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Reducing Activity of Interleukin-8 (IL-8) Extract of Sea cucumber (Holothuroidea sp.) in Experimental Animals Induced by Carrageenan

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Abstract

Interleukin-8 is a pro-inflammatory cytokine that plays a role in the inflammatory response. Sea cucumbers (Holothuria sp.) are used as alternative medicines that have anti-inflammatory activity. The study aims to determination of Sea cucumber (Holothuria sp.) as anti inflammation activty based on capability of reducing interleukin-8 (IL-8) on Rats induced by Carrageenan. Experimental research used was a post-test-only control group design. A total of 25 rats were divided into 5 groups, namely negative control, positive control (Na. Diclofenac), and treatment groups given Sea cucumber extract (doses of 45, 90, and 180 mg/ g BW). Carrageenan-induced mouse paw edema model was used for induction of inflammation. IL-8 of Paw tissue were measured by an ELISA reader at 450 nm wavelength. The result showed that treatrment groups has anti-inflammation activity based on IL-8 levels compared to negative and positive controls. The lowest levels of IL-8 showed at a dose of 90 mg/g BW. Based on One-way ANOVA, there were no significant differences of between groups. Sea cucumber has anti-inflammation activity by reducing IL-8 at all dose tested.
***Keywords**: anti-inflammatory, carrageenan, ELISA, interleukin-8 (IL-8), Sea cucumber extract.*

INTRODUCTION

Interleukin-8 is a pro inflammatory cytokine that plays a role in the inflammatory response. Inflammation is the body's response to pathogen attacks that activate white blood cells, the release of chemical immune systems such as cytokines and inflammatory mediators such as prostaglandins (Hermawati *et al.*, 2021). One of the pro inflammatory cytokines is interleukin-8 (IL-8) which is produced by mono nuclear phagocytes, monocytes, endothelial, lymphocytes with the activity of spurring acute phase protein and netrophil chemotaxis. High IL-8 is a defined as a pro inflammatory cytokine capable of inducing local and systemic inflammation in response to infenction or injury where its concentration will increase rapidly within 1 - 3 hours after the onset of infection (Pakpahan, 2016). Interleukin-8 has an important role related to inflammation. Clinically, inflammation is characterized by symptoms such as redness, feeling warm/hot, swelling, pain, and reduced or impaired function (Akrom and Hidayati, 2020).

Inflammation can be treated with anti-inflammatory agents such as NSAIDs (Non Steroidal Anti-Inflammatory Drugs), but have a risk of side effects that often occur, namely complications in the gastrointestinal tract. Some of the reported side effects of NSAID drugs include aspirin and ibuprofen causing mucosal injury, bleeding in gastric ulcers, disruption of intestinal passage, and gastric perforation (Govender and Brand, 2018). Diclofenac and meloxicam cause dyspepsia and gastrointestinal disorders (Hendera *et al.*, 2015). To reducethese side effects, traditional medicine from natural ingredients as an alternative can be utilized.

One of the natural ingredients have potential as anti-inflammatory is *Sea cucumber (Holothuroidea sp.)*. *Sea cucumber* is one of the marine biota that contains secondary metabolite compounds, namely flavonoids, saponins, and alkaloids (Mitu *et al.*, 2017). Several studies have reported that

the active compounds of sea cucumbers have anti-inflammatory activity and a large bioactive compound in *Sea cucumber* extract, namely saponins (Akerina and Anggari, 2021). Saponins and flavonoids can reduce COX-2 activity by inhibiting the activity of the cyclooxygenase enzyme in converting arachidonic acid into prostaglandins as mediators of inflammation. *Sea cucumber* extract was shown to suppress inflammation and improve the innate immune response (Janakiram *et al.*, 2015). Other ingredients in sea cucumbers, namely glycosaminoglycans, chondroitin sulfate, and omega 3 were found to have efficacy in reducing pain and improving joint function in people with osteoarthritis (Putra *et al.*, 2022) .

Based on previous research, this study aims to determination of anti-inflammation of *Sea cucumber* extract based on capability in decreased IL-8 levels. The anti-inflammatory activity was measured using carrageenan-induced rat paw edema assay (Adeyemi *et al.*, 2002) and IL-8 levels, as acute inflammation were measured using ELISA (Enzyme-Linked Immunosorbent Assay) with a microplate reader at a wavelength of 450 nm (Fadhila *et al.*, 2020).

RESEARCH METHODOLOGY

The protocols was assessed and evaluated by Health Research Ethics Committee of Dr. Moewardi Hospital with approvement number, No. 1.699/IX/HREC/2023. Experiment design used was post-test-only control group design.

Materials and Tools

The materials used were *Sea cucumber* extract from PT Akar Rimba Nusantara Medan, IL-8 kit (Abclonal®), carrageenan (Sigma Aldrich®), Na-diclofenac from Kimia Farma, CMC Na 0.5%, NaCl 0.9% sterile, PBS (Phosphate-buffered saline), protease inhibitor (Abclonal®), and male W*istar* rat aged 2-4 months obtained from STIKES Surakarta. The main equipment used were rat scales, test animal cages, ELISA reader (BioRad), centrifuge (*Gemmy*), glassware (*pyrex*), analytical balance (*Ohauss*), pletysmometer, micropipette (*socorex*), oral sonde needle (local).

Animal handling

Twenty five (25) male *Wistar* rats were acclimatized (adaptation) for 7 days in the Pharmacology Laboratory Faculty of Pharmacy Universitas Muhammadiyah Surakarta. The cage used is a single cage with dimension 42 x 31 x 13 cm and husks as a base in the cage. Rats must be healthy and have no abnormalities, especially in the legs. During the adaptation period, body weight is obsererved regularly and rats were given BRII pellet feed and mineral water. Cleaning of the cage, drinking water bottle at least once a week. *Bedding was* changed twice a week. Test animals were weighed and numbered on the tail, and marked on the right paw.

Preparation of 1% Carrageenan Suspension

Preparation of 1% carrageenan suspension was made by weighing 100 mg of carrageenan homogenized in 0.9% NaCl physiological saline solution up to 10 mL of volumetric flask. Heat until homogeneous.

Preparation of Diclofenac Sodium Suspension

Weighed 10 tablets of Na-diclofenac and averaged then crushed and weighed as much as 13.5 mg dissolved with *suspending agent* (CMC Na 0.5%) until homogeneous to a volume of 25,0 mL.

Preparation of *Sea cucumber* Extract

Extract of *Sea cucumber* (ESC) was carefully weighed as much as 450; 900; and 1080 mg then suspended with CMC Na 0.5% up to 25,0 mL.

Anti-inflammatory activity test

A total of 25 rats were divided into 5 groups. Group I as negative control (CMC Na), group II as positive control (Na. Diclofenac 1.35 mg/g BW), and group III, IV,

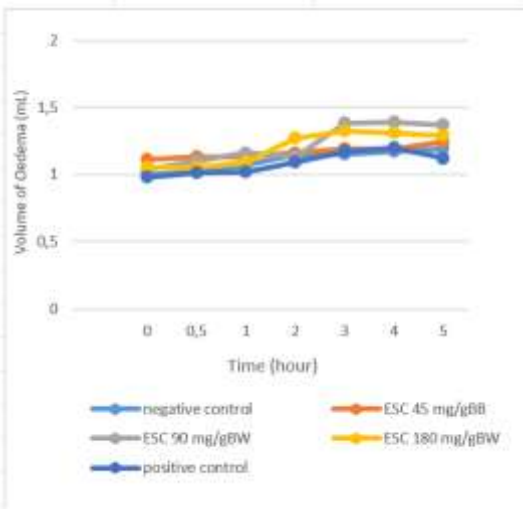

Figure 1. Graph volume of oedema volume after 5 hours of carrageenan induction

V as treatment groups given *Sea cucumber* extract (doses of 45, 90, and 180 mg/g BW). the dose of Na diclofenac and *Sea cucumber* extract was obtained from the conversion of the dose of human use in a day. Rats were fasted for 18-24 hours, mineral water still given and rats were weighed on the day of test. Each group of rats was given the test preparation orally according to the pre-determined dose. After 30 minutes, 0.15 mL of 1% carrageenan was injected subplantarly into the marked right paw. Paw edema was measured with a pletysmometer before and after carrageenan injection for 5 hours starting from the 0, 30, 60, 120, 180, 240, and 300 minutes.

Rats edema paw tissue was cut (Ghorbanzadeh *et al.*, 2015), one gram of paw tissue that had been cut into small pieces was homogenized in 9 mL of PBS solution and then centrifuged at 2400 rpm for 16 min**ute**s. The centrifuged supernatant was taken and added with 20 µL protease inhibitor in order to protect stability of protein during storage in -20^{o} C before measurement.

IL-8 Measurement with ELISA

Prepared all reagents, standard solutions and samples. Reagents are left at room temperature before use, the assay was performed at room temperature. A hundred microliter of samples or standard add to the appropriate number of wells then add 50 µL of enzyme solution to each well and mix well, but not blank well. Cover and incubate 1 hour at 37^{o}C in a humid chamber. Wash each well 5 times with 300 µL 1x wash solution per well. After the last wash invert the plate and dry by tapping on absorbent paper. After that, 500 µL substrate A add to each well followed by addition of 50 µL substrate B. Cover and incubate for 10-15 minutes at room temperature. Add 50 µL of stop solution to each well and mix well. Only wells containing IL-8 and enyme-conjugated antibody will show blue to yellow color change. Changes are measured sprectrophotometrically at a wavelenghth of 450 nm using ELISA Reader.

Statistical Analysis

IL-8 calculated using linear regression equation of calibration curve. IL-8 data then homogenicity and normality were conducted. In order to assessed the anti-inflammatory activity of sea cucumber, One-way-ANOVA was performed.

RESULT AND DISCUSSION

Oedema, formation induced by carrageenan, will produce acute inflammation and does not cause tissue damage (Sukmawati *et al.*, 2015). The advantage of using carrageenan is does not leave marks, can provide a more sensitive response to anti-inflammatory drugs that other irritants so it is suitable to be selected as an inductor of edema (Rifaldy *et al.*, 2019). An increase in paw volume after carrageenan induction indicates that the injection was successful.

In this study, rat paw tissue was used because the results of measurements with rat paw soft tissue were more potent than serum. Based on previous study, the anti-inflammatory effect in carrageenan-induced rats had no effect on serum levels after 5 hours of injection (Ghorbanzadeh *et al.*, 2015). However, there was a significant decrease using paw tissue samples after 5 hours of injection.

The data obtained showed a decrease in the volume of the rat's legs at the 5th hour at the positive control (diclofenac sodium) and the 90 and 180 mg/g BW extract administration groups. The maximum increase in edema occurred at the 4th hour. The negative control and extract dose of 45 mg/g BW did not show a decrease in edema up to 5 hours. The negative control group did not show anti-inflammatory activity. Meanwhile, at the dose of 45 mg/g BW was less effective in reducing edema due to inflammation. The accuracy of measurements of pletysmometer can be influenced by several factors such as the volume of mercury at each measurement, the position of the rat's paw, how to read the scale on the device, or the condition of the rats during measurement. The result showed that induction of 1% carrageenan in rat paws triggered acute inflammation characterized by redness and edema. Carrageenan will stimulate mast cell membrane phospholipids found in the connective tissue around the soles of the paw to secrete arachidonic acid with the help of the enzyme phospholipase A2 to produce various mediator products (Narande *et al.*, 2013). In addition, induction of carrageenan 1% through intraplantar on rat paw can trigger the occurance of local swelling local of the rat's leg accompanied by redness due to he accumulation of inflammatory mediators (Ghorbanzadeh *et al.*, 2015).

Furthermore, to evaluate anti-inflammatory effect the levels of IL-8 was measured. Samples from each group were measured for IL-8 levels by ELISA method. Calibration curve measured obtain linear coefficent with value of R^2 = 0.9377. Moreover, the value of slope is -0.0236x. The minus, indicate that more high concentration more lower the absorbtion value (**Figure 2**).

The IL-8 ELISA kit employs a competitive enzyme immunoassay technique utilizing anti-IL-8 monoclonal antibodies and an IL-8-HRP conjugate. The product of the enzyme-substrate reaction forms a blue complex, then turns yellow after adding the reaction stopping solution. Color intensity was measured spectrophotometrically at 450 nm in a microplate reader. The color intensity is inversely proportional to IL-8 concentration because IL-8 from the sample and the IL-8-HRP conjugate compete for binding of the anti-IL-8 antibody. The advantage of this technique is that sample solutions containing antibodies or antigens do not need to be purified (Santosa, 2020).

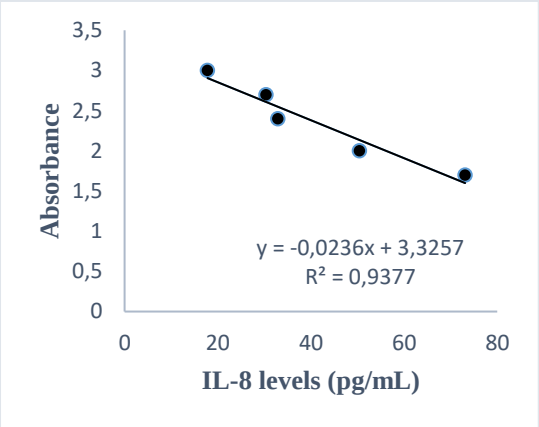

Figure 2. Interleukin-8 (IL-8) standard curve

The results of the anti-inflammatory activity of *Sea cucumber* extract on carrageenan-induced (Table **1**) showed a decrease in IL-8 levels compared to the negative control. These results are in accordance with other studies which state that sea cucumbers have potential as anti-inflammatory. This indicates that the potential anti-inflammatory activity in *Sea cucumber* extract plays a role in reducing the levels of pro-inflammatory cytokines, which in this case is IL-8.

The average IL-8 levels for each treatment can be seen in **Table 1**. The dose variation group of *Sea cucumber* extract showed that IL-8 levels were lower that the negative and positive controls. The lowest level was in the 90 mg/gBB dose of *Sea* cucumber extract with IL-8 levels 9.563 pg/mL.

Table 1. Percentage of Interleukin-8 (IL-8) Levels in Each Group (n=5) (Mean± SD)

Group	IL-8 level (pg/mL) (Mean± SD)[#]
Negative control	14.804 ± 2.298
Positive control	16.937 ± 4.309
ESC 45 mg/g BW	12.992 ± 2.984
ESC 90 mg/g BW	9.563 ± 5.974
ESC 180 mg/g BW	11.547 ± 2.853

[#]ANOVA test results show p>0,05

The decrease in IL-8 levels in the *Sea cucumber* extract group was due to the high content of saponin compounds in sea cucumbers (Sanjeewa and Herath, 2023). The mechanism of saponins in inhibiting pro inflammatory cytokine concentrations is by inhibiting vascular permeability and exudate formation. The content of other compounds in sea cucumbers reported by (Mitu *et al.*, 2017) contains secondary metabolite compounds, namely flavonoids, saponins, and alkaloids. The mechanism of action of flavonoids is by inhibiting the synthesis of eicosanoids so that there is a decrease in the content of arachidonic acid in the phospholipid membrane network of cells which results in inhibition of the release of a number of inflammatory mediators. IL-8 is widely used as a diagnostic and prognostic marker for inflammatory conditions (Bernhard *et al.*, 2021). Other chemical compounds in sea cucumber, namely glycosagminoglycans, chondroitin sulfate, and omega-3 were found to have efficacy in reducing pain and improving joint function in people with osteoarthritis (Putra *et al.*, 2022).

The insignificant results could be due to pro inflammatory cytokines in acute and chronic inflammation, not only IL-8. Pro inflammatory cytokines in the early phase when immunocompetent cells are IL-6 which will then be stimulated by TNF-α. IL-6 is reported to be a good indicator of inflammatory activity in various conditions (Tanaka *et al.*, 2014).

CONCLUSIONS

Sea cucumber has anti-inflammation activity by reducing IL-8 at all dose tested. The lowest levels of IL-8 achived at a dose of 90 mg/g BW of sea cucumber.

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