8, 4.0, 1.2 and in distilled water for selecting the solvent for further formulation development and physicochemical evaluation.

4.7.2 Compatibility study of drugs with polymers by FT-IR

The drug excipient interaction is possible in any formulation due to their intimate contact. Therefore, assessment of their compatibility is very important to identify product’s stability as well as its reproducibility with ensured therapeutic efficacy. Although polymers selected for pharmaceutical formulations bear no pharmacological significance i.e. inert in nature, but the polymers may participate in physical and chemical interactions and can cause serious degradation of active pharmaceutical ingredients, and hence it important to study the compatibility of drugs and polymers. The interaction between drug and polymer was examined by FT-IR (Fourier Transform Infrared) spectroscopy. IR spectra for active ingredient, polymers and their combination were recorded in FT-IR spectrophotometer (Thermo scientific Necolet IS 10) using KBr pellets. The scanning range was 400–4000 cm$^{-1}$.

4.8 Method of Analysis of *Gymnema sylvestre* Extract

4.8.1 Preparation of phosphate buffer pH 6.8

22.4 ml of 0.2 M NaOH and 50 ml of 0.2 M potassium dihydrogen phosphate were added into a 200 ml volumetric flask to prepare phosphate buffer pH 6.8 and water was added to make up volume.
4.8.2 Determination of $\lambda_{\text{max}}$ of *Gymnema sylvestre*

Approximately 10 mg extract of *Gymnema sylvestre* was added to volumetric flask having capacity of 100 ml. 10 ml of phosphate buffer pH 6.8 (PBS 6.8) was incorporated to dissolve the extract. A stock solution of 100 $\mu$g/ml was prepared by adding the PBS 6.8 up to the mark. Then this stock solution was again diluted with PBS 6.8 and was scanned for UV spectrum by using Shimadzu UV/Visible double beam spectrophotometer. The UV spectra was taken in a range of 200-600 nm.

4.8.3 Preparation of standard calibration curve of *Gymnema sylvestre* using UV

Calibration curve of *Gymnema sylvestre* was developed in PBS 6.8 at 278.5 nm. Stock solution containing 100 $\mu$g/ml was prepared and kept aside. 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 ml of stock solution were withdrawn and further diluted to 10 ml with buffer solution to obtain a concentrations range of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 $\mu$g/ml respectively. At 278.5 nm wavelength the absorbance of solutions were scanned by using UV-VIS spectrophotometer. A graph of Concentration vs Absorbance was plotted.

4.9 Formulation of Herbal Buccal Spray

4.9.1 Composition of buccal spray

- The formulation containing therapeutically active ingredient (drug substance) was dissolved in solution or mixtures of excipients like solvent, cosolvent, viscosity modifiers, penetration enhancer, preservatives, taste masking agent and sweeteners/flavors, emulsifying agents, buffering systems in non-pressurized dispensers that convey a spray holding metered dose of drug.
- The solvent mostly utilized are water and ethanol or isopropyl alcohol or their combination with various fatty acid oils.
- PEG (grade 400-1000), propylene glycol etc. can act as co-solvents whereas polyvinyl alcohol, miglyol, glycerin etc. are generally used as viscosity builders.
• Penetration enhancer can be selected from wide range of materials like surfactants, bile salts, fatty acids, salicylic acid etc. which are generally utilized for enhancement of buccal transmucosal absorption.
• Various flavors and sweeteners (e.g. aspartame, neotame, sucralose, saccharine sodium etc.) can be selected as per the requirement.

4.9.2 Preliminary Trial

4.9.2.1 Ex-vivo Transmucosal Diffusion Study using Different Penetration Enhancers (Shanker et al. 2009; Biswal et al. 2014)

Ex-vivo study was performed on goat buccal mucosa, obtained from local slaughterhouse, using Franz diffusion cell. The receiver and donor compartment were respectively filled with PBS 6.8 while former received 25 ml and later received just enough to moisten the inter compartment membrane (Goat buccal mucosa). The temperature of assembly was kept constant at 37°C ± 0.05°C and the content of receiver compartment was stirred constantly using a magnetic stirrer. Various penetration enhancers like ethanol, PEG 400, Linoleic acid, Tween 80, PG and Oleic acid were used for study. Every 3 min, 3 ml of sample was withdrawn for 15 min duration and volume made up using PBS 6.8. Absorbance was measured at 278.5 nm keeping PBS 6.8 as blank. The concentration of drug diffused was computed from the given equation.

\[ Y = 0.172X + 0.003 \]

Where, \( Y \) = Absorbance  
\( X \) = Concentration

4.9.3 Formulation Batches of Herbal Buccal Spray

The drug *Gymnema sylvestre* was dissolved in water. The oleic acid, linoleic acid were used as penetration enhancers. The ethanol was used as co-solvent and also enhances the permeability of the drug. The *Stevia rebaudiana* was added to the solution
as adjuvants. Along with that it also exerts antidiabetic activity. The Peppermint oil was added as a flavoring agent. All these excipients were used in formulation and all the batches were decided on trial and error method (Table 4.3).

### 4.9.3.1 Procedure to formulate the solution for Buccal Spray

In this formulation *Stevia rebaudiana* was added to the water and made the solution. Then drug was added slowly to the vehicle and dissolved by continuous stirring. The oleic acid was dissolved in ethanol. Both the solutions were mixed with continuous stirring followed by addition of peppermint oil.

#### Table 4.3 Formulation Batches for the Herbal Spray

<table>
<thead>
<tr>
<th>Sr No.</th>
<th>GSE (mg)/spray</th>
<th>Stevia (mg)</th>
<th>Oleic acid (ml)</th>
<th>Linoleic acid (ml)</th>
<th>Ethanol (ml)</th>
<th>Peppermint oil</th>
<th>Water (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>150</td>
<td>100</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>q.s.</td>
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<td>1</td>
<td>3</td>
<td>q.s.</td>
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<td>1</td>
<td>2</td>
<td>q.s.</td>
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<td>2</td>
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<td>2</td>
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<td>3</td>
<td>q.s.</td>
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<td>2</td>
<td>2</td>
<td>2</td>
<td>q.s.</td>
<td>20</td>
</tr>
</tbody>
</table>

**Dose Calculation:**

The container capacity is 30 ml and net volume filled in each container is 25 ml. Here each Spray delivers 0.125ml volume, so 25 ml can be sprayed for 200 times. The drug content desired to be delivered per spray should be multiplied by 200 times. Here, it was desired that one spray should deliver 150mg in all formulation batches.
4.10 Evaluation Parameters for Herbal Buccal Spray (Leon Lachman & Lieberman 1991)

- **Leak test:** Leak test will be performed twice.

**Immediate Leak test:** Final containers will allow to sink in warm water (around 50°C) for about 10 seconds, immediately after the filling. The bubbling in water will identified as a leakage in the container.

**Delayed Leak test:** Accurately weighed containers were kept at room temperature for 1 month. After this time period the containers were reweighed. The weight difference in the container indicated the leakage in the container.

- **Spray Pattern**
  
  The method of comparing spray pattern is based on the impingement of the spray on the piece of a paper. Clear colored patterns were observed on the paper when the spray was sprayed on it from 3 cm distance. The spray patterns were compared on the basis of their shape and width or diameter, using a graduated scale/ruler.

- **Pump discharge rate**
  
  Pump discharge rate of buccal spray was determined by spraying a known weight and discharging the contents for 10 times. By reweighing the container after ten sprays, the change in weight per unit spray i.e., the average weight dispensed per spray was calculated and considered as valve discharge rate, which can be expressed as grams per spray. After that it is converted in to ml with help of density of liquid.

- **Droplet size determination** (Bakshi et al. 2008)
  
  Droplet size distribution was examined by using optical microscopy. Glass slide was taken and the formulation was sprinkled. Minimum 100 particles were measured from various 25 regions under a binocular Labomed vision 2000 microscope of magnification 40X.
• **Dose uniformity**

One of the most critical quality control parameter of the Buccal spray is its dose uniformity i.e. uniformity of the drug content per unit spray. To investigate dose uniformity, one puff from product was sprayed in a beaker and that sprayed content was diluted with PBS 6.8. After making sufficient dilutions, absorbance was measured by 278.5 nm by UV/VIS spectrophotometer keeping PBS 6.8 as blank. Uniformity of the dose dispensed, was assured by measuring at least 3 dispensed doses per product.

**4.11 Stability study**

Stability is defined as the extent to which a product retains within specified limits and throughout its period of storage and use (i.e. its shelf life), the same properties and characteristics that it possessed at the time of manufacture. Stability testing is performed to ensure that drug products retain their fitness for use until the end of their expiration dates. The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors. Short term stability study was carried out for the formulation at 60±2°C and 75% RH for period of 6 months. After the period of each month, formulation checked for drug content per spray.