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RESEARCH ARTICLE

Antibacterial activity and stability of mouthwash formulated with ethanolic extract of sappan wood (*Caesalpinia sappan* L.) against *Streptococcus mutans* ATCC 25173 and *Pseudomonas aeruginosa* ATCC 10145

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Abstract

This study aimed to develop and evaluate a herbal mouthwash formulation containing an ethanolic extract of sappan wood (*Caesalpinia sappan* L.) as a potential natural alternative to chlorhexidine-based products. The extract and derived mouthwash were assessed for antibacterial activity against *Streptococcus mutans* Clarke and *Pseudomonas aeruginosa* (Schroeter) Migula using the agar well diffusion and minimum inhibitory concentration (MIC) methods at concentrations of 1 %, 3 % and 5 %. Positive controls included 0.2 % chlorhexidine and a commercial mouthwash, while negative controls consisted of mouthwash without extract and 96 % ethanol. The stability of the mouthwash was evaluated through a six-cycle cycling test by monitoring organoleptic properties, homogeneity, pH, sedimentation and redispersion. The mouthwash exhibited a maximum inhibition zone of 21.5 mm and 15.2 mm against *S. mutans* and *P. aeruginosa*, respectively, with MIC values of 1 % and 3 %. It remained physically stable during six storage cycles with consistent pH (4.6–6.5) and homogeneous dispersion. These results demonstrate that sappan wood extract possesses strong antibacterial activity and good formulation stability, supporting its potential as a safe and effective herbal mouthwash. The study highlights the novelty of exploring a plant-based mouthwash as a promising natural alternative to chlorhexidine, showing comparable *in vitro* antibacterial activity with potentially fewer adverse effects.

Keywords: *Caesalpinia sappan*; mouthwash; *Pseudomonas aeruginosa* ATCC 10145; sappan wood; *Streptococcus mutans* ATCC 25173

Introduction

Oral diseases are among the most widespread global health issues, imposing substantial health and economic impacts that significantly diminish the quality of life of affected individuals (1). This global concern is reflected in The Global Burden of Disease Study in 2016, which reported that dental and oral health problems, particularly dental caries, affect nearly half of the worlds' population. Similarly, data from the Basic Health Research Report in 2018 indicated that cavities and swollen gums are the most prevalent oral health issues in Indonesia (2). The main factor contributing to dental health problems is the formation and accumulation of dental plaque, which begins as a thin biofilm of bacteria adhering to the tooth surface (2). Over time, the bacterial colonies produce acids through carbohydrate metabolism, initiating enamel demineralisation that leads to dental caries and eventual tooth loss if left untreated. Continuous plaque accumulation may also result in gingivitis, characterised by inflammation of the gingival tissue, which can progress to periodontitis, a more severe condition that destroys connective tissue and alveolar bone (3).

Two major bacterial species are closely associated with oral infectious diseases. *Streptococcus mutans* Clarke plays a key role in dental plaque formation by producing acids that demineralise enamel and cause dental caries, while *Pseudomonas aeruginosa* (Schroeter) Migula, an opportunistic microorganism commonly found in subgingival plaque, increases the risk of periodontal infections and may also contribute to secondary respiratory complications (4, 5). To prevent these bacterial infections, maintaining oral hygiene through regular cleaning and the use of mouthwash is recommended. Although a 0.2 % chlorhexidine mouthwash effectively controls dental plaque, its long-term use may cause adverse effects such as tooth discolouration and salivary gland irritation. Therefore, herbal mouthwash formulations have been explored as safer and eco-friendly alternatives (6).

Sappan wood (*Caesalpinia sappan* L.), a member of the Leguminosae family, is widely used in traditional medicine and has shown potential as an active ingredient in herbal mouthwash formulations. This plant has long been utilised to treat various ailments such as malaria, tetanus, diarrhoea and dysentery (7). Several flavonoid compounds have been identified in sappan wood, including brazilin, protosappanin A, sappanone B and 4-O-methylsappanol, all known for their antibacterial activity (8–10).

Among these, brazilin is the predominant bioactive flavonoid responsible for inhibiting the growth of oral pathogenic microbes through multiple mechanisms, including interference with DNA and protein synthesis, disruption of bacterial membranes and inhibition of DNA gyrase activity (9, 11, 12). Previous studies have further substantiated the antibacterial potential of sappan wood extracts by demonstrating their effectiveness against both Gram-positive and Gram-negative oral pathogens. Research indicates that the ethanolic extract of sappan wood exhibited strong inhibitory activity against *S. mutans*, while subsequent work identified brazilin as the major compound responsible for this activity (4, 11). Moreover, research confirms that the ethanolic extract also inhibited the growth of *P. aeruginosa*, highlighting its broad-spectrum antibacterial potential relevant to oral health applications (13).

However, while several studies have investigated the antibacterial potential of sappan wood extracts, limited information is available regarding their formulation stability and comparative efficacy when developed into mouthwash preparations. This study presents a novel approach by formulating an ethanolic extract of sappan wood into a mouthwash and evaluating its antibacterial efficacy against *S. mutans* and *P. aeruginosa*. The main objectives were to determine the total flavonoid content, assess the antibacterial activity of the ethanolic extract and the formulated mouthwash and evaluate the product stability. For stability assessment, organoleptic parameters such as colour, aroma and appearance, along with pH, homogeneity, sedimentation volume and redispersion, were analysed to ensure the mouthwash maintained its effectiveness in inhibiting pathogenic bacterial growth and reducing the risk of dental caries.

Materials and Methods

Materials

Sappan wood (*C. sappan*) was obtained and identified from the Centre for Research and Development of Medicinal Plants and Traditional Medicines in Tawangmangu, Indonesia, during February-March, when the plants were in their mature growth phase. The plant was taxonomically identified under reference number KM.04.02/2/392/2022 and the authenticated herbarium specimen was deposited at the Teknobioindustri Laboratory, Faculty of Technobiology, Universitas Atma Jaya Yogyakarta. *S. mutans* ATCC 25173 and *P. aeruginosa* ATCC 10145 bacterial isolates were obtained from the Integrated Research Laboratory, Faculty of Dentistry, Universitas Gadjah Mada. Subsequently, the substances used included 96 % ethanol, distilled water, nutrient agar (NA) medium, nutrient broth (NB) medium, commercial mouthwash containing 0.2 % Chlorhexidine, 0.2 % Chlorhexidine solution and other reagents that were of analytical grade.

Samples preparation and simple standardisation

Sappan wood was dried in a hot-air oven at 50 °C for 24 hr, ground into powder using a blender and grinder and then sieved through a 61-mesh strainer to obtain fine simplicia powder (14, 15). Standardisation of sappan wood simplicia consisted of total ash, acid-insoluble ash, ethanol-soluble compound, water-soluble compound and water content, as well as the loss on drying. The results of simplicia standardisation were compared to the standard from Indonesian Herbal Pharmacopoeia 2nd Edition (16).

Extraction of sappan wood

The dried powdered sappan wood underwent extraction using the maceration method for 24 hr, followed by two additional remaceration cycles, each lasting 24 hr. Ethanol 96 % served as the solvent, with a sample-to-solvent ratio of 1:10. Subsequently, the samples were macerated in a shaker incubator at 30 °C and 150 rpm for 24 hr and the resulting filtrate was evaporated using a rotary evaporator set at 50 °C and 55 rpm. The weight of the thick extract was measured and the yield percentage was calculated (17). The extract concentrations of 1 %, 3 % and 5 % were selected based on preliminary evaluations and formulation feasibility, representing low, medium and high concentrations commonly applied in herbal mouthwash formulations to assess dose-dependent antibacterial effects (18).

Formulation of mouthwash base

The mouthwash base was prepared using a combination of safe and commonly used pharmaceutical excipients. The base formulation consisted of glycerin (15 %) as a humectant, propylene glycol (15 %) as a cosolvent, sorbitol (20 %) as a sweetening and flavouring agent, sodium benzoate (0.1 %) as a preservative and distilled water (100 %) as a solvent. These ingredients were thoroughly mixed to obtain a homogeneous solution, which served as the vehicle for incorporating the ethanolic extract of *C. sappan* at concentrations of 1 %, 3 % and 5 %.

Determination of total flavonoid content (TFC)

Determination of TFC was carried out using $AlCl_3$ and CH_3COOK reagents. The optimum wavelength was determined using a quercetin standard solution (15 ppm). Subsequently, the absorbance of the standard solution was measured in the wavelength range of 405-450 nm to obtain an optimum wavelength of 435 nm (19). The quercetin standard curve was made by measuring quercetin standard solution in various concentrations of 3, 6, 9, 12 and 15 ppm. The total flavonoid content of sappan wood extract and the derived mouthwash was measured by adding 0.5 mL of the sample with 0.1 mL of $AlCl_3$ 10 %, 0.1 mL of CH_3COOK and 4.3 mL of distilled water. The sample was incubated in a dark room for 30 min and the absorbance was measured at 435 nm. The total flavonoid content was determined by a linear equation from the quercetin standard curve and calculated by the following formula (19).

$$TFC = \frac{c \; x \; v \; x \; fp}{m} \quad \text{(Eqn. 1)}$$

where, c: Quercetin equivalent result (mg/mL); v: Sample volume (mL); fp: Dilution factor; m: Sample weight (mg)

Evaluation of antibacterial activity

The antibacterial activity of sappan wood ethanolic extract and the derived mouthwash was determined using the inhibition zone test with agar well diffusion method against *S. mutans* and *P. aeruginosa* (20). Subsequently, *S. mutans* and *P. aeruginosa* suspensions were inoculated on Nutrient Agar (NA) media and wells were made in the media. The wells were filled with 35 µL of the sample, which consisted of sappan wood ethanolic extract (1 %, 3 % and 5 %), a mouthwash of sappan wood extract (1 %, 3 % and 5 %), chlorhexidine 0.2 % and commercial mouthwash containing chlorhexidine 0.2 % as the positive control, including 96 % ethanol and mouthwash base as the negative control. The media were

incubated at 37 °C for 24 hr and the diameter of the inhibition zone formed was measured in triplicate for each treatment (20).

Determination of MIC

The MIC test was performed using the agar-dilution method. The test commenced by preparing *S. mutans* and *P. aeruginosa* starters, which were incubated at 37 °C for 24 hr. Subsequently, a 10 % stock solution was made for sappan wood extract and mouthwash with sappan wood extract. The MIC test solution consisted of a suspension of bacteria inoculated in a test tube containing 2 mL of nutrient broth (NB) media and the extract or mouthwash with a concentration series of 1 %, 3 % and 5 %. The positive controls used were 0.2 % chlorhexidine and commercial mouthwash containing 0.2 % chlorhexidine, while the negative control was an untreated bacterial suspension (21). The sample solution inoculated with the bacteria and treated with the extract or mouthwash was incubated at 37 °C for 24 hr. The sample tube solution was inoculated into a petri dish containing NA media. Inoculation of *S. mutans* was carried out by the pour plate method, while *P. aeruginosa* was performed by the spread plate method. All inoculated plates were incubated for 24 hr at 37 °C and observed to assess bacterial growth in each treatment (21).

Evaluation of mouthwash stability

Evaluation of mouthwash stability was carried out using a cycling test. This test was conducted for six cycles by storing mouthwash at 4 °C for 24 hr and continuing at 40 °C for another 24 hr. Meanwhile, one cycle was defined as 24 hr of storage at 4 °C, followed by 24 hr at 40 °C. The organoleptic evaluation was carried out by visual examination of its appearance, flavour, form and scent. Homogeneity was assessed by observing two parameters, namely, before and after shaking. The homogeneity was evaluated before shaking by observing whether the mouthwash formed two layers. Homogeneity after shaking was assessed with one drop of mouthwash poured onto an object glass to examine for undispersed coarse granules. The pH value was observed using a pH meter calibrated at values of 4 and 7 (6, 22, 23).

The sedimentation and redispersion tests were carried out at room temperature. The sedimentation test was conducted by adding 10 mL of mouthwash into a measuring cup and the changes in the volume were observed and recorded every cycle without stirring. The initial volume (Vo) was the initial volume of the mouthwash filled, while the final volume (Vu) was the volume of mouthwash supernatant obtained after sedimentation occurred. The sedimentation volume (F) was determined by dividing Vu by Vo. The redispersion test was carried out by placing 5 mL of mouthwash in a test tube and rotating it at 180°. The time of rotations needed to redisperse the sediment was calculated using a stopwatch. The stability of the mouthwash is deemed satisfactory when there are no significant physical changes observed and it remains stable for at least three cycles during the stability test (6, 22, 23).

Data analysis

Quantitative data were statistically analysed using a completely randomised design (CRD). Differences between treatments were evaluated using analysis of variance (ANOVA) and significant differences were further tested using the Duncan multiple range test (DMRT) at a significance level of $p < 0.05$. All analyses were performed using SPSS Statistics, version 25.0.

Results and Discussion

Sappan wood simplicia standardisation

The standardisation results in Table 1 showed that sappan wood simplicia fulfilled all the required parameters (24). These results indicated that sappan wood simplicia possessed good quality and can be used as an active component in the mouthwash formulation. Research indicates that the quality of heartwood material strongly influences the yield of secondary metabolites such as brazilin and total flavonoids, which determine antibacterial activity (9).

Table 1. Standardisation of sappan wood

Parameters	Result (%)	Standard (18)
Total ash content	0.97 ± 0.08	≤ 2 %
Acid-insoluble ash content	0 ± 0.00	≤ 0, 5 %
Ethanol-soluble compound content	10.05 ± 0.38	≥ 6 %
Water-soluble compound content	4.89 ± 0.02	≥ 4 %
Loss on drying	2.46 ± 0.12	≤ 5 %
Water content	6.35 ± 0.44	≤ 10 %

Extraction of sappan wood

As presented in Table 2, the extract yield was 1.5 times higher than the average of 12.05 % (25). The higher yield was due to remaceration, which prevents solvent saturation and increases the yield percentage. Remaceration was also used to extract active compounds (26). Research indicates that stepwise ethanolic extraction enhances the content of brazilin, the principal bioactive compound of sappan wood, responsible for both antibacterial and anti-inflammatory effects (26).

Table 2. Yield percentage of sappan wood ethanolic extract

Sample	Extraction yield (%; Mean ± SD, n = 3)
C. sappan ethanolic extract	19.17 ± 0.13

Total flavonoid content

The TFC of sappan wood ethanolic extract in Table 3 showed a significant difference compared to the previous study. The result was 3.8 times higher than that of previous researchers, who obtained a total flavonoid content of sappan wood ethanolic extract at 9.59 mg QE/g extract (27). This higher value was influenced by several factors, including the length of extraction time and the drying process of the simplicia used. Furthermore, a difference in the location of the growing sample used due to environmental influences can also affect the acquisition of total flavonoid levels (28). The high flavonoid level obtained in this study reinforces the hypothesis that these metabolites act as the main bioactive compounds responsible for the antibacterial activity.

The TFC was also determined in mouthwash formulations containing different concentrations of sappan wood extract (Table 3), confirming the successful incorporation of flavonoids into the formulations. Although the measured flavonoid levels increased numerically with higher concentrations of sappan wood ethanolic extract, the differences were not statistically significant ($p > 0.05$). This suggests that beyond a certain concentration, the matrix effect of the formulation may limit the detectable increase in total

flavonoids. Research indicates that higher extract concentrations tend to enhance the presence of active compounds, although the magnitude of increase may not always be statistically significant due to formulation stability and interaction factors (29). The TFC of the sappan wood ethanolic extract mouthwash showed its potential antibacterial activity against the tested bacteria. The flavonoid content in the mouthwash formulation was higher than that of the extract, likely due to the presence of glycerin and propylene glycol as co-solvents that enhance flavonoid solubility and stability (30).

Table 3. Total flavonoid content of sappan wood ethanolic extract and mouthwash with sappan wood ethanolic extract

Sample	Total flavonoid content (mg QE/g extract)
Sappan wood ethanolic extract	36.38 ± 1.81^{a}
Mouthwash with 1 % sappan wood extract	88.65 ± 0.41^{b}
Mouthwash with 3 % sappan wood extract	90.56 ± 1.20^{b}
Mouthwash with 5 % sappan wood extract	93.22 ± 0.72^{b}

Note: Values followed by the same letter are not significantly different in the Duncan test. $*p < 0.05$. Positive control: chlorhexidine 0.2 %; Negative control: ethanol 96 %.

Antibacterial activity of sappan wood ethanolic extract and mouthwash from sappan wood ethanolic extract

The antibacterial activity of sappan wood ethanolic extract and the derived mouthwash was determined based on the diameter of the inhibition zone, which was repeated three times. Table 4 shows that the average inhibition zone diameter for *S. mutans* and *P. aeruginosa* increased consistently with higher extract concentrations, indicating a clear positive correlation between extract concentration and antibacterial efficacy. This trend agrees that increasing extract concentration enhanced inhibition zone diameter due to the higher abundance of active compounds (31).

Table 4. Zone of inhibition of sappan wood ethanolic extract

Sample	Zone of Inhibition (mm)	
	S. mutans	***P. aeruginosa***
Extract 1 %	17.00 ± 0.30^{c}	12.50 ± 0.50^{c}
Extract 1 %	20.00 ± 0.50^{b}	14.50 ± 0.50^{b}
Extract 5 %	23.30 ± 0.30^{a}	16.70 ± 0.30^{a}
Chlorhexidine 0.2 %	20.20 ± 0.30^{b}	11.80 ± 0.50^{c}
Ethanol 96 %	0	0

Note: Values followed by the same letter are not significantly different in the Duncan test. $*p < 0.05$. Positive control: chlorhexidine 0.2 %; Negative control: ethanol 96 %.

The antibacterial activity of sappan wood ethanolic extract against *S. mutans* and *P. aeruginosa* increased with higher extract concentrations (1 %, 3 % and 5 %), as shown in Fig. 1 and 2. Although the extract concentrations differ from those of the positive control (chlorhexidine 0.2 %), the comparison was made to provide a practical reference for assessing the antibacterial potential of the extract under formulation-relevant conditions. These findings indicate that sappan wood ethanolic extract exhibits promising inhibitory activity against both *S. mutans* and *P. aeruginosa*, supporting its potential use as an alternative active ingredient in mouthwash formulations. The 96 % ethanol used as a negative control exhibited no inhibitory activity against *S. mutans* or *P. aeruginosa*, confirming that the inhibition zones observed at the three extract concentrations were solely due to the antibacterial compounds present in the sappan wood ethanolic extract. This antibacterial effect is primarily attributed to brazilin and sappanone B, the major bioactive constituents of *C. sappan*, which function as natural antibacterial agents. Brazilin disrupts bacterial membrane permeability and inhibits DNA gyrase, while other flavonoids in the extract may deactivate cell-wall proteins through complex formation with sulfhydryl groups. These findings are

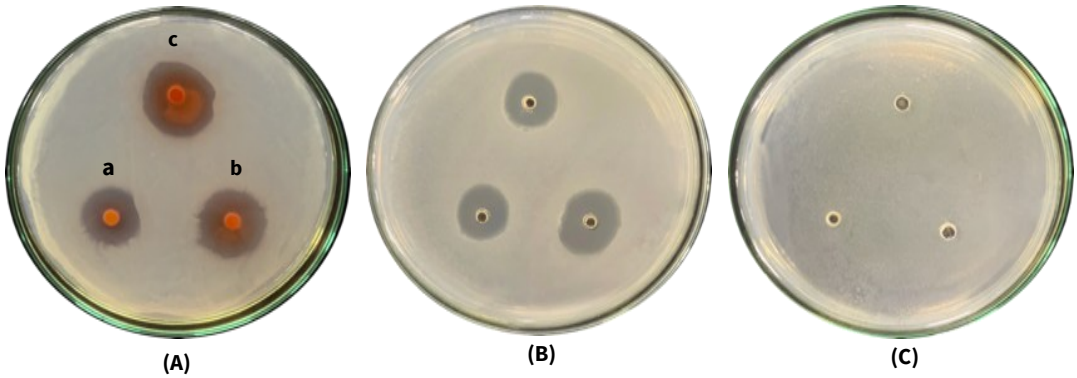

Fig. 1. Inhibition zones of *S. mutans* in the agar well diffusion assay. A. Sappan wood ethanolic extract at concentrations of (a) 1 %, (b) 3 % and (c) 5 %; B. Positive control, 0.2 % chlorhexidine; C. Negative control, 96 % ethanol.

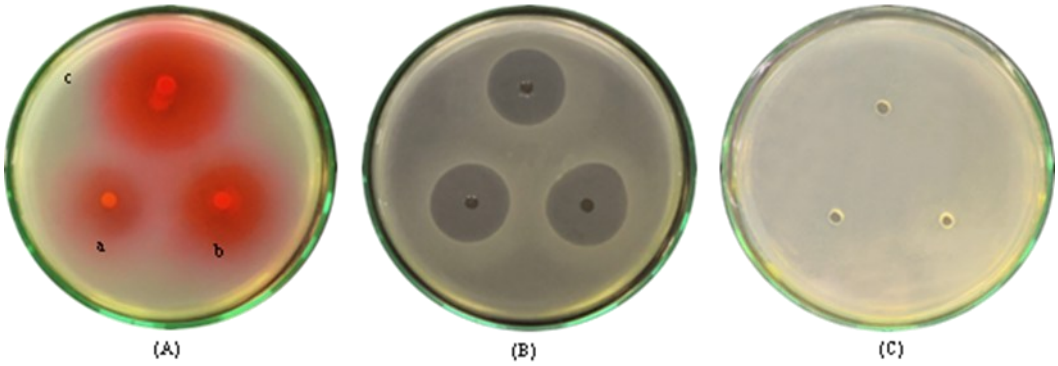

Fig. 2. Inhibition zones of *P. aeruginosa* in the agar well diffusion assay. A. Sappan wood ethanolic extract at concentrations of (a) 1 %, (b) 3 % and (c) 5 %; B. Positive control, 0.2 % chlorhexidine; C. Negative control, 96 % ethanol.

consistent with previous research that 96 % ethanol had no inhibitory effect on the growth of *S. mutans* and *P. aeruginosa* (32, 33).

The strength of the inhibition can be divided into four categories based on the diameter of the inhibition zone (34). These included weak, moderate, strong and very strong for diameters of 5 mm, 5–10 mm, 10–20 mm and 20 mm, respectively. Based on the category of inhibitory power, sappan wood ethanolic extracts at 1 % and 3 % concentrations, along with a positive control, had a strong inhibitory power against *S. mutans*. However, at 5 % concentration, the extract had a very strong inhibitory power against *S. mutans*. The results showed that the sappan wood ethanolic extract at 1 %, 3 % and 5 % concentrations and the positive control produced a strong inhibition zone diameter against *P. aeruginosa*. The results in Table 5 and Fig. 3 showed that the inhibition zone of the mouthwash containing 5 % sappan wood ethanolic extract against *S. mutans* was higher than that observed for lower extract concentrations, indicating a clear concentration-dependent antibacterial effect. Similarly, Fig. 4 demonstrates that the inhibition zone against *P. aeruginosa* increased progressively with increasing extract concentration. Based on the inhibition power category, mouthwash formulations containing 1 % and 3 % extract exhibited strong antibacterial activity against *S. mutans*, while the 5 % extract formulation showed a very strong inhibitory effect. All extract-based mouthwash formulations demonstrated strong inhibition against *P. aeruginosa*.

Table 5. Zone of inhibition of mouthwash from sappan wood ethanolic extract

Sample	Zone of inhibition (mm)	
	S. mutans	***P. aeruginosa***
Mouthwash with 1 % sappan wood extract	15.30 ± 0.30^{d}	10.70 ± 0.30^{c}
Mouthwash with 3 % sappan wood extract	17.20 ± 0.30^{c}	12.80 ± 0.30^{b}
Mouthwash with 5 % sappan wood extract	21.50 ± 0.50^{a}	15.20 ± 0.30^{a}
Commercial mouthwash containing chlorhexidine 0.2 %	20.70 ± 0.30^{b}	10.00 ± 0.00^{d}
Mouthwash base	0	0

Note: Values followed by the same letter are not significantly different in the Duncan test. $^{*}p < 0.05$. Positive control: Commercial mouthwash containing 0.2 % chlorhexidine; Negative control: Mouthwash base.

In this study, the sappan wood ethanolic extract played an important role as an antibacterial agent in mouthwash preparations. This was supported by the absence of an inhibition zone in the mouthwash base without extract (negative control), confirming that the antibacterial activity originated solely from the sappan wood extract, while the base components served only as carriers that supported the stability of active compounds. The inhibition zone results also demonstrated that the ethanolic extract formulation exhibited comparable antibacterial potential to a commercial mouthwash containing 0.2 % chlorhexidine. Although this study demonstrated antibacterial activity of sappan wood extract, further

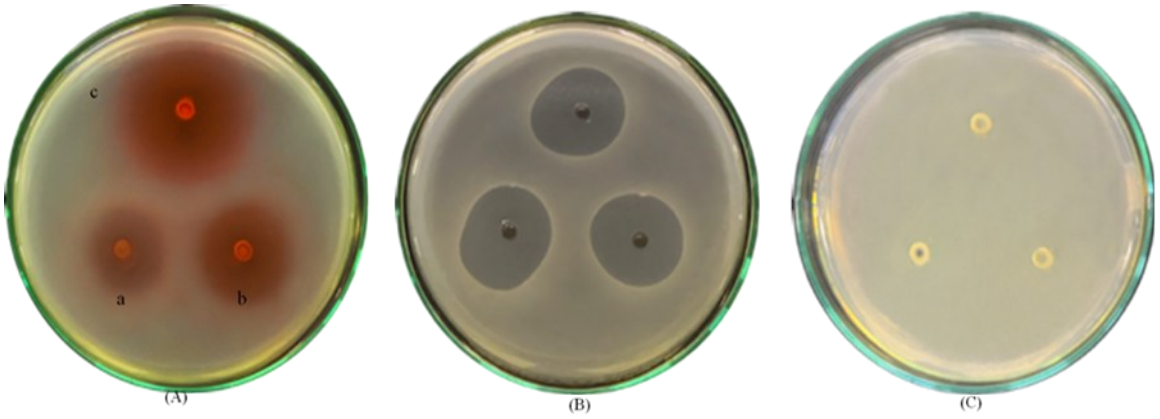

Fig. 3. Inhibition zones of *S. mutans* in the agar well diffusion assay. A. Mouthwash formulated with sappan wood ethanolic extract at concentrations of (a) 1 %, (b) 3 % and (c) 5 %; B. Positive control, commercial mouthwash containing 0.2 % chlorhexidine; C. Negative control, mouthwash base without extract.

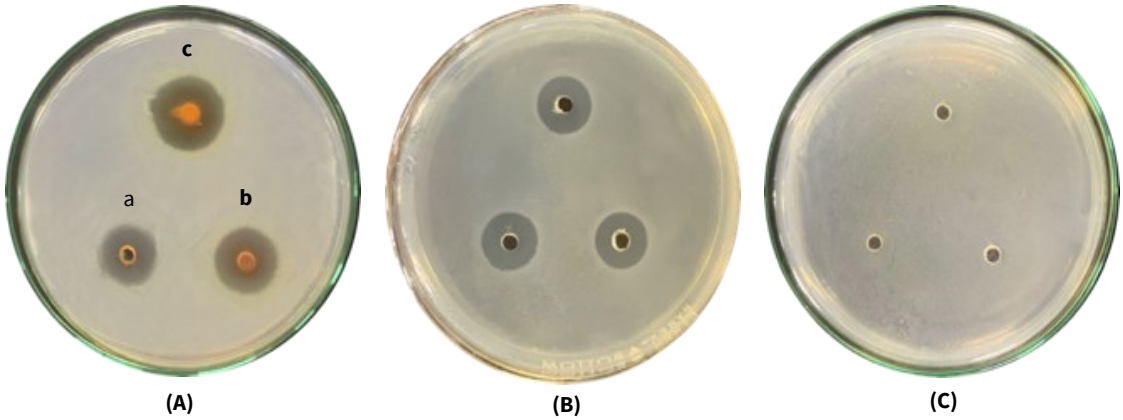

Fig. 4. Inhibition zones of *P. aeruginosa* in the agar well diffusion assay. A. Mouthwash formulated with sappan wood ethanolic extract at concentrations of (a) 1 %, (b) 3 % and (c) 5 %; B. Positive control, commercial mouthwash containing 0.2 % chlorhexidine; C. Negative control, mouthwash base without extract.

characterisation using HPLC-MS is recommended to identify the specific bioactive compounds responsible for the observed inhibition of pathogenic bacteria.

MIC of sappan wood ethanolic extract and mouthwash from sappan wood ethanolic extract

The results presented in Supplementary Table 1 and Fig. 5, showed that the samples of sappan wood ethanolic extract at 1 %, 3 % and 5 % concentrations, along with mouthwash formulations containing the same extract concentrations, exhibited no growth of *S. mutans* in the media. In contrast, bacterial growth of *P. aeruginosa* was still observed at the 1 % concentration. The results presented in Fig. 6 showed that *P. aeruginosa* growth was inhibited at 3 % and 5 % concentrations of sappan wood ethanolic extract and mouthwash. Based on these findings, the lowest concentrations of sappan wood ethanolic extract and mouthwash that inhibited the growth of *S. mutans* and *P. aeruginosa* were 1 % and 3 %, respectively. The MIC results of the positive controls, 0.2 % chlorhexidine and commercial mouthwash containing 0.2 % chlorhexidine, showed no bacterial growth against *S. mutans* and *P. aeruginosa*, whereas the negative control (bacterial suspension without extract) exhibited bacterial growth. The results showed that lower MIC values for *S. mutans* were similar to those obtained against the two types of pathogenic bacteria at a concentration of 1.56 %. Meanwhile, the MIC value for *P. aeruginosa* obtained was greater than previous studies (35). The difference in the MIC between the two bacteria was influenced by the nature of each bacterium due to differences in the structure of their cell walls.

Pseudomonas aeruginosa is a Gram-negative bacterium whose cell envelope consists of an outer membrane bound directly to the inner membrane. The outer membrane, composed of phospholipids and selective porin proteins, restricts the entry of large or hydrophobic molecules, making antibacterial substances less effective in penetrating the cell. In addition, *P. aeruginosa* possesses multiple efflux pump systems that actively expel antimicrobial agents from the periplasmic space, further reducing their intracellular concentration. These structural and functional characteristics contribute to the intrinsic resistance of *P. aeruginosa* compared to Gram-positive bacteria. Therefore, due to differences in cell wall composition and permeability, *P. aeruginosa* exhibited higher MIC values than *S. mutans* (36, 37).

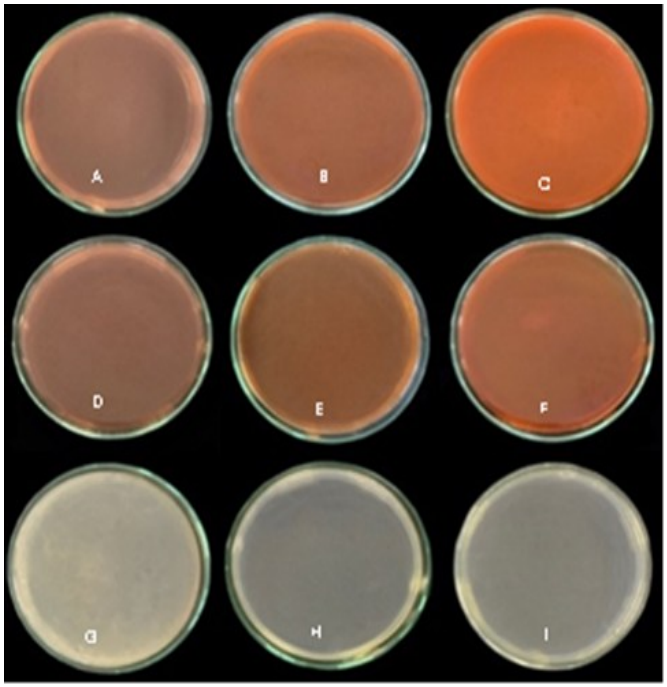

Fig. 5. MIC test of sappan wood ethanolic extract and mouthwash against *S. mutans*. Bacterial growth inhibition was observed after 24 hr of incubation at 37 °C. A–C. Sappan wood extract 1 %, 3 % and 5 %; D–F. mouthwash with 1 %, 3 % and 5 % sappan wood extract; G. *P. aeruginosa* suspension; H. Chlorhexidine 0.2 %; I. Mouthwash with chlorhexidine 0.2 %.

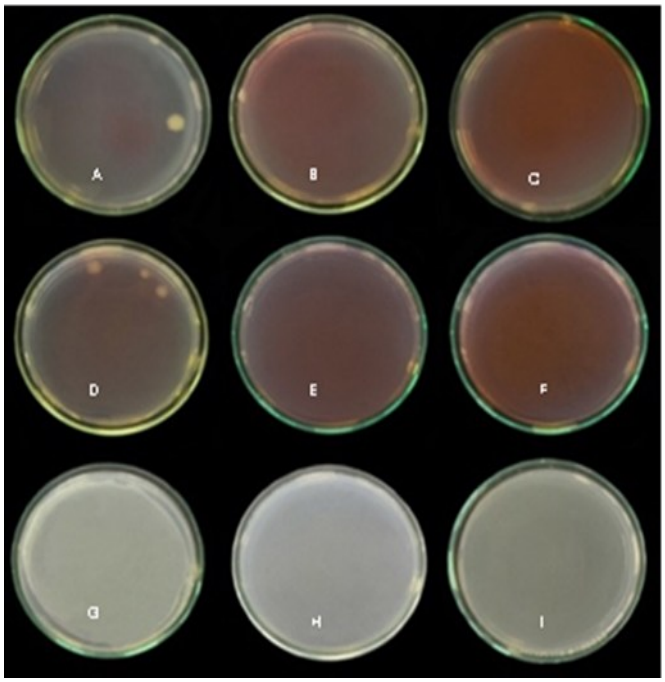

Fig. 6. MIC test of sappan wood ethanolic extract and mouthwash against *P. aeruginosa*. Bacterial growth inhibition was observed after 24 hr incubation at 37 °C. A–C. Sappan wood extract 1 %, 3 % and 5 %; D–F. Mouthwash with 1 %, 3 % and 5 % sappan wood extract; G. *P. aeruginosa* suspension; H. Chlorhexidine 0.2 %; I. Mouthwash with chlorhexidine 0.2 %.

Stability of mouthwash from sappan wood ethanolic extract

Mouthwash is an antiseptic oral preparation used to treat bad breath and clean oral cavity disorders such as gingivitis (38). The stability test determines the durability and characteristics of products when subjected to the limits set during storage (39). In this study, the stability test was carried out on the ethanol extract of sappan wood mouthwash, namely the cycling test for six cycles. Cycling tests were used to determine product stability during storage in the presence of temperature fluctuations (40). These tests observed several parameters, such as organoleptic parameters, homogeneity and pH values, including sedimentation volume and redispersion ability at room temperature. Organoleptic testing involved observing or visualising the physical properties of mouthwash preparations based on colour, shape, aroma and taste. Six cycles of organoleptic observations were performed and the results presented in Supplementary Table 2 showed that Cycle 0 represented the initial characteristics of the mouthwash prepared from sappan wood ethanolic extract. An organoleptic test on mouthwash preparations of sappan wood ethanolic extract at a concentration of 1 % (F_1), 3 % (F_2) and 5 % (F_3) showed that the preparation had a typical herbal scent and brown colour. There was also a slightly sweet taste in F_1, slightly bitter in F_2 and bitter in F_3. The F_1 preparation had a liquid solution form, while the F_2 and F_3 preparations formed a suspension, a liquid with a precipitate that forms when allowed to stand. Moreover, suspension is a liquid preparation with insoluble solid particles that can be dispersed in the liquid phase (41).

The organoleptic test results in Fig. 7 showed that mouthwash preparations of 1 % ethanolic extract of sappan wood (F_1) were consistent with the reference standard established (23). The mouthwash had a distinctive extract scent, brown colour, in the form of a solution and a slightly sweet taste. The taste parameters of the mouthwash preparations of 3 % (F_2) and 5 % (F_3) sappan wood ethanolic extract were significantly different, with both preparations obtaining a slightly bitter and bitter taste, respectively. This bitterness can be attributed to the active compound of sappan wood from the flavonoid compounds group, activated by the bitter taste receptors of the tongue (42). Therefore, the higher the concentration of the extract, the more bitter the taste produced. A mild bitterness at higher extract concentrations may affect taste acceptability and user compliance; hence, future formulation development should consider natural sweeteners or flavour-masking agents to improve palatability without compromising stability.

A homogeneity test was performed with parameters before and after shaking due to the presence of sediment in the F_2 and F_3 mouthwash preparations. This made it necessary to carry out shaking to determine whether the precipitate particles can be homogenised again after shaking. The mouthwash homogeneity after making was demonstrated in Cycle 0 and the results are presented in Supplementary Table 3. F_0 and F_1 mouthwash preparations had good homogeneity according to the reference standard (43). The F_2 and F_3 mouthwash preparations had sedimentation and were not homogeneous before shaking. However, both preparations produced homogeneous results after shaking because all particles were redispersed without any solidified sediment.

Research also examined the effects of sediments in F_2 and F_3 mouthwash preparations (40). The results showed that sediment formation occurred when the cosolvent present in the base became saturated, preventing complete dissolution of the mouthwash preparation base. The incomplete solubility was caused by a small amount of cosolvent or solubility-enhancing agent that cannot dissolve all of the solutes in the extract, resulting in sedimentation. The pH test was used to determine the degree of acidity in a mouthwash preparation. In this study, the test was performed using a pH meter calibrated in a buffer solution with values of 4 and 7. The pH of the mouthwash was repeated three times for each cycle, with a total of six cycles. Based on the results, cycle 0 displayed the initial pH of the mouthwash preparation when it was made. The pH test results in Fig. 8 showed that all treatments for mouthwash preparations had a pH value ranging from 4.5 to 10.5 according to SNI No. 12-3524-1995. The observed pH fluctuation across cycles was minimal (SD < 0.2), indicating consistent acidity and good formulation stability throughout the six-cycle storage test. Several commercial mouthwashes exhibited pH values below the critical threshold for enamel dissolution (pH < 5.5), which may promote enamel erosion and increase the risk of dental sensitivity, discolouration and caries with prolonged use (44).

The sedimentation test is carried out to determine the precipitation ratio that occurred during preparation and storage over a particular time. The deposition ratio will be used to calculate the sedimentation volume (F) value, which indicates the solubility of suspended particles in the carrier base (45). In this study, the sedimentation test parameters observed were the initial volume (Vo) of the mouthwash preparation that was filled first, followed by the volume of the supernatant formed after sedimentation (Vu). The value of Vu was observed every cycle at a contact Vo of 10 mL until the sixth cycle. The sedimentation parameter of the mouthwash was determined by measuring the F value, which was calculated at the end of each cycle by dividing Vu by Vo. The sedimentation test results in Supplementary Table 4 showed that F_0 and F did not have sediment, indicating a good sedimentation volume value, namely F = 1. These results were in accordance with the Indonesian pharmacopoeia 6th edition (46). Furthermore, F_2 and F_3 had an F-value that was approaching 1 during six cycles of observation. The variation of F values across cycles was minimal (SD < 0.03), confirming that the dispersion of suspended particles remained uniform and physically stable throughout storage. This indicated that the F value was in accordance with the Indonesian Pharmacopoeia 6th standards.

Based on the Indonesian Pharmacopoeia 6th standard, a good F value is F = 1 or close to 1. The F = 1 value indicates that the extract particles can be well dispersed on the carrier base, while the F value > 1 shows that the extract is not entirely dissolved in the base. Therefore, the volume of the sediment formed is greater than the volume of the supernatant. The redispersion test was carried out to determine the time required for the precipitate particles to be

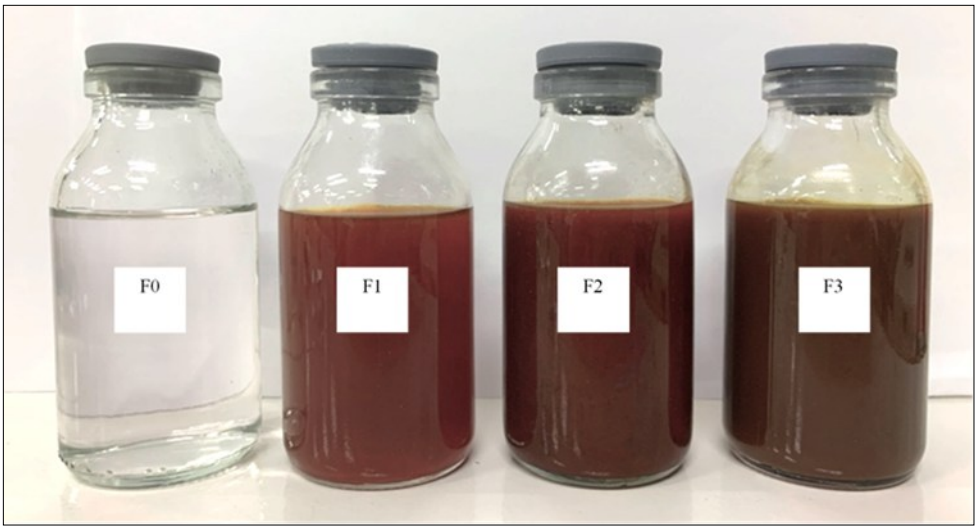

Fig. 7. Visual appearance of mouthwash formulations containing sappan wood ethanolic extract. F_0: mouthwash base (without extract), F_1: Formulation containing 1 % extract, F_2: Formulation containing 3 % extract and F_3: Formulation containing 5 % extract. The figure illustrates the colour and clarity differences among formulations observed after preparation.

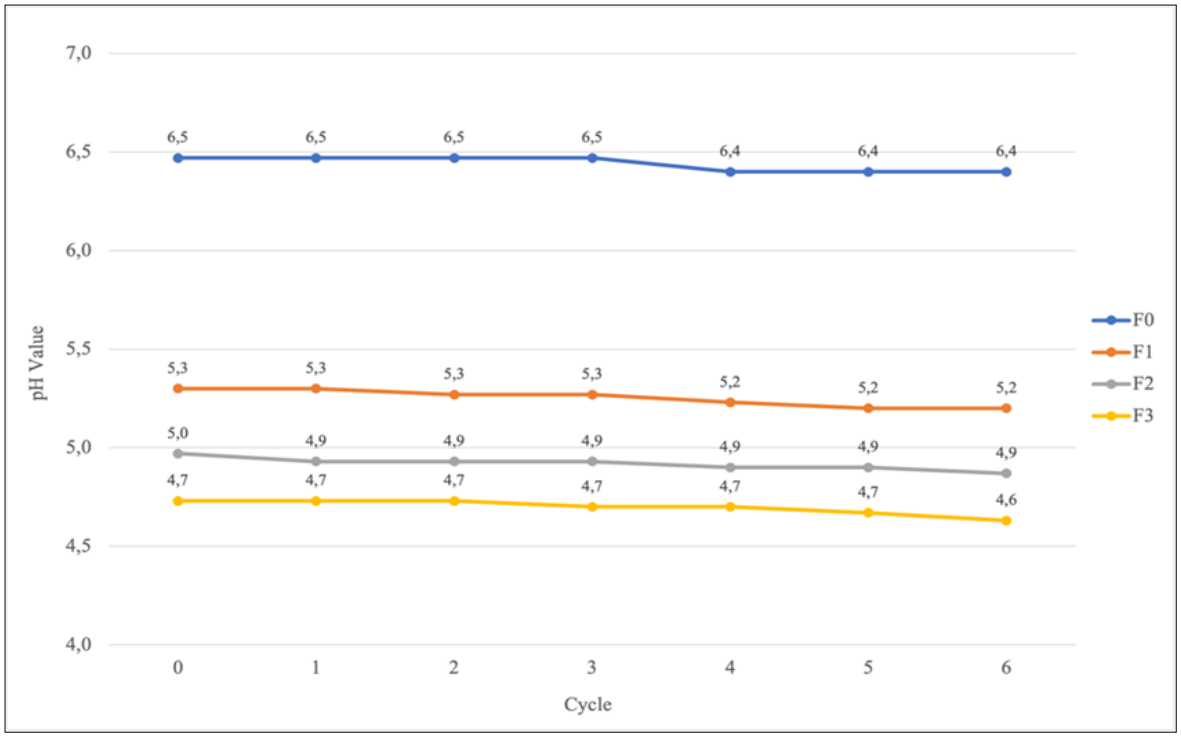

Fig. 8. pH observations of mouthwash formulations containing sappan wood ethanolic extract (F_0–F_3) over six storage cycles. F_0: mouthwash base (without extract), F_1: Formulation with 1 % extract, F_2: Formulation with 3 % extract and F_3: Formulation with 5 % extract. The figure shows the stability of pH values across six observation cycles.

redispersed after precipitation (41). In this study, the redispersion test was performed using the mouthwash that had been allowed to stand for one cycle and sedimentation was formed by shaking it and rotating the test tube 180 degrees. The time required for the sedimentation particles to be redispersed was calculated with a stopwatch. The dispersibility ability of the mouthwash preparation was observed at the end of each cycle throughout the six observation cycles. The redispersion test in Supplementary Table 5 revealed that all mouthwash preparations, including those that experienced sedimentation, namely F_2 and F_3, had redispersion times less than 30 sec. These results were in line with the standards set by the Indonesian Ministry of Health (46). Mouthwash preparations with a redispersion time of less than 30 sec indicated the ability of the precipitate particle to be well dispersed and not compacted (41).

Conclusion

The ethanolic extract of *C. sappan* contained 36.38 mg QE/g extract of total flavonoids, while the formulated mouthwash contained 88.65–93.22 mg QE/g extract. Both the extract and its mouthwash formulation exhibited strong antibacterial activity, with maximum inhibition zones of 23.30 mm and 16.70 mm against *S. mutans* and *P. aeruginosa*, respectively. The MIC values were 1 % for *S. mutans* and 3 % for *P. aeruginosa*. The mouthwash also showed good physical stability over six storage cycles, maintaining a pH of 4.6–6.5, homogeneous dispersion and no visible changes in colour, form, or odour. These results demonstrate the novelty and potential of *C. sappan* ethanolic extract as a natural antibacterial component in herbal mouthwash formulations, showing comparable efficacy to synthetic products. However, as this study was limited to *in vitro* evaluation, further *in vivo* and clinical investigations are required to confirm its safety, long-term stability and effectiveness under real-use conditions.

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Authors' contributions

MLMO designed and led the research, provided materials and tools, conducted statistical analysis and interpretation of the extraction, total flavonoid content, antibacterial activity and contributed to the writing process. EM designed and coordinated the research, supported statistical analysis, interpreted the findings on extraction, total flavonoid content, antibacterial activity and contributed to manuscript writing. SSW carried out the extraction procedures, analysed the results and contributed to discussions on total flavonoid content while assisting in the research and writing process. All authors reviewed and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors do not have any conflicts of interests to declare.

Ethical issues: None

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