The Effectiveness of *Clerodendrum* Paniculatum. L against TNF- α in Rats Induced by S. *Aureus*

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Abstract: *Staphylococus Aureus* is one of the mastitis causes which causes inflammation due to elevated levels of TNF-α. This study aimed to analyze the anti-inflammatory activity of bag flower leaf (*Clerodendrum Paniculatum L*) on TNF-α level in rats that is induced by *S. Aureus* bacteria. This study used post test only control group design with 15 samples of Strain Sprague Dawley rats divided into 3 groups. K (-) group as normal control group, K (+) was induced by *S. Aureus* bacteria and the treatment group was administered by extract 150mg / kg BB then it is measured TNF-α levels by the enzyme-linked immunosorbent assay (ELISA) test method. Data were analyzed by using bivariate analysis namely one way ANOVA test. The results showed that Bag Flower Extract contains secondary metabolites in the form of flavonoids and tannins. Giving bag flower leaf extract (*Clerodendrum Paniculatun L*) at 150 mg / kg BB has anti-inflammatory action by reducing TNF-α levels in rate which is induced by *staphylococcus aureus* (P <0, 0). 05). It was concluded that the administration of bag flower leaf extract could reduce TNF-α cytokines due to *S. Aureus* bacteria. It is expected that future researchers will use human subjects to analyze TNF-α levels so that later bag flower leaves can be used as complementary therapies in mastitis treatment due to *S. aureus* bacteria.

1 INTRODUCTION

Staphylococcus aureus is one of the main pathogens that are most often isolated from intramammary infections (IMI) throughout the world. *S. aureus* bacterial infections are becoming a serious problem today due to increased bacterial resistance to various types of antibiotics (Multi Drug Resistance / MDR). *S. aureus* resistant can cause the spread of other infections and cause other diseases associated with other infections. Although *S. aureus* can cause acute and clinical mastitis with macroscopic changes in milk, bacterial infections can develop against chronic and subclinical mastitis, without macroscopic changes in milk but with high somatic cell and bacterial counts in the mammary glands (Green *et al.*, 2012; Chinchali and Kaliwal, 2014)

The occurrence of mastitis begins with an increase of pressure in the duct (breast milk channel) due to breast milk stasis. If the milk is not removed immediately, there is excessive alveoli tension and it causes the epithelial cells that produce milk become flat and depressed, so that the connective tissue permeability will be increase. Some components (mainly immune proteins and sodium) from plasma enter to breast milk and subsequently into the tissues around cells so that it triggers an immune response(Acosta *et al.*, 2016),

Breast milk stasis, there is an inflammatory response, and tissue damage facilitate infection. Breast infections are usually caused by bacteria that are found in normal skin, namely *S. Aureus*. Other bacteria that cause mastitis are *Streptococcus beta-hemolitik* (such as Group A or Group B streptococcus) or *Escherichia coli*. These bacteria often originate from the baby's mouth which enters through the lactiferous duct into secretion lobe, through cracked nipples to the lymph glands around the duct (periductal) or through spread of hematogenous (blood vessel) (Schwartz *et al.*, 2002;

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Amir *et al.*, 2007; Indonesian Pediatric Society, 2013)

Tumor Necrosis Factor alpha (TNF α) is a pro-inflammatory cytokine that is involved in an inflammatory reaction and it plays a role in the pain emergence. This was first isolated by Carswell et al in 1975 toward an effort to identify the Tumor Necrosis Factors that is responsible for nicrosis from the Meth A. sarcoma. Most organs appear to be affected by TNF α, and there are many cytokine functions which are not fully understood (Sanchez, Ford and Yancey, 2005; Notebaert *et al.*, 2008; Lai *et al.*, 2017)

The various roles of TNF-α can be explained through their effects on endothelial vascular and endothelial leukocyte interactions. When it is exposed to TNF-α, endothelial cells will cause inflammatory reactions by expressing various adhesion molecules. TNF can also cause vasodilatation by inducing the expression of cyclooxygenase 2 (COX-2) and its relationship to the production of prostacyclin 2 (PGI2). It can explain erythema and heat which are signs of inflammation. Other signs of inflammation are tumors that occur through increased vascular permeability, vascular through TNF mediators and liquid passage and transendothian macromolecules which ultimately cause edema and inflammation (Nair, M.; Mahajan, 2006; Berthold-Losleben and Himmerich, 2008; Lai *et al.*, 2017)

Immune response that occurs due to the invasion of *staphylococous aureus* bacteria as antigens is when the entry of *s. aureus* into the body will be eliminated by neutrophils and macrophages as their role in the innate immune system. Macrophages can also act as Antigen Presenting Cells (APC). Bacteria will be phagocytosis in macrophages then recognized by Major Histocompatibility Complex II (MHC II) then presented in the form of peptide antigens. Then MHC II will bind to T lymphocytes. T lymphocytes have several surface molecules or Cluster of Differentiation (CD). Peptide agents that have been presented by MHC II will bind to the T helper lymphocytes (CD4) in section of T Cell Receptor (TCR). (Abbas, Licthman and Pillai, 2015; Tong *et al.*, 2015; Acosta *et al.*, 2016)

Bag flower leaf is one of the plant species included in the Clerodendrum genus which has a different species number of 580 species, and it is spread evenly in Asia, Africa, America and Australia and it has been used in traditional medicine in Asia and Africa. India, China, Korea, Thailand, and Japan are countries that have used several species of this genus in medical practice (Cucumber, Virus and Indonesia, 2009; Florence, Joselin and Jeeva, 2012; India Biodiversity Portal, 2017)

Research on the efficacy of bag flower leaf which aims to find out the antioxidant and anti-inflammatory activities of big flower leaf extract (*Cloredendrum Paniculatum L*) that is conducted Anti-inflammatory activity tests on animals, it showed the antioxidant activity results of bag flower leaf ethanol is very strong (IC_{50}<50 µg/ml) namely IC_{50}= 27,73376 µg/ml and it has anti-inflammatory activity at a dose of 50 mg / kg. Other research on the anti-inflammatory activity test of *Clerodendrum Paniculatum L.* which aims to evaluate the anti-inflammatory activity of various extracts of *Clerodendrum paniculatum* leaf shows the anti-inflammatory activity in best vitro at a dose level of 200 and 400 mg / kg. Indomethacin at a dose level of 10 mg / kg is used as a standard reference drug. Both extracts showed a significant dose reduction (P <0.001) in edema paw when it is compared to controls). The study results indicated that petroleum ether and chloroform extract from bag flower leaf have anti-inflammatory potential which provides a scientific basis for the traditional claims of *Clerodendrum Paniculatum Linn* leaves as anti-inflammatory drugs (Joseph, Bindhu and Aleykutty, 2013; Hafiz, Rosidah and Silalahi, 2016a).

This study is aimed to assess the level of TNF-α which is one of the pro-inflammatory cytokines that can trigger inflammation in mastitis caused by *S. Aureus* bacteria.

2 MATERIALS AND METHODS

2.1 Location and Research Design

This research will be carried out in the Laboratory of Hasanuddin University Hospital for ELISA examination and bacterial culture, Hasanuddin University Bio pharmacy Laboratory and Hasanuddin University Animal Laboratory. The research type used is the type of research used true experimental with post only test control group design.

2.2 Population and Sample

The population of this study were 15 Strain Sprague Dawley *(Rattus Novergicus*) white rats according to WHO standards and were randomly selected to avoid bias in the study. The rats that used in this study were rats weighing between 200-250

grams. Before being given treatment, rats were first adapted for 7th days in a cage with controlled temperature and adequate lighting. Rats were fed with pellets and water on an ad libitum basis. The use of experimental animals in this study was approved by the health research ethics commission, medical faculty of Hasanuddin University, Makassar (No. 1053 / H4.8.4.5.31 / PP36-KOMETIK / 2017).

2.3 Method of Data Collection

2.3.1 Sampling Criteria

Sampling criteria in this study namely inclusion criteria: female rat Strain Sprague Dawly weighing 200-250 grams with age 2-3 months, there is no anatomical abnormalities. Exclusion criteria: rats don't want to eat, rats that are sick during the adaptation process. The materials needed in this study were rat maintenance: cages, food containers. Rat treatment: scales, sterile syringes, feeding tube. Sample preparation: centrifuge, vortex, shaker, yellow and blue tip, micropipette for volume 2 µl - 1000 µl.

Female rat Strain Sprague Dawley each group consisted of 5 rats. Furthermore, rats were divided into 3 treatment groups, each consisting of five rats that is consisting of two control groups and one treatment group so that a total sample of 15 rats were obtained. To avoid bias factors due to weight variation, the grouping of samples is done randomly. To avoid drop outs sample, the sample size which is used was 6 per sample group so that the number of rats used was 15 rats for 3 groups.

Blood sampling was taken twice after the induction of *S. aureus* bacteria and after treatment in each group. 1 ml of blood is drawn in centrifuge at a speed of 5000 rpm for 5 minutes. Then do the separation of blood with serum. The serum is suctioned with a 1.0 µL dropper and placed in an effendorf tube. The serum collected was carried out by the ELISA method to obtain TNF-α level. Test reagent for TNF-α examination: TNF-α ELISA Kit RAT with catalogue number RTA00 .

2.3.2 Extraction Preparation

Bag flower leaves are obtained in the yard of community houses as much as ± 2 kg of raw leaves, then cleaned of dirt attached by using running water then the sample is cut into small pieces, then dried to contain water content below 10%, after that the bag flower leaves are sieved with mesh size 40 so that a smooth simplicia sample is obtained, after it, the sample is ready to be extracted by maceration method (Poorter *et al.*, 2012; Hafiz, Rosidah and Silalahi, 2016b).

Maceration is generally carried out by means of 10 parts of simplicity put into a vessel, then poured with 75 parts of the liquid solution, closed and left for 5 days which is protected from light, while repeatedly stirring after 5 days the juice is dispensed, the pulp is squeezed. The residue is added to solvent sufficiently and cleaned to obtain a total of 100 parts. The resulting maserat is then concentrated using a rotary evaporator until a thick extract is obtained, and then dried by using a water bath and desiccators (MOH RI, 2000).

2.3.3 Bacterial Culture

Staphylococcus aureus bacteria obtained from laboratory of Uhhas Hospital with the type of *staphylococcus aureus* bacteria then planted in BHIB medium and it is incubated for 18-24 hours at 37° C in incubator and then propagated by using Nutrient Agar (NA) medium which is then re-incubated for 18-25 hours. After bacterial incubation, it is done gram staining. Biochemical tests for *S. aureus* bacteria were planted in NA by planting on *DNAse agar* medium then mannitol salt agar, then bacitracin and Novobiocin tests were followed by callatase coagulase test. Then it is reincubated for 18-24 hours at 37° C. The bacteria that grew in biochemical test were matched with the identification table of *S. aureus* bacteria. To make a bacterial sample which is injected into rats by making a suspension in physiological Na Cl solution of 10 ml mixed with a colony of *S. aureus* bacterial which is golden yellow with turbidity level of Mc Farlan 2 x 10^8 CFU. The accuracy of Mc Farland turbidity level is measured by the Densi check tool (Das, Borah and Ahmed, 2013)

Figure 1 describe the research tools and materials like : ELISA-Test for TNF-α, Sprague Dawley Rats, Pagoda Leaves and Staphylococcus Aureus Bacteria

After getting the concentration of bacterial cells 10^8 cell / mL, then centrifuged at a speed of 10,000 rpm for 10 minutes at 25^0C. The obtained pellets were then suspended with 1 mL PBS. The suspension was then injected into experimental animals, namely in mammals of rat female Strain Sprague Dawly, precisely in the lactiferous duct section with a volume of 100 µL (0.2 ml).

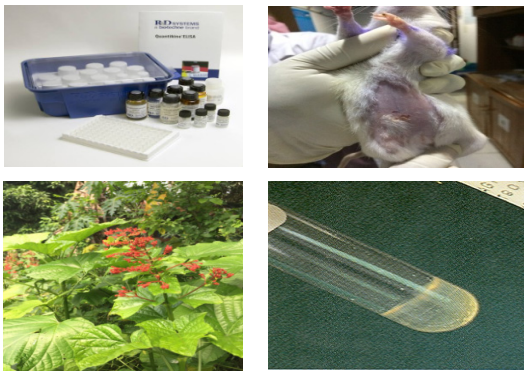

Figure 1. Research Tools and Materials

2.3.4 Data Analysis

Data analysis begins with test data normality distribution. To compare TNF-α level in the group after the induction of *S. Aureus* bacteria, and after the intervention in the treatment group was analyzed by using Paired T-Test. To compare changes in TNF-α level between the treatment and control group, statistical analysis used one way Anova test. The significance limit used in this study was 5% (p = 0.05).

3 RESULTS

Qualititative phytochemical test of bag flower leaf extract (*Clerodendrum Paniculatum L*)

Table 1 shows the phytochemical test results of secondary metabolite content in bag flower leaf extract (*Clerodendrum Paniculatum L*), there was secondary metabolite compounds include flavonoids and tannins.

Table 1. Qualitatively analysis of phytochemical content of sea leafy leaves (*Scaevola taccada (Gaertn) Roxb.*) Extracts

Metabolit Compunds	Analysis result
Alkaloid	-
Flavonoid	+
Steroid/triterpenoid	-
Saponin	-
Tanin	+

From several studies that conducted phytochemical testing of *Clerodendrum Paniculatum L* plants, there were differences in interpretation of the results of phytochemical screening that researchers conducted on several previous studies. Phytochemical screening results of bag flower leaf ethanol extract in this study were identified only 1 from 3 alkaloid solutions used namely Mayer solution. The difference in phytochemical screening results in this study can be caused by several factors, namely the treatment of the sample and the environmental conditions in which the bag flower leaves grow (Pidugu and Arun, 2012; Andriani *et al.*, 2017; Wang *et al.*, 2018)

However, differences phytochemical screening results conducted in this study with previous studies did not affect the value of the study, because the compound expected to exist in the ethanol extract of bag flower leaves that had an effect on anti-inflammatory activity was the flavonoid compound. The tannin compound itself in this study also influences anti-inflammatory activity and can function as an antibacterial and the compound does not differ from the results of previous studies that bag flower leaf extract has flavonoid compounds (Yadav *et al.*, 2014; Wang *et al.*, 2018)

3.1 TNF Levels - after Bacterial Induction and after Intervention

Table 2 shows that in negative control group there were no differences in mean TNF-α levels on rats that were not induced with *S. Aureus* bacteria with a p value = 0.825. In the positive control group there was no difference in the mean TNF-α levels after bacterial induction with p = 0.894. In the treatment group with a dose of bag flower leaf extract 150 mg / Kg BB there was a significant mean difference between TNF-α levels after bacterial induction and after administration of the intervention with a p value = 0.003.

Table 2. Difference in mean TNF-α level before induction of S. *Aureus* bacteria, after induction of S. *Aureus* bacteria and after administration of Bag Flower Leaf Extract

Group	After Induction Average ± SD	After Treatment Average ± SD	P Value
Control (-)	251,3 ± 8,3*	249 ± 20,2*	P = 0,825[a]
Control (+)	305,5 ± 22,9	303,3 ± 35,6	P = 0,894[a]
EDP 150 mg/Kg BB	319 ± 11,1	247,0 ± 25,9	P = 0,003[a]

Table 3. Difference in mean TNF-α level after administration of Bag Flower Leaf Extract

Group	Average ± SD	P Value
Control (-)	249 ± 20,2	
Control (+)	303,3 ± 35,6	0,008
EDP 150 mg/KgBB	247,0 ± 25,9	

[a]One Way Anova

Table 3 shows that the statistical test results obtained p value <0.008 so that there are significant differences in average TNF-α levels in the negative control group, positive control group, and EDP150 mg / Kg BB group after treatment. From the results of continued tests (post hoc) in table 4 shows the average comparison of TNF-α levels after giving treatment in the negative control group with a positive control group significantly different with a p value = 0.022. In the negative control and EDP treatment group 150 mg / kg BB there was no significant difference in the average TNF-α level after administration of the treatment with a p value> 0.05. In the positive control group and the EDP150 mg / Kg BB group, there was a significant difference in the mean TNF-α levels after administration of treatment with p = 0.016.

Table 4. Continued Test (Post Hoc) Differences in TNF-α Levels after Giving Bag Flower Leaf Extracts

Group	Control (-)	Control (+)	EDP150 mg/KgBB
Control (-)	-	0,022*	1,000
Control (+)	-	-	0,016*
EDP150 mg/KgBB	-	-	-

* Significantly different groups

4 DISCUSSION

This study showed that there were differences in the mean TNF-α level in rats between groups that were not induced by *S. Aureus* bacteria and those that were induced by *S. Aureus*. The statistical results in this study showed that the administration of bag flower leaf extract to rats at a dose of 150 mg / Kg BB was able to reduce levels of TNF-α in rat after being significantly induced by *S. aureus* bacteria.

This study was in line with several previous studies, namely mice infected with *Staphylococus aureus*, and then there was an increase in TNF-α production compared to mice that were not induced by *staphylococcus aureus*. (Mufidah & Rifa'i., 2015; Pereyra et al., 2017)

S. Aureus contains llipoteicoic acid which is found on the surface of bacteria which is recognized as toll-like receptors II (TLR II) which will further stimulate IL-12 production so as to stimulate INF- γ. This bacterium can also activate the adaptive immune response through superantigen induction. The interaction between superantigens and cells can lead to greater stimulation of T cells compared to other antigens. TNF-α cytokines and IL-1 have immunostimulatory activity and work synergistically with IFN-γ to enhance immune and inflammatory reactions. However, if the levels of cytokines are at high concentrations it can cause epithelial cell damage and cause toxic shock (Liang and Ji, 2007; Krakaeuer, 2011; Phuneerub *et al.*, 2015; Tong *et al.*, 2015).

In the phytochemical test results on bag flower leaf (*Clerodendrum Paniculatum L*), it is found secondary metabolite compounds in the form of flavonoids and tannins. The existence of secondary metabolites is an important factor through its mechanism of a bacterium. Flavonoids are the largest group of phenol compounds. Flavonoid compounds are good reducing compounds that inhibit oxidation reactions both enzymes and non-enzymes. Its mechanism as an antibacterial is to form complexes with extracellular and dissolved proteins and with microbial walls, flavonoids also play a direct role by interfering with the function of microorganism cells and inhibiting microbial cell cycles, denaturating bacterial cell proteins and damaging cell membranes that can result in lysis of bacterial cells (Parubak, 2013; Nanda, Bora and Tiwari, 2016; India Biodiversity Portal, 2017).

The work mechanism of tannin as an antibacterial is to inhibit the reverse transcriptase and DNA topoisomerase enzymes so that bacterial cells cannot be formed. Tannin has antibacterial activity related to its ability to inhibit and kill bacterial growth by reacting toward cell membranes, inactivation of essential enzymes in bacteria and destruction of functions and genetic material and interfering with the transport of proteins in the inner layers of cells. Tannins also have targets on cell wall polypeptides so that cell wall formation is less than perfect. This causes the bacterial cell become lysis due to osmotic and physical pressure so that the bacterial cell will die. Tannin acts as an antibacterial because it can form of protein complexes and hydrovobic interactions, if the hydrogen bond between tannin and protein enzymes contained in the protein is likely to be denatured so that bacterial

metabolism is disrupted (Daglia, 2012; Yadav *et al.*, 2014; Phuneerub *et al.*, 2015).

Bag flower leaf extract has active compounds namely flavonoids and tannins that can function as anti-inflammatory mediators that can reduce the release of proinflammatory cytokines in rats induced by *S. Aureus* bacteria. From previous studies, bag flower leaves that contain active compounds flavonoids, tannins and steroids can reduce inflammation that occurs in mice which is induced carrageen (Hafiz, Rosidah and Silalahi, 2016a).

Other studies on the methanol extract of mahogany with flavonoid active substances have also been shown to reduce TNF-α levels in mice induced by MLD-STZ. A similar study was carried out by using purple leaf extract (*Gratophyllum pictum L.*) with active flavonoid content, it has proven to reduce TNF-α level and NO in mice which is infected by *S. Aureus* bacteria(Suryani, Endang H and Aulanni'am, 2013; Tjahjani, Kristina and Endang Sri Lestari2, 2015; Bond, Morris and Nassar, 2017)

In this study, the treatment group with a dose of 150 mg / kg BW had a TNF-α value are below TNF-α level in the negative control group or it was not significantly different from the negative control group where p value> 0.005 so that the administration of bag flower leaf extract with a dose of 150 mg / kg body weight can reduce TNF-α levels in mice with normal conditions that are not induced by *S. Aureus* bacteria.

The content of secondary metabolites found in bag flower leaves (*Clerodendrum Paniculatum L*) which is derived from bag flower leaf extract is thought to act as an anti-inflammatory caused by *S. aureus* bacteria. The main bioactive component of phenols in flavonoids is quercetin. The mechanism of bag flower leaf extract as an anti-inflammatory can cause a decrease in TNF-α levels through inhibition of Nuclear Factor kappa B (NF-kB). NF-kB becomes active due to a stimulus from the Reactive Oxygen Synthase (ROS) agent which causes endothelial dysfunction, pathogen exposure, DNA damage and physical stress. NF-κB has the functions in controlling the genes expression of cytokines and proinflammatory chemokins TNF-α, IL-1β. The decrease in NF-kB activation is influenced by inhibitory effect of monocytes on p56 Protein Tyrosin Kinase (PTK) enzyme, which causes PTK to be inactive. Inactivated PTK causes NF-κB transcription factors to remain bound to NF-κB inhibitors so that it cannot trigger transcription and translation of TNF-α proinflammatory cytokines secreted by macrophages, thereby reducing TNF-α levels (Nair, M.; Mahajan, 2006; Fassihi and Sabet, 2008; Rathee *et al.*, 2009)

The dynamics of level changes in TNF-α between positive control and treatment group whereas in the treatment group after administration of bag flower leaf extract, it is decreased TNF-α levels after returning to the TNF-α level value equal to the negative control group. It showed that the content of compounds found in bag flower leaves can reduce the inflammatory effect by suppressing the release of TNF-α which is caused by *S.Aureus* bacteria.

5 CONCLUSIONS

This research shows that bag flower leaf extract contains secondary metabolite compounds including flavonoids and tannins which can reduce TNF-α level which are inflammatory mediators due to *S. Aureus* bacteria. Bag flower leaf extract with a dose of 150 mg / Kg BB is an effective dose to reduce TNF-α level in rats induced by *S. Aureus* bacteria. The administration of bag flower leaf extract can be a solution in the treatment of complementary therapies. It is necessary to evaluate the quantitative test of bag flower leaf to determine the activity of secondary metabolites and the percentage of compound content in grams.

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