In vivo Anti-arthritic Activity of Soft Coral *Lobophytum* sp. From Southeast Sulawesi in Freund's Complete Adjuvant Induced Arthritis

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Abstract

Rheumatoid arthritis (RA) is a systemic, progressive, chronic autoimmune inflammatory disease that affects tissues, organs, and damages synovial joints. The RA can be treated with DMARD (disease modifying anti rheumatic drugs), such as methotrexate. However, the use of this drug long-term can cause side effects. Soft coral Lobophytum sp. has secondary metabolite compounds such as flavonoids, alkaloids, and terpenoids which has an anti-inflammatory activity, which beneficial for anti-rheumatoid arthritis agent. This study aims to determine the potential of anti RA in Lobophytum sp. using in vitro and in vivo. The anti-inflammatory assay for anti RA in vitro was performed by stabilization of human red blood cell (HRBC) membrane. In addition, anti RA was performed in arthritis model mice by induction of complete Freund's adjuvant (CFA) by administering LEA once daily orally for 15 days. It was found that the ethyl acetate of Lobophytum sp. (LEA) reduced the haemolysis and increased the stability of HRBC membrane. Furthermore, LEA also showed anti RA by decreasing the edema in mouse paw at dose of 50 mg.kg^{-1} BW (LEA50), 100 mg.kg^{-1} BW (LEA100), and 200 mg.kg^{-1} BW (LEA200), respectively with 2.5 mg.kg^{-1} BW of methotrexate as positive control (C+) with P<0.05. Moreover, LEA200 demonstrated highest efficacy and showed no significant different with C+ (P>0.05). In conclusion, our research has shown that LEA has anti-inflammatory for treatment of rheumatoid arthritis from in vitro and in vivo studies.

Keywords: *Rheumatoid arthritis, Lobophytum sp., rheumatoid factor, HRBC*

Introduction

According to the World Health Organization (WHO), the incidence of rheumatoid arthritis (RA) in 2016 reaches 20% of the world's population that impacts approximately 0,5-1% of the adult population and presents two- to three-fold more frequently in women than in men; in Indonesia, in 2020, it will reach 23.3% of the Indonesian population. Based on data from the Southeast Sulawesi Provincial Health Service in 2019, the prevalence of RA was 22.5% (Jaya *et al*., 2022; Black *et al.*, 2023; Venetsanopoulou *et al.*, 2023). As the number of RA sufferers increases in Indonesia, the level of awareness and misunderstanding about this disease is relatively high. This situation explains the lack of knowledge among Indonesian people, especially sufferers, to know more about RA disease (Andri *et al.*, 2020).

RA is an autoimmune-systemic, progressive, and chronic inflammatory disease that affects many tissues and organs but principally damages synovial joints. RA is the most common cause of chronic joint inflammation. This inflammatory process produces an inflammatory response from the synovium (synovitis), causing hyperplasia of synovium cells, excess production of synovial fluid, and pannus formation in the synovium. This inflammatory process often leads to damage to joint cartilage and joint ankylosing (Jahid *et al*., 2023; Tanaka, 2020; Radu and Bungau, 2021). Rheumatoid factor (RF) is useful in RA diagnosis. In RA patients, the level of RF is highly detected (Ingegnoli *et al*., 2013; Motta *et al.*, 2023).

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In addition, T and B cells are key cells of adaptive immunity, while macrophages play an important role in the inflammatory reaction producing various pro-inflammatory cytokines and chemokines involved in the pathogenesis of RA, such as NO, TNF-α and IL-6 (Sun *et al.*, 2020).

The disease-modifying antirheumatic drugs (DMARD) are one of the class drugs that are often used for treating RA, such as methotrexate, leflunomide, and sulfasalazine. This class of drugs is a potent anti-inflammatory. These compounds are often combined with non-steroidal anti-inflammatory drugs or corticosteroids to strengthen their effects. However, the side effects resulting from the use of these drugs are dangerous because they can cause gastrointestinal bleeding, nausea, dyspepsia, and impaired kidney function (Dipiro *et al.*, 2009; Mysler *et al.*, 2021).

Indonesia has vast marine natural resources, including the coral reef ecosystem. The coral reef ecosystem is part of the marine ecosystem, a source of life for various marine biota. Soft corals are part of the coral reef ecosystem that can produce secondary metabolite compounds, which respond to the environment to survive (Susanto *et al.*, 2015; Hartini and Rosalina, 2018). *Lobophytum* sp. is one of the soft corals which mainly found in Indonesia's water. It contains secondary metabolites such as flavonoids, terpenoids, and saponins, which can be used as anti-inflammatories in treating rheumatoid arthritis (RA) by inhibiting the enzymes cyclooxygenase and lipoxygenase, which play a role in treating symptoms of inflammation and allergies (Ahmed *et al.*, 2017; Roy *et al.*, 2019).

The *in vitro* and *in vivo* methods can describe the molecular mechanisms of disease in RA. One of *in vitro* methods to determine the anti-rheumatoid activity is stabilization of human red blood cells (HRBC) membrane since it is very similar to the lysosomal membrane, which is one of the critical factors in reducing the inflammatory response by stabilization of the human red blood cell membrane through hypotonicity-induced membrane lysis is considered an in vitro measure of the anti-inflammatory activity of drugs and plant extracts (Ajithkumar *et al.*, 2020). Meanwhile, the one of the *in vivo* methods that can be conducted is by complete Freud's adjuvant (CFA)-induced arthritis model. CFA is considered the most reliable model for anti-arthritis drug estimation. CFA-induced RA is characterized by rapid activation of macrophages and secretion of various enzymes into the circulation (Li *et al.*, 2018; Grötsch *et al.*, 2019).

The studies of soft coral *Lobophytum* sp. for anti-rheumatoid arthritis is still limited, specifically in stabilizing the HRBC membrane and in animal model. Thus, investigation of anti RA in *Lobophytum* sp. might be alternative in discovering new agent with minimal side effect. Based on the description above, this study aims to determine the anti-rheumatoid arthritis activity of *Lobophytum* sp. ethyl acetate extract in vitro using the HRBC method with the complete Freund's adjuvant induction method in vivo.

Materials and Methods

The sample used in this research was *Lobophytum* sp. which was obtained from Bintang Samudra Marine Education Park, Soropia District, Konawe Regency, Southeast Sulawesi Province. The collection of samples was carried out by diving with SCUBA (Self Contained Underwater Breathing Apparatus) diving equipment and cutting the soft corals from the reef by letting around 10% of corals to let them to regenerate. Then, the samples were sorted, washed, and cut into small pieces for the extraction process.

The *Lobophytum* sp. were macerated for 3 x 24 h at room temperature with 99.5% v/v ethyl acetate (Merck®, Germany) with stirred every 24 h. Then, the macerate was filtered using Whatman filter paper (Cytiva, Sweden) and concentrated by using rotary vacuum evaporator (Buchi®, Switzerland) at 50°C until a crude extract was obtained.

HRBC membrane stabilization method

Fresh blood from healthy volunteers was collected and centrifuged at 3,000 rpm for 10 min at 25°C, followed by washing the cell pellet with isosaline and resuspending the cells in isosaline to obtain 10% v/v isosaline red blood cells. Next, the isosaline-cell suspension (0.5 ml) was mixed with phosphate buffer saline (PBS) pH 7.4 (1 ml), hyposaline solution (2 ml), and ethanol extract (1 ml at concentrations of 25, 50, 75, 100, 125, 150, and 200 $\mu g.ml^{-1}$), and incubated at 56°C for 30 min. Diclofenac sodium (Sigma-Aldrich, USA) was used as a positive control. The mixture was centrifuged at 5,000 rpm for 10 min. The supernatant was measured with a UV-VIS spectrophotometer (Shimadzu UV-1800, Japan) at 560 nm. Hemolysis and HRBC stability were calculated using the following formula:

$$\% \text{ Hemolysis} = \left[\left(\frac{\text{Sample absorbance}}{\text{Negative control absorbance}}\right) \times 100\right]$$

$$\% \text{ Stability} = 100 - \left[\left(\frac{\text{Sample absorbance}}{\text{Negative control absorbance}}\right) \times 100\right]$$

Induction of Arthritis

The test animals used in this study were 24 male white mice (*Mus musculus*) weighing 20-30 g.

Mice were first acclimatized for 7 days in a standard environment and given food and water ad libitum. Then, mice were induced with 0.1 ml of CFA (complete Freund's adjuvant) (Sigma-Aldrich, USA) intraplantarly, except for the normal group. The edema was measured at day 0 and day 17.

Experimental groups

A total number of twenty-four (24) mice divided into six groups, which group 1 was untreated group (normal control), group 2 and 3 were negative control (C-) and positive control (C+) group, which were rheumatoid modeling mice and treated with Na-CMC 0.5% and methotrexate 2.5 $mg.kg^{-1}$ BW (Sigma-Aldrich, USA), respectively. In addition, group 4-6 were rheumatoid modeling mice which treated with 50 $mg.kg^{-1}$ BW (LEA50), 100 $mg.kg^{-1}$ BW (LEA100), and 200 $mg.kg^{-1}$ BW (LEA200) of ethyl acetate extract of *Lobophytum* sp. They were treated once daily orally for 15 days (day-17 to 31).

Edema thickness measurement

Edema thickness measurements were performed on the day 17 to 31 by measuring the thickness of paw in CFA-induced mice with gauge meter (Sari *et al.*, 2010). The inhibition of RA (% RA inhibition) for each group was calculated as follows (Sari *et al.*, 2010):

$$\%\text{RA inhibition} = \frac{(edema\ volume\ of\ treatment\ group - edema\ volume\ of\ C-\ group)}{edema\ volume\ of\ C-\ group} \text{x}100$$

Rheumatoid factor assay

On the 17th day, a rheumatoid factor test was performed to confirm the rheumatoid arthritis model. Briefly, blood sampling was performed using the retro-orbital bleeds (ROB) method. Then, the blood was centrifuged for 30 min at 3,000 rpm at 4°C, and the supernatant was collected to be analyzed for rheumatoid factor by using a direct latex rheumatoid factor (RF) test to test for antigen agglutination. On the 31st day, the mice were sacrificed, and their blood was taken intracardially. Then, the blood was put into a sterile tube containing EDTA and centrifuged for 30 min at 3,000 rpm at 4°C for 10 min. Then, the blood was analyzed using a direct latex rheumatoid factor (RF) test to test for antigen agglutination.

Statistical analysis

The data obtained was presented as mean±SD and analyzed statistically using the paired t-test and One Way ANOVA (Analysis of Variance) statistical test with a significance of 0.05.

Result and Discussion

Determination of Lobophytum sp.

The soft coral *Lobophytum* sp., was collected from Soropia Water, Konawe Regency, Southeast Sulawesi. Then, they were determined at the Biology Education Department Laboratory, Faculty of Teacher Training and Education, Halu Oleo University, Kendari (no. 2515/UN29.18.1/PG//2021). Soft coral *Lobophytum* sp. was characterized by yellow, greenish-yellow, or gray colony color, finger-shaped upper surface, and wide capitulum located perpendicular to the capitulum surface (Manuputty, 2016).

Anti-inflammatory activity of Lobophytum sp. extract

The *in vitro* anti-inflammatory properties of *Lobophytum* sp. extract were performed by stabilization human red blood cell (HRBC) membrane. The HRBC membrane is analogous to the lysosomal membrane, and its stabilization implies that the extract can also stabilize the lysosomal membrane. Stabilization of the lysosomal membrane is essential in limiting the inflammatory response by preventing the release of lysosomal constituents from activated neutrophils, such as bactericidal enzymes and proteases, leading to further tissue inflammation and impairment of extracellular shedding. In this experiment, red blood cells from healthy volunteers and ethyl acetate extract of *Lobophytum* sp. (LEA) in various concentrations were incubated together in phosphate buffer saline (PBS) pH 7.4 and hyposaline solution. The lysis of HRBC was characterized by hemoglobin which detected at 560 nm. The ethyl acetate was chosen for solvent because of its high volatility, low boiling point, low solubility in water, can be easily removed from a mixture by evaporation, allowing for easy purification of the extracted compounds (Yeung and Stanley, 2010). The results showed that the ethyl acetate extract of *Lobophytum* sp. reduced the hemolysis and increase membrane stability in a dose-dependent manner similar to diclofenac sodium (Figure 1.).

The contents of flavonoid compounds, namely flavonol might contribute in the anti-rheumatoid arthritis activity of *Lobophytum* sp extract. Flavonoid compounds, as anti-inflammatory compounds, act through several pathways, such as inhibiting neutrophil degranulation, inhibiting the activity of cyclooxygenase (COX) and lipooxygenase enzymes so that prostaglandins are not formed, which are inflammatory mediators that cause RA (Pramitaningastuti and Anggraeny, 2017).

Rheumatoid arthritis modeling in mice

The administration of CFA stimulates the phagocytosis and cytokine secretion through

mononuclear phagocytosis, thereby various types of pro-inflammatory cytokines are released (Sudirman *et al.*, 2017). The mice were left until the day-16 to let the mice to experience arthritis with symptoms, namely swelling and redness of the toes, as well as changes in the shape of the soles of the feet (Sari *et al.*, 2010). On the day-17, the thickness of the mouse paw edema was measured using a thickness gauge meter and rheumatoid factor test was performed. The measurement of thickness of edema in mouse's paw aims to determine the effect of CFA in triggering the arthritis in mice, as shown in Figure 2.

The thickness of the edema demonstrates the effect of CFA in causing rheumatoid arthritis. The average thickness of mice paw edema in pre- and post-induction was showing the effect of CFA in inducing rheumatoid arthritis in mice (P<0.05).

Furthermore, the RA model was confirmed by rheumatoid factor (RF) test by using RF-Latex, which was RF test with agglutination latex. The immunoglobulin (IgG) of RF will react the serum and form the agglutination if detected as positive (Siregar, 2019; Beduleva, 2020).

The RF test carried out obtained negative results on the LEA sample. Based on Figure 3(a), the RF test results experienced agglutination because the RF reagent contained latex particles coated with human gamma globulin, which, when mixed with serum, the particles experienced agglutination. In contrast, the negative RF test results were caused by a reaction with antiglobulin and flavonoid compounds. as well as terpenoids contained in *Lobopyhtum* sp. inhibits the enzymes phospholipase and cyclooxygenase which cause the formation of arachidonic acid from phospholipids also to be inhibited (Ahmed *et al.*, 2017).

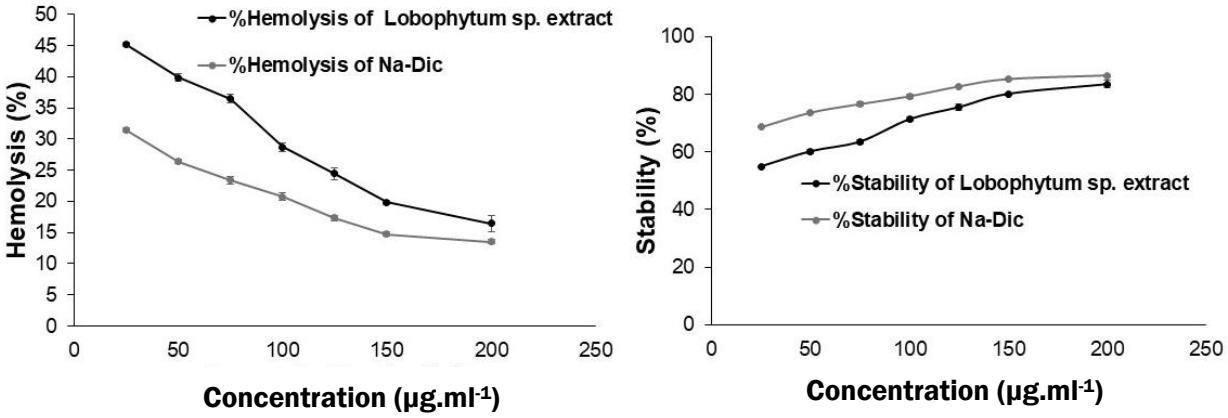

Figure 1. In vitro anti-inflammatory activity tests of ethyl acetate extract of *Lobophytum* sp. in human red blood cells (HRBC). (a) hemolysis activity; and (b) stability of activity. Diclofenac sodium was used as a positive control. Data are presented as mean±SD (n=3).

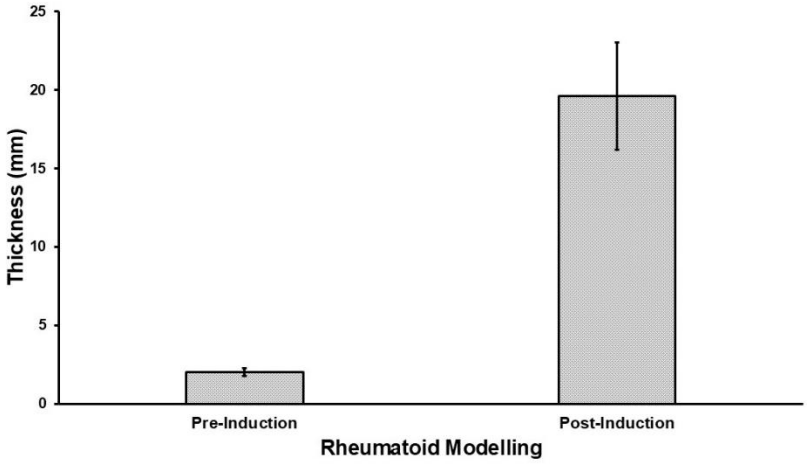

Figure 2. The edema's thickness of mice paws post-CFA induction. Data is obtained by measuring the thickness of the paws. Data are presented as mean±SD (*significantly different: P<0.05)

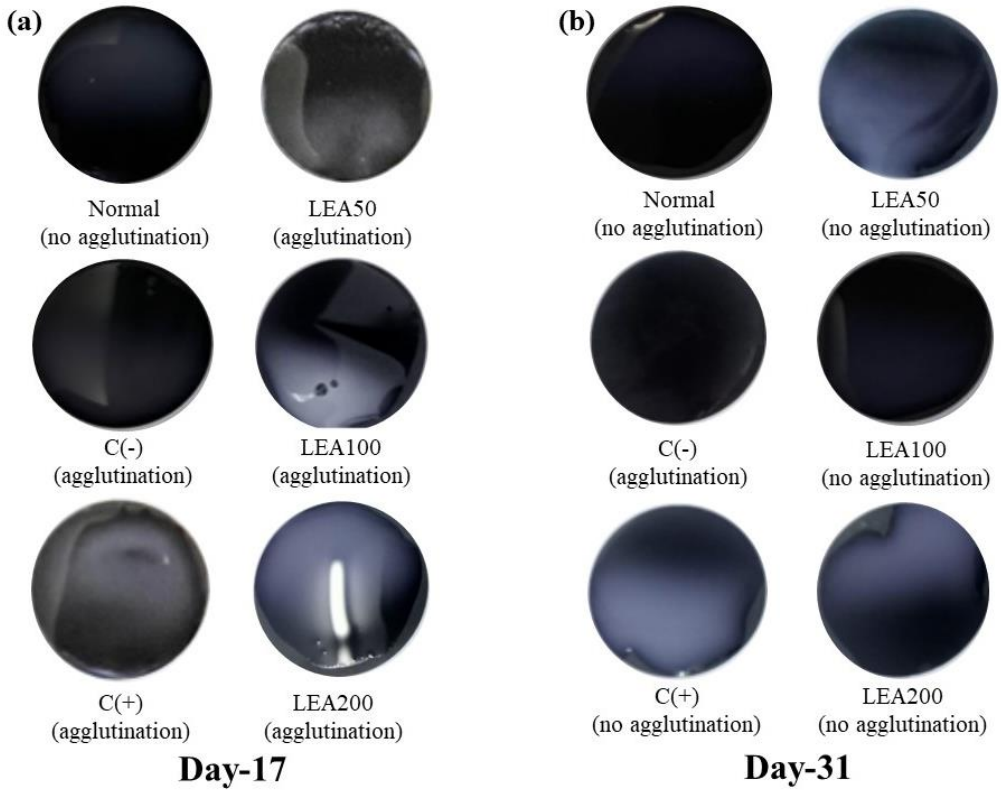

Figure 3. The Rheumatoid factor test in mouse blood is shown by agglutination on (a) day 17 and (b) day 31.

Anti-rheumatoid arthritis activity

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that initially develop in small joints, progress to larger joints, and eventually affects multiple organs. The bones and cartilage of the joint are often destroyed, and the tendons and ligaments weaken in RA patients. This joint damage leads to deformity and bone erosion. Common symptoms of RA include morning joint stiffness for more than 30 min, fatigue, fever, weight loss, painful, swollen, and warm joints, and rheumatoid nodules under the skin (Bullock *et al.*, 2018).

Complete Freund's Adjuvant (CFA) is widely agent used to induce the inflammatory and to study the antirheumatic activity of compounds. To evaluate the antirheumatic effect of the CFA-induced arthritis model can be seen in the reduction in animal body weight, reduced food consumption, body temperature, pain, and leg swelling (Zhang *et al.*, 2017; Navarro-Alvarez *et al.*, 2018). The administration of CFA stimulates phagocytosis and cytokine secretion by mononuclear phagocytosis, releasing metalloproteinase (MMPs) and other mediators, induces interleukin-1 (IL-1), IL-6, IL-8, IL-10, tumor necrosis factor-a (TNF-a), granulocyte-macrophage colony-stimulating factor (GM-CSF) and monocyte chemoattractant protein-3 (MCP-3) release (Sudirman *et al.*, 2017; Ismail *et al.*, 2022). The results showed that the decrease in the thickness of the edema formed on the mice's feet starts from the largest, namely positive control (C+), *Lobophytum* sp. ethyl acetate extract at dose 200, 100, and 50 mg.kg^{-1} BW (LEA200, LEA100, and LEA50), respectively (Figure 4.).

The anti-rheumatoid arthritis effect can be seen from the percentage of rheumatoid arthritis inhibition (Figure 5.). The most significant RA inhibition percentages were C+, LEA200, LEA100, and LEA50, respectively. Moreover, in Figure 3(b) also showed the treatment of LEA showed no agglutination which means the marker of RA is inhibited in the blood of mice. Methotrexate (MTX) as positive control is an anti-metabolite and folic acid analog that has the potential to inhibit dihydrofolate reductase so that it can inhibit purine and pyrimidine synthesis. MTX also reduces the growth and increases apoptosis of monocyte cells, reduces the secretion of IL-1 and IL-6, increases the production of interleukin receptor antagonists, increases the expression of IL-4 and IL-10, decreases the expression of pro-inflammatory cytokines, inhibits the synthesis of cyclooxygenase-2 (COX-2), and neutrophil chemotaxis. In addition, MTX can inhibit

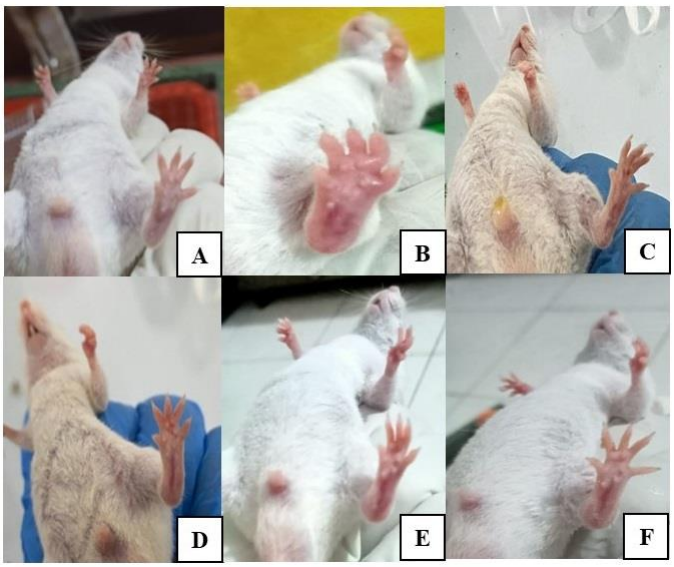

Figure 4. Rheumatoid arthritis model in mice on day 30 after being induced with CFA. (A) normal group; (B) negative control (C-), (C) positive control (C+), treatment group (D-F) LEA 50; 100; 200 mg.kg^{-1} BW (LEA50, LEA100, and LEA200).

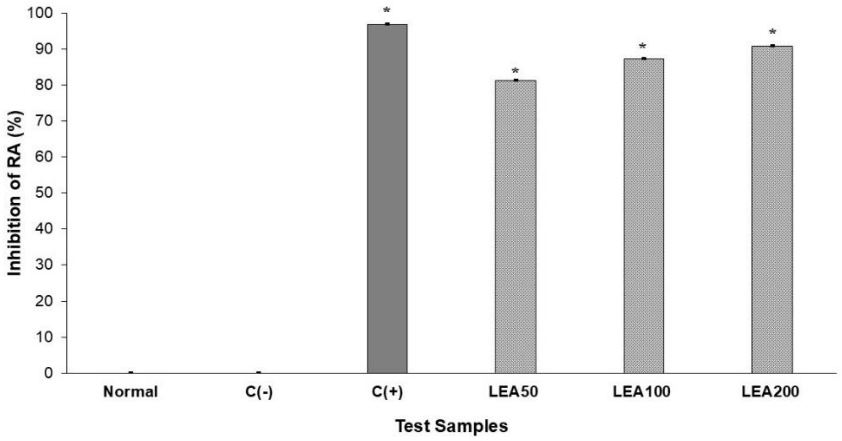

Figure 5. %Inhibition of RA. Data is presented as mean±SD (*P<0.05: significance difference to normal group)

synovial MMP production and stimulate the inhibition of the Tissue Inhibitor of Metalloproteinase (TIMP) (Friedman and Cronstein, 2019).

Based on the One-Way ANOVA test, a value was obtained (*P*<0.05) so that it can be interpreted that there is a significant effect from administration of soft coral *Lobophytum* sp. extract on reducing mouse paw edema. All doses used, which were LEA50, LEA100, and LEA200 were showing good result in suppressing the edema in mouse paw post induction with CFA. Moreover, LEA200 showed no significance different with methotrexate at dose of 2.5 mg.kg^{-1} BW, as positive control. The flavonoid compounds contained in the samples significantly inhibit RA, this result is supported by research conducted by Sun *et al*. (2020), four flavonoid compounds reduced the expression of NO, iNOS, TNF-α, IL-6, IFN-γ, and IL-2. In addition, flavonoid compounds also inhibit the nuclear factor-κB (NF-κB) pathway by reducing the levels of IKK, IκB and NF-κB phosphorylation, and downregulating the expression of iNOS, COX-2, and IL-6 mRNA, downregulating NO secretion and IL-6 (Sun *et al.*, 2020).

Conclusion

Ethyl acetate extract of *Lobophytum* sp. from Southeast Sulawesi's water showed anti-inflammatory properties *in vitro* and *in vivo* which

useful for treating rheumatoid arthritis. It reduced the volume of CFA-induced edema in mouse paw and has anti-rheumatoid arthritis activity by inhibiting rheumatoid factor with no agglutination shown. Apart from that, the extract also increases membrane stability, reducing hemolysis. These results indicated that *Lobophytum* sp. can be used as a new anti-inflammatory agent for rheumatoid arthritis at concentration of 200 $mg.kg^{-1}$ BW. All the results of this study as well as the available literature, show that *Lobophytum* sp. may be a promising agent for the treatment of RA. However, its mechanism for the treatment of RA is still unknown. Therefore, more research on animal and cellular models of RA is needed. Second, more experiments are needed to confirm its anti-rheumatoid arthritis mechanism. Third, further clinical trials are needed to investigate its safety and efficacy. Although this research still has a long way to go to guide clinical use, it is hoped that *Lobophytum* sp. will be a new and effective agent for RA.

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