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ULTRASTRUCTURAL EXAMINATIONS ON DIABETIC RAT SKIN TISSUE WITH TOPICAL APPLICATION OF *Salvia euphratica* ETHANOL EXTRACT

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Abstract: *Several medicinal plants to treat diabetic wound healing were researched and are still under investigation. A variety of processes contributed to diabetics such as; inhibition of inflammatory response, generation of reactive oxygen species, differentiation of the extracellular matrix and reduced collagen production. The purpose of this study was to examine the ultrastructural alterations in diabetic rat skin tissue treated with the ointment prepared with Salvia euphratica. Male Wistar albino rats were used in this study (n: 24), divided into 4 groups. Non-treated, diabetic, diabetic and cicatrizant treated, ointment prepared with ethanol extract of 1% Salvia euphratica topically applied for 14 days. A single dose of 45 mg/dL streptozotocin (i.p.) was given to rats to induce diabetes. Excisional wound model was created under anesthesia. A cicatrizant, fitocream, was used as positive control. Normal skin tissue was observed in non-treated group. Diabetic skin tissue revealed hyalinization of the cytoplasm and loss of cytoplasm. Ointment treated diabetic group revealed altered cellular elements suggests healing. At the wound area, number of fibroblast cells synthesizing connective tissue were increased and the collagen fibers were regularly oriented. Neutrophil and monocyte cells besides mast and macrophage cells were found in the capillaries of dermis in S. euphratica ointment treated group. Wound healing in diabetic tissues is known to be slow. Using phytocream therapy to expedite the healing process is a long time known phenomenon. Our observations demonstrated that Salvia euphratica promoted changes in skin tissue that may contribute to wound healing at the cellular level.*

Key words*: Diabetes, Salvia euphratica, Wound Healing, Ultrastructure, Phytotherapy*

1. Introduction

One of alternative method used for the treatment of wounds is phytotherapy [1]. Herbal medicinal products are being used as a primary source of healthcare and traditional medical practice in several communities [2]. The plant, its parts and their extracts are potentially used for wound treatment [3]. The use of herbal medicine demonstrated several healing properties on wounds such as improved tissue regeneration and anti-inflammatory response, increased wound contraction and higher collagen content according to the previous studies [4]. Medicinal herbs, which are subjected to multidisciplinary researches and are widely used in public, induce wound healing and regeneration in tissue [5]. The wound healing activities of several herbal remedies were investigated using different pharmacological models. However, many are still undiscovered [6].

As well-known, wound healing is slow and difficult in diabetic patients and wound remains open for prolonged periods and affects quality of life [7].Tissue repair occurs in order beginning with a 3 days lasting inflammatory response, followed by the formation of granulation tissue and finally remodeling phase that may take several months [8].

Salvia species have active secondary metabolites acting as free radical scavengers such as flavonoids, phenolic acid & terpenoids. Salvia species are applied for their anti-inflammatory, antidiabetic, anti-oxidative, anti-proliferative, antibacterial, antifungal, antiviral and cytotoxic effects [9]. Different Salvia species have been used in wound treatment studies. Narayan *et al.* (2011) in their study with extract of *Salvia splendens* reported improvement of dead tissue as a result of the application [10]. In a study with *Salvia hypoeuca*, it was observed that this plant extract provides new epithelial formation of wounds created in Albino Wistar rats [11]. Another study by Suntar *et al*. (2011), *Salvia cryptantha* plant ethanol extract treated group was determined to be a significant increase in wound contraction [12].

The present study was performed to examine the alterations arising from the application of endemic medicinal plant *Salvia euphratica* on experimental diabetic rat skin wound in the ultrastructural level.

2. Material and Methods

2.1. Experimental Animals

This study was done with the permission of ethical commission of Mersin University, Turkey. Male Wistar rats weighting 180–240 g were used (n:24). Their care were maintained in Mersin University Research Laboratory of Experimental Animals. Rats were kept in separate cages at room

temperature, humidity of % 65 and 12:12h light:dark photoperiod. They were fed with standard laboratory chow and had free access to water. Experimental groups were shown in Table 1.

Table 1. Groups of experimental animals

Groups	Number of experimental animals
1. Non –treated group	6
2. Diabetic control group-negative control	6
3. Diabetic and cicatrizant healing cream treated group-positive control	6
4. 1% (w/w) S. euphratica ointment treated diabetic group for 14 days	6

2. 2. Plant material and ointment processing

S. euphratica plant samples were collected from Van, Turkey by A. Kahraman. Air dried aerial parts of each plant material were powdered mechanically and macerated three times with ethanol. After filtration, solvents were evaporated under reduced pressure using vacuum evaporator at 35-40 °C and extracts were stored in the dark at 4 °C until use. Ointment base was prepared with glycol stearate: propylene glycol:liquid paraffin in the ratio of 3:6:1 [12] . Ointment prepared with 1% (w/w) *S. euphratica* extract was topically applied on the wound area for 14 days. Cicatrizant healing cream-fitocream including aqueous solution of *Triticum vulgare* was applied topically as a reference cream and positive control.

2.3. Induction of diabetes

A single dose of 45 mg/dL streptozotocin (Sigma Chemical Co.,USA) dissolved in citrate buffer (0.1 M, pH 4.5) was given to rats (i.p.) to induce diabetes. Blood glucose levels were measured using a rapid glucometer (Bayer, Germany). After STZ injection, animals with blood glucose levels above 300 mg/dL were used in the study.

2. 4. Anesthesia and Wound creation

Excisional wound model was created under intraperitoneal injection of xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (30 mg/kg) anesthesia. In order to create excisional wound, skin

tissue of 1. 5 cm diameters in a circular manner was removed by punch biopsy on the dorsal interscapular region of rats and left open [12].

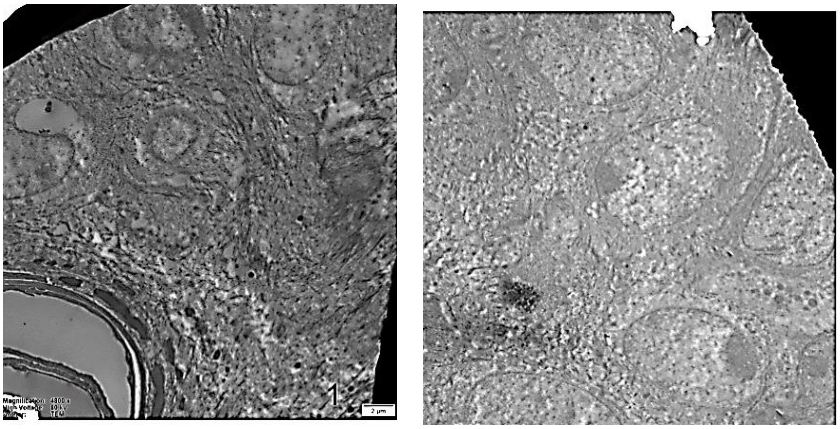
2.5. Preparation for Electron Microscopy

Rats were killed on the 14th day by excess sodium pentobarbitale anaesthesia. Skin samples were resected and rinsed in sodium phosphate buffer (0, 1 M, pH 7,4). Tissues were sliced into pieces of 1-2 mm^3 and immersed in 2. 5% glutaraldehyde for the first fixation. Following post fixation in 1% osmium tetroxide solution for 2h they were dehydrated by graded ethanol series of 70-100 %, placed into propylene oxide and embedded in araldite 502 [13]. Sections were cut with an ultramicrotome (Leica) stained with uranyl acetate-lead citrate and examined with transmission electron microscope (JEOL JEM 100 CXII) at 80-100 kV in Electron Microscope Laboratory of Ankara University, Faculty of Science, Department of Biology.

3. Results

Normal appearance of keratinocytes and other epithelioid cells were observed in non-treated group (Figs. 1, 2). Diabetic skin tissue sections revealed hyalinization (Fig. 3) and loss of cytoplasm (Figs.4, 5). and also nucleus of epithelioid cell was seen in heterochromatic appearance. After *S. euphratica* ointment application to diabetic rats it was found to have altered cellular elements suggesting wound healing at the cellular level. At the wound area it was seen that number of fibroblast cells synthesizing connective tissue were increased and active to synthesize collagen fibers. Distribution of collagen fibers were regular (Figs.6, 7). In the ointment group, Langerhans cells and keratinocytes were in normal appearance in Stratum spinosum. Intercellular connections were almost normal, Nucleus of cells were oval shaped with homogen euchromatic appearance (Fig 8). Electron lucent regions were observed next to the cell nuclei and ER cisternae were regular (Fig 9). Macrophages and fibroblasts were observed in dermis and monocyte was seen in capillaries (Fig. 10) may be differentiate into macrophage.

In the ointment treated sections; fibroblasts, macrophages and neutrophil cells were seen frequently (Figs.11-13). In the same sections mast cells (Fig.14). Diabetic and cicatrizant healing cream treated group demonstrated regular orientation of epithelial cells in epidermis layer, Langerhans cells and melanocytes between the epithelial cells of epidermis. In the same section, cisternae of ERs were dilated in fibroblast cells of dermis layer to synthesize collagen for healing (Figs.15-16).



Figures 1-2. Non-treated control group, normal appearance of keratinocytes and other epithelioid cells. Boarders of cells were regular, arrangement of cytoplasmic organelles were apparent.

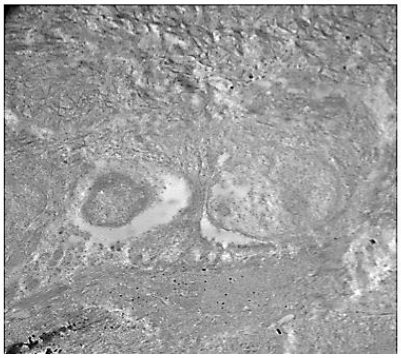
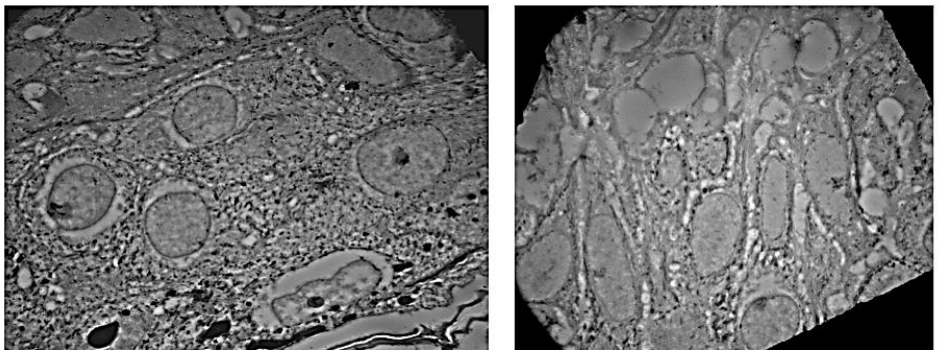
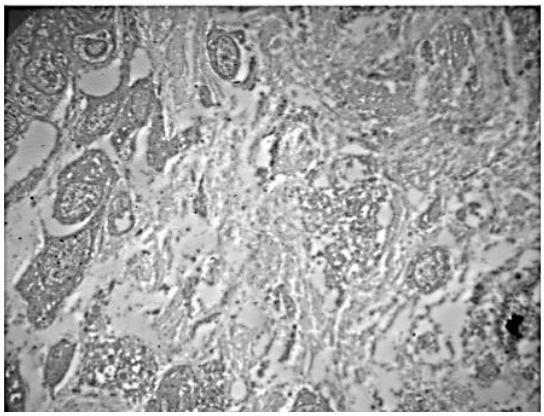
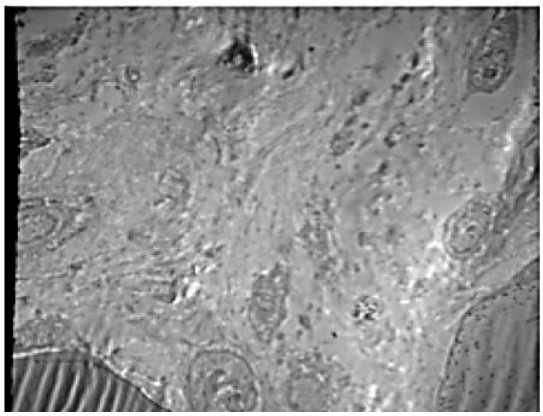


Figure 3. Diabetic group, nucleus of epithelioid cell was in heterochromatic appearance. Neighboring cell seem to be undergone pycnosis. Hyalinization was observed between epithelioid cells.

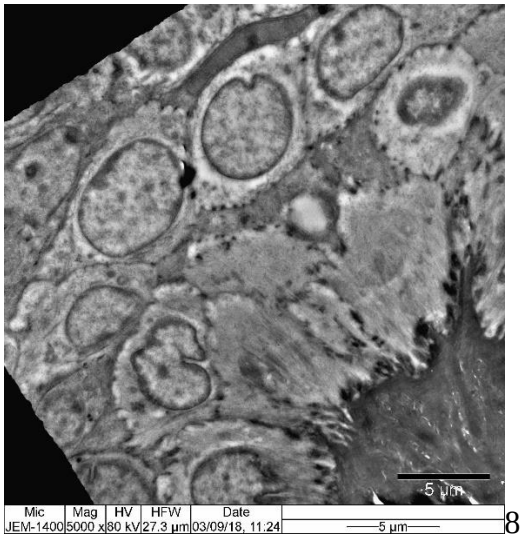


Figures 4, 5. Diabetic group, in the Stratum lucidum and Stratum granulosum layer, around the nucleus of epithelial cells, cytoplasmic material was hyalinized and an electron lucent region became evident. Apoptotic appearance in a few cells (Fig.4). Loss of cytoplasmic material and nucleus, loosening of intercellular connections and vacuole formation were observed in cells (Fig.5).





Figures 6, 7. *S. euphratica* extract applied for 14 days, distribution of collagen fibers were regular. Macrophage and other connective tissue cells were observed. Fibroblast cells were seem to be active (Fig.6). Collagen fibers were aligned parallel but distributed irregularly (Fig.7).



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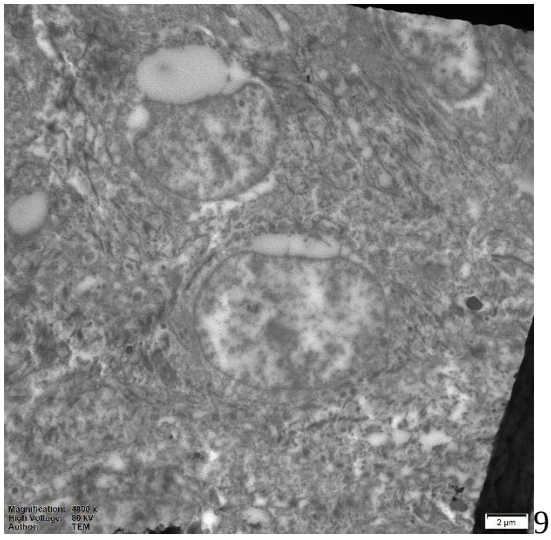

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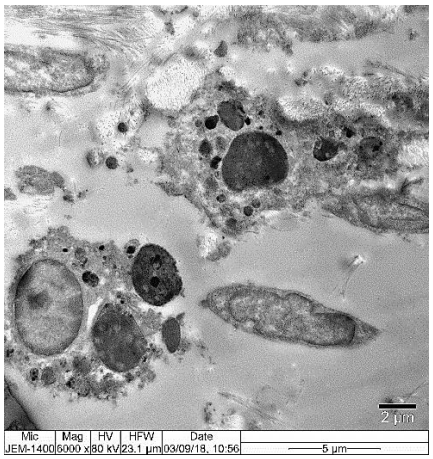
Figures 8, 9. *S. euphratica* applied group, Langerhans cells and keratinocytes were in normal appearance in Stratum spinosum. Intercellular connections were almost normal, Nucleus of cells were oval shaped with homogen euchromatic appearance (Fig 8). Electron lucent regions were observed next to the cell nuclei. ER cisternae were regular
(Fig 9).



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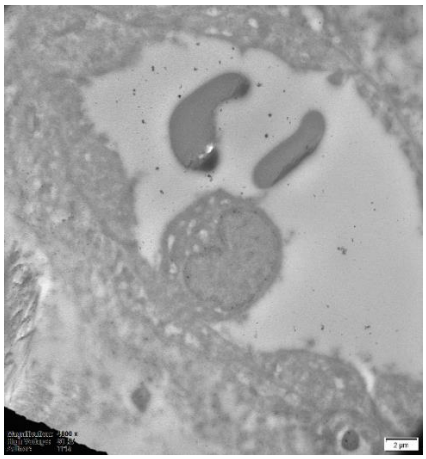
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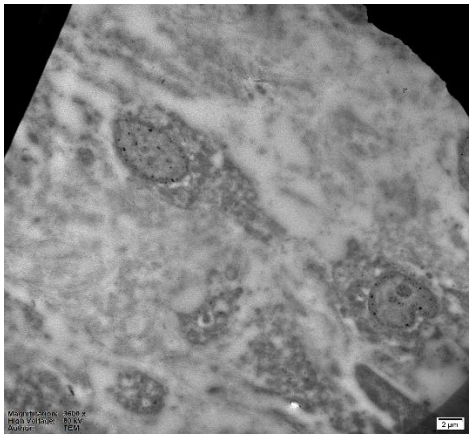
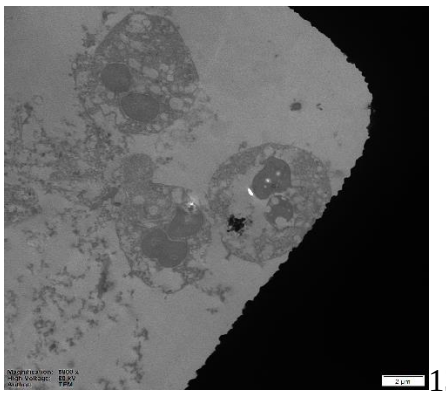
Figures 10, 11. *S. euphratica* applied group, macrophages and fibroblasts were observed in dermis (Fig. 10). Monocyte cell was seen in capillaries of dermis that may be differentiate into a macrophage as a tissue response (Fig. 11).



Figures 12. *S. euphratica* applied group, fibroblast cell synthesizing protein and active macrophage cells with phagosomes.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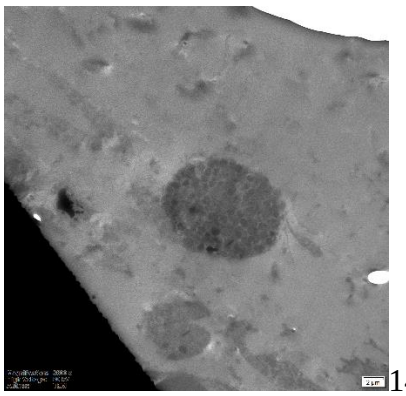
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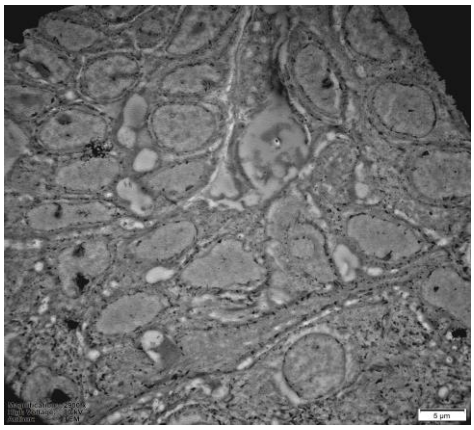
Figures 13-14. *S. euphratica* applied group, neutrophil cells (Fig. 13). In the same section mast cells (Fig.14)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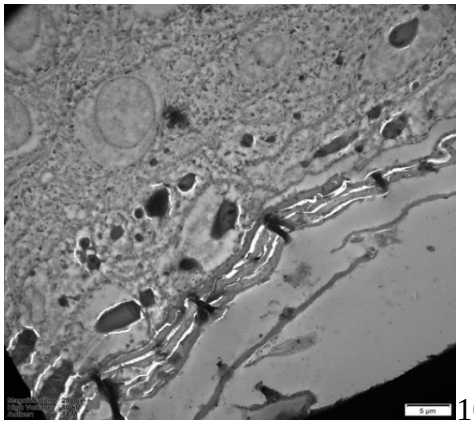
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Figures 15-16. Diabetic and cicatrizant healing cream treated group, regular orientation of epitheloid cells in epidermis layer, hyalinization of cytoplasm in a few cells was observed (Fig. 15). Cornified cells with the influence of fito-cream and keratinocytes and keratin layer was observed in Stratum corneum (Fig. 16).

4. Conclusion

Diabetes mellitus is one of the major factors leading to chronic wound healing problems. There is a complex relationship between diabetes and impaired wound healing. A series of processes take role in impaired wound healing in diabetics such as; dysfunction of immune system, neuropathy and vascular problems [7, 14]. According to a review by Dorai (2012), in the treatment of wounds, using herbal medicine is easily accessible, functional and moreover, an ongoing culture [4]. Another study of Medagama and Bandarar (2014) discussed in their study if the use of alternative medicines in the treatment of diabetes is effective [15].Many experimental wound models were created by the researchers. A study of Narayan et al. demonstrated that herbal ointment formulated with *Salvia splendens* had a wound healing effect on experimentally induced excisional skin wounds [10]. Salvia species were reported for their anti-inflammatory, antidiabetic, anti-oxidative, anti-proliferative, antibacterial, antifungal, antiviral effects [9]. Our study was constructed on this idea and a Salvia species; *Salvia euphratica* was used as a medicinal plant for observing wound healing activity in experimental diabetes.

Wound healing is a complex process involving multiple cellular and extracellular components, which are present in a normal healing process. It is known that inflammatory cells, fibroblasts and keratinocytes are important cell types during the healing process, modulating the reconstruction of the injured area. A study on effects of acute diabetes on rat cutaneous wound healing by Komesu et al. indicated that in diabetics wound healing phases; inflammation initial healing phase was slow and lasted longer [16]. They also found lower density of neutrophils in healing areas up to 3 days after surgery in diabetic animals and in addition, after day 3, when the neutrophils should leave the healing area, and be replaced by macrophages, compared to controls, diabetic animals showed higher numbers of neutrophils

[16]. Neutrophils are first defense cells of skin so, alterations in the number and function can be sign of a pathology [17]. We observed neutrophil and monocyte cells besides mast and macrophage cells in the capillaries of dermis layer of skin in ointment prepared with Salvia extract treated group. The presence of these cells indicates the process of wound healing. At the wound area treated with the herbal extract, it was examined that the number of fibroblast cells synthesizing connective tissue was increased and the collagen fibers were in a regularly oriented. In addition, the cytoplasm of some epithelial cells was seem to undergone melting and hyalinization, perhaps where the wound healing at the cellular level was not fully completed. As a result, we predict that topical application of herbal ointment prepared with *Salvia euphratica* ethanol extract depicted alterations on diabetic wound tissue at the cellular level.

Acknowledgment

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