CHAPTER III
EXPERIMENTAL
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This chapter covers the materials and methods, which include the raw materials used, sample preparation techniques, their characterization techniques and their application studies.

3.1. Materials Used

The chemicals used in the present study and the manufacturers are listed below:

<table>
<thead>
<tr>
<th>Materials</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Gelatin</td>
<td>E-Merck, India</td>
</tr>
<tr>
<td>(ii) Chitosan (Medium molecular weight)</td>
<td>Sigma-Aldrich Inc., USA</td>
</tr>
<tr>
<td>(iii) Gelatin Type-B (from bovine skin, 225 bloom)</td>
<td>Sigma-Aldrich Inc., USA</td>
</tr>
<tr>
<td>(iv) Tween 80</td>
<td>E-Merck, India</td>
</tr>
<tr>
<td>(v) Glacial Acetic acid</td>
<td>E-Merck, India</td>
</tr>
<tr>
<td>(vi) Urea GR grade</td>
<td>E-Merck, India</td>
</tr>
<tr>
<td>(v) Sulphuric Acid (98%)</td>
<td>E-Merck, India</td>
</tr>
<tr>
<td>(vi) Sodium sulphate (anhydrous)</td>
<td>Sd Fine chemicals, Mumbai</td>
</tr>
<tr>
<td>(vii) Sodium Hydroxide (pellets)</td>
<td>E-Merck, India</td>
</tr>
<tr>
<td>(viii) Genipin (Mw=226.23)</td>
<td>Challenge Bioproducts Co., Taiwan</td>
</tr>
</tbody>
</table>
(ix) Glutaraldehyde 25%(w/v) E-Merck, Germany
(x) Sodium chloride E-Merck, India
(xi) Potassium chloride E-MERCK, India
(xii) N, N-diethyl-m-toluimide (DEET) Sigma-Aldrich Inc., USA

Other reagents used for the study were of analytical grade.

3.2. Methods

3.2.1. Extraction of Essential Oil

Seeds of *Zanthoxylum Limonella* (locally called: Zabrang) a big tree available in the Solmara area of Tezpur, Assam, India were collected and dried in shed for one week. After air drying, steam distillation of *Z.Limonella* seeds was carried out to isolate the essential oil. Five hundred grams of dried and finely ground material of *Z. limonella* seeds were placed in an extraction column connected to a round-bottomed distillation flask containing distilled water. The flask was heated to about 100°C and allowed to reflux until distillation was completed. The liquid formed, together with distillate oil, was collected in a separating funnel. The mixture was allowed to settle and the water (lower) layer was slowly drawn off until only the oil layer remained. The volatile oil obtained was dried by treatment with anhydrous sodium sulphate and then collected. The dried oil was transferred into an ambered colored glass bottle and kept at 4°C for subsequent use.
3.2.2. Coacervation Behaviour Study

3.2.2.1. Simple Coacervation behavior of gelatin / or gelatin-chitosan with sodium sulphate

To optimize the coacervation process, the study of phase separation behaviour of aqueous gelatin or gelatin-chitosan solution in presence of sodium sulphate solution is essential. The coacervation process depends on several factors like polymer to salt ratio, temperature etc. The minimum temperature and polymer-salt ratio at which clear phase separation occurred indicate the optimum condition for coacervation. The temperature at which a homogenous solution separates into two phases namely, a polymer phase and a diluted one is known as the cloud point temperature.

a) Coacervation of gelatin by salting out method

A series of experiments were carried out to determine the cloud point temperature of a gelatin solution as a function of sodium sulphate concentration. Flask containing a certain amount of gelatin was immersed in a thermostatic water bath maintained at 5°C. A predetermined amount of aqueous sodium sulphate solution (10 % w/v) was added to the flask under stirring condition and the temperature of the water bath was gradually raised. The temperature at which the onset of phase separation started was recorded.
b) Coacervation of chitosan-gelatin by salting out method

0.25 % (v/v) aqueous solution of chitosan in 1% (v/v) acetic acid and 0.25% aqueous solution of gelatin in deionised water were prepared. The solution of chitosan and gelatin were mixed at different ratio (1: 0.5 – 2) at room temperature (~30°C) under stirring condition. Now a predetermined amount of aqueous sodium sulphate solution (20%w/v) was added to each polymer mixture containing chitosan and gelatin at different ratio at room temperature. The ratio of total polymer to sodium sulphate was varied from 1:2 to 1:30. The temperature was varied from 30°C to 50°C. The minimum temperature and polymer-salt ratio at which clear phase occurred was recorded.

3.2.2.2. Complex coacervation of chitosan and gelatin

Complex coacervation is a process where the phase separation is induced by the interaction of two oppositely charged macromolecules. It can be experimentally determined by measuring the coacervate yield and turbidity. Complex coacervation also depends on several factors like ratio of one polymer to other, pH, temperature, etc.

Coacervate Yield

0.25% (w/v) aqueous solution of chitosan in 1%(v/v) acetic acid and 0.25-1.0%(w/v) aqueous solution of gelatin in double distilled deionised (DDI) water were prepared. The prepared solution of chitosan and gelatin were mixed at different ratios at room temperature (~30°C) under stirring condition. The ratio of chitosan to gelatin was
varied from 1:1 to 1:40. The pH of the polymer solution was adjusted with either dil. HCl or NaOH solution and it was measured using digital pH meter. The investigated pH range was 5.0 to 6.0. This range was chosen as it was above the isoelectronic point of gelatin and below the pH at which precipitation for chitosan occurred. The charge and charge density of the polymer would vary with change of pH. Accordingly interaction between chitosan and gelatin would vary leading to formation of different coacervate yield. The dry coacervate yield was obtained by decanting the supernatant and drying the coacervate phase till attainment of constant weight.

**Turbidity measurement**

Mixture of chitosan and gelatin was taken in a beaker. The pH of the solution was kept initially low (below 5.0) using dil. HCl. The solution appeared clear. Now dil. NaOH was added drop wise from a burette. Turbidity would appear due to formation of coacervate particles in the continuous phase. The change in transmittance due to turbidity was monitored continuously using UV spectrophotometer at 450nm. The pH at which maximum absorbance noticed was recorded. The absorbance was expressed as 100 - %T (transmittance). This pH would produce maximum turbidity as well as maximum coacervate yield.

**3.2.3. Preparation of glutaraldehyde cross-linked gelatin microcapsules containing Zanthoxylum Limonella oil (ZLO) by simple coacervation technique**

To a reaction vessel, certain percentage (4-10 % w/v) of an aqueous solution (50ml) of gelatin was taken at room temperature (30°C). To this, a certain amount of essential oil (3-15 ml) was added under high agitation to form an emulsion. The
temperature of the vessel was then raised to 40°C. Coacervation was brought by gradual addition of aqueous sodium sulphate solution (20% w/v) for about 90 mins. The vessel was kept at this temperature for another 30 mins. The temperature of the vessel was then brought down slowly to about 5°C using ice crystals. The crosslinking of the polymer capsule was achieved by slow addition of certain amount of glutaraldehyde (1-10 mmol/g of gelatin) solution which consists of methanol 16.67%, acetic acid 5%, sulphuric acid 0.17% and glutaraldehyde 25%. The temperature of the vessel was then raised to 40°C and stirring was continued for about 3-4 h in order to complete the crosslinking reaction. The vessel was cooled to room temperature. The microcapsules were filtered, washed with 0.3% Tween 80 surfactant solution, dried and stored inside a glass bottle.

3.2.4. Preparation of genipin cross-linked chitosan-gelatin microcapsules containing ZLO using complex coacervation technique

To a beaker, certain amount of 2%(w/v) chitosan solution previously made in 1%(v/v) aqueous acetic acid and 2%(w/v) aqueous gelatin solution were taken. Total amount of polymer is kept constant at 1 gm. The mixture of polymer solution was stirred by mechanical stirrer under high agitation after adding one drop of silicon antifoaming agent at 40°C. The temperature was maintained at 40°C. To this, essential oil (1-10ml) was added under high agitation to form an emulsion. Using 0.1N NaOH, the pH of the emulsion was brought to the range of 5.4-5.8 to attain the maximum coacervation. Once the coacervation took place with the formation of microcapsules the system was brought to room temperature (30°C) to harden the microcapsules. The crosslinking of the polymer capsule was achieved by slow addition of certain amount of genipin (0.05-0.5
mmol/g of polymer) solution (0.5%w/v aq.solution). The temperature of the vessel was then raised to 40°C and stirring was continued for about 3-4 h in order to complete the crosslinking reaction. The vessel was then cooled to room temperature. The microcapsules were filtered, washed with 0.3% Tween 80 surfactant solution, dried and stored inside a refrigerator in a glass ampule.

3.2.5. Preparation of chitosan-gelatin microcapsules containing ZLO using salting out technique

2.50 g of chitosan flakes were dissolved in 100ml of 1%(w/v) acetic acid solution by stirring overnight in a conical flask until a clear solution was obtained. 4.0 g of gelatin (type B) was dissolved in 100ml double distilled water by heating for 1-2 hour to get a clear solution. Variable amounts of chitosan and gelatin solution were taken in a beaker at room temperature (~30°C) so that the weight ratios of chitosan to gelatin were 1/0, 1/1, 1/2, 2/1 and 0/1. The temperature of the beaker was raised to 40°C. To this mixture, a drop of silicon antifoaming agent and essential oil (1-10ml) were added under high agitation by mechanical stirring to form an emulsion. Initially the coacervation of chitosan was brought about by gradual addition of aqueous sodium sulphate solution (20%w/v) for about 2-2.5 h. In this stage ZLO encapsulated chitosan particles/microcapsules were formed. The pH of the entire mass of the beaker was then brought between 7.0-8.0 to induce interaction between chitosan, gelatin (type B) and genipin. The crosslinking of the chitosan-gelatin microcapsule was achieved by addition of certain amount of aqueous genipin solution (0.1-0.3 mmol/g of polymer). The temperature of the vessel was
maintained between 40-50°C and stirring was continued for another 3 hr. The vessel was then cooled to room temperature. The microcapsules were filtered, washed with 0.3% Tween 80 solution to remove excess oil adhered to the surface, dried and kept in a storage vial.

3.2.6. Preparation of chitosan microspheres containing urea using emulsification and cross-linking method

Emulsification and crosslinking method was adapted for the preparation of urea containing chitosan microspheres. 75ml paraffin oil and 25 ml petroleum ether were taken in a beaker. To this solution, 2.0g Tween 80 surfactant was added. They were mixed together and to the above solution, urea and chitosan mixture (mixed in another beaker) were added gradually with continuous stirring with the help of high speed homogenizer. A water in oil (W/O) emulsion resulted and then the temperature of the whole mass were brought to about 40°C after the addition of predetermined amount of crosslinking agent. Crosslinking reaction was allowed to occur for 3 h. The microspheres formed were separated from the paraffin oil and petroleum ether mixture by filtration. The filtered microspheres were washed thrice with petroleum ether, once with acetone and finally with water to remove surface urea (if any). The filtered and washed microspheres were dried under vacuum at about 60°C. The dried microspheres were kept in tightly stoppered bottle in vacuum desiccator.
3.2.7. Preparation of deacetylated chitosan

Chitosans with different degree of deacetylation (DDA)s were prepared from the procured chitosan sample. 10 g chitosan sample was refluxed with 100 ml of 40% (w/v) NaOH solution at 80°C for 4 h under nitrogen atmosphere. Similarly, another sample of chitosan was refluxed with NaOH solution at 80°C for 8 h to obtain chitosan with higher DDA. The refluxed samples were separated, washed with hot and cold distilled water till it was freed from alkali and finally dried in vacuum oven. Since reaction temperature affects the rate of deacetylation, deacetylation in chitosan samples was carried out at fixed temperature [1].

3.2.8. Determination of calibration curve for ZLO using UV-Visible spectrophotometer

Calibration curve for the system under study was not available in the literature and therefore a calibration curve was required to estimate the release rate of the encapsulated oil in the eluting solvent medium. It was observed that 1 gm of ZLO could be dissolved easily in 100 ml water containing 0.3 gm Tween 80.

A known concentration of essential oil in distilled water containing 0.3% Tween 80 was scanned in the range of 200-400 nm by using UV visible spectrophotometer. For ZLO having concentration in the range 0.005 to 0.1 gm/100ml, a sharp peak at 256 nm was noticed. The absorbance values at 256 nm obtained with the respective concentrations were recorded and plotted. From the calibration curve, the unknown concentration of ZLO was obtained by knowing the absorbance value.
3.2.9. Determination of % Oil loading, % Oil content and % Encapsulation efficiency

A known amount of accurately weighed crushed microcapsules was taken inside a volumetric flask containing a known amount of 0.3% aqueous Tween 80 solution and kept overnight with continuous stirring to ensure complete extraction of oil in Tween 80 solution. The encapsulation efficiency (%), oil content (%) and oil loading (%) were calculated by using the calibration curve and the following formulae.

\[
\text{Encapsulation efficiency} \, (\%) = \frac{w_1}{w_2} \times 100
\]

\[
\text{Oil content} \, (\%) = \frac{w_1}{w} \times 100
\]

\[
\text{Oil load} \, (\%) = \frac{w_2}{w_3} \times 100
\]

Where \(w\) = weight of microcapsules

\(w_1\) = actual amount of oil encapsulated in a known amount of microcapsules

\(w_2\) = amount of oil introduced in the same amount of microcapsules

\(w_3\) = total amount of polymer used including cross-linker

3.2.10. Determination of degree of deacetylation of chitosan

The degree of deacetylation of the original and alkali-treated chitosan samples were determined by using the following techniques.
**Linear Potentiometric Titration (LPT) method**

Approximately 0.1g of chitosan was dissolved in 25.0ml of standard 0.1M HCl solution. The solution was then topped up to 100ml with distilled water and calculated amount of KCl was added to adjust the ionic strength to 0.1M. The titrant was the solution of standard NaOH containing 0.1M KCl. The standard titrant solution was added to chitosan solution gradually. Both the volume of NaOH added and pH values of the solution were recorded using digital pH-meter. The differential and integral titration curves were drawn between pH and the volume of titrant added, which produced an integral curve with two inflexions. The differential volume ($\Delta V$) of alkali between first and second neutralization point corresponds to the acid consumed by amino groups present in the chitosan. The degree of deacetylation was calculated using the following equation [2]:

$$DDA = \left[ \frac{203 Q}{(1 + 42 Q)} \right] \times 100\% \text{ and } Q = \frac{N\Delta V}{m}$$

Where ‘m’ is the weight of chitosan sample and ‘N’ is the strength of NaOH used in titration.

**Infrared Spectroscopic method**

For determination of degree of deacetylation (DDA) of chitosan samples, both KBr disk and film samples were used. The KBr disk was prepared according to the method of Sabnis and Block [3] with slight modification. Approximately 20mg of chitosan powder and 120mg of KBr was blended and triturated with mortar and pestle for
approximately 10 min. The mixture was compacted to form a disk. The disk was conditioned in a desiccator placed in an oven at 80°C for 16h before analysis.

Chitosan solution in acetic acid (1% w/v) was prepared and casted on a glass plate. This was dried in a oven at 60°C for 12 h [4]. The chitosan films were deprotonated by washing 3-4 times with methanolic ammonia solution followed by distilled water and methanol. The chitosan films were kept in a desiccator placed in an oven at 80°C for 16h before scanning. The spectra of chitosan samples in the form of KBr disk and film were obtained using an IR instrument (Nicolet, model Impact-410) with a frequency range of 4000-400 cm⁻¹. The degree of deacetylation (DDA) was evaluated by recording absorbance at 1655 cm⁻¹ for amide-I and at 3450 cm⁻¹ for OH group in chitosan. The absorbance of chitosan was used to calculate the degree of deacetylation (DDA) using the following equation [5]:

\[
\text{DDA} = [1 - (A_{1655}/A_{3450})/1.33 \times 100] \%
\]

Where the factor 1.33 represents the ratio of \(A_{1655}/A_{3450}\) for fully N-acetylated chitosan.

**Elemental Analysis method**

The degree of deacetylation of chitosan samples was verified by elemental analysis. The following derived relationships between weight percent of elemental carbon and nitrogen and degree of deacetylation (DDA) were used [6].

\[
\text{DDA} = \left( \frac{9600}{364 \ W_C + 2400} \right) \times 100\%
\]

\[
\text{DDA} = \left( \frac{1400}{364 \ W_N} \right) \times 100\%
\]
Where 'W_C' and 'W_N' are the weight percent of carbon and nitrogen in the chitosan samples.

3.2.11. Molecular weight of Chitosan

The molecular weight of chitosan samples was determined using the Mark-Houwink-Sakurada (MHS) equation [7]

\[ [\eta] = K(M_w)^{\alpha} \]

\[ [\eta] \] and \( M_w \) are the intrinsic viscosity and molecular weight while \( K \) and \( \alpha \) are constants for given solute-solvent system and temperature. Six different concentrations (0.0156-0.5%, w/v) solutions of chitosan in 0.1M acetic acid-0.2M NaCl (1:1, v/v) were prepared. The solution was filtered to remove insoluble materials. The ubbelohde-type capillary viscometer was used to measure the flow time of the solutions through the capillary in a constant temperature bath at 25°C. Three measurements were made on each sample. The running times of the solution and solvent were recorded as seconds (sec) and used to calculate intrinsic viscosity \([\eta]\).

\[ \eta_{rel} (\text{Relative viscosity}) = \frac{t_{solution}}{t_{solvent}} \]

\[ \eta_{sp} (\text{Specific viscosity}) = \eta_{rel} - 1 \]

\[ \eta_{red} (\text{Reduced viscosity}) = \eta_{sp} / C \]

\[ \eta_{intrinsic} (\text{Inherent viscosity}) = (\ln \eta_{rel}) / C \]
\[ [\eta] = \lim_{C \to 0} \eta_{sp} / C = \lim_{C \to 0} (\ln \eta_{rel}) / C \]

Where C is concentration of chitosan solution (g/dl)

Both \( \eta_{sp} \) and \( \eta_{inh} \) were plotted on a same graph. The common intercept of the two plots on the ordinate at \( C=0 \) gives intrinsic viscosity, \([\eta]\) (dl/g). The intrinsic viscosity was obtained by extrapolating the reduced viscosity vs. concentration data to zero concentration.

The viscosity average molecular weight of chitosan samples was calculated using the MHS equation. The literature values of K and \( \alpha \) are \( 1.81 \times 10^{-5} \) and 0.93, respectively [8].

3.3. Release characteristics

3.3.1. Release characteristics of ZLO containing microcapsules

Oil release studies of encapsulated oil were evaluated using UV - visible spectrophotometer (UV-2001 Hitachi). A known quantity of microcapsules was placed into a known volume of 0.3% Tween 80 surfactant solution. The microcapsule-Tween 80 mixture was magnetically stirred at a constant rate and the temperature throughout was maintained at 30°C (room temperature). An aliquot sample of known volume (5 ml) was removed at appropriate time intervals, filtered and assayed spectrophotometrically at 256 nm for the determination of cumulative amount of oil release up to a time \( t \). Each
determination was carried out in triplicate. To maintain a constant volume, 5 ml of 0.3% Tween 80 solution was returned to the container.

3.3.2. Release characteristics of urea from chitosan/urea crosslinked microspheres

The conventional method for determining the dissolution for encapsulated urea is a static test in which a certain amount of urea containing microspheres were placed in a conical flask containing 100 ml of distilled water [9]. The temperature was controlled by using a water bath. The refractive index of the solution was measured at 25°C as a function of time (daily for 7 days). The amount of urea released in water could be known from the standard calibration curve (a correlation between refractive index and concentration).

3.4. Water Uptake or Swelling Studies

A known amount (0.4-0.5 g) of microcapsules / microspheres was immersed in distilled water for a stipulated time period to reach equilibrium. Before swelling in water, all the active agents from the microcapsules / microspheres were extracted in a suitable solvent and then dried. Water uptake at equilibrium was determined according to the following equation [10]:

\[
\text{Water uptake (\%)} = \frac{(W - W_0)}{W_0} \times 100
\]

Where \(W_0\) and \(W\) are the weights before and after immersion in water for a certain period, respectively.
3.5 Elemental Analysis Study

Elemental analysis for determination of degree of deacetylation was performed in a CHN elemental analyzer of Perkin Elmer (model CHN 2400).

3.6 Fourier transform infrared (FTIR) study

FTIR spectra were recorded using KBr pellet in a Nicholet (model Impact-410) spectrophotometer. Microcapsules/microspheres, physical mixtures of polymer with active component, active component alone, were finely grounded with KBr and FTIR spectra were recorded in the range of 4000-400cm⁻¹.

3.7 Scanning electron microscopic study

The samples were deposited on a brass/copper holder and sputtered with gold. Surface characteristics of the microcapsules / microspheres were studied using scanning electron microscope (model JEOL, JSM-6360) at an accelerated voltage of 10Kv/15kv and at room temperature.

3.8 Thermal properties study

Thermal properties of ZLO containing microcapsules, ZLO, gelatin, chitosan each individually and chitosan/gelatin mixture were evaluated by employing thermogravimetric analyzer (TGA) and differential scanning calorimeter (DSC). TGA study was carried out using thermogravimetric analyzer (model TA 50, shimadzu) at a heating rate of 10°C/min up to 600°C. DSC study was done in a differential scanning
calorimeter (model DSC-60, shimadzu) at a heating rate of 10°C/min up to 400°C. Both the study was done under nitrogen atmosphere.

3.9. Laboratory Evaluation of Mosquito repellency of ZLO containing microcapsules

A predetermined amount of microcapsules prepared under different conditions were sieved. Microcapsules of approximately similar sizes were considered for the formulations. The formulation comprises of petroleum jelly and 20% active repellent. Both were mixed thoroughly in an appropriate proportion to prepare the petroleum jelly based cream. The amount of microcapsules containing 20 % active repellent was calculated on the basis of oil content (%) of the corresponding microcapsules. Base cream (petroleum jelly) without repellent was used as control. In most of the commercial formulations, N, N, Diethyl-3-methyl-benzamide (DEET) were used as one of the ingredients. Therefore, another formulation consisting of petroleum gelly and DEET was prepared for comparison.

The repellent trials were conducted in a repellent test chamber (30 x 30 x 62.5 cm) under laboratory conditions. Before application of the repellents, hands were washed and cleaned thoroughly with rectified spirit. Male and female *Aedes (S.) albopictus* mosquito progenies obtained from laboratory colony was maintained in honey solution in a cloth cage (50 x 50 x 50 cm) under controlled temperature (28±2°C) and relative humidity range (75–80%). About 50 to 60 hungry (3 days old) female *Ae (S.) albopictus* were introduced into the repellent chamber through the hole on top. The test sample (1-2 g) was smeared on dorsal side of one hand (wrist to finger tips) of each of the subject.
After 30 min of application, the hand was placed inside the repellent chamber for 10 min through a hole up to wrist and plugged with cotton to prevent escape of mosquitoes in order to facilitate the female mosquitoes to bite on. The test was repeated at every 30 min interval. The interval between the application of repellent and the first two consecutive bites occurring within 30 min was considered as protection time against the bites afforded by each of the concentrations of the test repellents [11]. The test was replicated 3 times for each concentration of the repellents. Control readings were obtained by placing hand inside the repellent chamber without applying any repellent before the experiment.
References