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Research Article

Anti-Inflammatory Activity of Ethanolic Extract *Melophlus* sp. and *Callyspongia* sp. from Southeast Sulawesi

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Abstract

Marine sponges display significant potencies as an anti-inflammatory agent. Thus, this study aims to investigate the effect of anti-inflammatory of *Melophlus* sp. and Callyspongia sp. by decreasing plasma IL-1β levels of rats. This study was conducted by detecting chemical constituents of extracts and their anti-inflammatory by measuring the plasma IL-1β level of animals. Animals were acclimatized for seven days, followed at day-8 animals were induced by 1% carrageenan injection intraplantar. Animals were divided into ten groups (n=4) and treated orally according to groups which were C+, C-, M50, M100, M200, K+, K-, C50, C100, and C200. After 1 hour, blood was collected at the first, second, and third hour. Blood samples were then centrifugated and assayed with ELISA kit Rat IL-1β. Data collected were statistically analyzed. Both marine sponges contain flavonoid, saponin, alkaloid, and terpenoid. Tannin was only detected in *Melophlus* sp. Both marine sponges provided an effect in decreasing plasma IL-1β at the concentration of 100 ppm and 200 ppm. In conclusion, extracts of *Melophlus* sp. and *Callyspongia* sp. have anti-inflammatory activity with effective concentration are 100 ppm.

Keywords: Anti-Inflammatory, *Callyspongia* sp., *Melophlus* sp., Plasma IL-1β

Aktivitas Antiinflamasi Ekstrak Etanol *Melophlus* sp. dan *Callyspongia* sp. yang berasal dari Sulawesi Tenggara

Abstrak

Spons laut menunjukkan potensi besar sebagai agen antiinflamasi, sehingga penelitian ini bertujuan untuk menyelidiki efek antiinflamasi *Melophlus* sp. dan *Callyspongia* sp. dalam mengurangi kadar Interleukin-1β plasma tikus. Penelitian ini dilakukan dengan mendeteksi kandungan kimia ekstrak dan anti-inflamasi dengan mengukur tingkat IL-1β plasma hewan. Hewan diaklimatisasi selama 7 hari, diikuti pada hari ke-8 hewan diinduksi dengan injeksi karagenan 1% intraplantar. Hewan dibagi menjadi 10 kelompok (n = 4) dan diperlakukan secara oral sesuai kelompok, yaitu C+, C-, M50, M100, M200, K+, K-, C50, C100, and C200. Setelah 1 jam, darah dikumpulkan pada jam pertama, kedua, dan ketiga. Sampel darah kemudian disentrifugasi dan diuji dengan ELISA kit Rat IL-1β. Data yang dikumpulkan dianalisis secara statistik. Kedua spons laut mengandung flavonoid, saponin, alkaloid, dan terpenoid. Tannin hanya terdeteksi dalam *Melophlus* sp. Kedua spons laut memberikan efek dalam menurunkan IL-1β plasma pada konsentrasi 100 ppm dan 200 ppm. Kesimpulannya, ekstrak *Melophlus* sp. dan *Callyspongia* sp. memiliki aktivitas antiinflamasi dengan konsentrasi efektif 100 ppm.

Kata Kunci: Anti-inflamasi, *Callyspongia* sp., *Melophlus* sp., IL-1β plasma

1. Introduction

Inflammation is a host's response involving cells, vascular, protein, and mediators in the elimination of inflammatory agents such as infection, trauma, tissue necrosis, immune reaction, and systemic disease and to begin the process of tissue healing. Inflammation is a self-limiting condition if these factors are eliminated[1]—the pro-inflammatory cytokines such as IL-1β implicated in promoting the inflammatory process. Monocytes and macrophages secrete the IL-1β during cell injury, and infection leads to inflammation. The IL-1β is secreted by fibroblast and endothelial cells as well. The IL-1β increases the production of prostaglandin E2 (PGE2) involved in pathological pain.[2] In most cases, the inflammatory process can turn into permanent damage toward healthy tissue.[3] Thus, the anti-inflammatory agent needed to prevent these irreparable tissue damage.

Nowadays, few studies reported the identification of natural sources as anti-inflammatory agents, notably from marine biotas such as algae, sea cucumber, and marine sponges. Intensive studies of marine biotas are conducted domestically and abroad. The biotas contain more active compounds compared to terrestrial sources; one of them is marine sponges. About 10.000 species of marine sponges in the world and Indonesia alone are estimated at around 850 to 1.500 species of marine sponges.[4,5] Melophlus sp. and Callyspongia sp. are the marine sponge that can be found in the vast Indonesian sea. They can be utilized and developed as a novel anti-inflammatory agent.

Recent evidence suggests that *Melophlus* sp. (Figure 1 (a)) and *Callyspongia* sp. (Figure 1 (b)) contain secondary metabolites. Marine sponge *Melophlus* sp. contains alkaloids, flavonoids, saponins, tannins, and terpenoids.[6,7,8] The metabolites affect as antibacterial, antifungal, antiplasmodial, cytotoxic activity and anticancer, inhibiting aggregation of platelets, and antihyperlipidemic.[9,10] Also, with marine sponge *Callyspongia* sp. contains alkaloids, flavonoid, and terpenoid/ steroid, as well as callyaerins and diketopiperazine, which have activity as antimicrobials, antioxidants, antituberculosis, antitumor, anticancer, and anti-inflammatory.[7,10,11,12,13,14,15,16,17,18]

Melophlus sp. and Callyspongia sp. have abundant potential as anti-inflammatory agents due to their chemical constituents, which act as an anti-inflammatory. Many studies reported the activity of these marine sponges as anti-inflammatory agents; however, the study of a specific biomarker in inflammatory such as IL-1β still lacks, especially in Southeast Sulawesi's Sea.[17,19] Thus, the study aims to investigate the effect of ethanolic extract of *Melophlus* sp. and *Callyspongia* sp. in decreasing plasma IL-1β level, an inflammatory biomarker, toward Wistar male rat.

2. Method

2.1. Tools

The tools used include rotary vacuum evaporator (Rotavapor, Buchi®), blender (Philips), analytical balance (Precisa®), Erlenmeyer flask (pyrex), hot plate (Stuart®), waterbath, centrifuge, EDTA-tube, and ELISA Rat IL-1β (Elabscience®) Kit.

2.2. Materials

The materials used include marine sponge Melophlus sp. and Callyspongia sp., Wistar male rats, distilled water, 96% ethanol (Mercks®), 0.5% Na CMC (Mercks®), 1% carrageenan (Mercks®), and pellet chow. All solutions used were analytical grade.

2.3. Methods

2.3.1. Sponge Collection

Marine sponge *Melophlus* sp. and *Callyspongia* sp. obtained from Bintang Samudra Marine Edu-Park, Soropia Sub-District, Konawe District, Southeast Sulawesi at January 6th, 2019 (10.00-14.00 WITA). Marine sponge collected by diving with SCUBA (Self Contained Underwater Breathing Apparatus) at the reef slope (70º) at depth 10 m. The samples then put in an icebox filled with ice gell and moved into the freezer (-20ºC) for further evaluation.

2.3.2. Sponge Preparation and Extraction

Marine sponge *Melophlus* sp. (2.8 kg) and *Callyspongia* sp. (300 g) were wet sorted, running under running cold water and cut into pieces by using scissors. After that, each sponge macerated with 96% ethanol for three days in a sealed jar. After three days, the extract collected then concentrated by using a rotary evaporator at temperature 50ºC. The concentrated extract yielded 11.2 g and 1.3 g, respectively.

2.3.3. Animal Preparation

Wistar male rats (2-3 months) weigh 200-250 g, obtained from an animal farm in Surabaya. Animals (n=20) were put in a standard environment with controlled temperature (22±2ºC) for seven days for acclimatization purposes. Animals were free to access to food and water ad libitum. Animals were used in the study conducting following the Animal Ethics Committee of Halu Oleo University (No: 2685b/UN29.20/PPM/2018)

2.3.4. Chemical Screening

Chemical screening is conducted to determine the secondary metabolite contained in both marine sponge *Melophlus* sp. and *Callyspongia* sp. The secondary metabolites are flavonoid, saponin, tannin, alkaloid, and terpenoid.

1. Flavonoid

2 mL of sample put in the tube, added concentrated HCl and 0.2 g Magnesium powder. The result is positive if there is discoloration in dark red.

2. Saponin

2 mL of sample put in a tube added with distilled water and boiled for 2-3 minutes. Then, it cooled at the temperature room. The tube then shaken for 15 seconds. The result is positive if it forms stable foam for at least 10 minutes.

3. Tannin

1 mL of sample put in the tube and added 2-3 drops of $FeCl_3$ 1%. The result is positive if there is discoloration into bluish or greenish black.

4. Alkaloid

2 mL of sample added three drops of Dragendorff reagent. The result is positive is if it forms an orange deposit at the bottom of the tube.

5. Terpenoid

1 g of sample was added into ethanol and dissolved into the tube. Then, 0.5 mL acetate acid anhydrate and 2 mL concentrated HCl added into the tube. The result is positive if there is discoloration into the red.

2.3.1. Measurement of Plasma IL-1β Level *in vivo*

At day-8, animals were inducted with 100 µL 1% carrageenan injection intraplantar. Animals (n=40) then divided into 10 groups (n=4), which are C+, C-, M50, M100, M200, K+, K-, C50, C100, and C200. All groups treated orally, as following:

K+ : Positive Control (Diclofenac sodium (Flamar®) 3598 ppm)
K- : Na CMC 0.5% as vehicle (Negative Control)
M50 : Marine sponge Melpohlus sp. 50 ppm
M100 : Marine sponge Melpohlus sp. 100 ppm
M200 : Marine sponge Melpohlus sp. 200 ppm
C50 : Marine sponge Callyspongia sp. 50 ppm
C100 : Marine sponge Callyspongia sp. 100 ppm
C200 : Marine sponge Callyspongia sp. 200 ppm

After 1 hour, blood collected from the animals conducted. For the first and second hour measurement, blood collected by tail vein sampling, and for the third-hour measurement, blood collected by cardiac puncture. Thirty minutes after blood collection, blood samples were put in EDTA-tube and centrifuged at 1000 rpm for 15 minutes at temperature 4ºC. Plasma IL-1β levels assayed by ELISA kit Rat IL-1β.

2.3.2. Statistical Analytic

Decreased plasma IL-1β and varied concentrations of extract Melophlus sp. and Callyspongia sp. correlation were analyzed

statistically using SPSS. Data were analyzed by One-Way ANOVA test and followed by Post Hoc Test (LSD).

3. Result

Melophlus sp. and *Callyspongia* sp. contain secondary metabolites that exhibited in Table 1.

The anti-inflammatory activity of both sponges in 3 times measurement is exhibited in Figure 1 and Figure 2.

4. Discussion

Injection of 1% carrageenan induces an inflammatory response characterized by edema at the site of injection. Lipopolysaccharide (LPS) in carrageenan stimulates macrophage-mediated by inflammatory response to release pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, which exert a vascular endothelial effect at the inflammatory site [20]. The release of IL-1β by macrophages or monocytes occurs after stimulated by LPS that is recognized by TLR (Toll-Like Receptor), whose levels will elevate for 15 minutes to 3-4 hours and decreased gradually due to the half-time of IL-1β mRNA is short.[21] LPS, known as PAMP (Pathogen-Associated Molecular Patterns), binds to cellular receptor Pattern Recognition Receptors (PRRs) in phagocytes (macrophages, neutrophils, and dendritic cells) and epithelial cell thus innate immunity only recognize external antigen, not the body itself.[22]

Chemical screening showed that *Melophlus* sp. and *Callyspongia* sp. contain secondary metabolites, presented in Table 1. Both *Melophlus* sp. and *Callyspongia* sp. contain alkaloids, flavonoids, saponin, and terpenoid. Tannin was only detected in Melophlus sp. These results were along to a few pieces of evidence reported earlier.[6,7,8,10,18]

Also, *Melophlus* sp. containing tetramic acid (melophlin) and depsipeptides (papuamide E and F), agents that have cytotoxic activity as anticancer.[23,24,25,26] Marine sponge *Callyspongia* sp. contains callyspongidiol, which acts in dendritic cell (DC) activation, increasing IL-4 production, as a suppressor of IL-1β-releasing.[27] Besides that, callyspongia sp. contains diketopiperazine, which acts as an anti-inflammatory agent by inhibiting pro-inflammatory IL-1β-releasing from macrophage.[28]

Melophlin A decreased the activity of mitogen-activated protein (MAP) kinase, playing a role in the transduction of signals proliferation and differentiation of cancer cells. MAP kinase is a protein kinase that phosphorylates the NF-κB transcription factor, thereby inducing the expression of pro-inflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α. The pro-inflammatory cytokines production is inhibited indirectly by inhibiting MAP kinase.[29,30,31]

Alkaloids and terpenoids act as anti-inflammatory agents by inhibiting the activation of the NF-κB transcription factor. Thus, the pro-inflammatory cytokine will not be produced. Besides, alkaloid suppresses lymphocyte proliferation, NK, histamine-releasing by mast cell, secretion of cytokine IL-1, and activity of platelet-activating factor resulting in disruption of the inflammatory response.[32,33,34] Flavanoids inhibit the activity of cyclooxygenase-2 (COX-2) and lipoxygenase (LOX) enzymes, inhibit leukocyte migration and neutrophil

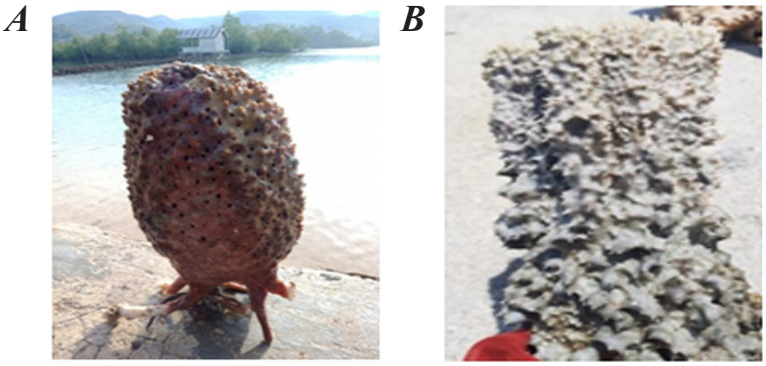

Figure 1. Marine Sponge: (a) *Melophlus* sp.; (b) *Callyspongia* sp.

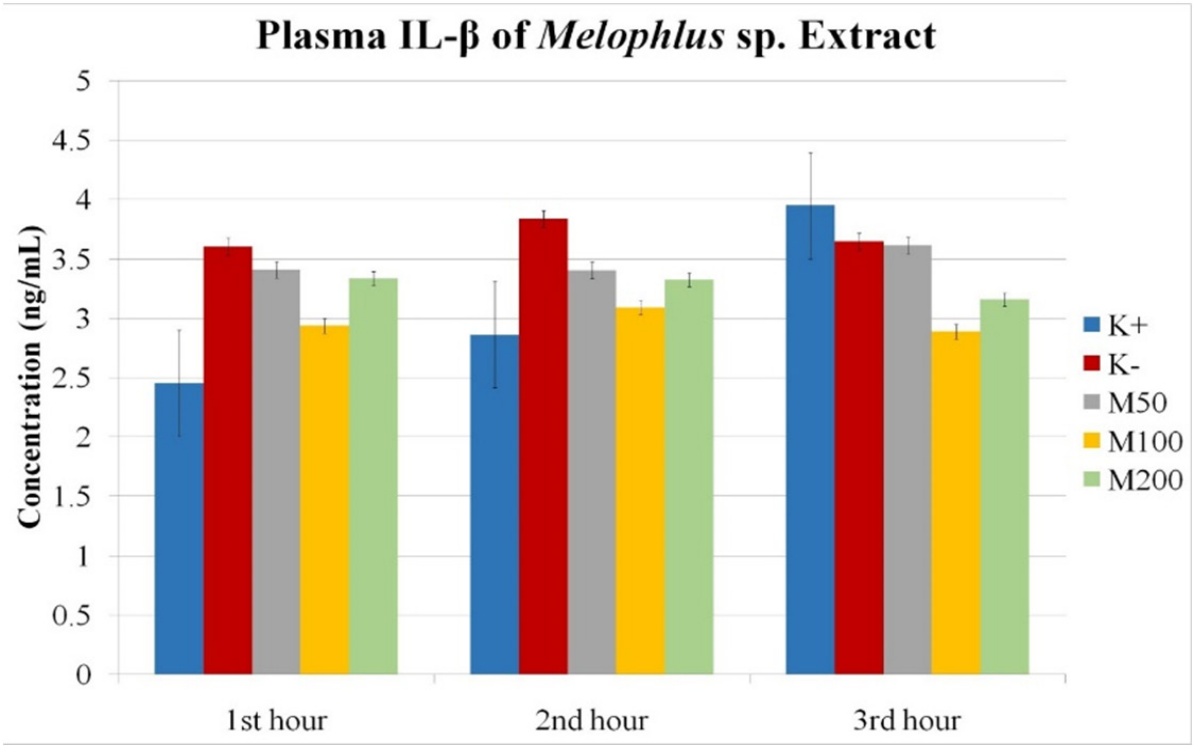

Figure 2. The Decreased of IL-1β at 1st Hour, 2nd Hour, and 3rd Hour post-inducing 1% Carrageenan at Wistar Male Rats Rats of ethanolic extract of *Melophlus* sp. Data is presented in mean ± SD (control + (K+), n = 4; control -, control – (K-), n = 4; M50, n = 4; M100, n = 4; and M200, n = 4)

degranulation, inhibit histamine-releasing by mast cell, and stabilize the reactive oxygen species (ROS).[35] Saponins are reported to have acted as anti-inflammatory agents. However, the mechanism is still unclear. Saponins inhibit the increase of vascular permeability, production of NO, PGE2, and TNF-α.[36] Saponins have characteristics detergent-like, resulting in inhibition of cytokine pro-IL-1β changes into the active form, IL-1β.[37] Moreover, tannins act as anti-inflammatory agents through the mechanism of inhibiting the expression of inflammatory mediators such as cytokines, inducible nitric oxide synthase (iNOS), and COX-2.[38]

The positive control used in the study was diclofenac sodium. Diclofenac sodium is NSAID (Nonsteroidal Anti-inflammatory Drugs), which acts as an anti-inflammatory, antipyretic, and an analgesic that absorbed at gastrointestinal wholly and rapidly. Diclofenac sodium inhibits cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) enzymes, thus inhibiting the synthesis of prostanoid like prostaglandin (PG)-E2, PGD2, PGF2, prostacyclin, and thromboxane (TXA2) from arachidonic acid.[39] Besides that, diclofenac sodium increases the production of IL-10, cytokine anti-inflammatory, and decreases pro-inflammatory cytokines TNF-α. The IL-10 inhibits inflammatory mediators-releasing from macrophage or monocyte induced by LPS, such as TNF-α, IL-1β, Il-6, IL-8, G-CSF, and GM-CSF.[40] Positive control on third-hour measurement showed elevated plasma IL-1β due to half-time of diclofenac sodium is 1-3 hours. Thus it cannot suppress IL-1β production from inhibition of IL-10.[41]

The measurement of plasma IL-1β level conducted using Kit ELISA Rat IL-1β. According to Figure 2, negative control (C-) provided the highest plasma IL-1β level

Table 1. Chemical screening of Melophlus sp. and Callyspongia sp.

Marine Sponge	Flavonoid	Saponin	Tannin	Alkaloid	Terpenoid
Melophlus sp.	+	+	+	+	+
Callyspongia sp.	+	+	-	+	+

compared to other groups, and the lowest plasma IL-1β level is positive control (C+) at first and second-hour measurement, followed by extract Melophlus sp. 100 ppm (M100), 200 ppm (M200), and 50 ppm (M50), consecutively. The third-hour measurement, a higher level of plasma IL-1β, was positive control (C+), and the lowest is M100, followed by M200 and M50.

Extract of *Melophlus* sp. 50 ppm (M50) compared negative control showed no significant difference ($p>0.05$) at first-hour, second-hour, and third-hour measurement concluded ethanolic extract of *Melophlus* sp. is ineffective as an anti-inflammatory agent (Figure 2). It showed a significant difference to M100 and M200 as well ($p<0.05$)

Extract of *Melophlus* sp. 100 ppm (M100) compared to negative control showed a significant difference ($p<0.05$) at first-hour measurement and second-hour measurement; it also did not show significant differences with positive control at second-hour measurement, but showed a significant difference with positive control at third-hour measurement. Thus it was concluded that M100 effectively decreased inflammatory response. Extract of Melophlus sp. 200 ppm (M200) compared to negative control showed a significant difference only at second-hour measurement, concluding that ethanolic extract of *Melophlus* sp. effective as an anti-inflammatory agent (Figure 2).

Both M100 and M200 showed decreased plasma IL-1β, which M100 decreased the plasma IL-1β at first and second-hour measurement, and M200 was only decreasing the plasma IL-1β at second-hour measurement. Both extracts compared statistically did not show significant differences in decreasing plasma IL-1β level ($p>0.05$) at first and second-hour measurement, thus concluding the lowest dose as the effective dose is M100.

According to Figure 3, the lowest to the highest level of plasma IL-1β were positive control (K+) followed by extract of *Callyspongia* sp. 200 ppm (C200), negative control (K-), extract of *Callyspongia* sp. 100 ppm (C100), and extract of *Callyspongia* sp. 50 ppm (C50) in sequence at first-hour measurement. The second-hour measurement, the lowest to highest plasma IL-1β levels, were K+, C200, C100, C50, and K-, respectively, and the third-hour measurement is C100,

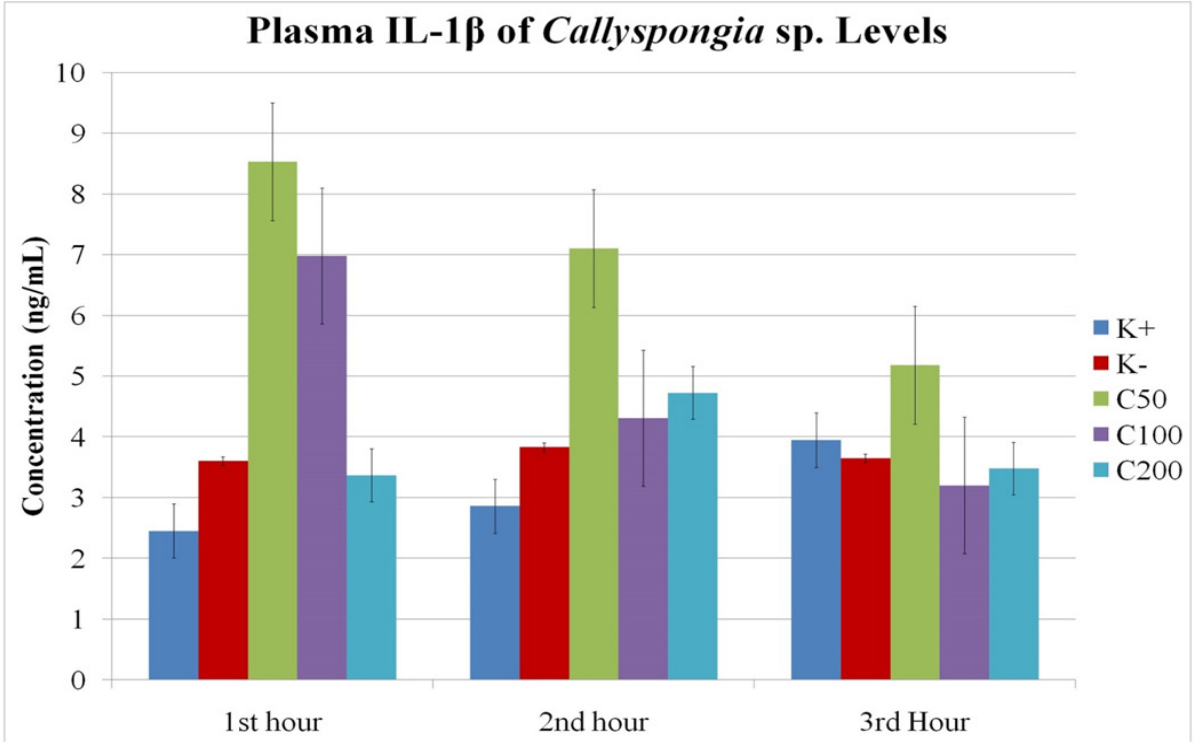

Figure 3. The Decreased of IL-1β at 1st Hour, 2nd Hour, and 3rd Hour post-inducing 1% Carrageenan at Wistar Male Rats of ethanolic extract of *Callyspongia* sp. Data is presented in mean ± SD (control + (K+), n = 4; control – (K-), n = 4; C50, n = 4; C100, n = 4; and C200, n = 4)

C200, K+, K-, and C50, respectively.

Despite the extract of *Callyspongia* sp. 50 ppm (C50) compared with negative control significant differences at first, second, and third-hour measurement of plasma IL-1β level ($p<0.05$), they were having higher levels than others, thus concluding that ethanolic extract of Callyspongia sp. 50 ppm has no activity as an anti-inflammatory by decreasing plasma IL-1β level (Figure 3).

Extract of *Calyspongia* sp. 100 ppm (C100) at first and second, and third-hour measurement showed significant differences to K+ and K- ($p<0.05$), although only the first-hour measurement showed that level of C100 was higher than K-. It means that C100 works as an anti-inflammatory by decreasing plasma IL-1β at second-hour post-treated with carrageenan. Although the C100 also works better than K+ at third-hour post-treated, it was not significant ($p>0.05$) (Figure 3). Extract of *Calyspongia* sp. 200 ppm (C200) showed a significant difference to K- at second and third-hour measurement ($p<0.05$), and works better than K+ at third-hour measurement, although it was not significant in decreasing plasma IL-1β levels ($p>0.05$) (Figure 3).

Extract of *Callyspongia* sp. 100 ppm and 200 ppm at second-hour measurement statistically analyzed showed no significant differences ($p>0.05$), concluding that the effective dose of the extract is the ethanolic extract of *Callyspongia* sp. 100 ppm.

5. Conclusion

Both ethanolic extract of *Melophlus* sp and *Callyspongia* sp. can decrease plasma IL-1β level, and their effective concentration is 100 ppm.

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