The Effect of Gracilaria Corticata and Scenedesmus Acuminates Extract Mixture on the Healing of Wounds Contaminated with Staphylococcus in the Rat Model

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Abstract: Introduction: Wound healing processes are dependent on the severity of the trauma, invasion of opportunistic microorganisms, and inflammatory, immunological, and metabolic responses. We tried to show the ability of algae to inhibit wound infection, which can lead to proper wound healing. Methods: Eighty rats were housed according to laboratory animal care protocols and divided into four groups at each operating time. Group I consisted of the non-treated animals. Group II was treated with 25% zinc oxide as a choice treatment. In the treated groups 3 and 4, an equal ratio of Gracilaria Corticata and Scenedesmus acuminate marine algae (mixed algae) was applied as 3% and 7% ointment pomade. Percentage of wound closure, number of bacteria in the wound surface, angiogenesis (Vascular endothelial growth factor; VEGF), the number of macrophages, collagen production level and transforming growth factor-beta (TGFβ), epithelialization, and fibrosis were evaluated. Results: Applying mixed algae extract 7% and zinc oxide 25% could result in a mild improvement in wound closure (df: 9, 48; F=5.97; p<0.0001). In addition, mixed algae 3%, mixed algae 7% and zinc oxide could reduce the rate of bacterial growth compared to non-treated animals (df: 3, 16; F=5.74; p=0.0007). However, these improvements do not seem to be clinically significant. Induction of angiogenesis, increase in macrophage infiltration rate, and expression of TGFβ are possible underlying mechanisms of mixed algae in accelerating wound healing process. Conclusion: The result showed that the administration of 3% and 7% mixed algae could mildly accelerate the wound healing process in a rat model of pelleted skin wound. However, it seems that its effect is not clinically significant compared to non-treated and zinc oxide treated animals.

Keywords: Gracilaria; Scenedesmus; Staphylococcus aureus; Wound Healing; CD68 protein, rat; Transforming Growth Factor beta; Vascular Endothelial Growth Factor A, rat

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1. Introduction

Wounds are a common problem of the skin. Wounds occur due to trauma injuries, leading to an opening of the epidermis and underlying dermis. To return the troubled utilitarian state of the skin and interrupted epidermal and connective tissue continuity back to normal state, the healing of the wound is important. Wound healing processes are dependent on the severity of the trauma, invasion of opportunistic microorganisms, and inflammatory, immunological, and metabolic responses. The healing process is dependent on the type of the infiltrated leukocytes, activated mast cells, the content of the extracellular matrix, and various inflammatory or regulatory mediators, which contribute to restoring tissue integrity (1, 2).

Resistance to routine antibiotics used to treat wound infections is a harmful consequence. Various types of antibiotics and the pathogens that infect the wound have developed more resistance against the routine antibiotics (3). Staphylo-
coccus aureus is a pathogenic bacterium that can infect the wound. The methicillin-resistant form of Staphylococcus aureus can lead to treatment failure. The formation of biofilm is a factor that reduces antibiotic penetration (4).

Investigation of various traditional plant extracts has demonstrated their healing ability for skin wounds. These pharmacological extracts promote wound healing by inducing epithelial cell and fibroblast proliferation, leukocyte migration, and cell differentiation and have additional advantages, such as antimicrobial activity against the infection (5). The use of medicinal plant extract remedies to promote effective wound healing develops every day.

Many years before, investigators found that plant extracts have antimicrobial and wound healing properties, which may be used as an efficient, safe, and economic wound healing medicine to heal infected injuries. Scenedesmus acuminatus, a colonial green alga, is usually seen floating on the river. Unlike colonial green algae, Scenedesmus makes only four-cell chains. The ends of the colony possess small oil vacuoles, which enable them to float in the water. Scenedesmus acuminatus contains many antioxidants, including glutathione (GSH), tocopherols, flavonoids, ascorbate, and polyphenols. Moreover, it also has many antioxidant enzymes, including glutathione reductase (GR), glutathione S transferase (GST), superoxide dismutase (SOD), ascorbate peroxidase (APX), and peroxidase (6). Some previous studies demonstrated that the fatty acid methyl esters (FAME) or pigment extract obtained from Scenedesmus exhibit anti-staphylococcus aureus and antifungal activity. These fatty acids from Scenedesmus can break the protective mechanisms of bacteria against antibiotics (7).

A bio-content analysis of the Gracilaria corticata revealed that tannins were the most abundant compounds. The cytotoxic results showed that this algae species could inhibit the growth of human colorectal adenocarcinoma cell line. Thus, this marine red algae could be a reservoir of antitumor food ingredients of bacteria against antibiotics (7).

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2. Methods

2.1. Preparation of the Algae extract

Gracilaria corticata and Scenedesmus acaminatus platensis were extracted from the Persian Gulf at 50-100 cm depth. After washing the algae to remove epiphytes and pollution, we dehydrated them with tissue paper and took them out to dry in the sun. We milled dried specimens with a pulverizer and produced the alcoholic extract using the soxhlet machine. A vacuum distiller evaporated the juices to remove the solvent. Then we preserved them in the refrigerator at 4℃. We mixed the extracts of Gracilaria corticata and Scenedesmus acaminatus platensis equally and used them in pharmacological investigations (9).

2.2. Experimental animals

Eighty 200±30 gr Wistar albino rats of both sexes were entered in the experiment. The rats were kept in optimized laboratory conditions (22-25 °C, humidity=60±5 percentage, and 15 h light) with free access to food and water. They were kept in similar cages and randomly selected for model induction and outcome assessment using a random numbers table. Animals were randomly divided into four groups (n=20 per group) and desired outcomes were assessed on days 3, 7, 14 and 21 after wound induction. Five animals were assessed in each follow-up time point. The experimental groups are introduced in table 1. All included animals survived during the study (mortality rate of zero). This experiment was done with the approval of the local ethic committee, under the code number 97001781 (IR.AJAUMS.REC.1400.313).

2.3. Wound induction model and treatments

Induction of wound was done in blinded manner, in which the caregiver was not aware of the experimental groups. Clipping and shaving were performed on the operation site, and a 1.5×1.5 cm full-thickness square wound was made. After creating wounds, we contaminated all back wounds of the rats with 50 µl of 2×10⁸ CFU/mL staphylococcus-containing suspension. Ointments including zinc oxide, mixed algae 3% and mixed algae 7% were applied to the wound area from the day after wound induction once daily for 21 days.

2.4. Outcomes

All outcome measurements were performed on days 3, 7, 14, and 21 after wound induction by an investigator who was blinded to the animal groups. In addition, animals were randomly selected for outcome assessments at each time-point.
2.5. Wound size

On day 1, before administration of treatments, the wound size was recorded through measurement of greatest width and length. Length and width were multiplied and square area was calculated. The wound size was assessed using the same method on days 3, 7, 14, and 21 after wound induction. The wound size in specific days was calculated as St/S0×100, where S0 is the wound area at the time before treatment and St is the wound area at the time t and reported as percentage. Clinically significant improvement was defined as at least 50% reduction in wound size compared to baseline.

2.6. Number of wound bacterial colonies

We put a swab on the wound and then placed it in 1 mL of sterile normal saline. The swab discharged the staphylococcus into the fluid. We diluted the liquid 10-fold with sterile normal saline. Then, 5 µL of the sample was added to a Mueller-Hinton agar plate and incubated for 24 hours. Finally, bacterial colonies were counted using an automatic colony counter (Scan 1200 Inter-science Company, France). The number of bacterial colonies was assessed on days 1, 3 and 7.

2.7. Histopathological evaluation of the wound healing

The wound area was cut around and gathered. The tissues were fixed in 10% formaldehyde for 36 hours after euthanasia of rats using overdosed anesthetic materials. After paraffin embedding, 5 µm sections were prepared using a microtome. The samples were stained using hematoxylin/eosin and Masson trichrome stain. The samples were analyzed by a pathologist who was blinded to the animals’ groups. The histological grading was made based on angiogenesis, the number of macrophages, collagen level, epithelization, and fibrosis. Each factor was given a score of 0-3 for grading. The absence of a phenomenon was scored 0; a mild presentation was scored 1, the moderate one was scored 2, and the severe one was scored 3 (16).

2.8. Immunohistochemical evaluation

All materials were purchased from Sigma/Aldrich Company (Germany). After fixing the samples and embedding them in paraffin, we cut them into 5-µm tissue sections. The endogenous peroxidase was quenched through exposure to 3% hydrogen peroxide. The excess antigens were blocked after dropping 1% bovine serum albumin on the sample. We incubated the slides with a primary antibody against transforming growth factor-beta (TGFβ), vascular endothelial growth factor (VEGF), or CD68 at 1:400 dilutions overnight. After washing the tissues, the slides were incubated with 1:400 dilutions of goat anti-mouse IgG antibody (Abcam) for 1 hour at room temperature. The slides were treated with Diaminobenzidine (DAB) for 5 minutes after washing. Counterstaining with hematoxylin was operated. The tissue slides were put in alcohol and then xylene. Then they were mounted on a mounting medium, and were studied to evaluate the rate of gene expressions.

2.9. Statistical Analysis

Data are presented as mean ± standard deviation. Two-way repeated measures ANOVA and Bonferroni post hoc test were used to assess the mean difference of normal quantitative variables across different time-points. Time trend of histopathological scores’ (ordinal variables) changes was evaluated using Friedman test. Finally for assessment of clinically significant changes we used Jonckheere–Terpstra test for trend. In this test, at least 50% decrease in wound area or wound bacterial colony was considered success rate. The p-values less than 0.05 were considered significant.

3. Results

3.1. Percent of wound closure

Two-way repeated measures ANOVA revealed that the wound closure was significantly different among studied groups (df: 9, 48; F=5.97; p<0.0001). Three-day follow ups showed that administration of zinc oxide ointment can accelerate wound closure compared to non-treated group (p=0.029). Seven days after wound induction, wound size in zinc oxide- (p=0.003) and mixed algae 7%-treated (p=0.0003) groups had significantly decreased compared to non-treated animals. While in 14-day follow-up, wound size had significantly decreased only in mixed algae 7% group (p=0.014) compared to non-treated group. In contrast, there was no significant difference between any of the groups on the 21st day (Figure 1). To assess clinically significant improvement, we compared the number of animals with at least 50% decrease in wound size between experimental groups. The results showed that

Table 1: Experimental groups

| Experimental group | Intervention                                          |
|--------------------|-------------------------------------------------------|
| Non-treated        | Wound induction without any treatment                 |
| Zinc oxide         | Wound induction + treated with 25% zinc oxide         |
| Mixed Alga 3%      | Wound induction + treated with Gracilaria Corticata mixed with Scenedesmus acuminatum marine algae extract 3% |
| Mixed Alga 7%      | Wound induction + treated with Gracilaria Corticata mixed with Scenedesmus acuminatum marine algae extract 7% |
on the 3rd and 7th days post-injury there were no rats with at least 50% decrease in wound size in non-treated and mixed algae 3% groups. While, 50% decrease in wound size was observed in 60% and 80% of animals in the zinc oxide and mixed algae 7% groups seven days post-injury. Notably, all animals reached at least 50% decrease in wound size by day 14. The non-parametric test for trend showed that clinically significant improvement (at least 50% decrease in wound size) is similar among studied groups (p=0.066) (Figure 1).

### 3.2. Number of bacterial colonies in wound culture

Mixed algae 3%, mixed algae 7% and zinc oxide could reduce the rate of bacterial growth compared non-treated animals (df: 3, 16; F=5.74; p=0.0007). On day 3, the efficacy of mixed algae 3% (p>0.999) and mixed algae 7% (p=0.257) was similar to zinc oxide. The inhibitory effect of mixed algae 7% was more predominant than zinc oxide (p=0.001) and mixed algae 3% (p<0.001) seven days after the initiation of treatment (Figure 1).

50% decrease in the number of bacterial colonies in wound culture was observed in all treated animals on days 3 and 7, while there were no animals with 50% decrease in the number of bacterial colonies in wound culture in non-treated groups. The non-parametric test for trend showed that success rate was significantly higher in zinc oxide- and mixed algae-treated animals compared to non-treated animals (p=0.0007) (Figure 1).

### 3.3. Histopathological results

#### Angiogenesis

Qualitative histopathological assessment showed a non-significant increase in angioblasts in the zinc oxide and mixed algae 3%-treated groups compared to non-treated animals on days 3, 7, 14 and 21. Proliferating angioblasts formed numerous new blood vessels in the granulation tissue in
Figure 2: Histological assessment of infected skin wounds of rats in different treatment groups on day 3 (A, B, C and D), day 7 (E, F, G and H), day 14 (I, J, K and L) and day 21 (M, N, O and P) post-surgery (Trichrome ×100). Star: wound surface; Black arrow: collagen fibers; Yellow arrow: new blood vessels, up-down arrow: epithelium. Error bar represents 200 µm. Non-treated: A, E, I and M; Zinc oxide: B, F, J and N; Mixed algae 3%: C, G, K and O; Mixed algae 7%: D, H, L and P.
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mixed algae 7%-treated animals on day 3, day 7, and day 14. Finally, the number of blood vessels were reduced in all groups 21 days post-operation (Figure 2).

Quantitative analysis based on Friedman test showed that zinc oxide and mixed algae 3% did not have a significant effect on angiogenesis score in any of the time points (p>0.05). While, mixed algae 7% increased angiogenesis score 3 days (p=0.028) and 7 days (p=0.002) after wound induction compared to non-treated animals. Angiogenesis score on day 21 did not significantly differ among study groups (p>0.05; Figure 2).

Immuno-histochemical evaluation showed that VEGF expression was changed in treated groups (df: 9, 48; F=43.26; p<0.0001). Post hoc analysis revealed that mixed algae 7% could increase the expression of VEGF from day 3 (p=0.041). On day 7, zinc oxide (p=0.006), mixed algae 3% (p=0.009), and mixed algae 7% (p=0.0001) could increase the level of VEGF. On day 14, only zinc oxide increased VEGF expression (p=0.038). Interestingly, on day 21, VEGF expression in zinc oxide (p=0.006) and mixed algae (p=0.026) groups was lower than the non-treated group (Figure 2).

Fibrosis
Qualitative report by a pathologist, few fibroblasts were observed in the wound area and no notable starting of fibrosis was observed in any of the groups on day 3. The fibroplasia of the mixed algae 7% group was slightly higher than the other groups on day 7. Although the fibrosis had increased 14 days and 21 days post-injury, there were no significant differences between groups. Quantitative analysis based on Friedman test showed that fibrosis score was not significantly different between experimental groups in any of the time points (p>0.05) (Figure 2).

Collagen synthesis
Qualitative assessment showed that mixed algae administration in both doses led to production of fine collagen bundles in the primary granulation tissue compared with the non-treated group on day 3 and day 7. Identical collagen synthesis was extensively observed on day 14 and day 21 in all groups. Quantitative analysis based on Friedman test showed that collagen synthesis score was not significantly different between experimental groups in all time points (p>0.05) (Figure 2).

Epithelial regeneration
No notable epithelial regeneration was observed on day 3. Seven days after treatment, the epithelial formation started with no significant difference between the groups. On day 21, epithelization was hyperplastic in both non-treated and 7% mixed algae groups. The highest epithelialization rate was observed in non-treated animals (Figure 2). Friedman test showed that epithelization was not significantly different between experimental groups in all time points (p>0.05) (Figure 2).

Macrophage infiltration
The highest rate of infiltration of macrophages was seen in the mixed algae 7%-treated group 3 days post-wound induction. Seven days after wound induction, the number of macrophages in the mixed algae-treated groups was higher than in the non-treated group. In addition, the number of macrophages in the 7% mixed algae-treated groups was higher than non-treated and group 3% mixed algae-treated groups 14-day post operation. Finally, macrophages were reduced in all groups 21 days after induction of wounds. However, the number of macrophages in the non-treated animals were much higher than in other groups (Figure 2). Friedman test revealed that macrophage infiltration score in zinc oxide (p=0.008) and mixed algae 7% (p=0.018) were significantly higher than non-treated animals. While on day 21, this score was significantly higher in non-treated animals (p for zinc oxide=0.026; p for algae 7%=0.020) (Figure 2). Immuno-histochemistry assay depicted that the percentage of macrophages (CD68-positive cells) in the granulation tissue was significantly different between studied groups (df: 9, 48; F=32.53; p<0.0001). Multiple comparison showed that macrophage infiltration in mixed algae 3% (p=0.0006) was significantly higher than the non-treated group on day 3. On day 7, the macrophage infiltration rate significantly decreased in mixed algae 3% (p=0.001) and its level remained low until the end of follow-up. On day 14, the macrophage infiltration rate had increased in zinc oxide (p=0.0001) and mixed algae 7% (p=0.001) groups, while on day 21 the macrophage infiltration rate was only high in zinc oxide (p=0.004) group (Figure 2).

TGFβ expression
Immunohistochemistry assay depicted that the expression of TGFβ was significantly different between studied groups (df: 9, 48; F=49.50; p<0.0001). Multiple comparison showed that TGFβ in mixed algae 7% (p=0.014) group was significantly higher than non-treated group on day 3. On day 7, TGFβ expression was significantly higher in mixed algae 3% (p=0.0002) group. On day 14, TGFβ expression had increased in zinc oxide (p=0.043) and mixed algae 3% (p=0.0001) groups. While on day 21, the level of TGFβ expression was not significantly different between treated groups compared to non-treated animals (p>0.05) (Figure 2).

4. Discussion
The results showed that the administration of mixed algae 3% and 7% could mildly accelerate the wound healing process in a rat model of pelleted skin wound. However, it seems that this effect is not clinically significant compared to non-treated and zinc oxide-treated animals. In addition, mixed algae have a possible anti-bacterial activity. Although, the effect was clinically significant compared to non-treated ani-
bacteria, viruses, and fungi. They can also reduce inflammation against bacteria, especially Gram-negative ones. Nine species of Gracilaria sp. can inhibit the growth of many pathogenic gram-negative and gram-positive bacteria. The phenolic compounds are a significant contributor to the antioxidant properties of the microalgae. After inducing a wound, TGFβ is secreted by macrophages, keratinocytes, and platelets. TGFβ is necessary for starting granulation, tissue formation, and fibrosis. In addition, TGFβ is indispensable for cell migration during wound repair.

The outputs of the present study may provide a vital insight into the field of natural drug discovery. A study in 2014 showed that the marine algae Gracilaria corticata and Sphyrula platensis were the best BioSources of active substances such as various phytochemicals, antioxidants, and antibiotics. Some studies revealed that topical application of algae extracts could increase wound contraction and reduce wound closure time. The decrease in wound size was significant in animals treated with Gracilaria compared to the control group until the 20th day. The extract induced whole healing compared to usual drugs. However, our results showed that, although the effect of mixed algae on wound closure was statistically significant compared to non-treated animals, its effect was not clinically significant. This difference implies the importance of assessment of clinical significance level in animal studies.

In this study, on day seven post-surgery, mixed algae at a concentration of 7% showed inhibitory effects on bacterial growth. Sargassum illicifolium components showed medium inhibitory potency on Staphylococcus aureus, and more inhibitory strength against Pseudomonas aeruginosa. It could increase fibroblast proliferation and migration. Adding a trace amount of Ag to red algae can produce a spherical shape with hydrodynamic nanoparticles. This shape and size increase fibroblast proliferation and migration. Adding a trace amount of Ag to red algae can produce a spherical shape with hydrodynamic nanoparticles. This shape and size increase fibroblast proliferation and migration.

Some studies on improving angiogenesis of the skin wounds using algae extract showed that the wound healing activity of Gracilaria extract in rats at the concentration of 200 mg/kg was better than that of standard ointment on the sixth day of the investigation. Consistently, our results also showed that the mixed algae 7% group had the most blood vessels and highest induction of angiogenesis. Also, in the last days of repair, which required less angiogenesis, the number of vessels was reduced.

One of the critical factors in wound repair is collagen production, which did not change significantly in different groups. Macrophages are identified as brown against a pale ground background. The presence of macrophages on days 3 and 7 after surgery is a hallmark for inducing proper repair because they help repair by secreting various repairing cytokines such as TGFβ and fibroblast growth factors. Although tissue engineering offers unlimited possibilities for regenerative medicine, several problems limit its clinical application. Insufficient oxygen delivery to 3D cultures is considered one of the most significant limitations for the practical application of tissue engineering in vitro. Simultaneously, tissue regeneration relies on necessary nutrients and bioactive molecules to control critical biological processes. Photosynthesis is the source of oxygen; thus, microalgae cab offers a new way to supply adequate oxygen for tissue engineering. Photosynthetic microalgae Chlamydomonas reinhardtii (C. reinhardtii) have been widely studied in tissue engineering in recent years. For example, Hopfner et al. cultured C. reinhardtii in scaffolds for tissue repair. Then the microalgae showed high biocompatibility and photosynthetic activity. In addition, C. reinhardtii could be cocultured with fibroblasts, reducing the hypoxia response of cells under hypoxic culture conditions. Based on the in vitro studies, when the microalgae scaffold was transplanted into a mouse skin defect, it was found that the microalgae survived for at least five days in vivo, and chimeric tissues composed of algae and mouse cells were formed. On this basis, scaffolds constructed by genetically modified microalgae have also been developed, which can also deliver recombinant molecules for gene therapy and essential oxygen supply. Chávez et al. created a genetically modified C. reinhardtii that constitutively secreted the human vascular endothelial growth factor VEGF-165 (VEGF) to the wound tissues in vivo. Other algal scaffolds have also been applied in tissue engineering. For instance, Chlorococcum littorale scaffolds could provide enough oxygen to sustain the survival of C2C12 or rat cardiac single-layer cell sheets. Arthrospira scaffolds could improve the adherence and proliferation ability of mesenchymal stem cells from C57/B16N mice liver.
5. Conclusion

The results showed that the administration of mixed algae 3% and 7% could mildly accelerate the wound healing process in a rat model of pelleted skin wound. However, it seems that this effect is not clinically significant compared to non-treated and zinc oxide-treated animals. In addition, mixed algae have a possible anti-bacterial activity. Although, the effect was clinically significant compared to non-treated animals, its antibacterial activity was the same as zinc oxide. Induction of angiogenesis, increase in macrophage infiltration rate, and expression of TGFβ are possible underlying mechanisms of mixed algae in accelerating wound healing process.

6. Declarations

6.1. Acknowledgments

Here we owe it to ourselves to thank the staff of the laboratory and clinic of the Islamic Azad University, Science and Research Branch.

6.2. The Ethics code and IRCT number

This experiment has received ethical approval under the code number 97001781(IR.AJAUMS.REC.1400.313).

6.3. Conflict of interest

No conflict of interest.

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