

Evaluation of wound healing potential of a sea cucumber (*Actinopyga mauritiana*) extract in mice (*Mus musculus*)

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Abstract. Sea cucumbers are a marine source with biological activities that have been used in food as well as medicine in many Asian countries. *Actinopyga mauritiana* is one of the sea cucumber species with limited research about its bioactive activities. In this study, the wound healing activity of *A. mauritiana* extract in the form of cream, gel, and serum formulations in mice was investigated. The histopathology test was performed to evaluate the fibroblast and collagen dense levels in wound healing. Phytochemical screening has been carried out by the qualitative test of alkaloids, flavonoids, glycosides, steroids, and saponins compounds. The *A. mauritiana* ethanol extract (SCAE) was added into cream, gel and serum formulations with various concentrations (3, 6, and 9%) and applied to the wounded area of mice. The wound healing activities and histopathology results indicated that serum 9%-SCAE performed the highest decrease of wound length 0.55 ± 0.32 compared to other groups with a density level of fibroblast and collagen are 10.00 ± 1.00 and 10.67 ± 0.00 , respectively. To the best of our knowledge, this is the first report on the wound healing activity of *A. mauritiana*, which demonstrated promising therapeutic agents for wound healing and cosmetics applications in the future.

Keywords: *Actinopyga mauritiana*, wound healing, cream, gel, serum

INTRODUCTION

Sea cucumbers are marine invertebrates that have been consumed in Asia for centuries [1]. Sea cucumber is known as nutritional seafood that contains polyunsaturated fatty acids, amino acids, and bioactive contents. Moreover, sea cucumbers have attracted high attention in the biomedical field since their healing tissue capabilities due to the presence of bioactive compounds [2]. They have been used as a traditional remedy for healing wound activity in East and SouthEast Asia. Their secondary metabolites have been identified to induce tissue repair and wound healing processes especially triterpene glycosides (saponins) and glycosaminoglycan [3].

Wound healing is the tissue injury that includes the cellular phase (granulation), narrowing of wound area (wound contraction), collagen deposition, epithelial covering (epithelialization), and scar remodeling [4]. The new tissue formation is a complex process involving several steps; inflammation, angiogenesis, granulation tissue formation, re-epithelialization, and extracellular matrix (ECM) reconstruction [5]. After the skin being injured, proliferation occurred in cells (fibroblasts, keratinocytes, macrophages, and other immunes) then cells migrated to the wound site and a complex healing process began. In the wound healing process, the cell's migration towards the wound is known as one of the key phases managed by various stimulatory factors of tissue micro environments [6].

In the skin tissue, fibroblasts are the most abundant cells that play a critical role in wound healing, such as: rupturing of fibrin clots, generation of ECM components, and generation of collagen structure [7], [8]. Collagen synthesis

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and granulation tissue formation also play an important role in wound contraction [9]. In contrast, free radicals inhibit the wound healing process that leads to impaired wound healing. It can disrupt dermal fibroblasts and keratinocytes functions by modifying or degrading ECM proteins. Moreover, ROS-mediated transcription can affect the secretion of proinflammatory cytokines and induction of matrix metalloproteinases [10].

Actinopyga mauritiana is a sea cucumber present in sand lagoons and seagrasses in the shallow reefs. This species belongs to the family the Holothuroidea and widely spread throughout the Red Sea to the Indo-Pacific Ocean [1], [11]. It has high protein and low-fat content and also becomes the highest commercial value among holothurians [1]. However, research on bioactivity from *A. mauritiana* is still limited. Since sea cucumbers have a high application to wound healing, thus this study aims to evaluate the wound healing potential of *A. mauritiana*. To the best of our knowledge, this is the first report of wound healing activities of ethanol extract of *A. mauritiana* sea cucumber on *Mus musculus* mice in cream, gel, and serum formulations.

METHODOLOGY

Sample preparation

Sea cucumbers *A. mauritiana* were obtained from Baitussalam, Aceh, Indonesia and were identified at the Research Center for Oceanography, Indonesian Institute of Sciences, Jakarta, Indonesia. 10,400 g of *A. mauritiana* were washed with distilled water, cleaned, and thinly sliced with a final weight of 5,300 g. The extraction process was done through maceration with 96% ethanol (2700 mL) for two days and filtered. This process repeated twice until the solution was clear. Then, the macerated solution was concentrated in a rotary evaporator at 40°C with 11.79 yield % or 625.2 g crude extract. The extract was labeled as SCAE (Sea Cucumber *A. mauritiana* ethanol extract). Phytochemical screening was performed on the crude extract with the following standard procedures [12].

Formulations

Cream formulation based on Young [13] with modification stearic acid (12 wt%) and cetyl alcohol (0.5 wt%), followed by nipagine (0.1 wt%), triethanolamine (1 wt%), and sodium metabisulfite (0.2 wt%) were dissolved in distilled water. The gel formulation was prepared with slight modification [14]. A 1.25 g CMC-Na, nipagin (0.1 wt%), 2.5 g glycerin, 1.25 g of propylene glycol were mixed in 125

mL of distilled water. Serum formulation was composed of diluted xanthan gum (0.5 wt%), methylparaben (0.18 wt%), and propylparaben (0.02 wt%) were mixed into ethanol and propylene glycol (15 wt%). Then, each cream gel, and serum formulation was added with 3, 6, and 9% of SCAE.

Animals

Male mice (*Mus Musculus*) strain Double Ditch Webster weights 20-25 g (6-8 weeks old) were selected and grouped into 10 groups (each group contained three mice). The mice were obtained from Balai Veteriner Medan which controlled under Institutional Animal Care and Use Committee (IACUC) guidelines of Indonesian Agency for Agricultural Research and Development. The animals were conditioned for a week for environmental conditions with ad libitum access to food and water. This study was approved by the Health Research Ethics Committee of Prima Indonesia University No 005/KEPK/UNPRI/I/2020.

Wound healing activity

To observe wound healing activity of sea cucumber extract, a previous method was followed with slight modification [15]. All of the experimental mice were anesthetized by local injection lidocaine hydrochloride (0.01 mL/100g BW). The hair on the dorsal side of mice was shaved and an excisional wound was inflicted (1 cm diameter and 0.2 cm depth). Then various concentrations of SCAE cream, gel, and serum (3%, 6%, and 9%) were applied to each group daily which was monitored for 14 days along with untreated group. For histopathology study, these mice were euthanized on the 15th day by inhalation of carbon dioxide followed by cervical dislocation [16]. The skin and subcutaneous tissue were dissected from the same wound area and incubated in 10% buffered formalin solution for 48 h. Each tissue was dehydrated in graded alcohol for 2 h and stained with hematoxylin and eosin according to standard procedure [15], [17].

Statistical analysis

All the results showed as mean $\pm$ Standard Error Mean. This study was measured by analysis of variance (ANOVA), followed by *Post-Hoc Duncan* and *LSD* tests. The values of $*P<0.05$ and $**P<0.005$ were considered to be statistically significant.

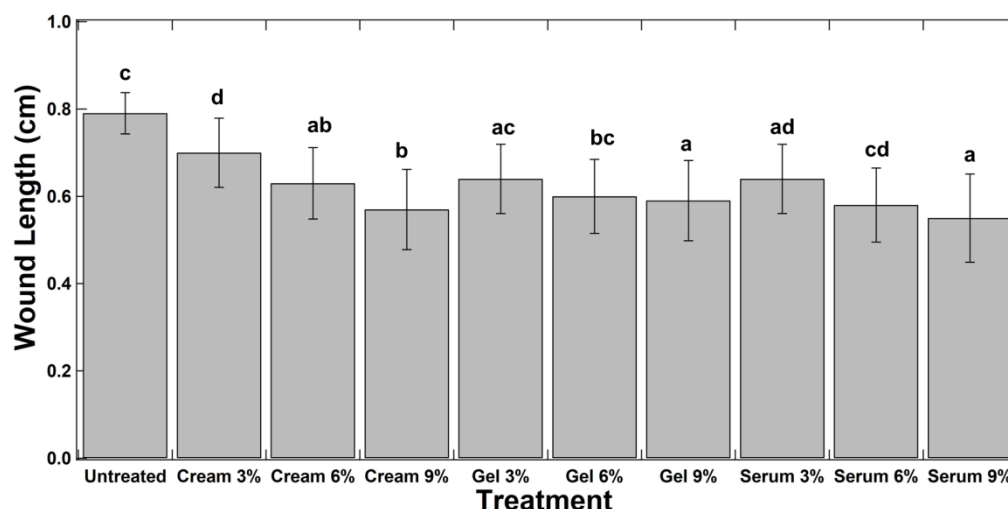


Figure 1. Reducing the length of the wound after cream, gel, and serum-SCAE treatments in 14 days on *Mus musculus*

RESULTS AND DISCUSSION

Phytochemical Screening

The result of phytochemical screening showed certain secondary metabolite compounds that present in SCAE as summarized in Table 1. The alkaloid tested by Mayer's and Dragendroff's reaction showed yellow-colored and orange precipitate, respectively.

Table. 1 Phytochemicals constituents of SCAE

Compounds	Result
Alkaloids	+
Flavonoids	+
Glycosides	+
Saponin	+
Steroid	+

+ = Present, - = Absent

Another test such as saponins exist with the appearance of stable foam after the reaction. Flavonoids were observed in the appearance of the orange color [18]. Molisch test showed the formation of a violet ring at the interface of the liquids as glycosides presence [19]. Steroids by Liebermann-Burchard reagent produce a greenish-blue color [20]. The crude extract of *A. mauritiana* contained several biocompounds that play an important role in the wound healing process. Flavonoids, part of phenolic compounds, known as best radical reducing power (e.g. DPPH and alkaloids) also have antioxidant activity and high anti-inflammatory

[21]. Antioxidants play a role in accelerating the wound healing process by preventing reactive oxygen species (ROS) from penetrating the wound area [22]. Glycoside acts as an inhibitor of the collagenase and elastase enzyme activity, thus accelerating the wound healing process [23]. Kim [24] found that saponins help re-epithelialize wounds and inhibit inflammatory reactions during the early phase. Steroids are believed to speed up wound healing [25].

Wound Healing

Figure 1 shows all formulations with different concentrations contributed to wound healing activities for 14 days. Serum 9%-SCAE treatment has significantly decreased the wound size 0.55 ± 0.32 cm and followed by cream 9%-SCAE 0.57 ± 0.29 cm, serum 6%-SCAE 0.58 ± 0.27 cm, and gel 9%-SCAE 0.59 ± 0.29 cm. The wound length reduction of three formulations showed significant improvement compared to the untreated group. The density level of fibroblast and collagen in the wounded areas listed in Table 2. As expected from the treatments, untreated wounds gave the least collagen level than the others. Interestingly, the dense level of collagen for each formulation shows different. The cream formulation has the lowest level of collagen, followed by gel, and serum. Serum formulation shows the best result in collagen amount in wound area which gives wound healing rate in serum formulation better than other formulations. Collagen dense levels in serum 3% -SCAE equivalent to 6% -SCAE gel and close to 9% -SCAE cream (Table 2).

Table 2. Wound reduction; a score of fibroblast and collagen dense level after received SCAE treatments.

Treatment	Length of wounds (cm)	Fibroblast	Collagen
Untreated	0.79±0.15 ^c	7.00±1.00 ^a	2.67±1.00 ^b
Cream 3%	0.70±0.25 ^d	6.33±0.58 ^a	4.67±0.58 ^c
Cream 6%	0.63±0.26 ^{ab}	7.33±0.58 ^b	9.00±0.58 ^a
Cream 9%	0.57±0.29 ^{ac}	9.00±0.00 ^c	9.33±1.00 ^{ab}
Gel 3%	0.64±0.25 ^d	6.33±0.58 ^a	6.00±0.58 ^d
Gel 6%	0.60±0.27 ^{cd}	8.67±0.58 ^d	8.67±0.58 ^a
Gel 9%	0.59±0.29 ^a	9.67±0.58 ^{ac}	10.33±0.58 ^a
Serum 3%	0.64±0.25 ^{ad}	6.67±0.00 ^a	8.67±0.58 ^{ac}
Serum 6%	0.58±0.27 ^{bc}	8.33±0.00 ^a	10.33±0.58 ^{ad}
Serum 9%	0.55±0.32 ^a	10.00±1.00 ^{ad}	10.67±0.00 ^a

Data are means ±SEM (n=3). ^{a-d}Within a column, means bearing different superscripts are significantly different (P<0.05).

According to Mazliadiyana et al. [26] and Chaphalkar et al. [27], the fatty acid compound in sea cucumber plays an important role in the wound healing process. It has been reported that *A. mauritiana* contains high oleic acid among other Holothurians [28]. Oleic acid has anti-inflammatory capability in cutaneous lesions closure [29]. In another study using different species, methanol extracts of *Holothuria atra* showed antioxidant, anti-inflammatory, and analgesic activities [30]. Visual observation in Figure 2 depicted the contribution of treatments on wound creation-evolution from zero to the 14th day. Histopathology test showed serum 9%-SCAE has the highest score of fibroblast and collagen with 10.00 ± 1.00 and 10.67 ± 0.00,

respectively (Table.2; Fig 3; Fig 4). It also showed a linear effect of SCAE concentration on wound size with incision length reduction. Fibroblasts play a vital role in the wound healing process that produces collagen during the tissue reconstruction process [31]. Fibroblasts have a contractile ability called myofibroblasts, which cause the wound edges to be drawn and closed [32]. The high dense fibroblasts induce collagen dense levels due to enhanced migration of fibroblasts in the treated area [33]. The proliferation occurred from the 7th to the 14th post-injury and the healing process will continue until the skin structure returns to normal.

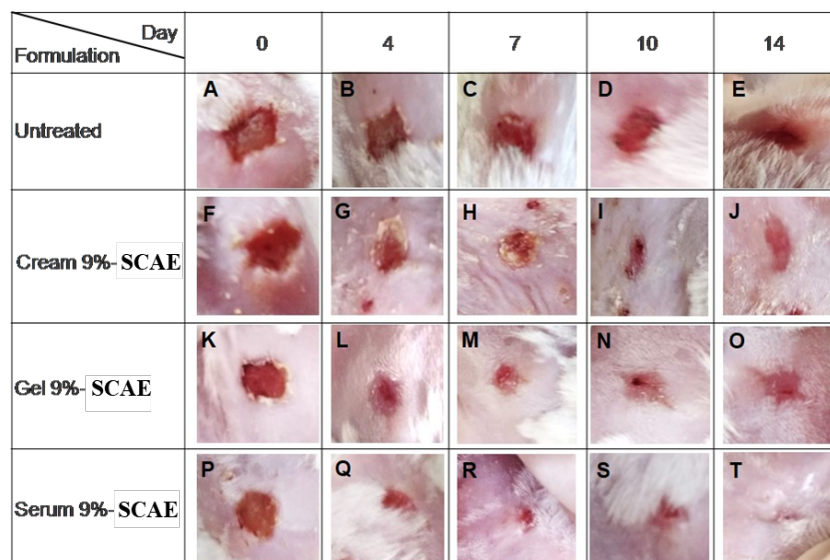


Figure 2. Photographic representation of wound healing from 0 to 14th day with SCAE treatment.

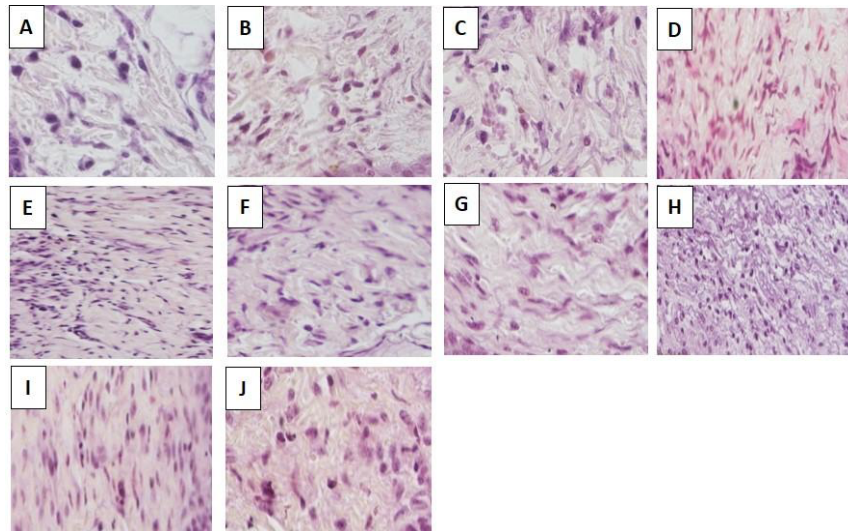


Figure 3. Microscopic images of fibroblast and collagen density at 400x magnification. (a) Untreated; (b) Cream 3%-SCAE ; (c) Cream 6%-SCAE; (d) Cream 9%-SCAE; (e) Gel 3%-SCAE; (f) Gel 6%-SCAE; (g) Gel 9%-SCAE;(h) Serum 3%-SCAE; (i) Serum 6%-SCAE; (j) Serum 9%-SCAE.

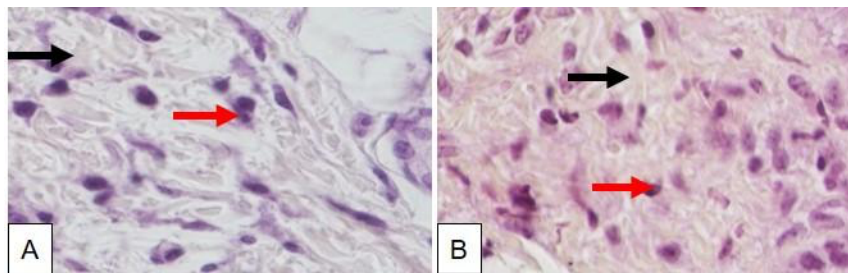


Figure 4. Density level of fibers (a.) Untreated; (b.) Serum9%- SCAE on the 14th day. The black arrow indicates collagen and the red arrow represent fibroblast.

Secondary metabolites such as steroids facilitated the anti-inflammatory effect [34], glycosides accelerated wound contraction [35], and flavonoids enhanced the number of fibroblasts [36]. Fibroblasts proliferation is stimulated by interleukin-1b (IL-1b), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF)[37]. Saponins increase fibroblast cell and macrophage number [38], [39] which is the major source of growth factors (FGF-2, GF, TGF- β) that contribute to proliferation [40]. TGF- β is charged in fibroblast activation and collagen deposition to form the epithelial layer. As a result, mucous epithelium and collagen layers were formed [41]. In addition, Bordbar et al [42] suggest that therapeutic effect of sea cucumber extract can be linked to the presence of bioactives such as saponins and glycosides as antimicrobial agents. It may contribute to inhibiting the secondary infections caused by bacteria and fungi in the wound.

CONCLUSION

A. Mauritiana is rich in secondary metabolites such as alkaloids, flavonoids, glycosides, saponins, and steroids. The result shows that SCAE has significant wound healing activity on mice particularly in serum formulations. The secondary metabolites as bioactive compounds could be the reason for the wound healing activity of SCAE. However further studies need to be carried out to isolate and investigate the efficacy of all identified compounds and also the application for human used.

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