CHAPTER 4

DEVELOPMENT AND CHARACTERIZATION OF WOUND DRESSING MATERIAL COATED WITH NATURAL EXTRACTS OF CALOTROPIS GIGANTEAN, EUCALYPTUS GLOBULES AND BUDS OF SYZYGIUM AROMATICUM SOLUTION ENHANCED WITH rhEGF (BURN - REGEN-D™ 60)

4.1 INTRODUCTION

During burn wound healing process, the dressing protects the injury and contributes to the recovery of epidermal tissues. This chapter describes the development and characterization of burn wound dressing material coated with natural extracts of calotropis gigantean, eucalyptus globules and buds of Syzygium aromaticum solution enhanced with rhEGF (burn-REGEN- D™ 60). It also explains elaborately about the wound healing efficacy of the developed wound dressing materials through \textit{in vivo} method for burn wound. All the above materials were chosen through their well-known eco-friendly, biocompatible and antibacterial properties. The materials and methodology used in extraction of natural extracts and preparation of coated wound dressing material are explained. The surface morphology and penetration of the coated natural extracts with growth factors were studied using Scanning Electron Microscopy (SEM).
Chemical bonding of the extracts coated with growth factors was identified with FTIR spectroscopy. The mechanical properties of the fabric such as areal density, thickness, tensile strength, tearing strength, abrasion resistance and flexural rigidity were characterized. The antimicrobial activity of developed wound dressing material with different natural extracts in different combination against two bacteria’s (Gram positive – *Staphylococcus aureus*, Gram negative – *Escherichia coli*) and *in vivo* evaluation using albino rats to heal burn wounds are assessed.

The results and interpretation of various parameters such as chemical bonding, microstructure, mechanical properties and their antimicrobial activity in developed wound dressing is briefed out in this chapter. The burn wound healing efficacy of Wistar albino rats is also discussed in detail.

### 4.2 MATERIALS

The materials used in the development of wound dressing material coated with natural extracts of calotropis gigantean, eucalyptus globules and buds of *Syzygium aromaticum* solution enhanced with rhEGF (burn-REGEN-D™ 60) are listed. The bamboo yarn (100%) of Ne 30s was sourced from S.V.TEX, Tirupur, Tamil Nadu, India and it was woven to gauze fabric. Leaves of Calotropis gigantea were sourced from Sadhumugai, Sathyamangalam. Leaves of Eucalyptus globulus were sourced from Orange Groove Estate, Ootacamund. The buds of syzygium aromaticum were sourced from a departmental store in Erode. The recombinant human Epidermal Growth Factor (rhEGF) as REGEN-D™ 60 was purchased from M/s. Bharat Biotech International Limited, Hyderabad, India. Deionized water was used for all experiments.
The microorganisms used in the experiment namely *Escherichia coli* and *Staphylococcus aureus* were provided by K.S.R Arts and Science College, Microbiological Culture Collection Center - Tiruchengode. Wistar albino rats were purchased from the animal house of Nandha College of Pharmacy, Erode. The chemicals and reagents used for work were of analytical grade. During all experiments de-ionized water was used.

### 4.3 METHODOLOGY FOR THE DEVELOPMENT OF WOUND DRESSING MATERIALS

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

![Flowchart of research methodology](image)

**Figure 4.1 Flowchart of research methodology**
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 4.1.

### 4.4 PREPARATION OF PLANT EXTRACTS

The collected fresh and healthy leaves of *Calotropis gigantean* (CG), *Eucalyptus globules* (EG) and buds of *Syzygium aromaticum* (SA) were washed with plain water. Then the leaves and buds were washed by distilled water and it was subjected to shade dry. The dried leaves and buds were grinded into a fine powder which is used for the study. The extraction process was carried out by taking each 3gm of dried powder and mixed with 50mL of 80% methanol. The container was closed completely and left ideal for overnight. After overnight of incubation, the extract was filtered through filter paper and to concentrate the extract, it was evaporated at room temperature. This methanol extract was used for the application on fabrics. The filtered liquid was measured and the pH of the solution was evaluated using pH meter or pH paper.

### 4.5 DEVELOPMENT OF COATED WOUND DRESSING MATERIAL

#### 4.5.1 Finishing of Fabrics

Calculated amount of extracts were taken as per the ratio of 20:20:20, 30:20:20, 20:30:40 in the order of EG: CG: SA (Table 4.1). The solution was taken as per the ratio and mixed thoroughly. The samples to be coated were put in to distilled water and were boiled at the temperature of 60°C for about ½ an hour. The boiled samples were taken out and the samples were put into extracted solution of predetermined ratio. The samples were coated with the extract and it was tend to padding mangle.
4.5.2 Incorporation of rhEGF on the Coated Fabric

The padded samples were spread on a dry surface and calculated amount of prepared drug solution (5gpl of REGEN-D™ 60 (Burn) in 100 mL of distilled water) were coated over the samples. Then the samples were dried in oven at 60ºC. The compositions of the extracts and drug are shown in the Table 4.1.

Table 4.1 Preparation ratio of Calotropis gigantean (CG), Eucalyptus globules (EG) and buds of Syzygium aromaticum (SA) and rhEGF (REGEN –D™ 60)

<table>
<thead>
<tr>
<th>Group</th>
<th>100 % Bamboo Yarn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>NES</td>
</tr>
<tr>
<td>CG (mL)</td>
<td>20</td>
</tr>
<tr>
<td>EG (mL)</td>
<td>20</td>
</tr>
<tr>
<td>SA (mL)</td>
<td>20</td>
</tr>
<tr>
<td>rhEGF (µg)</td>
<td>-</td>
</tr>
<tr>
<td>REGEN-D™ 60 (Burn)</td>
<td></td>
</tr>
</tbody>
</table>

4.6 CHARACTERIZATION OF DEVELOPED WOUND DRESSING MATERIAL

The chemical and physical structures of developed dressing material were characterized by using the modern analytical instruments namely FTIR and SEM.
4.7 FUNCTIONAL TESTING OF DEVELOPED WOUND DRESSING MATERIAL

4.7.1 Antibacterial Efficacy Test

As per AATCC 147 standard antibacterial activity was assessed. In this study two types of bacteria were used namely Gram-negative (Escherichia coli) and a Gram-positive bacteria (Staphylococcus aureus). The line of incubation of antimicrobial agent was shown by the presence of growth inhibition zones measured by using Muller- Hinton (HiMedia) (N Sukumar et al. 2012).

4.7.2 Fabric Properties

The bamboo gauze fabric specifications such as ends per inch, picks per inch, warp crimp and weft crimp were identified and cover factor of the fabric was calculated. As per the ASTM D1777-96 and ASTM D6828 standard methods, the thickness and stiffness of the samples were determined respectively. The areal density of the fabric was measured using GSM cutter method as per ASTM D377512.

4.8 IN VIVO EVALUATION OF DEVELOPED WOUND DRESSING MATERIAL

In vivo evaluation of developed dressing material was evaluated using healthy adult male Wistar albino rats initial weight of 150.71 ± 5 gm were taken for evaluation in this study. The rats were found to be hygienic and followed to have proper food at regular intervals and have proper digestion the life cycle of the rats have been monitored. The rats were weighed before and after treatment of wound healing process.
All animal procedures were according to guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals and Institutional Animal Ethics Committee (IAEC) approved by the Nanda College of Pharmacy, vide proposal No.NCP/IAEC/No: 8/2014 -15. (Appendix 1) Total of 30 rats were taken for study and they were divided into six groups. The burn wound model was performed on healthy rats.

4.8.1 Wound Creation

Wister Albino Rats were given anesthetic of minimum level of 0.05 mL and the hairs of the rats were shaved using shaver in position of lateral half way between the midline and it was left for 45min. A metal rod of 2cm diameter was heated to 90°C in boiling water for 15min and it was sterilized. Then the metal rod was taken and cooled down to 60°C and second degree wound was made on individual rats with the same temperature and pressure and the rats were allowed to take rest for about one day. The group I rats were not treated with any of the material and left open for natural healing. The group II rats were commercially available drug Silver sulphadiazine (0.5g of 1%) cream for every two days. The group III rats were treated with developed sample NES (Table 4.1). The group IV, V & VI rats were treated with the developed samples NES1, NES2 & NES3 respectively for every three days.

4.8.2 Wound Healing Observation

Treated wounds were subjected to periodical analysis on 0th, 3rd, 6th, 9th, 12th and 15th day. The wound healing rate was observed with the reduction in wound size. The size of the wound was measured by tracing with transparent butter paper. With Nikon digital camera (Model: Nikon Coolpix S6700 Point & Shoot Camera) the photography of wound closure was captured at the time interval mentioned earlier. The percentage of wound
closure was calculated by the initial and final area using graph paper during observation. The wound size reduction was calculated as follows:

\[
\% \text{ of Wound size reduction} = \frac{D_i - D_f}{D_i} \times 100
\]

Where \(D_i\) was the initial area of wound on 0th day

\(D_f\) was the area of wound at the time of treatment with developed material on 3\(^{rd}\), 6\(^{th}\), 9\(^{th}\), 12\(^{th}\) and 15\(^{th}\) day accordingly.

The experimental design for burn wound was designed as follows:

**Table 4.2 In vivo study design of developed wound dressing material**

<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>Silver sulphadiazine (0.5g of 1%) cream, topical – (Control)</td>
</tr>
<tr>
<td>3.</td>
<td>Group 3</td>
<td>Coated dressing material without rhEGF (REGEN-D™ 60 (Burn)) – (NES)</td>
</tr>
<tr>
<td>4.</td>
<td>Group 4</td>
<td>Coated dressing material with rhEGF (REGEN-D™ 60 (Burn)) – 5 µg (NES I)</td>
</tr>
<tr>
<td>5.</td>
<td>Group 5</td>
<td>Coated dressing material with rhEGF (REGEN-D™ 60 (Burn)) – 10 µg (NES II)</td>
</tr>
<tr>
<td>6.</td>
<td>Group 6</td>
<td>Coated dressing material with rhEGF (REGEN-D™ 60 (Burn)) – 15 µg (NES III)</td>
</tr>
</tbody>
</table>

Group 1 is negative control and Group 2 animals were treated with Silver sulphadiazine (positive control).
4.9 RESULTS AND DISCUSSION

4.9.1 Development of Natural Extract Coated Wound Dressing Material

The bamboo gauze fabric was desized and dried. The fabric was cut down into respective practical usage size and made ready for natural extract coating. The natural extracts of Calotropis Gigantean (CG), Eucalyptus Globules (EG) and buds of Syzygium Aromaticum (SA) at various compositions as shown in Table 4.1 were coated on the fabric in the respective laboratory condition and thus the required wound dressing material was developed.

4.9.2 FTIR Analysis of Developed Wound Dressing Material

FTIR spectroscopy is commonly used to investigate the conformation of blending of the different extracts on dressing material. In order to confirm the structural change in the developed wound dressing material with different composition were investigated by FTIR spectra. The FTIR spectra of natural extract coated dressing material with rhEGF-burn are shown in Figure 4.2 (a-d). Calotropis Gigantean had characteristics of vibration bands in their FTIR spectra such as absorption peaks of 3434 cm\(^{-1}\) (O-H), 2919 cm\(^{-1}\) (CH\(_3\)CH\(_2\)), 1637 cm\(^{-1}\) (C=O), 1424 cm\(^{-1}\) (C-H), 1104 cm\(^{-1}\) (C-O,C-C) and 1028 cm\(^{-1}\) (C-O) correspondingly (Ramamurthy & Kannan 2007).

Similarly the results of FTIR vibration bands of Eucalyptus Globules and Syzygium aromaticum showed that there is a clear and high intensive band at 3375 cm\(^{-1}\) which represent OH groups of alcoholic extract. The moderate band 2920-2852 cm\(^{-1}\) that represents frequency asymmetrical patterns of group CH\(_2\) and CH\(_3\) of alcoholic compound. The other band vibration observed at 1730 cm\(^{-1}\) represents the frequency patterns of ester
group C-O or aronic ketone group C=O. The band observed at 1643 cm\(^{-1}\)
which was also the patterns corresponds the frequency of the aromatic carbonyl group belonging to quinine (Khalid Abdul Kreem Mohammed et al. 2016) which was closer and similar to aromatic group (C=C). The vibration bands observed at 1452 cm\(^{-1}\) and 1402 cm\(^{-1}\) represents the frequency of pattern of group CH2- and stronger band observed at 1070 cm\(^{-1}\) which represents frequency of pattern of group C-O. Finally a moderate bands observed at 779 cm\(^{-1}\) and 919 cm\(^{-1}\) that represents the frequency of pattern of groups CH\(_2\) and C=C, respectively (Khalid Abdul Kreem Mohammed et al. 2016).

Figure 4.2 (a) FTIR image of NES

Figure 4.2 (a) shows the FTIR image of the developed wound dressing material NES. The characteristics vibration bands of coated extracts were shifted to absorption peaks of 3434 cm\(^{-1}\) and 3375 cm\(^{-1}\), 2919 cm\(^{-1}\) and 2920-2852 cm\(^{-1}\), 1730 cm\(^{-1}\) and 1637 cm\(^{-1}\), 1452 cm\(^{-1}\) and 1424 cm\(^{-1}\), 1104 cm\(^{-1}\), 1028 cm\(^{-1}\), 779 cm\(^{-1}\) and 919 cm\(^{-1}\) moved to 3332.25, 2919.45, 1652.69, 1456.47, 1107.32, 1027.20 and 663.64 cm\(^{-1}\) correspondingly.
Figure 4.2 (b) FTIR image of NES I

Figure 5.2 (b) shows the FTIR image of coated dressing material NES I. The characteristics vibration bands of coated extracts were obviously shifted to other positions where indicated OH group of alcoholic extract and CH₂ and CH₃ group of alcoholic extracts, such as absorption peaks of 3375 cm⁻¹, 2920-2852 cm⁻¹ and 1730 cm⁻¹ moved to 3332.69, 2908.65 and 1753.29 cm⁻¹ correspondently.

Figure 4.2 (c) FTIR image of NES II
Figure 5.2 (c) shows the FTIR image of natural extract coated wound dressing material NES II. The characteristics vibration bands of coated extracts were noticeably shifted to other positions where indicated alcoholic structure, such as absorption peaks of 3434 cm\(^{-1}\) and 3375 cm\(^{-1}\), 2919 cm\(^{-1}\) and 2920-2852 cm\(^{-1}\), 1730 cm\(^{-1}\) and 1637 cm\(^{-1}\), 1452 cm\(^{-1}\) and 1424 cm\(^{-1}\), 1104 cm\(^{-1}\), 1028 cm\(^{-1}\), 779 cm\(^{-1}\) and 919 cm\(^{-1}\) moved to 3300.20, 2699.01, 1764.87, 1406.11, 1101.35 and 935.48 cm\(^{-1}\) correspondingly.

![FTIR image of NES II](image_url)

**Figure 5.2 (d) FTIR image of NES III**

Figure 5.2 (d) shows the FTIR image of NES III dressing material. The characteristics vibration bands of the coated extracts were visibly shifted to other positions where indicated alcoholic structure, such as absorption peaks of 3434 cm\(^{-1}\), 2919 cm\(^{-1}\), 1730 cm\(^{-1}\), 1104 cm\(^{-1}\) and 919 cm\(^{-1}\) moved to 3926.35, 2893.12, 1734.01, 1143.79 and 912.33 cm\(^{-1}\) correspondingly.
4.9.3 Morphologies of the Coated Dressing Material

SEM observation was conducted to examine the surface morphology and internal penetration of the natural extracts coated over the fabric. The wound healing property which is considered as the important property for the developed dressing material can be justified with the uniform penetration of the extracts and this will be confirmed from the SEM image of the coated dressing materials. The wound dressing coated with different natural extracts exhibited a macroporous structure with open pores. Figure 4.3(a-d) shows SEM micrographs of the developed wound dressing materials. The SEM image visualizes the porous nature of the dressing material were not affected after coating with Calotropis gigantean, Eucalyptus globules and buds of Syzygium aromaticum solution.

Figure 4.3 (a) SEM image of NES (500 X magnification)
Figure 4.3 (a) shows the SEM image of NES sample coated with 20 mL of Calotropis gigantean, Eucalyptus globules and buds of Syzygium aromaticum solution without rhEGF-burn. The surface structure was uniformly coated with pores.

![SEM image of NES sample](image)

**Figure 4.3 (b) SEM image of NES I (500 X magnification)**

Figure 4.3 (b) shows the SEM image of NES I sample coated with 20 mL of Calotropis gigantean, Eucalyptus globules and buds of Syzygium aromaticum solution without rhEGF-burn (5 mg). The SEM image shows the extracts were coated uniformly.

Figure 4.3 (c) shows the SEM image of NES II sample coated with 30 mL of Calotropis gigantean, 20 ml of Eucalyptus globules and 20 mL buds of Syzygium aromaticum solution without rhEGF-burn (10 mg). SEM Image of CAC II dressing material shows regular pore size.
Figure 4.3 (c) SEM image of NES II (500 X magnification)

Figure 4.3 (d) SEM image of NES III (500 X magnification)

Figure 4.3 (d) shows the SEM image of NES III sample coated with 20 mL of Calotropis gigantean, 30 mL of Eucalyptus globules and 40 mL buds of Syzygium aromaticum solution without rhEGF-burn (15 mg). The morphological study of coated fabric reveals that natural extracts coated penetrates evenly over the surface of the gauze fabric samples. The porous
nature of the fabrics were not affected, was concluded from the images investigated after coating with the natural extract on the gauze fabric.

4.9.4 Areal Density of the Developed Dressing Material

The areal density of the developed dressing materials were measured before and after coating of the natural extracts. Figure 4.4 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.

![Areal Density of Coated Dressing Material](image)

Figure 4.4 Areal Density of Coated Dressing Material

4.9.5 Thickness of the Developed Wound Dressing Material

Figure 4.5 shows the results of the thickness of the control and coated fabric samples. 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.

**4.9.6 Bending Length of the Developed Wound Dressing Material**

The bending length of coated bamboo fabric were measured before and after coating. Figure 4.6 shows the results of the bending length 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 bending length values are statistically insignificant.

**4.9.7 Bending Modulus of the Developed Wound Dressing Material**

The bending modulus of coated bamboo fabric were measured before and after coating.
Figure 4.6 Bending Length of Coated Dressing Material

Figure 4.7 shows the results of the bending modulus 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 bending modulus values are statistically insignificant.

Figure 4.7 Bending Modulus of Coated Dressing Material
4.9.8  Flexural Rigidity of the Developed Wound Dressing Material

Figure 4.8 shows the results of the flexural rigidity of the 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 flexural rigidity values are statistically insignificant.

![Flexural Rigidity Chart](image)

**Figure 4.8 Flexural Rigidity of Coated Dressing Material**

4.9.9  Effect of Drug Release from the Coated Dressing Material

To determine drug, drug loaded material scaffolds (10 g) were immersed in Phosphate Buffered Saline (PBS) at 370°C. The buffer was removed and replaced with fresh buffer to approximate infinite sink conditions. The buffer was removed for every 24 hours from the system and the retention of drug (rhEGF) in the coated fabric samples were studied under UV spectra (324 nm). From this study all the natural extract coated fabrics are able to retain drug for certain period of time. The UV spectra observations of the coated samples are as follows:
1. NES fabric sample enhanced with 5mg of rhEGF was able to retain the drug up to 4 days.

2. NES I coated sample confirms the presence of drug (10 mg of rhEGF) up to 6 days.

3. In NES III sample (15 mg of rhEGF) UV spectra confirms the retaining capacity of drug up to 8 days.

Different concentrations of natural extracts were able to significantly retain the drug delivery or hold up the rate of release.

4.9.10 Antibacterial Activity of Coated Dressing Material

The anti-bacterial activity of the coated samples was tested against *Escherichia coli* and *Staphylococcus aureus* by agar diffusion method. The results of anti-bacterial activity of the coated samples the captured images of formation of zone of inhibition were shown in Figure 4.9. The test results of antibacterial activity of the natural extract coated samples were comparatively analyzed as shown in the Figure 4.10. The formation of zone of inhibition against *Escherichia coli* and *Staphylococcus aureus* in the sample NES 3 which is about 13 mm and 11 mm after 24 hrs of inhibition.

![Antibacterial Activity](image)

**Figure 4.9** Antibacterial activities of Developed Dressing Material
On its consolidation NES 3 shows better antibacterial property which was conformed with the evident from the zone of inhibition as shown in Figure 4.9. It also concludes that when the ratio of natural extracts and rhEGF was increased the better will be the antibacterial property.

The antibacterial activity of the coated dressing material indicate that the developed wound dressing materials can be suitable to be used as wound barrier.

![Figure 4.10](image)

**Figure 4.10** (a) Formation of zone of inhibition against *Staphylococcus aureus* and (b) Formation of zone of inhibition against *Escherichia coli*

### 4.9.11 Wound Closure Observation in Rats Treated with Developed Dressing Materials

A full-thickness secondary burn wound was created on the back of each rat. Figure 4.12 shows sample images of the animals from each group (wound without treatment, commercial product, NES, NES I, NES II and NES III) on 3rd, 9th and 15th day respectively after burn. The results confirmed that sample NES 3 has faster wound closure rate.
The images of wound size reduction after treatment with natural extract coated wound dressing on 0th, 3rd, 9th and 15th days are shown in Figure 4.12.

The comparative results of wound size reduction is shown in Figure 4.11 which confirms the wounds in sample NES 3 shows faster wound healing rate when compared with other compositions (Wounds not treated, treated with commercially available drug Silver sulphadiazine and other ratios of Calotropis gigantean (CG), Eucalyptus globules (EG) and buds of Syzygium aromaticum (SA) enhanced with rhEGF (REGEN-D™ 60 (Burn))).
<table>
<thead>
<tr>
<th>DAYS</th>
<th>DAY 0</th>
<th>DAY 3</th>
<th>DAY 9</th>
<th>DAY 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Treatment</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
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<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
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<tr>
<td>NES III</td>
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<td><img src="image22" alt="Image" /></td>
<td><img src="image23" alt="Image" /></td>
<td><img src="image24" alt="Image" /></td>
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</tbody>
</table>

**Figure 4.12 Sample photographs of burn wound on albino rats**

### 4.10 CONCLUSION

The wound dressing material coated with natural extracts were successfully developed using Calotropis gigantean, Eucalyptus globules and buds of Syzygium aromaticum solution (with and without rhEGF) for burn wound healing. Detailed FTIR studies indicated that the blending process of
the natural extracts does not cause chemical interactions. The coated gauze fabric enables retained release of rhEGF, confirmed by UV spectra measurements.

The samples coated with natural extracts exhibit good anti-bacterial activity against *Staphylococcus aureus* and *Escherichia coli*. The results of *In vivo* evaluation reveals that, the coated gauze fabrics were suitable for dressing the burn wounds and also it gives a better wound healing rate. It was also further proven that when ratio of the natural extracts was increased the wound healing rate was increased.