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Antioxidant and Antimicrobial Potential of Sappan Wood Extract against *Porphyromonas gingivalis*

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Abstract

Gingivitis inflammation triggered by microbial biofilms is one of the detrimental causes of periodontal diseases. When a periodontal disease occurs, tooth decay becomes inevitable, and can pose a serious threat to oral health, requiring appropriate treatment. Inflammation in periodontitis is mainly caused by oxidative stress; hence, antimicrobial and antioxidant-based therapies are suggested for periodontitis. Sappan wood is well-known for its brazilin compounds, which have the potential as herbal medicines and antibacterial agents to fight oral pathogens with minimal side effects. This study aimed to examine the antimicrobial and antioxidant activities of sappan wood extract (SE) as a candidate agent for preventing periodontal diseases. This study was conducted at Maranatha Christian University, S*ekolah Tinggi Farmasi Indonesia,* and Aretha Medika Utama from January–April 2023. The sappan wood was extracted using ethanol 96% and the antimicrobial evaluation was done by calculating the total colony of *P. gingivalis* while the antioxidant activity was evaluated by ABTS, H_2O_2, and FRAP Assays. This study revealed that SE was significantly effective in reducing the total colony of P. gingivalis up to 0 CFU/mL. The highest antioxidant activity of SE was found in 100 µg/mL with IC_{50} 19.06 µg/mL for ABTS, 90.99 µg/mL for H_2O_2, and 564 µM Fe (II) for FRAP. Thus, SE presents its strong antimicrobial and antioxidant activity potentials by in vitro evaluation that could be analyzed further for its utilization as a periodontal-preventing agent. This study also provides basic information for future implication of sappan wood extract as ingredients for dental care products.

Keywords: Antimicrobial, antioxidant, brazilin, periodontal diseases, sappan wood

Introduction

Periodontal diseases is one of the most common polymicrobial infections in humans, and they can progress and cause gum recession, bone damage, soft tissue damage, gradual osteoporosis, and tooth loss.[1] The first stage of periodontal disease is gingivitis, gingivitis is an inflammation of the gingiva tissue driven by microbial biofilm on the teeth and gingiva.[2] Chronic bacterial colonization by some pathogenic microorganisms, along with a self-destructive host inflammatory response, results in soft tissue deterioration and bone loss around the tooth. *Porphyromonas gingivalis* has been widely studied among bacteria and is considered to be responsible for the development of periodontal disease[3]. Destructive disease development appears to be influenced by a dysfunctional host response to the sub-gingival dental plaque. Much evidence has revealed that oxidative stress is associated with the–pathogenesis of periodontitis.[4] Dental plaque contains bacteria that cause the release of proinflammatory cytokine and the production of reactive oxidative stress (ROS). The excess production of ROS has harmful effects on the tissue structure.[5] Therefore, an antioxidant to counter the excess ROS production and an antimicrobial agent against the bacteria are needed to help enhance the treatment of diseases' development.

A main preventive strategy for chronic periodontitis is the prevention of gingivitis,

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which entails delaying the development of the microbial biofilm and/or regularly removing it.[2] Proper preventive treatment can help to suppress the cases of periodontal disease. Natural medicine has gained a lot of interest as a periodontitis prevention agent. Natural products show excellent characteristics such as diversity of chemical and biological properties and less toxicity. As a result, they are promising leads for the discovery of new medications.

Sappan wood (*Caesalpinia sappan* L.) or "Kayu Secang" in Indonesia is one plant that is utilized as a medicine to treat various diseases. *C. sappan* is a member of the Caesalpiniaceae family with an orange-red hard heartwood and a spiny trunk that, in addition to being used in turnery, produces a red dye.[8] The phytochemical compounds in sappan wood play a major role in its biological activity. The phytochemical analysis conducted by Widowati showed that sappan wood extract presents a high concentration of flavonoids, terpenoids, and phenols.[9] Amongst all, brazilin is the main compound present in sappan wood. Numerous studies have revealed that sappan woods possess various biological properties such as antioxidant, and antimicrobial against streptococcus pneumonia.[10]

Therefore, this study aims to examine the antimicrobial activity using total plate count (TPC) and antioxidant activity using 2,2'-azinobis-(3- ethylbenzothiazoline-6-sulfonate (ABTS), hydroperoxide (H_2O_2), and ferric reducing antioxidant power (FRAP) assays of Sappan wood Extract (SE) against *P. gingivalis.*

Methods

This study was an experimental laboratory method. This research was conducted from January 2023 to April 2023 in Maranatha Christian University, Sekolah Tinggi Farmasi Indonesia (STFI) and Aretha Medika Utama, Biomolecular and Biomedical Research Center Bandung. The Sappan wood (*C. sappan* L) extraction method used the maceration technique. The fresh sappan wood was dried and extracted using 96% ethanol. A total of 1 kg of ground sappan wood with a size of 60 mesh was immersed for 24 hours in 5 L of ethanol 96%. The filtrate was collected, then the sappan wood pulp was added with 5 L of ethanol, which was done up to 4 times immersion. The filtrate was evaporated to obtain sappan wood extract (SE).[9] To examine the antimicrobial activity of *P. gingivalis*, broth microdilution and total plate count (TPC) were used. *P. gingivalis* (ATCC® 33277™) was obtained from Aretha Medika Utama and was cultured prior to testing. For broth microdilution assay, a total of 100 µL of bacterial inoculum and 100 µL of Mueller Hinton Broth (MHB) (Himedia, M403-500G) were added to the wells to produce growth control. Subsequently, 100 µL MHB and 100 µL working solution for each concentration of the sappan woods extract were added to the well as a blank. Afterwards, 0.2% chlorhexidine (Minosep,11115) was added for the control well. Chlorhexidine was used as the positive control. The plates were placed at 37°C for 24 hours. For the total plate count assay, 100 µL of a 24-hour bacterial culture from broth microdilution assay was subjected to serial dilutions of 10^{-2} to 10^{-5} using PBS. A total of 100 µL of the dilution was plated on a Petri dish, and Mueller Hinton Agar (MHA) (Himedia, M096-500G) was added. The Petri dishes were placed in an incubator for 24 hours at 37°C. The Colony was formed after 24 hours and a colony counter (Funke Gerber 8500) was used.[11]

The ABTS assay was performed to examine the antioxidant activity. Briefly, In the sample well and the well blank, a total of 2 µL of the SE was added. In the sample well and control well, a total of 198 µL, 200 µL of ABTS reagent was added. For the blank well, 198 µL of DMSO (Merck, 1029522500) was added. The microplate was incubated for 6 minutes at 37°C. A microplate reader was used to measure the absorbance at λ=745 nm. The ABTS reducing activity was calculated using the equation:[12]

$$\text{ABTS activity (\%)} = \frac{A-B}{A} \; x \; 100$$

A: Control Absorbance
B: Sample Absorbance

The materials used in FRAP antioxidant assay included acetate buffer (pH 3,6), FeCl3 (Sigma-Aldrich, 12322-2.5L), and 2,4,6-Tripyridyl-s-Triazine (Sigma-Aldrich, 3682-35-7). Sappan wood extract was diluted to obtain the final concentration at 1.56, 3.13, 6.25, 12.50, 25, 50, and 100 µg/mL. As much as 7.5 µL of the SE was placed in the sample well and well blank. A total of 142.5 µL FRAP reagent was added to the sample well (SE). 142.5 µL of sample solvent (DMSO Merck, 1029522500) was added to the well blank. The microplates were next incubated for 6 minutes at 37°C. The absorbance was measured at λ=745 nm. The following equation was used to determine the reducing activity.[13]:

$$\text{FRAP activity} = \frac{A-B}{A} \; x \; 100$$

A: Control Absorbance
B: Sample Absorbance

The materials used in the H_2O_2 assay included: 1 mM ferrous ammonium sulfate (Sigma 7783859), dimethyl sulfoxide (DMSO) (Merck 1.02952), 1,10-phenanthroline (Sigma 131377), sulfuric acid (Merck 109981), hydrogen peroxide (Merck 1.08597.1000), and deionized water (ddH_2O). A total of 60 µL samples were added to the 96-well plate. Ferrous ammonium sulfate (12 µL in total) was added to the control well plate and the sample well. DMSO was added to the well blank (90 µL) and to the well control (30 µL). A total of 3 µL of H_2O_2 5 mM reagent was added to the well sample and incubation was carried out for 5 minutes in a dark place at room temperature. Afterward, 75 µL of 1 mM 1,10-phenanthroline was joined to both the sample and blank wells. The plate was left in a dark place at room temperature for 10 minutes. The absorbance was measured at λ=510 nm, and the absorbance values were used to calculate the scavenging activity using the calculation.[13]:

$$H_2O_2 \text{ activity } (\%) = \frac{A-B}{A} \; x \; 100$$

A: Control Absorbance
B: Sample Absorbance

The research was conducted in three replicates. The concentrations used for both assays were obtained from carried out optimation. To analyze the antimicrobial (total colony) and antioxidant activities of sappan wood extract, a One-way ANOVA and Tukey HSD post hoc were used to interpret the data with $p<0.05$. The mean ± standard deviation was used to express quantitative data. IBM SPSS Statistics v.25 was employed for all statistical analyses (IBM Corp., Armonk, NY, USA).

Results

The antimicrobial activity of SE towards the total colony of *P. gingivalis* is shown in Figure 1. The results demonstrate that SE significantly reduces the total colony of *P. gingivalis* compared to other groups ($p<0.05$). The lowest concentration of SE (12.5 µg/mL) had the highest total colony count $124.33x10^3$ CFU/mL among the treatments and it gradually decreased followed by a higher concentration. The most effective concentration was found in 1600 µg/mL with 0 CFU/mL. SE at 1600 µg/mL showed equivalent results with the positive control (Chlorhexidine 0.2%) that also exhibited 0 CFU/mL. This means that SE inhibits the growth of *P. gingivalis*.

The effects of SE on ABTS Reducing Activity are presented in Figure 2. Based on the result, it is evident that SE's antioxidant-reducing activity is closely related to the sample's concentration. The highest ABTS-reducing activity in SE was observed at a concentration of 100 µg/mL

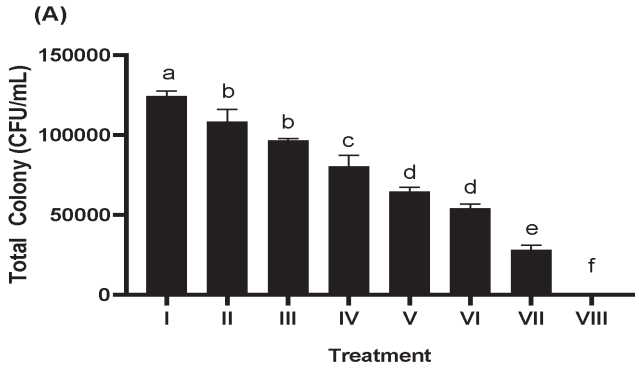

Figure 1 The Effect of Various Concentrations of SE toward Total Colony of *P. gingivalis*

*Data presented as mean ± standard deviation, the test was performed in three replicates. Different codes (a, b, c, d, e, f, g) show significant differences among concentrations. (I) 12.5 µg/mL, (II) 25 µg/mL, (III) 50 µg/mL, (IV) 100 µg/mL, (V) 200 µg/mL, (VI) 400 µg/mL, (VII) 800 µg/mL and (VIII) 1600 µg/mL SE concentration

Table 1 IC_{50} value of sappan wood extracts on ABTS and H_2O_2 Antioxidant Assay

Assay	Linear Regression	R2	IC_{50} (µg/mL)
ABTS	y=0.6018x + 38.529	0.99	19.06 ± 0.50
H_2O_2	y=0.3148x + 21.026	0.99	90.99 ± 0.91

* Linear regression was used to determine the coefficient of regression (R2) and the IC_{50} value.

(98.58%), with significant differences ($p<0.05$) among the treatments.

The FRAP-reducing activity is displayed in a concentration-dependent manner. The lowest FRAP-reducing activity was observed at a concentration of 1.56 µg/mL for SE, with a reducing activity of 157.33 µM Fe(II). The highest FRAP-reducing activity was observed at 100 µg/mL (Figure 3). However, no significant differences were found in the FRAP-reducing activity in concentrations of 3.13 µg/mL and 6.25 µg/mL. Significant differences were then

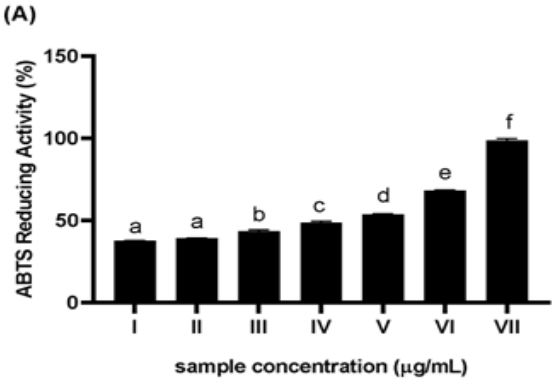

Figure 2 The Effect of Various Concentrations of SE toward ABTS-Reducing Assay

*Data presented as mean ± standard deviation, the test was performed in three replicates. Different codes (a, b, c, d, e, f) show significant differences among concentration. (I) 1.56 µg/mL, (II) 3.13 µg/mL, (III) 6.25 µg/mL, (IV) 12.50 µg/mL, (V) 25 µg/mL, (VI) 50 µg/mL, (VII) 100 µg/mL SE concentration

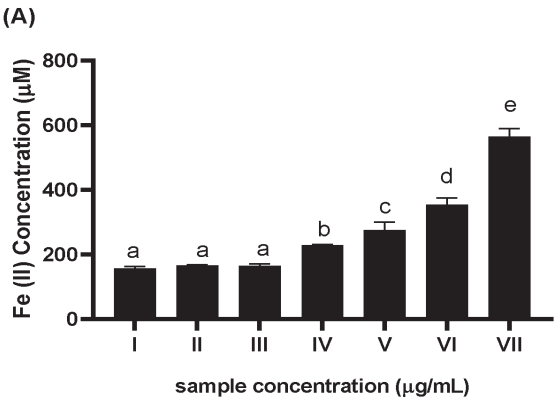

Figure 3 The Effect of Various Concentrations of SE on FRAP-Reducing Assay

*Data presented as mean ± standard deviation, the test was performed in three replicates. Different codes (a, b, c, d, e) show significant differences between concentration. (I) 1.56 µg/mL, (II) 3.13 µg/mL, (III) 6.25 µg/mL, (IV) 12.50 µg/mL, (V) 25 µg/mL, (VI) 50 µg/mL, (VII) 100 µg/mL SE concentration

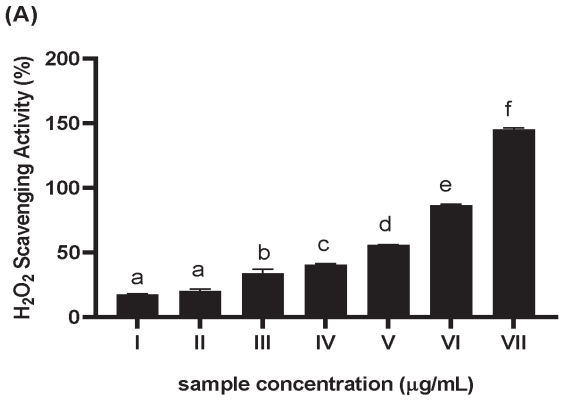

Figure 4 The Effect of Various Concentrations of SE toward H_2O_2 Scavenging Assay

*Data presented as mean ± standard deviation, the assay was done in triplicate. Different symbols (a, b, c, d, e, f, and g) show significant differences between concentration. (I) 1.56 µg/mL, (II) 3.13 µg/mL, (III) 6.25 µg/mL, (IV) 12.50 µg/mL, (V) 25 µg/mL, (VI) 50 µg/mL, (VII) 100 µg/mL. SE concentration

observed in 12.50 µg/mL ($p<0.05$).

The effect of SE on H_2O_2 Scavenging Assay is seen in Figure 4. The SE significantly ($P<0.05$) scavenged the H_2O_2 free radicals. The strongest H_2O_2 scavenging activity was found in the concentration 100 µg/mL with 145% scavenging activity. These findings indicate that sappan wood extract can scavenge the free radical in the H_2O_2 assay.

The inhibitory concentration of SE was evaluated using the ABTS and H_2O_2 assays, as presented in Table 1. The IC_{50} value was used to determine its antioxidant activity. Based on this study, it was found that sappan wood extract exhibits strong antioxidant activity (<50 µg/mL) in the ABTS reducing assay compared to H_2O_2 scavenging assay. However, in both assays, SE is still categorized as having strong antioxidant activity, as indicated by its IC_{50} value (<50–100 µg/mL).

Discussion

Around 20 and 50 percent of people worldwide are affected by periodontal diseases, which is common in both industrialized and developing nations.[14] Periodontal disease is then associated with several diseases such as cardiovascular disease, metabolic disease, chronic kidney disease, rheumatoid arthritis, cancer, respiratory diseases, and deterioration of cognitive ability.[15] Public health issues arise because of the high frequency of periodontal disease in adults, adolescents, and the elderly. To prevent periodontal diseases, the utilization of active ingredients derived from natural sources, such as plants, is currently popular. Researchers have studied a variety of plants that use to could be utilized as materials for traditional medical medicines for dental care. The biological activities of *C. sappan* including its antioxidant[16], anti-inflammatory, and antibacterial[6] activities have been extensively studied, but there have only been a few studies on how to prevent oral infections. Further scientific information on *C. sappan* wood extracts' antibacterial and antioxidant activity against *Porphyromonas gingivalis* is provided in the current study.

The results of this study reported that SE showed its antimicrobial activity significantly by inhibiting the growth of *P. gingivalis* (Figure 1). SE antimicrobial activity was evaluated using the total plate count method to calculate the total colony of *P. gingivalis*. The total colony value of *P. gingivalis* indicated that the sappan extracts were more effective as bactericidal at higher concentrations. This result is in line with Bukke et al., 2015 reported that methanol, aqueous, and petroleum ether extracts of heartwoods, bark, and leaves of *C. sappan* demonstrated inhibition against bacteria in concentration dependent-manner.[17]

The presence of phytochemical constituents in *C. sappan* heartwood has been shown to have antibacterial effects, as evidenced by the inhibition of bacterial growth. Another study reported that *C. sappan* exhibited antibacterial

effects against *Streptococcus mutans* and *Enterococcus faecalis*, as well as anticancer activity against A549 lung cancer with an IC_{50} value of 90,01µg/mL.[18] Moreover, a different study documented that pasteurized milk with added SE demonstrated antimicrobial activity by inhibiting bacterial growth to a level of 3.04 log CFU/mL.[11] This study also shows comparability with another study[6] that demonstrated *C. sappan* antibacterial activity against *P.gingivalis*. *C. sappan* extract is reported to exhibit inhibition against *S. mutans* with a MIC value of 1.95 mg/mL and MBC values of 62.5 mg/mL.[19]

Therefore, SE can combat pathogenic organisms without affecting the tissue structure, which is believed to be a primary cause of periodontal disease. Although this study exhibited equivalence with several prior studies, this study was hindered by some limitations because Minimum Inhibition Concentration (MIC) and Maximum Bactericidal Concentration (MBC) of the extracts haven't been able to be investigated.

To assess the antioxidant activity of SE, the ABTS, FRAP, and H_2O_2 assays were used. SE demonstrated antioxidant activity in a concentration-dependent manner.[20] The ABTS reduction test assesses the ability of antioxidant compounds to prevent the generation of ABTS+ radical cations. This study showed that SE has a high antioxidant activity in scavenging the free radicals of ABTS and H_2O with IC_{50} values of 19,06 µg/mL and 90.99 µg/mL, respectively, as seen in Table 1. The IC_{50} value of a compound is classified into three categories: IC_{50} value <50 µg/mL is considered a very strong antioxidant, IC_{50} 50–100 µg/mL is strong, IC_{50} 100–150 µg/mL is moderate, and IC_{50} 151–200 µg/mL as weak.[21] These results are consistent with a previous study reported that the ethanol extract of *C. sappan* has very strong activity using ABTS assay with an IC_{50} value close to Trolox as a comparison (26.70 ppm and 19.38 ppm, respectively), both of which are less than 50 ppm.[22]

In the FRAP assay, antioxidant compounds were tested for their ability to stabilize free radicals by donating electrons and converting Fe3+ to Fe2+. The absorbance value can be used to determine antioxidant activity and represents the amount of Fe2+ that has been reduced.[20] The results showed that the highest FRAP-reducing activity was found in 564 µM Fe(II). A previous study reported that ethanolic extracts of sappan woods exhibited strong FRAP activity with IC_{50} 11,37 ppm.[22] The first defense mechanism carried out by antioxidants is to suppress the formation of free radicals or oxidative stress in the body, neutralize free radicals, or enhance the ability of enzymes.[4]

Besides microbes, oxidative stress is one of the causes that is also associated with the etiology of periodontitis. The development of damaged tissues in periodontal disease seems to be driven by an abnormal cellular response to sub-gingival bacterial biofilm. The persistence of dental plaque in the periodontal pocket results in the mobilization of leukocytes and polymorphonuclear neutrophils (PMNs) from the blood circulation to the site of infection.[5] However, an over-activated of PMNs leads to excess production of reactive oxidative stress (ROS), which can damage the structure of periodontal tissue. The antioxidant status in periodontitis patients is important.[4] Therefore, antioxidants agents are needed to counter the harmful effects of ROS.

In addition, there are synergistic effects between the antimicrobial and antioxidant properties of SE due to its active compound present in the extracts. It has been observed that the presence of compounds like flavonoids and terpenoids, especially Brazilin in SE, is a possible reason for its scavenging effectivity and free radicals reducing activity.[23] Brazilin is the primary chemical compound found in sappan wood that exhibits anti-inflammatory and antibacterial properties, hypoglycemic effect, and cell protection from oxidative stress.[23,24] Another study reported that brazilin from *C. sappan* extract exhibits antioxidant activity with an EC_{50} of 60.5 µg/mL, antimicrobial activity against gram-negative bacterial biofilm, and anti-inflammatory activity by inhibiting protein denaturation up to 61.9%.[24] This suggests that SE can act as an antimicrobial to eliminate the bacterial biofilm and act as an antioxidant to neutralize the excess ROS caused by microbial biofilm as well. The differences in studies may be caused by several factors. Several factors that could influence antimicrobial activity capacity were concentrations of the antimicrobial, host factors, and bacterial condition.[25] The limitation of this study emerged because it hasn't been able to demonstrate a DPPH antioxidant assay.

However, as a conclusion, SE has exhibited the antimicrobial and antioxidant activity potential to act as a natural compound against *P. gingivalis*. These phytochemicals are potent as pharmaceutical compounds that have demonstrated important biological functions, such as antioxidant and antibacterial actions, providing essential information for their future

potential use as ingredients in dental care products. Further studies are needed to conduct a comprehensive phytochemical test and evaluate its anti-inflammatory properties, toxicity, and in vivo effects.

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