3. EXPERIMENTAL

3.A.1. Materials used

Table 2: List of materials used in the research along with supplier and grade.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Materials used</th>
<th>Grade</th>
<th>Manufacturer</th>
</tr>
</thead>
</table>
3.A.2. EQUIPMENTS

Table 3: List of equipments used in the research along with manufacturer.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Equipment</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>HPLC (Binary gradient Technique)</td>
<td>Shimadzu Corporation, Japan.</td>
</tr>
</tbody>
</table>
| 2.      | U.V. Spectrophotometer                    | UV 3000+
LabIndia, Mumbai                    |
| 3.      | FTIR Spectrometer                         | Brooker                                |
| 4.      | Refrigerated Centrifuge                   | Remi R21, Bangalore                    |
| 5.      | Orbital Shaker Incubator                  | Sakova, Bangalore                      |
| 6.      | Milllex-HIV 0.45 μm and 1 μm filter units | Millipore Molsheim, France.            |
| 7.      | Magnetic Stirrer 2m 2H                    | Remi Equipments, Bangalore.            |

3.A.3. Animals

Wistar albino rats weighing between 145-225 grams were purchased from Venkateshwara Enterprises, Bangalore, India. The animals were maintained under standard laboratory conditions, at temperature 25 ± 2°C, and a 12 h natural light period. Commercial pellet diet (Lipton India) and drinking water were provided *ad-libitum*. Animal experiments were carried out after obtaining the clearance from the Institutional Animal Ethical Committee.
3.B. Methods

3.B. 1. Size reduction of plant materials

Initially, seed husk of ispagol, kernel part of tamarind and pigeonpea seeds were grounded to get coarse powder was passed through sieve no. 10. This coarse powder was taken for further size reduction. The size reduction was done by manually triturating the husk in porcelain mortar & pestle. This process of size reduction was done in different batches and finally were mixed together to achieve uniform mixing. After complete size reduction, the material obtained was again sieved on sieve no. 80 and a fine powder was collected.

3.B. 2. Extraction of polymer

A general method of extraction of polymer from various plant sources have been adopted (figure 4). The dried powdered samples of ispagol husk, tamarind seed kernels and pigeonpea seeds were soaked for 24 hrs. in distill water to get pourable slurry. The slurry thus produced was poured into sufficient quantity of water and boiled for 20 min. and then allowed to cool at room temperature. Further this was subjected for centrifugation. The supernatant was separated and residue was washed successively with petroleum ether, diethyl ether and acetone to remove water insoluble fat and other matters from polymer. The precipitate obtained was dried at 50-60°C under vacuum to remove volatile matter and moisture. The dried material was further ground and sieved to obtain powder having uniform particle size, which was used for fabrication of wound dressing materials.\textsuperscript{95}
Figure 4: Scheme showing general methods of polymer extraction.
3. B. 3. Preliminary chemical tests

Purified polymer samples (SHL, TSP and PPP) were subjected to qualitative chemical analyses for the following constituents. 107

a) Detection of carbohydrates:

Polymer samples were dissolved/dispersed individually in 5ml of distilled water and filtered. The filtrate was used to test the presence of carbohydrates

i. Molisch’s test: To 2-3 ml of filtrate, add few drops of alpha- naphthol solution in alcohol, shake and add 2ml conc. sulphuric acid add carefully along the sides of the test tubes. A violet ring is formed at the junction of two liquids. It indicates the presence of carbohydrates.

Test for reducing sugars:

ii. Benedict’s test: Mix equal volume of Benedict’s reagent and polymer solution in a test tube. Heat in boiling water bath for 5min. Solution appears green, yellow or red depending on amount of present in test solution. It indicates presence of reducing sugar.

iii. Fehling’s test: Mix 1ml Fehling's A and 1ml Fehling's B solutions, boil for one minute. Add equal volume of test solution. Heat it in boiling water bath for 5-10 minutes. First yellow, then brick red precipitate is observed. It indicates presence of reducing sugar.

b) Detection of alkaloids:

Polymers were dispersed individually in dil. hydrochloride acid and filtered. Filtrates were tested carefully by following tests.

i. Mayer’s test: Treat 2-3 ml of filtrate with few drops of Mayer’s reagent (potassium mercuric iodide) gives cream precipitate. Indicates the presence of alkaloids.
ii. **Wagner's test**: Treat 2-3 ml of filtrate with few drops of Wagner's reagent (iodine in potassium iodide) gives brown/reddish brown precipitate, indicates the presence of alkaloids.

iii. **Dragendorff's test**: Treat 2-3 ml of filtrate with few drops of Dragendorff's reagent (solution of potassium bismuth iodide) gives orange brown precipitate; it indicates the presence of alkaloids.

iv. **Hager's test**: Treat 2-3 ml of filtrate with few drops of Hager's reagent (saturated picric acid solution) gives yellow colored precipitate, indicates the presence of alkaloids.

v. **Tannic acid test**: A freshly prepared solution of tannic acid (55w/w) gives a precipitate with most of the alkaloids which is soluble in dilute acid or ammonia solution. This test shows buff coloured precipitate with filtrate, it indicates presence of alkaloids.

c) **Detection of phytosterols**

i. **Salkowski's test**: To 2ml of polymer extracts add 2 ml of chloroform and 2ml of conc. sulphuric acid and shake well. Chloroform layer appears red and acid layer shows greenish yellow fluorescence. It indicates the presence of sterols and tri-terpence.

ii. **Libermann Burchard test**: Mix 2 ml extracts with chloroform solution. Add 1 ml of acetic anhydride and few drops of conc. sulphuric acid from the side of the test tube, first it shows red, green and then blue colour. It indicates that the presence of phytosterols.
d) Detection of glycosides

Polymer samples were dissolved / dispersed in water and hydrolyzed with dilute hydrochloric acid. The hydrolysate samples were subjected to glycoside tests.

Test for cardiac glycosides

i. Legal's test: To the hydrolysate add 1 ml of pyridine and 1 ml of sodium nitroprusside solution. Colour changes from pink to red. It shows the presence of glycosides.

ii. Liebermann’s test: Mix 3 ml. of hydrolysate with 3 ml. of acetic anhydride, heat and cool. Add few drops of conc. sulphuric acid. Appearance of blue colour it shows the presence of steroid.

iii. Keller Killiani test: To 2 ml. of extract add glacial acetic acid, one drop of 5% Ferric chloride and conc. sulphuric acid. A reddish brown colour appears at junction of two liquid layers and upper layer appears bluish green.

iv. Xanthydrol test: The extract heated with 0.1 to 5% solution of xanthydrol in glacial acetic acid containing 1% hydrochloric acid. A red colour produced, it indicates that the presence of 2-deoxysugar.

e) Detection of phenols and tannins

Ferric chloride test: The extract treated with 5% ferric chloride solution. The bluish black colour formed indicates that the presence of phenols.
f) Detection of amino acids and proteins

i. Millon’s test: Mix 3 ml of polymer extract with 5 ml of Millon’s reagent. The formation of white precipitate, which turns to red upon heating, indicates the presence of proteins.

ii. Ninhydrin test: (2, 2-Dihydroxyindane-1, 3-Dione) Heat 3 ml of polymer extract and 3 drops of 5% ninhydrin reagent in boiling water bath for 10 minutes. Formation of purple or blue colour indicates presence of amino acid.
3. B. 4. Fourier transforms infrared spectroscopy (FTIR) studies

The isolated polymers were purified and subjected for FTIR studies. In case of TSP and PPP, the plain polymers as well as cross-linked polymers were subjected to FTIR study. Whereas only plain seed husk of isapgol i.e., SHI was subjected for FTIR study. Polymeric samples were grounded with potassium bromide (KBr) and pellets were made by applying 6 tons of hydraulic pressure. The FTIR spectra were obtained on Nicolet, Model Impact 410, USA at University Scientific Instrumental Center (USIC), Karnataka University, Dharwad, India. The scanning was done in the wavelength region between 400 and 4000 cm\(^{-1}\).

3.B. 5. \(^{13}\)C Nuclear magnetic resonance spectroscopy (\(^{13}\)C NMR)

The isolated and purified polymers were also subjected to \(^{13}\)C NMR spectral study. Solid state \(^{13}\)C NMR spectra were recorded for polymeric samples using a NMR spectrometer (AMX 400) at Indian Institute of Sciences, Bangalore, India. Samples were ground in glass mortar and pestle. The powered sample was filled in sample holder without any solvent.
3.B.6. Fabrication of films

Polymeric films were prepared according to the method described by Kulkarni et al.\textsuperscript{108, 109} with modification (figure 5). Weighed quantity of polymer was dispersed in water in different concentrations. Concentration of polymer was chosen depending upon the type of polymer i.e., 1.5, 4.0 and 4.0 % w/v for SHI, TSP and PPP respectively. After formation of polymeric dispersion, the solution was poured in to glass plates placed on a leveled platform. The rate of evaporation from polymer dispersion poured on glass plate was controlled by inverting a cut funnel over the glass plates. After 7 days the dried films were taken out for further use.

\textbf{Figure 5: Schematic representation of fabrication of films.}
3. B. 7. Cross-linking of films

Polymeric films of TSP and PPP were cross-linked with epichlorohydrin as per the method described by Suathi et al.\textsuperscript{110} films were boiled in hydro alcoholic solution containing 5 % sodium sulfate and 1 % epichlorohydrin for 15 min to achieve cross-linking reaction. Cross-linked films were washed with deionized water (DI) several times and dried and stored in desiccators at room temperature.

3. B. 8. Loading of films with Povidone iodine

All the films were loaded with Povidone iodine (PI) by soaking films in PI solution for 12 hrs. Then PI loaded films were washed with DI water and dried in desiccators. Films loaded with PI were further subjected for screening for wound healing activity.


Polymeric films were cut in to small pieces of 5 mm circle and were fixed on a brass holder. SEM photographs were taken with a JSM 6400 Scanning Microscope (Japan) at the required magnification at room temperature. Working distance of 39 mm was maintained and the acceleration voltage applied was 5 KV with a secondary electron image (SEI) as a detector.

3. B. 10. Determination of tensile strength and percentage of elongation

Tensile strength and percentage of elongation were determined by using a universal testing machine (Shimadzu). Films of 10 mm width and 80 mm length were cut and fixed to the machine jaws. Then the load on the film was increased gradually to a maximum and the tensile strength and percentage of elongation was noted.
3.B. 11. Equilibrium swelling studies

Polymeric films of 10 mm diameters were made and kept in watch glass containing DI water. At definite intervals of time, films were taken out, blotted with Whatman’s filter paper and weighed on an electronic balance with 0.1 mg sensitivity.

3. B. 12. Water vapor transmission rate (WVTR)

Water vapor transmission studies were carried out using pre-weighed glass vials of 5 ml volume containing 1 ml distilled water. Films were fixed on the brim of the vials with an adhesive and stored in a desiccators containing anhydrous calcium chloride at room temperature for seven days (figure 6). The weight of the vials was noted down every 24 hrs. to calculate weight loss per day and WVTR were calculated as mentioned in earlier methods using equation no. 1.

\[
WVTR = \frac{WL}{S} \quad (1)
\]

Where, \( W \) is weight loss, \( L \) is thickness and \( S \) is the surface area of the film.

![Diagram](image.png)

Figure 6: Schematic representation of WVTR study.
3.B. 13. Antimicrobial study

Polymeric films and standard medicated WDM were cut into circular shape with a diameter of 10 mm and placed on nutrient agar medium containing standard bacterial inoculum. Anti-microbial activity was carried out by using Escherichia coli, Shigella dysenteriae, Pseudomonas aeruginosa and Bacillus subtilis. The anti-microbial activity was measured by measuring the zone of inhibition using a standard technique.\textsuperscript{111-113}

3. B. 14. Designing of a wound dressing patches

Designing of wound dressing patches were carried out as per the schematic diagram shown in figure 7. It is a bi-layered patch consisting of a circular porous adhesive backing membrane and a centrally placed inner wound dressing polymeric film cut into circular shape of 20 mm diameter.

\textbf{Figure 7: Descriptive diagram of the polymeric patch.}
3.B. 15. Wound healing studies

Animals were divided into three groups of six animals each. On day zero, animals were anaesthetized and secured to the operation table on its natural position. An ink impression was made on the dorsal thoracic central region 5 mm away from the ears by using round seal of 2.5 cm diameter as described by Morton and Malone.\textsuperscript{34} Skin of the impressed area was excised to the full thickness to obtain a wound area of about 500 mm\textsuperscript{2}. Wounds of the animals in the control groups were kept open without any treatment but occluded with only backing membrane whereas wounds of the animals in standard groups were applied with a Drez\textsuperscript{30} and wounds of the animals in test groups were applied with patch (figure 8). The physical attributes of healing which mainly contributes for wound closure in the first three weeks were studied by tracing the raw wound area on the tracing paper till complete epithelialization occurred as described by Lee and Tong.\textsuperscript{30}

Figure 8: Photographs of rat open wound (a) and wound treated with patch (b)