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Role of Surf Redfish (*Actinopyga mauritiana*) in Wound Incision Healing Activities of Diabetic Mice

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Abstract

It is estimated that 6% of the US population suffer from diabetes with 15% of them experience diabetic foot. Several studies have explored the various benefits of sea cucumber for human being, including its antioxidant nature. This study aimed to investigate the effect of surf redfish, one species of the sea cucumbers, in diabetic wound healing. This was an animal experimental study performed at the Pharmacology and Pharmaceutical laboratory of the Faculty of Pharmacy, Universitas Tjut Nyak Dhien Medan, Indonesia, during the period of February- July 2019. Twenty five mice in diabetic condition were divided into five groups: control, standard, ethanol extract of surf redfish-1, ethanol extract of surf redfish-2, and ethanol extract of surf redfish-3 3. The surf redfish was obtained from Lamreh Village in Aceh and the ethanol was extracted using the maceration method. The parameters used for evaluating the efficacy of surf redfish ethanol extract were blood glucose level, length of wound incision, and histopathological features. Surf redfish extract presented a significant reduction of blood glucose level which that followed the increase in the dose of the ethanol extract (p-value <0.05) where the most significant reduction was seen in the group with the highest dose on the last day. This trend was also seen for the length of the wound, where the reduction was significantly higher in treatment groups when compared to control (p-value <0.05). The histological study also confirmed the improvement in the clinical appearance of wound by showing significantly increased fibroblast cell number and collagen density (p-value <0.05). Overall, the surf redfish can help better incision's wound healing in diabetic condition.

Keywords: Diabetic, ethanol, incision, surf redfish, wound

Introduction

The wound is the loss or disintegration of the tissue. Once the wound arises, the tissue lose the organ's function and show a sympatric response, hemorrhage, bacterial contamination, and cell death. Wound healing is a complex process consisting of three phases: inflammation, proliferation, and maturation. At the begining, the wound in the skin is the closure of the epidermis by keratinocytes, and it also requires the formation of various extra-cellular matrix (ECM). Some classes of the ECM including collagen, elastin, glycosaminoglycan, proteoglycan, glycoprotein, and others.[1–3]

Based on the emergency department data in the US in 2005, there were 11.8 million wound injury cases, and more than 7.3 of these cases was laceration. Incision or Stab wound leads to more than 2 million admissions to the hospital. The prevalence of opened wound injury in Indonesia was 25.4%, and Central Sulawesi has the highest prevalence (33.3%). Meanwhile, based on age, the highest prevalence of open wound injury was found between 25–34 years old person (32%). [4]

These were not only a problem among the healthy person. The wound injury can be more severe among diabetic patients. Amount of 6% of people in the US has diabetes, and 15% of these people suffer from diabetic foot due to neuropathy, obesity, or ischemic condition due to smoking. Moreover, around 14–20% of diabetic foot patients required amputation. [5]

Recently, the nutritional intervention has become the most explored due to its pharmacological properties like improving immunity, anti-inflammation, and protection against diabetes mellitus. Sea cucumber has been used as a traditional medicine in Asia for a hundred years. The medical applications of the sea cucumber were noted legally in the "Compendium of Materia Medica (*Bencao Gangmu*)" at 1758.[6] Indonesia and Malaysia

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used the sea cucumber traditionally as the sea cucumber water (also known as "*air gamat"*) or as the sea cucumber oil (also known as "Minyak Gamat").[7]

Various benefits of the sea cucumber are due to various phytochemicals and nutrients, like vitamin, calcium, zinc, iron, magnesium, chondroitin sulfate, triterpene glycoside, and saponins. Saponin can reduce the blood glucose level and is potentially used as an anti-diabetic agent. Due to various mechanisms to reduce the blood glucose level, one of the mechanisms is improving the insulin function by increasing the insulin plasma level and releasing insulin from the pancreas.[7,8]

Several studies have been performed to explore the benefits of the sea cucumber. These studies were reported that the sea cucumber has antioxidant effects. It will reduce the damage of some organs in the body. On the other hand, the sea cucumber also has other benefits, including antimicrobial, inhibiting the growth of neoplasm cell, wound healing, and the anti-thrombotic effect that prevents cardiovascular and cerebrovascular disease. [9]

Flavonoid and saponin in surf redfish improve the wound healing acceleration because these phytochemicals may reduce the ROS formation and increase various enzymes levels that neuteriles free radicals like superoxide *dismutase* (SOD) and *catalase* (CAT).[10] Moreover, these phytochemicals also decrease blood glucose levels and inhibit cyclooxygenase and lipoxygenase enzymes, decreasing inflammation severity in the wound site.[5,11–13]

There are many species of sea cucumber. Some of the sea cucumber species have low toxicity but major health benefits. These species are *Stichopus hermanni, Thelenota ananas, Thelenota anax, Holothuria fuscogilva, Holothuria leucospilota, Holothuria atra, Holothuria scabra* and *Actinopyga mauritiana.* Meanwhile, *Actinopyga mauritiana,* commonly known as surf redfish, is also named "*teripang buntal*" or "*teripang ballang ulu*" in Indonesia. Surf redfish are widely found in some regions in Indonesia, but few studies explored the benefits of this sea cucumber species. Due to these reasons, this study was designed to explore the wound healing effect of the surf redfish using alloxan-induced diabetic mice (*Mus musculus*).

Methods

This study was an experimental study using mice (*Mus musculus*) as the animal trial. This study was performed in the Pharmacology and Pharmaceutical laboratory of Pharmacy Faculty, Universitas Tjut Nyak Dhien Medan, in February–July 2019. Moreover, this study has been approved by the Health Research Ethics Committee from Universitas Prima Indonesia with letter No. 015/KEPK/UNPRI/V/2020.

This study used some materials included: Surf redfish, carboxyl-methyl cellulose sodium (Na-CMC), local anesthetic cream (Emla ®), alcohol with various concentrations (70%, 80%, 90%, 95%, and 96%), ether, 10% neutral buffered formalin (NBF), normal saline, parafin, xylol, hematoxylin and eosin stain, distilled water, and Entellan ® (as mounting medium).

Surf redfish was collected from Lamreh's Village in Aceh. It was washed with distilled water. After that, the body content was removed by the incision of the lateral body. At last, it was chopped. Cold extraction methods extracted the chopped surf redfish. It was soaked into 96% ethanol; the ratio of the sample against ethanol was 25:75. The mixture of surf redfish and the ethanol was saved far from sunlight. It was stirred regularly for three days. After that, it was filtered, and the residue was repeatedly extracted 2 times. The filtrates from each extraction were collected and evaporated by a rotary evaporator at 40^{o}C. The concentrated form of the extract was known as Ethanol Extract of Surf Redfish. Moreover, the extract was screened to find phytochemicals including alkaloid, flavonoid, glycoside, saponin, and steroid.

After that, the surf redfish ethanol extract was formulated into suspension form. Amount of 300 mg, 200 mg, and 100 mg ethanol extract of surf redfish were added into 5 ml 0.5% carboxymethyl cellulose sodium using volumetric flask to form the suspension of Surf redfish dosage 300 mg/kgBW, 200 mg/kgBW, and 100 mg/kgBW, respectively. Furthermore, the suspension of glibenclamide (2 mg/kgBW) was made by mixing 5 mg glibenclamide into 25 mL 0.5% Carboxymethyl cellulose sodium using a volumetric flask. Moreover, it was continued to prepare the animal trial. Twenty-five mice were divided into five groups and received some treatments described in Table 1.

Before the treatments, the mice were induced by alloxan monohydrates and incised in the back as the wound. The mice were induced by 10% (w/v)Alloxan monohydrate at 150 mg/kgBW. Ten percent Alloxan monohydrate solution was made by dissolving 0.01 gram (10 mg) alloxan monohydrate into 100 mL normal saline using

a volumetric flask. It means that every milliliter of 10% alloxan monohydrate contained 0.1 mg alloxan monohydrate (10 mg/ 100 mL), thus to obtain the dose of 150 mg/kgBW alloxan, it required 1.5 mL/kgBW of 10% alloxan monohydrate (0.1 mg/mL x 150 mg/kgBW). Due to this reason, each mouse received 1.5 mL/kgBW of 10% alloxan monohydrate solution. After that, the mice were shaved at the back, and then the incision was made 1 cm in the shaved back under local Anastasia (Emla ®).[14,15]

Some parameters evaluated the wound healing activity, including length of incision, histology of skin tissue, and blood glucose level. Meanwhile, the blood glucose level of mice was measured before and after induction, 3rd, 6th, 9th, and 15th day after the induction. Moreover, the density of collagen and the number of fibroblast cells from skin tissue histology were evaluated at 400x magnification and Hematoxylin and Eosin staining. The skin tissue was gotten from the mice by excision. On the 15th day, the mice were sacrificed by inhaling an excess dose of Ether solution. Then the back of the mice was shaved and excised with the depth of tissue ±3 mm and length 2.5 cm. The gotten skin tissue was fixated into 10% *neutral buffer formalin* for 24 hours. After that, the fixated tissue was hydrated by various alcohol concentrations and concentrated alcohol around 2 hours for each alcohol. After that, the tissue was cleared by xylol for an hour. Then, it was infiltrated into paraffin at 60°C for an hour. Moreover, it was slashed 5 mm thick by a microtome, placed in object-glass, and coated by Entellan®.

The slide was deparaffinization and stained by hematoxylin-eosin. After that, the slide was stained by hematoxylin staining for 5 minutes and rinsed with distilled water for 10 minutes. Then the slide was stained with eosin staining for 2 minutes and followed by the various concentrations of alcohol solution, clearing with xylol, and coating the slide with a glass cover (mounting process) using Entellan®.

All data were analyzed descriptively. Moreover, these data were analyzed by one way ANOVA, followed by Post Hoc Test Tukey HSD if the data were distributed normally. Nevertheless, if the data were not distributed normally, Kruskal-Wallis analyzed these data and followed by Mann-Whitney.

Results

According to screening phytochemicals, the ethanol extract contained several phytochemicals, including alkaloid, flavonoid, glycoside, saponin, and steroid. Moreover, table 2 describes blood glucose levels among the treatment groups. Due to data distribution of blood glucose levels was not normal, the blood glucose level was analyzed by Kruskal-Wallis and followed by the Mann-Whitney test and described in Table 2. No significant differences were found on the 15th day after feeding the highest dosage of the extract against the standard. It also can significantly reduce blood glucose levels than the control group. At the highest dosage group, the decrease of the blood glucose level also occurred in the mid of the observation period. Meanwhile, the lower dosage of extract reduced the blood glucose level than the group of mice that did not receive any intervention; however, the lower dosage did not reduce the blood glucose level as low as the standard group.

Other than the blood glucose level, this study also evaluated incision length as the parameter for wound healing activity. The incision length of the treatment group was distributed normally,

Table 1 Treatment Groups of Mice

Groups	Treatment
Control	the mice received 1 ml 0.5% carboxymethyl cellulose sodium
Standard	The mice received 0.2 mL suspension of glibenclamide
Ethanol extract of surf redfish-1 (100 mg/kgBW)	The mice received 1 mL suspension of surf redfish dosage 100 mg/kgBW
Ethanol extract of surf redfish-2 (200 mg/kgBW)	The mice received 1 mlL suspension of surf redfish dosage 200 mg/kgBW
Ethanol extract of surf redfish-3 (300 mg/kgBW)	The mice received 1 mL suspension of surf redfish dosage 300 mg/kgBW

Table 2 Comparison of Blood Glucose Level among Groups of Treatment

Groups	Blood Glucose Level						
	Before Induction	1st day	3rd day	6th day	9th day	12th day	15th day
Control	57 (2)	509 (1)	527 (2)	544 (5)	559 (21)	578 (37)	592 (32)
Standard	55 (1)[a]	558 (5)[a]	317 (10)[a]	254 (4)[a]	209 (18)[a]	166 (34)[a]	151 (34)[a]
Ethanol extract of surf redfish-1	58 (4)[b]	560 (8)[a]	501 (70)[a, b]	479 (4)[a, b]	453 (23)[a, b]	430 (22)[a, b]	407 (26)[a, b]
Ethanol extract of surf redfish-2	58 (4)[b]	562 (38)[a]	504 (6)[a, b]	464 (7)[a, b]	375 (32)[a, b]	430 (28)[a, b]	330 (22)[a, b]
Ethanol extract of surf redfish-3	52 (6)[a]	564 (6)[a, b]	346 (7)[a, b]	256 (7)[a]	190 (9)[a, b]	191 (20)[a, b]	171 (49)[a]
P-value*	0.003	0.002	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05

* P-value was obtained from kkruskall-wallis test; [a]Significant differences against control at 0.05 by Mann-Whitney test; [b]Significant differences against standard at 0.05 by Mann-Whitney test

and then it was analyzed by the one-way ANOVA and followed by the post hoc test. Figure 1 describes that the incision length was reduced over the observation time. Meanwhile, the length dispersion in each of the groups was narrow. Moreover, the one way ANOVA was shown that the p-value was <0.05, while the post hoc test by Tukey HSD showed the significant difference of standard and ethanol extract of surf redfish groups along the period of observation, except the ethanol extract of surf redfish-2 at ninth day.

This study was also evaluated the histology view of wound site viz. density of collagen and number of fibroblast cells. The density of collagen was expressed as a score. Table 3 showed the density of the collagen density among treatment groups. The collagen density of a higher extract dosage was denser than the standard groups. Other groups than the control and lowest dosage also showed a significant difference against the control group. Hence, the two highest dosages of surf redfish ethanol extract significantly affect collagen density.

Furthermore, the number of fibroblast cells was expressed as a score. Table 4 compared the number of fibroblast cells as the score. There was a significant increase in fibroblast cells among the treatment groups than the control group. On the other hand, the ethanol extract of surf redfish also significantly increased the number

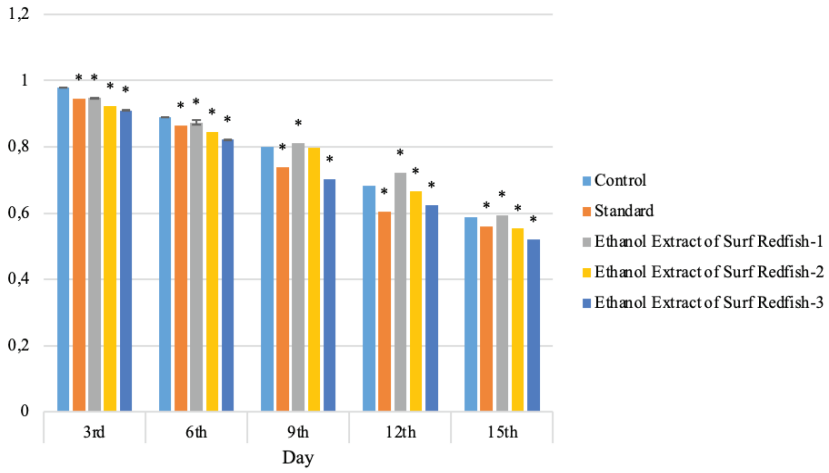

Figure 1 Comparison Length of Incision's Wound among the Treatment Groups
There is a significant difference against control groups at the 0.05 by Post Hoc Test Tukey HSD

Table 3 Comparison of Collagen Density among Treatment Groups

Groups	Score				P-Value*
	Median	Range	Min	Max	
Control	2	2	1	3	
Standard [a]	13	2	12	14	
Ethanol extract of surf redfish-1 [a]	3	2	2	4	< 0.05
Ethanol extract of surf redfish-2 [a, b]	16	2	15	17	
Ethanol extract of surf redfish-3 [a, b]	17	2	16	18	

* P-value was obtained by the Kruskal-Wallis test; [a] The score is significant difference against control at 0.05 by Mann-Whitney test; [b] The score is significant difference against the standard at 0.05 by Mann-Whitney test

Table 4 Comparison of Fibroblast Cells among Treatment Groups

Groups	Score				P-Value
	Median	Range	Min	Max	
Control	2	1	2	3	
Standard [a]	6	2	5	7	
Ethanol extract of surf redfish-1 [a, b]	4	1	4	5	< 0.05
Ethanol extract of surf redfish-2 [a, b]	11	3	10	13	
Ethanol extract of surf redfish-3 [a, b]	13	2	12	14	

* P-Value was obtained by the Kruskal-Wallis test; [a] The score is significant difference against control at 0.05 by Mann-Whitney test; [b] The score is significant difference against the standard at 0.05 by Mann-Whitney test

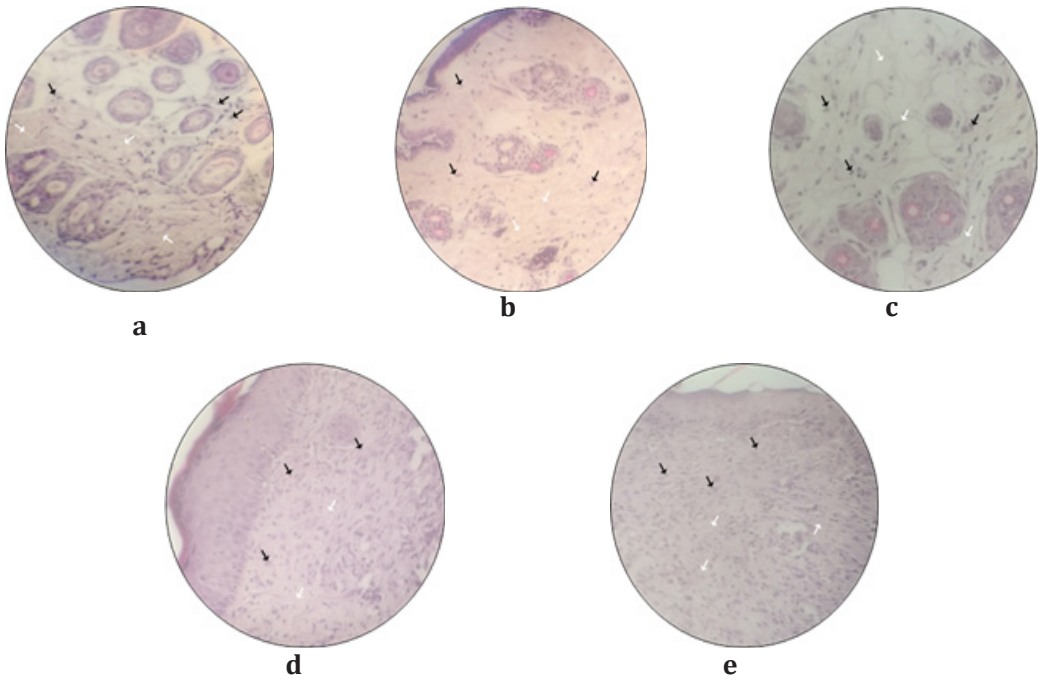

Figure 2 Histology Views of Skin Tissue from Control

(a) Standard (b) Ethanol Extract of Surf Redfish-1 (c), 2 (d), and 3 (e). Magnification: 400x. Stain: Hematoxylin and Eosin (HE)

of fibroblast cells than standard groups.

The histology view of some skin tissues is shown in Figure 2. Figure 2 shows the collagen fibre and the fibroblast in the dermis layer of the skin tissue. The white and black arrows indicate collagen fibre and fibroblast cells, respectively. Based on the histology view, the density of tissue becomes denser followed by the increase of the extract dosage; it showed that the rise in the dose gives better healing activity.

Discussion

This study answered the purpose of this study. The highest dose (300 mg/kgBW) of surf redfish ethanol extract showed incision wound healing best. It was indicated by improving collagen and fibroblast density in the highest dose of the surf redfish group. This improvement may be due to various phytochemicals like alkaloid, flavonoid, glycoside, saponin, and steroids. These phytochemicals may have an antioxidant effect and anti-diabetic effect. Hence, this surf redfish decreased the blood glucose level that accelerated the wound healing process, and its antioxidant effect prepares a good microenvironment for wound healing.

When the blood glucose level is high, it will damage the blood vessels. Consequently, the blood cannot perform its functions. Moreover, the blood vessels cannot perfuse the organ well, which reduces the oxygenation and nutrition delivery, or more severe can cause blockage of blood vessels. At last, it will cause a delay in the wound healing process.[16] On the other hand, the high blood glucose level can also enhance the anaerobic bacteria growth and complicate wound condition.[5,11]

Surf redfish ethanol extract has various phytochemicals, especially flavonoids and saponin. Flavonoids and saponin have antioxidants and anti-diabetic. Flavonoid reduces reactive oxygen species (ROS) formation by inhibiting the enzyme that contributes to ROS formation. Meanwhile, saponin decreases the impact of free radicals by donor the electron or hydrogen atom to free radicals. Moreover, saponin also increases the level of the enzyme that neutralizes free radicals like SOD and CAT.[10]

Flavonoids can reduce the formation of arachidonic acid by inhibiting cyclooxygenase and lipoxygenase enzymes. Due to the presence of these activities, these phytochemicals also have anti-inflammatory effects. Meanwhile, the saponin increase migration of macrophages into the wound tissues by releasing some type of cytokines. Additionally, saponin can also act as an antiseptic, disturbing the permeability of bacteria's membrane cells. Moreover, the protein and nucleotide in the bacteria flux out.[12,13]

Flavonoids and saponin reduce the blood glucose level, preventing worse blood vessel damage. Moreover, the flavonoid improves the blood vessels damage that has been occurred. On the other hand, saponin also increases the number of macrophages in the wound tissue. The macrophages will secrete some growth factors like fibroblast growth factor (FGF), platelet-derived growth factors (PDGF), transforming growth factor –β (TGF-β), and epidermal growth factor (EGF). These growth factors attract the fibroblast cells into the wound tissue, increase the density of collagen fibres, and form new blood vessels.[11]

The fibroblast cells which has a contractility activity is also called myofibroblast. It will pull the edge of the wound and bring it up, attaching the edge of the wound and accelerating the wound healing process. These cells produce collagen fibres. It forms granulation tissue that contains connective tissue matrix and, finally, forms a solid fibrosis tissue. The denser connective tissue in the wound increases the wound's contractility, so the wound will be brought up and smaller the wound.[17] Overall, It can be concluded that the surf redfish can improve the incision's wound healing in the diabetic condition

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