

Effectivity of *Holothuria scabra* and *Spirulina platensis* extract combination as an Antiinflammatory Agent Measured by Carrageenan-induced Rat Paw Edema

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Abstract

Sea cucumber, *Holothuria scabra*, can be found abundantly in Indonesian seas, which is also known to possess several medicinal properties. *Spirulina platensis* is another marine resources that has recently been extensively researched for its medicinal ability, such as anti-inflammatory effect. This study aims to evaluate the efficacy of *H. scabra* extract combined with *S. platensis* extract in reducing inflammation. This study uses male Wistar rats as the study animal. Inflammation was induced by injecting carrageenan solution into the mice paw. Combination of *H. scabra* and *S. platensis* extract with various combination ratio (1:1; 1:2; and 2:1) was applied to the mice paw. Diclofenac sodium was used as the standard control therapy. Edema inhibition rate and anti-inflammatory efficacy were measured by analyzing the edema size and calculating the edema difference. Combination of *H. scabra* and *S. platensis* with 1:1 ratio has the largest edema inhibition volume compared to the other treatments. *H. scabra* and *S. platensis* combination outperforms the positive diclofenac sodium control group in terms of edema inhibition. The highest anti-inflammatory effect is obtained in the combination of *H. scabra* and *S. platensis* with 1:2 ratio, however, the anti-inflammatory efficacy is not as potent as the positive control. The effectivity of *Holothuria scabra* and *Spirulina platensis* extract in reducing the edema might be caused by their ability to reduce the levels of several inflammatory markers, including IL-6, NO, MMP9, and COX-2. This result suggests that *H. scabra* and *S. platensis* combination has anti-inflammatory effect shown in mice paw edema model.

Keywords: sea cucumber; *Spirulina*, inflammation, diclofenac, edema inhibition rate

Introduction

Indonesia is known as one of the countries possessing extensive marine resources. Being located in the equator, with more than 78% areas comprised of shallow-water seas, Indonesia has a diverse amount of aquaculture species (Hutomo and Moosa, 2005; California Environmental Associates, 2018). Invertebrate species, such as shrimps, sea cucumbers and molluscs, can be harvested from seagrass meadows throughout Indonesia (Hutomo and Moosa, 2005). Indonesia is one of the biggest exporters of sea cucumbers, with more than 2 million kg.y⁻¹ from Indonesia, which has almost 10 million USD in value.

Sea cucumbers are mostly harvested and consumed because of their health benefits (Firdaus, 2019). In Asia, sea cucumbers has been consumed as food medicines and delicacies (Maziar Yahyav et al., 2012). Sea cucumbers are recognized as tonic and traditional remedy in Chinese and Malaysian literatures for its effectivity against various medical disorders (Bordbar et al., 2011).

Holothuria scabra, also known as *teripang gosok* or *teripang pasir* (sand sea cucumber), is one of the sea cucumbers harvested from Indonesian seas which has high economic values and are exploited commercially (Firdaus, 2019). This species is distributed in tropical waters of Africa, South China Sea, South Pacific, South-East Asia, and Indian Ocean (Bordbar et al., 2011). This species is usually exported as dried sea cucumbers to several countries in Asia, most notably China, Taiwan, Hongkong, Korea, and Singapore. In Indonesia, *H. scabra* is mostly sold as *haisom* (sea cucumber for cuisine purposes) (Firdaus, 2019).

H. scabra can be found in almost coastal areas, from shallow-water seas up to deeper seas in the coastal region. Dried sea cucumbers contain 82% protein, 1.7% fat, 8.9% water and 4.8% carbohydrate. Sea cucumbers also has several active ingredients, including triterpene glycosides (saponin), chondroitin sulphate, glycosaminoglycan, sterol, glucose sulphate triterpenes, sulphate polysaccharides, peptides, proteins (gelatin and collagens), hydrosalicylate, glycoproteins, lectin, phenols, and

flavonoids (Arifin *et al.*, 2013). The main saturated fatty acids contained in *H. scabra* is palmitic acid, and the main unsaturated fatty acids contained in *H. scabra* is arachidonic acid (Yahyav *et al.*, 2012).

The most important bioactive compounds contained in *H. scabra* with experimentally confirmed pharmacological activity are triterpene glycosides (scabrasides B, D, echinoside A, 24-dehydroechinoside A, HS-1, holothurins A, A1, A3, A4, B, B4, fuscocineroside C) (Khotimchenko, 2018). Nine triterpene glycosides, namely echinoside A, 24-dehydroechinoside A, HS-1, holothurins B, B4, A and A1, scabrasides D and B, have been isolated from *H. scabra*. *H. scabra* also contains a variety of triterpene glycosides, including scabraside D, fuscocineroside C, and 24-dehydroechinoside A (Khotimchenko, 2018).

Several studies found that *H. scabra* has medicinal properties and benefits. *H. scabra* n-hexane extracts has been shown to inhibit *Escherichia coli* bacterial growth (Arifin *et al.*, 2013). Another study found that *H. scabra* extract has anticancer activity, which is shown by its ability to induce apoptosis against human glioblastoma cell lines. *H. scabra* triterpene glycosides has been shown to exhibit anti-proliferative activity and cytotoxicity against several tumor cell lines, including mouse leukemic cells (P-388), lung human cancer cell (A-549), human colorectal cancer cell (HCT-116), gastric cancer cell (MKN28), and human breast cancer cell (MCF-7) (Khotimchenko, 2018).

Several sea cucumbers exhibit anti-inflammatory activity. An *in vivo* study found that dried extracts of several species of holothurians (*H. nobilis* and *H. axiologa*) exhibits anti-inflammatory activity in rats against acute carrageenan-induced paw inflammation; however, its activity is lower than the synthetic standard compound (Aspirin) (Bordbar *et al.*, 2011). *H. polii* has been shown to exhibit anti-inflammatory activity decreases inflammatory markers (interleukin-6, interleukin-1 β , nitric oxide and matrix metalloproteinase 9) in mouse mammary SCp2 cells and THP-1 human monocytic cells (Kareh *et al.*, 2018). As of our knowledge, Frodanol, extracted from the sea cucumber *Cucumaria frondosa*, is currently being the only patented anti-inflammatory compound extracted from sea cucumbers (Kareh *et al.*, 2018).

Spirulina platensis is a filamentous photoautotrophic green-blue algae that belongs to cyanobacteria (Suratno, 2010; Saranraj and Sivasakthi, 2014; Wollina *et al.*, 2018). *Spirulina* is one of the edible cyanobacterias, along with some other species such as *Nostoc* and *Aphanizomenon*

(Saranraj and Sivasakthi, 2014). *S. platensis* is studied because of its high nutritional content which can be used in the medical field (Saranraj and Sivasakthi, 2014).

S. platensis is mostly composed of proteins around 50-70% of its dry weight. The essential amino acids contained in *Spirulina* includes leucine, valine, and isoleucine (Sotiroudis and Sotiroudis, 2013). *Spirulina*'s green color comes from phycobiliproteins contained within *Spirulina* cells which serves as a light-collecting pigment for its photosynthesis activity. Phycobiliproteins consisted of three pigments: allophycocyanin, phycocyanin and phycoerythrin. From these three pigments, phycocyanin is the pigment that is mostly studied due to its potential benefits in the medicine field (Saranraj and Sivasakthi, 2014).

Several studies have found health benefits of *S. platensis* since they have antifungal activity against several fungi, including *Aspergillus fumigatus*, *Mucor vulgaris*, *Penicillium expansum*, *Fusarium solani* and *Fusarium oxysporum*; all of which contributes to the invasive mycoses infection (Otlu and Rudic, 2016). *Spirulina* is also found to be able to improve wound healing by increasing fibroblast proliferation and migration, enhanced wound closure rate, and inhibiting colonization of *Staphylococcus aureus* in wounds (Wollina *et al.*, 2018). Anticancer activities can also be found in *S. platensis*. One research found that *S. platensis* has anti skin tumor effects in mice that is exposed to ultraviolet B (UVB) rays (Yogianti *et al.*, 2014).

S. platensis has also been found to exhibit anti-inflammatory effect, as shown by several studies. One study found that *S. platensis* extract exerts anti-inflammatory activity in diabetic rats (Nasirian *et al.*, 2018). *S. platensis* has also been shown to decrease the inflammation as shown by the reduction of prostaglandin E2-induced paw edema in rats given 2 mg.kg⁻¹ BW of *S. platensis* extract.(Somchit *et al.*, 2014). Phycocyanin and β -carotene of *Spirulina* sp. might have important role in the anti-inflammatory effects (Wu *et al.*, 2016). The blue pigment phycocyanin in *S. platensis* works as anti-inflammatory agent, by acting as a selective inhibitor of the cyclooxygenase-2 (COX-2) enzyme, inhibiting the production of nitric oxide (NO) and prostaglandin E2.(Wollina *et al.*, 2018). Besides that, phycocyanin is also able to inhibit the expression of TNF- α , and IL-6 which contributes to inflammation (Wu *et al.*, 2016). This study aims to evaluate the effectivity of *H. scabra* and *S. platensis* combination in reducing inflammation, as shown by measuring edema size on carrageenan-induced inflammation in mice paw.

Materials and Methods

H. scabra samples were harvested from Karimunjawa Islands, Indonesia by local fishers. The samples then were cleaned thoroughly with running filtered tap-water to prevent any residues of contaminants in the sea cucumbers itself. The whole sea cucumber body then were cut into small pieces (around 2 cm³ in size). The small pieces then were subjected to a second wash using filtered tap-water, to make sure that any contaminants that were hidden in crevices which were not expelled at the first washing are removed from the sea cucumber. The clean, small cuts of *H. scabra* then were blended with a 100 mL of demineralized water until a slurry is formed.

Two hundred grams of *H. scabra* blended sample were [it into a container filled with 400 mL of n-hexane. The mixture was soaked for 24 hours, and then were shaken for 5 hours at 150 rpm shake rate.

This process was repeated three times until a clear filtrate is obtained. The filtrate was separated from the solids sediment using vacuum Büchner funnel. The solvent then was evaporated using rotary evaporator at 40°C temperature to obtain the crude extract. The crude extract was further processed using vacuum desiccator to obtain a thick extract.

The *S. platensis* algae used in this study were obtained from Karimunjawa Islands, Central Java, Indonesia. The raw *S. platensis* algae gathered from the Karimunjawa Sea were dried using oven drying method (heated for 7 hours in 80 degrees Celcius temperature using a laboratory-type oven) (Güroy et al., 2017) The dried *S. platensis* were crushed into fine powder. The powdered *S. platensis* microalgae was macerated in 95% ethanol solution with 1:10 concentration (one part of *S. platensis* powder macerated in 10 parts of ethanol). The maceration process was done for 5 days in a glass container. The glass container was stirred every day to make sure



Figure 1. (a) Measurement of mice paw volume before carrageenan induction; (b) Induction of carrageenan into the mice paw; (c) Topical application of *H. scabra* : *S. platensis* mixture (d) Measurement after one hour application of *H. scabra* : *S. platensis* mixture.

the uniformity of the maceration process. The solution then was filtered through Whatman filter paper, and was evaporated using rotary evaporator machine at the ethanol boiling point temperature until a thick extract was obtained.

The anti-inflammatory activity of *S. platensis* and *H. scabra* was measured using carrageenan-induced rat paw edema assay. The subjects of this study were twenty-five Wistar male mice with a weight of 250 ± 50 g, which fulfilled the inclusion criteria for healthy conditions (active moves). The mice included in this study exhibited no signs that met our exclusion criteria, behavioral changes (activities seemed weak and lazy). Mice were kept at a constant room temperature of $28.0 \pm 2.0^\circ$ C with fluorescent lighting that is turned on for 12 hours per day between 9.00 AM to 9.00 PM, with adequate food supply. The mice are obtained from a certified breeder with a pure-bred lab mice certificate.

The preparation of rat paw edema assay begins with thorough cleaning by using water and bristles of rat paw to prevent any contaminants. Sub-plantar injection of 100 μ L freshly prepared 0,1% carrageenan in distilled water was injected in the right hind paws of each rat. Paw thickness before and after carrageenan injection (before any treatment was applied) were measured and noted.

The mice in group A were treated with *H. scabra* and *S. platensis* extract with 1:1. The mice in group B were treated with *H. scabra* and *S. platensis* extract with 2:1 ratio. The mice in group C were treated with *H. scabra* and *S. platensis* extract with 1:2 ratio. We mixed the *H. scabra* and *S. platensis* extract according to the ratio for each group on separate containers. The positive control group were treated with one fingertip-unit (0.5 grams) of diclofenac sodium ointment as a standard therapy, and the negative control group were not treated (were only covered with dimethyl sulfoxide). All of the medications were applied topically in a thin layer to the mice paw. The edema volume and anti-inflammatory efficacy were tabulated and analyzed statistically using computerized software.

This research has been ethically approved by Bioethics Commission, Medical Faculty of Universitas Islam Sultan Agung Semarang with the ethical clearance number of 172/V/2020/Komisi Bioetik.

Results and Discussion

For the average edema Mice given *H. scabra* : *S. platensis* with 1:1 ratio had the highest average edema inhibition volume among all study groups. The edema inhibition obtained in this group was even

higher than the established medication (diclofenac sodium ointment). The positive control has almost similar edema inhibition volume as *H. scabra* : *S. platensis* with 1:2 ratio. This result shows that the topical application of *H. scabra* : *S. platensis* with 1:2 ratio has almost the similar efficacy as the established medication (diclofenac sodium ointment).

Mice given *H. scabra* : *S. platensis* with 2:1 ratio had lower edema inhibition rate compared to the two other therapeutic group and the positive control. However, statistical analysis shows that there was no significant difference in results among the study groups (Table 1.).

The anti-inflammatory efficacy was measured by first calculating the increase of rat paw volume. The rat paw volume increase was measured by dividing the delta volume of rat paw before and after carrageenan induction by the initial volume. We measured the anti-inflammatory efficacy by dividing area under curve (AUC) between the negative control group and the therapeutic group. The AUC of the negative control group serves as the baseline value. Diclofenac sodium which is give at the positive control group had the highest anti-inflammatory efficacy, followed by topical application of *H. scabra* : *S. platensis* combination extract with 1:2 ratio. The other two remaining *H. scabra* : *S. platensis* extract combination achieved less than 20% of anti-inflammatory efficacy. See Table 2.

The standard animal experimental model for testing anti-inflammatory drugs is by using carrageenan injection for inducing paw edema in rats (Amdekar *et al.*, 2012) Carrageenan causes the release of proinflammatory and inflammatory mediators, hence causing inflammatory reactions at the rat paw which results in the formation of edema. There are two phases in carrageenan-induced edema; the first phase of edema is caused by the release of histamine, serotonin and kinins shortly after carrageenan injection, and the second phase of edema is caused by the release of prostaglandin-like substances after approximately two to three hours after carrageenan injection. Carrageenan was used as it is not antigenic and does not cause any apparent systemic effects. The use of carrageenan injection also allows a high degree of reproducibility (Amdekar *et al.*, 2012; Solanki *et al.*, 2015).

In this study, we found that *Holothuria scabra* and *Spirulina platensis* 1:1 combination yields in the largest edema inhibition rate. The average edema inhibition rate on the 1:1 group reaches -0,108 mm³, which is larger than the other two combinations. This combination has better edema inhibition rate compared to 1:2 combination and 2:1 combination.

Table 1. Volume of edema in each study sample

Grouping	Initial Rat Paw Volume	Rat Paw Volume after Caraagennan injection	Rat Paw Volume after 60 mins after application	Edema Inhibition volume	Average edema inhibition volume
<i>H. scabra</i> : <i>S. platensis</i> extract 1:1	0,64	0,77	0,60	0,17	0,108
	0,60	0,65	0,60	0,05	
	0,72	0,84	0,70	0,14	
	0,70	0,78	0,70	0,08	
	0,60	0,70	0,60	0,10	
<i>H. scabra</i> : <i>S. platensis</i> extract 2:1	0,52	0,54	0,54	0,00	0,048
	0,55	0,62	0,58	0,04	
	0,68	0,71	0,66	0,05	
	0,56	0,66	0,60	0,06	
	0,49	0,68	0,59	0,09	
<i>H. scabra</i> : <i>S. platensis</i> extract 1:2	0,6	0,62	0,6	0,02	0,076
	0,51	0,7	0,6	0,1	
	0,3	0,55	0,4	0,15	
	0,56	0,61	0,57	0,04	
	0,54	0,62	0,55	0,07	
Positive control (diclofenac sodium ointment)	0,58	0,73	0,60	0,13	0,070
	0,46	0,65	0,58	0,07	
	0,57	0,59	0,58	0,01	
	0,54	0,66	0,61	0,05	
	0,50	0,67	0,58	0,09	
Negative control	0,53	0,55	0,53	0,02	0,034
	0,68	0,64	0,60	0,04	
	0,49	0,58	0,55	0,03	
	0,54	0,57	0,50	0,07	
	0,43	0,46	0,45	0,01	

Table 2. Anti-inflammatory efficacy of *H. scabra* and *S. platensis*.

Mice in each group	1:1 ratio	2:1 ratio	1:2 ratio	Positive Control	Negative Control
Mice 1	5,1	1,2	0,6	5,1	0,6
Mice 2	0,9	3	8,4	9,3	3,6
Mice 3	4,2	1,5	10,5	0,9	4,5
Mice 4	2,4	4,2	1,8	5,7	2,1
Mice 5	3	3	2,7	7,5	2,7
% Anti-inflammatory	15,56%	4,4%	77,8%	113,3%	0%

The diclofenac positive control group has 0,07 mm³ edema volume inhibition. The interesting result is that the 2:1 extract combination yields in the lowest rat paw edema inhibition. This combination also has the lowest anti-inflammatory efficacy, only 4,4%. Interestingly, the 1:1 extract ratio which has the highest edema inhibition level, only managed to reach 15.56% of anti-inflammatory efficacy, outnumbered by the 1:2 ratio.

The results of this study were similar to previous study conducted by Zuniarto *et al.* (2017). The authors compared the effectivity of *H. scabra* extract compared to Thrombophob® (heparin sodium) ointment on rat paw edema induced by carrageenan. The results of the study shows that lower concentration extract of *H. scabra* (15% and

30% extract) has better edema inhibition rate compared to heparin sodium ointment; whereas 60% *H. scabra* extract shows lower edema inhibition rate compared to heparin sodium ointment.(Zuniarto *et al.*, 2017). On a different experimental model, Kareh *et al.* (2018) recently found that *H. polii* has anti-proliferative and anti-inflammatory activity on human cultured cancer cells. In Indonesia, *H. scabra* is known as one of the sea cucumbers with high economical value. Various study has been done to identify bioactive compounds in sea cucumbers to further evaluate the potency of sea cucumber components.

The effect of *H. scabra* extract in reducing the inflammation might be attributed to the ability of *H. scabra* extract to reduce levels of Interleukin-6 (IL-6),

nitric oxide (NO), and matrix metalloproteinase 9 (MMP9). However, the exact bioactive compound that contributes to this inflammation reduction were still under further research, due to the active substances identification difficulty.(Kareh *et al.*, 2018).

The ability of *S. platensis* extract in reducing inflammation was attributed to several factors. Anti-inflammatory agents produced by *S. platensis* are phycocyanin and β -carotene.(Wu *et al.*, 2016). As an anti-inflammatory agent, phycocyanin contained in *S. platensis* works a selective inhibitor of the cyclooxygenase-2 (COX-2) enzyme which is regulated during the inflammatory process and has the ability to induce apoptosis in macrophages. Other studies also concluded that phycocyanin was able to inhibit the expression of iNOS, COX-2, TNF- α , and IL-6 while β -carotene was able to inhibit the production of iNOS, COX-2, TNF- α , and IL-1 β .(Wu *et al.*, 2016; Sorg *et al.*, 2017).

Conclusion

The combination of *H. scabra* : *S. platensis* extract with 1:2 ratio has the potential to be used as an anti-inflammatory agent with anti-inflammatory efficacy nearly similar to diclofenac sodium.

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