Histological Evaluation of local application of Phyllunthus amarus extracted powder and its combination with fibronectin protein on Wound Healing of rats

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ABSTRACT
Background: A wound is defined as a break or damage in the skin, resulting from physical or thermal damage or as a result of the presence of an underlying medical or physical condition. Herbal medicine can be called one of the branches of medicine in various forms. Phyllunthus amarus is a small herb well known for its medicinal properties and widely used worldwide. *P. amarus* is an important plant of Indian Ayurvedic system of medicine. Fibronectin is a major component of the extracellular matrix. It is secreted by various cells, primarily fibroblasts, as a soluble protein dimer and is then assembled into an insoluble matrix in a complex cell-mediated process.

Materials and methods: Forty rats will be subjected for a surgical operation of skin of the cheek of rats. The animals were divided into control group contain 10 rats the wound was left for spontaneous healing and experimental group which divided into the followings according to the applicable materials: Group I the wound defect treated with 1mg of phyllunthus amarus extracted powder daily by using spoon excavator. Group II the wound defect treated with 1mg of fibronectin protein daily by using spoon excavator. Group III the wound defect treated with 1mg combination of (0.5 mg phyllunthus amarus extracted powder &0.5mg fibronectin protein) daily. Every single group composed of 10 rats that study in two periods 5, 10 days (5 rats for each period), then the rats will sacrificed on each period. Histological assessment regarding the count of inflammatory cells was performed on all studied samples with assessment of epithelial thickness and clinical consideration for wound contraction.

Results: Histological findings of the study showed that re-epithelialization, wound contraction were accelerated after local application in combination group of phyllunthus amarus extracted powder with fibronectin protein at wound site as compared with other groups.

Conclusion: local application of phyllunthus amarus extracted powder and it’s combination with fibronectin protein was significantly effective in cheek skin wound healing.

Key words: Phyllunthus amarus extracted powder, fibronectin protein, cheek wound-healing, local application.


INTRODUCTION
Skin, is the largest soft outer covering organ in the body. It has several functions, the most important being to form a physical barrier to the environment, allowing and limiting the inward and outward passage of water, electrolytes, various substances and protection against microorganisms (1). Skin is composed of three primary layers. The epidermis; which provides water proofing and serves as a barrier to infection. The dermis; which serves as a location for the appendages of skin. The inner most layer is hypodermis (2).

Wound is generally a knowledge tissue damage resulting in the disruption of the original tissue architecture and homeostasis (3). Wound healing, as a normal biological process, achieved through four precisely and highly programmed phases: hemostasis, inflammation, proliferation, and remodeling. For a wound to heal successfully, all four phases must occur in the proper sequence and time frame (4). Phyllanthus amarus belongs to the family Euphorbiaceae is a small herb well known for its medicinal properties and widely used worldwide. *P. amarus* is an important plant of Indian Ayurvedic system of medicine which is used in the problems of stomach, genitourinary system, liver, kidney and spleen. It is bitter, astringent, stomachic, diuretic, febrifuge and antiseptic. The whole plant is used in gonorrhea, menorrhagia and other genital affections. It is useful in gastropathy, diarrhoea, dysentery, intermittent fevers, ophthalmopathy, scabies, ulcers and wounds (Jay et al., 2011).
Fibronectin is a high-molecular weight (~440kDa) glycoprotein of the extracellular matrix that binds to membrane-spanning receptor proteins called integrins. Pankov et al.; 2002. Fibronectin also binds to other extracellular matrix proteins such as collagen, fibrin, and heparan sulfate proteoglycans (e.g. syndecans). Fibronectin exists as a protein dimer, consisting of two nearly identical monomers linked by a pair of disulfide bonds (Pankov et al., 2002). The fibronectin protein is produced from a single gene, but alternative splicing of its pre-mRNA leads to the creation of several isoforms. Fibronectin is a major component of the extracellular matrix. It is secreted by various cells, primarily fibroblasts, as a soluble protein dimer and is then assembled into an insoluble matrix in a complex cell-mediated process. Fibronectin plays a major role in cell adhesion, growth, migration, and differentiation, and it is important for processes such as wound healing and embryonic development (Pankov et al., 2002). Alteration fibronectin expression, degradation, and organization has been associated with a number of pathologies including cancer and fibrosis (Williams et al., 2008).

MATERIALS AND METHODS

Materials
- Phyllanthus amarus extracted powder
- Fibronectin protein
- Anesthetic solution: Ketamine Hydrochloride
- (Ketamin 50mg/ml) {1 ml/kg body weight};
- Xylocain (10%) {1 ml/kg body weight}.
- Zylazine (20mg/ml).
- Formalin 10%, Ethanol alcohol 96%, Xylol, Paraffin wax.
- Hematoxylen and eosin (H&E).

Methods
Forty rats were subjected for a surgical operation of skin of the cheek of rats. The animals were divided into following groups:

A. Control group contain 10 rats the wound the wound was left for spontaneous healing.
B. Experimental group will be divided into the followings according to the applicable materials:
1- Group I the wound defect treated with 1mg of phyllanthus amarus extracted daily.
2- Group II the wound defect treated with 1mg of fibronectin protein daily.
3- Group III the wound defect treated with 1mg of combination of (0.5 mg phyllanthus amarus extracted powder &0.5mg fibronectin protein) daily.

Every single group composed of 10 rats that study in two periods 5, 10 days (5 rats for each period), then the rats will sacrificed on each period (Fadhil etal;2018). All tissue specimens, samples and controls,were fixed in 10% neutral formalin and processed in a routine paraffin blocks. Each formalin-fixed paraffin-embedded specimen had serial sections were prepared as follows: 5μm thickness sections were mounted on clean glass slides for routine Haematoxylin and Eosin staining (H&E), from each block of the studied sample (experimental and the control groups) for histo- pathological reexamination. Analysis of number of inflammatory cells, it was performed by counting inflammatory cells, in histological sections (H&E stained), for each animal and in four microscopic fields at x40. The image J was used to measure the thickness of the epidermis from the inner edge of the basal cell layer until the surface of corium, under power x40 (12). Clinically for the wound contraction every wound was monitored by the electronical vernea at day 5 and 10 postoperatively

RESULTS

Five day duration

Control group
Skin section of 5days duration, shows the new epithelium formation, fibroblasts and collagen fibers are noticed (Figure 1).

Experimental group
1-Phyllanthus amarus extracted powder
Microphotograph of 5ays duration at wound site shows the thin newly formed epithelium, loose fibrous connective tissue is detected in the dermis (Figure 2).

2-Fibronectin protein
Histological view of facial skin section in the dermis of 5days durations, shows numerous blood capillaries, surrounded by number of inflammatory cells, fibroblasts and remodeling collagen fibers (Figure 3).

3-Combination of phyllanthus amorus extracted powder and fibronectin protein
Histological view of 5 days combination group shows complete epithelization at wound defect with numerous blood vessels, collagen fibers and fibroblasts (figure 4).

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Figure 1: Skin section of 5 days duration, shows the new epithelium formation, fibroblasts and collagen fibers are noticed H&E20x.

Figure 2: Microphotograph of 5 days duration at wound site shows the thin newly formed epithelium, loose fibrous connective tissue is detected in the dermis H&E20x.

Figure 3: Histological view of cheek skin section in the dermis of 5 days durations, shows numerous blood capillaries, surrounded by number of inflammatory cells, fibroblasts and remodeling collagen fibers H&E20x.
Figure 4: Histological view of 5 days combination group shows complete epithelization at wound defect with numerous blood vessels, collagen fibers and fibroblasts H&E20x.

Ten days duration

Control group
View of cheek skin section of 10 days duration of control group, shows epithelium collagen fibers and fibroblasts, blood vessels and newly formed hair follicles (Figure 5).

Experimental group
1-Phyllunthus amarus extracted powder
Microphotograph of 10 days duration at wound site shows maturing fibrous connective tissue, blood vessels and newly formed hair follicles. (Figure 6).

2-Fibronectin protein
Histological view of facial skin section, of powder group after 10 days, shows that the wound surface is covered by thin epithelium, numerous congested blood vessels, remodeling fibers and fibroblasts with hair follicles (Figure 7).

3-Combination of phyllunthua smorus extracted powder and fibronectin protein
Histological view shows a complete thick epithelization at defect wound area with numerous blood vessels and hair follicles (figure 8).

Figure 5: View of cheek skin section of 10 days duration of control group, shows thin epithelium, collagen fibers and fibroblasts, blood vessels and newly formed hair follicles. H&E20x.
Figure 6: Microphotograph of 10 days duration at wound site of Phyllanthus amarus extracted powder shows maturing fibrous connective tissue, blood vessels and newly formed hair follicles. H&E20x.

Figure 7: Histological view of cheek skin section, of Fibronectin protein group after 10 days, shows that the wound surface is covered by thin epithelium, numerous congested blood vessels, remodeling fibers and fibroblasts with hair follicles. H&E 20x.

Figure 8: Histological view of a Combination group at 10 days shows a complete thick epithelialization at defect wound area with numerous blood vessels and hair follicles. H&E 20x.

Table 1: Descriptive statistics and duration difference of account of inflammatory cells (H&E) in each group

<table>
<thead>
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<th>Groups</th>
<th>Duration</th>
<th>Descriptive Statistics</th>
<th>Duration difference</th>
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<td></td>
<td></td>
<td>Mean</td>
<td>S.D.</td>
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<tr>
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<td>5 days</td>
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<td></td>
<td>10 days</td>
<td>5.43</td>
<td>0.18</td>
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<tr>
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<td>5 days</td>
<td>5.33</td>
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<td>10 days</td>
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<td>Fibronectin protein</td>
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<td>10 days</td>
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<td>Combination</td>
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<td>10 days</td>
<td>4.25</td>
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Table 2: Descriptive statistics of epithelial thickness with time of healing for all groups.

<table>
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<th>Duration</th>
<th>Groups</th>
<th>Mean</th>
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<th>Min.</th>
<th>Max.</th>
<th>F-test</th>
<th>p-value</th>
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<td>Combination</td>
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Table 3: Descriptive statistics of wound contraction in cm with time of healing for all groups.

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<th>Min.</th>
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<td>1.73</td>
<td>1.68</td>
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<tr>
<td></td>
<td>Fibronectin protein</td>
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<td>1.64</td>
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<td>Combination</td>
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DISCUSSION

Wound healing is a complex process that involves inflammation, granulation and tissue remodeling. Interactions of different cells, extracellular matrix proteins and their receptors are involved in wound healing, and are mediated by cytokines and growth factors (9).

The use of herbal therapies for caring of wounds and injuries has been popular since ancient civilizations. In contrast to only 1–3% of modern drugs being used for the treatment of wounds and skin disorders (10).

The results of this study showed clear acceleration of healing in the combination groups with (phyllunthus amarus extracted powder and fibronectin protein) in comparison with the other groups.

Inflammatory cells were reduced in combination groups with (phyllunthus amarus extracted powder and fibronectin protein), than in the other groups, and highly significant value between all groups (table1), then decreased with time, throughout healing intervals which reveals an acceleration in healing and there were an anti-inflammatory period shown in the experimental group than the control one. This result was coincide with Fawzi et al., 2018. At 5 days, histological findings showed, thin new epidermis covering wound surface in studied groups, and fibrous connective tissue, with fibroblasts and remodeling collagen fibers with areas of blood congestions, which was obviously seen in combination group where complete reepithelialization of the surface, presence of collagen fibers, inflammatory cell infiltration was evident, this agreement with Al zamily et al.(12). At10 days, reepithelialization was complete. The underlying dermis showed remodeling immature collagen fibers, inflammatory cells are few, agreed with findings of Hussein et al. (11). Cellular fibrous connective tissue with congested blood vessels and infiltration of few inflammatory cells covered by thick, larger cellular epidermis was detected in the present study (13). The results of control group showed gradual increase in epithelization. While for the experimental group the process of wound surface epithelization was enhanced and accelerated by combination group (phyllunthus amarus extracted powder and fibronectin protein) application, with highly significant value between the groups in the healing interval period of 5 and 10 days. Also the results of epithelial thickness for both control and experimental groups reach its peak at 10 days and this result was disagreement with (Al-Wattar, 2013) In this study wound contraction was accelerated in all experimental group and in 5 and 10 duration as a compared to the control group (table 3). This result is agree with Lee et al., 2008 and Heng 2011.
As conclusions; local application of phyllunthus amarus extracted powder and fibronectin protein represents simple and inexpensive model of wound healing enhancement and the combination of phyllunthus amarus extracted powder and fibronectin powder are more effective in enhancement of wound healing regarding histological assessment. Inflammatory cells had highest mean values, and these values decreased with time. Besides highly significant difference was recorded between the studied groups at 5 and 10 days.

REFERENCES