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Research Article
Anti-inflammatory Effect of Red Macroalgae Bulung Sangu (*Gracilaria* sp.) Extract in UVB-Irradiated Mice

[1]Maria Malida Vernandes Sasadara, [1]I. Gede Putu Wirawan, [2]I. Made Jawi, [1]Made Sritamin, [2]Ni Nyoman Ayu Dewi and [3]Anak Agung Ayu Mirah Adi

[1]Faculty of Agriculture, Udayana University, Bali, Indonesia
[2]Faculty of Medicine, Udayana University, Bali, Indonesia
[3]Faculty of Veterinary Medicine, Udayana University, Bali, Indonesia

Abstract

Background and Objective: Bulung Sangu, like many other macroalgae, is a source of beneficial phytochemical for health. This study was aimed to determine the anti-inflammatory effect of bulung sangu ethanol extract cream. **Materials and Methods:** The compounds of bulung sangu ethanol extract were identified by using gas chromatography. The antioxidant activity of the extract was examined by the DPPH assay. The anti-inflammatory effect was analyzed *in vivo* against ultraviolet B (UVB) induction through variables of epidermal thickening and epidermal erosion scores. **Results:** Our results showed that bulung sangu ethanol extract contained 18 compounds, in which, 11 compounds considered active as antioxidant and/or anti-inflammatory. Cream extract in both concentrations showed scavenging for more than 50%, with a concentration of 10% cream extract exhibited higher antioxidant activity compared to 5%. The *in vivo* assay showed a reduction of epidermal thickness and epidermal erosion in the application of both concentrations. The concentration of 10% cream extract showed higher reduction compared to 5% with results produced resembling normal. **Conclusion:** It can be concluded that bulung sangu displayed a potential source for being developed for the health and medicine aspect, especially for various activities supported by antioxidants and anti-inflammatory.

Key words: Antioxidant, anti-inflammatory, bulung sangu, Gracilaria, photoprotection, photoaging skin, UV-B

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Corresponding Author: I. Gede Putu Wirawan, Faculty of Agriculture, Udayana University, Bali, Indonesia

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Bulung sangu, a member of the *Gracilaria* genus, is a red microalga widespread in Bali. Bulung sangu (*Gracilaria* sp.) is local Balinese seaweed usually consumed as vegetables. It is not cultivated but grows wildly around the waters especially in South Bali such as Serangan Islands and Sawangan, Nusa Dua.

Bulung sangu, like many other macroalgae, is a source of beneficial phytochemical for health. Macroalgae is a source of important bioactive metabolites for drug development. Many macroalgae compounds are used to treat diseases like cancer, inflammation, pain, arthritis, Acquired Immune Deficiency Syndrome (AIDS) as well as bacterial, fungal and viral infection[1]. Macroalgae is rich in long-chained polyunsaturated fatty acids including eicosapentaenoic. Macroalgae also contain organic compounds classified as polar phenols, phenol derivates such as phlorotannins and phloroglucinols and non-polar phenols such as sterols, triterpenes tocopherols and pigments[2].

In our previous study, it was found that the ethanol extract of bulung sangu contains carotenoids and chlorophyll pigments[3]. Bulung sangu also possesses antioxidant and anti-cholesterol effect[4,5]. This potential activity can be further explored to assess its activity as other pharmacological agents such as anti-inflammatory agents, considering that one of the anti-inflammatory mechanisms is through antioxidant activity. Several studies also reported the effect of *Gracilaria* sp. as an anti-inflammatory agent[6-8].

Inflammation is an immune system response to harmful stimulation such as pathogens, cell damage, toxic compounds, or radiation[9]. In these conditions, the body will eliminate harmful stimulants and initiate the recovery process[10]. Inflammation can arise from physical stimulation such as tremors and radiation exposure, chemical stimulation such as toxins and chemicals, as well as biologics such as cell damage received[11]. Inflammation is pathogenesis in many chronic diseases such as cardiovascular disorders and bowel disease, diabetes, arthritis and cancer. Inflammation can be initiated by free radicals that will induce oxidative stress. Oxidative stress is pathogenesis in several diseases such as cardiovascular disease, cancer, diabetes, hypertension, aging and atherosclerosis[12].

The UV radiation is one of inflammation stimulation. UV radiation has been known as one of the causes of various skin disorders, including cancer, due to its ability to induce DNA mutation. The UV radiation is the most likely risk factor to be modified in several diseases such as skin cancer and other skin disorders influenced by the environment. However, UV light is also needed for the human body to mediate the natural synthesis of vitamin D and endorphins in the skin. High UV exposure can affect skin health, causing atrophy, pigment changes, wrinkles and malignancy[13].

Prompted by these data we identify chemical compounds in bulung sangu ethanol extract followed by formulation in a topical preparation. It was also evaluated the *in vitro* antioxidant capacity of the extract using the DPPH assay. Importantly, we investigated the *in vivo* anti-inflammatory effect of bulung sangu ethanol extract cream using UVB-induced skin inflammation. The effect of inflammation response was observed from epidermal thickening and epidermal erosion parameters.

MATERIALS AND METHODS

Study area: The research was carried out in July 2019-April 2020 on the beach of Serangan Island and in the Central Laboratory of Genetic Resources, Udayana University, Bali, Indonesia.

Macroalgae material and preparation of the extract: Fresh bulung sangu was collected from Serangan Island, Bali. The sample was cleaned from impurities and washed with running water, then chopped to reduce the size. The chopped sample was then dried in an oven at 40°C for 3-7 days until a constant weight was obtained. Extraction of the bioactive algal extract has been carried out as follows: the finely powdered algal material (750 g) was macerated by 96% ethanol (1.5 L) at room temperature for 3 days with regular shaking. After filtration, the organic solvent was evaporated under vacuum at 50°C to furnish dry ethanol extract. The crude extract was then stored at -20°C.

Gas chromatography: Gas chromatography analysis was performed using Agilent 7890B MSD 5977B, with Wakosil ODS/5C18-200 silica column with a size of 4.6×200 mm. The injector temperature was set at 290°C for 27 min. N2 was used as carrier gas at a constant flow of 1.0 mL min^{-1}. A volume of 2 µL was injected in the splitless mode and the purge time was 1 min.

Formulation of cream: Bulung sangu ethanol extract cream (BSC) formulated in o/w cream type. The cream formula is presented in Table 1. The cream used 5 and 10% Bulung sangu ethanol extract of the total weight of the cream. The oil phase was heated to 70°C, while the water phase was heated to 60°C. The water phase was added to the oil phase and stirred

Table 1: Cream formula

Oil phase (%)	Water phase (%)
Vaseline album 6.2	Triethanolamine 0.2
Mineral oil 13.8	Xanthan gum 0.2
Isopropyl myristate 1.5	Nipagin 0.01
Stearic acid 7.5	Extract 5/10
Glycerol monostearate 5	Aquadest ad 100
Nipasol 0.05	

constantly counterclockwise. The extract was dissolved in aqua dest at 35 then added to the mixture of the oil phase and water phase. Aqua dest was added to reach 100% of the formula weight.

Measurement of antioxidant activity: Antioxidant activity was measured using the DPPH assay. The standard curve was made by using DPPH solutions in series concentrations of 0, 2, 4, 8 and 12 mg L^{-1}. About 0.5 mg of each cream was diluted in 1 mL methanol. An amount of 1.5 mL of test solution was added with 1 mL of DPPH solution in methanol, then incubated for 30 min. The absorbance of the solution was read with a UV-Vis spectrophotometer at a wavelength of 517 nm. Methanol was used as the control.

The percentage of antioxidant activity was calculated by the formula:

$$\text{Antioxidant activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where, Control absorbance ($A_{control}$) is absorbance of methanol in DPPH solution[14].

Animal's experiments: Total 32 BALB/C mice weighting 20-25 g were obtained from Integrated Biomedical Laboratory, Faculty of Medicine, Udayana University were then placed under controlled environmental conditions: temperature of 22±2°C, 55±5% relative humidity and a constant light/dark cycle. A standard diet with pellets and water were supplied *ad libitum*. All experiments were performed under the Animal Ethics Committees of Veterinary Medicine Faculty, Udayana University.

Mice were divided into four groups: the control group (without UVB radiation and treatment/KN), the mock-treated group (KP) received UVB radiation without treatment of extract cream, the 5% BSC-treated group (K5) received both UVB radiation and treatment of 5% BSC and the 10% BSC-treated group (K10) received both UVB radiation and treatment of 10% BSC. Protocol for UVB radiation referred to Wiraguna *et al.*[15].

Examination of anti-inflammatory effect: Histological preparation referred to Wiraguna *et al.*[15] with modification. The tissue was sliced into 5 µm thick and stained with hematoxylin-eosin (HE). Inflammatory responses were observed through two variables of histopathological lesion of epidermal: epidermal thickening and epidermal erosion scores. Epidermis thickness was digitally calculated as micrometers (µm), under a microscope at 400 times magnification. Epidermal erosion was scored referring to Sari *et al.*[16] with modification. Score 0-1 referred to epidermal erosion less than or equal to 1% ($x \leq 1\%$), score 1.1-2 referred to epidermal erosion more than 1% to less than or equal to 25% ($1\% < \times \leq 25\%$), score 2.1-3 referred to epidermal erosion more than 25% to less than or equal to 50% ($25\% < \times \leq 50\%$), score 3.1-4 referred to epidermal erosion more than 50% to less than or equal to 75% ($50\% < \times \leq 75\%$) and score 4.1-5 referred to epidermal erosion more than 75% to less than or equal to 100% ($75\% < \times \leq 100\%$).

Statistical analysis: Data of epidermal thickening was analyzed by using One-Way ANOVA followed by Post Hoc Test Fisher's Least Significant Difference (LDS). Data of epidermal erosion score was analyzed using Kruskal-Wallis followed by Mann-Whitney Post Hoc Test. Data on antioxidant activity was analyzed using an unpaired t-test. All statistical analyzes used 95% confidence level.

RESULTS

Chromatography analysis: The GC chromatogram analysis of the ethanolic extract of bulung sangu (Fig. 1) showed eighteen peaks, which indicated the presence of phytochemicals constituents.

GC of the eighteen compounds identified, the most prevailing compounds were n-hexadecanoic acid (16.884 48.220%), [1,2,4]triazolol[4,3-a]quinolone (16.380%) and phytol (7.630%). Information on compound names, retention time (Rt) and the area under the curve (AUC) as shown in Table 2, sorted from the highest to the lowest AUC. Bulung sangu extract contains phenolic, terpenoids, alkaloids and fatty acids besides other compounds. Bulung sangu extract contained n-hexadecanoic acid or palmitic acid as the most abundant compound followed by [1,2,4]triazolol[4,3-a]quinolone and phytol.

***In vitro* antioxidant activity assay:** The bulung sangu ethanol extract cream (BSC) both in 5 and 10% concentrations

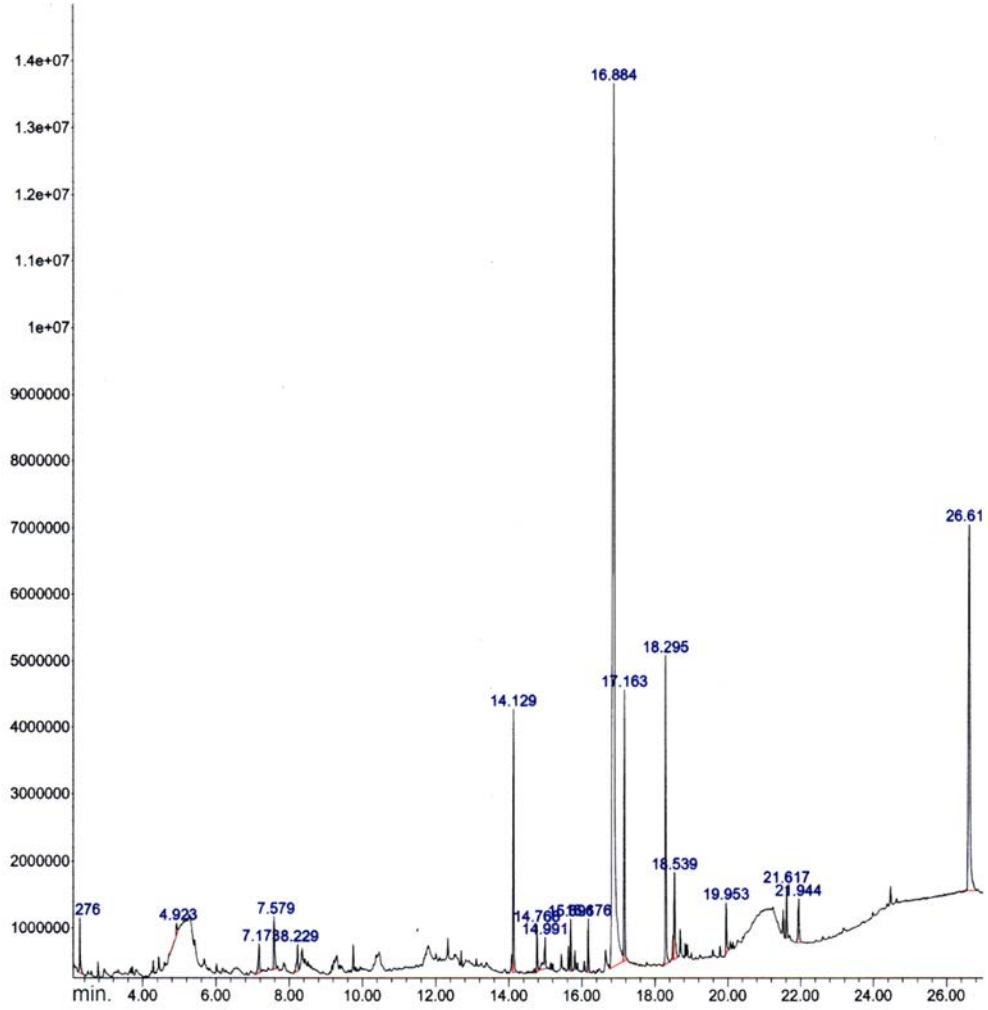

Fig. 1: Chromatogram of bulung sangu ethanol extract, The highest peak (RT 16.884) indicating the presence of n-hexadecanoic acid as the most abundant compound of the ethanolic extract of bulung sangu

Table 2: The compounds produced from the gas chromatography analysis of bulung sangu ethanol extract

Rt (min.)	AUC (%)	Compound
16.884	48.220	n-Hexadecanoic acid
26.618	16.380	[1,2,4]triazolo[4,3-a]quinoline
18.295	7.630	Phytol
17.163	6.260	Hexadecanoic acid, ethyl ester
14.129	5.830	Heptadecane
18.539	2.550	Oleic acid
7.579	1.600	Homopiperazine, Arginine
21.944	1.330	Phenylephrine
21.617	1.260	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
14.991	1.260	6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuren-2(4H)-one
2.276	1.170	Hexanal
19.953	1.140	Arachidonic acid
16.176	1.100	N-(6-Methyl-8-oxo-6,7,8,9-tetrahydro-5-oxa-9-azabenzocyclohepten-3-y-1)acetamide
15.696	1.100	6,10,14-trimethyl-2-Pentadecanone
14.768	1.030	Tetradecanoic acid
7.173	0.980	Benzoic Acid
4.923	0.250	Glycerin
8.229	0.083	1H-Pyrrole-2,5-dione,3-ethyl-4-methyl

Rt: Retention time, AUC: Area under curve

showed antioxidant activity of 55.50±17.52% and 55.58±17.56%, respectively (Table 3). Either 5 or 10% BSC produced inhibition activity more than 50%, but the mean difference between the two concentrations is not high or about 0.08%. Statistically, there was a significant effect of 5 and 10% BSC treatment on antioxidant activity.

Anti-inflammatory effect of BSC: Histopathological images of mice skin section showed epidermal thickening (Fig. 2) and epidermal erosion (Fig. 3). Normal condition without UV radiation or extract cream treatment resulted in the epidermal thickness of 661.17±24.892 µm as shown in the control group (KN), while radiated condition without any protection treatment resulted in 1148.56±136.749 µm of epidermal thickening as shown in the mock-treated group (KP) (Table 3). Histological images visually showed differences in the epidermal thickness of control (Fig. 2a) and mock-treated group (Fig. 2b). UV Radiation-induced damaged reaching 73%

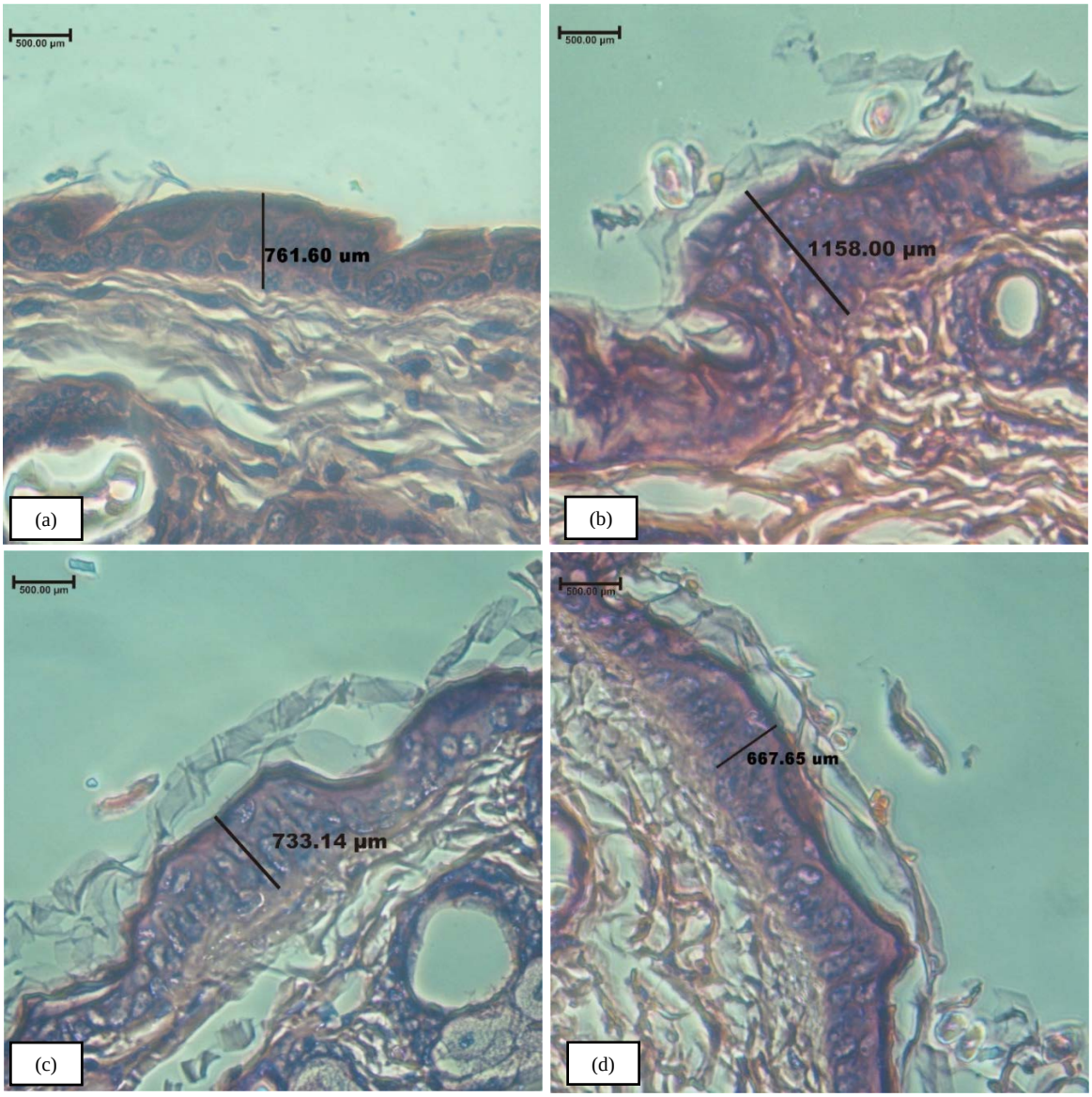

Fig. 2(a-d): The UVB irradiation on the dorsal skin of mice causes an increase in epidermal thickness and the application of BSC can decrease the epidermal thickness

(a) The control group showed normal epidermal skin, (b) the UVB exposure showed severe epidermal thickness, (c) the UVB exposure with topical application of BSC 5% and (d) the UVB exposure with topical application of BSC 10% showed decreased of the epidermal thickness (HE stained, magnification 400×, epidermal thickness are indicated in each figure)

in the mock-treated group. Treatment of 5 and 10% (Fig. 2c-d) BSC produced epidermal thickening of 736.07±39.032 and 676.88±16.593 μm, respectively (Table 3). These results statistically similar to the control group, indicating the protection activity of 5% and 10% BSC protect epidermal skin to resemble normal conditions.

Table 3: Percentage of Antioxidant Activity, epidermal thickening and epidermal erosion observed in all animal groups

Experimental group	Antioxidant activity (%)	Epidermal thickening (Mean±SD)	Epidermal erosion (Mean±SD)
Control group (KN)	-	661.17±24.892	0.11±0.035
Mock-treated group (KP)	-	1148.56±136.749**	2.93±0.212**
5% BSC-treated group (K5)	55.50±17.52	736.07±39.032*	1.49±0.084**
10% BSC-treated group (K10)	55.58±17.56	676.88±16.593*	1.21±0.125**

Values are expressed in Mean±SD, *p>0.05 compared with control group (KN), **p<0.05 compared with control group (KN)

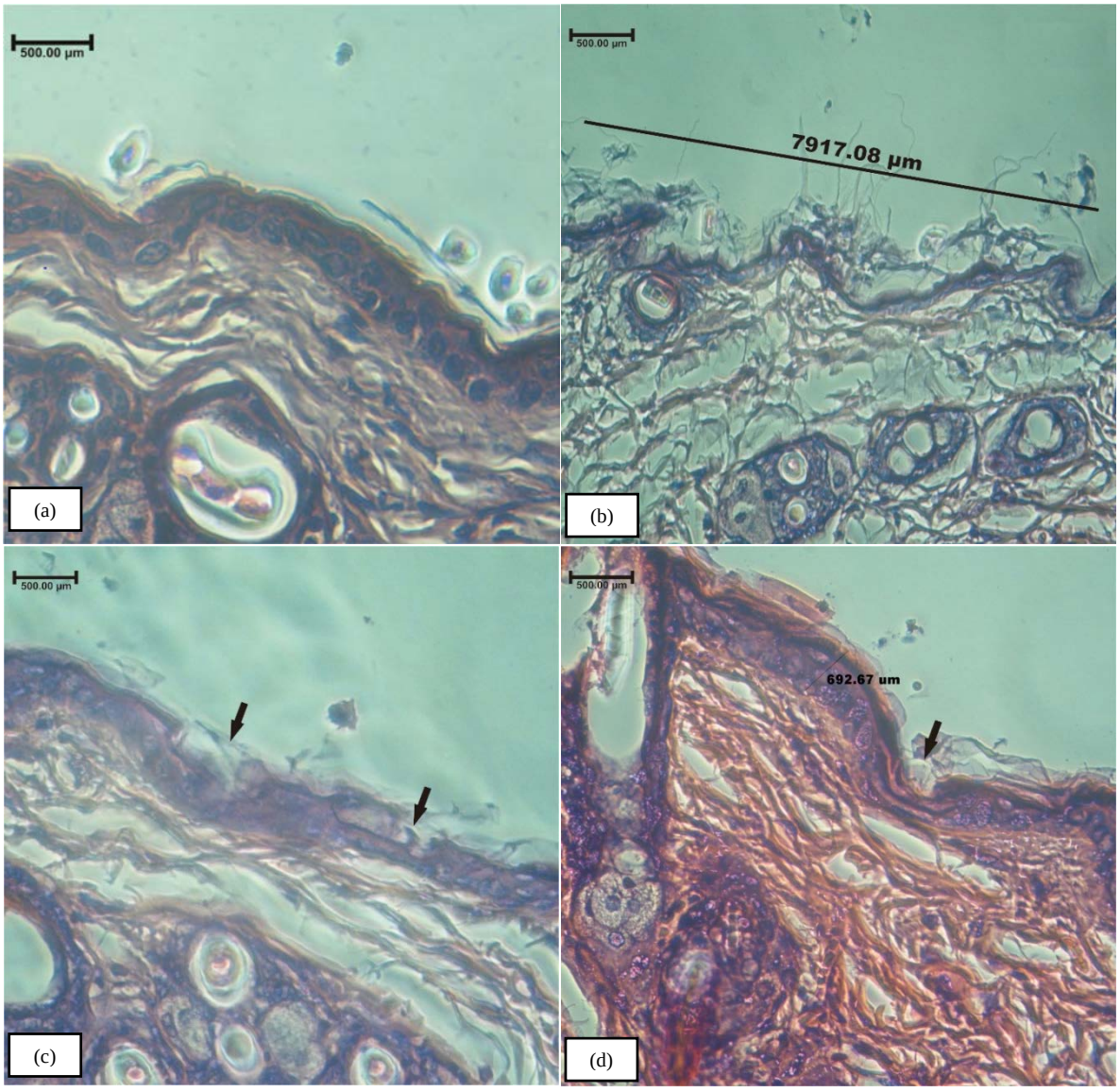

Fig. 3(a-d): The topical application of BSC cream in mice can reduce the severity of epidermal erosion due to UVB irradiation
(a) Control group with an intact epidermal layer, (b) UVB irradiation with severe epidermal erosion, (c) UVB exposure with topical application of BSC 5%, (d) UVB exposure with topical application of BSC 10% showed less severe epidermal erosion, (HE stained; magnification 400×, the severity of the epidermal erosion area is pointed at each figure)

Similar to epidermal thickening, the epidermal erosion parameter indicating the potential anti-inflammatory activity of BSC. The control group (Fig. 3a) showed a healthy condition without severe epidermal erosion with an epidermal erosion score of 0.11±0.035 (Table 3). On the contrary, the mock-treated group (Fig. 3b) showed severe epidermal erosion reaching 64% with an epidermal erosion score of 2.93±0.212 (Table 3). Treatment of either 5 or 10% (Fig. 3c-d) of BSC resulted in protection with a small amount of multifocal erosion with an epidermal erosion score of 1.49±0.084 and 1.21±0.125 (Table 3) and as shown in histopathological images. Application of 5 and 10% of BSC cream exhibit protection in epidermal erosion, even though the results statistically different to the control group (KN).

DISCUSSION

Chromatography analysis exhibited that bulung sangu ethanol extract contained bioactive compounds with potential therapeutic interest. Bulung sangu contained several classes of beneficial constituents which reported for exhibit antioxidant and/or anti-inflammatory response such as phenolic, terpenoid, alkaloid and fatty acid compounds, as also reported in other species of macroalgae[1,2,17-19]. Red macroalga of the *Gracilaria* genus reported for showing antioxidant properties[20-24]. Nine out of eighteen compounds detected in bulung sangu extract reported for having antioxidant properties in various studies, such as n-hexadecanoic acid[25,26]. [1,2,4]triazolo[4,3-a]quinolone[27,28], phytol[29], hexadecanoic acid, ethyl ester[25,26], heptadecane[30], 6-Hydroxy-4,4,7a-trimethyl- 5,6,7,7a-tetrahydro benzofuran-2(4H)-one[29], Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester[26], benzoic acid[31] and glycerin[32].

Ultraviolet (UV) radiation is one of the factors initiating the inflammatory response and DNA damage and induces several cutaneous lesions such as photo-aging and photo-carcinogenesis[33]. The thickening of the epidermis as a result of the proliferation of the epidermal layer is one of the inflammatory responses due to UVB exposure[13,34]. It seems that in more severe situations, this thickening is followed by necrosis to the erosion of the epidermal layer, as shown in the untreated group (Fig. 3b). UV radiation produces various reactive oxygen species (ROS) that are then converted to free radicals and initiating inflammation response[35], through inducing peroxidation contributing to an increase of phospholipase activity[36].

Marine algae are important sources of active compounds and new chemical substances, which are recognized as having a number of biological activities, including anti-inflammatory[37]. Bulung sangu showed effect as an anti-inflammatory against UV radiation as shown that the application of bulung sangu cream extract topically protects skin epidermal from thickness and erosion. The presence of n-hexadecanoic acid, as the most abundant compound in bulung sangu ethanol extract, may be responsible for the anti-inflammatory properties found in the extract through the inhibition of phospholipase A2 (PLA2)[38]. Moreover, bulung sangu as other *Gracilaria* reported for showing anti-oxidative activity[20-24], in which this activity may due to the presence of eight active compounds in bulung sangu extract that are reported for having antioxidant activity[25-32]. Histopathological images of epidermal skin treated with bulung sangu cream extract showed epidermal with thickness and erosion resemble normal condition with no UV treatment. These results showed the high potency of bulung sangu as an anti-inflammatory agent. As bulung sangu, several other red macroalgae from the Gracilaria genus reported for having anti-inflammatory properties[6-8]. Aparna *et al.*[38] reported the ability of n-hexadecanoic acid to competitively inhibit phospholipase A2 (PLA2) enzymes through binding to the active site hereafter inhibits the binding of substrate and enzyme, thus reducing the availability of arachidonic acid, as the precursor of prostaglandin. According to Hruza and Pentland[36], peroxidation induced by phospholipase activity is considered to initiate a stage of UVB-induced inflammation. In several pathological conditions, PLA2 was involved in the inflammation process of the various stems and organs[39].

Several types of prostaglandin cause different actions such as prostaglandin E2 (PGE2) which mediate inflammation after damage or infection[40] and prostaglandin H2 (PGH2) which is converted to thromboxane A2 promoting proliferation, increase macrophage function and modulates the expression of TNH-α and Il-β[41], in which all of them are components of pro-inflammatory response.

The decrease in epidermal thickness and epidermal erosion are also predicted as the effect given by the antioxidant composition of the bulung sangu ethanol extract. ROS production is also mediating the inflammatory response. ROS including hydroxyl free radicals, superoxide, nitric oxide and peroxyl radicals, subsequently turn other molecules into radicals and trigger a chained radical reaction. UVB radiation causes the increase of NADPH oxidase furthermore increase in ROS production that will activate the signaling pathway in epidermal keratinocytes and dermal fibroblasts, following by activation of inflammatory genes[35].

Keratinocytes signaling pathway activation impacts the production of TNF-α, IL-1, PGE2, IL-6, IL-8 and metalloproteinase (MMP). ROS will also cause damage to the

lipid component in the cellular membrane. The damage occurs increase the amount of cellular debris and macromolecule damage and change the composition of the extracellular matrix activate innate immune response following by activation and migration of immune cell. In keratinocytes, ROS produced by UV radiation able to promote auto-phosphorylation in Epidermal Growth Factor (EGF) receptor and activate The Mitogen-Activated Protein Kinase (MAPK) pathway[35].

Epidermal thickening or hyperkeratosis is one of the inflammatory response induced by UVB radiation. UVB radiation induces cytokine cascade, vasoactive mediator and neuroactive in skin furthermore cause hyperkeratosis. The increase of cell division after UV exposure accumulates epidermal keratinocyte following by epidermal thickening[13]. UV radiation affects mostly in epidermal keratinocytes. Production of various inflammatory cytokines including IL-1, IL-6, IL-8, IL-10 and TNF-α induce changes of skin immunity cell[42]. Moreover, inflammatory cytokines such as EGF, TNF and IFN-γ will be able to induce activation of keratin K6, K16 and K17 induced hyperkeratosis[43].

Beside of n-hexadecanoic acid, eight other compounds of bulung sangu ethanol extract reported for having anti-inflammatory properties, those are [1,2,4]triazolo[4,3-a]quinolone[44], phytol[45], heptadecane[30], 6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydro benzofuran-2(4H)-one[46], Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester[47], benzoic acid[12,48] and glycerin[49].

Altogether, the topical administration of bulung sangu ethanol extract showed an anti-inflammatory response observed through the variables of epidermal thickening and epidermis erosion score. The response is produced by the various compound in bulung sangu ethanol extract detected in gas chromatogram. Most compounds are reported for having anti-inflammatory and/or antioxidant activity. Inhibition of free radicals contributed to the decrease of ROS accumulation resulted in the restriction of exaggerated inflammatory response. Besides, anti-inflammatory compounds of bulung sangu ethanol extract give anti-inflammatory responses through various mechanisms. Further research is still needed to unravel the anti-inflammatory mechanism of bulung sangu ethanol extract. Furthermore, evaluation of the safety aspect is also important, considering anti-inflammatory agent tends to show several side effects that must be taken into account.

CONCLUSION

Our study demonstrated that ethanol extract of bulung sangu formulated in 5 and 10% cream preparation exhibited antioxidant activity, inhibiting more than 50% radical assayed by the DPPH method. Moreover, topical administration showed a protective effect in UVB-induced skin inflammation. Protection produced resembles normal condition without any radiation treatment. The high level of *in vitro* antioxidant activity and the presence of abundance n-hexadecanoic acid from our extract are probably co-responsible for the anti-inflammatory processes. The results provide a basis to further investigate the potential role of bulung sangu as an adjuvant therapeutic tool for the management of inflammatory-related diseases.

SIGNIFICANCE STATEMENT

This study discovered the constituent of bulung sangu (*Gracilaria* sp.) ethanol extract collected in Serangan, Bali that can be beneficial for an antioxidant and anti-inflammatory agent. This study will help the researchers to uncover the critical areas of bulung sangu potency, especially for medicines and cosmetics development, that has not been discovered before. Thus a new theory on beneficial constituents of bulung sangu and its effect in anti-oxidative and anti-inflammatory activity may have arrived at its development as a source of cosmetics, medicines and adjuvant therapeutic tool for the management of inflammatory-related diseases

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