Introduction

In the developed countries, about 2% of the population experienced a chronic wound in their lifetime. The number of chronic wounds is expected to increase due to the increase of lifestyle diseases such as diabetes, obesity and cardiovascular diseases (Gottrup, 2004). Worldwide, cases of diabetes have been estimated to be 246 million in 2007 and this number is projected to reach 380 million by 2025 (Tajbakhsh et al., 2011a). Foot ulcerations are one of the most common complications in patients with diabetes (Boulton et al., 2005). Approximately 15% of diabetic foot ulcers (DFUs) result in lower-extremity amputation (Sanders, 1994). DFUs complications are growing at double digit rates (Tajbakhsh et al., 2011b), so efforts should be directed to reverse the rising trend in the rate of amputation. Even with good standard wound care, the healing rate for DFUs is poor and slow (24.2% over 12-weeks and 30.9% over 20 weeks) (Margolis et al., 1999). Therefore, healing diabetic ulcers continues to be a challenge and tough to manage (Falanga, 2004). Though the exact pathogenesis of poor wound healing in diabetic wounds is not clearly understood, many studies evidenced several abnormalities in the various phases of the wound healing process (Singer and Clark, 1999). A variety of connective tissue abnormalities is known to be associated with diabetes mellitus and contribute to the impaired wound healing (Spanheimer et al., 1988). In these abnormalities, the collagen content of the skin is decreased as a result of reduced biosynthesis and/or accelerated degradation of newly synthesized collagen (Chithra et al., 1998). Recently, focus on medicinal plants has increased all over the world and many evidences have been collected to show enormous potential of medicinal plants in various traditional medicine. Many medicinal plants have a very important role in the process of wound healing because they promote the natural repair mechanisms (Shenoy et al., 2009). Plant based therapy not only accelerates healing process but also maintains the aesthetics (Kumar et al., 2007). More than 70% of
wound healing pharma-products are plant based, 20% are mineral based and the remaining is animal based materials. The plant based materials are used as first aid-antiseptic coagulants and wound wash (Dahanukar et al., 2000; Biswas and Mukherjee, 2003). On the other hand, marine algae which known as seaweeds are one of the marine biological resources with great pharmaceutical and biomedical potentials (Morrissey et al., 2001; Smit, 2004). Brown algae are a group of seaweeds that considered as a promising object in the medicine. They are recognized as rich source of alginates, which have therapeutic potential for wound treatment (Sedlarik, 1993). In spite of tremendous advances in the pharmaceutical drug industry, the availability of drugs capable of stimulating the process of wound repair is still limited (Udupa et al., 1995). Moreover, the management of chronic wounds is another major problem due to the high cost of therapy and the presence of unwanted side effects (Suh et al., 1998). In the case of diabetic wounds, an important consideration in the drug is their ability to promote healing and control infection. Therefore, the current study aimed to investigate the effectiveness of a combination of some selected medicinal plants with the brown algae that is widely spread among the Egyptian coasts, on wound treatment in healthy and alloxan-induced diabetic rats.

Materials and methods

Experimental medicinal extract mixture

The experimental medicinal extract mixture (EMEM) was composed of the brown algae, Cystoseira trinodis, and three medicinal plants (garlic, liquorice and ginger), which were chosen according to Michael (2013). The algal samples were collected from the coast of the Egyptian Red Sea. The four selected medicinal plants were purchased from the market.

Preparation of extract

Samples of algae were washed with sea water, tap water then distilled water adlibitum to remove the epiphytes and other wastes. All plants and algae were dried in hot air oven at 45°C until complete dryness and each plant was extracted separately. For ethanol extract, each dried plant material was ground to fine powder, macerated and soaked in 70% ethanol for 24 hrs, and then the extract was filtered. This was repeated three times for the complete extraction of ethanol and water-soluble compounds. All the three ethanol extracts were pooled and evaporated from crude extract by rotatory evaporator at 40°C under negative pressure until it became free of ethanol. Ethanolic extracts of all components of EMEM were pooled; moisture was adjusted to have a semisolid past-like pharmaceutical form, and stored in dark containers at 4°C until use as a mixed crude extract according to Chiheb et al. (2009).

Experimental animals

Twenty four male Wistar rats, 3 months old, weighing about 130-140 g were enrolled in the current study. The animals were obtained from the Animal House Colony of the National Research Center, Dokki, Cairo, Egypt. The animals were acclimated in cages for two weeks before starting the experiment. They were fed commercial rat feed and tap water. The animals were maintained under controlled conditions and received human care in compliance with the guidelines of the Scientific Ethical Committee, Faculty of Veterinary Medicine, and Suez Canal University.

Induction of diabetes

Diabetes was induced in 12 hrs fasted rats by a single intra-peritoneal injection of freshly prepared 20% alloxan monohydrate solution (Sigma Chem. Co., St. Louis, MO, USA) in a dose of 120 mg/kg body weight. Blood glucose level was estimated, using Glucometer® apparatus, 48 hrs after alloxan injection. 12 rats with glucose levels greater than 400 mg/dl were used in the study.

Epithelial wounding model

Wounds were created before beginning of the experiment for 12 healthy rats, and then on the 3rd day after induction of diabetes in the later 12 rats. The wounding model was induced as described by Carvalho et al. (2006). The animals were lightly anesthetized with diethyl ether. The dorsal region of each rat was shaved and cleaned with 70% ethyl alcohol. In the mid-region, between the infra-scapular line and the base of the tail, a circular area
of the skin was removed with a 5 mm diameter punch as shown in (Fig. 1). Wounds were uniform in diameter, depth, and location. Incision of the muscle layer was avoided and tension of skin kept constant during the procedure.

Topical application of vehicles

The diabetic and normal rats were assigned to evaluate wound healing activity of EMEM. The animals were divided into four groups (6 rats/group) as following. Group 1 (control, untreated normal rats (UN); group 2: treated normal rats (TN); group 3 (control diabetic); untreated diabetic rats (UD) and group 4 (Model, treated diabetic rats (TD). Wounds of groups 2 and 4 were dressed topically with the prepared crude extract. Dressings were applied once daily to the wound and then observed daily until complete wound healing occurs.

Estimation of wound contraction

Wound contraction was measured by digitized planimetry of the wound margins. The areas of the wounds were measured using Scion image software (Scion, MD) at days 0, 3, 7, 10, 14, 17 and 21 after wound induction. The percentage of closure was calculated according to the following formula:

\[
\text{Closure (\%)} = \left( \frac{\text{area at wounding day} - \text{area at biopsy day}}{\text{area at wounding day}} \right) \times 100
\]

Histopathological examinations

Specimens from the healed wounds of each rat were taken and fixed in 10% buffered formalin solution. Sections of the Specimens were made at a thickness of 5μl and, stained with hematoxylin and eosin (H & E) for histopathological studies.

Statistical analysis

All data were expressed as mean ±SD and one way ANOVA analysis was applied to determine the significance of the difference. If ANOVA showed statistical differences, the differences in averages among groups were analyzed by Dunnett’s multiple comparison. Significance between groups was accepted when P<0.05.

Results

Wound healing activity

The wound areas in the UN, TN, UD and TD animals were measured twice weekly up to 21 days (complete wound healing in TD group) (Table. 1). By comparing the wound area of UN and UD rats, it was larger in the UD rats, which exhibited delayed healing. In UN group, the wound area decreased sharply after the 3rd day and became
undetectable by the 17th day, while it decreased slowly in UD rats showing approximately large area on 21st day (8.7±2.2 mm²). In TD rats, the topical application of EMEM started to accelerate wound healing on the 7th day giving lower wound area of 13.5±2.8 mm² compared with that of UD group (15.5±3.1 mm²). The progression of healing activity increased rapidly throughout the following days and completely healed on the 21st day for TD group. When the measurements of the contracted area were subjected to statistical analysis, significant differences (p<0.05) were observed among the diabetic groups (treated and untreated) at 7, 14 and 17 days. On the other hand, there were no significant differences between treated and untreated normal rats (Table 1). This was obvious when the normal groups (UN and TN) showed approximately close wound area throughout the period of the experiment with slightly progress in the treated group, but they have the same time of complete healing (21 days). As shown in Fig. 2, the rate of wound contraction expressed in terms of the percentage of wound area that had healed. It was observed that up to the 14th day, all groups except UD group showed relatively close percentage of wound contraction with high values and more or less consistent increasing rate during interval period. Afterward, the normal groups (treated and untreated) preceded the TD in complete closure by three days whereas UD showed impaired healing throughout the period of the experiment achieving only 54.7 % of wound closure on the 21st day. As shown in Fig. 3, the gross appearance of excised wounds of TD rats demonstrated improving wound healing process better than the UN and UD rats. The healing potential of EMEM was clearly highlighted by

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<th>Group</th>
<th>Days</th>
<th>Wound area (mm²)</th>
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<tr>
<td>UN</td>
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<td>UD</td>
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<td>TD</td>
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Fig. 2. Rate of wound contraction in normal and diabetic rats (treated and untreated).
the full thickness coverage of the wound area by an organized epidermis in the presence of mature scar tissue in the dermis. The wound of UD rats appeared to be unclean with a biofilm glaze on the surface giving poor healing process and retarding contraction.

Histological evaluation of healed wounds

In both TN and UN groups, wounds were reepithelialised and necrotic tissue were replaced with granulation tissue. Both groups were demonstrated similar wound healing activity. The wounds of UD rats demonstrated skin ulcer with excessive muscle fiber necrosis and accumulation of fibrin exudates at the 3rd day post-wound excision (Fig. 4A). The wound revealed marked delayed healing activity with minimal re-epithelialization and marked necrotic tissues which were not completely replaced with granulation tissue (Fig. 4, B and C). Healing signs of TD group at the 3rd day post wounding revealed marked debridement of the of necrotic tissues (Fig. 4D). At the 7th day post wounding, faster granulation tissue formation containing more proliferating blood capillaries (angiogenesis) and collagen fiber with marked collagen deposition were also noticed. At the 14th day, epithelial tissue regeneration was also markedly increased at the wound edges showing less scar width at the wound enclosure (Fig. 4E). Signs of complete healing activity with complete epithelialization were noticed at the 21st (Fig. 4F).

Discussion

Martin (1991) mentioned that, the wound is a disruption in the continuity of cells and wound healing is an interaction of complex cascade of cellular and biochemical processes leading to the restoration of structural and functional integrity with regain of strength of injured tissues. Wound healing is characterized by three phases; inflammatory, proliferative and maturational. The first phase is characterized by homeostasis and inflammation, while the proliferative phase includes angiogenesis, collagen deposition, granulation tissue formation and epithelialization, which discussed by Porras-Reyes et al. (1993). Leite et al. (2002) reported that, in angiogenesis, new blood vessels grow from the endothelial cells, while in the granulation tissue formation fibroblasts grow and form a new extracellular matrix by excreting collagen and fibronectin and in the epithelialization, epithelial cells crawl across the wound bed to cover it. Moreover, in the maturational phase, the wound undergoes contraction resulting in a smaller amount of apparent scar tissue (Greenhalg, 2003). Wound healing process depends upon the reparative capacity of the tissue, types and extent of damaged tissues and the general states of health. Diabetes mellitus is one of the initial barrier and an important delaying agent to wound healing (Haber and Weinstein, 1992). Greenhalgh (2003) mentioned that, increased blood glucose level causes cell walls to become rigid, impairing blood flow through the critical small vessels at the wound surface which leads to impeding red blood cell permeability and oxygen release, decreased collagen and fibronectin synthesis and suppress the immune system which is necessary for inflammatory phase of wound healing. In the present study, the effect of diabetes was obvious when the UD rats showed delayed healing compared to the UN rats. Therefore, use of agents expediting healing in such condition was required, results which were in agreement with those recorded by Leite et al. (2002). Shenoy et al. (2009) stated that, plant products are potential wound healing agents, and largely preferred because of their widespread availability, non-toxicity, absence of unwanted side effects, and effectiveness.
as crude preparations. Hence crude extract composed of many plant material have been used in this study to formulate a potent therapy for the difficult wounds. The results of the present study revealed that topical application of our experimental extract EMEM on the excised wounds in alloxan induced diabetic rats promote wound healing and reduced the epithelialization period, results which were in agreement with those discussed by Marwah et al. (2007). Shenoy et al. (2009) stated that, although the active principal agents responsible for the wound healing activity of EMEM and their mechanisms of action have not so far been elucidated, the enhanced capacity of wound healing shown by EMEM could be explained on the basis of many actions. However, phytochemical screening of the selected plants is previously known from previous studies. Several phytoconstituents like alkaloids, saponins, flavonoids, tannin and steroids are known to promote wound healing process (Shafaghat et al., 2010). Garlic, liquorice and ginger have been documented to be rich in all of the aforementioned phytoconstituents. The antioxidant and anti-inflammatory activities of flavonoids were believed to be one of the important mechanisms in wound healing (Marwah et al., 2007). In the presence of tannin, it improved the regeneration and organization of the new tissue and hastened the wound healing process (Leite et al., 2002).

Conclusion

In the present study, topical application of EMEM promoted wound healing activity by increasing cel-
lular proliferation, granulation tissue formation and collagen synthesis in diabetic rat model. Wound healing properties of EMEM may be attributed to the individual or combined action of phytoconstituents like alginates, saponins, alkaloids, tannins and terpenoids. These compounds may mediate the beneficial effects of our extract. However, further investigations are necessary to determine the bioactive constituents present in the extracts to prove its potential in clinical studies.

Acknowledgment

The authors are grateful to the pharmacist, Mr. Mohammad M. Khalil for his financial assistance, providing facilities for this work as well as his help during preparation of the plant extracts.

References


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