WOUND HEALING, ANTIMICROBIAL AND ANTI FUNGAL ACTIVITIES OF ETHANOLIC LEAF EXTRACT OF AEGLE MARMELOS IN RATS

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Abstract: Aegle marmelos is a medicinal plant commonly known as bale. This plant contains different components of therapeutic value. The ethanolic extract of Aegle marmelos showed the presence of phytoconstituents like flavonoids, triterpenoids, coumarins, sterols, triterpenoids, alkaloids with other constituents. In present investigation, antibacterial, antifungal and wound healing effects of ethanolic extract of leaves of Aegle marmelos has been studied. This extract was formulated 4% Aegle marmelos ethanolic leaf extract ointment and was studied for its wound healing property against an excision wound on the skin of Wistar albino rats. The activity was compared with 2% framycetin ointment. Study shows faster wound healing with 4% Aegle marmelos ethanolic leaf extract ointment. On 12th day biochemical parameters like hydroxyproline, collagen and hexosamine were determined from excised tissue and they were significantly increased in extract treated groups. Healed skin was also subjected to histopathological studies to examine the microscopic changes and it also supported the wound healing on application of extract ointment in treated group. Histopathological observations showed increase in granulation and rapid collagen turnover. It was concluded that wound healing activity of ethanolic leaf extract of a Aegle marmelos was better and faster than framycetin ointment.

Key words: Aegle marmelos, Wound healing

INTRODUCTION

In recent years, a number of antibiotics have lost their effectiveness due to the development of resistant strains, mostly through the expression of resistance genes. In addition to this problem, antibiotics are sometimes associated with adverse effects including hypersensitivity, immune suppression and allergic reactions. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infection diseases from various sources such as medicinal plants [1]. Anti-microbial agents are undeniably one of the most important therapeutic discoveries of the 20th years. However, with the ‘antibiotic era’ scarcely five decades vintage, mankind is now faced with the global difficulty of appearing resistance in virtually all pathogens [2].Wound healing is the process of repair that follows injury to the skin and other soft tissues. Following injury, an inflammatory response occurs and the cells below the dermis begin to increase collagen production [3] later, the epithelial tissue is regenerated. Wound healing consists of an orderly progression of events that re-establish the integrity of the damaged tissue [4].

A wound can be defined as a break in the continuity of the soft tissues like skin, mucous membranes, and tissue surface. An external wound is the wound with
a varying degree of damage of the tissue including the skin. An internal wound damages the underlying tissue to varying degree, leaving the skin intact. Wound healing can be defined as the physiological process by which the body replaces and restores function to damaged tissue [5]. The mechanism of wound healing occurs by regeneration and repairs. In regeneration, damaged tissue is replaced in identical replication of cells. In repair, damaged tissue is replaced by connective tissue which then forms a scar. Wound healing occurs by the process of vascular responses (homeostasis), inflammation, proliferation and maturation.

Several studies showed a hypoglycemic antiseptic, antioxidant, antimicrobial, antidepressant, sedative, antinociceptive, hepatoprotective, and anti-inflammatory effects [6] of the *Aegle marmelos*. Fruits and seeds [7], leaves [8] and root [9] show antimicrobial [10] and wound healing activity in dermal wounds. Hence, the present study was conducted to investigate the efficacy of topical application of *Aegle marmelos* ethanolic extract ointment and measure the percentage of wound contraction, histopathology and estimation of biochemical parameters in wound healing.

**MATERIALS AND METHODS**

**Plant material:** Plant material of *Aegle marmelos* leaves were collected from Madikonda, Warangal, Andhra Pradesh and authenticated by Prof. M.A. Singara Charya, Kakatiya University, Warangal.

**Preparation of extract:** The leaves were dried in the shade and powdered by a mechanical grinder. The powder was initially defatted and extracted with ethanol by using a Soxhlet extractor for 72 hours at warmth not exceeding the boiling point of the solvent. The extract was filtered using whatman filter paper (No 1) and then concentrated in a vacuum and dried at 45°C for ethanol elimination. The extracts were kept in a sterile container under refrigeration situation of about 2-8°C.

**Test microorganisms:** Bacterial and fungal isolates used in the present study *Bacillus sphericus, Micrococcus luteus, Pseudomonas aeruginosa, Bacillus cereus, Proteus vulgaris, Escherichia coli, Bacillus subtilis, Salmonella paratyphi, Bacillus megaterium, Proteus mirabilis* were obtained from Department of Microbiology Kakatiya Univ. Andhra Pradesh. The bacterial isolates were first sub cultured in a nutrient broth and incubated at 37°C for 18 h while the fungal isolates were sub cultured on a Malt extract agar for 72 h at 25°C.

**Antibacterial activity:** Muller Hinton Agar was prepared according to the manufacturer’s instructions. The medium was sterilized by autoclaving at 121°C for 15 minutes at 15 psi pressure and was used to determine the antibacterial activity of *Aegle marmelos* ethanol leaf extract. Sterile molten cool (45°C) agar was poured aseptically into sterile petri plates (15 ml each) and the plates were allowed to solidify at room temperature in sterile condition. After solidification and drying, the plates were seeded with appropriate micro organisms by streaking evenly on to the surface of the medium with a sterile spreader and wells (6 mm diameter) were cut out from the agar plates using a sterile stainless steel bore and filled with 60 µl of the each extract solution in respective wells. Streptomycin was used as standard control respectively. Then the plates were incubated at 37°C for 24 hrs in the next day the zones of inhibition were measured with a measuring scale. This experiment was carried out in triplicate for their confirmation. The results were read by the presence or absence of zone of inhibition.

**Acute dermal toxicity studies:** Female albino rats 18-20 g weight and age of 3 months are use to determine acute dermal toxicity of ethanolic leaf extract of *aegle marmelos*. Highest concentration is apply on shaved dorsal side of rats according to the OECD guidelines and observe that the dose was safe and lower dose is consider for further study [11].

**Animals:** Wistar albino rats weighing about 150–250 gm (Mahaveer agencies, Hyderabad) were used for the study. They were fed with standard rat pellet diet. They were housed in polypropylene cages maintained under standard conditions (12 hour light-dark cycle; 25 ± 3°C; 35-60% humidity). The animals were left for 3 days at room conditions for acclimatization. They were maintained on standard pellet diet and water ad libitum throughout the experiment. Animals were devided in to three groups and minimum of six animals were used in each group.

The study was permitted by the Institutional Animal Ethics Committee (42/SPIPS/IAEC/13) and was performed according to the international rules relating to animal experiments.

**Wound-healing activity:** Excision wound models were used to evaluate the wound-healing activity of
leaf extracts of *Aegle marmelos*. Group I is controle group left open, group II is applied with ointment base, group III is treated with framycetin ointment, group IV extract ointment is applied daily on wound.

**Chemicals**: Framycetin, Wool fat, Hard Paraffin, Cetostearyl alcohol and White Soft Paraffin

**Preparation of ointment by fusion method:**

(a) **Preparation of simple ointment**: Wool fat-2.5 gm; hard paraffin-2.5 gm; cetostearyl alcohol-2.5 gm; white soft paraffin - 42.5 gm. Each ingredient was mixed and heated gently with stirring then cooled. The base was then packed in a wide mouth container.

(b) **Preparation of 4% ointment**: 2 gm ethanol leaf extract of *Aegle marmelos* was added slowly to the above melted ingredients and stirred thoroughly until the mass cools down and a homogeneous product is formed. The ointment was then packed in a wide mouth container.

(c) **Preparation of 2% ointment**: 1gm framycetin was added slowly to the above melted ingredients and stirred thoroughly until the mass cools down and a homogeneous product is formed. The ointment was then packed in a wide mouth container.

**Excision wound model**: The excision model of Morton and Malone [12] was used to check the wound contraction and wound closure durable time. Each group of six animals was anaesthetized under light ether anesthesia. The hair on the back of the rat was removed by shaving with an aseptic surgical blade. A circular wound was created on the dorsal interscapular region of each animal by excising the skin with a 300 mm² biopsy punch; the wounds were left open. The base and 4% *Aegle marmelos* ethanolic leaf extract ointments and 2%framycetin standard ointment were applied topically twice a day on the wound till they completely healed. The progressive changes in wound were monitored planimetrically by tracing the wound margin on graph paper every alternate day. Epithelialisation time was noted as a number of days after wounding required for the scar to fall off leaving no raw wound behind. From the healed wound, a specimen sample of tissue is isolated from each group of rats for histopathological examination [13].

**Histopathological studies of excision wound**: Sample tissues were fixed in 10% formalin and embedded in paraffin wax. Serial sections (5 µm thickness) of paraffin-embedded tissues were cut. The tissues were stained with haematoxylin and eosin, which were examined by light microscope [14,15]. Percentage of wound contraction is evaluated in the skin tissues.

**Estimation of biochemical parameters**: Circular wound area was excised and evaluated for various biochemical parameters at the end of the study. Especially Collagen content, Hydroxyproline [16] and Hexosamine [17] was estimated for evaluating the healing properties of Aegle marmelos ethanolic leaf extract.

**Statistical analysis**: The values were calculated as mean ± S.E.M. The significance of the difference of the mean value with respect to control group was analyzed by one way ANOVA followed by Dunnet’s t-test using statistica 8.0. Statistically significant at a level of P<0.05 or above was considered to be significant.

**RESULTS AND DISCUSSION**

Wound healing involves a highly dynamic integrated series of cellular physiological and biochemical processes that occurs in living organisms. [18,19]. Skin serves for a number of vital physiological functions to maintain homeostasis. The functional properties of skin are often underappreciated until substantial loss of the skin occurs. The existence of undifferentiated cells in the skin suggests that skin has the potential to regenerate, but the context of molecular signals after tissue injury promotes scar repair. The phytochemical screening of the ethanol leaf extract revealed the presence of alkaloids, terpenoids, saponins, tannin, flavonoids, and steroids in (Table -1). The majority of world population relies on traditional medicine for their health care [20]. This is also the case in the treatment of wounds. Many research proposed that wound healing can be improved by herbal drugs having antiseptic, antibacterial, antioxidant and anti-inflammatory properties. [21,22].

On the basis of earlier reported pharmacological activities, the plants have significant antibacterial activity [23], anti-inflammatory activity and antioxidant [24] which are all important in the process of wound healing. *B. pilosa* has previously been found to contain favonoids, alkaloids, essential
oils and polyacetylenes [25,26,]. Alkaloids and flavonoids have been demonstrated to play key role in promoting wound healing [27-29]. The leaves extract exhibited the antibacterial effect against different pathogenic bacteria was evaluated for its antibacterial activity with streptomycin as reference standard. The antibacterial studies revealed that the leaf extract has antibacterial activity against both gram positive and negative bacteria. The standard streptomycin showed the all most same level of inhibition against Micrococcus luteus. The inhibition zone around each well amended with Leaf extract or streptomycin reveals that the highest antimicrobial activity was observed against Micrococcus luteus (22 mm), followed by, Bacillus subtilis (20 mm) Pseudomonas aeruginosa (18) Escherichia coli (18) . The lowest activity levels were observed against Bacillus sphericus (8 mm each).The antibacterial activity reveals that the produced extract has a broad spectrum activity against gram positive and gram negative bacteria.(Table 2).

The antifungal activities determined against five pathogenic fungi (Table 2). The highest activity was

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>STD</th>
<th>Ethanol Extracts</th>
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</thead>
<tbody>
<tr>
<td>Bacillus sphericus</td>
<td>22</td>
<td>8</td>
</tr>
<tr>
<td>Micrococcus luteus</td>
<td>30</td>
<td>22</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>28</td>
<td>10</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>22</td>
<td>10</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>Salmonella paratyphi</td>
<td>28</td>
<td>16</td>
</tr>
<tr>
<td>Bacillus megaterium</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>28</td>
<td>12</td>
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</table>

<table>
<thead>
<tr>
<th>Test Organisms</th>
<th>Ethanol Extracts</th>
<th>Standard</th>
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</thead>
<tbody>
<tr>
<td>Aspergillus fumigatus</td>
<td>07</td>
<td>7</td>
</tr>
<tr>
<td>Aspergillus flavus</td>
<td>03</td>
<td>14</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>Candida</td>
<td>06</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 3: Antifungal activity of ethanoic extract of Aegle marmelos.

<table>
<thead>
<tr>
<th>Post Wounding Days</th>
<th>Wound area (mm2) mean± SEM and percentage of wound contraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Base</td>
</tr>
<tr>
<td>0</td>
<td>0.00%</td>
</tr>
<tr>
<td>1</td>
<td>0.10%</td>
</tr>
<tr>
<td>3</td>
<td>0.12%</td>
</tr>
<tr>
<td>6</td>
<td>7.02%</td>
</tr>
<tr>
<td>9</td>
<td>20.2%</td>
</tr>
<tr>
<td>12</td>
<td>45.03%</td>
</tr>
<tr>
<td>14</td>
<td>70.24%</td>
</tr>
</tbody>
</table>

Table 4: Effect of Aegle marmelos leaf extract on wound healing by exision wound method in albino rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Hydroxyproline (μg/gm)</th>
<th>Collagen (μg/gm)</th>
<th>Hexosamine (mg/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>40.02±2.21</td>
<td>320.25±6.20</td>
<td>67.0±0.63</td>
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<tr>
<td>Standard</td>
<td>65.60±2.80</td>
<td>590.43±6.20</td>
<td>20.73±1.50</td>
</tr>
<tr>
<td>Extract</td>
<td>80.03±4.19</td>
<td>606.21±2.20</td>
<td>22.18±1.02</td>
</tr>
</tbody>
</table>

Table 5: Effect of ethanolic leaf extract of Aegle marmelos on biochemical parameters of wound healing.
Fig. 3: Photographical representation of wound contraction on different days A: Treatment group B: Control group

Fig. 4: Hematoxylin and eosin stained sections of the granulation tissue in treated group and control group at different time intervals. Fibroblasts (FB), collagen (c), vascularisation with larger blood vessels (BV) and inflammatory cells (IC).

Treated Groups

Control Groups
7.0 mm diameter of zone inhibition observed against *Aspergillus fumigatus* followed by 6.0 mm diameter of zone inhibition against *Candida*.

Acute dermal toxicity is determined by apply concentrated extract on shaved portion (300 mm²) on rat. No irritation or noticeable redness is observed. Wound healing activity is determined by excision wound model method and wound of 300 mm² is induced in rat and apply ointment regularly two times a day. The 4% *Aegle marmelos* ethanolic leaf extract ointment exhibited significant wound healing activity as compared to control in excision wound model. It is observed that the wound contracting ability of the 4% (w/w) extract ointment treated groups showed significant wound healing from sixth day onwards. The wound closure time was lesser as well as the percentage of wound contraction was more with the 4% (w/w) *Aegle marmelos* leaf extract ointment treated group than compared with control group. The wound were completely healed and percentage of wound contraction is observe in treated group is 12 days in standard group 12 ± 2 days where as in the control animals 24 ± 2 days (Fig. 3). 100% wound contraction is observe with standard (2% framycetin ointment) and test group (Fig. 2) than compare with control group. In control group percentage of wound contraction is 70% to 100% wound healing is observed with 4% ethanolic leaf extract of *Aegle marmelos* in rats with in twelve days, where as in standard treated group is 14 days wound healing is faster in 4% (w/w) *Aegle marmelos* leaf extract ointment than compare with the standard ointment (2% framycetin ointment).

Histological examination also provided additional evidence for the wound healing study, which was based on excision and restored incision model. Increase in collagen mass due to enhanced migration of fibroblasts and epithelial cells was observed in treated groups [30]. During the wound healing process the tissues were collected on 3, 6, 9, 12 days and histopathological studies were conducted. Study shows that the treatment with 4% ethanolic leaf extract of *Aegle marmelos* ointment and 2% standard drug framycetin ointment treated animals shows reduction of congestion, oedema, mononuclear leukocyte infiltration and necrosis. Extract treated animals showed mild vascular proliferation and reduction of accessory skin structures and increase in the dermal collagen content. Antimicrobial property of *Aegle marmelos* massively reduced the bacterial population, thereby indirectly reducing the inflammatory cells on the wound site. Early dermal and epidermal regeneration in the treated group confirmed that the ointment containing the *Aegle marmelos* extract had a positive effect toward cellular proliferation, granulation tissue formation, marked epithelialization, a moderate amount of extracellular matrix synthesis, and new blood vessel formation. Incomplete epithelialization with less extracellular matrix synthesis, clumps of degenerating neutrophils, necrotic changes, and the persistence of inflammatory exudates was observed in the upper dermis with loss of epidermis in control rats up to Day 12 (Fig 4).

On 12th day biochemical parameters are evaluated in excision wound model. There was a significant increase in the hydroxyproline content that is 80.03 ± 4.19 ìg/gm in ethanolic extract treated group which was much higher than control and standard drug treated group. Both later groups showed the values of 40.02 ± 2.21 and 65.60 ± 2.80 ìg/gm. Increase in hydroxyproline content is responsible for increase in collagen levels. Control animals showed much lesser collagen content which was 320.25 ± 6.20 ìg/gm, in standard 590.43 ± 6.20 ìg/gm, but the ethanolic extracts treated groups revealed 606.24 ± 2.20 concentration of collagen respectively. For healing property the hexosamine content was evaluated in the animal tissues which showed 22.18 ± 1.02 mg/gm in ethanolic extract treated group, while it was 6.7 and 20.73 mg/gm in control and standard drug treated group respectively. The values were statistically significant at P<0.05 when compared to untreated control group (Table 5). In the present experiment, the extract increases not only granulation and hexosamine formation but also, showed significant increase in hydroxyproline content of the granulation tissue of the excision wound which indicated rapid collagen formation and rapid healing of wounds. Considering the obtained results we can assume that the ethanolic leaf extract of *Aegle marmelos* might become a useful component for healing the wounds. Thus, further efforts will be put forth towards emphasizing its active components responsible for its wound healing potential.

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