CHAPTER 5

DEVELOPMENT AND CHARACTERIZATION OF WOUND DRESSING MATERIAL COATED WITH NATURAL EXTRACTS OF ALOE VERA, PIPER BETEL AND NEEM LEAF SOLUTION ENHANCED WITH rhEGF (REGEN-D™ 150)

5.1 INTRODUCTION

This chapter reveals the development and characterization of wound dressing material coated with the natural extracts of aloe vera, piper betel and neem leaf solution with enhancement of recombinant human Epidermal Growth Factor (rhEGF). Above materials were chosen for appreciable value of well known biodegradable, biocompatible and antibacterial properties. The materials and methodology used in the development of natural extract coated dressing material were explained.

The microstructure of the coated fabric was characterized using SEM images. Chemical bonding of developed wound dressing was identified with FTIR spectroscopy. The areal density, thickness, tensile strength, tearing strength, abrasion resistance and flexural rigidity of the developed wound dressing material were measured as per the laboratory standard. Antimicrobial activity of the coated fabric against bacteria *Staphylococcus saprophyticus* was assessed in this chapter. To gain further knowledge about
developed wound dressing material with respect to diabetic wound healing \textit{in vivo} study was undertaken in Wistar albino rats.

This chapter also explains the results and interpretation of various parameters such as microstructure of the natural extract coated wound dressing material through SEM, chemical bonding by FTIR, drug content and their antimicrobial activity. The healing efficacy of coated dressing material on diabetic induced wound in Wistar albino rats were also discussed in detail.

5.2 MATERIALS FOR DEVELOPMENT OF WOUND DRESSING MATERIAL

The materials used in the development of wound dressing material coated with natural extracts of aloe vera, piper betel and neem leaf solution with enhancement of recombinant human Epidermal Growth Factor (rhEGF) are explained. 30° Ne Bamboo yarn (100 %) was purchased from M/s. Cheran spinning mills, Salem, Tamilnadu, India. The yarns were weaved as fabric in M/s. Sun Power Bit Looms, Pallipalayam, Tamilnadu, India. Leaves of Aloe vera, Piper betel and neem are collected from farm fields around Salem, Tamilnadu. The recombinant human Epidermal Growth Factor (rhEGF) as REGEN-D™ 150 was purchased from M/s. Dr. Reddys, Hyderabad, India.

The antimicrobial activity of the coated dressing materials were investigated using gram positive \textit{Staphylococcus saprophyticus} bacteria. From the animal house of Nandha College of Pharmacy, Erode, Wister albino rats were purchased for \textit{in vivo} evaluation. The diabetes was inducted into the rats by injecting Streptozotocin (STZ) injection purchased from M/s. SIGMA-Aldrich, Mumbai (Nachiappan Sukumar et al. 2014). Analytical grade was followed in the usage of all other chemicals and reagents.
5.3 METHODOLOGY FOR DEVELOPMENT OF WOUND DRESSING MATERIAL

This section describes the methodology flow for the development of natural extracts coated wound dressing material.

Figure 5.1 Flowchart of research methodology

- **Sourced Bamboo yarn (100%)** of Ne 30s Yarn conversion to fabric
- **Preparation of Natural extracts** from Aloe vera, Piper betal and neem leaf solution
- Development of Wound dressing Materials
- Drug loading of rhEGF (REGEN – DTM150) on prepared Wound dressing Materials at different concentration level
- Characterization of Wound dressing Materials
  - Scanning Electron Microscope
  - Fourier-transform infrared spectroscopy
- Functional Testing of drug loaded dressing materials
  - Physical properties of the fabric
  - Bacteriological study of the coated fabric
- *In vivo* evaluation of wound dressing materials on diabetes induced Wistar albino rats
In addition, an experimental methodology for characterization, investigation of functional testing and in vivo evaluation of the developed wound dressing materials are also briefed in this section. The flow chart for development of dressing material is given in Figure 5.1.

5.4 PREPARATION OF PLANT EXTRACTS

In the preparation of plant extracts initially the selected leaves of Aloe Vera, Piper Betal and Neem leaf were collected and washed with plain water. Washed leaves were shade dried. Shade dried leaves were grinded into a fine powder which is used for the study.

5.4.1 Aloe vera Extracts Preparation

300 mL of ethanol is taken in the round bottom flask along with 30 g of Aloe vera leaves powder. The round bottom flask is gradually heated for two hours to carry out process of extraction from Aloe vera. The evaporated ethanol is being cooled by the circulation of water and condensed to settle as liquid in the round bottom flask at the bottom. Once the condensed ethanol reaches a particular level in the soxhlet apparatus, they get siphoned.

5.4.2 Piper betal Extracts Preparation

300 ml of ethanol is taken in the round bottom flask along with 30 g of Piper betal leaf powder. The round bottom flask is gradually heated for two hours to carry out process of extraction from Piper betal. The evaporated ethanol is being cooled by the circulation of water and condensed to settle as liquid in the round bottom flask at the bottom. Once the condensed ethanol reaches a particular level in the soxhlet apparatus, they get siphoned.
evaporation and condensation of solvent is taken in beaker as Piper betal extract.

5.4.3 Neem Leaf Extracts Preparation

300 mL of ethanol is taken in the round bottom flask along with 30 g of Neem leaf powder. The round bottom flask is gradually heated for two hours to carry out process of extraction from Neem leaf. The evaporated ethanol is being cooled by the circulation of water and condensed to settle as liquid in the round bottom flask at the bottom. Once the condensed ethanol reaches a particular level in the soxhlet apparatus, they get siphoned evaporation and condensation of solvent is taken in beaker as Neem leaf extract.

5.5 DEVELOPMENT OF COATED WOUND DRESSING MATERIAL

The procedure for the development of coated wound dressing material was explained briefly in chapter 3.5. The composition for preparation of natural extract coated dressing materials were listed in Table 5.1.

5.6 CHARACTERIZATION OF FINISHED FABRIC

The chemical and physical structures of developed dressing material were characterized by using the modern analytical instruments namely FTIR and SEM.
Table 5.1 Preparation ratio of Aloe vera, Piper betel and Neem leaf solution and rhEGF (REGEN –D™ 150)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Sample code</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>APN</td>
<td>Aloe Vera leaves Solution – 20 mL Piper Betel leaves Solution – 20 mL Neem leaf – 20 mL</td>
</tr>
<tr>
<td>2.</td>
<td>APN 1</td>
<td>Aloe Vera leaves Solution – 20 mL Piper Betel leaves Solution – 20 mL Neem leaf – 30 mL rhEGF –5 mg</td>
</tr>
<tr>
<td>3.</td>
<td>APN 2</td>
<td>Aloe Vera leaves Solution – 20 mL Piper Betel leaves Solution – 30 mL Neem leaf – 20 mL rhEGF –10 mg</td>
</tr>
<tr>
<td>4.</td>
<td>APN 3</td>
<td>Aloe Vera leaves Solution – 30 mL Piper Betel leaves Solution – 20 mL Neem leaf – 20 mL rhEGF –15 mg</td>
</tr>
</tbody>
</table>

5.7 FUNCTIONAL TESTING OF COATED DRESSING MATERIAL

The developed dressing materials are evaluated for fabric properties such as ends per inch and picks per inch were counted using the counting glass and areal density, tearing strength, tensile strength, abrasion resistance, fabric thickness and fabric stiffness were measured as per the standard mentioned in chapter 3.7.2.

The bacteriological activity of developed dressing materials were tested using Muller- Hinton (HiMedia) (Sukumar et al. 2012) Staphylococcus saprophyticus (S. saprophyticus) were used as gram positive in the tests. Positive control used was Amoxicillin antibiotic.
5.8 \textit{IN VIVO EVALUATION OF DEVELOPED DRESSING MATERIALS}

Type 1 diabetes induced Wistar albino rats were used in \textit{In vivo} evaluation of natural extract coated dressing materials. The animal study was approved by the ethics board and complied with Nandha College of Pharmacy institutional guidelines with the vide proposal number No.NCP/IAEC/No: 8/2014 -15 (Appendix 1). A gentle surgical technique was carried out under sterile conditions for all the operations.

5.8.1 \textit{Type 1 Diabetes Model}

A single i.p injection of 62 mg/kg Streptozotocin (STZ) dissolved in sodium citrate buffer (pH. 4.5) was injected on Male Wistar albino rats, weighing 180 ± 40 g. After 27 days of span period using Contour\textsuperscript{TM} TS Blood glucose monitoring system (M/s. Bayer Pharmaceuticals (India) Pvt. Ltd.), the whole-blood glucose obtained from rat tail-vein was monitored. Room temperature was maintained with 65% relative humidity in an animal house and allowed free access to food and water. The whole-blood glucose levels higher than 300 mg/dl in the STZ-treated rats were considered diabetic condition and used for this study (Sukumar 2015).

5.8.2 \textit{In vivo Study Design}

In this study a total of 30 rats divided into six groups was used. Each group consisting of five animals was treated with different composition of natural extracts coated on the fabric as shown in Table 5.2.
Table 5.2 *In vivo* study design

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Coding</th>
<th>Group details</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Group 1</td>
<td>Natural healing – (Negative Control)</td>
</tr>
<tr>
<td>2.</td>
<td>Group 2</td>
<td>Povidone Iodine Ointment USP – (Control)</td>
</tr>
<tr>
<td>3.</td>
<td>Group 3</td>
<td>Material without rhEGF (REGEN-D™ 150) – (APN)</td>
</tr>
<tr>
<td>4.</td>
<td>Group 4</td>
<td>Material with rhEGF (REGEN-D™ 150) – 5 µg (APN I)</td>
</tr>
<tr>
<td>5.</td>
<td>Group 5</td>
<td>Material with rhEGF (REGEN-D™ 150) – 10 µg (APN II)</td>
</tr>
<tr>
<td>6.</td>
<td>Group 6</td>
<td>Material with rhEGF (REGEN-D™ 150) – 15 µg (APN III)</td>
</tr>
</tbody>
</table>

The animals in Group 2 animals were treated with commercial medicine for every three days while the others were treated every 7 days with developed dressing materials. The wound closure was measured on 0, 2nd, 6th, 9th, 12th, 15th, 18th and 21st day after wounding.

5.8.3 *Excision Wound Creation*

Diethyl ether (molar mass of 74.12 g/mol) was used to anesthetize the diabetic induced animals with confirmed glucose levels above 300 mg/dl. Hair removal cream was used to remove the dorsal skin of the animals and disinfected using ethanol (concentration of 96%) and each diabetes rat was created with a full-thickness skin wound (approximately 2.00 cm long and 0.3 cm wide) on its back. Animals after recovery from anesthesia were housed individually in properly disinfected cages. The percentage of wound closure was calculated using the formula 3.8.2 (Chapter 3). (Sukumar N 2015)
5.9 RESULTS AND DISCUSSION

5.9.1 Development of Natural Extract Coated Wound Dressing Material

The woven bamboo fabric was desized and dried. The fabric was cut down into suitable size and made ready for coating with natural extract. The natural extracts of aloe vera, piper betel and neem leaf solution with enhancement of recombinant human Epidermal Growth Factor (rhEGF) at various contents as shown in Table 5.1 were coated on the fabric in the respective laboratory condition and thus the required wound dressing material was developed.

5.9.2 FTIR Analysis of Developed Wound Dressing Material

The FTIR spectra of the coated dressing materials are shown in Figure 5.2 (a-d). The characteristic vibration bands of neem leaf extracts were obviously shifted to other positions indicated namely C–H bending (985.56–1472.55 cm\(^{-1}\)), C–H stretching (2845.77–2909.42 cm\(^{-1}\)), O–CH\(_3\) (1475.12 cm\(^{-1}\)), C=C, ketone (1575.73–1748.35 cm\(^{-1}\)), carboxylic (1319.22–1717.49 cm\(^{-1}\)), amides (1575.73 cm\(^{-1}\)), aromatic (754.12–762.79 cm\(^{-1}\)), C–O–C stretching (1164.92 cm\(^{-1}\)), sulfur compounds (1097.42–1339.47 cm\(^{-1}\)), alcohols and phenols (1271–3627.85 cm\(^{-1}\)) (Novie Febriana et al. 2010).

The peak presented between 1415 and 1260 cm\(^{-1}\) can be assigned to –CH, –CH\(_2\) bending vibrations. Significant differences are observed in the 1700–1400 cm\(^{-1}\) regions of the spectra and the –NH bending vibration band is observed at 1643 cm\(^{-1}\) for Piper Betel and aloe vera (Maria L Ojeda-Martínez et al. 2015).
Figure 5.2 (a) FTIR image of APN

Figure 5.2 (a) shows the FTIR image developed wound dressing material APN. The characteristic vibration bands of coated extracts shifted to other positions, such as absorption peaks of C–H bending (985.56–1472.55 cm\(^{-1}\)), C–H stretching (2845.77–2909.42 cm\(^{-1}\)), carboxylic (1319.22–1717.49 cm\(^{-1}\)), amides (1575.73 cm\(^{-1}\)), C–O–C stretching (1164.92 cm\(^{-1}\)), alcohols and phenols (1271–3627.85 cm\(^{-1}\)) moved to 1483.2, 2900.92, 1399.56, 1527.62, 1150.52 and 1273.02 cm\(^{-1}\) correspondingly. The characteristic vibration bands of –NH bending vibration band is observed to 1662.64 cm\(^{-1}\).

Figure 5.2 (b) shows the FTIR image developed wound dressing material APN 1. The characteristic vibration bands of coated extracts shifted to other positions, such as absorption peaks of C–H bending (985.56–1472.55 cm\(^{-1}\)), C–H stretching (2845.77–2909.42 cm\(^{-1}\)), carboxylic (1319.22–1717.49 cm\(^{-1}\)), amides (1575.73 cm\(^{-1}\)), C–O–C stretching
(1164.92 cm\(^{-1}\)), alcohols and phenols (1271–3627.85 cm\(^{-1}\)) moved to 1091.71, 2895.15, 1377.17, 1595.13, 1145.72 and 1260.73 cm\(^{-1}\) correspondingly. The characteristic vibration bands of –NH bending vibration band is observed to 1672.84 cm\(^{-1}\).

Figure 5.2 (b) FTIR image of APN 1

Figure 5.2 (c) FTIR image of APN 2
Figure 5.2 (c) shows the FTIR image developed wound dressing material APN 2. The characteristics vibration bands were obviously moved to 997.20, 2887.44, 1390.68, 1593.20, 1176.72 and 1260.73 cm$^{-1}$ correspondingly. The characteristic vibration bands of –NH bending vibration band is observed to 1652.28 cm$^{-1}$.

Figure 5.2 (d) shows the FTIR image developed wound dressing material APN 3. The characteristics vibration bands were obviously moved to 1048.61, 2861.32, 1299.26, 1558.76, 1162.81 and 1248.73 cm$^{-1}$ correspondingly. The characteristic vibration bands of –NH bending vibration band is observed to 1664.28 cm$^{-1}$.

![Figure 5.2 (d) FTIR image of APN 3](image)

5.9.3 Morphologies Structure of Developed Dressing Material

The morphology of the coated fabric samples were studied using SEM. The extent of penetration of extract coated on the fabric reveals that the different extracts prepared coated were penetrated interior into the fabric
which plays a major role in wound healing property of the developed wound dressing material. The wound dressing coated with different natural extracts exhibited a macro porous structure with open pores and pore sizes varied from 10 to 100 μm. Figure 5.3 (a-d) shows SEM micrographs of the developed wound dressing material coated with the natural extracts. The morphological study of coated fabric reveals that extracts coated binds evenly over the surface of the fabric samples. The images clearly shows that porous nature of the fabrics were not affected after coating with natural extracts of aloe vera, piper betel and neem leaf solution.

Figure 5.3 (a) SEM image of APN (2.59 KX magnification)

Figure 5.3 (a) shows the SEM image of APN sample coated with natural extracts and without rhEGF. The surface was found to be uniformly coated and folds with small pores.
Figure 5.3 (b) SEM image of APN 1 (1.75 KX magnification)

Figure 5.3 (b) shows the SEM image of sample coated with natural extracts along with 5 µ gram of rhEGF. The surface was found to be rough and of micro pores structure.

Figure 5.3 (c) SEM image APN 2 (1.45 KX magnification)
Figure 5.3 (c) shows the SEM image of the sample APN 2 loaded with 10 µg of rhEGF. The drug and the natural extracts were found to be even on surface and micro pores are visible.

![SEM Image of APN 2](image)

**Figure 5.3 (d) SEM image of APN 3 (2.48 KX magnification)**

Figure 5.3 (d) shows the SEM image of sample coated with natural extracts and 15 µg of rhEGF. The natural extracts were visible found to be on the surface and also even penetration through pores were confirmed.

5.9.4 **Tensile Strength of the Developed Coated Material**

The tensile strength of coated bamboo fabrics were measured before and after coating.
Figure 5.4 Tensile Strength of Dressing Material

Figure 5.4 shows the results of the tensile strength of control and coated fabric samples. The line chart describes that coated fabric samples exhibit a marginally higher values in all composition of natural extracts. Even though the ‘t’ test values reveals that this marginal raise in tensile strength values are statistically insignificant.

5.9.5 Tearing Strength of the Developed Coated Material

The tearing strength of coated bamboo fabric were measured before and after coating with natural extracts.
Figure 5.5 Tearing Strength of Dressing Material

Figure 5.5 shows the results of the tearing strength of the developed dressing material. The line chart describes that coated fabric samples exhibit a very minimum higher values in all composition of natural extracts. Even though the ‘t’ test values reveals that this marginal raise in tearing strength values are statistically insignificant.

5.9.6 Areal Density of the Developed Coated Material

The areal density of the developed dressing materials were measured before and after coating of the natural extracts. Figure 5.6 shows the results of the areal density of the control and coated fabric samples. The bar diagram reveals that coated fabric samples exhibit a very negligible higher values in all composition of natural extracts. Even though the ‘t’ test values reveals that this marginal raise in areal density values are statistically insignificant.
Figure 5.6 Areal Density of Dressing Material

5.9.7 Thickness of the Developed Coated Dressing Material

Figure 5.7 shows the results of the thickness of the control and coated fabric samples.

Figure 5.7 Thickness of Dressing Material
The bar diagram shows that coated fabric samples exhibit a slight variation in all composition of natural extracts. Even though the ‘t’ test values reveals that the raise in thickness values are statistically insignificant.

5.9.8 Abrasion resistance of the Coated Wound Dressing Material

The abrasion resistance of the natural extract coated dressing materials were measured before and after coating.

![Abraision resistance graph](image)

**Figure 5.8 Abrasion Resistance of Dressing Material**

Figure 5.8 shows the results of the percentage of abrasion resistance of the control and coated fabric samples. The bar diagram shows that coated fabric samples has a minimum variation in all composition of natural extracts. Even though the ‘t’ test values reveals that the raise in abrasion resistance values are statistically insignificant.
5.9.9 Antibacterial Activity of Developed Wound Dressing Material

The antimicrobial efficacy of the developed fabrics were studied against *Escherichia coli* and *Staphylococcus aureus* through agar diffusion test method. The samples were grouped in the way that group 1 was untreated fabric, group 2 was fabric treated with the commercially available drug Povidone-Iodine (PVP-I) (Control Group) and group 3, 4, 5 and 6 (already named as APN, APN I, APN II and APN III respectively) were fabrics coated with the ratio mentioned in Table 5.1.

The comparative analysis of the test results of antibacterial activity is shown in the figure 5.9. The zone of inhibition is high against *Escherichia coli* and *Staphylococcus aureus* in the sample CAC III which is about 18 mm and 22 mm after 24 hrs of inhibition.

![Antibacterial Activity](image)

**Figure 5.9 Antibacterial Activity of Developed Wound Dressing Material**

From all the samples APN III shows better antibacterial property which is evident from the zone of inhibition. This also concludes that as the ratio of natural extracts & rhEGF is increased the better will be the
antibacterial property. These results indicate that the developed wound dressing materials may be suitable to be used as wound barrier.

5.9.10 Wound Closure in Rats Treated with Developed Wound Dressing Materials

In wound closure study, a full – thickness wound was created on the rat for the size approximately 2.0 cm long and 0.3 cm wide. Figure 5.10 shows the comparison results of wound closure rate in which the animals from each group (wound with no treatment, positive control, APN, APN I, APN II and APN III) on 3rd, 5th, 7th, 10th, 12th, 15th, 17th and 21st day after wound creation. Among these dressings, the wound of the rats treated with APN III, seemed to be healed than other samples at different days after creating wound.

Figure 5.10 Wound closure Rate (%)

Figure 5.11 shows the sample photographs of wound on albino rats after 0th, 6th, 9th and 15th days. The size reduction of wounds shows faster wound healing rate when compared with other compositions (Wounds not
treated, treated with commercially available drug Povidone-Iodine (PVP-I) and other ratios of Aloe vera, Piper betal and Neem leaf extracts enhanced with rhEGF).

<table>
<thead>
<tr>
<th>Day Group</th>
<th>0</th>
<th>3</th>
<th>9</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without treatment</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>Control Group</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
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</tr>
<tr>
<td>APN</td>
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<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td>APN I</td>
<td><img src="image13.png" alt="Image" /></td>
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<td><img src="image15.png" alt="Image" /></td>
<td><img src="image16.png" alt="Image" /></td>
</tr>
<tr>
<td>APN II</td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
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</tr>
<tr>
<td>APN III</td>
<td><img src="image21.png" alt="Image" /></td>
<td><img src="image22.png" alt="Image" /></td>
<td><img src="image23.png" alt="Image" /></td>
<td><img src="image24.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 5.11 Sample photographs of diabetic wound on albino rats**
The samples APN II and APN III shows higher wound closure rate in minimum duration than positive control and negative control. The reason for quicker wound healing of wound may be the proportion of drug loaded (rhEGF) in this particular sample.

5.10 CONCLUSION

A novel series of blended natural extracts were successfully coated and a dressing material was developed with the enhancement of growth factor. An increase in the natural extracts content resulted in an increase in affinity towards the antibacterial activity. It was investigated that the effect of natural extract coated dressing material on the wound healing process by using diabetic rats. The antimicrobial efficacy was tested against gram positive \textit{S.saprophyticus} bacteria. The bacterial sensitivity test revealed that the developed natural extracts coated dressing material have excellent bacterial resistance against \textit{Escherichia coli} and \textit{Staphylococcus aureus} bacteria. The feasibility of developed wound dressing material’s wound healing ability was also investigated and the results are shown as \textit{in vivo} models using Wistar albino rats. The results confirmed that the natural extracts coated dressing material can promote the healing process in the diabetic type of wound in rats.