# ANTIMICROBIAL ACTIVITY OF SAPPAN WOOD *(Caesalpinia Sappan L)* EXTRACT AGAINST *Haemophilus Influenza* AND ITS IRON CHELATION ACTIVITIES IN VITRO

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**Abstract**

*Haemophilus influenzae is a normal bacterium in the upper respiratory tract of humans, it can be opportunistic and cause pathogenic conditions to cause Acute Respiratory Tract Infections (ARI). This study aimed to determine the antimicrobial activities of Sappan wood extract (SWE) against Haemophilus influenza. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of SWE for Haemophilus influenzae at $10^3$ and $10^5$ CFU/mL were assessed by macrodilution method, with four treatment groups including SWE group, a positive control group, a negative control group, and antibiotic's control group. AAS measured the reduction of free iron concentration in Haemophilus Test Medium Broth (HTMB) after the addition of bacteria and SWE. The data were analyzed descriptively. The result showed that the MIC value for SWE against Haemophilus influenzae at $10^3$ and $10^5$ CFU/mL was 16ppm and the MBC value was 32 ppm. The SWE at 16 ppm and 32 ppm could inhibit 98.64 % and 100 % of the Haemophillus infleunza in $10^{-3}$ and $10^{-5}$ dilutions, respectively and also the reduction of iron concentration as the effect of SWE to chelate iron. The iron-chelating property of SWE and the absolute dependency of Haemophilus influenzae to iron can be an important mechanism for the antimicrobial activity to Haemophilus influenzae. SWE is a potential herbal remedy to be developed as therapeutic agent for Haemophilus influenzae infectious disease.*

**Key words:** Sappan Wood Extract (SWE), *Haemophilus influenzae*, MIC, MBC, iron chelation, Antimicrobial

## INTRODUCTION

*Haemophilus influenzae* is a coccobacillus, gram-negative, non-motile bacteria which generally can be found in the upper respiratory tract [21]. However, *Haemophilus influenzae* can be an opportunistic bacteria and potentially caused a pathogenic condition through the virulence factors in the state of decreased body immune response and the bacterial infection by the air-borne droplet and secretion contact [2]. The most common disease caused by *Haemophilus influenzae* infection is Acute Respiratory Tract Infection, such as Pneumonia [9]. Acute Respiratory Tract Infection is an infectious disease as the primary cause of the high morbidity and mortality rate in the world, especially in the developing countries, such as Indonesia [23]. The data from Indonesia Ministry of Health showed that 197.000 death in Indonesia caused by pneumonia with *Haemophilus influenzae* as an etiologic agent occurred in 2011 [9]. Other than Acute Respiratory Tract

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Infection, *Haemophilus influenzae* can cause several pathogenic conditions including many body organs, such as meningitis, otitis media, cellulitis, bacteremia, and septic arthritis [21].

Nowadays, the increased Acute Respiratory Tract Infection incidence with *Haemophilus influenzae* as an etiologic agent cause the increased of antibiotic usage [7]. In Indonesia, the high availability of antibiotic and easy to access many types of antibiotics cause an increased resistance phenomenon. Until now, a resistance of antibiotic to *Haemophilus influenzae* occurs because the ability of *Haemophilus influenzae* to produce a β-lactamase enzyme [7, 24]. This occurrence affects the action of penicillin that will be less effective for killing bacteria. Therefore, study about antimicrobial agent which can inhibit the growth and kill the bacteria is necessary to be developed.

Based on the data from the previous report, 80% of people in the world use herbal medicine as the primary method to fulfill health needs [4]. Indonesia is a country with high biodiversity, and more than 80% of the world's medicinal plant species were found in the tropical forest of Indonesia [5]. One of the plant species used as an herbal agent in Indonesia is secang (Sappan wood; *Caesalpinia sappan L.*). Sappan Wood grows in many areas of Indonesia and is easy to get [11]. Sappan wood usually used by people to relieve pain because of circulation disturbance, hemorrhagic condition, diarrhea, edema and as an antiseptic [16].

The previous studies showed that sappan wood extract could inhibit the bacterial and fungal growth. A review stated that sappan wood ethanol extract could inhibit the growth of several gram-positive bacteria, such as *Staphylococcus aureus, Bacillus subtilis* and gram-negative bacteria, such as *Klebsiella pneumoniae, Escherichia coli,* and *Proteus vulgaris* [13]. The antimicrobial effect of sappan wood extract is better than other plants, likes *Mimosa pudica L.* because more phenolic, alkaloid, and tannin bioactive substances were found [15]. Brazillin, a pigment compound of sappanwood plant derived from phenolic substance, was proved to chelate iron based on the redox properties [12, 15, 16]. *Haemophilus influenzae* needs exogenous iron to support bacterial growth [14, 22]. This statement is a primary reason to organize this in vitro study and prove the antimicrobial sappan wood extract activity to *Haemophilus influenzae* as the aim of this study. We expect that the result of this study can be a primary data to support the development of antimicrobial study as a causative therapy for *Haemophilus influenzae.*

## MATERIALS AND METHODS

### Sample Collection

The *Haemophilus influenzae* got from Eijkman Microbiology Laboratorium, Faculty of Medicine, Padjadjaran University. Sappan wood (*Caesalpinia sappan L.*) got from Wanagama forest, Yogyakarta, Indonesia and then processed in pre-extraction and extraction methods to get the powder. This study used experimental laboratory design and was held in Eijkman Microbiology Laboratorium, Faculty of Medicine, Padjadjaran University and Regional health laboratory (Laboratorium Kesehatan Daerah Labkesda) Bandung. To evaluate the activity of Sappanwood *(Caesalpinia sappan L.)*, this study adopted three methods; broth macrodilution agar well diffusion and measurement of reduced iron concentration by Atomic Absorption Spectrophotometry (AAS).

### Extraction and fractionation of *C. sappan* L.

The extraction and fractionation methods were adopted by Safitri *et al.* The sappan woods (*C. sappan* L.) were dried in the open air and sheltered from direct sunlight. Once dried, the bulbs were crushed using blender to obtain fine powder. Sappan wood powder was then weighed as much as 1 kg, placed in a Buchner funnel, then was macerated using 15 l of ethanol solvent for 24 h, and was repeated up to 3 times. The macerate was filtered using Whatman filter paper No. 2, and then, it was concentrated using a rotary evaporator at 60°C to obtain dry extract. To remove the oil (non-polar compounds) fluids, liquid extraction was conducted using 500 ml of n-hexane solvent, and then, it was evaporated.

**Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assay**

The Minimal Inhibitory Concentration (MIC) of sappan wood for antimicrobial activity was assessed by the macrodilution method. Sappan wood extract was made as an antimicrobial stock solution of 4096 ppm in dimetylsulfoxide (DMSO). Stock solutions were first diluted 10-fold in Haemophilus Test Medium Broth (HTMB) and then were diluted two-fold in 1,8 mL Haemophilus Test Medium Broth (HTMB) until 11 different concentrations of sappan wood extract were formed. To these solutions, 0.2 mL of bacterial suspensions (10% of final solution) of $10^3$ and $10^5$ CFU/mL were added to each concentration. The bacterial suspension was ajust to Mcfarland standard 0,5 by using direct visual measurement and then was diluted until it reaches $10^3$ and $10^5$ CFU/mL of bacterial suspension. Minimum Inhibitory Concentration (MIC) was determined as the lowest concentration of the antimicrobial compound that inhibits the growth of a microorganism after 24 hours incubated [3]. According to Manual of Clinical Microbiology, MIC determination could be based on turbidity visual inspection or calculation of ≥80% growth inhibition [8]. To ensure the appropriate interpretation of the result, some control groups were prepared, those are positive control (bacterial suspension+HTM broth medium), negative control (broth medium) and antibiotic control (bacterial suspension+HTM broth medium+antibiotic standard ampicillin 1 ppm and cefotaxime 2 ppm). All test and control tubes were incubated at 33-37ºC for 20-24 hours in 5% $CO_2$.

**Total Plate Count (TPC)**

The reduction of the population of bacteria Minimum Bactericidal Concentration (MBC) was evaluated by colony number through Total Plate Count (TPC). All of the sample solution in macrodilution method after being incubated for 20-24 hours were 10-fold diluted five times (for 105 CFU/mL) and three times (for 103 SFU/mL) in aquades. Each dilution was spread in chocolate agar. These positive, negative and antibiotic control solutions were also spread in chocolate agar. The chocolate agar plates were inverted and incubated at 33-37ºC for 20-24 hours in 5% $CO_2$.

**Agar Well Diffusion Method**

Agar well diffusion method was used to measure the diameter of inhibition zone caused by secang heartwood solutions in different concentrations to *Haemophilus influenzae* in the range of MIC-MBC samples. Inoculum 108 CFU/mL were prepared and inoculated in chocolate agar. Then, 6 mm wells were formed in the chocolate agar. Subsequently, wells were filled with 100 µL of secang heartwood extract solution in the range of MIC and MBC and allowed to diffuse to chocolate agar. The antibiotic control group was used as a standard disc (ampicillin 10 µg and cefotaxime 30µg) dissolved in the 100 µL aquades. These are equal to 100 ppm ampicillin concentration and 300 ppm cefotaxime concentration. The chocolate agar plates were inverted and incubated at 33-37ºC for 20-24 hours in 5% $CO_2$. The inhibitory response was classification by those are potent response (++++, if the diameter >30 mm), strong response (+++, if the diameter is ranged as 21-30 mm), moderate response (++, if the diameter is ranged as 16-20 mm), weak response (+, if the diameter is ranged as 10-15 mm) and little or no response (-, if the diameter <10 mm) [10].

**Atomic Absorption Spectrophotometry**

The last method was measurement of reduced iron concentration by Atomic Absorption Spectrophotometry (AAS). This method was used to evaluate the dependence of *Haemophilus influenzae* growth to iron. There would be 4 different groups to be evaluated; Haemophilus Test Medium Broth (HTMB) + hemadex (source of iron), HTMB + *Haemophilus influenzae* + hemadex, HTMB + sappan wood extract solution + hemadex, HTMB + *Haemophilus influenzae* + sappan wood extract solution in the range of MIC and MBC + hemadex. The free iron concentration in each group were measured by Atomic Absorption Spectrophotometry (AAS).

**Data Analysis**

The result of quantitative data, such as Minimal Inhibitory Concentration (MIC), Minimal Bactericidal Concentration (MBC)

and the percentage of reduced iron concentration, were analyzed descriptively where the concentration of sappan wood extract that has the effect of inhibiting the growth and killing *Haemophilus influenzae* was analyzed descriptively. The data of diameter of inhibition zone was analyzed descriptively and showed in table 2 in the form of mean±SD calculation using IBM SPSS Statistics 25. The data of reduced iron concentrations in broth medium that is capable of inhibiting the growth of *Haemophilus influenzae* through the iron-chelating effect of sappan wood on *Haemophilus influenzae* was analyzed descriptively.

## RESULTS AND DISCUSSIONS

### MIC and MBC of Sappan Wood (*Caesalpinia sappan* L.) Extract against *Haemophilus influenzae*

MIC and MBC can be seen from bacterial growth and the percentage of bacterial reduction number after being treated with antimicrobial agent as presented at the following tables.

Table 1 showed that *Haemophilus influenzae* growth at $10^3$ and $10^5$ dilution of inoculum (bacterial suspension $10^5$ and $10^3$ CFU/mL) was still seen after the addition of Sappan wood extract (SWE) at 16 ppm concentration, whereas the bacterial growth was not seen after the addition of SWE at 32 ppm concentration. The result showed that SWE at 16 ppm concentration still exhibit the existence of *Haemophilus influenzae* growth at both inoculums of $10^{-3}$ dan $10^{-5}$ dilutions. The MIC of SWE is probably 16 ppm based on the growth of bacterial colonies. However, MIC of SWE will be proved by the result of the percentage of bacterial reduction number that indicates the inhibition of bacterial growth appropriate to MIC criteria. Whereas SWE concentration that able to kill *Haemophilus influenzae* at $10^{-3}$ dan $10^{-5}$ dilution of inoculum is 32 ppm. The MBC value of SWE against *Haemophilus influenzae* is 32 ppm based on the growth of bacterial colonies.

Table 1 - MIC and MBC of Sappan wood (*Caesalpinia sappan* L.) Extract (SWE) against *Haemophilus influenzae* at $10^{-3}$ and $10^{-5}$ dilution of inoculum

| Sample | Bacterial growth at $10^{-3}$ dilution of inoculum | | | Bacterial growth at $10^{-5}$ dilution of inoculum | | |
|---|---|---|---|---|---|---|
| | S | D | T | S | D | T |
| Negative control | - | - | - | - | - | - |
| Positive control | + | + | + | + | + | + |
| Ampicillin | - | - | - | - | - | - |
| Cefotaxime | - | - | - | - | - | - |
| SWE (ppm) | | | | | | |
| 4 | + | + | + | + | + | + |
| 8 | + | + | + | + | + | + |
| 16 | + | + | + | + | + | + |
| 32 | - | - | - | - | - | - |
| 64 | - | - | - | - | - | - |
| 128 | - | - | - | - | - | - |
| 256 | - | - | - | - | - | - |
| 512 | - | - | - | - | - | - |
| 1024 | - | - | - | - | - | - |
| 2048 | - | - | - | - | - | - |
| 4096 | - | - | - | - | - | - |

Annotation : S : Simplo, D : Duplo, T : Triplo, Negative control : HTMB
Positive control : HTMB + *Haemophilus influenza*

Table 2 showed that the percentage of *Haemophilus influenzae* colony reduction number at $10^{-3}$ and $10^{-5}$ dilution of inoculum (bacterial suspension $10^5$ and $10^3$ CFU/mL) with SWE 16 ppm concentration are 98.64% and 99.29%, respectively. The reduction of bacterial colony number also showed in SWE 8 ppm at the same bacterial dilution of inoculum with the percentage values are 42.40% and 47.41%. The reduction of bacterial colony number that was added after the addition of SWE at 16 ppm concentration is indicated to an inhibition of bacterial growth that is more than 80%. From the

result, we conclude that **SWE** 16 ppm concentration is categorized as MIC value of SWE. Whereas, the percentage of *Haemophilus influenzae* colony reduction number at $10^{-3}$ and $10^{-5}$ dilution of inoculum (bacterial suspension $10^5$ and $10^3$ CFU/mL) with SWE 32 ppm concentration is 100%. There is a total reduction of bacterial colony number that was added after the addition of SWE at 32 ppm concentration so that SWE 32 ppm concentration is able to kill the bacteria. SWE 32 ppm concentration can be categorized as MBC value of SWE that result the 100 % reduction in TPC method.

Table 2 – Percentage of *Haemophilus influenzae* reduction numbers in $10^{-3}$ and $10^{-5}$ inoculum dilution

| Treatment (ppm) | *Haemophilus influenzae* colonies reduction (%) in $10^{-3}$ inoculum dilution | *Haemophilus influenzae* colonies reduction (%) in $10^{-5}$ inoculum dilution |
|---|---|---|
| Negative control | 0 | 0 |
| Positive control | 0 | 0 |
| Ampicillin | 100% | 100% |
| Cefotaxime | 100% | 100% |
| SWE (ppm) | | |
| 4 | 0% | 0% |
| 8 | 42.40% | 47.41% |
| 16 | 98.64% | 99.29% |
| 32 | 100% | 100% |
| 64 | 100% | 100% |
| 128 | 100% | 100% |
| 256 | 100% | 100% |
| 512 | 100% | 100% |
| 1024 | 100% | 100% |
| 2048 | 100% | 100% |
| 4096 | 100% | 100% |

Annotation : Negative control : HTMB, Positive control : HTMB + *Haemophilus influenza*

**The Diameter of Inhibition Zone of Secang Heartwood (*Caesalpinia sappan L.*) Extract for *Haemophilus influenza***

Table 3 showed that the antimicrobial activity of SWE against *Haemophilus influenzae* at Mcfarland standard 0,5 inoculum (equal to $10^8$ CFU/mL) according to the diameter of inhibition zone was identified at 256 ppm with the mean diameter of inhibition zone is 11.14 mm, at 512 ppm with the mean diameter of inhibition zone is 15.59 mm and at 1024 ppm with the mean diameter of inhibition zone is 19.83 mm. We classified that the mean diameter of inhibition zone by SWE for *Haemophilus influenzae* at 256 ppm has weak response, whereas the mean diameter of inhibition zone at 512 and 1024 ppm has moderate response. We also classified that the mean diameter of inhibition zone by SWE for *Haemophilus influenzae* started at 256 ppm is susceptible to SWE. The result of antibiotic control group showed the greater mean diameter of inhibition zone and classified as potent inhibitory response.

Table 3 – The Diameter of inhibition zone of $10^{-3}$ sappan wood extrac against *Haemophilus influenzae* at $10^8$ CFU/mL inoculum

| **Treatment (ppm)** | **Diameter of Inhibition Zone** (Mean±SD) |
|---|---|
| Negative Control | 0±0 |
| Positive Control | 0±0 |
| Ampicillin | 31.37±2.43 |
| Cefotaxime | 39.19±0.49 |
| SWE (ppm) | |
| 64 | 0±0 |
| 128 | 0±0 |
| 256 | 11.14±0.74 |
| 512 | 15.59±0.04 |
| 1024 | 19.83±1.21 |

Annotation : Negative Control: Haemophilus Test Medium Broth (HTMB)
SD : Standard Deviation

**The Activity of Sappan Wood Extract as an Iron-Chelating Agent to Inhibit the growth of *Haemophilus influenza***

The reduction of iron concentration can be seen on table 4 and 5. Table 4 showed that iron chelation effect of SWE is indicated through the comparison between the group of sample HTMB with and without SWE. Iron concentration of a group of sample HTMB without SWE is higher than a group of the sample with SWE. SWE at 16 ppm added to HTMB can decrease 8.95% of iron concentration. Whereas SWE at 32 ppm added to HTMB can decrease 16% of iron concentration.

Table 5 showed that iron concentration in HTMB that was added by 10-3 dilution of inoculum is lower than HTMB that was added by 10-5 dilution of inoculum. SWE 16 ppm has the lower chelating effect than 32 ppm because of the lower concentration of the chelating substance contained.

The comparison between the group of HTMB that was added by *Haemophilus influenzae* at $10^{-3}$ and $10^{-5}$ dilution of inoculum (bacterial suspension 105 and 103 CFU/mL) and the group of HTMB without bacterial adition showed that the different iron concentration. Iron concentration of the group of HTMB that was added by the bacteria is lower than a group of HTMB without bacteria. Moreover, the result of iron concentration in HTMB that was added by bacteria and SWE is lower than HTMB and bacteria without SWE.

Table 4 – Iron (Fe) Concentration in HTMB and SWE

| Sample | Iron (Fe) Concentration (ppm) |
|---|---|
| HTMB + Hemadex | 191.25 |
| HTMB + SWE 16 ppm + Hemadex | 182.3 (8.95%) |
| HTMB + SWE 32 ppm + Hemadex | 175.25 (16%) |

Table 5 – Iron (Fe) Concentration in HTMB and SWE After Bacterial Inoculation

| Sample | Iron (Fe) Concentration ppm | |
|---|---|---|
| | $10^{-3}$ dilution of inoculum | $10^{-5}$ dilution of inoculum |
| HTMB + Bacteria + Hemadex | 168.9 (11.69%) | 176.2 (7.87%) |
| HTMB + Bacteria + SWE 16 ppm (MIC) + Hemadex | 159.55 (16.58%) | 163.5 (14.51%) |
| HTMB + Bacteria + SWE 32 ppm (MBC) + Hemadex | 137.6 (28.05%) | 155.9 (18.48%) |

## DISCUSSIONS

This study hypothesized that the process of growth inhibition of *Haemophilus influenzae* after the addition of SWE at certain concentration was due to the iron-chelating effect of SWE. HTMB is enriched by iron, in the form of heme derived from bovine blood, as the vital nutrient component to support *Haemophilus influenzae* growth aerobically and establish an infection process [17, 20].

*Haemophilus influenzae* has developed a complex process of iron uptake to acquire this for its survival [20]. One of the important mechanism of iron uptake by *Haemophilus influenzae* is the utilization of siderophore (LMW-iron chelator) to uptake iron [20]. The competition between SWE and *Haemophilus influenzae* to bind the iron probably exist. Phenolic compounds in SWE have iron-chelating properties for binding iron in HTMB as a *Haemophilus influenzae* specific medium. However, SWE may have better affinity than siderophore in an iron-chelator property so that SWE may cause reduction of iron level in HTMB. Thus, the iron-chelating property of SWE and the dependency of *Haemophilus influenzae* to iron as a nutrient source can be one of the important mechanism for the antimicrobial activity to

*Haemophilus influenzae* so that SWE can be an option as the herbal remedy that useful to be developed as a therapeutic agent for *Haemophilus influenzae* infectious disease. Several studies have a different hypothesis about the mechanism of antimicrobial activity of SWE to bacteria. A study explained that alkaloid and tannins might undergo the disintegration of bacterial colonies due to their interference with bacterial cell wall so that the bacterial growth could be inhibited [13]. Other studies pronounced that the bactericidal effect of brazillin could be caused by the inhibition of DNA and protein synthesis of bacteria [15, 16].

The antimicrobial activity of SWE for *Haemophilus influenzae* is highly stronger than other plant extract and plant oil as previous studies reported. This may be due to different plant with different bioactive substances and different processing methods. This study was used brazilin component as the main bioactive substance that has antimicrobial activity from SWE with the MIC, and MBC values are 16 and 32 ppm, respectively. Brazillin is homoisoflavonoid, a form of phenolic compounds (flavonoid) [15]. A study stated that a specific binding site could cause the activity of trace metal chelation, such as iron, in flavonoid on flavonoid, called catechol moiety [18]. The reduction of iron concentration may be caused by the ability of iron to bind with catechol moiety on flavonoid-contained SWE. Other study showed that serrulate diterpenoid as a bioactive substance of Eremophila neglecta leaf extract had shown the MIC and MBC values are not identified at the maximum concentration tested (more than 200 ppm) by the same method of this study [1]. Whereas, Other study showed essential oils from Citrus hystrix (makrut lime) has shown the MIC, and MBC values are 0.06-0.50 and 0.06-1.10 mg/ml (or equivalent with 60-500 and 60-1100 ppm) by the same method of this study [19].

This study result also showed the antimicrobial activity of SWE compared to standard antibiotic control groups. Two main antibiotics, Ampicillin and Cefotaxime, were used in this study as commonly used for the treatment of *Haemophilus influenzae* infection [17]. However, these two antibiotics gave the better sensitivity than SWE groups. Ampicillin at 1 ppm concentration and Cefotaxime at 2 ppm concentration exhibited the inhibition of *Haemophilus influenzae* growth and 100% reduction of *Haemophilus influenzae* colony number. It means that the lower concentration of these two antibiotics is needed to kill *Haemophilus influenzae* than SWE. Ampicillin and cefotaxime can kill *Haemophilus influenzae* by inhibiting bacterial cell wall synthesis [2]. Although these two antibiotics have better antimicrobial activity to *Haemophilus influenzae* than SWE, these antibiotics still have side effects that can be harmful to patients [6]. SWE was not shown any side effects instead. A study showed that SWE did not produce any acute or subacute toxicity in both male and female rats with body and organs weight, hematological, biochemical and histopathological parameters [15]. So, it can be a reason that SWE has the potential to be developed as an antimicrobial drug for *Haemophilus influenzae*.

## CONCLUSIONS

Antimicrobial activities of SWE has been proved by the MIC and MBC values for SWE against *Haemophilus influenzae* at 103 and 105 CFU/mL were 16ppm and 32ppm, and also the diameter of inhibition zone at 108 CFU/mL appeared at 256 ppm (Mean±SD=11.14mm±0.74), 512 ppm (Mean±SD=15.59mm±0.04) and 1024 ppm (Mean±SD=19.83mm±1.21). Antimicrobial activities of SWE are probably related to the ability of iron to bind with catechol moiety on flavonoid-contained SWE that is matched to the reduced iron concentration measured. This study can be a basis for the future study of causative therapy for *Haemophilus influenzae*.

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