Chapter 3

EXPERIMENTAL
### EXPERIMENTAL

#### 3.1 Materials

The different materials which are used for conduct of experiments have been arranged or procured from different sources shown in Table 1.

**Table 1. Materials used in experiment.**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Drug</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Ranitidine hydrochloride</td>
<td>Hetero drugs, Hyderabad, India</td>
</tr>
<tr>
<td>2.</td>
<td><em>Trigonella foenum graecum</em></td>
<td>Local market of Kanpur, India</td>
</tr>
<tr>
<td>3.</td>
<td><em>Tamarindus indica</em> seeds</td>
<td>Local market of Kanpur, India</td>
</tr>
<tr>
<td>4.</td>
<td>Eudragit RS 100</td>
<td>Evonik, Mumbai, India</td>
</tr>
<tr>
<td>5.</td>
<td>Tween 80</td>
<td>Central Drug House Ltd, Mumbai, India</td>
</tr>
<tr>
<td>6.</td>
<td>Acetone</td>
<td>Ozone International, Mumbai, India</td>
</tr>
<tr>
<td>7.</td>
<td>Dichloromethane</td>
<td>Central Drug House Ltd, Mumbai, India</td>
</tr>
<tr>
<td>8.</td>
<td>Isopropyl alcohol</td>
<td>E. Merck (India) Ltd, India</td>
</tr>
<tr>
<td>9.</td>
<td>Hydrochloric acid</td>
<td>S D Fine-Chem Ltd, Mumbai, India</td>
</tr>
<tr>
<td>10.</td>
<td>Sodium bicarbonate</td>
<td>Rankem Laboratory Chemicals, New Delhi, India</td>
</tr>
<tr>
<td>11.</td>
<td>Castor oil</td>
<td>Ozone International, Mumbai, India</td>
</tr>
<tr>
<td>12.</td>
<td>Span 80</td>
<td>Loba Chemie Pvt. Ltd, Mumbai, India</td>
</tr>
<tr>
<td>13.</td>
<td>Epichlorhydrin</td>
<td>A. B. Entreprises, Mumbai, India</td>
</tr>
<tr>
<td>14.</td>
<td>Potassium bromide</td>
<td>Thomas Baker (Chemicals) Pvt. Ltd, Mumbai, India</td>
</tr>
<tr>
<td>15.</td>
<td>Liquid paraffin</td>
<td>Thomas Baker (Chemicals) Pvt. Ltd, Mumbai, India</td>
</tr>
<tr>
<td>16.</td>
<td>Phenol</td>
<td>Loba Chemie Pvt. Ltd, Mumbai, India</td>
</tr>
<tr>
<td>17.</td>
<td>Sodium Phosphite</td>
<td>Loba Chemie Pvt. Ltd, Mumbai, India</td>
</tr>
<tr>
<td>18.</td>
<td>Ruthenium red</td>
<td>Loba Chemie Pvt. Ltd, Mumbai, India</td>
</tr>
<tr>
<td>19.</td>
<td>Lead Acetate</td>
<td>Thomas Baker (Chemicals) Pvt. Ltd, Mumbai, India</td>
</tr>
<tr>
<td>20.</td>
<td>Glucose</td>
<td>S D Fine-Chem Ltd, Mumbai, India</td>
</tr>
<tr>
<td>21.</td>
<td>Conc Sulphuric acid</td>
<td>Thomas Baker (Chemicals) Pvt. Ltd, Mumbai, India</td>
</tr>
</tbody>
</table>

The different equipments used to carry out the experiment are shown in Table 2.
Table 2. Equipments used in experiments.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Instrument</th>
<th>Make and model</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Rotary vacuum evaporator</td>
<td>Shalimar Motor Works, New Delhi</td>
</tr>
<tr>
<td>2.</td>
<td>SEM apparatus</td>
<td>Leo-435 VP, UK</td>
</tr>
<tr>
<td>3.</td>
<td>Bulk density apparatus</td>
<td>Harrison’s Pharma Machinery Pvt. Ltd, New Delhi</td>
</tr>
<tr>
<td>4.</td>
<td>Moisture analyzer</td>
<td>Harrison’s Pharma Machinery Pvt. Ltd, New Delhi</td>
</tr>
<tr>
<td>5.</td>
<td>Optical microscopy</td>
<td>Radical Instruments, Ambala Cant, India</td>
</tr>
<tr>
<td>6.</td>
<td>UV-visible spectrophotometer</td>
<td>Shimadzu 1700, Japan</td>
</tr>
<tr>
<td>7.</td>
<td>Nephlo-Turbidity Meter</td>
<td>Systronic, India</td>
</tr>
<tr>
<td>8.</td>
<td>Refractometer</td>
<td>Contech Instruments, Mumbai, India</td>
</tr>
<tr>
<td>9.</td>
<td>R/S-CPS-Plus Brookfield Rheometer</td>
<td>Brookfield Engineering Labs, U.S.A</td>
</tr>
<tr>
<td>10.</td>
<td>magnetic stirrer</td>
<td>Harrison’s Pharma Machinery Pvt. Ltd, New Delhi</td>
</tr>
<tr>
<td>11.</td>
<td>High speed mechanical stirrer</td>
<td>Remi Laboratory, India</td>
</tr>
<tr>
<td>12.</td>
<td>FTIR</td>
<td>Shimadzu 84005, Japan</td>
</tr>
<tr>
<td>13.</td>
<td>X-ray diffractometer</td>
<td>Bruker AXS D8 advance, Germany</td>
</tr>
<tr>
<td>14.</td>
<td>Digital phase contrast microscope</td>
<td>Redical instruments, Ambala Cant, India</td>
</tr>
<tr>
<td>15.</td>
<td>USP dissolution apparatus (type II)</td>
<td>Harrison’s Pharma Machinery Pvt. Ltd, New Delhi</td>
</tr>
<tr>
<td>16.</td>
<td>Ultrasonicator</td>
<td>Leela Electronics, Mumbai, India</td>
</tr>
<tr>
<td>17.</td>
<td>Centrifuge</td>
<td>Remi Laboratory, India</td>
</tr>
</tbody>
</table>
3.2. Methods

3.2.1. Polysaccharide extraction from TI seeds

Polysaccharide was extracted as per previously reported methods in various publications with little modifications (Sharma et al., 2013, Baveja et al., 1989; Nair & Fahsa, 2013; Chandramouli et al., 2012a). Briefly, an accurate quantity (200 g) of seeds of TI was weighed and washed with distilled water to get rid of sticking substances. The TI seed were processed by separating the brown peels from the kernel seed with the blander and plastic sieve were used to separate the seed. The seeds were then crushed lightly and about three volumes of water were separately added in crushed seeds. The obtained content was heated at 60°C for about 4 h on a water bath to get the slurry and the slurry was filtered. The filtrate was then diluted with water (three volumes). The diluted filtrate was kept undisturbed entire night in a refrigerator to settle down the most of the un-dissolved portion. The clear supernatant fraction thus obtained was decanted and transferred a rotary vacuum evaporator at 60±1°C to concentrate. The concentrate was cooled and precipitated in acetone (three volumes). The precipitate was repeatedly washed with acetone and dried at 50±1°C for about two days. Afterwards, the protein contaminations were removed by Sevag method. The dried substance was grounded by using a mechanical grinder and passed through sieve(#80 mesh). The dried polysaccharide powders were preserved in desiccators.

3.2.2. Polysaccharide extraction from TFG seeds

Similarly as above section, the polysaccharides were extracted from the TFG seeds and were kept in a desiccator for further use.

3.2.3. Preliminary Phytochemical Screening of Extracts

The extracts of TI and TFG were evaluated for the following constituents (Lala, 1981; Baveja et al., 1989; Verma et al., 2014; Kokate, 1991).
(a) Tests for alkaloids:

1. **Dragendorff’s test:** To 1 ml Dragendorff’s reagent, 1 ml of extract was added. The presence of alkaloids is indicated by the appearance of orange red precipitate.

2. **Wagner’s test:** To 2 ml wagner’s reagent, 1 ml of extract was added. The presence of alkaloids is indicated by the appearance of reddish brown precipitate.

3. **Mayer’s test:** To 2 ml mayer’s reagent, 1 ml of extract was added. The presence of alkaloids is indicated by the appearance of dull white precipitate.

4. **Hager’s test:** To 3 ml Hager’s reagent, 1 ml of extract was added. The presence of alkaloids is indicated by the appearance of yellow precipitate.

(b) Tests for carbohydrates:

1. **Molisch test:** To 1 ml of α-naphthol solution and concentrated sulphuric acid, 1 ml of extract was added. The presence of carbohydrate is indicated by the appearance of Purple or reddish violet color.

2. **Fehling’s test:** To 1 ml of Fehling’s solution A and B, 1 ml of extract was added. The presence of carbohydrate is indicated by the appearance of brick red precipitate upon heating.

3. **Benedict’s test:** To 5 ml of Benedict’s reagent, 1 ml of extract was added and boiled for 2 minutes then cooled. The presence of carbohydrate is indicated by the appearance of red precipitate.

(c) Tests for proteins:

1. **Biuret test:** To 1 ml of 40% sodium hydroxide solution and 2 drops of 1% copper sulphate solution, 1 ml of extract was added. The presence of proteins was indicated by formation of violet color.

2. **Xanthoprotein test:** To 1 ml of extract, 1 ml of the conc. nitric acid was added and the white precipitate, if any was boiled and cooled. Added 20% of sodium
hydroxide was added to it. The presence of aromatic amino acids is indicated by orange color.

3. **Lead Acetate test:** To the extract added 1 ml of lead acetate solution. Formation of a white precipitate indicates the presence of proteins.

**(d) Test for amino acids:**

1. **Ninhydrin test:** Added two drops of freshly prepared 0.2% ninhydrin reagent to the extract solution and heated. Development of violet color reveals the presence of amino acids.

**(e) Tests for steroids:**

1. **Libermann-Burchard test:** To 2 ml of chloroform, the extract was dissolved and added with 11 drops of acetic anhydride and 3 drops of concentrated sulphuric acid. The presence of steroids was indicated by the solution becomes red, then blue and finally bluish green.

2. **Salkowski test:** To 2 ml of concentrated sulfuric acid (H₂SO₄), the 2 ml of extract was dissolved. The steroid components in the tested extract was represented by the formation of bluish red to cherry red color in layer of chloroform and green fluorescence in the layer of acid.

**(f) Test for flavonoids:**

1. **Shinoda test:** To 1 ml of magnesium and 1-3 drops of concentrated hydrochloric acid, 1 ml of the extract added. The presence of flavonoids was showed by the formation of red color.

**(g) Tests for phenolic compounds:**

1. To 1 ml of the extract added ferric chloride, formation of a dark blue or greenish black color product shows the presence of phenolic compounds.

2. To the extract added potassium dichromate solution, formation of a precipitate shows the presence of phenolic compounds.
(h) **Tests for glycosides:**

1. **Legal test:** In pyridine and sodium nitroprusside solution, the extract was dissolved for making it alkaline. The presence of glycosides was exhibited by formation of pink red color.

2. **Baljet test:** To 1 ml of the test extract added 1 ml sodium picrate solution and the change of yellow to orange color reveals the presence of glycosides.

(i) **Test for terpenoids:**

   **Salkowski test:** 5 ml of extract was mixed in 2 ml of chloroform and 3 ml conc. sulphuric acid was carefully added for the formation of layer. The presence of terpenoids can be indicated by reddish brown coloration of the interface.

(j) **Test for saponins:**

   In a test tube containing about 5 ml of extract, a drop of sodium bicarbonate (NaCo$_3$) solution was added then the test tube was vigorously shaken and left for 3 minutes. Formation of honeycomb like froth indicates the presence of saponins.

(k) **Test for tannins:**

   To 1-2 ml of extract, few drops of 5% w/v FeCl$_3$ solution were added. A green colour indicates the presence of tannins.

3.2.4. **Confirmatory tests**

   After the preliminary test, the confirmatory tests were performed to confirm the presence of carbohydrate, polysaccharide and mucilage.

   (a) **Ruthenium test:** A drop of ruthenium red solution was added to a small quantity of extract placed on a glass slide and observed under microscope (Kokate, 1991).

   (b) **Iodine test:** A small quantity of 0.2 N iodine solution was added to the small quantity of powder,
(c) **Enzyme test:** A small quantity of benzidine (in 90% alcohol) was added to the small pinch of powder and allowed to stand for few minutes.

### 3.2.5. Microbial properties of polysaccharides

The total microbial (bacterial and fungal) load per gram of extracted polysaccharides was analysed. Moreover, the specific tests for presence of *P. aeruginosa*, *S. typhi*, *S. aureus* and *E. coli* were done according to the standard protocols (Indian Pharmacopoeia, 1996; WHO, 1998).

### 3.2.6. Solubility analysis

The solubility characteristics of TI and TFG polysaccharides were performed using different solvents such as water, chloroform, acetone, ethanol and methanol (Phani Kumar et al., 2011).

### 3.2.7. Polysaccharide Characterization

#### 3.2.7.1. Organoleptic evaluation

Extracted polysaccharides of TFG and TI were evaluated for organoleptic properties such as colour, odour and taste etc. (Kokate, 2006; Sahu VK et al., 2017).

#### 3.2.7.2. pH Determination of polysaccharides

In order to determine the pH of the both polysaccharide, 1% w/v solution of each polysaccharide was made ready in distilled water separately.

The pH of polysaccharide solution was measured using a digital pH meter. The pH meter was calibrated by observing the reading of a standard buffer solution with a known pH at the room temperature. Buffer solutions (pH 4, 7 and 9.2) were used as standards for the calibration and then the electrode on an adjustable arm was positioned in the sample solution to watch the pH of the solution directly (Razavi et al., 2014; Verma et al., 2014).

#### 3.2.7.3. Micromeritics evaluation

Bulk density of a powder is defined as the ratio of mass of untapped powder sample and its untapped volume including the contribution of the inter-particulate void volume.
The bulk density of powder depends mainly on the particle size, particle shape and the trend of particles to stick with each another. Bulk density of TFG & TI polysaccharides powder were determined by measuring the volume of a known weight (W) of powder sample that may have been passed through a sieve 25 into a graduated cylinder. Mathematically bulk density can be represented by equation below (Razavi et al., 2014).

\[ \text{Bulk density } (B_d) = \frac{W}{V_0} \]

Where, W is the weight of powder and \( V_0 \) is the initial bulk volume of powder.

Tapped density was determined by mechanically tapping a graduated measuring cylinder containing a powder sample. Tapped density of TFG & TI polysaccharide powder was determined by placing a known weight of powder (W) in to graduated cylinder and then the cylinders were placed into a mechanical tap density tester. After observing the volume, the cylinder was mechanically tapped for 100 times or until the powder achieves a constant tapped volume \( (V_f) \) (Pabon et al., 1992). Mathematically tapped density can be represented by equation below:

\[ \text{Tapped density } (T_d) = \frac{W}{V_f} \]

Where, W is the weight of powder and \( V_f \) is the final volume of tapped powder.

Angle of repose was determined by using funnel method. In this method, the funnel is kept vertically in a stand at a specified height (i.e. 2.0 cm) above a graph paper placed on a horizontal surface. The polysaccharide powder was poured into the funnel keeping the orifice of the funnel blocked by the thumb. Then the orifice of the funnel was opened to release the powder on the paper to form a smooth conical heap. The radius of the heap base \( (r) \) and the height of the heap \( (h) \) were measured. Angle of repose can be mathematically represented by equation below.

\[ \text{Angle of repose } (\theta) = \tan^{-1} \left( \frac{h}{r} \right) \]

Where, \( h \) is the height of the heap and \( r \) is the radius of the heap base.

Carr’s Compressibility index and Hausner ratio are the parameters used to study the flow behaviour of powders. These are the simple, fast and popular parameters for predicting
flow characteristics (Bajpai et al., 2008). These are indirectly related to the relative flow property, cohesiveness (i.e. inter-particulate interactions) and particle size (Malviya, 2011).

Carr’s index can be mathematically calculated by equation below:

\[ \text{Carr’s index (\%)} = \left( \frac{T_d - B_d}{T_d} \right) \times 100 \]

Where \( T_d \) is the tapped density of the powder and \( B_d \) is the bulk density of the powder.

Hausner ratio was calculated using the equations below:

\[ \text{Hausner Ratio} = \frac{V_0}{V_f} \]

Where, \( V_0 \) is the initial bulk volume of powder and \( V_f \) is final volume of Tapped powder.

3.2.7.4. Surface Characteristics of polysaccharide using Scanning Electron Microscopy (SEM)

To study the surface characteristics of both the extracted polysaccharides, SEM technique was used. SEM is one of the advanced technologies which are used to determine the surface characteristics of a material. The dry powder of polysaccharide was kept on an aluminum stub with both sided adhesive tape and evaporated with carbon and then sputtered with gold to make the samples electrically connected. Layering of Carbon was made to a thickness of approximately 10 nm and gold was layered to approximately 25 nm. The photographs were taken at different resolution by using SEM.

3.2.7.5. Determination of glucose in extracted polysaccharide

Total glucose content in both the extracted plant polysaccharide was estimated by using Phenol-sulfuric acid method (Dubois et al., 1956; Harshal and Priscilla, 2011). Briefly, two milliliters of standard grade sugar solutions (ranging from 10 and 100 µg/ml concentrations of sugar) were prepared in distilled water. Similarly, test solutions 100 µg/ml concentration of both the polysaccharide were prepared by dissolving 200 µg into 2 ml of distilled water separately. All standard and test solution were transferred into test tubes. To these solutions 1 ml of phenol solution (5%w/v) and concentrated sulfuric acid (5 ml) was added. All the mixture was shaken vigorously and equilibrated at 30°C till estimation. The absorbance of the characteristic yellow-orange colour was measured by
using UV-visible spectrophotometer at 488 nm. Blanks were prepared by substituting distilled water for the sugar solution. Sugar content in both test solution were calculated in comparison to the standard analytical curve of sugar.

### 3.2.7.6. Molecular weight determination

Molecular weight of both extracted polysaccharide was determined by equation as reported in previous publications (Kar and Arslan, 1999).

\[
\frac{1}{M_{w, \text{ave}}} = \lim_{c \to 0} \left( \frac{HC}{\tau} \right)
\]

Where \( M_{w, \text{ave}} \) is the average molecular weight (kg/kg mol), \( \tau \) is the turbidity of solution (m\(^{-1}\)) which was measured by Nephlo-Turbidity Meter. \( H \) can be calculated by equation given by below (Allock and Lampe, 1981).

\[
H = \frac{32\pi^3 n_o^2}{3\lambda^4 N_o} \left( n - n_o \right)^2
\]

Where \( n_o \) the refractive index of the solvent (0.1 M sodium phosphate buffer, pH 7.0), \( n \) the refractive index of the polysaccharide solutions, \( \lambda \) wavelength of light (0.5893x10\(^{-6}\) m), \( N_o \) avogadro number (6.023 x 10\(^{23}\)) and \( C \) is the concentration of polysaccharide solution (2.5, 5, 10, 15 & 20 kg/m\(^3\)).

The refractive index of polysaccharide solutions (TI and TFG) were determined by refractometer using sodium vapour lamp. The intensity of light scattered through polysaccharide solution was measured as percentage of light transmitted, as compared to that through 0.1M sodium phosphate buffer. \( M_{w, \text{ave}} \) was determined by plotting a graph between \( HC/\tau \) versus the concentration of polysaccharide solutions.

### 3.2.7.7. Rheological Characterization

Brookfield Rheometer was used for determination of rheological behavior of TFG and TI polysaccharides. It comprises of concentric cylinder, measuring cones and plates geometry. C-25 Din measuring system was used for the study. Temperature was regulated by using thermo regulator (Brookfield PTR-I) and obtained rheological data were recorded (RHEO 2000 version 2.8) with the software provided with the instrument. All the rheological measurements were made in triplicate and the average of data were reported. Solution of different concentrations (1.0 to 4% w/v) of TFG and TI was prepared in
deionised water, under slow stirring for 4 h using a magnetic stirrer. Resulted solution was stored overnight for complete hydration. Flow behaviour of both the polysaccharide solution were observed under a range of shear rate ($s^{-1}$) from 10 to 100 in 100 sec followed by immediately decrease from 100 to 10 in 100 sec. Sample was kept in measuring gap between the rotating plate or cone and the stationary lower plate, permitted to equilibrate for 5-10 min. Flow behaviour index ($n$) and consistency coefficient ($k$) were calculated from curve by fitting the power law model equation given below.

$$F = k\gamma^n$$

Where $F$ is shear stress (Pa), $\gamma$ is shear rate ($s^{-1}$), $k$ is consistency coefficient (Pas$^n$) and $n$ is flow behaviour index (dimensionless).

In the case of Newtonian liquids the value of flow behaviours index ($n$) is equal to one while if $n$ is not equal to one the the flow is termed as non-newtonian. Moreover, if the value of $n< 1$, the flow shows non- Newtonian pseudo plastic flow and if the value of $n> 1$ the flow is termed as non Newtonian dilatants flow (Saikia et al., 2013).

### 3.2.7.8. Assessment of In vitro cytotoxicity

In order to performe cell toxicity of extracted polysaccharides, MTT assay was performed by using rat macrophage cells (Sahu VK et al., 2016). The fresh cell lines were maintained in essential media supplemented with streptomycin (100 U/mL, 10% fetal bovine serum (FBS) and penicillin (100 U/mL) in a humidified atmosphere at 37±0.5°C of temperature. Different concentrations (ranging from 10 to 30 µg/ml) of aqueous solutions of extracted polysaccharides (TFG and TI) was prepared in sterile double distilled water. The cells were incubated for specific time period with different dilution of sample solutions of polysaccharide into 24-well plates in 100 µl of respective media. Plating density of cells was maintained 1x10$^5$/well. After specific time period, sample solutions were removed from the cell and washed with phosphate buffer saline (pH 7.4). After that 200 µl/well (5mg/mL) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) was added in phosphate buffer saline solution and incubated for 4 h. Cells viability were determined by the absorbance at 570 nm. Finally, percentage cell viability was calculated in respect of positive control by using following equation.

$$\% \text{ cell viability} = \left( \frac{A_{\text{test}}}{A_{\text{control}}} \right) \times 100$$
Where, $A_{\text{test}}$ is the absorbance of test sample and $A_{\text{control}}$ is the absorbance of positive control.

3.2.7.9. Drug excipients physical compatibility studies

RH and all the excipients were subjected to compatibility studies with the preliminary identification testing as specified in the individual monographs (Indian Pharmacopoeia, 1996). The Ranitidine hydrochloride was mixed with different excipients in equimolar ratio in the glass bottles and kept at various temperature and humidity conditions such as 30C/65%RH, 40C/75%RH as well as 60C/80%RH for 30 days in open as well as closed bottles. The samples were analyzed at specified time intervals for any visible changes in physical properties.

3.2.8. Analytical method development for quantification of RH

**Primary stock solution:** Primary Stock solution of ranitidine hydrochloride at 1mg/ml concentration was prepared. Accurately weighed quantity of pure drug Ranitidine HCl (100mg) was dissolved in 0.1M HCl (pH 1.2) and volume was made up to 100ml with HCl solution.

**Sample solution:** Aliquots were prepared from the stock solution to get concentrations of 25, 50, 75, 100 and 125 µg/ml by proper dilution with same 0.1M HCl solution.

The absorbance of prepared drug solutions was measured at 314nm using UV-spectrophotometer against an appropriate blank

**Blank solution:** 0.1 M HCl (pH 1.2) alone was used as blank without drug for calibration curve of RH.

3.2.9. Formulation and Characterization of RH loaded microsphere

3.2.9.1. Preparation of RH loaded TI microspheres

Microspheres were prepared by using cross-linking emulsification method (Sharma et al. 2013; Hamdi et al., 2001). Aqueous phase was consisting of different drug and polysaccharide ratio as shown in Table 3. Aqueous phase was prepared by dissolving RH
into required amount of distilled water to get 20mg/ml drug solution. Varied amount of extracted polysaccharide (as mucoadhesive polymer) and Eudragit RS 100 (as rate controlling polymer) were dispersed into drug solution to make 2% w/v dispersion containing both excipients (TI polysaccharide and Eudragit RS 100) (Table 3). Sodium bicarbonate (0.1:1 with TI polysaccharide) was dissolved into aqueous phase. Aqueous phase was placed for 4 h under magnetic stirring at 500 rpm for entire hydration of polysaccharide. Hydrated Aqueous phase was dispersed into castor oil by use of span 80 (1.0% v/v) as an emulsifying agent. The aqueous and oil phase ratio was placed in the ratio of 1:10 throughout the whole experiment. The emulsion was homogenized with an addition of 0.2 mL H₂SO₄ using high speed mechanical stirrer at 2000 rpm. Epichlorohydrin (4% v/v) was added as a crosslinking agent. Stirring was continued for 18 h at 45°C. Finally, microspheres were collected by centrifugation followed by its washing using isopropyl alcohol and dried at 60°C for 5 h.

*Percentage of TI polysaccharide and Eudragit RS 100 was for total content of both excipients to make 2% w/v dispersion into aqueous phase

3.2.9.2. Preparation of RH loaded TFG microspheres

RH loaded Microspheres were prepared similarly as TI microspheres by using crosslinking emulsification method. The different drug and polysaccharide ratio used were as shown in table 4.

3.2.10. Characterization of microspheres

3.2.10.1. FT-IR study

The FTIR spectra of drug (RH), extracted polysaccharides and drug loaded microspheres were recorded using a Fourier transform infrared spectrophotometer (FTIR; Shimadzu 84005, Japan), between ranges 400 to 4500 cm⁻¹. The samples were gently triturated with small amount of potassium bromide (KBr) powder (300 mg) and compressed into pellets/discs by applying 6000 kg/cm² pressure, using a manual hydraulic presser (Freitas et al., 2005).
3.2.10.2. Powder X-ray diffraction (PXRD) study

To determine the changes in the crystalline nature of the drug, PXRD diffractograms of ranitidine hydrochloride (RH), extracted TFG polysaccharide and prepared microspheres were recorded by using X-ray diffractometer equipped with Cu-Kα radiation (40 kV, 60 mA). Each sample was scanned at a scanning speed of 2°/min and a chart speed of 2°/2 cm per 20 (Razavi et al., 2014).

3.2.10.3. Morphology

Surface characteristics of prepared microspheres were evaluated by scanning electron microscopy (SEM, LEO-430, UK). The samples were prepared by lightly dusting the samples of microspheres on a double sided tape on an aluminium stub. The stubs were then coated with gold to a thickness of ~ 300 Å using a sputtering coater (POLARON model SC-76430) under reduced pressure (0.133 Pa) and examined in microscope fitted with a scanning electron detector and the micrographs of prepared polysaccharide based microsphere samples were taken (Tanwar et al., 2007).

3.2.10.4. Particle size analysis

The particle size of microspheres was analyzed by the help of optical microscopy fixed with an ocular and stage micrometer (Tanwar et al., 2007). A suspension of microspheres was prepared in liquid paraffin. Few drops of suspension was transferred onto glass slide and covered with a cover slip. The geometric diameters of at least 300 particles were examined in all measurements. The geometric diameter of microspheres of each batch was measured and the average diameter was calculated.

3.2.10.5. Swelling ratio

Swelling behavior of prepared microspheres was evaluated in phosphate buffer (pH 6.8) by using previously reported methods (Harikarnpakdee et al. 2006; Bal et al., 2012; Saikia et al., 2013). Microspheres were spread over the series of 10 glass slide and few drops of phosphate buffer (pH 6.8) were added over the microspheres for swelling of microspheres. Diameter of microspheres was periodically measured at the interval of 2 min by using a digital phase contrast microscope with attached software for measurement of size. The particles size of microspheres was determined until erosion of the particle surface.
The determination of percentage of swelling was carried out at different time intervals by the difference between diameter of microspheres at time t ($D_t$) and initial time ($t = 0$ [$D_0$]) as calculated from the following equation and averaged from three determinations was calculated

\[
\text{Percentage of Swelling} = \left( \frac{D_t - D_0}{D_0} \right) \times 100
\]

(8)

Where $D_t$ is the mean diameter of microspheres at time t ($t = 2, 4, 6, 8, \ldots$ min). $D_0$ is the diameter of microspheres at $t = 0$.

A percentage swelling Vs time curve was prepared to study the swelling behaviour of each batch.

3.2.10.6. Muco-adhesion study

In-vitro muco-adhesion ability of prepared drug loaded microspheres was evaluated by using rat stomach mucosa (Junginger et al. 2007; Saikia et al., 2013) with slight modification. Briefly, rat stomach mucosa (2x2 cm$^2$) was collected immediately after the scarification of animal. Microspheres from each batch were spread onto the rinsed stomach mucosa and kept inside a stability chamber under the specified conditions (i.e. relative humidity 80%; temperature 25$^\circ$C) for 15 min. After that the microspheres were washed with phosphate buffer (pH 6.8, temperature 37$^\circ$ C) with flow speed of 2ml/min using simple pneumatic syringe. Finally, time required for the complete washing of microspheres from mucosal surface was recorded and results were reported as an average of triplicates.

3.2.10.7. In vitro Buoyancy study

In order to determine the floating properties of prepared microspheres in-vitro buoyancy study was performed (Dey et al. 2016). Microspheres (300mg) were spread over the surface of a USP dissolution apparatus (type II) filled with 900 ml of media (0.1 N hydrochloric acid containing 0.02% v/v tween 80) at 37±0.5$^\circ$C. The medium was agitated with a paddle rotating at 100 rpm for 12 h. The fraction of microspheres floated on the surface and the fraction settled down were recorded separately. The separated microspheres after weighing were dried. In vitro (%) Buoyancy percentage was calculated as the ratio of
the mass of the microspheres that remained floating to the total mass of the microspheres and expressed as a percentage (Srivastava et al., 2005).

### 3.2.10.8. Encapsulation efficiency

Drug Encapsulation efficiency is defined as the amount of added drug (in % w/w) encapsulated in the formulation of microspheres (Dey et al. 2016). Encapsulation efficiency was calculated in terms of the ratio of drug in the final formulation to the amount of added drug. An accurately weighed amount (10 mg) of the formulation of microspheres was dispersed in 10 ml of phosphate buffer (pH 6.8) and ultrasonicated for 3 consecutive periods of 5 minutes each, with 5 minutes of resting period. The sample was then left to equilibrate for 24 hours at room temperature. Debris of microspheres was removed by filtration and centrifugation. The absorbance of the filtrate was measured after suitable dilution using UV-visible spectrophotometer at 313 nm. The drug encapsulation efficiency of each sample was determined in triplicate. Drug encapsulation efficiency was determined using the formula given in following equation.

\[
\text{Encapsulation efficiency} \% = \left( \frac{W_A}{W_T} \right) \times 100
\]

Where \( W_A \) is the actual amount of drug and \( W_T \) is theoretical amount of drug present in microspheres.

### 3.2.10.9. Accelerated stability studies

Both the TFG and TI microsphere samples were subjected to accelerated and real time stability studies according to ICH Q1A (R2) guidelines. The optimized RH-loaded microsphere formulations under a sealed condition were kept at refrigerated temperature (5 ± 3°C) and in stability chamber maintained at 25 ± 2 °C/60 ± 5% RH tests (Phani Kumar et al., 2011). The stability samples were examined periodically for any modification in particle size and drug content for a total period of six months.

### 3.2.10.10. In-vitro dissolution studies

In vitro release study of drug from microspheres is conducted by using modified dissolution method (Tanwar et al., 2007). Accurately weighed quantity of prepared microspheres equivalent to 300 mg of RH was suspended in 900 ml of 0.1M HCl (pH 1.2).
Temperature of the buffer was maintained at 37±0.5°C on magnetic stirrer (50 rpm). At various pre-determined time intervals, 1 ml aliquot were withdrawn and replaced with fresh dissolution medium. The aliquot was filtered through a 0.45 µ membrane filter, diluted to a suitable concentration with the dissolution medium and centrifuged for 15 min at 2000 rpm. The supernatant was removed and analysed for drug content by using UV spectrophotometer at 313 nm. The cumulative % release was computed using the regression equation resulted from the standard analytical curve of ranitidine hydrochloride.

The amount of RH was quantified as percentage using the following equation:

\[
\text{% RH released} = \frac{\text{Amount of RH in dissolution medium}}{\text{Total amount of RH}} \times 100
\]

3.2.10.11. Drug Release Kinetic Modelling of Floating microspheres of Ranitidine

Further, in order to determine the exact mechanism of drug release from the microspheres, the in vitro dissolution release data were fitted into following mathematical kinetic models representing: zero-order, first-order, Higuchi’s and Korsmeyer-Peppas equations.

The zero order is used to describe concentration independent drug release rate of the formulation and calculated as cumulative amount of drug released vs. Time:

\[
C = k_0 t
\]

Where \(k_0\) is the zero-order rate constant expressed in units of concentration/time and \(t\) is the time in hours.

First order drug release model describes a concentration dependent drug release from the system and calculated as log cumulative percent drug remaining to release vs. time in hours:

\[
\log C = \log C_0 - \frac{kt}{2.303}
\]

Where \(C_0\) is the initial concentration of drug, \(C\) is the concentration of drug released in time \(t\) (hours) and \(k\) is the first order constant.
Higuchi’s model is based on Fickian diffusion as a square root of time dependent process from a swellable insoluble matrix and calculated as cumulative percentage of drug released vs. square root of time:

\[ Q = kt^{\frac{1}{2}} \]

Where \( Q \) is cumulative percentage of drug released, \( t \) is the time in hours and \( k \) is the constant reflecting the design variables of the system (Adhikari, 2011).

**Korsmeyer-Peppas** exponential model

\[ \frac{M_t}{M_\infty} = Kt^n, \]

where, \( \frac{M_t}{M_\infty} \) is fraction of drug released after time ‘\( t \)’ and ‘\( K \)’ is kinetic constant and ‘\( n \)’ is release exponent which characterizes the drug transport mechanism (Korsmeyer et al., 1983).

Value of kinetic rate constant (\( k \)), correlation coefficient (\( r^2 \)) and release exponent (\( n \)) were determined to investigate the best fit model.