

## **Effect of Sappan Wood Ethanol Extract in CRP Level and Phagocytic Index Between Group of Mice Infected with *S. aureus* and *E. coli***

**Martina Kurnia Rohmah<sup>1</sup>, A'yunil Hisbiyah<sup>1</sup>, Amellya Octivani<sup>2</sup>, Farida Anwari<sup>2</sup>**

<sup>1</sup>Department of Pharmacy, Universitas Anwar Medika, Sidoarjo, Indonesia, 61263

<sup>2</sup>Department of Medical Laboratory Technique, Universitas Anwar Medika, Sidoarjo, Indonesia, 61263

✉ [martina.kurniarohmah@gmail.com](mailto:martina.kurniarohmah@gmail.com)

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### **ABSTRACT**

*S.aureus* and *E.coli* are pathogenic bacterial that cause many infectious disease in the world. Immunomodulator is needed to prepare the immune system to be able against the infection. Some parameters usually used to assess the immunomodulatory activity such as C-Reactive Protein (CRP) and phagocytic index. This study aims to investigate the difference effect of sappan'wood extract in CRP level and phagocytic index between group mice infected with *S. aureus* and *E. coli*. Two treatment groups of mice were prepared for *S. aureus* and *E. coli* test. Each group subjected to 7 treatments i.e. (1) Normal mice<sup>1</sup>(CMC-Na 1 %- Merck® 217277), <sup>1</sup>(2) Negative Control (bacterial infection + CMC-Na 1 %), <sup>1</sup>(3) Positive Control (bacterial infection + Imboost force®<sup>1</sup>treatment, PT SOHO Industri Pharmasi), (4) Bacterial infection and EESW treatment 25 mg/kg BW, (5) 50 mg/kg BW, (6) 100 mg/kg BW, and (7) 200 mg/kg BW. Mice blood was taken to detect the CRP and phagocytic index after treatment. The T test showed that there was a significant difference between CRP levels ( $p < 0.05$ ) and phagocytic index ( $p < 0.05$ ) of *S. aureus* and *E. coli* group. EESW 200 mg/kg BW reduced CRP level to 11 mg/dL (*S. aureus*) and 6 mg/dL in (*E. coli*). EESW 200 mg/kg BW increased phagocytosis to 1.54 folds (*S. aureus*) and 4.62 folds (*E. coli*). Sappan wood ethanol extract effect to CRP level and phagocytic index in mice group infected with *E. coli* is better than *S. aureus* infection group.

**Keywords:** CRP; *E.coli*; Phagocytic index; Sappan wood; *S. aureus*

### **INTRODUCTION**

Infectious diseases are disorders caused by pathogenic microorganisms that can be transmitted between humans and increase the potential for disease and death. Bacteria cause many infectious disease in the world. Infection of bacteria has a high impact in health problem (Susmitha et.al., 2022). Although bacterial infections are easier to treat than viral infections, bacterial resistance is one of the problems that has not been resolved (Doron, S & Gorbach, 2020). Immunity needs to be increased to prevent infection and the pathogenesis of infectious diseases (Reyes-Silveyra & Mikler, 2016). Immunomodulators are active ingredients that can optimize the regulation of the immune system to increase immunity (Gond et

al., 2022).

*S. aureus* and *E. coli* are pathogenic bacteria that causes various human disease. *S. aureus* is a Gram-positive and most dangerous among *Staphylococcus* species. This bacteria secrete enterotoxin that cause human skin infection, pneumonia, hearth valve, and bone infections (Ibrahim, 2020). *E.coli* (Gram-negative bacteria) cause some infectious diseases including gastroenteritis, pneumonia, bacteremia, and peritonitis (Mueller et al., 2023). The basic difference between both bacteria is in cell wall structure of cell wall. There are differences metabolic physiological and immune response between gram-positive and negative. Serum metabolite level (ketone body and 3-hydroxybutyrate) of *S. aureus* significantly increases. It

caused by some bacterial exo-metabolomes and high fatty acid oxidation. It indicates high virulence and severe changes in liver cellular response. Different from *S. aureus* infection, *E. coli* induces immune response such as Toll Like Receptor 4 (TLR4) and reduces metabolic activity (Hoerr et al., 2012).

Immune system has two ways to response infections including innate and adaptive immunity. Innate immunity is first immune response to defence infection. There are many parameter that used in innate immunity activity including C-Reactive Protein (CRP) and phagocytosis activity. CRP is protein in acute phase that most synthesized in hepatocytes. CRP increases infection and inflammation until 1.000 folds (Sproston & Ashworth, 2018). CRP can become a marker in *S. aureus* (Mölkänen et al., 2016) and *E. coli* infection (Narayan Swamy et al., 2022). Phagocytosis is a removal mechanism the pathogen by ingestion the pathogene molecule. During infection and inflammation, phagocytosis response increase caused by bacterial product, cytokines and inflammatory mediator. Reduction of phagocytosis usually show the reduction of immune defense that seen by phagocytic index (Gordon, 2016).

Sappan wood usually used as herbal medicine. Sappan wood has antibacterial activity in *S. aureus* (Hemthanon & Ungcharoenwiwat, 2022) and *E. coli* (Widigdyo et al., 2017). Sappan wood has immunomodulatory activity (Sunitha et al., 2015). It contains phytochemical substances including brazilin (major component), brazilide A, brazilein, protosappanin A, B, C, D, dan E etc (Muti et al., 2021). Brazilin (specific color of Sappan wood) has antioxidant effect (IC<sub>50</sub> 57,2 µM) and potential effect on anti-inflammation (Vij et al., 2023). Brazilin also has anti-inflammatory effect, inhibits LPS (Lippo Poly Saccharide), prostaglandin E<sub>2</sub>, TNF-α and IL-1β (Arjin et al., 2021). This research aims to study the effect of Sappan wood ethanol extract in CRP level and phagocytic index between groups of mice infected with *S. aureus* and *E. coli*.

## METHODS

Mice (*Mus musculus*) Balb/C strain (Male, 25 days, 20-40 g, n=70) was used in this research. The protocol of this research has ethics approve from Faculty of Dental Medicine Health Research Ethical Clearance Commission (Certificate number 217/HRECC.FODM/V/2022). Semi-quantitative phytochemical screening was carried out to detect the

presence of secondary metabolite in sappan wood extract. The parameter measured in this research were CRP level and phagocytic index using post-test only control group design. The mice divided into 2 groups: *S. aureus* and *E. coli* infected mice. Each group consists of 7 treatments including (1) Normal mice (CMC-Na 1 %- Merck® 217277), (2) Negative Control (bacterial infection + CMC-Na 1 %), (3) Positive Control (bacterial infection + Imboost force® treatment, PT. SOHO Industri Pharmasi), (4) Bacterial infection and EESW treatment 25 mg/kg BW, (5) 50 mg/kg BW, (6) 100 mg/kg BW, and (7) 200 mg/kg BW. Each treatment group consist of 5 mice as replication data.

## Preparation of Sappan Wood Extract

Sappan wood (500 g sappan wood powder from Materia Medica, Batu) was macerated using using 3.750 mL of 96 % ethanol (PT Brataco, Bandung). Maceration and re-maceration process was carried out for 5 day while stirring occasionally 3 times a day. The obtained filtrate was concentrated at 65 °C using rotary evaporator (IKA Scientific®, No. 0010012324, Germany). We made EESW filtrate in 4 concentrations including 25, 50, 100, and 200 mg/kg BW.

## Phytochemical Screening of Sappan Wood Extract

Phytochemical screening of Sappan wood ethanol extract was carried out using qualitative assay to determine the presence of flavonoid, polyphenols, alkaloid, tannin, saponin, and steroid compounds. Flavonoid was detected using Mg powder (PT Multi Medika Laboratory) and concentrated with HCl. The alkaloid was tested using Dragendorff reagent (Matelab Trifa.Solusindo). Tannin was tested using iron (III) chloride 10 % (Merck KGaA, Germany). Saponin was tested using HCl 1 N (Merck KGaA, Germany). Polyphenol was tested using FeCl<sub>3</sub> 1 % (Merck KGaA, Germany). Steroid was tested HCl and H<sub>2</sub>SO<sub>4</sub> (Merck KGaA, Germany). The CRP test kit (Glory Diagnostic®, Spain).

## *S.aureus* and *E.coli* Preparation

The bacterial suspension (*S. aureus* and *E. coli*) was prepared at a concentration of 10 CFU/mL. Bacterial suspension (1 mL) was added with sterile distilled water (9 mL) then diluted by taking 1 mL of suspension from tube 1 and added with 9 mL of distilled water to reach the concentration of 10<sup>9</sup>

CFU/mL.

### Mice Preparation and Infection

Mice was given pellets that contains fiber, protein, fat, minerals, and water *ad libitum*. Mice was infected by *S. aureus* and *E. coli* ( $10^9$  FU/mL by oral. After 24 hours, CRP level was tested to confirm effect of infection. Each group of mice were subjected to treatments procedure for 7 days. Each day the mice blood was collected from jugular vein. The blood serum was separated using centrifuge.

### CRP Test

The presence of CRP in sample were detected using qualitative test. About 50 mL CRP latex were drop on the slide followed by 50 mL serum sample. CRP test was positive if agglutination existed and vice versa. Sample with positive CRP will be followed semi-quantitatively CRP test using serum, NaCl and CRP latex by dilution method. Titter of CRP was calculated by multiplying the CRP constant value (6 mg/L) with the highest dilution number.

### Phagocytosis Activity Test

Carbon clearance method was used to determine phagocytosis activity using UV-Vis Spectrophotometer (Thermo-Scientific®, USA) at 650 nm wavelength (Nimal et al., 2015). Constant carbon elimination speed value was measured followed by calculation of phagocytic index using formulas from several previous studies (Reze & Rosidah, 2020) (Rahman et al., 2016).

## RESULTS AND DISCUSSION

### Qualitative Phytochemical Screening of Sappan Wood Ethanol Extract

The result of phytochemical screening showed

that Sappan wood ethanol extract contain flavonoids, polyphenols, alkaloids, saponins, and tannins, but no steroid and terpenoid were detected. The phytochemical compounds detected in sappan wood extract was showed in Table 1.

This research uses ethanol which is a polar solvent. Flavonoids, polyphenols, saponins and tannins are polar compounds and can dissolve in ethanol (Stern et al., 2022). Alkaloid can dissolve in semi-polar solvents, so they are also found in the ethanol extract of Sappan wood. Other compounds such as steroids and terpenoids in this study are not soluble in ethanol because the compounds are non-polar. This fits with the like dissolves like theory.

Flavonoids, polyphenols, alkaloids, saponins, and tannins have immunomodulator effect. Flavonoids modulate some immune activity such as inhibit TLR mediated inflammation, inhibit ROS, and inhibit release of inflammatory cytokine (Bernard et al., 2015). Flavonoids inhibit the secretion of lysozymes,  $\beta$ -glucuronidase and arachidonic acid. It causes reduces inflammatory reactions. It also induces anti-inflammatory cytokines such as  $\text{TNF}\alpha$ ,  $\text{IL-1}\beta$ , and  $\text{IL-8}$  (Al-Khayri et al., 2022). Flavonoid stimulates NK cells, dendritic cell maturation reduction, promote macrophage conversion from M1 to M2. Flavonoid also induce Treg cell proliferation, increase CTL and B cell activation (Han et al., 2022). Alkaloids regulate proliferation of thymic and splenic lymphocytes. It also increase anti-inflammatory cytokine secretion (Jiang et al., 2021). Polyphenols pro inflammatory cytokine suppression, natural killer cells and macrophages mediation, and improve commensal bacteria (Mamun et al., 2024). Tannins reduce Th-derived cytokines level ( $\text{IL-17}$ ,  $\text{IFN-}\gamma$ , and  $\text{IL-4}$ ) by oral administration (Piazza et al., 2022).

**Table 1.** Phytochemical Screening of Sappan Wood Ethanol Extract

Compound	Result	Observation
<b>Positive</b>		
Flavonoid	+	Orange precipitation
Polyphenol	+	Dark blue solution
Alkaloid	+	Orange precipitation
Saponin	+	Presence of foam
Tanin	+	Bluish green color
<b>Negative</b>		
Steroid	-	Discoloration
Terpenoid	-	No brownish-red

### Effect of Sappan Wood Ethanol Effect on CRP Levels between Group Mice Infected with *S. aureus* and *E. coli*

The first parameter that used to assess immunomodulator activity in this research is CRP. The result of CRP level assessment listed in Table 2. Statistical analysis showed that there were significant differences among CRP level of 7 treatments of both *S. aureus* ( $p=0.000$ ) and *E. coli* ( $p=0.002$ ) infection groups. CRP level is zero in health mice, increased in negative control (infected mice) and low in positive control (drug treatment after infection). CRP level of EESW 25 kg/kg BB still same with negative control ( $p>0.05$ ) in both *S. aureus* and *E. coli* infection, but not EESW 50, 100, and 200 mg/kg BW. The CRP level of EESW 100 and 200 mg/kg BW were not different with positive control ( $p>0.05$ ). T test showed that there was significant different between CRP level of *S. aureus* and *E. coli* infection groups ( $p<0.05$ ). CRP level between *S. aureus* and *E. coli* treatment group was showed on graph in Figure 1.

Data showed that EESW has potential in immunomodulator and anti-inflammatory activity through CRP reduction effectively on 100 and 200 mg/kg BW in both *S. aureus* and *E. coli*. Immunomodulatory effect of Sappan wood ethanol extract on CRP level reduction in *E. coli* infection was better than *S. aureus*. EESW 100 mg/kg BW reduces CRP level until 12 mg/dL in *E. coli* infection and 22 mg/dL in *S. aureus* infection. EESW 200 mg/kg BW reduces CRP level until 6 mg/dL in *E. coli* infection and 11 mg/dL in *S. aureus* infection.

CRP is synthesized primarily in hepatocytes and other cell such as lymphocyte, monocyte, muscle, endothelial cell etc (Sproston & Ashworth, 2018). It binds to damage tissue, antigen and to pathogenic organism in a calcium-dependent manner. CRP activates complement, binds to Fc receptor (FcR) and

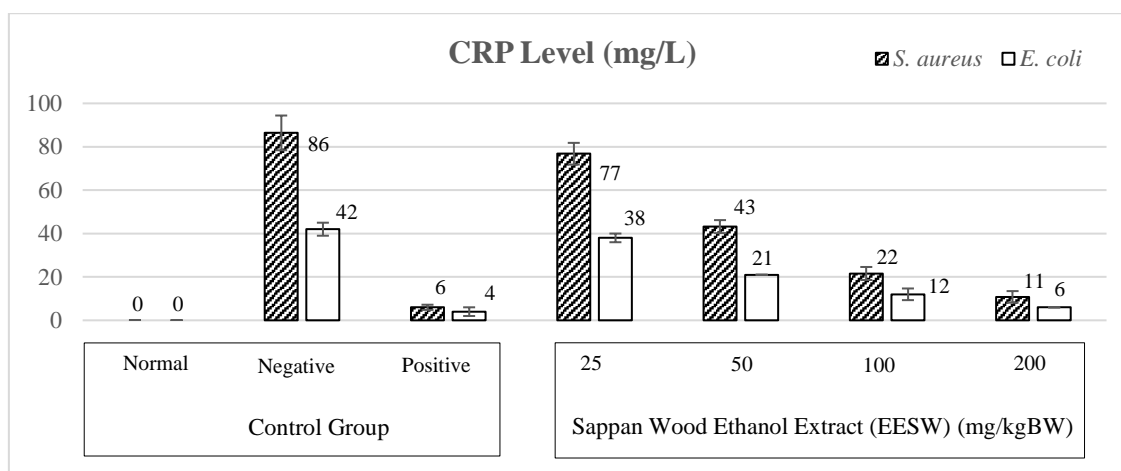
activates opsonin for various pathogens. CRP and FcR induces proinflammatory cytokine and enhance the inflammatory response. CRP was measured to monitor various inflammatory state and response of immune system (Du Clos, 2000). CRP increased in infection and inflammation (Sproston & Ashworth, 2018). CRP level is also as a predictive factor of Sepsis mortality (Lubis et al., 2020).

CRP level of *S. aureus* (Gram-positive bacteria) infection was higher than *E. coli* (Gram-negative bacteria). CRP level of *S. aureus* infection was 8,7 mg/dL (87 mg/L) (Sproston & Ashworth, 2018), whereas *E. coli* infection was 6,2 mg/dL (62 mg/L) (Park et al., 2005). Based on CRP level interpretation, the CRP level of *S. aureus* and *E. coli* was moderate elevation between 1.0–10.0 mg/dL or 10–100 mg/L (Nehring et al., 2023). Acute inflammatory response of gram-negative versus gram positive bacterial study showed that pro-inflammatory cytokine serum concentration in Gram-positive bacteria is higher than negative bacteria. *S. aureus* induces lipopolysaccharide more than *E. coli*. The levels of Interleukin 1Ra (IL-1Ra), IL-10, IL-8, and TNF $\alpha$  in patients with sepsis with Gram-negative bacteria infection were not different from those with sepsis with Gram-positive bacterial infection, but the concentrations were higher in patients with Gram-positive bacterial infections. However, plasma concentrations of IL-18 and IL-1 $\beta$  were significantly higher in Gram-positive infection patients (Feezor et al., 2003). CRP level after *Echinacea purpurea* (positive control) decreased CRP level in *S. aureus* and *E. coli* infection successively 6 mg/L and 4 mg/L (minor elevation). *Echinacea purpurea* has returned CRP level at the normal condition (0 mg/L) after oxidative stress. It also may afford protection against oxidative stress in tissues and modulate immune responses (Ezz, 2011).

**Table 2.** CRP Level of *S. aureus* and *E. coli* – Infected Mice after Treatment

Treatment Group	Mean of CRP level (mg/L) $\pm$ SD	
	<i>S. aureus</i>	<i>E. coli</i>
1. Normal (Health Mice)	0 <sup>a</sup> $\pm$ 0	0 <sup>a</sup> $\pm$ 0
2. Negative Control (Infected Mice)	86 <sup>b</sup> $\pm$ 8	42 <sup>b</sup> $\pm$ 3
3. Positive Control (Infected Mice + <i>Echinacea</i> Immune booster)	6 <sup>a</sup> $\pm$ 1	4 <sup>c</sup> $\pm$ 2
4. Infected Mice + EESW 25 mg/kg BW	77 <sup>b</sup> $\pm$ 5	38 <sup>b</sup> $\pm$ 2
5. Infected Mice + EESW 50 mg/kg BW	43 <sup>d</sup> $\pm$ 3	21 <sup>d</sup> $\pm$ 0
6. Infected Mice + EESW 100 mg/kg BW	22 <sup>a</sup> $\pm$ 3	12 <sup>c</sup> $\pm$ 3
7. Infected Mice + EESW 200 mg/kg BW	11 <sup>a</sup> $\pm$ 3	6 <sup>c</sup> $\pm$ 0

Note: Different notations (a, b, c, and d) showed the different effects.



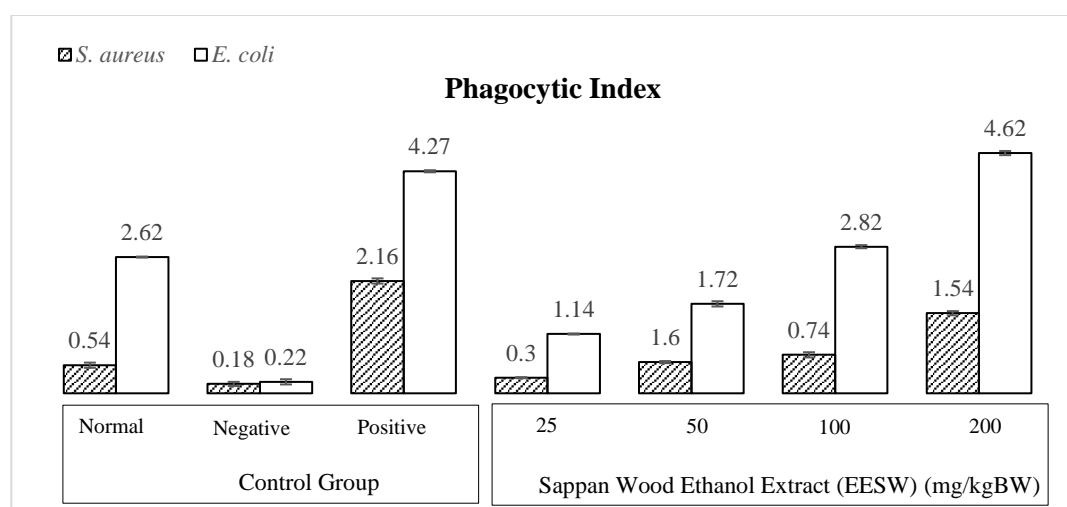
**Figure 1.** CRP Level Comparison Between *S. aureus* and *E. coli* Treatment Group.

EESW 50, 100, and 200 mg/kg BW could reduce CRP level. EESW has immunomodulator and anti-inflammatory activity through CRP reduction in both *S. aureus* and *E. coli* effectively on 100 and 200 mg/kg BW. Immunomodulatory effect of EESW CRP level reduction in *E. coli* infection was better than *S. aureus*. EESW 100 mg/kg BW reduce CRP level until 12 mg/dL in *E. coli* infection and 22 mg/dL in *S. aureus* infection. EESW 200 mg/kg BW reduce CRP level until 6 mg/dL in *E. coli* infection and 11 mg/dL in *S. aureus* infection. Based on these data, EESW has similar activity with positive control in CRP level reduction. EESW reduce CRP level in moderate elevation in *S. aureus* infection and until minor elevation in *E. coli* infection. This result was associated with proinflammatory and CRP level in both infections. CRP level and proinflammatory cytokine of *S. aureus* (Gram-positive bacteria) infection was higher than *E. coli* (Gram-negative bacteria). In

previous study showed that Sappanwood ethanol extract 100 mg/kg BW also has antioxidant and hepatoprotective activity in Diabetic Rats (Holidah et al., 2022). Previous study showed that Sappan wood (*Caesalpinia sappan* L. extract reduces necrosis, inflammatory cell infiltration, and myocardial interstitial edema of rats induced by isoproterenol at 100 and 200 mg/kg BW (Nugraheni & Saputri, 2017).

#### Effect of Sappan Wood Ethanol Extract on Phagocytosis Index Between Group of Mice Infected with *S. aureus* and *E. coli*

Phagocytosis is one of innate immune response that promotes pathogen elimination and limit pathogen growth. Phagocytosis is indicated by phagocytic index. The phagocytic index ( $\alpha$ ) is the average number of bacteria ingested per macrophage. The phagocytic index data are shown in Figure 2.



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



The statistical analysis showed that there were significant differences among treatments in *S. aureus* ( $p=0.000$ ) and *E. coli* ( $p=0.000$ ). The phagocytic index of *E. coli* was higher than *S. aureus* infection. Phagocytic index of negative control was lower than normal control, but the phagocytic index of positive control was higher than normal. The phagocytic index of the infection group with EESW 25, 50, 100, and 200 mg/kg BW treatment were higher than negative control. EESW 100 mg/kg BW increased phagocytic index to 0.74 folds (*S. aureus*) and 2.82 folds (*E. coli*). EESW 200 mg/kg BW increased phagocytic index to 1.54 folds (*S. aureus*) and 4.62 folds (*E. coli*).

Phagocytosis is an immunological process that can engulf and eliminate particles larger than 0.5  $\mu\text{m}$  in diameter, including foreign substances, microorganisms, and apoptotic cells (Uribe-Quero & Rosales, 2017). The phagocytic index is the average number of bacteria ingested per macrophage (Jäger et al., 2021). Phagocytic number showed the number of bacteria ingested per macrophage. In this research showed the low phagocytic index of *S. aureus* and *E. coli* infection successively until 0.18 and 0.22 folds. This phagocytic index was lower than phagocytic index of normal control 0.54 and 2.62 folds. This data indicated that *S. aureus* and *E. coli* infection decrease phagocytosis. In the previous study showed that *S. aureus* secretes the 16 kD Extracellular fibrinogen binding protein (Efb) that effectively blocks phagocytosis in mouse peritonitis model (Ko et al., 2013). Previous studies showed that neutrophils in infected patients were significantly lower compared with healthy adults. After 24 hours after differentiation and maturation during infection, neutrophils will spontaneously apoptosis so that the phagocytes produced in the bone marrow are released in an immature condition. This causes the phagocytic index in infectious conditions to quickly fall (Yang et al., 2021).

Phagocytic activity shown through the phagocytic index indicates the condition of the immune system when infection occurs. The phagocytic index in *S. aureus* and *E. coli* infection groups with immune booster was 2.16 and 4.27 times respectively. This phagocyte index was better than the negative control which did not receive immune booster treatment with *Echinacea purpurea* L. Previous research showed that Sappan wood increased phagocytosis and the release of TNF- $\alpha$ , IL-6, and IL-

1 $\beta$  (pro-inflammatory cytokines) (Sudeep et al., 2023). The phagocytic index of Sappan wood was higher than the negative control (see Figure 2). Increasing EESW concentrations correlate with higher phagocytic index. EESW 100 mg/kg BW increased the phagocytic index up to 0.74-fold in *S. aureus* infections and 2.82-fold in *E. coli*. EESW 200 mg/kg BW increased the phagocytic index up to 1.54-fold in *S. aureus* infection and 4.62-fold in *E. coli*. This difference may correspond to the phagocytic activity against bacteria.

Previous research showed that neutrophil phagocytosis against *E. coli* (46.91–83.09 %) was higher than *S. aureus* infection (33.92–69.48 %) (Yang et al., 2021). *Caesalpinia sappan* L. ethanol extract 25 mg/kg BW increased the phagocytic index in infected mice by more than 80-fold (Mathew, 2015). *Caesalpinia sappan* L. also increased Interleukine-10 in candidiasis. IL-10 increases phagocytosis and neutrophil recruitment thereby mediating inflammation (Wahyu et al., 2021). *Caesalpinia sappan* L. ethanol extract 100 and 200 mg/kg BW increased the number of Kupffer cells (liver macrophages) (Erick Khristian, Ratu Safitri, Mohammad Ghozali, 2022).

## CONCLUSION

Sappan wood ethanol extract has immunomodulatory effect marked by CRP reduction and phagocytosis enhancement. Immunomodulatory effect of Sappan wood ethanol extract in group of mice infected with *E. coli* was better than the group of mice infected with *S. aureus*. Sappan wood reduced CRP in moderate elevation in *S. aureus* mice group and minor elevation in *E. coli* mice group. Sappan wood also increased phagocytic index in *E. coli* mice group better than *S. aureus* mice group. This result associated with bacterial virulence and innate immune response of body against bacterial infection.

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## CONFLICT OF INTEREST

All authors have not conflict of interest in this research

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