4.1 MATERIAL

Chemicals: Lecithin soya 30% was purchased from Vinayak Ingredients (India) Private Limited, Mumbai. Cholesterol was purchased from S.D. Fine Chem., Mumbai and all other chemicals are of analytical grade.

4.2 METHOD

4.2.1 Preparation of aqueous extract of *Tecomella undulata*

For preparation of aqueous extract, powder of bark (500 g) was decocted with distilled water for 1 hour and Extract was filtered using Whatman filter paper (size no.1). Solvent was evaporated in water bath and aqueous extract was concentrated.

4.2.2 Method of preparation of Phytosomal syrup of *Tecomella undulata*

**Step-1: Preparation of thin layer of Phospholipid mixture**

Phytosomes of *Tecomella undulata* was prepared by solvent evaporation method. Accurately weighed quantity of lecithin and cholesterol were dissolved in chloroform (10 ml) in round bottom flask (RBF) and sonicated in rotary evaporator (45-50°C) for 10 minutes for removal of organic solvent. After complete removal of solvent thin layer of phospholipids mixture was formed.

**Step-2: Preparation of Phytosomes**

Thin film of phospholipids was hydrated with 10 ml aqueous extract of Tecomella undulata at 37-40°C. After complete hydration, mixture of lipid and plant extract was sonicated for 20 minutes by using Ultrasonic Probe Sonicator.

**Step-3: Preparation of syrup base (simple syrup IP)**

667 mg. Sucrose was added to 10 ml. purified water in tared beaker and heated the mixture on water bath until sucrose was completely dissolved. Filtered hot syrup through cotton wool and cool the solution. Sodium benzoate (1 gm), Methyl parabene (1.5 gm), Propyl parabene (400 mg) and mixed Phosphate buffer pH 6.8 (dihydrogen phosphate and Potassium dihydrogen phosphate) were added in prepared Simple syrup IP.

**Step-4: preparation of phytosomal syrup:**

Prepared solution of phytosomes (prepared in step 2) was mixed with simple syrup (prepared in step 3), Sorbitan monolaurate 20 (0.5 ml) was added in solution and stirred for one hour on magnetic stirrer at 50 rpm on room (24-25°C).
Table 4.1: Optimization and Preparation of Phytosomes

<table>
<thead>
<tr>
<th>S. N.</th>
<th>Formulation</th>
<th>Cholesterol (mg)</th>
<th>Lecithin (mg)</th>
<th>Chloroform (ml)</th>
<th>Aq. extract (T. undulata) (ml)</th>
<th>Cholesterol: lipid ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TU1</td>
<td>15</td>
<td>30</td>
<td>5</td>
<td>10</td>
<td>1.5:3</td>
</tr>
<tr>
<td>2</td>
<td>TU2</td>
<td>15</td>
<td>35</td>
<td>5</td>
<td>10</td>
<td>1.5:3.5</td>
</tr>
<tr>
<td>3</td>
<td>TU3</td>
<td>15</td>
<td>40</td>
<td>5</td>
<td>10</td>
<td>1.5:4</td>
</tr>
<tr>
<td>4</td>
<td>TU4</td>
<td>15</td>
<td>45</td>
<td>5</td>
<td>10</td>
<td>1.5:4.5</td>
</tr>
<tr>
<td>5</td>
<td>TU5</td>
<td>15</td>
<td>50</td>
<td>5</td>
<td>10</td>
<td>1.5:5</td>
</tr>
<tr>
<td>6</td>
<td>TU6</td>
<td>15</td>
<td>55</td>
<td>5</td>
<td>10</td>
<td>1.5:5.5</td>
</tr>
<tr>
<td>7</td>
<td>TU7</td>
<td>15</td>
<td>60</td>
<td>5</td>
<td>10</td>
<td>1.5:6</td>
</tr>
<tr>
<td>8</td>
<td>TU8</td>
<td>15</td>
<td>65</td>
<td>5</td>
<td>10</td>
<td>1.5:6.5</td>
</tr>
<tr>
<td>9</td>
<td>TU9</td>
<td>15</td>
<td>70</td>
<td>5</td>
<td>10</td>
<td>1.5:7</td>
</tr>
<tr>
<td>10</td>
<td>TU10</td>
<td>15</td>
<td>75</td>
<td>5</td>
<td>10</td>
<td>1.5:7.5</td>
</tr>
</tbody>
</table>

4.3 CHARACTERIZATION

4.3.1 Morphology of formulation
The morphology of prepared formulation was evaluated by the inverted microscope and Transmission Electron Microscopy (TEM).

A drop of phytosomal syrup was applied to a grid covered with a thick film. After leaving for five minutes to allow adsorption of phytosomal syrup to the grid, the excess was removed by a filter. 1% phosphotungstic acid was dropped onto the grid. Then the grid was air-dried for approximately 10 minutes and examined under a transmission electron microscope. The sample shape of formulation was photographed.144-145

4.3.2 Size Analysis of formulation
Particle size and size distribution of formulations were measured by the photon correlation spectroscopy (PCS) (Mastersizer 2000, Malvern England). A drop of phytosomal syrup was diluted in 10 mM of sodium chloride solution. Then solution was
put into the cuvette and placed in the Mastersizer™ machine. A magnetically stirred cell dispersion unit was employed with medium speed stirring in order to keep the formulation dispersed during size measurement. These set up conditions permitted accurate measurement of particles having a size range between 0.20 and 20,000 nm. The data was obtained from the program of PCS. 145

4.3.3 Entrapment efficiency (Percent drug remaining entrapped)

The sample of each batch were withdrawn, rehydrated with 10 ml NaCl (0.9%) and analyzed for PDR. Formulation was subjected to centrifugation on ultracentrifuge at 45,000 rpm for 45 min. The supernatant was collected and the pellets of Phytosomal syrup were redispersed with appropriate dilution with NaCl (0.9%) solution to remove drug adsorbed onto phytosomal syrup. Centrifugation was repeated for further 45 min and the supernatant was again collected and added to the first supernatant to comprise the unentrapped fraction of the drug. The pellets of Phytosomal syrup were solubilized using Triton-X 100 (1% w/v) and the released phytoconstituents (phytosoal syrup-entrapped fraction) was found. The drug released was analyzed by HPTLC as Phytosomal syrup entrapped fraction.145 Entrapment efficiency was expressed at the “Percent drug Remaining Entrapped”. PDR was calculated as follows –

\[
\text{Entrapment Efficiency (EE) (\%)} = \frac{\text{Amount of Tecomin entrapped in phytosomal syrup}}{\text{Overall amount of the Tecomin in formulation}} \times 100\%
\]

4.3.4 Zeta potential study

The measurements were made with a zetasizer 2000 DTS52013 (Malvern England) at 25°C. The formulation was diluted with distilled water, loaded into capillary cell mounted on the apparatus and all experiment was performed at least in triplicate.146-147

4.3.5 In-vitro Release Study

The in vitro release of prepared formulation was studied by using simple diffusion cell apparatus. The diffusion cell apparatus consist of glass tube with an inner diameter of 2.5 cm open at both ends, on end of the tube is tied with sigma dialysis membrane, which serves as a donor compartment. Phytosomal syrup equivalent to 100 mg of Tecomella
Tecomella undulata was taken in test tube and placed in 100 ml of phosphate buffer. The medium was stirrer and the temperature at 35-37°C. 10 ml of samples were withdrawn periodically and maintained the sink conditions. Then the samples were assayed spectrophotometrically, at 251 nm using phosphate buffer as blank. The releases of all prepared formulations were compared with pure Tecomella undulata extract.\(^{148-149}\)

The in-vitro release results of tecomella phytosoal syrup were expressed as the mean ± SD. Multiple comparisons of means (Tukey Test) was applied to substantiate statistical differences between groups. Significance was tested at the 0.001 level of probability (p).\(^{150-151}\)

### 4.3.6 Drug Release Kinetics study

In the present study, the prepared formulations were evaluated for phytoconstituents release kinetics. The drug release data were plotted using various kinetic equations (Zero order, first order, Higuchi’s kinetics, Korsmeyer’s equation, and Hixson-Crowell Cube root law) to evaluate the drug release mechanism and kinetics.

The data obtained from in vitro drug release studies were plotted as cumulative amount of drug released vs time, Zero order equation (eq.1), log cumulative percentage of drug remaining vs time, first order (eq.2), cumulative percentage of drug released vs square root of time, Higuchi’s model (eq.3)

\[
C = K_0t 
\]  

(1)

\(K_0\) is the zero order rate constant expressed in units of concentration/time and \(t\) is the time in hours. A graph of concentration vs. time would yield a straight line with a slope equal to \(K_0\) and intercept the origin of the axes.

\[
Log C = Log C_0 - kt/2.303 
\]  

(2)

Where \(C_0\) is the initial concentration of drug, \(k\) is the first order constant, and \(t\) is the time.

\[
Q = Kt^{1/2} 
\]  

(3)

Where \(K\) is the constant reflecting the design variables of the system and \(t\) is the time in hours. Hence, drug release rate is proportional to the reciprocal of the square root time. To evaluate the drug release with change in the surface area and the diameter of the particles, the data were also plotted using the Hixson-Crowell cube root law:
\[ 3 \ Q_0 - 3 \ Q_t = k_{HC} \ X_t \]  

(4)

Where \( Q_t \) is the amount of drug released in time \( t \), \( Q_0 \) is the initial amount of the drug in the microsphere, and \( k_{HC} \) is the rate constant for the Hixson-Crowell rate equation, as the cube root of the percentage of drug remaining in the matrix vs. time.

**Mechanism of Drug Release:** Log cumulative percentage of drug released vs log time (Koresmeyer et al. equation, Eq. 5) was plotted to evaluate the mechanism of drug release from microspheres and the exponent \( n \) was calculated through the slope of the straight line

\[ M_t / M = Kt^n \]  

(5)

Where \( M_t/M \) is the fractional solute release, \( t \) is the release time, \( K \) is a kinetic constant characteristic of the drug/polymer system, and \( n \) is an exponent that characterizes the mechanism of release of tracers.

If the exponent \( n = 0.45 \), then the drug release mechanism is Fickian diffusion, and if \( 0.45 < n < 0.89 \), then it is non-Fickian or anomalous diffusion.\(^{152,153}\)
4.4 RESULTS

4.4.1 Selection of method of Preparation
Solvent evaporation method was selected for the preparation of phytosomes containing plant extracts.

4.4.2 Optimization of lipid and Lecithin Ratio and preparation of phytosomal syrup
Ten formulation batches of aqueous extracts of Tecomella undulata bark with the different ratio of lipid & lecithin were prepared (step 2) (table 4.1), and phytosomal syrup was prepared by mixing the phytosomes and simple syrup according to procedure. Prepared formulations of phytosomal syrup were tested for in-vitro release profile and best formulation batch was selected on the basis of various in-vitro evaluations.
Various in vitro studies performed for optimization of batches were, digital microscopy, TEM, Size analysis, zeta potential study, in vitro drug release, particle size and entrapment efficiency.

4.4.3 Visualization study

4.4.3.1 Digital Microscopy of prepared batches
Photomicrography of syrup of TU4 was shown in figure 4.1. It was shown that formulation prepared from the ratio of 1.5:4.5 (cholesterol:lecithin) seemed to be unilamellar.

Figure 4.1: Digital photograph of prepared TU4 phytosomal syrup
4.4.3.2 Transmission electron microscopy (TEM) analysis

TEM provided the evidence of vesicle formation and their morphology evaluation showed small, spherical, and unilamellar vesicles (Figure 4.2).

![TEM image of vesicles](image)

Figure 4.2: TEM of syrup of TU4

4.4.4 Quantification of TU4

HPTLC analysis was used for quantification of tecomella phytoconstituents (glycoside). Tecomin was taken as marker compound and standard curve of tecomin was prepared by using HPTLC & UV spectrophotometer method. Following values for Y and R were obtained from HPTLC method:

\[ Y = 86.111x + 4836.3 \] and \( R^2 = 0.9987 \)

Following values for Y and R were obtained from UV spectroscopy method:

\[ Y = 0.0899x + 0.0773 \] and \( R^2 = 0.9834 \)

4.4.5 Size analysis of phytosomal syrup

The average particle size of the syrup of TU4 was 183.9 nm (Figure 4.3)
Figure 4.3 (i): Report of Particle Size of syrup of TU4
4.4.6 Zeta potential

The presence and magnitude, or absence, of a charge on colloidal particle is an important factor in the stability of colloidal system. Zeta potential of TU4 was near to zero which suggests that particles require only a minute charge for stabilization.
Figure 4.4 Zeta Potential study of syrup of TU4
4.4.7 Separation of entrapped and unentrapped plant extract

The entrapment efficiency of prepared phytosomal syrup formulations were found to be up to 90% (Figure 4.5).

![Figure 4.5: Entrapment efficiency of prepared formulations](image)

4.4.8 *In-Vitro* Release Studies

Drug release of various prepared syrup formulations was showed in figure 4.6. Prepared batches showed % cumulative release between 55 to 88.7%.

![Figure 4.6: *In-vitro* Drug release kinetics of prepared formulations](image)
4.4.9 Drug release kinetics study

Various plots of kinetic equations (Higuchi matrix plot, first order plot, zero order plot, korsemeyer peppas plot) were plotted to evaluate the drug release mechanism and kinetics.

The kinetic treatment reflected that release data of TU4 showed R value of 0.997 for first order, 0.818 for zero order equation, 0.7753 for korsenmeyer peppas and 0.949 for higuchi respectively, indicating that release of plant phytoconstituents follows first order kinetic further in higuchi, zero order and korsenmeyer peppas.

(A) Higuchi Plot (UT4)

(B) First Order plot (UT4)
Figure 4.7: Release kinetics of syrup of TU4: (A) Higuchi matrix plot, (B) first order plot, (C) zero order plot, (D) Korsemeyer Peppas plot.


4.5 DISCUSSION

Prepared Phytosomal syrup was evaluated for their morphology, release character, drug entrapment efficiency, size of particles and charge on surface. It was concluded that prepared Phytosomal syrup of TU4 was showed good entrapment and release property, having size less than 200 nm and having unilamellar vesicle. Kinetic stability in majority of the preparations is related to the presence of charge on membrane, and consequently to the existence of electrostatic forces of repulsion that balance the London dispersion forces. Vesicles repel each other when their double layer overlaps. The origin of repulsive force is entropic. Thus surface charge produces an energy barrier and when this barrier far exceeds the thermal energy (expressed as kT), the primary minimum becomes inaccessible and the system is kinetically stable.