

In-vivo and *in-silico* immunomodulator activity of *Caesalpinia sappan* L. wood ethanol extract in *Rattus norvegicus* infected by *E. coli*



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ABSTRACT

Introduction: Immunomodulators from natural compounds have a good potential in curing infectious diseases including *Escherichia coli* (*E. coli*) infection in the gastrointestinal and urinary tract. In Indonesia, Sappan wood (*Caesalpinia sappan* L.) is known as a traditional herbal drink that can be used to increase immunity. *Caesalpinia sappan* L. has a specific red natural pigment called brazilin. This study aimed to determine the immunomodulatory activity of *Caesalpinia sappan* L. in *Rattus norvegicus* infected with *E. coli*.

Method: This study was a true experimental with a post-test-only control design (*in vivo*) that used *Rattus norvegicus* infected by *E. coli* (10^9 CFU/ml). This study consisted of normal control (non-infected group), negative control (placebo), positive control (immune booster), and 96% Ethanol Extract of Sappan Wood (SWEE) 25, 50, 100, and 200 mg/kg BW. The treatment was given for 7 days after *E. coli* infection. *In silico* study, we used brazilin as a ligand and pro-inflammatory cytokine (TNF α and IL-1 β) as the target. It was conducted using a molecular docking web server (Docking Server).

Results: *In vivo* study showed that *Caesalpinia sappan* L. decreased C-reactive protein (CRP) levels and increased phagocytic index. SWEE 100 and 200 mg/kg BW was significantly different from the negative control ($p < 0.05$) but not the positive control ($p > 0.05$). SWEE 100 and 200 mg/kg BW increased phagocytic index by 2.82 and 4.62 folds. The molecular docking showed that brazilin binds to TNF α , and IL-1 β with total energy binding respectively -5.05 and -3.14 kcal/mol. The estimation inhibition constant (Ki) of brazilin into TNF α and IL-1 β respectively 199.52 and 4.99 μ M.

Conclusion: *Caesalpinia sappan* L. has immunomodulatory activity through CRP level suppression and phagocytosis induction, especially at 100 and 200 mg/kg BW. Brazilin as a specific pigment of *Caesalpinia sappan* L. also has been proven to inhibit pro-inflammatory cytokine TNF α and IL-1 β .

Keywords: *Caesalpinia sappan*, CRP, phagocytic index, TNF α , IL-1 β .

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INTRODUCTION

Immunity plays a main role in the protection of the human body from infectious and pathogenic microorganisms.¹ Immunity is supported by the immune system which consists of innate and adaptive immunity. Innate immunity is the first immunological mechanism with a rapid immune response and has no immunological memory. On the other hand, adaptive immunity is antigen-dependent and antigen-specific with memory capacity and a more efficient immune response upon subsequent exposure to the antigen.² Innate immunity consists of some immune elements such as cellular elements (macrophage, dendritic cells, mast cells, neutrophils, NK cells, and

non-hematopoietic cells) and humoral elements (complement, LPS binding protein, CRP, acute-phase reactants, antimicrobial peptides, and mannose-binding lectin). Adaptive immunity some immune elements such as cellular elements (T and B lymphocytes) and humoral elements (immunoglobulin).³

Infection is one of the factors that can be persistent in the body and induce immune responses. The antigen induces an immune response and inflammation pathway, causing cell damage, and tissue injury. Antigen also causes the failure of endogenous anti-inflammatory mechanisms.⁴ Bacteria have antigenic components that can trigger an infection, immune response,

inflammation, and organ dysfunction. *E. coli* is a Gram-negative bacterium that causes gastroenteritis, renal failure, septic shock, and extraintestinal illnesses such as pneumonia, bacteremia, and peritonitis.⁵ In the infection and immune response mechanism, there are many markers including C-reactive protein (CRP), phagocytic index, and inflammation mediator (TNF α and IL-1 β). CRP is an acute inflammatory protein that is synthesized in hepatocytes, muscle cells, macrophages, endothelial cells, lymphocytes, and adipocytes. CRP increases up to 1.000 at infection or inflammation.⁶ CRP can become a marker in *E. coli* infection.⁷ Phagocytosis is one of the immune responses to

bacteria. Phagocytosis is a complex process that removes the pathogen and is usually followed by immune response and inflammation.⁸ Phagocytic function usually decreases in infection. Phagocytic function can see by phagocytic index. In the molecular mechanism, phagocytosis is indicated by TNF α and IL-1 β expression as inflammation mediators.⁹

Immunomodulator is an active compound that can modulate immune system that is preceding changes the immune response result helps the tissue damage and immoderate response that is obtained by natural and unnatural (human-made) from. Immunomodulator modulate humoral immunity, cellular, non-specific protection constituent and this are medicinal agents and depend on the dose and exhibit immunomodulatory results including stimulation (immunostimulant) and suppression (immunosuppressant).¹⁰ Natural compounds of plant have contributed enormously to immunomodulatory therapeutics. Since ancient times, natural medicines have constituted treatments with minimal side effects.¹¹

Caesalpinia sappan L. is used as traditional medicine including as immunomodulator in *Staphylococcus aureus*.¹² *Caesalpinia sappan* L. has antibacterial activity in *E. coli* infection.¹³ *Caesalpinia sappan* L. contains phytochemical properties such as brazilin, brazilein, brazilide A, 2, 4, 5-Trihydroxybenzaldehyde, euxanthone, 3, 8, 9-Trihydroxy-6H-benzo [c] chromen-6-one, sappanone B, 3-deoxysappanone B, (E)-3-(3,4-dihydroxybenzylidene)-7-hydroxychroman-4-one, sappanchalcone, 3-deoxysappanchalcone, butein, protosappanin A, B, C, D, dan E. Brazilin is the major compound in *Caesalpinia sappan* L. that becomes as specific color in this plant that has a potential effect on anti-inflammation. Brazilin has antioxidant activity with IC₅₀ 57,2 μ M. As an antiinflammation agent, brazilin inhibits Lippo Poly Saccharide (LPS)-induces NO production, prostaglandin E₂, TNF- α and IL-1 β .¹⁴

This study aims to investigate the immunomodulatory activity of *Caesalpinia sappan* L. ethanol extract on rat-infected models. *Rattus norvegicus* were infected by *E. coli* as gram-negative

bacteria using CRP and phagocytic index parameter *in vivo*. This research also wants to know the profile of molecular docking (*in silico*) between brazilin as a ligand and some inflammation mediators including TNF α and IL-1 β .

MATERIAL AND METHODS

Rattus norvegicus (Male, 2 Month, 2750375 grams, n=35) were used in this pre-clinical research. The research has received an ethics certificate from Research Ethical Clearance Commission, Faculty of Dental Medicine, Universitas Airlangga (Number: 217/HRECC.FODM/V/2022). *Rattus norvegicus* was infected by *E. coli* (10⁻⁹ CFU/ml). This research was divided into 7 treatment groups, including: (1) Normal control (no infection but only CMC-Na 1% treatment - Merck® 217277, Merck KGaA, Germany), (2) Negative control (bacterial infection and CMC-Na 1% treatment), (3) Positive control (bacterial infection and Imboost force® treatment, 3526003999-KDO-005457985, PT. SOHO Industri Pharmasi, Jakarta), (4) Bacterial infection and SWEE treatment 25 mg/kg BW, (5) SWEE 50 mg/kg BW, (6) 100 mg/kg BW, and (7) 200 mg/kg BW. Each treatment was repeated 5 times.

Immunomodulatory activity test of this study was carried out *in vivo* and *in silico*. The parameters of the *in vivo* immunomodulatory test were CRP level (mg/L) and phagocytic index (folds). The parameters of the *in-silico* test were free energy binding (kcal/mol), interaction surface, and inhibition constant (μ M) between ligand and receptor target. The receptor targets of the test were TNF α and IL-1 β .

Sample Preparation

The 500 grams of *Caesalpinia sappan* L. wood powder (Materia Medica Batu Jawa Timur) was extracted using 3750 ml of 96% ethanol (3413101000-FKS-183074369, PT Brataco, Bandung) by maceration method for 5 days and stirred 3 times every day. The extract was filtered until got the filtrate. The filtrate was concentrated using a rotary evaporator (IKA Scientific®, No. 0010012324, Germany) at 65° C until a thick extract. We made 4 concentrations of SWEE such as 25, 50, 100, and 200 mg/kg BW.

Phytochemical Assay

Phytochemical tests were carried out to investigate the *Caesalpinia sappan* L. content including flavonoid, alkaloid, polyphenols, saponin, tannin, and steroid qualitatively. The alkaloid test was carried out using Dragendorf (3417012020-RID-071732686, Matelab Trifa Solusindo), Flavonoid test using Mg powder (3719598000-AL2-040056030, PT. Multi Medika Laboratory) and concentrated HCl. Test Saponification was carried out using HCl reagent 1N (Merck®1090571000, Merck KGaA, Germany). Meanwhile, the tannin test was carried out using FeCl₃ reagent 10% (Merck®1039430250, Merck KGaA, Germany).

E. coli Preparation

E. coli suspension (1 ml) was added to 9 ml of sterile distilled water. The concentration of bacteria was 10⁻¹ CFU/ml (tube 1). This bacterial suspension was diluted by taking 1 ml from tube 1 and adding 9 ml of sterilized distilled water continuously until 10⁻⁹ CFU/ml.

Animal Preparation and Infection

Rattus norvegicus were infected with *E. coli* (10⁻⁹ CFU/ml) per oral. Infection can be confirmed by CRP released. After 24 hours infection, *Rattus norvegicus* were treated with 7 treatments (5 times replication) for 7 days and followed by an immunomodulatory activity test (CRP level, phagocytic index, and *in silico* study). Feed was given *ad libitum* in the form of pellets containing protein, fat, fiber, minerals, and water. Blood was taken through the jugular vein. The collected blood was separated by centrifugation method until we got the serum. Serum was used for CRP and Phagocytosis test.

CRP Level Test

CRP test was carried out in 2 stages, qualitative and semiquantitative test. We dripped 50 ml CRP latex reagent (Glory Diagnostic®, GD-CRP 100, LOT 24895, Kemenkes RI AKL 20305710497, Spain) on the slide followed by dripping 50 ml serum sample. In the qualitative test, we used agglutination as a parameter. If there is agglutination, then the sample is declared positive for containing CRP and vice versa. If the qualitative test results

showed positive results, then proceed with a semi-quantitative test to determine the CRP level. In the semi-quantitative test, it used the 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).¹⁵

Phagocytic Index Test

Phagocytosis activity was viewed by carbon clearance method using UV-Vis Spectrophotometer (Thermo-Scientific®, 840-209700, Massachusetts, USA) at 650 nm wavelength.

Constant carbon elimination speed (K) = $\frac{\log A(n) - \log A(n-1)}{t(n-1) - t(n)}$ ¹⁶

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)

Phagocytic Index (α) =

$\frac{\text{Constant carbon elimination of sample}}{\text{Constant carbon elimination of negative control}}$ ¹⁷

Molecular Docking

The potential analysis of brazilin as an immunomodulatory activity substance was analyzed by molecular docking method. The molecular docking study was performed by a docking server. We used Brazilin of *Caesalpinia sappan* L. as a ligand that was gotten from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). We used native ligands (TAPI-0 and Anakinra), TNF α , and IL-1 β as receptors that were downloaded from Protein Data Bank (www.pdb.org).

Statistical Analysis

The Data of this research was analyzed using Analysis of Variance (ANOVA) with LSD Post hoc test.

RESULTS

Qualitative Phytochemical Screening

Based on the qualitative phytochemical screening, it was found that SWEE contained secondary metabolites such as flavonoids, alkaloids, polyphenols, saponins, and tannins (Table 1).

Table 1. Qualitative phytochemical screening of ethanol extract of *Caesalpinia sappan* L. (SWEE)

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

Table 2. CRP Level of *Rattus norvegicus* infected by *E. coli* after treatment

Treatment Group	CRP Level (Mean \pm SD)
Normal Control	0 ^a \pm 0
Negative Control	43 ^b \pm 11
Positive Control	8 ^c \pm 3
SWEE 25 mg/kg BW	43 ^b \pm 11
SWEE 50 mg/kg BW	24 ^d \pm 0
SWEE 100 mg/kg BW	11 ^c \pm 3
SWEE 200 mg/kg BW	6 ^c \pm 0

Statistical analysis of treatment used the ANOVA and LSD post hoc test with a significant value was $p < 0.05$. Different notations (a, b, c, and d) showed the different effects of the treatment.

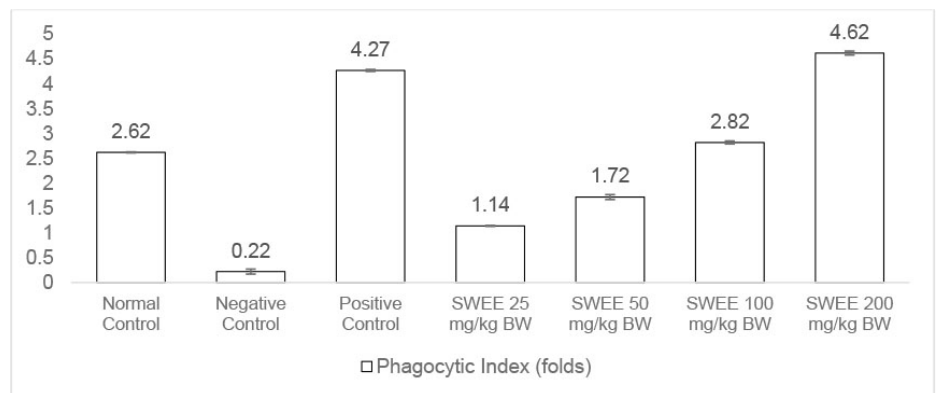


Figure 1. Phagocytic index of *Rattus norvegicus* infected *E. coli* after treatment.

CRP Level

CRP is an acute-phase protein that is produced in the inflammation mechanism. Semi-quantitative CRP level was measured after infection but before treatment (pre-test) aimed to make sure that infection and inflammation existed. CRP levels in *E. coli* infection were successively 96 mg/L and 48 mg/L. CRP level after infection and after treatment was measured to know the anti-inflammatory activity of the treatment agent through CRP inhibition. CRP levels of *E. coli* infection in all treatments are shown in Table 2.

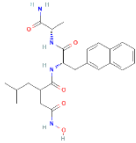
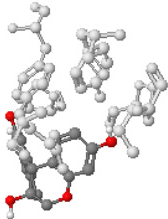
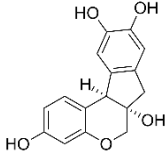
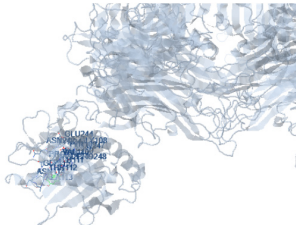
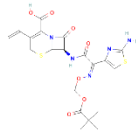
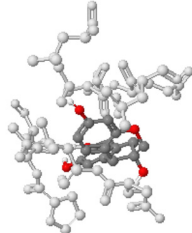
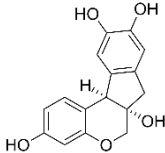
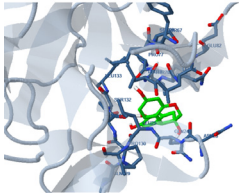
One-way ANOVA test showed that there were significant differences among 7 treatments ($p = 0.002$). LSD post hoc analysis showed an activity comparison of each treatment. SWEE 25 mg/kg BW was not different from the negative control

($p > 0.05$) in *E. coli* infection groups. SWEE 50, 100, and 200 mg/kg BW was significantly different from negative control ($p < 0.05$) in *E. coli* infection groups. SWEE 100 and 200 mg/kg BW were not different with positive control ($p > 0.05$). The activity of positive control treatment using Immune booster decreases CRP level. Based on this data, SWEE 100 and 200 mg/kg BW are the two most effective concentrations in reducing CRP levels in *E. coli* infections.

Phagocytic Index

Phagocytosis is a biological mechanism that follows inflammatory pathway activation, which promotes pathogen elimination and inhibits pathogen growth. Phagocytic activity can be observed through the carbon clearance method

Table 3. The result of molecular docking

Receptor	Ligand	Est. Free Energy of Binding (kcal/mol)	Interaction Surface	Est. Inhibition Constant (Ki) (μM)	Amino Acid (Hydrogen Bond)	3D Molecular Docking Interaction
TNF α (3it8)	Native Ligand (TAPI-0) PubChem 	- 6.02	318.99	212.15	Proline Tyrosine Glutamine Isoleucine	
	Brazilin PubChem 	- 5.05	433.77	199.52	Proline Tyrosine Glutamine Isoleucine	
IL-1 β (4gaf)	Native Ligand (Anakinra) PubChem 	- 3.42	542.145	5.03	Leucine Serine	
	Brazilin 	- 3.14	538.145	4.99	Leucine Serine	

with the phagocytic index parameter. The phagocytic index is the average number of bacteria ingested per macrophage. The phagocytic index (α) of *E. coli* infection in all treatments are shown in [Figure 1](#).

One-way ANOVA and LSD post hoc test showed that there were significant differences among treatments in *E. coli* infection with significant values successively $p=0.000$. The phagocytic index of the infection group with SWEE 25, 50, 100, and 200 mg/kg BW treatment were higher than negative control. The higher concentration of SWEE correlated with the higher phagocytic index. SWEE 100 mg/kg BW increased phagocytic index until 2.82 folds. SWEE 200 mg/kg BW increased phagocytic index until 4.62 folds.

Molecular Docking Result

Molecular docking showed that there was an interaction between Brazilin and inflammatory mediators including TNF α and IL-1 β . The result of molecular docking is shown in [Table 3](#).

DISCUSSION

The biological activity of plants was associated with compounds that were contained. Qualitative phytochemical screening of SWEE contained secondary metabolites such as flavonoids, alkaloids, polyphenols, saponins, and tannins. SWEE did not contain steroids and terpenoids. Based on the like-dissolved-like theory, the polar compounds could be dissolved in a polar solvent. Ethanol is a polar solvent.

Some polar compounds such as flavonoids, polyphenols, saponins, and alkaloids (semi-polar) were dissolved in ethanol solvent. Some nonpolar compounds such as steroids and terpenoids were not dissolved in ethanol solvent.

Some secondary metabolites of plants such as flavonoids, alkaloids, polyphenols, saponins, and tannins have immunomodulatory activity. Flavonoids have specific immunomodulatory activity in several cancers. It can suppress mTOR activity and are consequently able to induce the T regulatory subset.¹⁸ Flavonoid induce immune and anti-inflammatory responses.¹⁹ Flavonoid also inhibit various transcriptional factor, modulate activation of immune cells, enhance regulatory T cell generation, inhibition pro-inflammatory

cytokine production, down regulation of chemokines, and reduction of reactive oxygen and nitrogen species.²⁰ Alkaloids exhibit anti-inflammatory action via Nuclear Factor - $\kappa\beta$ (NF- $\kappa\beta$) and Cyclooxygenase-2 (COX-2).²¹ Alkaloid can reduce the IFN γ activity and the expression of CD4+.²² Alkaloid has potential as immunosuppressant in autoimmune disorder.²³ Polyphenols inactive NF- $\kappa\beta$, modulate MAPK and arachidonic acids pathway, suppress pro-inflammatory gene expression, and activate anti-inflammation properties.²⁴ Saponin against superoxide anion radical and attenuated the release of inflammatory mediators such as NO, TNF- α , and COX-2.²⁵ Tannins alleviate inflammation through multiple signaling pathways. It inhibits SMAD2-dependent gene transcription in response to TGF- β , STAT3, and NF- $\kappa\beta$ pathway. It also inhibits pro-inflammatory cytokines such as IL-6, IL-1, and TNF- β .²⁶

The first parameter of anti-inflammatory activity in this research was CRP level. CRP is an acute inflammatory protein that increases up to 1.000-fold at sites of infection, immune response and inflammation.⁶ In Cases of *S. aureus* Bacteremia (SAB) cohort study on patient showed that the mean of CRP level in uncomplicated and complicated SAB successively were 127 mg/L and 160 mg/L.²⁷ In *E. coli* urinary tract infections showed that CRP level of patient is > 100 mg/L.⁷ The CRP level of *Rattus norvegicus* infected *E. coli* in this research was 48 mg/L.

CRP level after treatment with immune booster that contain *Echinacea purpurea* (positive control) decreased CRP level from 48 to 8 mg/L. This immune booster has returned CRP level at the normal condition (0 mg/L in normal control). In previous study showed that *Echinacea* can return CRP level to normal condition after oxidative stress induction.²⁸ *Echinacea purpurea* activate neutrophils, macrophages, polymorphonuclear leukocytes (PMN), and natural killer (NK) cells. *Echinacea purpurea* also stimulated lipopolysaccharide (LPS) and increase phagocytic activity. It also can up-regulate some genes involve in immune cell activation including Chemokine (C-C motif) ligand 4 (CCL4), interleukine-7

receptor (ILR7), nuclear factor of activated T-cells.²⁹

SWEE treatment showed CRP reduction in 50, 100, and 200 mg/kg BW. It was proven from the ANOVA and LSD post hoc (see Table 2). SWEE 25 mg/kg BB was not different from the negative control ($p > 0.05$). SWEE 50, 100, and 200 mg/kg BW was significantly different from negative control ($p < 0.05$). SWEE 100 and 200 mg/kg BW were not different with positive control ($p > 0.05$). SWEE has anti-inflammatory activity through CRP levels reduction in *E. coli* infection effectively on 100 and 200 mg/kg BW. Based on these data, SWEE has similar activity with positive control in CRP level reduction. At the dose of 100 mg/kg BW, ethanol extract of *Caesalpinia sappan* L. also has antioxidant and hepatoprotective activity in Diabetic Rats.³⁰ At the doses of 100 and 200 mg/kg BW, *Caesalpinia sappan* L. extract reduces myocardial interstitial edema, necrosis, inflammatory cell infiltration of rats induced by isoproterenol.³¹

The second parameter of this research was a phagocytic index (α) that showed the phagocytosis process. Phagocytosis is an immune response by consuming and lysing microorganisms with a size $d > 0.5 \mu\text{m}$.³² The phagocytic index is the average number of bacteria ingested per macrophage.³³ High phagocytic number showed the high number of bacteria ingested per macrophage. In this research, *E. coli* infection decrease phagocytic index until 0.22 folds (infected group). It was lower than normal group (2.62 folds). The study showed that neutrophil phagocytosis in infected patients (*E. coli*) was significantly lower compared with healthy adults. The neutrophil phagocytosis was negatively correlated with neutrophils count and percentage because neutrophil spontaneously apoptosis at 24 hours after differentiation and maturation. This condition induced the bone marrow to produce immatures and low functional phagocyte.³⁴ The phagocytic index of positive control was higher than negative control. The number of phagocytic in *E. coli* infection with immune booster was 4.27 folds. Immune booster that contained *Echinacea* increased phagocytosis. In the previous study showed that *Echinacea purpurea* (L.) significantly increased

phagocytosis and cytokine release (TNF- α , IL-6, and IL-1 β) in the cells.³⁵

The phagocytic index of SWEE (25, 50, 100, and 200 mg/kg BW) were higher than negative control (see Figure 1). The higher concentration of SWEE correlated with the higher phagocytic index. SWEE 100 mg/kg BW increased phagocytic index until 2.82 folds. SWEE 200 mg/kg BW increased phagocytic index until 4.62 folds. In the previous study showed that 25 mg/kg BB of *Caesalpinia sappan* L. ethanol extract increased phagocytic index in non-infected mice until higher than 80 folds.³⁶ *Caesalpinia sappan* L. extract also increased anti-inflammatory cytokine Interleukine-10 (IL-10) in candidiasis. IL-10 increased phagocytosis and neutrophil recruitment so as to mediate inflammation.³⁷ Ethanol extract of *Caesalpinia sappan* L. 100 and 200 mg/kg BW increased Kupffer cell (liver macrophage) number.³⁸

Molecular activity of *Caesalpinia sappan* L. also observed by molecular docking study. Brazilin as a major compound of *Caesalpinia sappan* L. was used as ligand with TNF α and IL-1 β as target receptors. TNF α and IL-1 β were usually used as inflammatory markers. TNF α is a pro-inflammatory cytokine that produced by macrophage and other cells belonging innate immunity and involved in the complex chemotactic process of adaptive immunity. TNF α is involved in pathological process of disease.³⁹ IL-1 β is produced by endothelial cells and macrophages and has the function of inducing the chemokine and secondary cytokine secretion, inductions of neutrophil release, and stimulating T-cell and macrophage.⁴⁰

The parameter in molecular docking study was binding free energy, inhibition constant (Ki), bond hydrogen, and other patterns of interaction with amino acid residues on the active site of the receptor. Binding free energy is often used to determine the affinity of biomolecular interactions and drug efficacy.⁴¹ The more negative the value of the 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.⁴² Inhibition constant (Ki) represent a dissociation constant

for binding of an inhibitor to an enzyme. The binding equilibrium describe by K_i values depends on the kinetic mechanism of inhibition.⁴³ Hydrogen (H)-bond is possible interaction that 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. Hydrogen (H)-bonds potentiate diverse cellular functions by facilitating molecular interactions.⁴⁴

Molecular docking showed that brazilin has anti-inflammatory activity by TNF- α and IL-1 β inhibition. Brazilin and TNF- α has strong binding with free energy of banding was -5.05 kcal/mol, whereas native ligand and TNF- α has free energy of banding was -6.02 kcal/mol. The inhibition constant of brazilin at TNF- α was 199.52 μ M, whereas inhibition constant of native ligand was 212.15 μ M. Some amino acids interaction with hydrogen bond between brazilin and TNF- α were proline, tyrosine, glutamine, and isoleucine. Brazilin and IL-1 β has strong binding with free energy of binding was -3.14 kcal/mol, whereas native ligand and IL-1 β has free energy of binding was -3.42 kcal/mol. The inhibition constant of brazilin at IL-1 β was 4.99 μ M, whereas inhibition constant of native ligand was 5.03 μ M. Some amino acids interaction with hydrogen bond between brazilin and IL-1 β were leucine and serine.

In the previous study showed some inhibition activity of brazilin on pro-inflammatory cytokine that involved in anti-inflammatory activity. Brazilin decreased mRNA expression levels of inflammatory cytokines such as IL-1 α , IL-1 β , IL-6, IL-8 and TNF- α in a concentration dependent manner.⁴⁵ Brazilin (50 – 300 mM) also showed dose dependent inhibition of NO, prostaglandin E2 (PGE2), TNF- α , IL-1 β production and iNOS expression in LPS stimulated macrophage.⁴⁶

In the last, it was summarized that there was normalized action of SWEE as immunomodulatory activity in *E. coli* infection. In the infection condition, bacteremia (*E. coli*) that stimulated immune response and induce phagocyte activation to produce pro-inflammatory

cytokine. Phagocyte spontaneously apoptosis at 24 hours after differentiation and maturation. This condition induced the bone marrow to produce immatures and low functional phagocyte (low phagocytosis activity). Pro-inflammatory cytokine induced CRP release from hepatocyte (high CRP condition). In this condition, SWEE reduced CRP level reduction and stimulated phagocytosis activity. The molecular docking show that SWEE inhibited proinflammatory cytokine TNF- α and IL-1 β . This condition can reduce inflammation.

The limitation of this research is the phytochemical tests which were carried out qualitatively. For future research, it is necessary to carry out quantitative phytochemical analysis and isolate brazilin as an active compound. Brazilin isolate can be tested further to investigate its immunomodulatory potential as a single compound in vivo.

CONCLUSION

Ethanol extract from *Caesalpinia sappan* L. has immunomodulatory activity by CRP levels reduction and phagocytosis stimulation in *E. coli* infection condition. The molecular study shows that brazilin as a major compound of *Caesalpinia sappan* L. bind and inhibit pro-inflammatory cytokines including TNF α and IL-1 β . This research needs to be continued by molecular anti-inflammatory investigation from fractioned or isolated active compound of *Caesalpinia sappan* L.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ETHICAL STATEMENT

This research was approved by the ethics committee of the Research Ethical Clearance Commission, Faculty of Dental Medicine, Universitas Airlangga (Number: 217/HRECC.FODM/V/2022).

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AUTHORS' CONTRIBUTION

All authors work equally in doing this research and writing this research article concepts and investigation, laboratory work, factual studies, and paper preparation.

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