# CHAPTER VI

## 6.0. MATERIALS AND METHODS

### 6.1. MATERIALS

#### 6.1.1. List of materials used

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Chemicals/Materials</th>
<th>Manufacturer/Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cefixime</td>
<td>Kniss Laboratories Pvt Ltd., Chennai, India.</td>
</tr>
<tr>
<td>2.</td>
<td>Chitosan (specifications: molecular weight: 100,000-300,000 Da, deacetylation degree &gt;80%)</td>
<td>Sigma Aldrich, Bangalore, India.</td>
</tr>
<tr>
<td>3.</td>
<td>Gum kondagogu (grade-1)</td>
<td>Girijan Co-operative Corp Ltd., Hyderabad, India.</td>
</tr>
<tr>
<td>4.</td>
<td><em>Basella alba</em> leaves, fenugreek seeds</td>
<td>Local market, Tirupati, Andhra Pradesh.</td>
</tr>
<tr>
<td>5.</td>
<td>Potassium-dihydrogen orthophosphate AR</td>
<td>S.D. Fine Chem Ltd., Mumbai</td>
</tr>
<tr>
<td>6.</td>
<td>Sodium hydroxide pellets AR</td>
<td>Qualigens Pvt. Ltd., Mumbai</td>
</tr>
<tr>
<td>7.</td>
<td>Concentrated hydrochloric acid LR</td>
<td>Qualigens Pvt. Ltd., Mumbai</td>
</tr>
<tr>
<td>8.</td>
<td>Acetonitrile (HPLC grade)</td>
<td>Rankem Ltd., New Delhi</td>
</tr>
<tr>
<td>9.</td>
<td>Tween 80 LR Rankem</td>
<td>Rankem Ltd., New Delhi</td>
</tr>
<tr>
<td>10.</td>
<td>Ethanol (HPLC grade)</td>
<td>E. Merck (India) Ltd., Mumbai</td>
</tr>
</tbody>
</table>
6.1.2. List of equipment used

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Equipment</th>
<th>Manufacturer/Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>pH meter</td>
<td>Ion 510 bench pH, Eutech Instruments, Mumbai</td>
</tr>
<tr>
<td>2.</td>
<td>Magnetic stirrer</td>
<td>Remi Electrotechnik Ltd., Vasai, India</td>
</tr>
<tr>
<td>3.</td>
<td>UV-VIS Spectrophotometer</td>
<td>Lambda 35, Perkin Elmer, India</td>
</tr>
<tr>
<td>4.</td>
<td>Digital oven</td>
<td>HICON, Grover Enterprises, New Delhi</td>
</tr>
<tr>
<td>5.</td>
<td>Digital electronic balance</td>
<td>Sartorius BT 224 S, Bangalore</td>
</tr>
<tr>
<td>6.</td>
<td>FTIR spectrophotometer</td>
<td>Shimadzu FTIR 84000 S, Japan</td>
</tr>
<tr>
<td>7.</td>
<td>Particle size analyser</td>
<td>Horiba SZ-100, Japan</td>
</tr>
<tr>
<td>8.</td>
<td>Deep freezer</td>
<td>Labline, Mumbai</td>
</tr>
<tr>
<td>9.</td>
<td>Freeze dryer</td>
<td>SKL-12 N, Esquire Biotech, Chennai</td>
</tr>
<tr>
<td>10.</td>
<td>Incubator</td>
<td>Jindal Scientific Instruments, New Delhi</td>
</tr>
<tr>
<td>11.</td>
<td>Orbital shaker</td>
<td>IKA KS 4000 i control, Chennai</td>
</tr>
<tr>
<td>13.</td>
<td>Autoclave</td>
<td>HICON, Grover Enterprises, New Delhi</td>
</tr>
<tr>
<td>14.</td>
<td>Scanning Electron Microscopy (SEM)</td>
<td>SEM, JFC 1200 fine coater, Japan</td>
</tr>
<tr>
<td>15.</td>
<td>Transmission Electron Microscopy (TEM)</td>
<td>TEM, Philips CM-10, USA</td>
</tr>
<tr>
<td>16.</td>
<td>Atomic Force Microscopy</td>
<td>AFM (NT-MDT), USA</td>
</tr>
<tr>
<td>17.</td>
<td>X-ray diffractometer</td>
<td>Shimadzu XRD-7000, Japan</td>
</tr>
<tr>
<td>18.</td>
<td>GC-MS analyser</td>
<td>Agilent Technologies, Palo Alto, USA</td>
</tr>
<tr>
<td>19.</td>
<td>NMR spectroscopy</td>
<td>Bruker AC-300 Spectrometer, Germany</td>
</tr>
<tr>
<td>20.</td>
<td>Stability Chamber</td>
<td>Grover Enterprises, New Delhi</td>
</tr>
</tbody>
</table>
6.2. METHODS

6.2.1. Collection and authentication of the plant

The plant parts of Cochlospermum gossypium DC. were obtained from Girijan Co-operative Corp Ltd, Hyderabad, India. The plant parts of Trigonella foenum-graecum L., Azadirachta indica A. Juss., Basella alba L. were collected from in and around Tirupati, Andhra Pradesh and were authenticated by Professor K. Madhava Chetty, Plant Taxonomist, S.V. University, Tirupati, A. P., India.

6.2.2. Isolation and purification of water soluble fractions of gum and mucilages

6.2.2.1. Extraction and purification of gum kondagogu [GK] \textsuperscript{[134]}

The exudates of GK were powdered in a high-speed mechanical blender and sieved using a bin (meshsize - 180 µm), so as to obtain a fine and uniform sample. Gum kondagogu powder (0.5% w/v) was accurately weighed and transferred into a clean glass beaker containing 500 ml of deionized water. The gum solution was kept on a magnetic stirrer at room temperature and gently stirred up to 12 h and allowed to stand at room temperature for 12 h, to remove any undissolved matter. The gum solution was filtered using #G-2 sintered glass funnel to remove extraneous material. The gum was then precipitated by using acetone followed by centrifugation at 3000 rpm. The extracted gum was filtered and the water was evaporated in oven at 45°C. The obtained powder was re-dissolved in 100 ml of water, filtered and centrifuged for 10 min at 3000 rpm. The supernatant clear solution was collected, evaporated and dried. This process of purification was repeated thrice. The purified solid mass was freeze dried, grounded and passed through sieve no. 80 and stored in an airtight glass container until further use.
6.2.2.2. Extraction and purification of *Trigonella foenum-graecum* (Fenugreek) seed mucilage [FG] \[^{[135]}\]

The high quality fenugreek seeds were obtained from market and washed with water to remove the dirt and debris. The seeds (250 g) were soaked in double distilled water (500 ml) overnight and then heated at 50°C for 2 h. The mucilage was extracted using a multi-layer muslin cloth bag to remove the marc from the solution. Acetone (in the quantities of three times the volume of filtrate) was added to precipitate the mucilage. The mucilage was separated, dried in an oven at 40°C, collected, powdered, passed through a # 80 sieve and stored in desiccator at 30°C & 45% relative humidity till use. The obtained powder was re-dissolved in 100 ml of water, filtered and centrifuged for 20 min at 3000 rpm. The supernatant clear solution was collected, evaporated and dried. This process of purification was repeated thrice. The purified solid mass was dried by freeze drying and obtained powder was stored in an airtight container.

6.2.2.3. Extraction and purification of *Azadirachta indica* fruit mucilage [AI] \[^{[136,137]}\]

The fresh *Azadirachta indica* fruits was collected and washed with water. The outer shells are removed and seeds with mucilage are to be placed in water for 5-6 h, boiled for 2 h and left to stand for 1 h to allow complete release of the mucilage into the water. The material was filtered through a muslin cloth and hot distilled water (50 ml) was added through the sides of the marc and squeezed well in order to remove the mucilage completely. Three times the volume of acetone was added to the filtrate in order to precipitate the mucilage and kept aside in a refrigerator for one day or effective settling. It was filtered, dried completely in an incubator at 37°C, powdered and weighed. The water soluble fraction was extracted by suspending the powder in 100 ml of distilled water and centrifuged at 3000 rpm and supernatant was collected, freeze dried and stored in a dessicator.
6.2.2.4. Extraction and purification of *Basella alba leaf* mucilage [BA] \cite{138}

*Basella alba* leaves (250 g) was powdered in a mechanical blender and soaked in distilled water (500 ml) for 24 h in a round bottomed flask and then heated at 50°C for 2 h. The mucilage was extracted using a multi-layer muslin cloth to remove the marc from the solution. Acetone (in the quantities of three times the volume of filtrate) was added to precipitate the mucilage. The mucilage was separated, dried in an oven at 40°C, collected, powdered, passed through a # 80 sieve and stored in desiccator at 30°C & 45% relative humidity till use. The obtained powder was re-dissolved in 100 ml of water, filtered and centrifuged for 20 min at 3000 rpm. The supernatant clear solution was collected, evaporated and dried. This process of purification was repeated thrice. The purified solid mass was dried by freeze drying and obtained powder was stored in an airtight container.

![Fig. 16: Gum and mucilages isolated from](image)

a b c d

Fig. 16: Gum and mucilages isolated from a) GK b) FG c) AI d) BA

6.2.3. Characterisation of gum and mucilages

6.2.3.1. Chemical characterisation of gum and mucilages

Preliminary confirmatory test for dried gum and mucilage powder was carried out to confirm the presence of carbohydrates, mucilage, polysaccharides, enzymes \cite{139-143}. 
Table 3: Preliminary confirmatory test for gum and mucilages

<table>
<thead>
<tr>
<th>Test</th>
<th>Observation</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molisch’s test: To 100 mg of dried mucilage powder, Molisch’s reagent and conc. H₂SO₄ was added on the side of a test tube</td>
<td>Violet green colour at the junction between two layers</td>
<td>Presence of carbohydrates</td>
</tr>
<tr>
<td>Ruthenium test: Small quantity of dried mucilage powder was mounted on a slide with ruthenium red solution, and was observed it under microscope</td>
<td>Pink colour develops</td>
<td>Presence of mucilage</td>
</tr>
<tr>
<td>Iodine test: To 100 mg of dried mucilage powder, 1 ml of 0.2 N iodine solution was added</td>
<td>No colour change</td>
<td>Presence of polysaccharides</td>
</tr>
<tr>
<td>Enzyme test: To 100 mg of dried mucilage powder in 20 ml distilled water; 0.5 ml of benzidine in alcohol (90%) was added. The above solution was shaken and allow to stand for few min</td>
<td>No blue colour produced</td>
<td>Absence of enzymes</td>
</tr>
</tbody>
</table>

i) Test for sulphates

To a little amount of the sample, few drops of 5% barium chloride solution was added resulting in formation of white precipitate insoluble in HCl indicates the presence of sulphates.

ii) Test for chlorides

To a few ml of the sample solution prepared in HNO₃, a few drops of AgNO₃ solution was added, results in formation of white precipitate insoluble in dilute ammonia solution indicates the presence of chlorides.
iii) Test for tannins

The substance was mixed with basic lead acetate solution. Formation of white precipitate indicates the presence of tannins.

iv) Test for phenols

The substance was mixed with few drops of ferric chloride solution. Formation of red colouration indicates the presence of phenols.

v) Test for saponins

Foam Test: The extract (2 g) was shaken vigorously with 20 ml of water and observed for persistent foam, which indicates the presence of saponins.

vi) Test for flavonoids

Shinoda Test: To the dry extract (2 g), 5 ml of ethanol (95% v/v), 5 drops of hydrochloric acid and 0.5 g of magnesium turnings were added. Appearance of pink colour indicates the presence of flavonoids.

vii) Test for alkaloids

To 10 g of dry extracts, 20 ml of dilute hydrochloric acid was added, shaken well and filtered. The following tests were performed using the filtrate.

Mayer’s Test: To 3 ml of the filtrates, 1 ml of Mayer’s reagent (potassium mercuric iodide) was added. The appearance of white precipitate indicates the presence of alkaloids.

6.2.3.2. Physical characterisation of gum and mucilages [144-147]

The dried mucilage was studied for organoleptic properties such as colour, percentage yield, chemical test, loss on drying, solubility, viscosity, pH, swelling index and flow properties.
i) Percentage yield

Dried mucilage was weighed and the percentage yield was calculated.

ii) Loss on Drying

This parameter was used for determination of moisture content. Loss on drying is the loss in weight in % w/w determined as per the standard procedure. Pre-weighed quantity of powder was placed in hot air oven and the weight of the powder was noted every 15 min until constant weight. This was repeated for three times and the loss in weight was calculated in percentage.

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

iii) Determination of zeta potential, pH, viscosity

1% w/v solution of sample was prepared in distilled water and zeta potential was determined using Nanoparticle analyser SZ-100. The pH and viscosity of same solution (1% w/v) was determined using digital pH meter and Brookfield viscometer. All measurements were performed in triplicates (n=3) and the standard deviation (SD) was recorded.

iv) Density

A 0.5% w/v solution of dried mucilage was prepared and transferred to a density measurable bottle. An empty bottle with distilled water was weighed. The density of the dried mucilage was calculated.

v) Melting point

Melting point was determined to find out the temperature at which the sample melts and it was used to find out the purity of the sample. The presence of any contamination will indicate difference in the melting point of the sample. It was determined by melting point apparatus.
vi) Determination of percentage swelling index

The swelling index of sample was determined by placing one gram of powder in a measuring cylinder. The initial volume of the powder in a measuring cylinder was noted. The volume was made up to 100 ml with distilled water at room temperature. The cylinder was shaken gently and set aside for 24 h. The volume occupied by the sample sediment was noted after 24 h\textsuperscript{[148]}. The swelling index of the sample was calculated by the formula,

\[
\% \text{ S.I} = \frac{\text{wt}-\text{wo/wo}}{\text{wo}} \times 100
\]

Where, S.I. = Swelling index

Wt = Height occupied by swollen sample after 24 h

Wo = Initial height of the powder in graduated cylinder

vii) Total ash

Total ash was determined by placing 3 g of mucilage in a crucible, the material was spread as an even layer and ignited by gradually increasing the temperature to 550°C until it was white, indicating the absence of carbon. The crucible was cooled in a desiccator, weighed and the content of total ash in mg/g of air dried material was calculated\textsuperscript{[149]}.

viii) Acid insoluble ash

Acid insoluble ash was determined by boiling the ash with 25 ml of dilute hydrochloric acid for 5 min and then filtered through an ash less filter paper. The filter paper was then ignited in the silica crucible, cooled and weighed.

ix) Determination of flow properties\textsuperscript{[150-153]}

The dried gum and mucilages was tested for the flow properties like angle of repose, bulk densities, compressibility index and Hausner's ratio. All these evaluations were carried out in triplicates as per procedures described in official books.
Materials and Methods

a) Bulk density and bulkiness

The bulk density was calculated by dividing the weight of the sample by bulk volume of the sample contained in the cylinder. Reciprocal of bulk density gives the bulkiness.

b) Tapped density

Tapped density is the ratio of weight of dry powder to its tapped volume. The weighed quantity of dry powder was taken in graduated cylinder. The cylinder was placed on the tap density apparatus.

c) Compressibility index and Hausner’s ratio

The compressibility index and Hausner’s ratio were determined by using both bulk density and tapped density of powder. The percent compressibility index (I) of powder was calculated using the following formula

\[
\text{Compressibility index} = \frac{\text{Tapped density} - \text{Bulk density}}{\text{Tapped density}} \times 100
\]

\[
\text{Hausner’s ratio} = \frac{\text{Tapped density}}{\text{Bulk density}}
\]

d) Angle of repose

The angle of repose (θ) was determined using fixed funnel method. The height of the funnel was adjusted in such a way that the tip of the funnel just touched the apex of the pile of powder sample. The powder was allowed to flow through the funnel freely on to the surface. It can be calculated by,

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

\[
h = \text{Height of the pile}
\]

\[
r = \text{Radius of the pile}
\]
x) Microbiological assay

Gum and mucilages needs freedom from pathogenic microorganisms. Suitable culture media are selected and prepared as described in Indian Pharmacopoeia 2007.

a) *Salmonella Typhi*

10 mg sample was taken and dissolved in 1 ml of DMSO. 100 µl was taken from each and spread onto petri plate containing SS-agar media incubated over night at 37°C. The plates were observed for bacterial growth.

b) *Escherichia coli*

10 mg sample was taken and dissolved in 1 ml of DMSO. 100 µl was taken from each and spread onto petri plate containing Macconkey agar media incubated over night at 37°C. The plates were observed for bacterial growth.

c) *Pseudomonas aeruginosa*

10 mg sample was taken and dissolved in 1 ml of DMSO. 100 µl was taken from each and spread onto petri plate containing cetrimide agar media incubated over night at 37°C. The plates were observed for bacterial growth.

d) Fungi (*Candida albicans*)

10 mg sample was taken and dissolved in 1 ml of DMSO. 100 µl was taken from each and spread onto petri plate containing potato dextrose agar media incubated over night at 37°C. The plates were observed for fungal growth.

6.2.3.3. Structural elucidation of gum and mucilages

a) Acid-base hydrolysis

The pH of the sample solution (300 mg/ml) was adjusted to 2.0 by adding 0.5M sulphuric acid. The solution was heated to 100°C for 24 h. After cooling, the pH of
solution was adjusted to 10 by the addition of 0.5M NaOH and heated to 100°C for 24 h. After cooling the solution was neutralized with barium carbonate and filtered. The solution was then dialyzed for 24 h against de-ionized water and freeze dried. The obtained hydrolysed dry samples were subjected for FTIR and Gas chromatography-mass spectrometry (GC-MS) studies.

b) FTIR, GC-MS analysis of hydrolysed fraction and XRD

The FT-IR spectrum of gum and mucilages was recorded on FT-IR spectrophotometer. The sample were mixed with KBr in ratio of (1:4) and pressed into pellets under mechanical pressure using hydraulic press. The scans were obtained with a spectral resolution of 2 cm⁻¹ from wave number 4000 to 200 cm⁻¹. The GC-MS analysis was carried out using HP-5 conventional capillary column (30m x 0.25 mm with internal diameter of 0.25 µm) coupled to ion trap mass spectrometry functioned at 70ev. The columns were automated from 50 to 250°C at 50°C/min. X-ray diffraction (XRD) patterns of gum and mucilages were carried out using Shimadzu, XRD 6000 equipment, with nickel filtered tube CuKα1 at a voltage of 45 kV and current of 45 mA. The scanned angle was set at 20 from 5° to 90° and scanned rate was 1°/min.

6.2.4. Drug-polymer compatibility studies

The compatibility studies of cefixime in combination with different polymers in 1:1 ratio (physical mixture) were carried out by FT-IR, ¹H NMR, XRD and DSC studies.

i) Fourier Transform InfraRed spectroscopy (FT-IR)

FT-IR spectra of cefixime alone were recorded and their physical mixtures with polymers were recorded using FT-IR spectrophotometer (method described in section 6.2.3.3 (b)).
ii) $^1$H NMR

The proton NMR spectrum of deacetylated gum was recorded in an NMR spectroscopy (varian, UNITY-400, Switzerland). 100 mg of sample was dissolved in D$_2$O and chemical shifts were reported in ppm relative to an internal standard TMS (tetramethylsilane) for $^1$H NMR. The proton NMR spectrum was obtained at a base frequency of 400 MHz and with 16 transitions at a delay time 2 seconds. The existence of an interaction was detected by the alteration, shift or disappearance of a characteristic peak of the drug $^{[156]}$.

iii) X-ray diffraction analysis (XRD)

In X-ray studies, an automatic x-ray diffractometer equipped with a PW R30 x-ray generator was used. The dry sample powder was pressed into pellets and X-ray diffraction spectra were recorded $^{[157]}$.

iv) Differential scanning calorimetric analysis (DSC)

Differential scanning calorimetric analysis was used to characterize the thermal behavior of the drug, mucilages, gum and their physical mixtures. Sample was crimped in standard aluminium 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 calibration reference standard $^{[158]}$.

6.2.5. Standard calibration curve of cefixime by UV-Vis spectrophotometer

10 mg of cefixime dissolved in 10 ml of 0.05M Phosphate buffer pH 7.4. From this stock solution, 1 ml was withdrawn and further diluted to 10 ml to obtain a concentration of 100 µg/ml. Aliquots of 2, 4, 6, 8, 10, 12, 14 and 16 ml were pipetted out and made up to 10 ml with buffer in order to obtain a concentration range of 2-16
µg/ml and solutions were scanned in the range of 400 nm to 200 nm. A standard curve of concentration vs. absorbance was plotted

6.2.6. Preliminary parameters for nanoparticle preparation

The different concentration ranges of mucilages and gum (0.01-1.0% w/v) were subjected in suitable media (distilled water and 0.1% acetic acid in case of chitosan) at room temperature. Opalescent suspension formation was determined by visual examination of the suspension under light alternatively against white and black backgrounds. The pH of the solution also plays important role in opalescent suspension formation. The selected ranges of mucilages and gum were subjected to the different pH range (4.5-8.0) to study the effects of pH on opalescent suspension formation. The pH of different solutions were adjusted by addition of 0.1N hydrochloric acid/0.1N sodium hydroxide and pH was measured by pH meter. The effect of pH on opalescent suspension was examined under light and dark background. All measurements were performed in triplicate (n=3) and the standard deviation (SD) was recorded.

6.2.7. Formulation of nanoparticles

Chitosan was dissolved in distilled water with 0.1% acetic acid was kept under magnetic stirring until chitosan dissolves completely and pH of the solution was adjusted to 5.5 using 1N sodium hydroxide. Simultaneously, anionic polymer solution (GK, FG, AI and BA) was dissolved in distilled water and kept under magnetic stirring and pH of the solution was adjusted to 5.2 using 0.1N hydrochloric acid. The drug was dissolved in 7.4 phosphate buffer to this 0.5% tween 80 was added and sonicated to get clear drug solution as drug was poorly soluble. The prepared drug solution was added to the chitosan solution with constant stirring at 3500 rpm to this anionic polymer solution was added slowly drop wise with the help of syringe for 2 h and the solution was centrifuged at 12000 rpm for 45 min and then freeze dried and the nanoparticles were stored in desiccator.
Table 4: Composition of different formulations of cefixime nanoparticles

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefixime (mg)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Anionic polymer solution</td>
<td>0.02%</td>
<td>0.04%</td>
<td>0.02%</td>
<td>0.04%</td>
</tr>
<tr>
<td>(GK, FG, AI and BA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chitosan (C)</td>
<td>0.02%</td>
<td>0.02%</td>
<td>0.04%</td>
<td>0.04%</td>
</tr>
</tbody>
</table>

6.2.8. Evaluation of prepared nanoparticles

6.2.8.1. Particle size, poly dispersity index and zeta potential

Nanoparticles size distribution and zeta potential was determined using Nanoparticle analyser SZ-100. The size distribution analysis was performed at a scattering angle of 90° and at a temperature of 25°C using samples appropriately diluted with isopropanol. The poly dispersity index (PDI) is a dimensionless measure for the broadness of a particle size distribution and can be used for the nanoparticle dispersion. PDI between 0.03 and 0.06 can be denoted as monodisperse, between 0.1 and 0.2 as narrowly distributed and between 0.25-0.5 as broadly distributed and value above 0.5 indicated extremely broad size distribution that cannot be described by means of PDI \[^{163}\]. The zeta potential was measured using a disposable zeta cuvette using samples appropriately diluted with double distilled water. For each sample, the mean diameter/zeta potential ± standard deviation of three determinations was calculated.

6.2.8.2. Determination of percentage entrapment efficiency, percentage drug content and percentage yield

The percentage entrapment efficiency of drug loaded nanoparticles was determined by the separation of drug-loaded nanoparticles from the aqueous medium containing free drug by cooling centrifugation at 12000 rpm for 45 min. The amount of
free drug in the supernatant was determined spectrophotometrically by measuring the absorbance in UV-Vis spectrophotometer at 287 nm \[164, 165\]. The entrapment efficiency of the nanoparticles was determined in triplicates and calculated as follows;

\[
\text{% Entrapment efficiency} = \left( \frac{\text{Actual drug content}}{\text{Theoretical drug content}} \right) \times 100
\]

\[
\text{% Drug content} = \left( \frac{\text{Weight of drug in nanoparticles}}{\text{Weight of nanoparticles}} \right) \times 100
\]

\[
\text{% Yield} = \left( \frac{\text{Weight of nanoparticles recovered}}{\text{Weight of the polymer and drug fed initially}} \right) \times 100
\]

6.2.8.3. Morphology studies \[166\]

a) Scanning Electron Microscopy (SEM)

The surface morphology of nanoparticles was recorded by using SEM. Appropriate samples were mounted on an aluminum stub with double-sided adhesive tape. The tape was first firmly attached to the stub and the sample powder was scattered carefully over its surface. The stub with the specimen was then sputter coated with a thin layer of gold to make the specimens conductive. The processed specimen was subjected to SEM analysis.

b) Transmission Electron Microscopic (TEM)

TEM was used to study the surface morphology of nanoparticles. Samples of the nanoparticles suspension (5-10 µl) were dropped onto formvar-coated copper grids. After complete drying, the samples were stained using 2% w/v phosphotungstic acid. Digital Micrograph and soft imaging viewer software were used to perform the image capture.
c) Atomic force microscopy (AFM)

The morphological characteristics of cefixime nanoparticles were observed using AFM. A small quantity of sample was dissolved suitably in isopropyl alcohol and deposited on a glass slide and dried overnight to form a thin film and was analysed by AFM (NT-MDT).

6.2.8.4. In-vitro drug release by diffusion bag technique

In-vitro drug release studies for the nanoparticles were performed using diffusion bag technique. The formulation (equivalent to 10 mg) dispersed in 5 ml of dissolution medium was placed in a dialysis bag (MWCO: 12–14 kDa, surface area of 22.5 cm²) immersed in USP Apparatus I with a dissolution medium (300 ml) of 0.05M potassium phosphate buffer of pH 7.4 stirred at 37 ± 2°C maintained at 100 rpm. 1 ml of sample was withdrawn at regular intervals of time and an equal volume of buffer solution was added to maintain the constant volume of dissolution medium. The amount of drug released was measured spectrophotometrically using UV at 287 nm. All measurements were performed in triplicates (n=3) and SD was calculated [167, 168].

6.2.8.5. Drug release kinetics

The in-vitro drug release data obtained were extrapolated by various mathematical models such as zero order, first order, Higuchi, Korsmeyer-Peppa’s and Hixson-Crowell equation to know the mechanism of drug release for all the formulations. The equation with high regression coefficient (R²) for formulation will be the best fit of release data. Zero order equation describes that the system where the release rate is independent of the concentration of the dissolved species. The first order equation describes the release from the system where the dissolution rate dependent on concentration of the dissolving species. Higuchi square root equation describes the
release from the systems where the solid drug is dispersed in an insoluble matrix, and the rate of drug release is related to the rate of drug diffusion. The Hixson-Crowell cube root law describes the release from the systems where there is change in surface area and diameter of the particle. Korsmeyer-Peppa’s equation is used when the release mechanism is not well known or when more than one type of release phenomenon could be involved. If $n=0.5$ indicates pure fickian diffusion, $n=0.5-1$ indicates anomalous non-fickian diffusion and $n=1$ indicates zero order release \cite{169,171}.

6.2.8.6. *In-vitro* antimicrobial efficiency by disc agar diffusion technique and broth dilution technique

The antibiotic-resistant profile of sample was determined by disc diffusion method to determine the zone of inhibition. The study was carried out on Muller Hinton Agar (MHA) medium. The inoculums were then spread on to solid plates with sterile swab moistened with bacterial suspension (*Salmonella Typhi*). 20 µl of pure cefixime and formulations were serially diluted to a different concentration of 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml and 1000 µg/ml was added to respective disc placed on MHA plates. These plates were incubated for 24 h at 37°C. Then the activity was determined by measuring the diameter of zone of inhibition \cite{172}.

The MIC50 of pure cefixime and formulations was determined using broth dilution technique by adding 5 ml of sterilized nutrient broth in each tube. To this 100 µl of bacterial culture (*Salmonella Typhi*) was added and then 100 µl of different concentration of samples (62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml and 1000 µg/ml) was added. OD was measured at 620 nm \cite{173}.
6.2.8.7. Accelerated stability studies

Stability is a very important criterion for formulation development. Stability helps in assessment of shelf life. Different regulatory bodies like ICH have guidelines and stringent norms of real time stability data that needs to be established. This stability data gives information about shipping and storage of samples. Accelerated stability studies were conducted for the optimised formulation as per ICH guidelines. Nanoparticles were transferred to 5 ml glass vials sealed with plastic caps and were kept in stability chamber at a storage conditions of 40°C ± 2°C/75% RH ± 5% RH for a time period of six months. The samples were analysed for change in particle size, zeta potential, entrapment efficiency, drug content and morphology by SEM.

6.2.8.8. In-vivo studies

Animals

Adult male Sprague Dawley (SD) rats were obtained from Centre for Toxicology and Developmental Research (CEFT, SRU). Animals were housed in groups (n=4/cage) on soft bedding with food and water available ad libitum, in a temperature-controlled environment with a light-dark cycle of 12:12 h. All animals were allowed to habituate to the housing facilities for at least 1 week prior to study. Guidelines of “Guide for the Care and Use of Laboratory Animals” (Institute of Laboratory Animal Resources, National Academic Press 1996; NIH publication number #85-23, revised 1996) were strictly followed throughout the study. All experimental procedures were approved by the Institutional Animal Ethical Committee (IAEC), Sri Ramachandra University, constituted as per the directions of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India, (IAEC NO: IAEC/XL III/ SRU/428/2015, IAEC/XL VI/ SRU/453/2015).
6.2.8.8.1. Acute oral toxicity studies (OECD 425 guidelines)

Procedure

Description of the method

Female Sprague Dawley rats, with 8-12 weeks old age (average wt. 150-200 g) animals were randomly selected, marked to permit individual identification and kept in their cages for at least 5 days prior to dosing to allow for acclimatization to the laboratory conditions. Animals were fasted overnight prior to dosing. In the present study, the formulation was suspended in 0.5% CMC and then administered.

Principle of the main test

Single animals was dosed orally in sequence usually at 48 h intervals using oral gavage after being fasted overnight, but free access to water. Animals were fed 3-4 h after dosing. The first animal was dosed a step below the best preliminary estimate of the LD50. As per the guideline if the animal survives, second animal receives a higher dose. If the first animal dies or appears moribund, the second animal receives a lower dose using the test sequence 1.75, 5.5, 17.5, 55, 175, 550 and 2000 mg/kg body weight with a progression factor of 3.2. Since there was no toxicity information on the test item, 175 mg/kg body weight was selected as starting dose. The time interval between the administration of dose to animals was determined by observing the survival and severity of toxic signs of the animals during the first 48 h of dosing [174]. The testing was stopped when one of the following criteria was met:

a) 3 consecutive animals survive at the upper bound;

b) 5 reversals occur in any 6 consecutive animals tested;

c) At least 4 animals have followed the first reversal and the specified likelihood-ratios exceed the critical value.
When stopping criteria was met, the LD50 was calculated from the animal outcomes at test termination using computer software AOT425statpgm (version: 1.0).

**Observations**

- Animals were observed for mortality and morbidity following test item administration and thereafter twice a day till necropsy.
- Animals were observed for clinical signs at 30 min, 1, 2 and 4 h after test item administration and once a day for 14 days.
- Body weight was recorded once before dosing (day 0) and thereafter on day 7 and day 14.
- All gross pathological changes were recorded for each animal.

**6.2.8.8.2. Pharmacokinetic analysis**

Sprague Dawley rats (male) weighing 150-200 g was used for the pharmacokinetic studies. All the rats were fasted for 12 h before the experiment but had free access to water. The free cefixime and selected formulation (20 mg/kg body weight dispersed in 0.5% CMC) were administered to 6 rats in each group using oral gavage. Blood samples (0.5 ml) were taken at 0, 0.25, 0.75, 1, 2, 4, 6, 8, 24 and 48 h after administration, plasma was separated and stored frozen at -80ºC until further analysis \[175\].

**Plasma analysis**

Plasma concentrations of cefixime were quantified using HPLC method. The chromatographic system consisted of Shimadzu LC-20 AD, SPD-M201 230V UV/visible detector. Separation was achieved using C18 column at ambient temperature with a mobile phase comprising of methanol: phosphate buffer solution \[35: 65 \text{ v/v, pH}=2.75 \text{ adjusted with phosphoric acid}\] at a flow rate of 1 ml/min. The
sample extracts were prepared by mixing 100 µl of rat plasma with 200 µl of methanol and vortexed for 3 min then centrifuged at 12000 rpm for 10 min. The supernatant was filtered through 0.2 µm syringe filter and 50 µl of this filtrate was injected into the HPLC system under the chromatographic conditions employed. Cefixime was eluted at a retention time of 4.31 ± 0.61 min. The plasma concentration of cefixime was found to be linear over the range of 0.040 µg/ml to 20 µg/ml (R² > 0.999). LOD and LOQ were detected as 0.0185 µg/ml and 0.040 µg/ml respectively\textsuperscript{176}.

**Data analysis**

The data of plasma cefixime concentration-time was analysed based on non-compartmental pharmacokinetics using the WinNonlin (Version 4.0) computer software.

**6.2.8.8.3. In-vivo efficacy studies in rat model**

**Procedure**

*Salmonella enterica* serovar Typhimurium is a widely used strain to induce acute systemic salmonellosis infection in rat model. Bacteria was grown in Luria-Bertani broth overnight at 37°C and bacterial subcultures were grown until log phase, then harvested and suspended in PBS. Serial ten-fold dilutions were made, plated out in nutrient agar and incubated overnight at 37°C. The total number of colony forming units (CFU) was calculated\textsuperscript{177}.

Thirty healthy male Sprague Dawley rats, with 12-14 weeks (average wt. 280-350 g) were divided into four groups of six to eight rats, each tested for anti-microbial efficacy.
Materials and Methods

Group I: Non-infected (n=6); intraperitoneally injected with 1 ml of PBS and orally administered with 0.25% CMC (10 ml/kg body weight);

Group II: Infected (n=8); intraperitoneally injected with $2 \times 10^7$ CFU in 1 ml of PBS and orally administered with 0.25% CMC;

Group III: Free cefixime (n=8); administered with 20mg/kg body weight of cefixime in 0.25% CMC orally and bacteria ($2 \times 10^7$ CFU/ml) via intraperitoneal injection;

Group IV: Formulation (n=8); administered with 10 mg/kg body weight of formulation dispersed in 0.25% CMC orally and bacteria ($2 \times 10^7$ CFU/ml) via intraperitoneal injection.

The infected rats were orally treated with free cefixime and formulation 2 h of post-infection once-daily for three days.

Observations

- Body weight was measured every day and body temperature was performed 2 h following the treatment on day 1, 3 and 5 using telithermometer. The blood and faeces were microbiologically tested for the presence of viable bacteria.

- On day 6, animals were euthanized and the liver, colon, spleen and intestine of each animal was removed and homogenized in 3 ml of PBS aseptically. The homogenates were serially diluted and were plated onto Salmonella Shigella agar (SS agar) plates. The plates were incubated overnight, and the number of viable bacteria (CFU/mg tissue) was counted.

- To analyse haematological changes generated by *Salmonella* Typhimurium infection, post-mortem blood samples were taken and the haematological parameters were analysed using automated analyser.
6.2.9. Statistical analysis

The results were expressed as mean ± standard deviation (SD) for each parameter analyzed. The pharmacokinetic results were analyzed statistically using student’s independent sample t-test and expressed as one-way $P$-value. A nonparametric Kruskal-Wallis statistical analysis was performed to determine significance among the different experimental groups for in-vivo efficacy studies. The significance was set at $P$-value less than or equal to 0.05, and for those cases in which there were significant differences, the Mann-Whitney post hoc test was applied. All statistical analysis were performed with SPSS software.