# Anti-inflammatory Activity of Ethanol Extract of Sappan Wood *(Caesalpinia sappan* L.) *on Mice Infected by Staphylococcus aureus and Escherichia coli*

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## Abstract

**Background:** Sappan wood *(Caesalpinia sappan* L.) is distributed in Southeast Asia and used as traditional medicine. Sappan wood contains phytochemical properties such as brazilin, brazilein, Sappanone B, protosappanin etc. This study aims to investigate about the anti-inflammatory activity of ethanol extract of Sappan wood in mice infected by *Staphylococcus aureus* and *Escherichia coli*.

**Methods:** Seven group of mice were treated respectively such as normal control, negative control (placebo), positive control (immune booster), and 96 % ethanol extract of Sappan wood (SWEE) 25, 50, 100, and 200 mg/kg body weight that was given for 7 days after infection. Blood samples was taken to measure the C-reactive protein (CRP) level and phagocytic index. Molecular docking studies were carried out using a webserver.

**Results:** The results showed that ethanol extract of Sappan wood decrease CRP level and increase phagocytic index until 48 mg/L and 1.54 folds (*Staphylococcus aureus* infection) and 12 mg/L and 4.62 folds (*Escherichia coli* infection). The molecular docking showed that brazilin has the potential to bind to TNFα, and IL-1β with total energy binding respectively -

5,05, and -3,14 kcal/mol. The estimation inhibition constant (Ki) of brazilin into TNFα, and IL-1β respectively 199,52 and 4,99 μM. Brazilin has less potential to bind to IL-6.

**Conclusion:** The ethanol extract of Sappan wood has anti-inflammatory activity through decreasing CRP level and increasing phagocytic index. Brazilin as a major compound of Sappan wood can bind and inhibit pro-inflammatory cytokine including TNFα and IL-1β.

**Keywords**: Anti-inflammation, CRP, phagocytic index, brazilin

## Introduction

Inflammation is immune response mechanism that can be triggered by many factors such as pathogens, toxic compound, and damaged cells. These factors can induce acute and/or chronic inflammatory responses in human organs. It can potentially cause tissue damage or disease (1). Inflammation is part of the pathogenesis of chronic disease. Although inflammation occurs in various organs under different physiological conditions, inflammation has the same general mechanisms including receptor recognition by stimulant, inflammatory pathway activation, releasing of inflammatory markers, and inflammatory cells recruitment (2).

Infection is one of factor that induce inflammation. Infection or antigen can be persistence in the body and cause inflammation. The antigen induces immune response and inflammation pathway, cause cell damage, and tissue injury. Antigen also cause the failure of endogenous anti-inflammatory mechanisms (3). *Staphylococcus aureus (S. aureus)* and *Escherichia coli (E. coli)* infections are quite common infections in humans. *S. aureus* is a gram-positive bacterial that most notorious as human pathogen and cause skin illness and wound infection to fatal sepsis (multiorgan failure) (4). *E. coli* is a gram-negative bacterial that cause gastroenteritis, renal failure, septic shock and extraintestinal illness such pneumonia, bacteremia, and peritonitis (5).

In inflammation mechanism, there are many markers including C-Reactive Protein (CRP), phagocytic index, inflammation mediator (TNFα, IL-1β, and IL-6). CRP is an acute inflammatory protein that is synthesized primarily in hepatocytes but also muscle cells, macrophages, endothelial cell, lymphocyte, and adipocyte. CRP increase up to 1.000 at infection or inflammation (6). CRP can become marker in *S. aureus* (7) and *E. coli* infection (8). Phagocytosis is a complex process that remove the pathogen and usually followed by inflammation (9) Phagocytic function usually decreases in infection. Phagocytic function can

seem by phagocytic index. In the molecular mechanism, phagocytosis is indicated by TNFα, IL-1β, and IL-6 expression as inflammation mediators (10).

Sappan wood *(Caesalpinia sappan* L.) is used as traditional medicine. Sappan wood has potential activity that influence on health including on combat the cardiovascular and vascular disease (11), gastroprotective (12), antibacterial (13) antiviral (14), antidiabetic (15) etc. Sappan wood contains phytochemical properties such as brazilin, brazilein, Sappanone B, protosappanin etc. Brazilin is the major compound in Sappan wood that become as specific colour in this plant. Brazilin has antioxidant activity with IC50 57,2 μM. As antiinflammation agent, brazilin inhibit Lippo Poly Saccharide (LPS)-induces NO production, prostaglandin E2, TNF-α and IL-1β (16).

This study aims to investigate the anti-inflammatory effect of Sappan wood ethanol extract on mice infected model. Mice was infected by *S. aureus* as gram-positive and *E. coli* as gram-negative bacterial. In this research, we also want to compare the anti-inflammatory effectiveness of Sappan wood between *S. aureus* and *E. coli* infection condition. We also want to know how the profile of molecular docking between brazilin as a ligand and some inflammation mediators including TNFα, IL-1β, and IL-6.

**Methods**

This research was pretest - posttest control group experiment consists of 7 treatment groups including normal control, negative control (CMC-Na 1%), positive control (immune booster), and 96% ethanol extract of Sappan Wood (EESW) 25, 50, 100, and 200 mg/kg BW. This research is a pre-clinical study using Mice *(Mus musculus)* Balb/C strain and has receive an ethics certificate from Research Ethical Clearance Commission, Faculty of Dental Medicine, Universitas Airlangga (Number: 217/HRECC.FODM/V/2022).

The 500 g of Sappan wood powder (Materia Medica Batu Jawa Timur) was extracted using 3750 ml of 96% ethanol by maceration method during 5 days and stirred 3 times every day. The extract was filtered until got the filtrate. The filtrate was concentrated using rotary evaporator (IKA Scientific) at $65^0$ C until obtain a thick extract. We made 4 concentrations of EESW such as 25, 50, 100, and 200 mg/kg BW. Phytochemical tests were carried out to investigate the Sappan wood content quantitively including flavonoid, alkaloid, tannin polyphenols, anthraquinone, saponin, steroid, and terpenoid.

*S. aureus* and *E. coli* were grown respectively on nutrient rich agar media (Merck) in incubator for 1 x 24 h. The first, we prepared $10^{-1}$ CFU/ml (Tube 1) by adding 1 ml of bacterial suspension into 9 ml of sterile distilled water. The dilution was carried out by taking 1 ml from Tube 1 and adding 9 ml of sterilized distilled water continuously until we get $10^{-9}$ CFU/ml. Mice that have acclimatized for 7 days were infected with *S. aures* and *E. coli* ($10^{-9}$ CFU/ml). After 24 hours, mice were treated by 7 treatments during 7 days. Blood was taken though jugular vein. The collected blood was separated by centrifugation method until we got the serum. Serum was used to CRP and Phagocytosis test.

CRP test was carried out in 2 stages, qualitative and semiquantitative test. We dripped 50 ml CRP latex reagent (Glory Diagnostic) on the slide and followed by dripping of 50 ml serum sample. If the results show that there was agglutination, the test was followed by semi quantitative test using dilution method (serum in NaCl) and CRP latex reagent. CRP titer was calculated by multiplying the highest dilution number by 6 mg/L (constant CRP value).

Phagocytosis activity was viewed by carbon clearance method using UV-Vis Spectrophotometer (Thermo Scientific) at 650 nm wavelength.

Constant carbon elimination speed (K)= $\frac{Log\ A\ (n) - Log\ A\ (n-1)}{t(n-1) - t\ (n)}$ (17)

Information:

K : Constant carbon elimination speed.

A(n) : Absorbance at time-n

t : time (0,5,10,15,20) minute

n : observation (n= 1,2,3,4,5)

$$\text{Phagocytic Index } (\alpha) = \frac{\text{Constant carbon elimination of sample}}{\text{Constant carbon elimination of negative control}} \quad (18)$$

Data obtained by in vitro experiment were CRP level and phagocytic index. The data was analyzed using One Way Analysis of Variant (ANOVA) using signification number p = 0,05.

Potential analysis of brazilin as anti-inflammatory substance was analyzed by molecular docking method. Molecular docking study was performed by docking server. We used Brazilin from *Caesalpinia sappan* L. as a ligand that was gotten from Pubchem (https://pubchem.ncbi.nlm.nih.gov/). We used TNFα, IL-1β, and IL-6 as receptor that was downloaded from Protein Data Bank (www.pdb.org) with PDB ID code respectively 3it8, 4gaf, and 5fuc.

## Results

### Phytochemical Screening

Based on the phytochemical screening, we known that EESW contains the secondary metabolite that was shown in Tabel 1.

**Table 1.** Phytochemical Screening of Ethanol Extract of Sappan Wood (EESW)

| Group of Compound | Observation | Result |
|---|---|---|
| Flavonoid | Orange color | + |
| Alkaloid | Orange precipitation (Dragendorff) | + |
| Polyphenol | Dark blue color | + |
| Saponin | Stable foam | + |
| Tanin | Bluish green color | + |
| Steroid | Discoloration | - |
| Terpenoid | No brownish red color | - |

## CRP Level

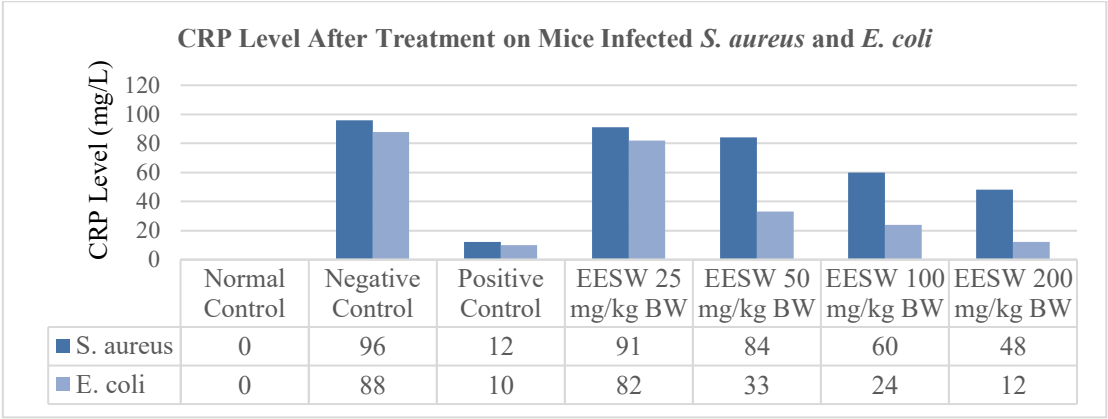
One Way ANOVA result shown that there were significantly different among treatment with significant value was p= 0.000 (p<0.05). Data of CRP level after treatment between *S. aures* and *E. coli* infection in all treatment were shown in Tabel 2 and Figure 1.

**Table 2.** CRP Level After Treatment on Mice Infected *S. aureus* and *E. coli*

| Treatment Group | Mean (mg/L) ± SD | |
|---|---|---|
| | ***S. aureus* Infection** | ***E. coli* Infection** |
| Normal Control | $0^{a} \pm 0$ | $0^{a} \pm 0$ |
| Negative Control | $96^{b} \pm 0.04$ | $88^{b} \pm 0.12$ |
| Positive Control | $12^{c} \pm 0.05$ | $10^{c} \pm 0.02$ |
| EESW 25 mg/kg BW | $91^{b} \pm 0.15$ | $82^{b} \pm 0.15$ |
| EESW 50 mg/kg BW | $84^{b} \pm 0.1$ | $33^{b} \pm 0.11$ |
| EESW 100 mg/kg BW | $60^{d} \pm 0.05$ | $24^{d} \pm 0.08$ |
| EESW 200 mg/kg BW | $48^{d} \pm 0.04$ | $12^{c} \pm 0.02$ |

Different notation (a,b,c,d) indicated the significant differences between treatments (p <0.05)

**Figure 1.** CRP Level After Treatment on Mice Infected *S. aureus* and *E. coli*




## Phagocytic Index

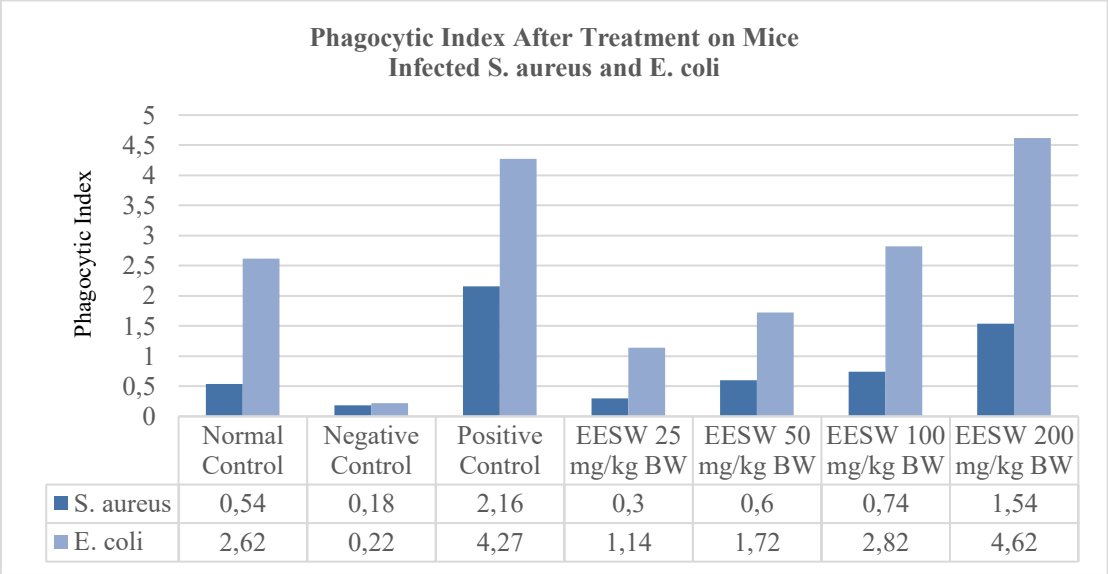
One Way ANOVA result shown that there were significantly different among treatment with significant value was p= 0.000 (p<0.05). Data of phagocytic index (α) after treatment between *S. aures* and *E. coli* infection in all treatment were shown in Tabel 3 and Figure 2.

**Table 3.** Phagocytic Index After Treatment on Mice Infected *S. aureus* and *E. coli*

| Treatment Group | Mean ± SD | |
|---|---|---|
| | ***S. aureus* Infection** | ***E. coli* Infection** |
| Normal Control | $0.54^{a} \pm 0.05$ | $2.62^{a} \pm 0.01$ |
| Negative Control | $0.18^{b} \pm 0.04$ | $0.22^{b} \pm 0.05$ |
| Positive Control | $2.16^{c} \pm 0.05$ | $4.27^{c} \pm 0.02$ |
| EESW 25 mg/kg BW | $0.3^{a} \pm 0.01$ | $1.14^{ab} \pm 0.01$ |
| EESW 50 mg/kg BW | $0.6^{a} \pm 0.02$ | $1.72^{ab} \pm 0.05$ |
| EESW 100 mg/kg BW | $0.74^{a} \pm 0.05$ | $2.82^{a} \pm 0.03$ |
| EESW 200 mg/kg BW | $1.54^{ac} \pm 0.04$ | $4.62^{c} \pm 0.04$ |

Different notation (a,b,c,) indicated the significant differences between treatments (p <0.05)

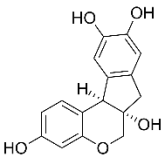
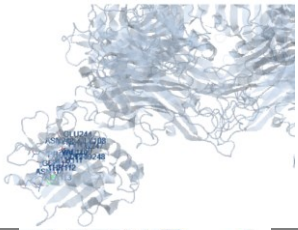
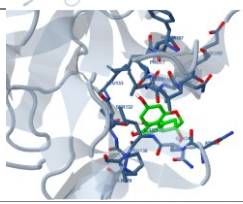
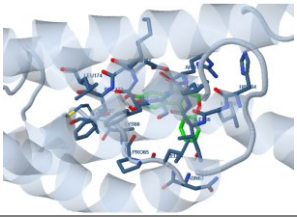
**Figure 2.** Phagocytic Index After Treatment on Mice Infected *S. aureus* and *E. coli*




## Molecular Docking Result

Molecular docking shown that there was interaction between Brazilin and inflammatory mediator including TNFα and IL-1β. The result of molecular docking was shown at Table 4.

**Table 4.** The Result of Molecular Docking

| Ligan | Receptor | Est. Free Energy of Binding (kcal/mol) | Interaction Surface | Est. Inhibition Constant (Ki) (µM) | Amino Acid (Hydrogen Bond) | 3D Molecular Docking Interaction |
|---|---|---|---|---|---|---|
| **Brazilin**  | TNFα (3it8) | - 5.05 | 433.77 | 199.52 | Proline Tyrosine Glutamine Isoleucine |  |
| | IL-1β (4gaf) | - 3.14 | 538.145 | 4.99 | Leucine Serine |  |
| | IL-6 (5fuc) | + 14.23 | 546.758 | - | Leucine Arginine Asparagine Glutamine |  |

**Discussion**

Anti-inflammatory activity test of plant is associated with natural compound that contains in there. Phytochemical screening in ethanol extract of Sappan wood is a confirmatory qualitative of natural compound through main classes of compound identification. Based on phytochemical screening shown that ethanol extract of Sappan wood contain flavonoid, alkaloid, polyphenol, and tannin. The ethanol extract of Sappan wood was not contain steroid and terpenoid. Based on the theory like dissolves like, the types of compounds that can be dissolved in polar solvents are polar compound. Polyphenol are a large group of naturally occurring phenols. The type of polyphenol including flavonoid and tannin. Alkaloid is semi polar. Steroid and terpenoid were not detected in phytochemical screening because steroid and terpenoid were non polar compound. Flavonoid compound of Sappan wood have biological effect in the body including brazilin, brazilein, and Sappanone.

The first parameter of anti-inflammatory activity in this research is CRP level. In *S. aureus* and *E. coli* infection, the CRP level increase respectively up to 96 and 88 mg/L (9.6 and 8.8 m/dL). Infection of *S. aureus* and *E. coli* in 24 hours can increase CRP until moderate elevation (1.0 – 10.0 mg/dL) (19). Infection of gram-positive bacteria increase CRP level was higher than gram-negative bacteria (20). CRP level was associated with cytokine response in the body. Plasma concentrations of TNFα, IL-1Ra, IL-8, and IL-10 did not differ between patients with sepsis due to gram-negative and gram-positive bacteria. However, plasma concentrations of IL-1β, IL-6, and IL-18 were significantly higher in patients with gram-positive sepsis (21).

As a parameter, CRP levels should be able to fall due to anti-inflammatory activity. The positive control (immune booster) can decrease CRP level in *S. aureus* and *E. coli* infection respectively until 12 mg/L and 10 mg/L (1.2 and 1.0 mg/dL). Based on the CRP level interpretation, immune booster can decrease CRP level until normal/ minor elevation. The

Ethanol extract of Sappan wood (200 mg/kg BW) can decrease CRP level in *S. aureus* and *E. coli* infection respectively until 48 mg/L and 12 mg/L (4.8 and 1.2 mg/dL). Based on the CRP level interpretation, EESW can decrease CRP level until normal/ minor elevation in *E. coli* but moderate in *S. aureus* infection. If it is associated with cytokine responses in gram-negative and positive bacterial infections, the anti-inflammatory ability of EESW in *E. coli* (gram-negative) infections is better than in *S. aureus* (gram-positive) infections.

The second parameter of this research is phagocytic index (α) that shown the phagocytosis process. Phagocytosis is a cellular process for ingesting and eliminating particles ($d > 0.5$ μm) including microorganisms, foreign substances, and apoptotic cells (22). The phagocytic index of *S. aures* and *E. coli* infection respectively are 0.18 and 0.22. Phagocytic index of *E. coli* infection is higher than *S. aureus* infection. The phagocytic index shown the immunological response to eliminate *S. aures* and *E. coli.* Phagocytosis plays a role in defense against pathogen and successive process of healing (inflammation). Immune booster has immunostimulant to increase phagocytosis process that eliminate pathogen in *S. aureus* and *E. coli* respectively until 2.16 and 4.27. Ethanol extract of Sappan wood (200 mg/kg BW) also increased phagocytosis process in *S. aureus* and *E. coli* respectively until 1.54 and 4.62. EESW can decrease inflammation though increasing of phagocytosis. The success of the process of eliminating pathogens through phagocytosis can affect the decrease in the level of inflammation in the body. On the other hand, an emphasis on inflammatory mechanisms to reduce tissue damage is needed through the inhibition of pro-inflammatory cytokines.

Parameters analyzed is the bond free energy (ΔG), bond hydrogen, and other patterns of interaction with amino acid residues on the active site of the receptor. Bond hydrogen is a possible interaction stabilizes the ligand binding to the receptor. Interaction through hydrogen bonds between test ligands with the same amino acid residues with natural ligands show similarities the type of interaction in this case describes activity similarity. All test compounds

have a value of bond free energy (ΔG) which negative. The more negative the value of free energy bond indicates the level of stability between the ligand and the target protein (receptor) so that the bond formed will be more and more strong. In addition to bond strength, the ability to inhibit the active compound on the target is needed. Inhibition of pro-inflammatory cytokines can be seen from the inhibition constant (Ki) of Brazilin on TNFα and IL-1β. The smaller the Ki is needed to inhibit its binding partners activity.

Inhibition of pro-inflammatory cytokine is a key in inflammation suppression. The molecular study of this research shown that Sappan wood has potential role in pro-inflammatory cytokine inhibition. Brazilin as a major compound of Sappan wood (23) have an ability to bind and inhibit some pro-inflammatory cytokine including TNFα and IL-1β. The estimation of free energy of binding between brazilin and pro-inflammatory cytokine (TNFα and IL-1β) respectively were -5.05 and -3.14 with inhibition constant (Ki) respectively were 199.52 and 4.99. In the other hand, interaction between brazilin and IL-6 is not supported by good free energy of binding and Ki. Estimation of free energy of binding in brazilin and IL-6 interaction is +14.23 without Ki (0).

There are many types of proinflammatory cytokines in the body which depend on the cell source and function of each cytokine including TNFα, IL-1β, and IL-6. TNFα is produced by macrophage and T cell that has the function such as induction of secondary cytokine secretion, stimulation of NK cells and macrophage and induce the fever and apoptosis. IL-1β is produced by endothelial cell and macrophage that has the function to induce the chemokine and secondary cytokine secretion, inductions of neutrophil release, and stimulate T-cell and macrophage. IL-6 is produces by T-cells, endothelial cell, and macrophage that has the function to induce the fever, regulate the growth and differentiation of T and B cell, induce the soluble TNF receptor and IL-1R release, and induce acute phase and inhibit the neutrophil apoptosis.

## Conclusion

Ethanol extract of Sappan wood has anti-inflammatory activity through decreasing CRP level from moderate elevation to minor elevation. Ethanol extract of Sappan wood also decrease inflammation though increasing of phagocytosis that can eliminate the pathogen. Molecular study shows that brazilin as a major compound of Sappan wood can bind and inhibit pro-inflammatory cytokine including TNFα and IL-1β. The anti-inflammatory ability of Sappan wood on gram-negative bacterial infections is better than gram-positive. This research needs to be continued by study about fractionation/ isolation and their molecular activity in inflammatory mechanism.

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## Author Contribution

MKR planned the research, coordinated to collect the data, performed the analysis, and wrote the manuscript. NT were involved in giving critical revision. MO, RK and CW were involved in data collection, analyzed, and wrote the manuscript.

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