10. FORMULATION OF OINTMENT

10.1. Introduction

Herbal drugs are formulated in the form of ointment and are used topically for several purposes, e.g. as protectants, antiseptics, emollients, antipruritic, keratolytics and astringents. Ointment bases are always anhydrous and generally contain one or more medicaments in suspension or solution or dispersion. Ointment bases may be hydrocarbon (oleaginous), absorption, water removable and water soluble type. On the basis of their level of action, they are classified as: epidermatic, endodermatic and diadermatic (Carter, 1987). A wound healing ointment is aimed to heal the incised and excised wounds. In an earlier study, medicinal plants have been reported to be very beneficial in wound care, promoting the rate of wound healing with minimal pain, discomfort, and scarring to the patient (Odimegwu et al., 2008).

The objective of the study was to formulate and evaluate the original herbal extract ointment and Nanotised herbal extract from the local medicinal plant for its wound healing property.

Ointments are semisolid preparations that are thicker than creams but thinner than pastes. The ointment formulation consists of active and inactive ingredients added to a base. For herbal formulations, an ointment is made by emulsifying oil phase in water while the mixture is warm, then letting the formulation congeal at room temperature. During the preparation, the oil is heated to approximately 70°C and the water to 75°C. Usually the water phase
contains an extract of the herb to be incorporated into the mixture. When added together and mixed in the presence of an emulsifying agent, the two phases quickly form a stable emulsion. The mixing continues at room temperature until the formulation begins to congeal. Upon mixing the two phases together, the mixture initially looks milky in appearance, and unless colouring agents are added to the formulation, the final ointment is normally white to off-white in colour. Since the ointment is kept over a period of up to several weeks, preservatives such as benzoic acid or its salt, sodium benzoate, must be used in ointment preparations. Dispensation may be in a plastic, but preferably, glass jar. Shelf life can be improved by storing the ointment in a refrigerator or in a cool place. If heat is deleterious to the herbal components, then the ointment can be prepared without heat by incorporation method. A possible method for incorporating an aqueous herbal extract into an ointment base is to mix the extract with a small quantity of lanolin (wool fat) using a porcelain mortar and then incorporating the resulting mixture into white petrolatum (petroleum jelly) using the same mortar for mixing.

The herbal extract and nano herbal extract were incorporated to the base separately and named as extract ointment (EO) and Nano ointment (NO).

The ointment of the *Urena lobata* was prepared by using the simple ointment base I.P.
Table: 10.1 Composition for the formulation of *Urena lobata*

<table>
<thead>
<tr>
<th>Extract Ointment (EO)</th>
<th>Quantity (g)</th>
<th>Nano extract Ointment (NO)</th>
<th>Quantity (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>White bees wax</td>
<td>2</td>
<td>White bees wax</td>
<td>2</td>
</tr>
<tr>
<td>Hard paraffin</td>
<td>3</td>
<td>Hard paraffin</td>
<td>3</td>
</tr>
<tr>
<td>Cetosteryl alcohol</td>
<td>5</td>
<td>Cetosteryl alcohol</td>
<td>5</td>
</tr>
<tr>
<td>White soft paraffin</td>
<td>90</td>
<td>White soft paraffin</td>
<td>90</td>
</tr>
<tr>
<td><em>Urena lobata</em> alcoholic extract</td>
<td>5</td>
<td><em>Urena lobata</em> Nanotised alcoholic extract</td>
<td>5</td>
</tr>
</tbody>
</table>

**Procedure**

The extract and the nano extract separately were incorporated into the molten simple ointment base and allowed to congeal by stirring. After the ointment was formulated, they were packed in collapsible tube separately.

**10.1 Ointment formulation of original and nano extracts**
10.2. Quality control of the formulated Ointment

**Physical evaluations:** Preliminary evaluation of formulations at different concentrations was carried out as follows:

**Organoleptic parameters:** Organoleptic parameters like colour, odour of the formulations were carried out by visual examination.

**Loss on drying**

This is employed in IP and USP. Although the loss in weight, in the sample so tested, principally is due to water and small amount of other volatile material will be contribute the weight loss 1gm of ointment is placed digital moisture balance instrument set the temperature 105°C and run the instrument up to constant weight. Finally read out the percentage loss on drying automatically.

**pH:** The pH of various formulations was determined by using Digital pH meter (Digital pH meter 335, Systronics, Noroda, Ahmedabad). The 0.5 g of the weighed formulation was dispersed in 50 mL of distilled water and the pH was (Panigrahi et al., 1997) noted.

**Homogeneity:** All the developed ointments were tested for homogeneity by visual inspection. They were tested for their appearance with no lumps (Panigrahi et al., 1997).

**Viscosity:** The measurement of viscosity of prepared ointments was carried out with Brookfield Viscometer (model LV-DV-II, Helipath spindle type S-96). The values of each formulation were done in triplicate and average values were depicted in Table 10.2. The
viscosity values are expressed as Mean ± Standard deviation (Kim et al., 2003).

**Spreadability:** Spreadability of the formulation was determined by an apparatus suggested by Mutimer et al. (1956) which was suitably modified in the laboratory and used for the study. The experiment was performed as described by Wood et al. (1963). Spreadability was determined by using the formula

\[ S = \frac{M \times L}{T} \]

Where \( S \) = spreadability, \( M \) = Weight tied to upper slide, \( L \) = Length of glass slides and \( T \) = Time taken to separate the slides completely from each other. In this present experiment, \( M = 80 \) g, \( L = 10 \) cm and \( T \) was recorded (Ehrlich and Hunt, 1968).

**Acute skin irritation study:** This test was performed on albino rats weighing between 150-200g. The animals were given standard animal feed and had free access to water *ad libitum*. The total mass was separated into four groups, each batch containing five animals. Dorsal hair at the back of the rats were removed one day prior to the commencement of the study and kept individually in cages to avoid contact with the other rats. Two groups of each were used for control and standard irritant. Other two groups were used as test. The 50mg of the each formulation were applied over one square centimetre area of whole and abraded skin to different animals. Aqueous solution of 0.8 % formalin was used as standard irritant. The animals were observed for seven days for any signs of oedema and erythema (Marzulli and Maibach, 1997).
**Stability studies:** The stability studies were carried out in all formulations at different temperature conditions (4°C, 25°C and 37°C) for 3 months. All the evaluation parameters i.e., pH, viscosity, spreadability, consistency and phase separation were studied at different time intervals i.e., 15, 30, 60 and 90th days (Shinde *et al.*, 2005; Mohanta *et al.*, 2007).

**Table: 10.2 Stability studies of the ointment of the Urena lobata extracts**

<table>
<thead>
<tr>
<th>Physicochemical parameters</th>
<th>Formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour</td>
<td>Light green</td>
</tr>
<tr>
<td>Odour</td>
<td>Characteristic</td>
</tr>
<tr>
<td>Loss on Drying</td>
<td>9.5 % w/w</td>
</tr>
<tr>
<td>pH</td>
<td>6.5 (5.95 - 6.56)</td>
</tr>
<tr>
<td>Spreadability(Seconds)</td>
<td>15 (between 18 sec)</td>
</tr>
<tr>
<td>Diffusion study</td>
<td>0.9 cm (between 1 cm)</td>
</tr>
<tr>
<td>Skin irritation study</td>
<td>No skin irritation was observed</td>
</tr>
<tr>
<td>Storage( 4°C,24°C,37°C)</td>
<td>Stable</td>
</tr>
</tbody>
</table>

**In-vitro drug release study:**

In *In-vitro* diffusion study of the ointment was carried out on Franz diffusion cell having 57 ml capacity. Whatman filter paper no.41 was used as diffusion membrane. Pieces of Whatman filter paper no.41 were soaked in phosphate buffer (PB) pH 6.0 for 24 hours, prior to experiment. Diffusion cell was filled with phosphate buffer pH 6.0, Whatman filter paper no.41 was mounted on cell. The
temperature was maintained at 37 ± 0.5°C. The formulation was spread on the filter paper as a thin layer. The time point for ointment was different. A sample of 1ml was withdrawn at predetermined time intervals, the solution was filtered with 0.45 micron filter paper and make up the volume with 5 ml of PB pH 6.0 and equivalent amount of fresh dissolution fluid equilibrated at same temperature was replaced. The sample was diluted to 5 ml with PB at pH 6.0. The standard also was prepared as the same concentration of that of sample. The amount of drug permeated was determined using a UV- spectrophotometer at 340 nm. (Linearity range = 10-20µg/ml, R2 = 0.9988)

Weight of the ointment-10 mg

Volume of solution (7.4 pH): 100 ml

Amount of sample withdrawn: 5 ml

Absorbance: 304 nm

Table: 10.3 In-vitro drug release study

<table>
<thead>
<tr>
<th>NANO OINTMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time</strong></td>
</tr>
<tr>
<td>15 mts</td>
</tr>
<tr>
<td>30 mts</td>
</tr>
<tr>
<td>45 mts</td>
</tr>
<tr>
<td>1 hour</td>
</tr>
<tr>
<td>2 hours</td>
</tr>
<tr>
<td>3 hours</td>
</tr>
<tr>
<td>4 hours</td>
</tr>
</tbody>
</table>
EXTRACT OINTMENT

<table>
<thead>
<tr>
<th>Time</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 mts</td>
<td>0.232</td>
</tr>
<tr>
<td>30 mts</td>
<td>0.262</td>
</tr>
<tr>
<td>45 mts</td>
<td>0.284</td>
</tr>
<tr>
<td>1 hr</td>
<td>0.362</td>
</tr>
<tr>
<td>2 hours</td>
<td>0.488</td>
</tr>
<tr>
<td>3 hours</td>
<td>0.52</td>
</tr>
<tr>
<td>4 hours</td>
<td>0.612</td>
</tr>
<tr>
<td>5 hours</td>
<td>0.632</td>
</tr>
<tr>
<td>6 hours</td>
<td>0.702</td>
</tr>
</tbody>
</table>

Fig 10.2 Percentage drug release of the extract ointment and nano ointment
10.3. RESULTS AND DISCUSSION

The various physicochemical parameters utilized to evaluate the prepared ointment formulations are shown in Table 10.2.

- The pH of the formulations lies in the normal pH range of the human skin (6.5 ± 1).
- All the formulations did not produce any skin irritation, i.e., erythema and edema for about a week when applied over the skin.
- The rheological behaviours of the different formulations of ointments in Rotational Brookfield Viscometer indicated that the speed of spindle increases viscosity decreases. A comparative study of viscosity and spreadability showed that the viscosity of the formulations increases, spreadability decreases and vice versa.
- These formulations did not produce any skin irritation for about a week when applied over the skin.
- From the stability studies, Ointments showed no changes in pH, viscosity, spreadability, consistency and phase separation after keeping at different temperatures for 90 days.

Discussion

The mechanical evaluation parameters like pH, viscosity, spreadability, homogeneity are important tests to evaluate pharmaceutical ointment formulations. The result of all the formulations near to pH 6.5 ± 1 indicates better chemical compatibility of ointments with skin. The results of viscosity gives
an idea about measurement of strength and the result of spreadability denote the extent of area to which the prepared formulations readily spreads on application to skin or affected part and homogeneity confirms no lumps. The results of stability study indicate that there was no change in results of evaluation parameters of prepared ointments during the treatment period. The absence of erythema and edema for about a week when the ointments are applied over the skin for skin irritation test indicates patient compliance and fewer side effects. The results of the physical evaluation of ointment preparation with ethanolic extract and the nano extract of *Urena lobata* indicated the suitability of method for the production of ointments (Ansel *et al.*, 2005).
References:


11. WOUND HEALING STUDY OF THE FORMULATIONS OF

*Urena lobata*

11.1. Introduction

Wound healing processes are well organized biochemical and cellular events leading to the growth and regeneration of wounded tissue in a special manner. Healing of wounds is an important biological process involving tissue repair and regeneration. It involves the activity of an intricate network of blood cells, cytokines, and growth factors which ultimately leads to the restoration to normal condition of the injured skin or tissue (Clark, 1991). The aim of wound care is to promote wound healing in the shortest time possible, with minimal pain, discomfort, and scarring to the patient and must occur in a physiologic environment conducive to tissue repair and regeneration (Bowler *et al.*, 2001).

Wound healing are conveniently classified into any of three types, healing by first intention, healing by second intention and healing by third intention, depending on the nature of the edges of the healed wounds. Whereas the edges of wounds healed by first intention are smoothly closed that no scar is left, wounds healed by second intention involve formation of granulation tissues which fill up the gaps between the wound edges and are associated with significant loss of tissue, leaving little scars. Wounds healed by third intention are usually those wounds left for three to five days until granulation bed falls before they are sutured resulting in extensive scars formation (Thomas, 1997). Four distinct stages of
wound healing have also been identified—-inflammatory, debridement, proliferation, and remodelling maturation stages.

Wound healing processes are known to be influenced by among other factors by infections, nutritional status, drugs and hormones, type and sites of wound, and wasting diseases like diabetes (Karl et al, 1995). In folklore medicine, medicinal plants have been used widely in facilitating wound healing with high degree of successes. This has inspired many researches which are aimed at validating the claims and discovering mechanisms which possibly explains the potentials of these herbs on wound repair processes.

In our investigation the two different formulations in the form of Ointments (original extract ointment (EO) and nanotised extract ointment (NO) from the plant *Urena lobata* were evaluated using the incision and excision models with Swiss albino rats.

Chemicals

All chemicals and reagents used were of analytical grade.

Experimental animals

Swiss Albino rats of 200 to 250 g body weight were used in the study. Animals were procured from BiogenLaboratory Animal Facility (CPCSEA -Reg no. 971 /bc/ 06), Bangalore.

All animals were kept in polyacrylic cages and maintained under standard housing conditions (room temperature 24-27°C and humidity 60-65% with 12:12 light: dark cycles. Food was provided in the form of dry pellets and water ad libitum.
All experiments involving animals complies with the ethical standards of animal handling and approved by Institutional Animal Ethics Committee (IAEC/XXXIV/SRU/284/2013).

Model design

The design of wound healing activity was performed by two models-

1) Excision wound model
2) Incision wound model

11.2. Materials and Methods

Male Wister rats were used for excision and incision wound models and the ointments were applied topically and animal were divided into the following groups.

Group- I : No wound was created and served as control. (6 animals)

Group- II: wound was created and served as positive control. (6 animals)

Group- III: 5%w/w Extract ointment (EO) was applied once daily.

  i) Excision-4 animals
  ii) Incision-4 animals

Group- IV: 5%w/w Nanotised extract ointment (NO) was applied once daily.

  i) Excision- 4 animals
  ii) Incision- 4 animals

Group- V: Betadine ointment (5 gm) was applied once daily

  i) Excision-4animals
  ii) Incision-4 animals
1) Excision wound model

The back of each rat was shaven under ether anaesthesia and prepared for operation. Thereafter open circular wound of 500 mm² area was produced in each rat by excising the skin. For this purpose a marker was used to mark the area to be excised.

The wounded animals were kept separately.

Rats wound were left undressed to the open environment; this model was used to monitor wound contraction and epithelisation time. The standard drug (Betadine ointment (0.5 gm), simple ointment; ethanolic extract ointment (EO) 5 %w/w and 3 %w/w Nanotised extract ointment (NO) were applied everyday till the wound was completely healed.

Incision Wound Model

Animals were grouped which was divided into four groups same as followed in excision wound model. The incision wound model was studied under light ether anaesthesia the animal was secured to operation table in its natural position. A longitudinal paravertebral incision of 6 cm long was made through the skin and cutaneous tissue on the back. After complete haemostasis, the wound was closed by means of interrupted sutures placed at equidistance points about 1 cm apart. Animals were treated daily with drugs, as mentioned above under excision wound model from 0th day to 9th post wounding day. Wounds were cleaned with 70 % alcohol soaked with cotton swabs. They were kept in separate cages.
All the sutures were removed on the 9th post wounding day. On 10th day the tensile strength was measured by continuous constant water supply technique.

All the above mentioned treatments were started from the day of operation and continued till 20th day of healing. On 2nd, 4th, 8th, 10th, 12th, 14th, 16th, 18th and 20th days the wound area of each rat was traced on a graph paper. Animals were sacrificed by cervical dislocation on day 30. Liver, spleen, stomach and skin were collected and processed for histopathological studies.

The following physical and biochemical parameters were studied:

11.2.1. Physical Evaluation
- Body weight
- Wound size and area
- Tensile strength

11.2.2. Antioxidant Studies
- Superoxide Dismutase (SOD) activity (Marklund and Marklund, 1974)
- Glutathione Peroxidase activity (GPX) (Moren et al., 1973)
- Estimation of Reduced glutathione (GSH) (Moren et al., 1979)
- Activity of Catalase (Asru K Sinha, 1987)

11.2.3. Biochemical Studies
- Myeloperoxidase activity (Bradley et al., 1982)
- Total protein by Biuret method (Biuret et al., 1948)
- Hydroxy proline content (R.E.Newman et al., 1950)
11.2.1 Physical evaluation

1. Measurement of wound area

The progressive changes in wound area were measured planimetrically by tracing the wound margin on a graph paper every alternate day. The changes in healing of wound i.e. the measurement of wound on graph paper was expressed as unit (mm²). Wound contraction was expressed as percentage reduction of original wound size.

\[
\text{Wound area - Unhealed Area} \times 100
\]

\[
\text{Wound area}
\]

2. Measurement of Wound Breaking Strength of Incised Wounds

Measurement of wound breaking strength was performed by using the following method with certain modifications. A board was placed on the table, on which the anaesthetized animal was made to lie on its abdomen. Two clamps were clamped on either sides of healed wound at a distance 0.5 cm. The left clamp was fastened tightly to stand by means of thread. The right clamp was connected to a leak proof polythene container through a pulley, by means of a thread. A reservoir containing water was placed at a suitable height and connected to a polythene bag by means of a rubber tube. The position of the board was adjusted so that, the polythene bag was hanging freely. Water was added to polythene bag rapidly at constant rate from the reservoir until the wound opened. Amount of water in polythene bag was measured (in ml) and was considered as tensile strength of the wound.
11.2.2 Antioxidant Studies

1. Superoxide Dismutase (SOD)

0.05 ml of sample is added to 0.3 ml of sodium pyrophosphate buffer (0.025 M, pH 8.3), 0.025 ml of phenazonium methosulphate (186 µM) and 0.075 ml of Nitroblue tetrazolium chloride (300 µM in buffer of pH 8.3). The reaction was started by addition of 0.075 ml of reduced nicotinamide adenine dinucleotide (780 µM in buffer of pH 8.3). After incubation at 30º C for 90 seconds, the reaction mixture was stirred vigorously and shaken with 2.0 ml of n-butanol. The mixture was allowed to stand for 10 minutes and centrifuged. 1.5 ml of n-butanol alone was served as blank. The colour intensity of the chromogen was read at 560 nm using thermo scientific multiscan spectrometer, USA (Kakkar et al., 1984).

Enzyme activity (1 unit) = 50% inhibition/minute

2. Glutathione Peroxidase (GPX)

Glutathione peroxidise (GPX) was assayed by taking 200 µl of tris HCl buffer (0.4 M), 200µl K.EDTA (0.4 mM) along with 100 µl of sodium azide and 200 µl of sample and mixed well. Thereafter, 200µl of reduced glutathione solution (2 mM) followed by 0.1ml of H₂O₂ were added and allowed to incubate for 10 min at 37º C. The overall reaction was then arrested by adding 0.5 ml of 10 % trichloroacetic acid (TCA). The precipitate was removed by centrifugation at 1500 rpm for 10 minutes. To 0.2 ml of the supernatant, 0.5 ml of saline and 1.0 ml of di-thio nitro benzoic acid (DTNB) were added and the colour intensity formed was absorbed at
412 nm using Thermo Scientific Multiskan Spectrophotometer, USA, (Rotruck, et al., 1973)

3. Reduced Glutathione (GSH)

Reduced glutathione was assayed by taking 0.25 ml of sample to equal volume of ice cold 5% TCA. The precipitate was removed by centrifugation at 3500 rpm for 10 minutes. 1 ml of the supernatant was mixed with 0.25 ml of 0.2 M phosphate buffer, pH 8.0 and 0.5 ml of DTNB (0.6 mM in 0.2 M phosphate buffer, pH 8.0) were added and mixed well. The absorbance was read at 412 nm using Thermo Scientific Multiskan Spectrophotometer, USA (Moren et al., 1979).

4. Activity of Catalase:

The assay mixture contained 100 µl of sample, 0.4 ml of H₂O₂ (2 mM) and 0.5 ml of phosphate buffer (10 mM, pH 7.4). The above mixture was stirred well and incubated at 37°C for 5 min and then dichromate acetic acid reagent (5% potassium dichromate in water, glacial acetic acid mixed in 1:3 ratio) was added and absorbance was taken at 570 nm using Thermo Scientific Multiskan spectrophotometer, USA. 2 ml of dichromate acetic acid reagent acts as blank (Asru K Sinha, 1987).

5. Myeloperoxidase activity

1 ml of sample was mixed with equal volume of hexadecyl trimethyl ammonium bromide (HTAB) buffer and centrifuge at 4°C for 15 minutes at 3000 rpm. Then 0.5 ml of supernatant was taken and added to 3.5 ml of phosphate buffer (pH 6.0) followed by 1.5 ml
of dinizidine. The absorbance was read at 450nm at 0 sec and 60 sec against blank using Thermo Scientific Multiskan spectrophotometer, USA (Bradley et al., 1982). The results were expressed in terms of units (Nanomoles H$_2$O$_2$ lib/min/mg protein).

6. **Total Protein by Biuret method**

0.6 ml of saline was mixed with 50 µl of sample followed by addition of 1.25 ml of working biuret reagent. The tubes were incubated at room temperature for 15 min. The color intensity was read at 540nm using Thermo Scientific Multiskan spectrophotometer, USA, (Lubran et al., 1948).

7. **Hydroxyproline (HPR) (R.E.Newman et.al., 1950).**

To each tube, 0.3 mL of hydrolysate, 2.5 N NaOH, 0.01 M CuSO$_4$, and 6 % H$_2$O$_2$ were added. Tubes were shaken vigorously and placed immediately in water bath at 80° C. After 15 minutes, tubes were removed and cooled for 5 minutes in cold water. 0.6 mL of freshly prepared 5 % solution of paradimethyl aminobenzaldehyde in n-propanol and 1.2 ml of 3 N H$_2$SO$_4$ was added. The test tubes were once again placed in a hot water bath at 75° C for 15 minutes and then cooled for 5 minutes under running stream of water. Color intensity was measured at 540 nm against the blank. Hydroxyproline content in the tissue was estimated as per standard curve prepared with standard 4-Hydroxy-L-proline (HiMedia Laboratories Pvt. Ltd., Mumbai, India), from 75 to 900 g/0.3 mL using 3 mg/mL working solution.
Results

Table: 11.1 Tensile Strength

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tensile strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>286±11.25</td>
</tr>
<tr>
<td>Standard</td>
<td>398±10.55</td>
</tr>
<tr>
<td>NO</td>
<td>499±12.00**</td>
</tr>
<tr>
<td>EO</td>
<td>510±8.6**</td>
</tr>
</tbody>
</table>

NO= Nano extract ointment
EO= original extract ointment

All values are mean SEM ± n =6, **P < 0.001 indicates extremely significant compared to the control
**Fig: 11.1 Tensile strength of the tissue of wound treated rats**

![Tensile Strength Graph]

**Table: 11.2 Comparison of wound area Using “Kruskal-Wallis Test”**

<table>
<thead>
<tr>
<th>Days</th>
<th>Groups</th>
<th>Mean</th>
<th>SD</th>
<th>Chi Square</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 2</td>
<td>136.166</td>
<td>95.1155</td>
<td>5.261*</td>
<td>0.154*</td>
</tr>
<tr>
<td></td>
<td>Group 3</td>
<td>173.148</td>
<td>55.4694</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group 4</td>
<td>252.703</td>
<td>112.347</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group 5</td>
<td>188.750</td>
<td>63.6295</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group 2</td>
<td>104.777</td>
<td>64.0765</td>
<td>2.842*</td>
<td>0.417*</td>
</tr>
<tr>
<td></td>
<td>Group 3</td>
<td>143.036</td>
<td>63.6552</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group 4</td>
<td>161.333</td>
<td>77.7134</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Group 5</td>
<td>113.041</td>
<td>37.1494</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day</td>
<td>Group 2</td>
<td>Group 3</td>
<td>Group 4</td>
<td>Group 5</td>
<td>p value</td>
</tr>
<tr>
<td>--------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>Day 7</td>
<td>60.7777</td>
<td>87.5554</td>
<td>83.0740</td>
<td>68.3376</td>
<td>1.580*</td>
</tr>
<tr>
<td></td>
<td>46.6074</td>
<td>55.7997</td>
<td>42.9695</td>
<td>24.4996</td>
<td></td>
</tr>
<tr>
<td>Day 10</td>
<td>29.6111</td>
<td>54.7036</td>
<td>53.1481</td>
<td>41.5416</td>
<td>5.070*</td>
</tr>
<tr>
<td></td>
<td>13.0833</td>
<td>30.8330</td>
<td>28.2970</td>
<td>12.6866</td>
<td></td>
</tr>
<tr>
<td>Day 13</td>
<td>23.1666</td>
<td>37.8148</td>
<td>36.9999</td>
<td>27.0833</td>
<td>4.972**</td>
</tr>
<tr>
<td></td>
<td>8.79582</td>
<td>23.0579</td>
<td>15.6720</td>
<td>10.9047</td>
<td></td>
</tr>
<tr>
<td>Day 16</td>
<td>11.6666</td>
<td>22.4443</td>
<td>17.18512</td>
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<td>10.48**</td>
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<td>9.14694</td>
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<td>5.648**</td>
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<td>3.93775</td>
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</table>

*p value < 0.05-Significant  ** p value< 0.01-Highly significant
1. Effect of Extract ointment and Nano ointment on Protein and Hydroxy Proline content on Dry Connective Tissue

**Table: 11.3 Estimation of Protein & Hydroxy proline**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Protein mg / g dry tissue</th>
<th>Hydroxyproline g / mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>198.1 ± 12.4</td>
<td>132.2 ± 10.6</td>
</tr>
<tr>
<td>Std</td>
<td>272.3 ± 11.3*</td>
<td>197.2 ± 9.02**</td>
</tr>
<tr>
<td>NO</td>
<td>297.5 ± 15.0*</td>
<td>193.1 ± 10.2**</td>
</tr>
<tr>
<td>EO</td>
<td>305.6 ± 11.3*</td>
<td>203.6 ± 9.9**</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of 6 rats in each group. * <0.05, ** <0.01 compared to respective control group (statistical analysis was done by using Mann Whitney test).

1. Effect of Extract ointment and Nano ointment on Protein and Myeloperoxidase content on Wet granulation Tissue

**Table: 11.4 Estimation of Protein & Myeloperoxidase**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Protein mg / g wet tissue</th>
<th>Myeloperoxidase mU/ mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>38.6 ± 2.25</td>
<td>22.1 ± 0.57</td>
</tr>
<tr>
<td>Std</td>
<td>53.7 ± 2.12*</td>
<td>17.9 ± 0.29**</td>
</tr>
<tr>
<td>NO</td>
<td>62.1 ± 2.98*</td>
<td>13.8 ± 0.37**</td>
</tr>
<tr>
<td>EO</td>
<td>66.20 ± 2.66*</td>
<td>12.9 ± 0.29**</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of 6 rats in each group. * <0.05, and ** <0.001 compared to respective control group (statistical analysis was done by using Mann Whitney test).
Results and Discussion

Collagen is the predominant extracellular protein in the granulation tissue of a healing wound and there is a rapid increase in the synthesis of this protein in the wound area soon after an injury. Breakdown of collagen liberates free hydroxyproline and its peptides. The observed increase in hydroxyproline, specific marker of collagen and an important component of extracellular granulation tissue matrix in the wounds treated with Urenalobata clearly indicated the rapid collagen turnover and accumulation, explains the increased rate of wound contraction (Geethalakshmi et al 2013).

Studies on the antioxidant, free radicals and MPO status revealed that Urenalobata had significant antioxidant activity, reduced MPO and free radical stress facilitated in prevention of inflammation and oxidative damage thereby promoting healing process (Murthy et al 2013).
The wounds treated with ointments showed significant increase in tensile strength which confirms collagen formation thereby promotes wound healing (Pickett BP et al 1996).

The estimation of antioxidants such as GSH, SOD, CAT is relevant because the antioxidants have been reported to hasten wound healing (Murthy et al 2013).

Scavenging of free radical and reactive oxygen species promote contraction of the wound and increasing the formation of capillary vessels and fibroblasts (Murthy et al 2013).

Increasing total protein confirms the tissue synthesis and fighting infection and body tissue (James TJ et al 2000).

**Table 11.5 Antioxidant Studies**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LPO (mg/dl)</th>
<th>SOD (IU/mg protein)</th>
<th>CAT(µM/mg tissue)</th>
<th>GSH(µg/mg tissue)</th>
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<tr>
<td>NC</td>
<td>12 ± 1.58</td>
<td>5.59 ±1.17</td>
<td>26.94 ± 5.28</td>
<td>3.24 ± 0.29</td>
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<tr>
<td>PC</td>
<td>14.05 ± 0.81</td>
<td>1.70 ±0.48</td>
<td>12.90 ± 2.01</td>
<td>1.32 ±0.32</td>
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<tr>
<td>STD</td>
<td>10.22 ± 1.06***</td>
<td>3.49± 1.80**</td>
<td>25.08 ±3.45***</td>
<td>2.51 ± 0.45*</td>
</tr>
<tr>
<td>NO</td>
<td>8.04 ± 1.16***</td>
<td>2.80±1.73***</td>
<td>19.85 ±5.14***</td>
<td>1.88 ±0.33***</td>
</tr>
<tr>
<td>EO</td>
<td>9.25 ±1.54***</td>
<td>3.21±0.60***</td>
<td>20.81 ±2.57***</td>
<td>2.52 ± 0.22***</td>
</tr>
</tbody>
</table>

Values are mean SEM of 6 rats in each group. *-P value is <0. 05,

**-P value is <0. 01 and ***-P value is <0. 001 compared to control by Krushkal walli’s test**
Fig: 11.3 LPO on wound treated rats

Values are expressed in mean± SEM of 6 rats in each group

Fig: 11.4 SOD on wound treated rats

Values are expressed in mean± SEM of 6 rats in each group
Fig: 11.5 Catalase on wound treated rats

Values are expressed in mean± SEM of 6 rats in each group

Fig: 11.6 GSH on wound treated rats

Values are expressed in mean± SEM of 6 rats in each group
11.3. Histopathology

Procedure:

Wound was created in the rat skin by incision and excision method. On day 30, all rats from each group were euthanized using anesthetic ether. The skin from the incised and excised region were collected and fixed in 10% neutral buffered formalin for 48 hours. The skin tissues were subjected to dehydration in series of graded alcohol and embedded in paraffin wax. Tissue sections of 4 to 5 micron thickness were obtained and stained with Hematoxylin and Eosin (Bancroft and Gamble, 2008) for light microscopic examination. The samples were also stained with Masson’s trichome staining for specific evaluation of fibrous tissue proliferation.

Histopathology studies of treated rats for both extract and nano ointment

Fig: 11.7

Control group MST 4x  Control group Skin H & E 10x
Fig: 11.7.1

*Induced group – Epithelial erosion and mononuclear infiltrates in the dermis region*

H& E 40 X

Fig: 11.7.2

*Induced group – Epithelial erosion dermal Infiltration and increased fibroblast Proliferation H& E 10 x*

Fig: 11.7.3

*Induced group region of the skin - MST 10 x*
Fig: 11.7.4

Moderate degree of erosion with dermis infiltration - H& E 40 x

Fig: 11.7.5

Nano extract ointment – neovascularisation and Infiltration in dermis H & E 40 x

Fig: 11.7.6

Nano extract ointment MST 47 X
Fig: 11.7.7

Nano extract ointment – H & E 10 X

Fig: 11.7.8

Extract ointment – Dermal fibrosis with infiltration H & E 40X

Fig: 11.7.9

Extract ointment - Inflammatory cells in the dermis - H & E 40X
Histopathology report on the evaluation of wound healing activity of herbal ointment in rats

Wound was created in the rat skin by incision and excision method. On day 30, all rats from each group were euthanized using anesthetic ether. The skin from the incised and excised region were collected and fixed in 10 % neutral buffered formalin for 48 hours. The skin tissues were subjected to dehydration in series of graded alcohol and embedded in paraffin wax. Tissue sections of 4 to 5 micron thickness were obtained and stained with Hematoxylin and Eosin (Bancroft and Gamble, 2008) for light microscopic
examination. The samples were also stained with masson’s trichome (MST) staining for specific evaluation of fibrous tissue proliferation.

The skin from the control group revealed normal histology of epidermis, dermis and hypodermis.

The H&E stained skin section from the induced group revealed moderate degree of epithelial erosions, ballooning degeneration of epithelium, incoherent and flaky layers of keratin, moderate degree of polymorphonuclear leucocytes, mononuclear cells infiltration in the dermis and increased fibroblast proliferation along with neovascularization.

The skin tissues of standard group revealed absence of epithelial erosions, re-epithelisation but increased mononuclear cells infiltration along with fibroblast proliferation in the healed wound region of skin.

The skin treated with Nano herbal and extracts ointment revealed re-epithelisation, thin epidermis, mild to moderate degree of dermal fibrosis with cutaneous adnexal atrophy (loss of hair follicles and sebaceous glands) in wound healed region along with neovascularization and mild degree of inflammatory infiltrates in the dermis region composed of mononuclear cells.

The skin wound treated with nanoherbal and extract ointment revealed re-epithelisation, fibrosis in the healed region and the inflammatory infiltration persists but the proportion of
inflammatory cells was reduced than those observed in the standard and induced group.

These histopathology findings suggest that nanoherbal and extract ointment treated group revealed better wound healing property as evident by fibrosis and the reduced inflammatory infiltrates when compared to that of the standard. The fibrosis was further confirmed by Masson’s trichome staining in skin tissues of induced, standard, nanoherbal and extract ointment treated groups.
**CERTIFICATE**

INSTITUTIONAL ANIMAL ETHICS COMMITTEE

<table>
<thead>
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<th>IAEC Number</th>
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<tr>
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<td>06.07.2013</td>
</tr>
<tr>
<td>Date of Expiry</td>
<td>05.07.2014</td>
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This is to certify that the project entitled “Development and Pre clinical Evaluation of Herbal formulation for wound healing” submitted by R. Thirumalaikumaran, Assistant Professor, Faculty of Pharmacy, Sri Ramachandra University has been reviewed by the members of XXXIV Minutes of IAEC held on 06.07.2013 at Sri Ramachandra University. The proposal has been APPROVED/ Approved with Modifications/ Disapproved.

Details of the animals approved for individual study:

<table>
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<tr>
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<th>AGE/B.W</th>
<th># ANIMALS</th>
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<td>Rat</td>
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Details of the animals approved for the entire study:

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<th>AGE</th>
<th># ANIMALS</th>
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<tr>
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</tbody>
</table>

**TOTAL: 42 (FORTY TWO) MALE SPRAGUE DAWLEY RATS ONLY**

**Signature with Date**

Prof. Venkataraman S (Chairman IAEC)

**Signature with Date**

Dr. Kathivelan C (CPCSEA Nominee)
SCORING OF WOUND FROM 0\textsuperscript{TH} DAY TO 21\textsuperscript{ST} DAY

Each area represents values in sq.cm
Each row represents groups
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References:

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