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Evaluation of Antioxidant and Wound Healing Activities of *Spirulina* sp. Extract

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Abstract

Spirulina spp. is an alga from phylum Cyanobacteria that have multi-benefit due to their potential compounds like protein, carbohydrate, lipid, mineral, pigment, and phenolic compounds. Several types of research already describe *Spirulina* spp. capacity as an antioxidant, anti-inflammation, and antimicrobial agent, thus expand its application as a food source, cosmeceutical, and pharmaceutical compound. This study observed and evaluated *Spirulina* spp. extract as a potential source for cosmeceutical compound antioxidant and wound healing activities. *Spirulina* spp. was extracted with a variable solvent like water, ethanol, acetic acid, and buffer for 1 and 3 hours by the maceration method. *Spirulina* extract which give the highest antioxidants activity, was evaluated for wound healing, toxicity, and irritation. The result showed that *Spirulina* spp. water extracted for one hour using the maceration method have the highest antioxidant capacity with IC_{50} 45.21 mg/mL, AEAC 0.12 mg Vit. C/g sample, phycocyanin content 6.478 µg/ml, and chlorophyll content 2.642 µg/ml. Treatment with 1% *Spirulina* extracts exhibits a higher wound healing effect rather than 10% *Spirulina* spp. extract, control group, and povidone-iodine 10%, on Wistar rat skin. *Spirulina* spp. water extract showed capacity as a whitening agent with inhibitory activity on tyrosinase were IC_{50} 0.51 mg/mL. *Spirulina* spp. water extract had no irritation and toxicity effect on rabbit skin and mouse, respectively. This study showed that *Spirulina* spp. water extract is a potential source for antioxidant activity, wound healing capacity, and whitening agent; without toxicity and irritation effect. The result showed that *Spirulina* spp. extract could be potential value in cosmeceutical application.

Keywords: *Alga; wound healing; antioxidant*

1. Introduction

Spirulina spp. is Cyanobacteria, which contains several compounds like protein, lipid, polysaccharide, and pigment. Pigments or chromophores in *Spirulina* are integration of a complex protein and non-protein like phycoerythrin, allophycocyanin, phycocyanin, and chlorophyll. Phycocyanin (PC) is the major pigment that gives green-blue colour on *Spirulina*. Phycocyanin is formed by subunit α and β protein, and has different isomeric linear tetrapyrrole that contain double bonds (bilin chromophore). The bilin chromophore is attached to polypeptide through thioether linkage to cys-residue. The apoprotein contains 20 amino acid and able to chelate Fe^{+2} and Hg^{+2} [1,2].

Free radicals from internal and external sources can attack biological molecules in the human body. The imbalance between the antioxidant system and free radical content will lead to inflammation and several diseases like atherosclerosis, neurodegenerative diseases, cancer, and diabetes mellitus. Several studies on phycocyanin antioxidant potential already described by Romay et al. (2003) [3]. Thus, the capability related to phycocyanin characteristic structure and component which has a conjugation of the double bond on tetra-pyrrole and residue of amino acid from phycobiliprotein like cysteine, methionine, tryptophan, tyrosine, and histidine [1,3].

Phycocyanin also gives antioxidant results when tested with ABTS and DPPH as an antioxidant method assay. It means that phycocyanin have the same way as an antioxidant as Trolox and ascorbic acid [1,2]. Besides phycocyanin, *Spirulina* also contains chlorophyll, carotenoid, phenolic compounds, polysaccharide, fatty acid, and vitamin, which contribute to its antioxidant capacity [2,4].

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On the wound condition, ROS were produced on a stress level to eliminate infections agent by denaturing the pathogen. But when ROS accumulate, it can also denature cell and have an adverse effect on cell mitosis and fibroblast for wound enclosure. Wound condition also has a risk for external ROS, which can penetrate to membrane cells and induce apoptosis. *Spirulina* extract balance the ROS level needed to enhance regeneration of wounded skin or wound healing [5]. *Spirulina* enhance proliferation and migration of human dermal fibroblast cell line [6]. *Spirulina* extract also enhance re-epithelialization and improve vascularization of wound healing [7].

Spirulina also can inhibit tyrosinase activities [8]. Tyrosinase catalyses two melanin synthase reactions, which are hydroxylation from L-tyrosine to 3,4-dihydroxy-L-phenylalanine (L-dopa) and oxidation of L-dopa to dopaquinone, then become melanin. Sunlight with UV light increases the synthesis of tyrosinase and melanosome. Melanosome will become melanin and then migrate to keratin, where melanin's degradation occurs and lead the skin to become tanning. The degradation of melanin can be inhibited by inhibition of tyrosinase. It can be used as a skin whitening [9].

Spirulina proved as Generally Regarded as Safe (GRAS) and already used as food, supplement, and cosmetic compound. Here, we observed and evaluated the potential of *Spirulina* spp. from Indonesia as a cosmeceutical compound. The evaluation will be closure on *Spirulina* extracts' antioxidant activity, wound healing capacity, and tyrosine activity. The acute dermal toxicity and irritation test are conducted to determine the effect through skin contact and become a reference regarding product safety.

2. Experimental

2.1. Organism and Materials

Dry biomass of *Spirulina* spp. was procured from PT. Neo Alga Surakarta, Indonesia. Here in after, we stated it as *Spirulina* NAS. Wistar rat, Swiss Webster mice and albino rabbit which were used in wound healing assay, acute toxicity, irritation and dermal toxicity, respectively were provided by School of Pharmacy, Institut Teknologi Bandung.

The solvent as acetic acid, ethanol, citric acid/sodium hydroxide buffer pH 5, di-sodium hydrogen phosphate/potassium dihydrogen phosphate buffer pH 7, and tris-HCl buffer pH 10 were purchased from Merck. The water as aquabidest destilata purchased from Ikapharmindo Putramas. The ascorbic acid and DPPH were purchased from Sigma.

2.2. Animals

The test animals were 20 male Wistar rat (150-200 g), 10 female Swiss Webster mice (20-40 g), and 3 albino rabbit (1.5-2 kg). The animals were obtained from the Animal Laboratory of School of Pharmacy, Institut Teknologi Bandung. All animal experimental protocols have been approved by the Institutional Animal Ethics Committee (IAEC) in accordance with the Committee's guidelines for the Objective of Supervision and Control of Animal Experiments.

2.3. Spirulina Extraction

Spirulina NAS extracted by maceration of 10 g powder in 1000 mL of different solvents (water, ethanol, acetic acid 40%, acetic acid 80%, buffer solution pH 5, buffer solution pH 7, buffer solution pH 10) for 1 and 3 hours. The mixture then precipitated to separate cell debris, followed by freeze-drying the filtrate.

2.4. Biochemical Composition

Spirulina biochemical compositions of water and ash content were analysed by gravimetric method; fat content by Soxhlet hydrolysis; protein by Kjeltech; carbohydrate by differences; omega 3 and 6 by GC; Pb and Cd by ICP-OES; and Hg by AAS.

The examination for *Spirulina* NAS extract on total flavonoid content was based on Indonesian Herbal Pharmacopoeias [10].

2.5. Antioxidant Activity

Antioxidant activity were analysed with Ascorbic acid Equivalent Antioxidant Capacity (AEAC) method using DPPH as free radical and ascorbic acid as standard. The absorbance was recorded on wavelength 517 nm and water was used as blank.

2.6. Wound Healing Assay

Before the treatment, the dorsal fur was removed. Atropine injection with a dosage of 0.04 mg/kg body weight was given as premedication. Anaesthesia by using the mixture of ketamine injection with dosage 100 mg/kg body weight and xylazine injection with dosage 5 mg/kg body weight by intramuscular. The treated area's mouse fur was removed, and the dorsal skin was cleaned by alcohol 70%. On each mouse, laceration wound with 1 cm wide and 2 cm depth

were performed. For three (3) days upward, analgesic paracetamol is given orally with a dosage of 54 mg/200 g body weight. The group was divided into four groups: control group (I), *Spirulina* NAS extract 1% group (II), *Spirulina* NAS extract 10% group (III), and comparative group (IV). The comparative groups were treated with wound healing ointment, which contains povidone-iodine 10%. The percentage of wound closure was calculated by the reduction of the length of the wound area. The examinations, including wound contraction, erythema, and eschar formation.

2.7. Tyrosinase Assay of Spirulina Extract

Screening for tyrosinase inhibitor evaluated according to Masuda et al. [11].

2.8. Acute Skin Irritation and Acute Dermal Toxicity Test

An acute skin irritation and acute dermal toxicity test for the *Spirulina* extract was according to the Organization for Economic Cooperation and Development methodology used for determining the degree of acute dermal irritation/corrosion [12]. An acute skin irritation assay was performed. Irritation assay was using rabbit skin to mimic human skin. The assay was performed with three steps, which were preliminary, first confirmation, and second confirmation. The rabbit's skin was shaved into 3 x 3 cm^2 and abrasion until the stratum corneum. *Spirulina* extract was prepared by dissolving 8 gram *Spirulina* extract into 8 mL water, and the dose for each treatment was 500 µL. For the treatment, 500 µL of *Spirulina* extract was dropped into cotton gauze and stick it on the abrasion skin for four hours. The treatments were evaluated for erythema and oedema with an interval time of 4 hours, 24 hours, 48 hours, and 72 hours. If there was no detected erythema and oedema until 72 hours, the first confirmation steps were taken while the preliminary step was prolonged for 7 days and 14 days. If there was no detected erythema and oedema until 72 hours on the first confirmation step, the second confirmation step was taken while the preliminary step was prolonged for seven days and 14 days. The result is described by the score number.

Furthermore, acute dermal toxicity test was done using mouse, with a dose of 5 g/kg body weight with volume 1 mL/200 g body weight, on five female mice. The mouse's skin was shaved for a 10% body surface. *Spirulina* extract was prepared by dissolving 8 gram *Spirulina* extract into 8 mL water. For the treatment, *Spirulina* extract was dropped into cotton gauze and stick it on the shaved skin. The treatments were evaluated, with an interval time of 30 minutes, 1 hour, 4 hours, and 24 hours. After 24 hours, cotton gauze was removed, and the skin was evaluated. Examinations were performed on the body weight, behaviour, and the death of mice on the 14^{th} days after the treatment. If the mouse died, the incision was performed to observe the causes of death. The not-affected mouse will be dissected, and the organ will be excised for microscopic observation on the 14^{th} days after the treatment.

2.9. Statistical Analysis

The computations were performed by SPSS 20 program. The data were presented as means ± standard deviations. The significance between control and experimental groups was assessed by ANOVA test. A probability value of < 0.05 was considered to be statistical significant.

3. Results and discussion

3.1. Spirulina composition

Biochemical compositions of *Spirulina* were influenced by nutrient of medium, culture condition, harvest time, rearing condition, and drying method [13]. Biochemical compositions of *Spirulina* NAS were describe in Table 1., as we compare with several publication and review by [2,13,14] which obtained the amounts of protein content in *Spirulina* were ranged between 50-70%; carbohydrate 15-25%; crude lipid 5-7%; ash content 3-6% and water content 4-6%. For PUFA, *Spirulina* has a high amount of polyunsaturated fatty acids (PUFAs), 1.5–2.0% of 5–6% total lipid [14]. Biochemical content of *Spirulina* NAS was between the reported ranges.

Spirulina can neutralize or to chelate toxic minerals from water; thus, the metal content of *Spirulina* should be monitored [15]. Regulation for *Spirulina* extract by FDA is state on section 21CFR73.530 and listed on Table 1. *Spirulina* NAS has heavy metal below the permitted limit by Food and Drug Administration (FDA).

3.2. Spirulina NAS extraction

The extraction process consists of the separation of a substance from a matrix based on diffusion using an appropriate solvent [15]. *Spirulina NAS* were extracted with several solvents for 1- and 3-hours extraction by maceration to achieve the excellent condition for its antioxidant activity. Maceration is generally used by small and large scales industries. *Spirulina* has several compositions which act as an antioxidant like phycocyanin, carotenoid, chlorophyll, sulfated polysaccharide (Calcium Spirulina-Ca Sp), tocopherol-Vitamin E, phenolic compound (flavonoid), vitamin C, and fatty acid

[2,4], which can be extracted by ethanol and water. Previous research conducted by Herrero et al. (2004) [17] were extracting *S. platensis* by Pressurized Liquid Extraction (PLE) with four different solvents (hexane, light petroleum, ethanol, and water). All extracts demonstrated a significant antioxidant activity as tested using electro-chromatography with diode array detection (MEKC-DAD). Organic solvents used to extract the pigments from algae biomass include acetone, ethanol, methanol, petroleum ether, or chloroform. Chlorophyll can be extracted with acetone [18].

Table 1. *Spirulina* NAS composition

Parameter	This research		IPGSR [14]	Food and Drug Administrations [16]
	Unit	Result		
Moisture content	g/100g	6.66	6	
Ash content	g/100g	11.54	9	
Fat content	g/100g	5.04	6	
Protein	g/100g	53.60	61	
Carbohydrate	g/100g	23.16	14	
Omega 3	mg/100g	840.53		
Omega 6	mg/100g	835.42		
Pb (LOD 0,08)	mg/kg	n.d.		< 2 mg/kg
Cd (LOD 0,02)	mg/kg	n.d.		-
Hg	mg/kg	0.02		< 1 mg/kg

n.d. = not detected

IPGSR - Institute of Post-graduate Studies and Research Laboratory, University of Malaya

In this research, we used water, acetic acid, ethanol, and buffer as a solvent for extraction. We used water because phycobiliproteins, including phycocyanin, are water-soluble proteins [19,20]. Water extraction method is able to extract phenolic compounds. Previous research by Herrero et al. (2007) [21] showed that *Spirulina* aqueous extract contain phenolic compound and fatty acid derivatives.

Research by Yudiati et al. (2011) [22] showed that *Spirulina* that macerated with ethanol as solvent gives antioxidant activity 323.70 ppm. The *Spirulina* methanol extract compounds were beta carotene, chlorophyll, alkaloid, flavonoid, steroid, and terpenoid.

Here, we analysed the extracted compound as the whole compound for its synergy on antioxidant activity. The highest antioxidant activity based on IC_{50} result was using water as solvent for one-hour maceration (Table 2). Antioxidant activity with acetic acid as the solvent gives higher results than ethanol. Acetic acid is a hydrophilic polar protic solvent. It dissolves polar compounds such as inorganic salts and sugars; and non-polar compounds such as oils and polar solutes. Acid functional groups were known to be more polar rather than alcohol. Antioxidant activity with acetic acid as solvent was highest on three hour maceration, but the phycocyanin and chlorophyll were the lowest. The result showed, on the acetic acid extract, that the main contributed compound on its antioxidant activities wasn't phycocyanin and chlorophyll, which were also shown by the transparent coloured extract (picture weren't shown).

Antioxidant activity with buffer pH 10 as a solvent gives better results rather than buffer pH 5 and 7. Phycobiliproteins are acidic and hydrophilic. *Spirulina* sp. phycoyanin have isoelectric point around 4.6-5.2 [19]. Therefore, pH 10 will make charge differences on protein which lead the phycobiliproteins soluble to the solvent. The phycobiliprotein as protein was influenced by the pH condition.

In our research, the water solvent that we used was aquabidest destilata, which have been demineralized and had a neutral pH. The antioxidant activity of *Spirulina* NAS extracted with water was higher than buffer 7 (di-sodium hydrogen phosphate/potassium dihydrogen phosphate), on one and three hour maceration. This is in according to Ridlo et al. [23] research which resulted in *Spirulina* sp. extract having a higher antioxidant activity value (IC 50) in water compared to buffer 7. We assumed on the *Spirulina* NAS water extracted, several compounds which were water-soluble also contributed to the antioxidant activities.

Research by Silveira et al. [24] observed that phycocyanin content on *Spirulina* extracted with water did not differ too much from sodium phosphate buffer pH 7. In our research, phycocyanin content of *Spirulina* NAS extracted with water was higher than buffer 7 on one-hour maceration, but lowered on three-hour maceration.

The antioxidant activity on three-hour maceration with buffer pH 5 and 7 gives lower results than one hour. The long extraction time can trigger hydrolysis or denaturation on the active compound, leading to decreasing antioxidant activities. Those results are similar to the research of Purba et al. [25] on the effect of length maceration on *Gracilaria* sp. The extracted color of *Spirulina* NAS, which was extracted with a buffer for three hours, was lighter than water extracted. The three hours buffer extracted were also given precipitation, if left aside for a while (picture weren't shown). In this research, the antioxidant activity was influenced by the type of solvent and extraction time.

Phycocyanin concentration was strongly influenced by variable biomass, solvent ratio [24], cellular disruption method, type of solvent, and extraction time [26]. The differences of water concentration as solvent had no significant effect on the C-Phycocyanin concentration and yield [27]. Extraction *Spirulina platensis* by maceration for 12 hours with solvent water, ethanol 6% and methanol 6% gives phycocyanin concentration (mg/ml) 0.516, 0.877, and 0.834, respectively [28]. The phycocyanin that extracted with water (1:100) for 24 hours by maceration-technical rupture gives a concentration of 0.178 mg/ml [27]. The phycocyanin and chlorophyll content of *Spirulina* NAS were extracted with several solvents for one and three hours. The result showed that the time variable did not give any differences for water and acetic acid 10% as a solvent. The phycocyanin and chlorophyll content for ethanol as a solvent were unidentified. On this research, phycocyanin and chlorophyll content was influenced by type of solvent.

The phycocyanin and chlorophyll content on *Spirulina* NAS was stabile with water as solvent for one and three hour. *Spirulina* NAS which was water extracted for one hour, given the highest antioxidant activity result (IC_{50}) result. Therefore for further research we used *Spirulina* NAS water extracted for one hour.

Further evaluation was conduct on *Spirulina* NAS water extracted for the flavonoid content. Flavonoid is a phenolic compound that has antioxidant activity. Flavonoid scavenges free radical by direct chelation, hydrogen transfer, or single electron transfer. Those mechanisms were influenced by its configuration and the total amount of hydroxyl group [29]. Flavonoid can be extracted by polar solvents like methanol, ethanol, and water.

Spirulina NAS flavonoid content by maceration with water was 1.09 ± 0.03 %. Previous research by Dianursanti et al. (2020) [30] on *Spirulina* flavonoid content by maceration (2%) with ethanol as solvent was 2.68%.

Table-2: Spirulina NAS and extraction with several solvents

Solvents	IC 50 (mg/ml)	AEAC (mg Vit C/g sample)	Phycocyanin (µg/ml)	Chlorophyll (µg/ml)
1 hour extraction				
Water	45.21	0.12	6.478	2.642
Acetic acid 10%	204.34	0.05	1.985	0.365
Buffer pH 7	134.93	0.06	2.682	3.338
Buffer pH 10	82.62	0.09	10.125	1.907
3 hours extraction				
Water	78.87	0.06	6.478	2.642
Ethanol 10%	194.00	0.05	0	0
Acetic acid 10%	68.98	0.20	1.985	0.365
Acetic acid 80%	72.71	0.19	0	8.223
Buffer pH 5	1034.69	0.003	8.228	0.2691
Buffer pH 7	19360.47	0.003	10.125	1.907
Buffer pH 10	86.00	0.08	3.338	2.882

3.3. Wound healing activity

Spirulina NAS water extracted were further evaluated for its potency on wound healing. *Spirulina* NAS water extracted were chosen because it gives highest antioxidant activity. Several studies has been observed the potential *Spirulina* as a wound healing agent [6,7,31–34] and correlation of antioxidant activity with wound healing activity. Besides balancing the ROS level, *Spirulina* compound also enhance re-epithelialization [5,7]. Previous research by Syarina *et al.* (2015) showed that aqueous extract of *Spirulina* gives significant wound healing activity on normal adult Human primary Dermal Fibroblast cell (HDF) rather than ethanolic and methanolic extract [35]. Phytochemical compound from *Spirulina* NAS water extracted was proven compositing of chlorophyll, phycocyanin and

flavonoid. Chlorophyll has been found to accelerate wound healing progress by more than 25%. Since chlorophyll stimulates tissue growth; it prevents the advancement of bacteria and speeds up the wound healing process [4]. Phycocyanin accelerated wound healing by increased fibroblast proliferation in a dose dependent manner and induced cellular migration to recover wound area [36]. Flavonoids, alkaloids, and triterpenoids promote the wound healing process mainly due to their antimicrobial and wound contraction property and increased rate of epithelisation [34].

Observations of acceleration for wound healing were done for 14 days for its wound length (Table 3). In the control group (group I), the contraction on wound length was 37.5%. On the comparative product, the contraction on wound length was 22.5%. For *Spirulina* NAS extract 1% and 10%, the contraction on wound length were reached 62.5% and 52.5%, respectively. Statistic results with Anova-one way showed that *Spirulina* NAS extract 1% give significant differences compared with the control group on third and fifth days. For *Spirulina* NAS extract 10%, it gives significant differences compared with the control group only on fifth day. It was found that *Spirulina* NAS extract 1% exhibit the fastest highest wound closure on skin cells compared to *Spirulina* NAS extract 10% and the reference group. Research by Gunes et al. (2017) also showed that skin cream with 1.125% *Spirulina* crude extract was more efficient and exhibited a highest proliferative effect on skin cells rather than 0.5% [31]. The wound healing agent's effect on wound closure on the epidermis was influenced by its biochemical properties like the particle size, type of bioactive compound, concentration of the bioactive compound, and characteristic of bioactive compound. There was no evidence of erythema, eschar formation, and inflammation cell on the treated groups with *Spirulina* NAS extract (Figure 1).

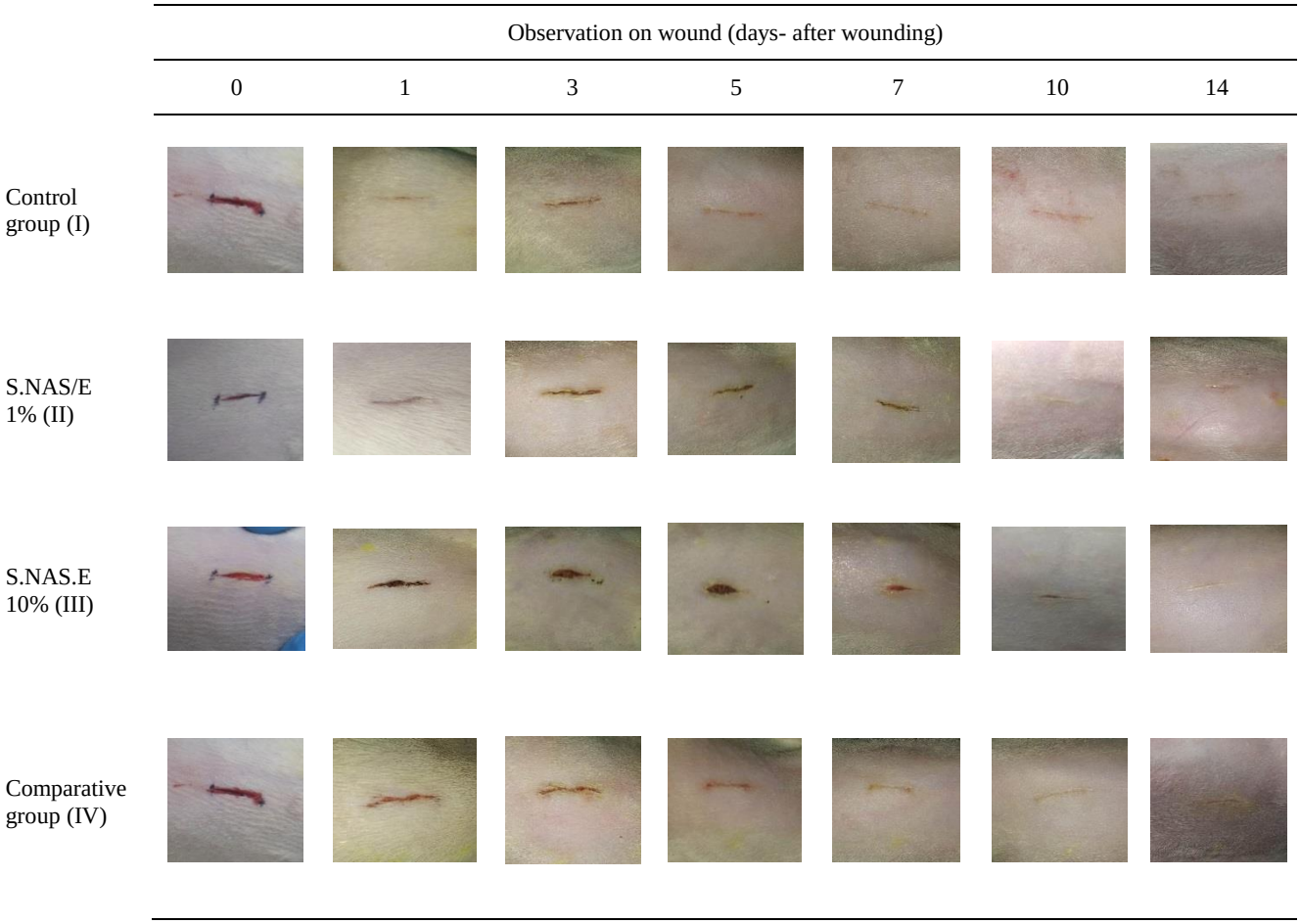

Figure-1: Dermal wound healing in mice models. Wound healing was measured by quantifying the total wound area over a period of two week.

Table-3: Wound healing assay of *Spirulina* NAS extract

Group	Wound length (cm on day-)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Control group (I)	1.00 ± 0.0	0.98 ± 0.05	0.95 ± 0.10	0.95 ± 0.1	0.93 ± 0.10	0.90 ± 0.12	0.78 ± 0.26	0.78 ± 0.26	0.73 ± 0.31	0.65 ± 0.31	0.65 ± 0.31	0.65 ± 0.31	0.63 ± 0.29	0.63 ± 0.29
S.NAS/E 1% (II)	1.00 ± 0.0	0.80 ± 0.0	0.78 ± 0.05*	0.75 ± 0.06	0.68 ± 0.13*	0.65 ± 0.10	0.60 ± 0.12	0.55 ± 0.06	0.53 ± 0.05	0.50 ± 0.08	0.48 ± 0.10	0.48 ± 0.10	0.40 ± 0.14	0.38 ± 0.10
S.NAS.E 10% (III)	1.00 ± 0.0	0.88 ± 0.0	0.88 ± 0.13	0.83 ± 0.10	0.65 ± 0.1*	0.63 ± 0.10	0.63 ± 0.10	0.58 ± 0.10	0.58 ± 0.10	0.58 ± 0.10	0.58 ± 0.10	0.55 ± 0.10	0.48 ± 0.10	0.48 ± 0.10
Comparative group (IV)	1.00 ± 0.0	0.95 ± 0.06	0.95 ± 0.06	0.93 ± 0.10	0.93 ± 0.10	0.90 ± 0.14	0.83 ± 0.21	0.83 ± 0.21	0.83 ± 0.21	0.83 ± 0.21	0.83 ± 0.21	0.80 ± 0.18	0.78 ± 0.15	0.78 ± 0.15

Note:
n = 5-6 rats/group.
The data expressed as mean ± standard deviation
* means significantly different compared to control group (P<0.05)
S.NAS.E = *Spirulina* NAS Extract

3.4. Tyrosinase activity

Correlation of antioxidant and melanogenesis was proposed by Lin et al. (2018) [37]. Tyrosinase inhibition by *Spirulina* extract confirms the potential of *Spirulina* extract as a whitening compound for skin. Jian et al. (2019) [38] have proposed recent claims for polyphenol extract (gallic acid) of *Spirulina* as a tyrosinase inhibitor.

Tyrosinase as metalloenzyme can be inhibit by phenolic compound like chalcone [39], flavone [11], flavonoid [40], catechin, gallic acid [41], vanilic acid, cinnamic acid, caffeic acid and ferulic acid [42]. Phenolic compound inhibitory effect influenced by its hydroxyl group on aromatic rings, which can act as chelator of cooper on the active site of tyrosinase, thus preventing dopachrome formation [39,41]. Phycocyanin regulation on tyrosinase inhibition was observed by Wu et al. (2011) [43]. *Spirulina platensis* ethanol extract showed IC_{50} on level 9.232 mg/mL [44]. *Spirulina* NAS water extract containing flavonoid, phycocyanin and chlorophyll. The *Spirulina* NAS water extract showed inhibitory activity of tyrosinase on IC_{50} value 507.687 ppm (0.51 mg/mL).

3.5. Acute Skin Irritation Test

Spirulina biochemical compounds as wound healing agent may penetrate the skin layer and must be screening of its toxicity and irritation for human skin. *In vivo* models can be alternative to animal testing for the evaluation of toxicity and irritation of *Spirulina* biochemical compounds as wound healing agent.

Spirulina NAS extract was sticky to the skin; therefore, it was attached to the skin. *Spirulina* NAS extracts wash off made redness on the skin that was shown on a preliminary step on irritation screening from the treated group with *Spirulina* after 4 hours of treatment. After 24 hours of treatment, the treated group were showed similar results as an indication with the control group. *Spirulina* NAS extract-treated group wasn't shown any irritation reaction after 14 days examination on the preliminary step. Moreover, the confirmation step also gave similar results with initial steps. *Spirulina* NAS extract-treated group also wasn't shown any irritation reaction after 14 days examination on the confirmation step. *Spirulina* NAS extract water doesn't give any irritation reaction after 14 days examination (Table 4.); therefore, it is considered as a non-irritant compound.

3.6. Acute Dermal Toxicity

Observation for 24 hours on toxicity screening did not show any reduction of motoric activities above platform activities. There was no evidence of straub, piloerection, ptosis, lacrimation, catalepsies, salivation, tremor, convulsion, and writhing. Pineal reflects, cornea reflects, and respirations were normal for all treated mouse. The incline of body weight was found on some treated mouse (Figure 2). There was no evidence of death on treated mouse with dosage 5 g/kg *Spirulina* NAS extract by dermal treatment, during examination for 14 days (Table 5). Macroscopic observation did not show any organ abnormality for all treated mouse after 14^{th} days treatment with *Spirulina* NAS extract. It points out that *Spirulina* NAS extract dermal treatment with dosage 5 g/kg was practically not toxic.

Table 4. Acute Skin Irritation test of *Spirulina* NAS extract

Group	Observation	Observed parameter	
		Erythema	Oedema
1 (Preliminary step)	4 hours	0	0
	24 hours	0	0
	48 hours	0	0
	72 hours	0	0
	7 days	0	0
2 (First confirmation step)	4 hours	0	0
	24 hours	0	0
	48 hours	0	0
	72 hours	0	0
	7 days	0	0
3 (Second confirmation step)	4 hours	0	0
	24 hours	0	0
	48 hours	0	0
	72 hours	0	0
	7 days	0	0

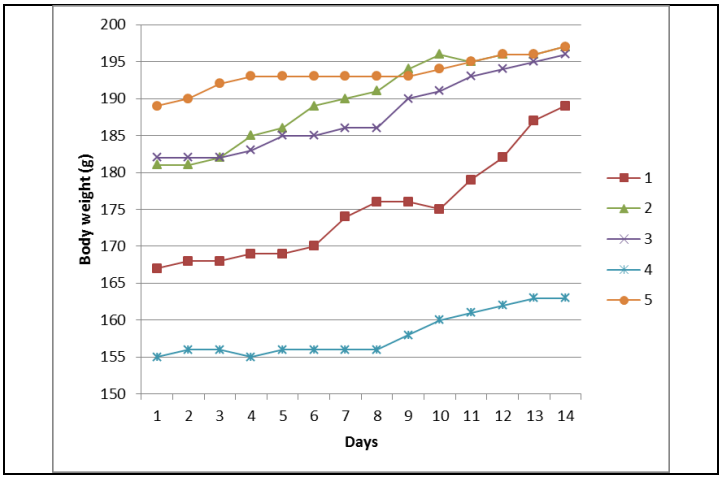

Figure 2. Development of mouse body weight during examination for 14 days

Table 5. Acute Dermal Toxicity study of *Spirulina* NAS extract

Dosage	N	Cumulative death													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
5 g/kg bw	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0

4. Conclusion

Spirulina NAS extraction by maceration method was better with water as the solvent rather than acetic acid 10%, ethanol 10%, buffer pH 5, 7, and 10. *Spirulina* NAS water extract has phycocyanin, chlorophyll, and flavonoid content, which acts for antioxidant capacity, wound healing capacity, and tyrosinase inhibitor. The water extract *Spirulina* NAS water extract 1% has wound contraction 62.5%, better than povidone-iodine 10% with 22.5%. *Spirulina* NAS water extract gives no irritation and toxicity effect on rabbit skin and mice, respectively.

5. Conflicts of interest

There are no conflicts to declare.

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