Anti-Inflammatory Effect of Beta-Beta Wood Ethanolic Extract (Lunasia amara Blanco.) in Mice Model of Rheumatoid Arthritis

Hasnaeni1, Sudarsono2, Nurrochmad A3*, Widyarini S4

1Faculty of Pharmacy, Universitas Muslim Indonesia, Jl. Urip Sumoharjo Km. 5, Makassar, Indonesia-90231.
2Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Sekip Utara Yogyakarta, Indonesia-55281.
3Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Sekip Utara Yogyakarta, Indonesia-55281.
4Department of Pathology Anatomy, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Karangmalang Yogyakarta, Indonesia-55281.

Available Online: 25th October, 2016

ABSTRACT
Rheumatoid Arthritis (RA) causes damage to the joints that are affected by chronic inflammatory diseases. The aim of present study the anti-inflammatory effect of ethanolic extract Beta-beta wood (Lunasia amara Blanco.) in Complete Freund’s Adjuvant (CFA)-induced arthritis in mice. The ethanolic extract of L. amara (ELA) was orally administered once daily for 60 days after sub-plantar hind paw administration with CFA. The ability of the plant extract to reduce swelling as a sign of arthritic inflammation was assessed of volume of hind paw swelling. Tumor necrosis alpha (TNF-α), interleukin 6 (IL-6) and paw histopathology were also determined. The administration of ELA (50; 100, and 250 mg/kg BW) significant reduced volume of edema in mice-treated CFA (P<0.05) and the activity were equal to diclofenac sodium, 2 mg/kg BW. The reduction of edema were last until day 60. The administration of ELA was also significantly decreased the level of serum TNF-α and IL-6 in arthritis model in mice (P<0.05), but still less than diclofenac sodium. Histopathological examination indicated that ELA decrease edema and infiltration of inflammatory cells and synovial hyperplasia as well as protected joint destruction without osteoclast. The result suggests that ethanolic extract of L. amara might be beneficial for the treatment of chronic inflammatory disorder like rheumatoid arthritis.

Keywords: Beta-beta wood (Lunasia amara Blanco.), rheumatoid arthritis, CFA-induced arthritis, Tumor Necrosis Factor alpha (TNF-α), Interleukin (IL-6)

INTRODUCTION
Rheumatoid Arthritis (RA) is chronic inflammatory disease that affects the joints destructive about 0.5 - 1% of the world's population1. Inflammatory disease is very common throughout the world. Joint inflammation in caused by the infiltration of lymphocytes pannus formation and fibrin into the joint2,3,4. RA is usually significant impact on disability and reduced quality of life1. The prevalence of RA in Indonesia known about 0.2% for population of rural areas and 0.3% for the population of the city region5. There are no systematic research on the prevalence, spectrum and patterns of chronic joint disease, but the most common cause of the onset of disability in adults in developing countries6. RA animal model designed to test the potential therapeutic. This model is necessary to evaluate the safety, efficacy and toxicity of treatment for RA. The adjuvant arthritis (CFA) as an experimental model resembles the pathology of RA in histological, pannus formation and a number of angiogenic mediators, including cytokines and growth factors7. The treatment of RA is generally by the using of anti-inflammatory drugs, but no progress can be achieved for a permanent cure. The greatest losses obtained from the synthesis of the potent drug is its toxicity and the reappearance of symptoms after discontinuation of the drug. Various characteristics that are not favorable of drug use today for example the drug's effectiveness is limited in reducing the development of RA disease, a serious side effect, toxic effects of high and high costs1,8,2. Anti-inflammatory drugs reduce inflammation symptoms are temporary, but the disease still develops over time. Besides NSAIDs may induce gastric and toxic to the liver. Medicines for modifying desease anti-rheumatic drugs (DMARDs) e.g methotrexate. The toxicity of Methotrexate is similar to antineoplastic drugs should be restricted in the long term2. Thus the development and discovery of RA drugs derived from nature is very important for treatment of RA diseases in human. Beta-beta (L. amara) is a plant family Rutaceae and has been used traditionally in South Sulawesi Indonesia as a single extract and in a mixture of several herbs. These were used for leg swelling, skin diseases and inflammation or irritation of the eyes. Beta-beta wood (L. amara) is also known in various Indonesian areas, such as in Papua is

*Author for Correspondence: ariefnr@ugm.ac.id
used for ulcers tropical whereas in South Sulawesi this plant with its popular name is Sanrego used as aphrodisiac and also used as an anti-pyretic (fever), help diabetic people and for inflammation (wound healing). In our knowledge of the pharmacological activity of beta-beta wood (L.amara) are still limited, but the pharmacological activity in particular as anti-inflammatory various members of family Rutaceae plant species have been widely known. Plants such as Ruta graveolens L. (Rutaceae) can reduce levels of TNF-α and IL-6 in the inflammatory state. Nitidum Zanthoxylum (Rutaceae) reportedly has the effect of anti-rheumatoid arthritis associated with active compounds such as coumarin. Extract Chloroxylon sweitenia (Rutaceae) also have anti-inflammatory activity at doses that differ in standard animal models.

Several studies have linked the biological activity of plants L. amara, the effect aphrodisiac. Similarly, this plant has an effect on microbial tuberculosis and the results of molecular docking to have anti-cancer effects. In present study, the potential of anti-inflammatory of the ethanolic extract from L. amara in rheumatoid arthritis mice models was done. Study was conducted on mice models of RA induced-CFA and measurement of volume of edema by the use of plethysmometer and the level TNF-α and IL-6 also determined. The histopathological of joint and index arthritis, inflammation also observed. Based on the previous study, L. Amara may offered promising agent for treatment chronic inflammatory disorder like rheumatoid arthritis.

**MATERIAL AND METHODS**

**Plant Material**
The plant (Lunasia amara Blanco.) was collected from South Sulawesi Province, Indonesia. The plant material was identified at Departement of Pharmaceutical Biology Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia, the voucher specimen has been deposited at this Department. The dried wood were powdered and then stored in an airtight container for further use for extraction.

**Extraction**
The extraction process of the L. amara was done by maceration technique. The powder of 1 kg of L. amara was macerated with ethanol 96% for 72 hours. The maceration was performed three times. The ethanolic extracts were filtered before evaporated to dry under reduced pressure at low temperature with a rotary evaporator (Heidolph Instruments GmbH & Co, Schwabach, Germany). The ethanolic extract of L. Amara (ELA) was further lyophilized by a freeze dryer (VirTis BTK, SP Scientific, Gardiner, NY, USA) and use for further study.

**Experimental animal**

Figure 1: Effect of ethanolic extract of L. Amara (ELA) on volume of edema volume in arthritis mice model incuced by CFA. Data represent mean ± standard error of mean. *P<0.05, significantly different with CFA-treated group.
Figure 2: Effect of ethanolic extract of *L. Amara* (ELA) on serum TNF-α in arthritis mice model induced by CFA. Data represent mean ± standard error of mean. *P*<0.05, significantly different with normal group; *P*<0.05, significantly different with CFA-treated group.

Figure 3: Effect of ethanolic extract of *L. Amara* (ELA) on serum IL-6 in arthritis mice model induced by CFA. Data represent mean ± standard error of mean. *P*<0.05, significantly different with normal group; #*P*<0.05, significantly different with CFA-treated group.
Male Balb/c mice 20-30 g BW, weight 20-30 g, 6-8 weeks old were housed under laboratory condition at temperature 25°C, relative humidity ± 70%, within 12 hour light/dark cycle. The mice were purchased from Animal Experimental Unit, Universitas Gadjah Mada. The food and drink for all the experimental animals were given by ad libitum. These animals were maintained under temperature (25±2°C), relative humidity ± 65-75%. Mice were acclimatized to laboratory condition for 1 week before commencement of all experiment. All experiments were conducted in accordance with the guide for the care and use of laboratory animals by the commission Ethical Clearance LPPT of Universitas Gadjah Mada in numbers: 158/KEC-LPPT/IV/2014.

**Evaluation of arthritis**

Healthy forty two mice were divided were divided into 6 groups each containing 5 animals. Group I was a normal; group II, negative control group were induced by CFA 0.1 ml (single dose of CFA 0.1 ml for each mice sub-plantar of right hind paw) without treatment; group III was administered diclofenac sodium-treated group in mice-induced CFA; group IV was administered ethanolic extract of Lamara (ELA, 50 mg/kg BW) in mice-induced CFA; group V was administered ethanolic extract of Lamara (ELA, 100 mg/kg BW) in mice-induced CFA; group VI was administered ethanolic extract of Lamara (ELA, 250 mg/kg BW). The administration of diclofenac sodium and ethanolic extract of L. amara was orally administered once daily for 60 days. Arthritis index was measure by edema volume using plethysmometer on day 0, 7, 14, 21, and 56 after the induction of CFA. At the end of experiment (day 60), blood were collected by cardiac puncture and afterthat animals were sacrificed and removed the liver, spleen and kidneys. Finally, mice were cut the right leg including hind paw of mice for histological observation. Blood was centrifuged at 2000 x g for 15 min and stored temperature at −20°C for cytokines determination. The liver and kidney were weight and kept in 10% formaline for histopathology observation. The legs of mice-induced CFA were cut and kept in 10% formalin for determine of index arthritis, inflammation score and joint damage. For the osteoclasts observation were performed by Tartrate-Resistant Acid Phosphatase (TRAP) staining.

**Examination of cytokine pro-inflammatory**

The levels of cytokines of TNF-α and IL-6 were determined by ELISA. Cytokine pro-inflammatory tumor necrosis alpha (TNF-α) were measured by enzyme linked immunosorbent assay (ELISA) kit from eBioscience (catalog No. E09479-1645) and for interleukin 6 (IL-6) measured by ELISA kit from eBioscinece (catalog No. E09358-1647).

**Histological examination**

On the last day (day 60) mice were fasted overnight before giving up and cutting the legs of mice induced. It were fixed in 10% buffer formalin solution. The paws were then decalcified in 10% EDTA for 15-30 days at 4°C, embedded in paraffin, and sectioned in a mid-sagittal plane. The tarsocral joint were stained with hematoxylin eosin (HE) and TRAP staining. The histological damage evaluated microscopically was defined according to system evaluated cartilage and bone destruction by edema, periarticular, osteoblast and osteoclast formation.

**Statistical Analysis**

All values were presented as mean ± the SEM. The statistical significance of differences between the groups were assessed with a one-way ANOVA or non-parametric Kruskal-Wallis test, followed by Bonferroni post-hoc or Mann Whitney analysis test analysis using GraphPad InStat 3 (GraphPad Software, Inc., USA). P<0.05, considered statistically significant.

**RESULTS**

**Evaluation of arthritis**

Evaluation of arthritis was examined by edema of hind paw of mice induced by CFA. As shown in figure 1, the volume of edema as a sign of inflammatory was increased on day 7 after the induction of CFA 0.1%. The administration of ELA (50; 100, and 250 mg/kg BW) significant reduced volume of edema in mice-treated CFA (P<0.05) and the activity were equal to diclofenac sodium, 2 mg/kg BW. The reduction of edema were last until day 60.

**Examination of cytokine pro-inflammatory of extract Lamasia amara wood**

The cytokines inflammatory marker TNF-α and IL-6 in serum also determined. As shown in figure 2 and 3, the administration of ELA (50; 100, and 250 mg/kg BW) significant reduced the level of TNF-α and IL-6 in arthritis model in mice (P<0.05), but still less than diclofenac sodium. The administration of ELA in serial doses trend to increase the activity, although statistically not significant.

**Histological examination**

The joint damage and osteoclast formation in joint right hind paw were examined by HE and TRAP staining. The induction of CFA lead to induced chronic inflammatory and edema and formation of osteoclast last to 60 days. The histological examination shown the CFA also induced necrosis in mice hind paw. The administration of ELA (50; 100, and 250 mg/kg BW) and diclofenac sodium qualitatively reduced the edema and also the formation of osteoclast.

**DISCUSSION**

The present study was conducted to determine the anti-inflammatory activity of the ethanolic extract of Beta-beta wood (L. amara). The chronic inflammatory was conducted in mice models of RA-induced by CFA. The CFA induced arthritis model is widely used for pharmacological evaluation of antiarthritic agents as it shares a number of clinical and immunological features with human arthritis17,20. Along with measurement of joint swelling, the key cytokines primarily secreted by macrophages, TNF-α and IL-6 were also evaluated. The results shown that all doses of ELA could reduce the joint swelling in CFA-treated mice and equal with diclofenac sodium. As well as the results demonstrated that there was an increase in serum TNF-α and IL-6 in mice induced by CFA. ELA have activity decreased level TNF-α and IL-6. RA is caused by a number of pro-inflammatory molecules that are released by macrophages. Include in this reactive
oxygen species and eicosanoid such as prostaglandin, leukotrienes and cytokin (IL-1β, IL-6 and TNF-α)\textsuperscript{21-23}. Treatment of ELA produced a equal inhibition of pro-inflammatory cytokine expression as compared to diclofenac sodium especially in higher doses. Tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6) are a proinflammatory cytokine that plays a pivotal role in regulating the inflammatory response in rheumatoid arthritis (RA)\textsuperscript{22}.\textsuperscript{23} Previous study also reported that LPS like CFA is a potent stimulator of TNF-α release from Kupffer cells, TNF-α plays an important role in the chronic inflammatory in liver\textsuperscript{24}. The histological examination of the hind paw also evaluated in mice. Several line of evidence indicated that a large proportion of the CD11b+ cells consisted of osteoclast precursors, provide evidence of a possible link between the myelopoietic events and joint involvement, as has been described in experimental arthritis in TNF-α transgenic mice\textsuperscript{25-27}. Rheumatoid arthritis causes damage to cartilage and bone\textsuperscript{28}. Causes damage to cartilage and bone characterized by osteoclast. Osteoclast is the absorption of bone cells. Osteoclast characterized by the expression of tartrate-resistant acid phosphatase (TRAP) were detected using histochemical method\textsuperscript{29}. Histopathologic on the inflamed tissue or in CFA-treated mice characterized by increase in the number of inflammatory cells and edema, occurs pericondritis and discovered the presence of osteoclasts. Inflammatory cells are formed in all of the groups in general CFA induced high to necrosis. This indicates that arthritis occurs in all group induced CFA. In observation of preparations with HE staining seen that occured edema and inflammation accompanied induced necrosis in CFA-treated mice, while the edema and inflammation was reduced in ELA-treated mice. The results showed the presence of edema and inflammation are more severe in CFA-treated mice compare to ELA-treated mice. The result suggests that ethanolic extract of beta-beta wood (L. amara) might be beneficial in the treatment of chronic inflammatory disorder including RA. L. Amara exerts potent anti-inflammatory action in the arthritis model and was found to be effective in chronic inflammatory condition including inhibit cytokines that involved in development of RA. This study supports the utilizing beta-beta wood (L. amara) as anti-inflammatory and potential in chronic inflammatory conditions such as RA disease.

**CONFLICT OF INTEREST**
We declare that we have no conflict of interest.

**Figure 4:** Histopathological examination of right hind paw of mice induced by CFA using HE staining. Normal right hind paw (A); presence the edema in right hind paw induced by CFA (B); presence the pericondritis in right hind paw induced by CFA (C); necrosis and chronic inflammatory in right hind paw induced by CFA (C). Histopathological examination of joint damage with no osteoclast (E) and osteoclast formation (F)(red arrow) using TRAP.
ACKNOWLEDGEMENTS

The first author is grateful to The Ministry of Research, Technology and Higher Education for Scholarship BPPDN Assistance. The authors also acknowledge the support extended by the Dean, Faculty of Pharmacy, Universitas Gadjah Mada for carrying out the work and to Dr. Joko Santoso for authenticated the plant material.

REFERENCES

6. Mothana RAA. Anti-inflammatory, antinociceptive and antioxidant activities of the endemic Soqotraen Boswellia elongata Ball. f. and Jatropha unicosifolia Ball. f. in different experimental models. Food and Chemical Toxicology 2011; 49: 2594–2599.

