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Immunomodulator activity test of ethanol extract of Sappan Wood (*Caesalpinia Sappan L.)* in mice (*Mus Musculus*) infected by *Staphylococcus aureus*

Prueba de actividad inmunomoduladora del extracto etanólico de madera de Sappan (*Caesalpinia Sappan L.*) en ratones (*Mus Musculus*) infectados por *Staphylococcus aureus*

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SUMMARY

Introduction: *The potential of immunomodulators from a natural product is widely studied as a choice of natural immune support supplements. In Indonesia, Caesalpinia sappan (Sappan wood) is a traditional herbal drink to boost the immune system. Sappan wood contains potential compounds as immunomodulators, such as brazilin, sappanchalcone, and phenol. This study aims to determine the immunomodulatory activity of Sappan wood infected by Staphylococcus aureus.*
Methods: *This study was a true experimental design that used Mus musculus infected by Staphylococcus aureus (10^{-9} CFU/mL). This study consisted of 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. The treatment was given for 7 days after the Staphylococcus aureus infection.*
Results: *One-way ANOVA analysis showed that the administration of 96 % ethanol extract from Sappan wood on phagocytic activity, C-Reactive Protein (CRP), and total leukocyte levels was significantly different from negative control ($p=0.0001$), but not to normal and positive control ($p<0.05$). The ethanol extract of Sappan wood at 100 and 200 mg/ kg body weight increases the phagocytic index to 1.32 and 1.54 folds. It also can reduce CRP levels to 60 and 48 mg/L. The lower CRP level indicates lower inflammation.*
Conclusion: *Sappan wood has immunomodulatory activity through increased phagocytic activity, decreased CRP levels, and normalized total leukocyte levels, especially at 100 and 200 mg/kg body weight. The extract can enhance total leukocyte levels and positively affect CRP levels.*

Keywords*:* C-Reactive protein*, immunomodulator, leukocytes, phagocytosis, Sappan wood.*

RESUMEN

Introducción: *El potencial de los inmunomoduladores de un producto natural es ampliamente estudiado como una opción de suplementos naturales de apoyo inmunológico. En Indonesia, Caesalpinia sappan*

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(madera de Sappan) es una bebida tradicional a base de hierbas para estimular el sistema inmunológico. La madera de sappan contiene compuestos potenciales inmunomoduladores, como la brasilina, la sappanchalcona y el fenol. Este estudio tiene como objetivo determinar la actividad inmunomoduladora de la madera de Sappan infectada por Staphylococcus aureus.

Métodos: *Este estudio fue experimental que utilizó Mus musculus infectado por Staphylococcus aureus (10-9 UFC/mL). Consistió en un control normal, un control negativo (placebo), un control positivo (refuerzo inmunitario) y 25, 50, 100 y 200 mg/kg de peso corporal del extracto etanólico de madera de Sappan (SWEE) al 96 %. El tratamiento se administró durante 7 días después de la infección por Staphylococcus aureus.*

Resultados: *El análisis ANOVA de una vía mostró que la administración de extracto etanólico al 96 % de madera de Sappan sobre la actividad fagocítica, la proteína C reactiva (PCR) y los niveles totales de leucocitos fue significativamente diferente del control negativo (p=0,0001), pero no un control normal y positivo (p<0,05). El extracto etanólico de madera de Sappan a 100 y 200 mg/kg de peso corporal aumenta el índice fagocítico hasta 1,32 y 1,54 veces. También puede reducir los niveles de CRP a 60 y 48 mg/L. El nivel más bajo de PCR indica una inflamación más baja.*

Conclusión: *La madera de sappan tiene actividad inmunomoduladora a través de una mayor actividad fagocítica, disminución de los niveles de PCR y normalización de los niveles totales de leucocitos, especialmente a 100 y 200 mg/kg de peso corporal. El extracto puede mejorar los niveles totales de leucocitos y afectar positivamente los niveles de PCR.*

Palabras clave: *Proteína C reactiva, inmunomodulador, leucocitos, fagocitosis, Sappan wood.*

INTRODUCTION

The immune system has the basic function of protecting against foreign pathogens and infectious agents, consisting of innate and adaptive immunity with various cells and molecules involved (1-3). Innate immunity is a non-specific immune response as a first-line defense such as physical barriers, anatomical barriers, epithelial and phagocytic cell enzymes, phagocyte, inflammations-relate serum proteins, surface and phagocyte granule antimicrobial peptides, the immune receptor on cells, and cells that release cytokines and inflammatory mediators (4-6). Innate immunity is followed by adaptive immunity, a specific immune response, and complex. Adaptive immunity consists of antibody response (B cell) and cell-mediated response (T helper and Cytotoxic T lymphocyte) (7).

Immunomodulators are synthetic or natural substances that can stimulate, suppress, or modulate any aspect of the immune system, including innate and adaptive immunity. In clinical medicine, immunomodulators are usually used to treat infection, reconstitute immunodeficiency, and suppress excessive immune function (8-10). Immunomodulators consist of immunoadjuvants, immunostimulants, and immunosuppressants. Immunoadjuvant is used for enhancing the efficacy of vaccines (specific immunostimulants). Immunostimulant is used to enhance the immune system and against infection. Immunosuppressant suppresses excessive immune function (11).

Currently, immunomodulator from natural compounds (phytochemical) is preferable because it has lower toxicity (12,13). The most common phytochemicals that can modulate immune response are flavonoids, flavanols, quinones, glycosides, polysaccharides, terpenoids, alkaloids, phenolics, saponins, various bitter, vitamin C, etc. (14).

Caesalpinia sappan (Sappan wood) is a traditional medicinal plant used in Asia, including Indonesia. Indonesians consume Sappan wood to increase stamina and maintain a healthy body. Sappan wood has antioxidant, antibacterial, antiviral, antifungal, cardiovascular protection, anti-inflammatory, antidiabetic, anticancer, anti-malarial, and gastroprotective roles (15). Sappan wood contains homoisoflavonoid and phenolics such as 4-O-methylsappanol, protosappanin A, protosappanin B, protosappanin E, brazilin, brazilein, caesalpin, brazilide A, neosappanone A, caesalpin P, sappanchalcone, 3-deoxysappanone,10 7,3',4'-trihydroxy-3- benzyl-2H-chromene (16). Purifie brazilin (10 mg/kg body weight) isolated from Sappan wood reduced the arthritis index score, and acute inflammation was administered every three days for 21 days. Other research show that 124 mg Sappanchalcone from the dried heartwood of Sappan wood regulates the level of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 (17). In addition, Sappan wood (25 mg/ kg body weight) has an immunomodulatory effect on murine peritoneal macrophages (18).

Staphylococcus aureus can cause some disturbances to cells that play a role in the immune system, one of which is the virulent *Staphylococcus aureus*

in the form of A protein that can cause binding to immunoglobulin receptors (19). In addition, *Staphylococcus aureus* causes excessive production of proinflammatory cytokines, resulting in inflammation and systemic shock (20). Leucocidin enzymes can destroy leukocytes, neutrophils, and macrophages. Coagulase enzymes can accelerate the formation of fibrin so that it blocks the phagocytosis process. *Staphylococcus aureus* has also increased C-Reactive Protein levels (CRP) *in vitro* (21).

Leukocytes are an important part of the body's defense system, fighting infection-causing microorganisms, tumor cells, and harmful foreign substances. Leukocytes protect the body against various diseases using phagocytes and produce antibodies. The main cells that play a role in non-specific defense are mononuclear (monocytes and macrophages). Both types of cells are derived from white blood cells. Early phagocytosis of bacterial invasion can prevent disease (1).

C-Reactive Protein (CRP) is an alpha globulin present in the serum as a non-specific immune response when inflammation occurs. CRP is an acute phase protein that can increase in value up to 1 000 times at the site of inflammation or infection. An immunomodulator is a drug that can be used to restore and repair an impaired immune system (22). Based on the above background, this research aims to determine the immunomodulatory activity of Sappan wood in *Mus musculus* infected by *Staphylococcus aureus*.

METHODS

This research was an true experimental design that used Mus musculus (mice) with pre and post-design. The mice were infected by *Staphylococcus aureus* (10^{-9} CFU/mL). This research was divided into 7 groups: normal control, negative control (CMC-Na 1 %), positive control (immune booster), and 96 % ethanol extract of Sappan wood 25, 50, 100, and 200 mg/kg body weight. The treatment was given for 7 days after the *Staphylococcus aureus* infection. This research is a pre-clinical study using experimental animals and has received an ethics certificate numbered 632/RSAM/III/2021.

The extraction of Sappan wood was carried out using the maceration method. The 500 g of extracted sample was placed into a vessel and dissolved in 3 750 mL of 96 % ethanol as solvent. Maceration was carried out for 5 days with stirring 3 times a day. The extract was filtered to separate the filtrate. The filtrate was then concentrated with a rotary evaporator at a temperature of 65°C to obtain a thick extract of Sappan wood. Phytochemical tests were carried out to determine the content of Sappan wood extract, including flavonoids, alkaloids, tannins, polyphenols, anthraquinones, saponins, steroids, and terpenoids. Sappan wood ethanol extract solution was made with 4 concentrations: 25, 50, 100, and 200 mg/kg body weight. The treatment was adjusted according to the body weight of each mouse.

Staphylococcus aureus was grown on nutrient agar media and incubated for 1 x 24 h. First, the preparation of 10^{-9} CFU/mL bacterial suspension was done by adding 1 mL of bacterial suspension into 9 mL of sterile distilled water in the 1st test tube to form a bacterial concentration of 10^{-1} CFU/mL. Then, the dilution was carried out by taking 1 mL in the 1st tube and adding 9 mL of sterilized distilled water to form a suspension with a concentration of 10^{-9} CFU/mL.

Mice that had been acclimatized for 7 days were then infected with *Staphylococcus aureus*. After 1x24 h, the mice were grouped into 7 test groups: normal control, negative control (CMC-Na 1 %), positive control (immune booster), and 96 % ethanol extract of Sappan wood 25, 50, 100, and 200 mg/kg body weight. The treatment was given for 7 days.

Parameters in this immunomodulatory activity test included total leukocyte count, phagocytosis index, and CRP levels. Measurement of leukocyte count, index, and CRP levels before and after treatment (pre and post-design). The total leukocyte was measured by using Hematology Analyzer. The phagocytosis index was measured using the Carbon Clearance method on a UV-Vis Spectrophotometer with a wavelength of 650 nm. CRP levels were measured using a semi-quantitative method. The data that has been obtained was analyzed statistically using a t-test and one-way ANOVA.

RESULTS

Phytochemical Test of Sappan Wood Ethanol Extract

Based on the phytochemical test, it is known that the ethanol extract of Sappan wood contains the group of compounds shown in Table 1.

Table 1

Phytochemical Test of Sappan Wood Ethanol Extract

Group of Compounds	Results
Flavonoid	+
Alkaloid	+
Polyphenol	+
Saponin	+
Anthraquinone	+
Steroid	-
Terpenoid	-

Phagocytic Activity Test

Phagocytic activity was measured from the phagocytic index using the carbon clearance method. The result phagocytic activity test is shown in Table 2 and Figure 1.

Based on paired sample t-test, post and pre-test is significantly different with significant value p=0.007 ($p<0.05$) and t-count > t-table (3.959 > 2.364). This result shows that the administration of Sappan wood ethanol extract increases the phagocytic index.

The enhancement of the phagocytic index before and after treatment can be seen in the graph in Figure 1.

Table 2

Phagocytic Activity Test Pre and Post Design

Treatment Groups	Phagocytic Index	
	Pre (Mean ± SD)	Post (Mean ± SD)
Normal Control	1.4 ± 0.017	1.9 ± 0.008
CMC Na 1 %/ infected only (Negative Control)	1.4 ± 0.003	1.6 ± 0.013
Immuno Booster (Positive Control)	1.6 ± 0.021	3.8 ± 0.015
SWEE 25 mg/ kg body weight	1.5 ± 0.011	2.1 ± 0.015
SWEE 50 mg/ kg body weight	1.7 ± 0.001	2.6 ± 0.003
SWEE 100 mg/ kg body weight	1.6 ± 0.008	2.9 ± 0.011
SWEE 200 mg/ kg body weight	1.7 ± 0.013	3.2 ± 0.007

SWEE: Sappan Wood Ethanol Extract

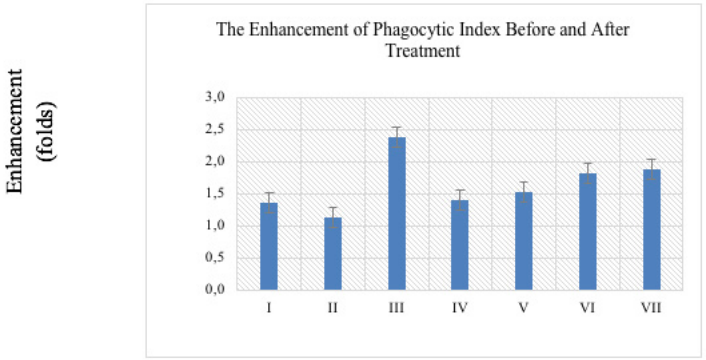

Figure 1. The Enhancement of Phagocytic Index Before and After Treatment. Treatment I: Normal Control, II: CMC Na 1 %/ infected only (Negative Control), III: Immuno Booster (Positive Control), IV: SWEE 25 mg/ kg body weight, V: SWEE 25 mg/ kg body weight, VI: SWEE 25 mg/ kg body weight, VII: SWEE 25 mg/ kg body weight.

The graph shows that an immune booster can increase the phagocytic index 2.4 folds, while the administration of ethanol extract of Sappan wood 25, 50, 100, and 200 mg/ kg body weight successively increased the phagocytic index by 1.4, 1.5, 1.8 and 1.9 folds respectively. This result differs from the negative control group with *Staphylococcus aureus* infection only without treatment. The phagocytic index is quite low.

One-way ANOVA show there are significant differences among 7 treatments with significant value p=0.0001 and F-count > F table (639.48 > 2.29). The result of one-way ANOVA is shown in Table 3.

The statistical test shows that the phagocytic index significantly differs between the infection condition (negative control) and the normal condition. The phagocytic index in normal conditions is higher than in infection conditions. The administration of an immune booster (positive control) is proven to increase phagocytic index 2.4 folds and is significantly different from the normal and negative control. The administration of ethanol extract from Sappan wood is proven to increase phagocytic index on 100 and 200 mg/kg body weight until 1.32 and 1.54 folds. It is significantly different from the normal and negative control.

Table 3

The Difference in Phagocytosis Index Before and After Treatment

Treatment Groups	Mean ± SD
Normal Control	0.54a ± 0.05
CMC Na 1 %/ infected only (Negative Control)	0.18b ± 0.04
Immuno Booster (Positive Control)	2.16c ± 0.05
SWEE 25 mg/ kg body weight	0.6a ± 0.1
SWEE 50 mg/ kg body weight	0.74a ± 0.05
SWEE 100 mg/ kg body weight	1.32d ± 0.04
SWEE 200 mg/ kg body weight	1.54e ± 0.01

SWEE: Sappan Wood Ethanol Extract

C-Reactive Protein (CRP) Test

The graph of CRP levels after each treatment is shown in Figure 2.

CRP levels in the normal group, negative control, positive control, SWEE 25, 50, 100, and 200 mg/ kg body weight were 0, 96, 12, 96, 84, 60, and 48 mg/L. CRP is not produced in the normal condition, but high in infection (negative control). The immune booster can decrease CRP level to 12 mg/L. The ethanol extract of Sappan wood at 100 and 200 mg/kg body weight can decrease CRP levels to 60 and 48 mg/L. The lower CRP level indicates lower inflammation.

Total Leukocyte Level

The total leukocyte was measured on the last day after treatment. The total leukocyte is shown in Figure 3. The result shows that the normal condition's leukocyte level is ± 1.5 x 10^3 cell/ mm^3. *Staphylococcus aureus* infection increases leukocyte infection until ± 16.7 x 10^3 cell/ mm^3. The leukocyte

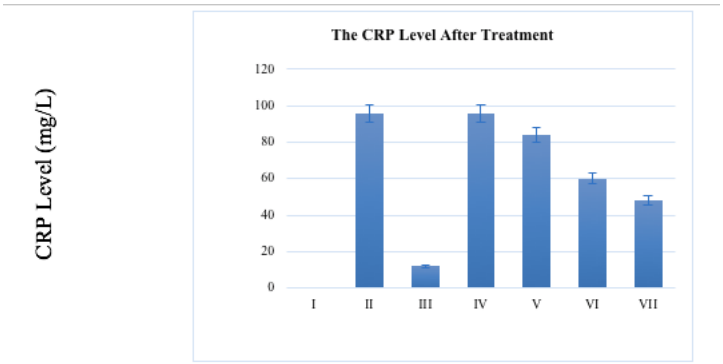

Figure 2. The CRP Level After Treatment and *Staphylococcus aureus* infection. Treatment I: Normal Control, II: CMC Na 1 %/ infected only (Negative Control), III: Immuno Booster (Positive Control), IV: SWEE 25 mg/ kg body weight, V: SWEE 25 mg/ kg body weight, VI: SWEE 25 mg/ kg body weight, VII: SWEE 25 mg/ kg body weight.

level if positive control is ± 11.2 x 10^3 cell/ mm^3. The leukocyte ethanol extract of Sappan wood 25, 50, 100, and 200 mg/ kg body weight respectively are ± 15.8 x 10^3, 14.2 x 10^3, 13.6 x 10^3, and 12.2 x 10^3 cell/ mm^3. The leukocyte level of ethanol extract of Sappan wood is in the normal range.

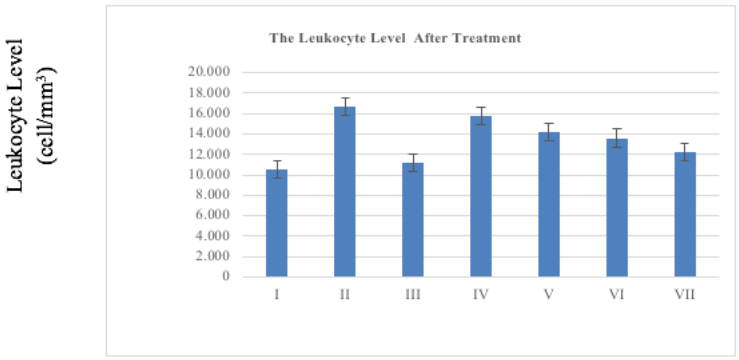

Figure 3. The Leukocyte Level After Treatment and *Staphylococcus aureus* infection. Treatment I: Normal Control, II: CMC Na 1 %/ infected only (Negative Control), III: Immuno Booster (Positive Control), IV: SWEE 25 mg/ kg body weight, V: SWEE 25 mg/ kg body weight, VI: SWEE 25 mg/ kg body weight, VII: SWEE 25 mg/ kg body weight.

DISCUSSION

Based on the phytochemical test in this research, we know that Sappan wood contains flavonoids, alkaloids, polyphenols, saponin, and anthraquinone but not steroids and terpenoids. Based on the literature, ethanol 95 % Sappan wood extract contains an alkaloid, flavonoid, anthraquinones, coumarin, saponin, tannin, and cardiac glycoside but does not contains steroid and terpenoid (23). Sappan wood also contains phenolic compounds, mainly including phenolic acids, flavonoids, tannins, coumarins, lignans, quinones, stilbenes, and curcuminoids (24). Sappan wood also contains several aromatic compounds such as brazilin, sappanchalcone, caesalpin, protosappanin A and B, homo-isoflavonoids β-sitosterol, mono-hydroxybrazilin, and quercetin (25).

Phagocytic tests show that ethanol extract of Sappan wood 100 and 200 mg/kg body weight significantly increases phagocytic index until 1.32 and 1.54 folds. Meanwhile, the immune booster (positive control) can increase the phagocytic index by 2.16 folds. Phagocytosis is a complex process for the ingestion and elimination of pathogens and apoptotic cells, tissue homeostasis, control the important aspect of inflammation and immune response. The phagocytic cells in the immune system such as monocytes, macrophages, neutrophils, dendritic cells, osteoclasts, and eosinophils. Phagocytosis is a fundamental process in immunity (26). The CRP test showed that Sappan wood ethanol extract reduced inflammation with a lower level than the infection condition (negative control). Along with an increase in the phagocytic index and a decrease in inflammation after the ethanol extract of Sappan wood treatment, the total leukocyte level gradually returns to normal after exceeding normal.

In this research, *Staphylococcus aureus* infection (negative control) shows that the phagocytic index is lower than the immune booster and treatment of Sappan wood. It is caused by the pathogenicity of *Staphylococcus aureus* and can decrease phagocytosis. The virulence factor of this bacteria causes this pathogen to attach to a tissue's cells, escape the host immune system, and have some factors that decrease phagocytosis, factors that interact with anti-staphylococcal antibodies, and factors that elaborate proteases, exotoxins, and enzyme in the immune system. *Staphylococcus aureus* is a pyogenic pathogen capable of tissue invasion and evasion of phagocytosis by neutrophils. Staphylococcal lipoteichoic acid is involved in the synthesis of inflammatory cytokines by monocyte/ macrophages (27). *Staphylococcus*

aureus can activate pro-inflammatory cytokines, including IL-6 and IL-8 (28).

The previous research showed that 25 mg/kg body weight of ethanol extract of Caesalpinia Sappan had immunomodulatory activity on the peritoneal macrophage of albino mice. It significantly enhanced the phagocytic activity of macrophages (18). Caesalpinia Sappan extracts dose-dependently inhibited the expression of proinflammatory cytokines IL-1β and TNF-α in IL-1β-stimulated chondrocytes and LPS-stimulated THP-1 macrophages (29). Some of the compounds in Sappan wood can act as immunomodulators, such as polyphenol, flavonoid, quercetin, brazilin, coumarin, episappanol, protosappanin C, brazilin, isoprotosappanin B, and sappanol.

This research shows that the ethanol extract of Sappan wood contains polyphenols and flavonoids. Polyphenol successfully isolated from Sappan wood is sappanchalcone, caesalpiniaphenol G, and quercetin (30). Polyphenols can increase phagocytosis and optimize macrophage function (31).

The previous research shows that levels of flavonoids in Sappan wood extract using the maceration method is 0.0539 % (32). The total flavonoid content correlates with the phagocytic capacity of macrophages (33). Flavonoids are also anti-inflammatory in several chronic diseases such as cancer, diabetes mellitus, cardiovascular disease, and neuroinflammation (34).

Quercetin is a type of flavonoid that has immunomodulatory activity. Quercetin promotes some gene expressions involved in phagocytoses, such as CORO1A, CYBA, LAMP1, RAB7A, RAC1, and PAK1 genes (35). In addition, quercetin inhibits the production of inflammation-producing enzymes such as cyclooxygenase (COX) and lipoxygenase (LOX), inhibits proinflammatory cytokine production against H_2O_2-induced inflammation, and suppresses many inflammatory pathways (36).

Brazilin, the major compound of Sappan wood, was reported to have anti-inflammatory. Brazilin suppressed the release of IL-1ß, TNF-α, NO, and PGE2, suggesting that these effects are mediated by Heme oxygenase-1. This result correlates well with studies on Caesalpinia sappan extract and brazilin in mouse macrophages, demonstrating that the potential anti-inflammatory effects of the agents involve the inhibition of PGE2 production (37).

Several compounds that have other immunomodulatory effects in Sappan wood include coumarin, episappanol, protosappanin C, brazilin, isoprotosappanin B, and sappanol. Coumarin increases phagocytosis activity. Episappanol, protosappanin C, brazilin, isoprotosappanin B, and sappanol significantly inhibited the secretion of the proinflammatory cytokines such as interleukin (IL-6) and tumor necrosis factor-alpha (TNF-α). Sappanol increased the secretion of the anti-inflammatory IL-10 (29).

CONCLUSION

In conclusion, Sappan wood ethanol extract significantly increases the phagocytic index and suppresses C-reactive protein (inflammatory acute-phase reactant). The phagocytic index and CRP level of 100 and 200 mg/kg body weight ethanol extract of Sappan wood were significantly different from the negative control (infection). This condition indicates the work of the immune system and inflammation was quickly completed so that the total leukocytes on the 7th day also gradually return to normal.

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