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Research Article

ANTI-INFLAMMATORY ACTIVITY OF MARINE SPONGE *CALLYSPONGIA* SP. AND ITS ACUTE TOXICITY

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ABSTRACT

Objective: This study aims to investigate the anti-inflammatory effect of the ethanolic extract of *Callyspongia* sp. using stabilization of the human red blood cell (HRBC) membrane method and its acute toxicity using brine shrimp lethality test (BSLT) method.

Methods: *Callyspongia* sp. was macerated with 96% ethanol. Extract characterized and screened for the secondary metabolite. Anti-inflammatory activity by stabilization of the HRBC membrane method with a varied dose of 50 ppm; 100 ppm; 200 ppm; 400 ppm; 800 ppm; 1600 ppm; and 3200 ppm. Solutions observed using a photometer to describing stability and ability in preventing membranes hemolytic and statistically analyzed using SPSS. Acute toxicity carried out by the BSLT method and analyzed using Minitab®ver. 17.2.1.

Results: The phytochemical screening was indicating that *Callyspongia* sp. contains flavonoid, alkaloid, and terpenoid. The results of the anti-inflammatory activity test showed that the percentage value of stability and hemolysis of extracts with doses of 50, 100, 200, 400, 800, 1600, and 3200 ppm were 55% and 45%, 63% and 37%, 70% and 30%, 74% and 26%, 80% and 20%, 87% and 13%, and 97% and 3%, respectively. It showed that extract of sponge *Callyspongia* sp. in all varied dose has activity in stabilizing the HRBC membrane thus can be potential as an anti-inflammatory. The results of acute toxicity assay showed that the value of LC_{50} was 1281.45 µg/ml and categorized as nontoxic to *Artemia salina* Leach.

Conclusion: Various concentrations of *Callyspongia* sp. effective as an anti-inflammatory in stabilizing HRBC, and categorized as safe.

Keywords: Anti-inflammatory, *Callyspongia* sp., Red blood cell membranes.

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INTRODUCTION

Inflammation is a response of host against to antigen which can cause tissue injury such as infection and burns hence the antigen will not spread. Inflammation is characterized by cardinal signs which are rubor, tumor, calor, dolor, and functiolesia. Inflammation is an important in the healing process however it can disturb activities if suffered for long term. Thus, the anti-inflammation agent is needed to decrease and stop the inflammation process [1].

Callyspongia sp. is one of marine sponges which can be found in Indonesia's abundant sea. *Callyspongia* sp. has activity as antibacterial, antifungal, promoting antitumor, antiretroviral, and anti-inflammation [2,3]. Previous studies showed that *Callyspongia* sp. contains triterpenoid, steroid, alkaloid, and flavonoid [4-6]. Flavonoid and terpenoid contained in *Callyspongia* sp. potentially have anti-inflammatory activity by its ability to stabilize the membranes. Human red blood cell (HRBC) membranes can be used as a parameter for *in vitro* anti-inflammatory activity due to its similarity with lysosome membranes, which are responsible for the inflammatory process [1,7].

Callyspongia sp. can be developed as a new alternative in treating inflammation because commercial anti-inflammation drugs such as diclofenac sodium if consumed for long term has side effects. However, each study that involved natural products that have potency as drug or empirical used as drug, need pre-clinical toxicity acute assay to predict the safety following other pharmacological tests [8]. Many previous studies focus in researching marine sponges in each aspect, yet remains have no study reported about anti-inflammatory properties of *Callyspongia* sp. using stabilization of the HRBC membrane method. Therefore, this study aims to investigate the anti-inflammatory activity of the ethanolic extract of *Callyspongia* sp. with stabilization of the HRBC membrane method and its acute toxicity acute to *Artemia salina* Leach larvae.

METHODS

Marine sponge extract

Marine sponge of *Callyspongia* sp. was determined at Faculty of Fisheries and Aquaculture, Universitas Halu Oleo, Kendari (No. 0087/UN29.12.1.UPP/2018). Marine sponge obtained from Bintang Samudra Edu-Marine Park, Konawe Regency, Southeast Sulawesi with a total of 2.8 kg. Marine sponge then wet sorted and chopped into pieces.

The pieces were macerated with 96% ethanol for 3×24 h. Filtrated obtained was concentrated using rotary vacuum evaporator (Rotavapor, Buchi®) at temperature 60°C and water bath (60°C) yielded concentrated extract 31.52 g (1.12%).

Characterization of extract

Characterization of extract conducted was moisture content and ash content.

Moisture content

2 g of extract was put in the oven (105°C) for 3–5 h then, cooled in desiccators for 30 min. Extract then weighed until constant.

Ash content

2 g of extract put in weighed Kruz (A_0), flamed slowly and raised temperature until 600±25°C. The sample then cooled in desiccators and weighed (A_1).

Phytochemical screening

Phytochemical screenings conducted were flavonoid test, alkaloid test, and terpenoid/steroid test.

Flavonoid test

2 ml of extract put in the tube, added with Mg powder and 1 ml HCl. Thereafter, 1 ml amyl alcohol added.

Alkaloid test

1 mg of extract put in tube added 0.5 ml HCl 2%+2–3 drops Dragendorff reagent.

Terpenoid/steroid test

2 ml of extract put in the tube added Liebermann–Burchard reagent.

Ethical approval

This study conducted accordance ethic issued by the Ethical Committee of Health Research, Halu Oleo University No. 916/UN29.20/PPM/2018.

Anti-inflammatory activity by HRBC stabilization method

1 ml of each varied dose of ethanolic extract of *Callyspongia* sp. (50; 100; 200; 400; 800; 1600; and 3200 ppm) as samples and diclofenac sodium as positive control was added into 1 ml phosphate buffer pH 7.4 (0.15 M), 0.5 ml of red blood cell, and 2 ml of hyposaline solution into tube. Negative control used is 1 ml, isosaline was added into 1 ml phosphate buffer pH 7.4 (0.15 M), 0.5 of red blood cell, and 2 ml of hyposaline.

Each solution incubated at 56°C temperature for 30 min. Afterward, solutions centrifuged at 5000 rpm for 10 min. Hemoglobin was measured using photometer 5010 from the supernatant obtained (λ=560 nm).

Table 1: Characterization of marine sponge *Callyspongia* sp.

Extract characterization		
S. No.	**Moisture content (%)**	**Ash content (%)**
1.	4.5	17

The percentage of hemolysis of HRBC as calculated as follows [9]:

$$\% \text{ Hemolysis} = \left(\frac{\text{Optical density of sample}}{\text{Optical density of control}} \right) \times 100$$

The percentage of stability of HRBC as calculated as follows [9]:

$$\% \text{ Stability} = 100 - \left(\frac{\text{Optical density of sample}}{\text{Optical density of control}} \right) \times 100$$

Toxicity acute assay by brine shrimp lethality test method

10 of *A. salina* larvae in seawater were put in the tube and added 2.5 ml of varied dose sample (25; 50; 500; 1000; and 2000 ppm). Control used is 2.5 ml Blanco solution added 2.5 ml seawater. Each sample replicated 3 times. Then, we observed for 24 h to count the number of live larvae and dead larvae.

$$\text{Percentage of lethality calculated as follow} = \frac{\text{Total of death larvae}}{\text{Total larvae}} \times 100\%$$

If control gives death of larvae, the formula can be corrected using

$$\text{Abbot formula} = \frac{\text{Total of death larvaae (Sample} - \text{Control)}}{\text{Total larvae}} \times 100\%$$

Data collected then analyze using probit analysis with Minitab®ver. 17.2.1

Statistical analysis

Data collected were analyzed by SPSS with Kolmogorov–Smirnov test and Levene test, followed by One-Way ANOVA test (confidence interval=95%) and Least Significant Difference test. Probability level $p<0.05$ indicates significant difference and vice versa.

RESULTS AND DISCUSSION

Characterization of extract

Based on the study conducted, found that moisture content and ash content of marine sponge *Callyspongia* sp. were 4.5% and 17% consecutively. Information about the extract characterization is presented in Table 1.

Moisture content obtained from this study was 4.5% compared with literature; the ideal moisture content of extract is must <10%. Moisture content must comply with specified standards due to the effect of the durability of extract and avoid activity from microbes [10,11].

Table 2: Phytochemical screening of marine sponge *Callyspongia* sp.

Phytochemical screening	Reagent	Reference	Result	Conclusion
Alkaloid	HCl 2%+Dragendorff	Formed red brick, red, or orange color deposit [13]	Formed red color deposit	Positive 
Flavonoid	Mg+concentrated HCl+amyl alcohol	Discoloration into red, orange, or yellow [13]	Discoloration into red	Positive 
Terpenoid/Steroid	Liebermann–Burchard	Discoloration into green or blue for steroid; and red or violet for terpenoid [14]	Discoloration into red	Positive 

Ash content obtained from this study was 17% and if compared with literature is must be <7%. Higher content of ash of extract describes extract contains a higher level of minerals and metals contamination [10,12].

Phytochemical screening

The information about the secondary metabolite of marine sponge *Callyspongia* sp. is presented in Table 2. It concluded that *Callyspongia* sp. contains flavonoid, alkaloid, and terpenoid. The study was in line with the previous study showed that flavonoid, alkaloid, and terpenoid were secondary metabolites that provide anti-inflammatory properties with their own mechanisms [15].

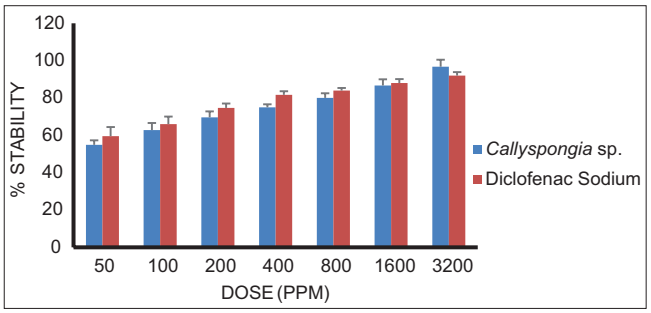

Fig. 1: Percentage of human red blood cell membrane stability. *n=3, data presented in mean±standard deviation. Statistical analysis shows an insignificant value between extract and control (p>0.05)

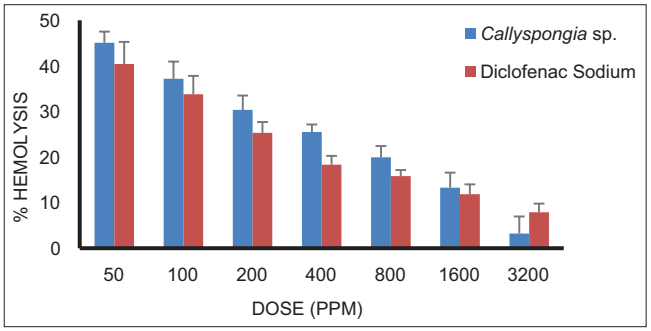

Fig. 2: Percentage of human red blood cell membrane hemolysis. *n=3, data presented in mean±standard deviation. Statistical analysis shows an insignificant value between extract and control (p>0.05)

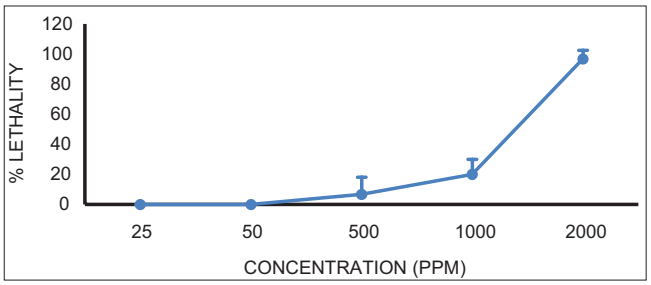

Fig. 3: Percentage of lethality *Artemia salina* larvae. *Values are presented in mean±standard deviation, n=3 with various doses 25 ppm, 50 ppm, 500 ppm, 1000 ppm, and 2000 ppm

Anti-inflammatory activity

Anti-inflammatory activity ethanolic extract of *Callyspongia* sp. conducted to investigate the ability of extract in stabilizing red blood cell membranes. Stabilization of red blood cell membrane method used due to similarity with the lysosome membrane which is involved in inflammation response [1]. Stabilization of lysosome membrane has a role in inhibiting inflammation response due to inflammation mediators released by lysosome that can affect tissue damage and induce inflammation response [16].

The ability of the ethanolic extract of *Callyspongia* sp. in preventing red blood cell hemolysis in the stabilization of red blood cell membrane method is observed from the values of measured absorbance. The value absorbance describes the lysis of the red blood cell membrane. The greater the absorbance value, the greater the lysis occurs and vice versa. The study was done in triplication, with data obtained were presented in mean±standard deviation (SD).

Ethanolic extract of *Callyspongia* sp. at varied dose 50 ppm; 100 ppm; 200 ppm; 400 ppm; 800 ppm; 1600 ppm; and 3200 ppm has activity in stabilizing red blood cell (Figs. 1 and 2). This is proven by the stability percentage value of extract compared with positive control was almost similar to control with value 55–97% and 60–92%, respectively. Besides that, the hemolysis percentage value of extract compared with control was almost similar to value 45–3% and 40–8%, respectively. The maximum dose of 3200 ppm gave a higher effect in stabilizing and preventing hemolysis of the red blood cell membrane with value 97% and 3% consecutively. The minimum dose 50 ppm gave minimum effect in stabilizing and preventing hemolysis of red blood cell membrane with value 55% and 45% consecutively. This illustrates the potency of ethanolic extract of *Callyspongia* sp. as an anti-inflammatory due to its ability to stabilizing and preventing hemolysis of red blood cell membranes [17].

Data collected were statistically analyzed showed that varied dose of extract at 50 ppm; 100 ppm; 200 ppm; 400 ppm; 800 ppm; 1600 ppm; and 3200 ppm compared to positive control (diclofenac sodium) showed no significant difference (p>0.05) in stabilizing the membranes thus concluded that extract and positive control with the same dose above shows similar activity in the stabilization of red blood cell membrane and preventing hemolysis.

The potency of *Callyspongia* sp. in stabilizing membrane possibly due to the presence of secondary metabolite contained in *Callyspongia* sp. such as flavonoid, alkaloid, and terpenoid/steroid. Mechanism of flavonoid is by stabilizing lysosome membrane *in vitro* and *in vivo* by inhibiting cyclooxygenase enzyme and lipoxygenase enzyme who responsible for converting arachidonic acid into prostaglandin and leukotriene [1,18-20]. Alkaloid has ability as anti-inflammatory by preventing the synthesis or action of certain proinflammatory cytokines, suppressing the histamine release, and nitric oxide production [15].

Besides of flavonoid and alkaloid, terpenoid either suspected has a role in stabilizing lysosome membrane by inhibiting cyclooxygenase enzyme

Table 3: Percentage of lethality *Artemia salina* larvae of marine sponge *Callyspongia* sp. and its LC_{50}

Varied dose (µg/ml)	Larvae death			Average	Lethality (%)	LC_{50} (µg/ml)
25 ppm	0	0	0	0	0	1281.45
50 ppm	0	0	0	0	0	
500 ppm	0	0	2	0.6	6	
1000 ppm	2	3	1	2	20	
2000 ppm	9	10	10	9.6	96	
Control	0	0	0	0	0	0

in converting arachidonic acid into prostaglandin as an inflammatory mediator [1].

Acute toxicity

The acute toxicity test was analyzed by probit analyze for observing the correlation of concentration and the mortality rate of larvae. The lethal concentration of the ethanolic extract of *Callyspongia* sp. was more than equal 500 ppm. Dose 25 ppm and 50 ppm showed no lethality to *A. salina* larvae similar to control. Information is presented in Table 3 and Fig. 3.

The LC_{50} of the ethanolic extract of *Callyspongia* sp. was 1281.45 µg/ml (Table 3). The LC_{50} of *A. salina* is declared as toxic if the value of LC_{50} ≤1000 µg/ml, and vice versa declared as nontoxic (safe) if the LC_{50} ≥1000 µg/ml [21]. Based on the value of LC_{50} of the extract concluded that extract *Callyspongia* sp. is regarded as safe or nontoxic.

CONCLUSION

Secondary metabolite found in *Callyspongia* sp. is flavonoid, alkaloid, and terpenoid/steroid. Ethanolic extract of *Callyspongia* sp. has anti-inflammatory by stabilizing membrane at dose 50 ppm; 100 ppm; 200 ppm; 400 ppm; 800 ppm; 1600 ppm; and 3200 ppm. Moreover, *Callyspongia* sp. is nontoxic that the value of LC_{50} is 1281.45 µg/ml.

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AUTHORS' CONTRIBUTIONS

Study concepts: IS, AF, and BS. Study design: IS, AF, and WW. Data acquisition: AF, WW, FM. Quality control of data and algorithms: AF, WW, FM, and LOMJP. Data analysis and interpretation: IS, AF, WW, FM, and BS. Manuscript preparation: IS, AF, LOMJP, and BS. Manuscript editing: IS, AF, and LOMJP. All authors reviewed the manuscript.

CONFLICTS OF INTEREST

The authors declare there are no conflicts of interest.

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