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The Effect of Pagoda Leaves Extracts on the Level of IL-6 of Female Rats Induced by Staphylococcus Aureus

Fitriana Ibrahim[1(CA)], Prihantono[2], Mardiana Ahmad[3], Nurul Aini Siagian[4]

[1(CA)]Midwifery School, Hasanuddin University, Indonesia; pipit_ibrahim@yahoo.com (Corresponding Author)

[2]Department of Oncology, Faculty of Medicine, Hasanuddin University, Indonesia

[3]Muhammadiyah Midwifery Academy of Palopo, Indonesia

4Institute of Health Science "Deli Husada Delitua" of Medan, Indonesia

ABSTRACT

The extract of Pagoda leaves can play roles as an anti-inflammatory, antioxidant, and antibacteria. Interleukin 6 (IL-6 henceforth) is the primary mediator in responding to the inflammation, while mastitis is caused by bacteria often found in the increased level of IL-6. This research aimed to unveil the effect of giving the extracts of Pagoda leaves on the level of IL-6 of female rats (Sprague dawley therefore) induced by *Staphylococcus aureus*. This true experimental research employing pre-test and post-test control design used eighteen rats chosen randomly. All rats were divided into three groups, namely negative control (placebo), positive control (amoxicillin 9.6 mg/ml/kgBB/hari/oral) and treatment group (amoxicillin 9.6 mg/ml/KgBB + EDP 50 mg/ml/KgBB/hari/oral). All groups were induced by *S.aureus* $0.2x10^8$ CFU. Treatment was given for five days. Investigating the level of IL-6 was done using ELISA method. The data were anaylized using ANOVA formula followed by post hoc. The empirical findings showed that there was a difference in terms of the level of IL-6 in all of the groups ±24 before and after the induction of S.aureus. The result of investigating the level of IL-6 in the post-test was significant ($\rho = 0.00$), meaning that there was a significant difference, in which the negative control group, the level of IL-6 was insacalating; while it was dipping in both the positive and treatment groups. However, the treatment group experienced the most significant drop in terms of the level of IL-6. It can be concluded that Pagoda leaves can be used as a complementary therapy for mastitis illness caused by the S.aureus bacteria.

Keywords: Interleukin 6, Mastitis, Pagoda leaves, Staphylococcus Aureus

INTRODUCTION

Mastitis is an inflammation occurring in both or one of the breast areas. It is supposed to be similar to breast infection. This sort of illness may happen to all population, either with or without breastfeeding. Two primary causes of mastitis are static mother's milk and bacteria infection. It was reported that around 33% of women breasfeeding in the world have been with this illness, while it was around 3-20% occurring in Indonesia every year[(1),(2)].

Staphylococcus aureus is gram positive bactertia yielding yellow pigment, characterized by facultative aerob, non spora generation and not motile, generally growing either in a couple or a group[(3)]. *S.aureus* is the main pathogen causing various deseases, ranging from skin and tissue infection, shock, not to mention pneumonia neurosis[(4)]. Numerous research examining Staphylococcus Aureus induced into mammary rats show the symstoms of mastitis on the mice, resulting in lower weights and the damage of breast gland tissue[(5),(6)].

IL-6 is a glycoprotein fosforilized containing 185 amino acid, including sitokin pleiotropic involved in different process of physiology and pathofhysiology, such as inflammation, bone metabolism, protein synthesis, C-reactive, and carsinogen. Sitokin and receptor belonging to the type of polypeptide mediate the process of inflammation[(7)]. IL-6 is pro-infmmatary sitokin released by immune cells, known to play central role in self-defence. This sort of Sitokin is a superior mediator in responding to a local inflammation. In the normal condition, the level of IL-6 is very low, yet during inflammation, more cells will produce IL-6[(7),(8)].

In Indonesia, especially South Sulawesi, there were found a myriad number of Pagoda leaves (*clerodendrum paniculatum.L*) around housing. The chemical compounds found in the leaves include flavonoid, terpenes, tannin, alkaloid, sterol, and glycoside. Flavonoid is polyphenol compound found in trees having biological effects, such as anti-microba, antiviral, anti-hypertension, antioxidant, antiplatelet, sitotoksik, and anti-inflammatory[(10),(11)]. Several research discover that the flavonoid found in methanol and chloroform extracts of

Pagoda leaves is an effective anti-inflammatory against the *S.aureus* bacteria[12]. Other research gound that extracts of Pagoda leaves have anti-inflammatory activities of around 50 mg/kg[13].

This study aimed to prove the effectiveness of Pagoda leaves extracts on the level of IL-6 of female rats (*Sprague dawley*) induced by *staphylococcus aureus* bacteria.

METHODS

The type of research used in this study is true experimental or pure experimentation that is experiments in a laboratory, with the design of the pre and posttest control design. Eighteen female rats (*strain Sprague dawley*) were divided into three grups, namely negative control group (given 1 ml/250grBB of *aqua pro injection water*), positive control group (antibiotic *amoxicillin* with dosage of 9.6 mg/ml/250gramBB for five days), treatment group (given 9.6 mg/ml/250gramBB antibiotic *amoxicillin* for five days plus 50 mg/ml/kgBB extract of Pagoda leaves). The sample was taken drawing on the *research guidelines for evaluating the savety and efficaty of herbal medicine* that meets the WHO standard. This study already gained ethical permission from the ethical health research commission of the Faculty of Doctor of Hasanuddin University.

Before the treatment, histopathology test was done on a rat as a parameter for the appropriateness of injection and the thrive of the bacteria on the targeted area, *duktus laktiferus*. The rats were adapted for seven days, and in the next following day was taken the blood to figure out the level of IL-6 in blood serum of rats before the treatment followed by the injection of *s.aureus* bacteria *of* $0.2x10^8$ CFU on the same day to prevent the penetration of other bacteria though the spot where the blood was taken. On day nine or about ±24 hours after the injection of the blood, the second blood sample was taken, and the same was also done on day fourteen after the treatment. histopathology test was peformed for each group. One rat from each group was killed through eter anaesthesia on the fourteeth day. The *Mammae* was cut open to take the sample of breast tissue, after which the hematocrit and the blood serum were separated, and from the latter was tested the level of IL-6.

Pagoda leaves collected from the area of Belopa of Luwu district was around 5kg row leaves, which were cleansed using flowing water and were cut into some small pieces, and which were dried by means of *herb dryer* to ensure the level of water in them was under 10%. Then, the leaves were blended to gain the sample of smooth simplisia, which was subsequently extracted by means of macerating method, in which 70% of ethanol solvent was used. In the process, the sampel was soaked using the ethanol 70% for about 15 minutes, and another 2litre of the ethanol 70% was added on the soaked leaves in the temperature room for 3 x 24hours while stirred at once. The macerate was subsequently filtered using filter paper. It was then poured into an evavorating flask, to which waterbath, later, was fully poured. Then, all sets of intruments, including the rotavapor, were installed. It was then evaporated to be thick and dried using steambath water. The thick extracts yielded were poured into a vial followed by basic phytochemical test.

S.aureus bacteria was kept in BHIB and incubated for 18-24 hours at the temperature of 37°C in the incubator. The bacteria were then planted in the *Nutrient Agar* (NA) and reincubated for 18-24hours at 37°C. after the incubation, the bacteria gram were collored. The colony growing in the NA was biochemically tested for *S.aureus* bacteria by planting on the medium of *DNAse agar* then on *manitol salt agar*, followed by *bacitracin* and *Novobiocin tests*, and *katalase koagulase test*. It was then reincubated for 18-24hours at 37°C. The bacteria thriving in the biochemical test were suited using a table indentifying the bacteria of *S.aureus*. to make bacterium sample injected into the rats was by making suspension solution of physiology NaCl of around 10ml mixed with the colony of *S.aureus* of gold yellow color on the level of turbidity *Mc Farland* 2 x 10^8 CFU. The accuracy of the level of *Mc Farland* turbidity was measured using Densicheck tool.

Dilution test of RD1-54 as much as 50 µL was poured into all wells. Each well was given 50 µL standard and controlled, and the sample was mixed smoothly for one minute and tipped using adhesive. Then it was incubated for two hours at a room temperature. Each well was apirated and washed five times. Cleansing was done by means of 500 µL *wash buffer*, and in the last cleansing, the wash buffer was thrown away and dried using drying paper. *Conjugate rat* IL-6 of 100 µL was poured into each well, closed using adhesive, and incubated for two hours at a room temperature. Then, a five-time cleansing was performed. After thatn, 100 µL of *substrate solution* was added into each well, incubated for 30 minutes at a room temperature and soundproof. 100 µL *stop solution* was also added to each well and mixed. Within 30 minutes, it was read at wave length 450 nm.

The results of measuring the level of IL-6 in the rat serum of control and treatment group were analyzed statistically using the Anova test formula at the significant value of 0.05, and interval confidence of 95%, followed by the analyses of *post hoc*.

RESULTS

Table 1. Results of phytochemical test of chemical compounds of Pagoda leaves (*Clerodendrum paniculatum.L*)

Name of Sample	Identification of chemical compound						
	Alkaloid			Flavonoid	Steroid/ tripernoid	Saponin	Tannin
	Drag	LB	Mayer				
Extract of Pagoda Leaves (C*lerodendrum paniculatum.L*)	-	-	+	+	-	-	+

Table 2. Mean difference of the level of IL – 6 in each of the female rat (*Sprague Dawley*) before, after being induced with the *Staphylococcus aureus* and after the treatment

Groups	Level of IL-6 (pg/ml)			p-Value
	Before	After induction	After treatment	
	Mean±SD	Mean±SD	Mean±SD	
Negative control group (n =6)	725.0 ± 16.9	913.8 ± 17.3	992.9 ± 9.7	0.00 [a]
Positive control group (Antibiotic flukloksasilin 9,6 mg/250 GRBB) (n =6)	722.0 ± 24.9	915.3 ± 28.1	834.6 ± 31.7	0.00 [a]
Treatment (Antibiotic flukloksasilin 9,6 mg/250 GRBB + EDP 50 mg/kgBB (n =6)	717.0 ± 22.54	914.6 ± 21.8	753.3 ± 28	0.00 [a]
p-value	0.84 [b]	0.99 [b]	0.00 [b]	

* ANOVA ------ [a] Repeated ANOVA ------- [b] One Way Anova

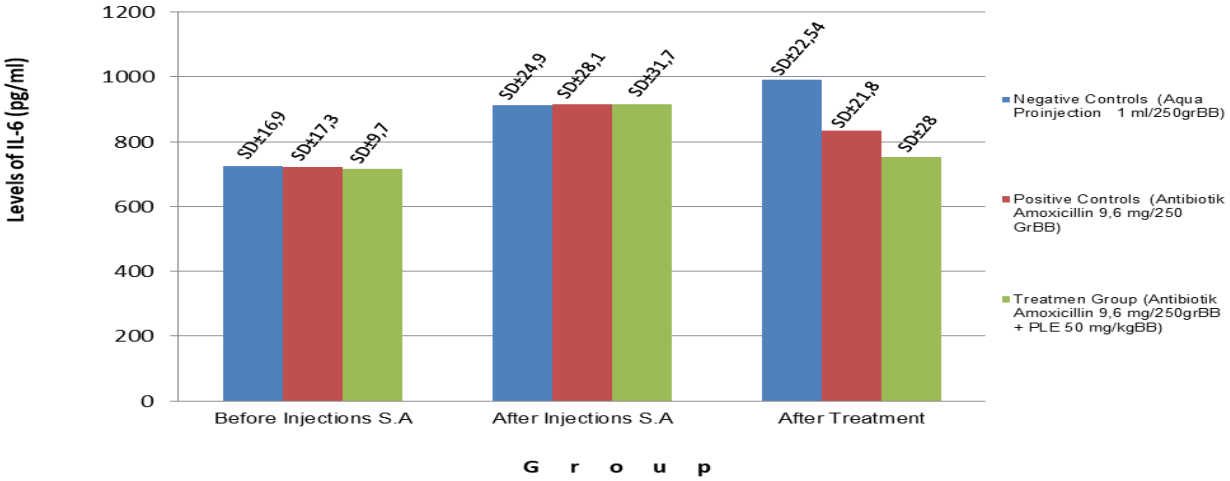


Figure 1. Trend of the level of IL-6 in each rat (Sprague Dawley) before, after the induction of S.aureus and after the treatment

Table 3. An analysis of different level of IL-6 before, after the induction of *S.aureus* and after the treatment

Measurement	Mean ± SD	Mean difference	p-value*	p-value
Before treatment				
Negative control group	725.0 ± 16.9	2.7	1.00	0.84
Positive control group	722.3 ± 24.9			
Negative control group	725.0 ± 16.9	7.4	1.00	
Treatment group	717.6 ± 22.54			
Positive control group	722.3 ± 24.9	4.7	1.00	
Treatment group	717.6 ± 22.54			
After the induction of *S. aureus* bacteria				
Negative control group	913.8 ± 17.3	-1.5	1.00	0.99
Positive control group	915.3 ± 28.1			
Negative control group	913.8 ± 17.3	-0.8	1.00	
Treatment group	914.6 ± 21.8			
Positive control group	915.3 ± 28.1	-0.7	1.00	
Treatment group	914.6 ± 21.8			
After the treatment				
Negative control group	992.9 ± 9.7	158.3	0.00	0.00
Positive control group	834.6 ± 31.7			
Negative control group	992.9 ± 9.7	239.6	0.00	
Treatment group	753.3 ± 28			
Positive control group	834.6 ± 31.7	81.3	0.00	
Treatment group	753.3 ± 28			

The result of ANOVA test ([a]repetead ANOVA) was (p = 0.00), meaning that there was a difference on the level of IL-6 between the negative control group, positive control group and treatment group ± 24hours before and after the induction of the *S.aureus* and after the *treatment.* [b]One Way ANOVA test showed that the level of

IL-6 in the *pretest* for all groups was (p = 0.84), after the induction of *S.aureus* was found (p = 0.99), which means that there was a significant difference on the level of IL-6 in each group. In the posttest measuring the level of IL-6, the value of (p = 0.00) was found, meaning that there was a significant difference, that is in Negative control group, the level of IL-6 was increasing, while in the Positive control group and treatment group, it was lowering, yet the later group experience the most significant drop in the level of IL-6.

The level of IL-6 in each group before induced the bacteria was not significantly different, yet after the induction, it was increasing in each group. On the other hand, after the treatment, the level of IL-6 in both Positive control group and treatment group was decreasing, but it was incrasing in the negative control group.

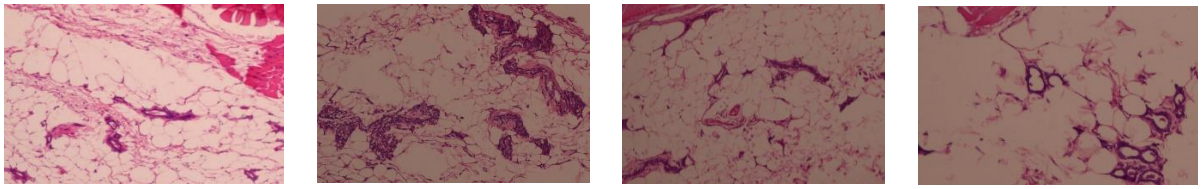
One Way Anova + post hoc Bonferroni, significant value $p \geq 0.05$. there was no difference in the level of sitokin IL-6, ± 24hours before and after the induction of *staphylococcus aureus* for all groups. There was a difference in the level of IL-6 after given the treatment in all groups.

There was a difference in the level of IL-6 in the negative and positive control groups before, after the induction of *s. aureus* and after the treatment. There was no difference in the level of IL-6 before and after the treatment in the treatment group at the significant value (p = 0.101).

Tabel 4. An analysis of difference in the level of IL-6 between group of female rats (*Sprague Dawley*) before, after the induction of *staphylococcus aureus* bacteria and after the treatment.

Measurement	Mean ± SD	Mean difference	p-value*	p-value
Negative control group				
Before	725.0 ± 16.9	-188.8	0.000	0.000
After the induction of *S.aureus*	913.8 ± 17.3			
before	725.0 ± 16.9	-267.9	0.000	
After the treatment	992.9 ± 9.7			
After the induction of *S.aureus*	913.8 ± 17.3	-79.1	0.000	
After treatment	992.9 ± 9.7			
Positive control group				
Before	722.3 ± 24.9	-192.7	0.000	0. 001
After the induction of *S.aureus*	915 .0 ± 28.1			
before	722.3 ± 24.9	-112.3	0.000	
After the treatment	834.6 ± 31.7			
After the induction of *S.aureus*	915 .0 ± 28.1	80.4	0.001	
After treatment	834.6 ± 31.7			
Treament group				
Before	717.6 ± 22.54	-197	0.000	0.000
After the induction of *S.aureus*	914.6 ± 21.8			
before	717.6 ± 22.54	-35.7	0.101	
After the treatment	753.3 ± 28			
After the induction of *S.aureus*	914.6 ± 21.8	161.3	0.000	
After treatment	753.3 ± 28			

*Repeated ANOVA + post hoc Bonferroni, significant value $p \leq 0.05$.



A B C D

Note:

(A) = Microscopic Picture of breast tissue of rats in the normal control group: it presents the ducts, blood vessels, and connective tissue of normal breasts.

(B) = Microscopic Picture of breast tissue of rats in the negative control group: it presents inflammation cells of polymorphonuclear (PMN), which were absent in the cells of normal breast tissue.

(C) = Microscopic Picture of breast tissue of rats in the positive control group: the tissue of rats' breast given antibiotic amoxicillin was found several inflamed cells (PMN), particularly in epithel cells and lactiferous ducts, yet with a significant number of difference from the Negative control group.

(D) = Microscopic Picture of breast tissue of rats in the treatment group: there were still some inflamed cells (PMN) found around the epithel cells and ducts, yet in a singnifcantly lower number from that of positive control group.

Figure 2. Histopathology test results.

DISCUSSION

The results of phytochemical test showed that the Pagoda leaves contained chemical compounds, flavonoid and tannin**.** The findings differed from the several previous research findings showing the chemical compounds found in the leaves, namely flavonoid, tannin, saponin, glikosida, steroid and terpenoid[14-16]. This can be understood due to numerous factors, such as treatment to the sample and distinct structure of soil in different regions.

The distinct results of screening did not give any significant impacts on the value of the research, because the intended compounds were actually expected from the extract of etanol of Pagoda leaves, which is flavonoid having dominant anti-inflammatory activities. It was justified by the previous studies which unveiled that the flavonoid found in the extract of metanol and chloroform of Pagoda leaves was an effective anti-inflammatory againes the bacteria, such as *Staphylococcus Aureus, Pseudomonas aeruginosa* and *Candida albicans*[10,12,16]. Flavonoid has a polar characteristics; hence, this compound allows other compounds to penetrate bacterium walls. Meanwhile, tannin could penetrate the lysis bacteria cell wall caused by flavonoid compound and coagulate the protoplasma of the bacteria cells[11,17,18,19].

In the nevative control group, the average level of IL-6 continued to escalate both before, after the injection of *S.aureus* and after given standardized water + feed for five days. This was due to the fact that IL-6 is one of the primary and most important mediators in inducing the controlling the synthesis of protein at an acute phase. During the inflammation, the concentrate of plasma of IL-6 could be detected within one hour and could remain for ten days. In addition, IL-6 will continuously increase in the serum of the patient experiencing the infection of unrinary and mastitis (20)–(22). The increased level of IL-6 can be caused by the response of immune cellular due to the injection of *s.aureus*, so there is a response to this bacterium. As a response to the various s.aureus bacteria and other gram positive bacteria, the reaction of sitokin pro-inflamatory IL-6 and TNF-α will be boosted; the pro-inflamatory was disynthesized and systemic inflammatory would occur. IFN-γ also known as *macrophage activating factor* (MAF) plays important roles in infectious persistence affected by the duraction of macrophage activities[23].

In the positive control group, after the induction of the bacteria, the average level of IL-6 was increasing. After given the antibiotic, amoxillin for five days, it was lowering to 834,6 pg/ml. The average level of IL-6 decreased was caused by the mechanical work of antibiotic, amoxillin, for killing the bacteria cells, bacteria protein, nucleat acid protein and nucleat bacteria and prevented the primary channel of bactria metabolism. The genetic materials of the bacteria died out because of the amoxicillin, which caused the the death of the bacteria, so the inflammatory was lowering as well as the level of IL-6[24]. A study showed that giving antibiotic along with emptying the breasts will fasten the process of recovery compared to breast emptying alone. One small experiment comparing amoxillin with cephradine found that there was no significant difference in terms of symptom and abcess. However, the researchers suggest that the use of amoxicillin can be used to relieve the symptom of mastitis for the women after delivery[25-27].

In the treatment group, the level of IL-6 was escalating after the injection of *S.aureus*, which, however, was lowering after given the treatment, the extracts of Pagoda leaves 50 mg/kgBB + *amoxicillin* 9.6 gr/ 250 grBB for five days. The average decreased level of IL-6 in the treatment group was caused by the mechanical work of the antibiotic, *amoxicillin*, mixed with the extract of Pagoda leaves containing fitochemical compounds, such as flavonoid and tannin, which, if working together, may have biological effects as an antioxidant, antibacterial and anti-inflammatory, so the increased level of IL-6 caused by the infection of *S.aureus* bacteria in the rats was decreasing and becoming normal[24,28]. The compunds of flavonoid and tannin found in the Pagoda leaves were responsible for the activities of the anti-inflammatory. This mechanism involves the activity of pro-inflammatory COX-2 or nitrate oxide sintase (iNOS) through the flavonoid compound. Other research found the fact that flavonoid (6-DMT) enforced the activity of HMC-1 cells with PMA by inhibiting the activity of anaplastik limfoma kinase (ALK) and mitogen activated protein kinase (MAPKs), which minimize the production and expression of gen TNF-alpha and IL-6. This shows that 6-DMT can play a role in managing the mast cells mediating the inflammatoru responses[19,28].

The results of this sudy discovered the difference on the level of IL-6 in each group, treatment group with average of 992.9 pg/ml; negative control group with average of 834.6 pg/ml; and the average of both positive control group and treatment group was 753.3 ± 28 pg/ml. The extracts of Pagoda leaves as complementary supplement can significantly minimize the production of IL-6 on the female rats infected by *S.aureus* as well as encouraged the performance of macrophage doing the phagocytosis. This findings accorded with the previous studies, which found that flavonoid contained in the extract of methanol and chloroform of Pagoda leaves was an effective anti-inflammatory against the *S.aureus* bacteria. Other research also showed that the extracts of Pagoda leaves have 50mg/kg anti-infmmatory activities[12,13].

The extracts of Pagoda leaves were very effective in diminishing the Staphylococcus aureus, Bacillus subtilis, Escherichia coli and Klebsiella pneumoniae. The study show that Clerodendron paniculatum have better antimacroba activities than other species. The extracts of Pagoda leaves can stimulate limfosit B and limfosit T and activate the neutrophil to perform phagocytosis. During the temporary induction of of IL-6, it will soon

participate in the defence of thebody against the threats in the cells, such as infection and trauma, and at the same time, IL-6 will give signals that stimulate wideer spectrum involved in the increase of immune system, inflammatory chronic and cancer[29,30].

Mastitis is the inflammatory of breasts of mother breasfeeding caused by the static of mother'smilk. The breast inflammatory causes crack around the nipple through which the pathogen penetrates, causing inflammation or infection. In this regard is the sitokin yielded by tumor or immune cells. Sitokin is one of the solvent mediators, which helps the communication of cells in responding to the immune system needed in the early reaction of inflammatory. IL-6 is the pro-inflammatory involved in the imunne phagocytosis of *S.aureus*[31-33].

During the infection of *S.aureus*, pro-inflammatory sitokin, such as IL-1β, and IL-6, IFN-ɣ and TNF-α were synthesized and turned into systemic inflammatory. After secreting the sitokin, the activity of cells T helper 1 (Th1) and T helper 2 (Th2) begin. The bacteria activities or IFN-ɣ will transmit the profile of changes of cells secretion thrpugh the production of organic nitrate. The presence pf *S.aureus* on the cells causes the secretion of sitokin and inflammatory infection or the death of cells through opoptosis. The signals from sitokin injected by the interaction of host and bacteria are crucial in the spread of illnesses[8].

Research findings show that the extracts of Pagoda leaves have pharmacotherapy effects on the mastitis illness or other infection caused by *S.aureus* bacteria. Pagoda leaves are one of the medical herbs that have been empirically used. The leaves used in this study could minimize the level of IL-6. The reason for the inclusion fo Pagoda leaves into the medical herbs is due to its ability to empower the body organs, get rid of the toxins or deseases and promote the increase of immune system. One of the conditions of the medical herbs to be categorized as complementary therapy or alternative cure in the improving the immune system is modulating the response of pathogen – management of T cells[34,35].

CONCLUSION

The extracts of Pagoda leaves (*Clerodendrum paniculatum*. L) were effective in stabilizing the level of sitokin IL-6 in the female rats (strain *Sprague Dawley*) induced by the *staphylococcus aureus* bacteria. They can also be capitalized on as the complementary therapy to cure mastitis caused by *staphylococcus aureus.*

This study suggests that future studies can deal with chlinical test on humans regarding the effects of the extracts of Pagoda leaves as an anti-inflammatory, immunomodulator antibakteria, particularly on the case of mastitis during mother breastfeeding of related cases of infections caused by the bacteria.

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