ORIGINAL ARTICLE

Ethanolic extract of *Gracilaria* spp. Attenuates the inflammatory stage of oral mucosa wound healing: An *in vivo* study

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ABSTRACT

Millions of bacteria present in the mouth cavity contribute to the challenging management of oral mucosa injury. On the other hand, *Gracilaria* spp. (red algae) is one of the widely cultivated algae that have a strong potential as a wound-healing agent for oral mucosa injury. This study aimed to investigate the wound-healing property of the red algae by observing its effect on polymorphonuclear (PMN), a neutrophil that is usually recruited during the initial wound healing. The extract was obtained through maceration and used as bioactive ingredient in gel preparation. *Rattus norvegicus* with incision wounds in the oral mucosa was used as the animal model. Our results revealed that rats treated with the red algae gel had significantly lower PMN on the injury site ($P < 0.01$) as observed on days 1, 3, and 5. Identification using gas chromatography–mass spectrometry showed that the extract was rich in hexadecenoic acid and glycerol. The brine shrimp lethality test suggested low cytotoxicity of this extract with LC_{50} = 10694.93 mg/mL. In conclusion, the extract could be potentially used as bioactive ingredient in gel formulation for topical management of oral mucosa wounds. Further, research to confirm these findings is warranted.

Key words: Glycerol, hexadecenoic acid, polymorphonuclear, red algae, wound healing

INTRODUCTION

Oral mucosa injury might pose a greater risk of infection because of the location susceptability to bacterial and other pathogenic infections. Injuries to the oral mucosa can result from both physical and chemical sources, as well as mechanical trauma. Wound healing is defined as the interaction of cellular and metabolic responses to restore tissue integrity and function following an injury. Effective wound healing within the oral mucosa is crucial in preventing the infiltration of bacteria and other pathogenic microorganisms into the tissue, thus mitigating the potential for chronic tissue damage resulting from trauma and infection.[1-3]

There are three phases involved during the wound healing process, namely inflammation, proliferation, and lastly, maturation. The inflammatory phase of wound healing begins with the occurrence of hemostasis, which is characterized by narrowing blood vessels and the formation of fibrin clots. This phase is governed by different

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growth factors and cytokines, as well as the involvement of polymorphonuclear (PMN) and monocytes in the chemotaxis process.[4] PMNs absorb bacteria and foreign objects, and monocytes differentiate into macrophages to phagocytize necrotic tissue and foreign particles, after which macrophages produce growth factors that encourage granulation tissue creation.[4] Millions of microorganisms that colonize the mouth cavity pose a hurdle to wound healing.[1]

Red algae, *Gracilaria* sp., are well-known for their considerable commercial importance and as an agarophyte and food. Polysaccharides, fatty acids, amino acids, and phytochemicals, such as alkaloids, flavonoids, tannins, steroids, triterpenoids, saponins, and phenol, are all found in red algae.[5-7] These components suggest the potential of red algae in accelerating wound healing because it has activity as antibacterial and anti-inflammatory agent.[8] However, none of the reported studies evaluate the algae effect on PMN.[9,10] Thus, the study reported herein had the objective to examine *in vivo* the activity of red algae on wound healing by observing the cell viability of PMN.

MATERIALS AND METHODS

Red algae collection and identification

Red algae were collected from Karawang, West Java, Indonesia, using a purposive random sampling technique. Taxonomic identification carried out at the herbarium laboratory in Indonesian National Research and Innovation Agency revealed the red algae as *Glacilaria* sp. (No. B-896/V/DI.05.07/3/2022).

Extraction and phytochemical analysis

The air-dried red algae sample was crushed into fine powder and subsequently immersed in ethanol 96% in a sealed container for maceration before left for 24 h. Thereafter, the filtrate was collected while the residue was re-macerated twice using the same procedure. The filtrate obtained from each maceration was combined and concentrated with rotary evaporator. A phytochemical test was performed on the resultant extract through qualitative analysis for the detection of alkaloids, phenols, flavonoids, terpenoids, saponins, tannins, and steroids, as suggested by a previous study.[11] A quantitative phytochemical test was also carried out on the extract for total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC) using colorimetric techniques that have been described in detail previously.[12,13] The absorbance was measured on ultraviolet (UV) visible spectrometer (Shimadzu UVmini-1240, Kyoto, Japan).

Identification of phytocompound

Gas chromatography–mass spectrometry (GC-MS) was used to identify the components of the ethanol extract from *Gracilaria* spp. The column (SPB-50) length was 30 m with 0.25 film thickness and 0.25 mm inside diameter. The injection temperature was set at 230°C, while the interface temperature was set at 250°C. The temperature of the ion source was fixed at 200°C. The carrier gas used was helium, and the flow rate was kept constant at 1 mL/min. Compound identification was based on the comparison of mass spectrum data with the National Centre for Biotechnology Information database.

Gel preparation

The preparation of topical gel followed our previous study.[14] Red algae extract was dissolved in water so that the concentration reached 5% and added with Na-carboxymethyl cellulose (3 g) before stirred at 100°C and 400 rpm until the mixture was homogeneous and mucilage formed. Thereafter, the mixture was added with 3 g glycerin, 1.5 propylene glycol, and 30 g distilled water while stirring for another 15 min. The gel was allowed to cool and stored in the vial. For control, the gel was prepared with the addition of red algae extract.

Animal and ethical clearance

Before the animal experiment was carried out, the protocol has been approved by the Ethics Commission of the Faculty of Dentistry, Universitas Syiah Kuala (227/KE/FKG/2020). A total of 10 male Wistar rats weighed 200–250 g, aged 2–3 months, and being in apparently healthy condition were procured from the Faculty of Veterinary, Universitas Syiah Kuala. The animal was first acclimated for 7 days (12-h day/night cycle) at room temperature and fed with standard feed *ad libitum*. The animal was evenly divided into treatment (n = 5) and control groups (n = 5) with randomization carried out using web-based-free software.

Oral mucosa incision

To induce anesthesia and produce sedation, intramuscular injections of 0.5 mL of xylazine hydrochloride and 1 mL of ketamine hydrochloride were administered to the animal. Subsequently, the incision with a dimension of 11 mm × 5 mm × 2 mm (length × width × depth) was made on the oral mucosa.

Treatment and observation

Red algae extract gel was applied to the rats in the treatment group using sterile cotton bud to the wound area and hold still for 1 min. The gel administration was performed twice a day (09.00 and 17.00 o'clock). The control group was treated with gel that did not contain the red algae extract. Observation was carried out on days 1, 3, and 5, where the rats were euthanized through cervical dislocation under anesthesia. The mucosal tissue was collected and prepared for histological slides. Histological examination was carried out on the PMN cells on Olympus XC10 series microscope with ×400.

Brine shrimp lethality test

The cytotoxicity was assessed with Brine Shrimp Lethality Test (BSLT) because its growth mimics cellular proliferation, though careful interpretation is required as the method might not be representative to mammalian physiology.[15] Dissolved ethanolic extract from *Gracilaria* spp. was prepared with a concentration variation of 1000–50 mg/L by adding dimethyl sulfoxide 5%. Each concentration was then added to a small container where larvae of *Arthemia salina* L. were present (n = 10), before being incubated for 24 h. Larvae exposed with saline water were taken as control. The results were then expressed as median lethality concentration (LC_{50}), obtained from linear regression.

Data analysis

Homogeneity was tested based on Shapiro–Wilk equation. All treated groups were compared to control when analyzed with statistical analysis, namely, analysis of variance with Tukey *post hoc*. P value of lower than 0.05 indicates statistical significance. SPSS v. 24.0 (SPSS Inc., Chicago, IL, USA) was utilized when handling the statistical analysis.

Table 1: Results from qualitative phytochemical test on the red algae extract

Metabolite	Remark
Alkaloid	+
Steroid	+
Triterpenoid	+
Saponin	−
Flavonoid	+
Phenolic	+
Tannin	+

(+) present, (−) not present

Table 2: The total phenolic, flavonoid, and tannin contents of the red alga extract

Variable	Value	Percentage
Flavonoid total (mgQE/g extract)	1.0	0.01
Gallic acid total (mgGAE/g extract)	17.39	0.069
Tannic acid total (mgTAE/g extract)	42	0.42

RESULTS AND DISCUSSION

Phytochemical content

Alkaloids, steroids, triterpenoids, flavonoids, phenolics, and tannins were found in the extract as suggested by the phytochemical test presented in Table 1. The extract has TFC, TPC, and TTC of 1.0 mgQE/g extract, 17.39 mgGAE/g extract, and 42 mgTAE/g extract, respectively [Table 2]. Based on the relative abundance in the GC-MS results, the red algae extract is predominated by glycerol (36.81%) and hexadecenoic acid (20.74) [Table 3]. Most of the compounds are fatty acids including hexadecenoic acid; 2-hydroxy-1-(hydroxymethyl) ethyl palmitate; 2-hydroxy-1-(palmitoxymethyl) ethyl palmitate; (9e)-9-octadecanoic acid; and (9e)-9-octadecanoic acid. A diterpene, phytol, is also identified in the extract with relative abundance of 1.51% [Table 3]. The phytochemical profile supports the wound-healing properties of the extract, such as glycerol and hexadecenoic acid.[16-18]

Infiltration of PMN leukocytes is observed on day 1 suggesting the initiation of inflammatory response, in which this activity is further reduced on day 3 [Figure 1]. Quantitatively, the reduction of PMN cells is observed on day 1, day 3, and day 5 with statistical significance at $P < 0.01$ [Figure 2]. Activation of the immune system following the injury causes inflammation and recruitment of immune cells, including PMN cells, to the wound location. This inflammatory cascade is a crucial stage in wound healing that facilitates the phagocytosis of the debris and opportunistic pathogens.[19,20] Nonetheless, excessive neutrophil activity may cause chronic inflammation and imbalance of oxidative stress concomitant to the

Table 3: Phytocompounds in red algae extract identified by the gas chromatography–mass spectrometry

Phytochemicals	Retention (min)	Similarity (%)	Relative abundance (%)
Glycerol	10.39	83	36.81
Hexadecenoic acid	28.82	99	20.74
Cholesterol	36.00	99	7.40
2-Hydroxy-1-(hydroxymethyl) ethyl palmitate	31.28	38	4.39
2-Hydroxy-1-(palmitoxymethyl) ethyl palmitate	31.49	70	4.38
(9e)-9-Octadecanoic acid	29.79	99	4.35
E, e-10,12-hexadecadien-1-ol acetat	30.60	96	3.27
Glycerol tricaprilate	35.70	50	2.20
Sarcosine, n-(1-naphthoyl_-octyl ester	38.25	59	2.17
Phytol	39.42	76	1.51
(9e)-9-Octadecanoic acid	32.17	92	1.38
Beta-d-glucopyranose, 1,6-anhydro	18.79	53	1.13
2-aminoethanethiol hydrogen sulfate (ester)	30.04	96	1.00

Table 4: Results from the Brine Shrimp Lethality Test on red algae extract

Concentration (mg/L)	Log concentration	Mean larva died	Mortality (%)	Probit	Linear equation	LC_{50} (mg/L)
1000	3.0	9.3	93.3	6.5	y=0.77x+1.91; R^2=0.89	10,694.93
500	2.7	1.3	13.3	3.9		
250	2.4	1.3	13.3	3.9		
100	2.0	0.7	6.7	3.5		
50	1.7	0.7	3.3	3.1		

LC: Lethality concentration

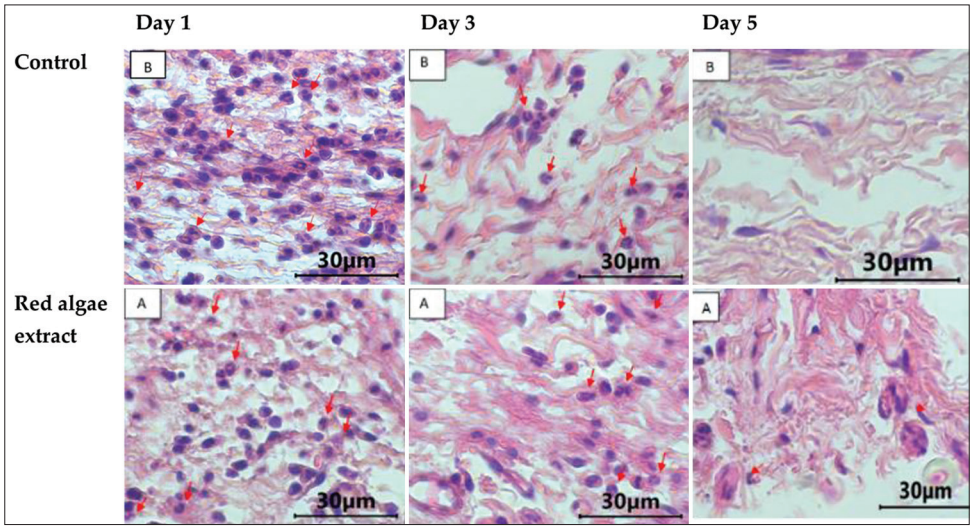

Figure 1: Histopathological images of polymorphonuclears cell counts as observed on day 1, 3, and day 5, in control and in sample treated with red algae extract

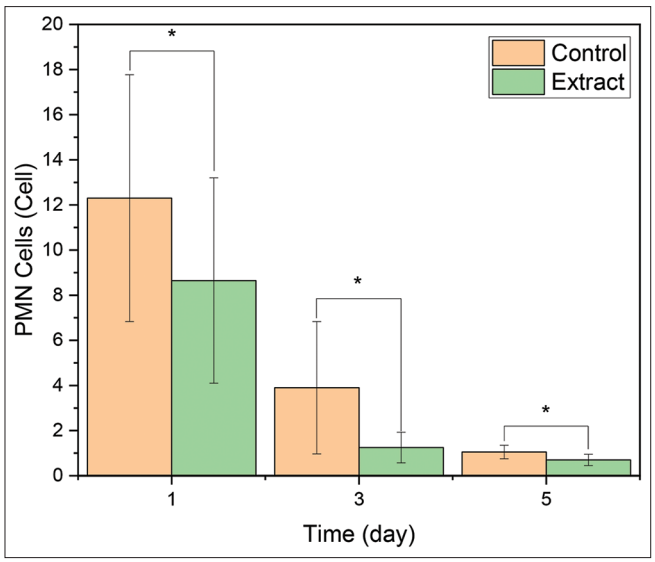

Figure 2: Polymorphonuclear cell count as observed on day 1, 3, and day 5, in control (a) and in the sample treated with red algae extract (b). *Significant at $P < 0.01$ based on Tukey *post hoc*

release of reactive oxygen species by neutrophils which could damage the healthy surrounding tissue. Herein, throughout the observation, the number of PMN cells remained higher in control as compared to the sample treated with red algae extract. This could be attributed to the activity of hexadecenoic acid predominantly contained in the extract which could prevent PMN migration. A study suggested that hexadecenoic acid may disrupt phosphoinositide 3-kinase activation or its downstream signaling events.[21]

It is worth noting that the number of PMN recruited to the wound site corresponds well to bacterial compounds such as lipopolysaccharides and formyl methionyl peptides.[22] Therefore, we speculated that the reduced of bacteria viability deriving from the red algae extract exposure lead to lower PMN. This is corroborated by the phytochemical profile and the content of flavonoids, phenols, and tannins which have antibacterial potential.[8,23]

Brine shrimp lethality test-based cytotoxicity

The results from BSLT on red algae extract are presented in Table 4. The LC_{50} was found to be 10694.93 mg/L estimated from a linear regression $y = 0.77x + 1.91$ ($R^2 = 0.89$). The value suggested that the red algae extract was noncytotoxic.[15] Moreover, the value is distantly lower than other previously reported medicinal plant extracts.[24,25] This might suggest that the reduction of PMN is not because of the extract cytotoxicity, but rather because of the cell migration inhibition. Regardless, analysis using more standardized protocol for cytotoxicity is still required.[26]

CONCLUSION

Findings from this study suggest that the *Gracilaria* spp. cultivated in Karawang, Indonesia has the ability to facilitate the wound healing process in mucosal tissue. Such bioactivity might be derived from the rich content of fatty acid, namely hexadecenoic acid that could attenuate the prolonged inflammation by inhibiting the migration of PMN.

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Conflicts of interest
There are no conflicts of interest.

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