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Antioxidant and antibacterial activity of sappan wood (*Caesalpinia sappan* L.) kombucha

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ABSTRACT

Sappan wood (*Caesalpinia sappan* Linn) is well known for its antioxidants, anti-inflammatories, and antibacterials. Unfortunately, this plant has not been used properly. In this study, sappan wood is processed into a kombucha drink through fermentation. Kombucha fermentation gives sappan wood drink a distinct and pleasant flavor while improving quality. The study aimed to increase the functional value of kombucha using sappan wood as raw material. Kombucha was prepared with sappan wood powder (16 mesh size) at various concentrations (0.4, 0.8, 1.2, 1.6, and 2% (w/v)) and 10% (w/v) sugar concentration. The effect of various sappan wood concentrations as a substrate for kombucha demonstrated a significant ($p<0.05$) reduction in pH and improvement in total phenol content, IC_{50} value of antioxidant activity, and antibacterial activity against *E. coli*. The best sappan wood kombucha treatment was obtained at a concentration of 1.2% with pH of 3.85, total phenol of 874.44 mg GAE/mL, total sugar of 6.62%, IC_{50} value of 85.43 ppm, antibacterial activity against *E. coli* of 8.57 mm, and antibacterial activity against *S. aureus* of 7.99 mm.

Introduction

Sappan (*Caesalpinia sappan* L.) is a medicinal plant known as secang wood in Indonesia. Sappan plants can grow widely in tropical countries, such as Indonesia, Brazil, China, Malaysia, Sri Lanka, etc. (Amar and Lev, 2017). The wood part of sappan plants is mostly used as a primary or additional ingredient in functional drinks (Al-Mahbub and Swasono, 2017). According to historical records, sappan wood is used as a traditional medicine to cure diarrhea, epilepsy, diabetes, heart disease, venereal diseases, obesity, and as an anti-inflammatory (Amar and Lev, 2017). Nirmal et al. (2015) summarized other benefits of sappan wood, including antioxidant that effectively counteracts free radicals, anti-acne drugs, vasorelaxation, hepatoprotective, hypoglycemic, and antibacterial activity.

Due to its potential, sappan wood requires additional processing to maximize use and diversify products, such as fermentation into kombucha. Kombucha is a fermented beverage made from tea and sugar with the addition of Symbiotic Culture of Bacteria and Yeast (SCOBY), which has a sour taste, slightly sweet, and has a carbonation effect because of the CO_2 content (Alcohol and Tobacco Tax and Trade Bureau, 2015; Kaashyap et al., 2021]. Various health benefits can be obtained when consuming kombucha regularly, including detoxifying the blood, lowering harmful cholesterol levels, lowering blood pressure, as an anti-inflammatory, improving liver function, etc. (Jayabalan et al., 2014).

Current research about kombucha is not only based on tea from *Camellia sinensis* plant as a substrate, but also using other alternatives. Sinamo et al. (2022), previously investigated the effect of sugar concentration and fermentation time on the physicochemical properties of sappan wood kombucha. The optimal sugar concentration of 20% and fermentation time for 10 days were obtained by measuring pH, total acid, total soluble solids, viscosity, and Lactic Acid Bacteria (LAB) color tests. However, this research did not mention the concentration of sappan wood used and numerous important analyses need to be carried out regarding the efficacy of kombucha and sappan wood on its own. Using sappan wood as a kombucha substrate aims to increase the functional

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value of kombucha drinks, particularly the antioxidant and antibacterial activities. The antioxidant index of sappan wood extract is higher than some commercial antioxidants, making it a potential free radical scavenger. Based on the results of the antioxidant effects comparison research with Peroxide value (POV) and Thiobarbituric Acid (TBA) methods, the antioxidative index of 200 ppm ethyl acetate fraction from sappan wood 75% ethanol extract in palm oils is higher than commercial antioxidants (BHA, α-Tocopherol) and almost the same as Butylated hydroxytoluene (BHT) (Lim et al., 1996). Sappan wood also contains many bioactive compounds that have antibacterial functions, especially homoisoflavonoids, phenols, flavonols, vitamins, minerals, and organic acids (Syamsunarno et al., 2021).

Therefore, further research needs to be done related to the best concentration of sappan wood for making kombucha, paying attention to its antioxidant, antibacterial activity, and chemical characteristics. To assess the effectiveness of kombucha fermentation, chemical characteristics, including pH, total phenolic content, and total sugar content, were analyzed.

Research and methods

Material

The materials needed for making kombucha were dried shaved sappan wood from a local market, black tea (as contro)l, kombucha starter (fermented for 14 days), refined sugar (Gulaku), and mineral water (Aqua). The chemicals used include distilled water, methanol P.A (Merck), DPPH reagent 0.2 mM (Sigma Aldrich) in methanol, anthrone reagent 0.1% (Merck) in concentrated H_2SO_4 (Merck), Na2Co3 7.5% (Merck), Folin-Ciocalteu 10% (Sigma Aldrich), $CaCO_3$ (Merck), antibacterial disc paper (Oxoid), Nutrient Broth (NB) (Merck), Nutrient Agar (NA) (Merck), isolates of bacteria *Staphylococcus aureus* and *Escherichia coli* from the laboratory of Universitas Brawijaya.

Kombucha starter making

1000 mL of water was brought to a boil over medium heat and 4 g of black tea (0.4% w/v) was added. After simmering for 5 minutes, 100 g of granulated sugar (10% w/v) was added and boiled further for 1 minute, stireed occasionally. Hot tea solution was poured into sterile jars and tightly closed. After it reaches 25ºC, 100 mL (10% v/v) of kombucha black tea starter was added and stirred until homogeneous. Next, the jars were covered with a sterile cloth, tied with rubber bands, and fermented for 14 days at room temperature in a closed place (not exposed to direct light). The method was modified from our previous study (Zubaidah et al., 2022).

Sappan wood kombucha fermentation

The sappan wood was grounded to a 16-mesh coarse powder using a hammer mill. Shaved sappan wood that does not pass through the sieve was processed further with a grinder machine. Then, weighed 2, 4, 6, 8, and 10 g (or 0.4%, 0.8%, 1.2%, 1.6%, and 2% (w/v)) and each simmer in 500 mL of mineral water using medium heat for 5 minutes. 50 g (10% w/v) of sugar was added and boiled further for 1 minute, stirred occasionally. Then, sappan wood solution was poured into sterile glass jars while hot and tightly closed. After the temperature reaches 25ºC, 50 mL (10% v/v) of kombucha starter was added and stirred until homogeneous. The jars are covered with a sterile cloth, tied with rubber bands, and then fermented for 12 days at room temperature (24-26ºC) in a closed place (not exposed to direct light). The same procedure was performed to make black tea kombucha as a control in this study, using 2 g of black tea (0.4% w/v). Analysis was conducted after adding kombucha starter on day 0 and on day 12 after fermentation. The method was modified from Nummer, (2013) and Kombucha Brewers International (2022).

pH measurement

The pH of kombucha was measured before fermentation (D-0) and after fermentation (D-12) using a pH/ORP meter (Edge dedicated pH, HI2002-02).

Total phenolic content assay

The Folin-Ciocalteau method (modified from Singleton and Rossi, 1965) was used to measure total phenolic content. 1 mL of the sample was weighed and diluted in distilled water using 10 mL measuring flask. Next, 0.5 mL of diluted samples were taken into a test tube, added 2.5 ml of 10% Folin-Ciocalteu reagent (v/v), homogenized using vortex, added 2 mL of 7.5% Na_2CO_3 solution (w/v), and mixed again with vortex. The sample was incubated in a closed room (not exposed to direct light) at room temperature for 30 minutes. Then, the sample absorbance was measured using a UV-Vis spectrophotometer at ʎ of 752.7 nm, with blanks sample (i.e., distilled water with reagents). The absorbance results were substituted into the standard curve equation for gallic acid and

calculated using the formula for total phenol with units of mg GAE/mL.

Total sugar content assay

The procedure of total sugar analysis used the Anthrone method with a standard solution of anhydrous glucose (modified method from Dreywood, 1946). A 10 g sample was dissolved in 100 mL of distilled water, added with 2 g of $CaCO_3$, homogenized, covered with aluminum foil, and heated in a water bath (95^{o}C for 30 minutes). The solution was filtered and centrifuged for 15 min at 3000 rpm. 1 mL of filtrate was diluted into 100 mL of distilled water, then 1 mL was taken into a test tube. 5 mL of 0.1% Anthrone reagent (in concentrated H_2SO_4 (w/v)) was added, covered with aluminum foil, vortex, heated (95^{o}C for 30 min), and the absorbance was measured at ʎ of 630 nm. The absorbance result was substituted into the standard curve equation and calculated using the total sugar formula.

Antioxidant activity assay

DPPH antioxidant activity assay was conducted to analyze the increase in antioxidant activity of sappan wood kombucha (modified method from Molyneux, 2004). A sample of 1 mL was dissolved with methanol P.A using a 10 mL measuring flask. Stock solutions were diluted to 60 ppm, 70 ppm, 80 ppm, 90 ppm, and 100 ppm. 2 mL of sample and methanol P.A was taken into a test tube and added with 2 mL of 0.2 mM DPPH. The sample solution was incubated at a room temperature for 30 minutes in a dark place and then measured the absorbance at ʎ of 517 nm. The antioxidant activity and the IC50 value were calculated using a standard curve..

Antibacterial activity assay

The disc diffusion method was performed to obtain the inhibitory zone against *E. coli* and *S. aureus* (modification method from Bauer et al., 1966). First, *E. coli* and *S. aureus* bacteria cultures were prepared in NB media and incubated for 24 hours at 37^{o}C. Next, 100 µL of bacterial culture was taken and inoculated on a petri dish containing sterile NA media using the spread plate technique. Sterile disc paper dripped with 20 µL of samples (conditions were dry for 30 minutes) was attached to the surface of each isolate of *E. coli* and *S. aureus* on NA media and allowed to dry under aseptic conditions. Then, it was incubated for 12 hours at 37^{o}C and the inhibitory zone was measured in mm.

Statistical analysis

The data was statistically analyzed using the analysis of variance (ANOVA) method and Fisher test with a 95% confidence level using the Minitab-19 software. The best treatment was determined using the Multiple Criteria Decision Making (MCDM) method and the Simple Additive Weighting (SAW) technique (Anvari et al., 2018). The assessment parameters were defined as criteria, and their weights were determined based on interest (decrease in pH = 3, increase in total phenol = 4, total sugar content = 2, antioxidant activity = 5, antibacterial activity against *E. coli* = 5, antibacterial activity against *S. aureus* = 5). Next, the performance rating value was calculated by normalizing each alternative value in each attribute (treatment) and then calculating the preference weight value. The results of the preference calculation were ranked to obtain the best treatment.

Results and Discussion

Chemical characteristic of sappan wood kombucha before fermentation (D-0)

Based on Table 1, the statistical analysis resulted in no significant difference ($p<0.05$) in the initial pH of kombucha from any variation in sappan wood concentration. The initial pH of kombucha depends on the pH of the starter used and the type of raw material (Suhardini and Zubaidah, 2016). Since sappan wood has a neutral pH, the initial pH of sappan wood kombucha results from adding a starter instead of a variation in sappan wood concentration (Ulma et al., 2018).

The total phenolic content of sappan wood kombucha before fermentation ranges from 359.94 to 1097.90 mg GAE/mL, with the lowest total phenolic content was found at a concentration of 0.4% and the highest total phenolic content at a concentration of 2%. Statistically, there was a significant difference between the concentrations of sappan wood, indicating that increasing concentration of sappan wood was parallel to a significant increase in total phenolic content.

Table 1 shows that the total sugar content of sappan wood kombucha before fermentation ranges from 8.27 to 9.22%. The ANOVA test results showed no significant difference in total sugar content due to variation in the concentrations of sappan wood. Sappan wood is a plant stem component composed of cellulose, hemicellulose, and lignin (Bechtold et al., 2023), and cannot be used as a sweetener.

Table 1. Average pH, total phenol, and total sugar of kombucha with variations in the concentration of sappan wood during fermentation

Sappan Wood Concentration (%)	pH			Total Phenolic Content (mg GAE/mL)			Total Sugar Content (%)		
	D-0	D+12	Change	D-0	D+12	Change	D-0	D+12	Change
0.4	4.26 ± 0.62	3.89 ± 0.45	-0.37 ± 0.17^{b}	359.94 ± 18.82^{e}	373.19 ± 18.51^{e}	13.24 ± 6.24^{b}	8.42 ± 2.06	6.37 ± 0.52	-2.05 ± 2.14
0.8	4.28 ± 0.66	3.91 ± 0.43	-0.37 ± 0.23^{b}	554.65 ± 18.76^{d}	626.22 ± 47.51^{d}	71.57 ± 34.38^{a}	9.22 ± 2.07	7.09 ± 0.73	-2.14 ± 2.30
1.2	4.35 ± 0.60	3.85 ± 0.37	-0.50 ± 0.23^{a}	779.51 ± 18.51^{c}	874.44 ± 39.92^{c}	94.93 ± 28.82^{a}	8.77 ± 1.69	6.62 ± 0.91	-2.15 ± 2.36
1.6	4.30 ± 0.58	3.80 ± 0.38	-0.51 ± 0.21^{a}	1011.62 ± 56.13^{b}	1091.96 ± 33.51^{b}	80.35 ± 26.19^{a}	8.73 ± 1.79	7.55 ± 1.82	-1.18 ± 0.31
2	4.34 ± 0.64	3.82 ± 0.36	-0.52 ± 0.27^{a}	1097.90 ± 7.60^{a}	1178.32 ± 22.08^{a}	80.42 ± 14.50^{a}	8.27 ± 0.37	6.75 ± 0.70	-1.52 ± 1.04

Note: The data was the average of three replications± SD. Numbers followed by different letters showed a significant difference in Fisher's test (95% confidence).

pH and total sugar content of sappan wood kombucha after fermentation

The average pH value and total sugar content of sappan wood kombucha decreased after 12 days of fermentation (Table 1).

On day 0, the pH value of sappan wood kombucha ranged from 4.26-4.35 and decreased after fermentation for 12 days to 3.80-3.91. The decrease in pH was caused by bacterial and yeast metabolism, which transforms the existing substrate into different kinds of organic acids (Kaczmarczyk and Lochyński, 2014). The organic acids formed include gluconic acid, lactic acid, acetic acid, malic acid, tartaric acid, malonic acid, citric acid, and oxalic acid (Jayabalan and Waisundara, 2019). The presence of vitamin B-complex, including niacin 39.9 mg/g, thiamin 9.3 mg/g, and riboflavin 8.3 mg/g (Senthilkumar, 2011), in sappan wood substrate can also support the kombucha microorganisms' metabolism during fermentation as co-enzyme to catalyze the organic acids formation (Nirmal, 2015).

The formation of organic acids will increase the level of free hydrogen ions in kombucha, causing in a decrease in pH ranged from 2.0 to 4.0 (Laureys et al., 2020). A further decrease in pH (<2.0) will be inhibited by the presence and accumulation of CO_2 and amphiprotic hydrocarbonate anion HCO_3 resulting from fermentation. Both of these compounds will react with hydrogen ions in organic acids and act as buffers to prevent further pH drops (Ayed et al., 2017).

The decrease in pH value increases with the high concentration of sappan wood. This is because sappan wood contains micronutrients in the form of ascorbic acid (0.987 mg/g) and gallic acid (0.519 mg/g) (Mekala and Radha, 2016). The presence of these micronutrients may contribute to decrease the pH of kombucha due to its acidic properties. Moreover, organic acids accumulated in kombucha can lower the pH. Previous research confirmed that the higher the organic acid content, the lower the pH of kombucha (Sinir et al., 2019). In addition, a low pH (2.0-4.0) is required in the final kombucha product to prevent unwanted mold contamination and maintain the stability of phenolic compounds and vitamins (Laureys et al., 2020).

Table 1 also shows that the initial total sugar content of kombucha before fermentation ranging from 8.27 to 9.2%. However, after 12 days of fermentation, the total sugar content of sappan wood kombucha decreased to 6.37-7.55%, giving change values ranging from 1.18 to 2.15%.

Ater fermentation, total sugar content decreased due to kombucha microorganism's metabolism. During fermentation, yeast and Gluconobacter bacteria in SCOBY metabolized sucrose in the medium into monosaccharide sugars (i.e., glucose and fructose) (Gaggia et al., 2019). Some monosaccharide sugars will be further broken down by other yeasts, Acetic Acid Bacteria (AAB), and LAB into ethanol, glycerol, CO_2, organic acids, vitamins, etc. (Jayabalan et al., 2014). AAB will break down some of these metabolites into cellulose strings, which will bind to each other to form macrofibrils and produce cellulose disks similar to nata (Emiljanowicz and Malinowska-Pańczyk, 2019). The conversion of carbohydrate sugar compounds into carbohydrate non-sugar components decreases the total sugar content of kombucha compared to before fermentation (Yasni, 2013).

The ANOVA test results indicate that variations in sappan wood concentration have no

significant difference in total sugar content of kombucha. The total sugar content in sappan wood kombucha differs because the metabolism of the sugar substrate varies depending on the fermentation conditions of bacteria and yeasts (Ulma et al., 2018). Different substrates used in making kombucha affect microbial growth and metabolisms during fermentation (Kaewkod et al., 2019). In sappan wood kombucha, the higher concentration of sappan wood does not have a significant effect on the total sugar kombucha because it does not contribute many carbohydrates that can be metabolized by kombucha microbes. Previous study reported that sappan wood contains a small amount of carbohydrates (i.e.., in the form of cellulose, hemicellulose, and lignin), which can only be digested by certain microbes (Mu'nisa et al., 2017).

Total phenol content of sappan wood kombucha after fermentation

An increase in the total phenolic content occurred in all treatments after fermentation. Based on Table 1, the total phenol content of kombucha with variations of sappan wood concentration on day 0 ranged from 359.94-1097.90 mg GAE/mL. After fermentation for 12 days, the total phenolic content increased to 373.19-1178.32 mg GAE/mL. The increase in total phenolic content is due to the enzymes of kombucha microorganisms degrading complex polyphenolic compounds into several simpler phenolic compounds (Ettayebi et al., 2003; Senthilkumar et al., 2011). Kombucha microbes also produce amylase and cellulase enzymes that can break down the bonds between phenol and material tissue structures by oxidation reactions (Zhao et al., 2020).

The statistical result showed a significant difference between the concentrations of sappan wood kombucha before and after fermentation. On day 0 of fermentation, the highest and lowest total phenol levels were found at a concentration of 2% (1097.90 mg GAE/mL) and 0.4% (359.94 mg GAE/mL), respectively. Based on the result of day-12 fermentation, the higher the concentration of sappan wood will result in higher phenolic content. Similarly, the total phenolic content in kombucha was influenced by the type and concentration of the substrate (Zubaidah et al., 2022). Sappan wood contains 150 mg/g of phenolic compounds, depending on the wood part (Senthilkumar et al., 2011). Some of identified compounds include Caesalpiniaphenol A-F, Caesalpiniaphenol G-H, Epicaesalpin J, 7,10,11-Trihydroxydraca-enone, and brazilin (Mu'nisa et al., 2017; Al-Mahbub and Swasono, 2017). Meanwhile, the type of phenolic compounds in sappan wood kombucha still requires further analysis based on microbes' metabolisms, and there is a possibility that fermentation will create new phenolic compounds. As the concentration of sappan wood increases, the total phenol content in kombucha increases, potentially leading to an increase in the antimicrobial level. Sappan wood contains various antimicrobial compounds dominated by brazilin (Nirmal et al., 2015). The presence of antimicrobial compounds can inhibit SCOBY metabolism by breaking down polyphenolic compounds during kombucha fermentation.

Antioxidant activity of sappan wood kombucha

Based on Table 2, variations in the sappan wood concentration significantly affect the IC50 value ($p>0.05$). The antioxidant activity of sappan wood kombucha on day 12 was lower than that of on day 0. The IC50 value indicates the concentration required to stabilize DPPH by 50%. A decrease in IC50 value after 12 days of fermentation shows that fermentation can increase the antioxidant activity of sappan wood kombucha. This can occur due to microbial metabolism, which increases the bioactive compounds in kombucha. Some bioactive compounds have the ability as antioxidant activity and can affect the structure of the components of antioxidant compounds. This aligns with a previous study stating that the antioxidant activity of kombucha is influenced by its content, such as phenolic compounds, organic acids, vitamins, and minerals (Chen and Liu, 2000).

Table 2. Antioxidant Activity (IC50) of Kombucha with Variation in Sappan Wood Concentration During Fermentation

Sappan Wood Concentration (%)	Antioxidant Activity (IC50) (ppm)				Change (ppm)
	D-0	Category	D+12	Category	
0.4	143.76 ± 9.01^{a}	Medium	126.88 ± 4.78^{a}	Medium	-16.88a
0.8	112.07 ± 0.20^{b}	Medium	97.47 ± 0.43^{b}	Strong	-14.61a
1.2	103.30 ± 3.10^{c}	Medium	85.43 ± 3.55^{c}	Strong	-17.87a
1.6	83.55 ± 2.62^{d}	Strong	75.87 ± 1.00^{d}	Strong	-7.68b
2	77.60 ± 1.93^{d}	Strong	70.58 ± 3.28^{d}	Strong	-7.02b

Note: The data was the average result of three replications ± SD. Numbers followed by different letters showed a significant difference in Fisher's test (95% confidence).

The antioxidant activity of sappan wood kombucha increased with the increasing concentration of sappan wood. This is influenced by the content of compounds in sappan wood that act as antioxidants. It was discovered that sappan wood contains phenolic and flavonoid compounds, which function as antioxidants (Yodha et al., 2021). Phenolic compounds in sappan wood include tannins (Kimestri et al., 2018) and Caesalpiniaphenol (Cuong et al., 2012). While flavonoid compounds in sappan wood include natural dyes, namely brazilin, brazilein, and 3'-0-methylbrazilin (Dapson and Bain, 2015; Settharaksa et al., 2019), and sappanone (Zhao et al., 2020). Increasing the concentration of sappan wood is correlated with an increase in organic compounds, hence increasing the antioxidant activity of the kombucha. The fermentation process allows the synthesis of new compounds from the microbe's metabolism. Diverse compounds have been discovered by GC-MS analysis, including organic acids, phenolic acids, alcohols, and sugars (Villarreal-Soto et al., 2019).

Antibacterial activity of sappan wood kombucha

a. *Antibacterial activity against Escherichia coli*

The antibacterial activity of kombucha with variations in the sappan wood concentration against Gram-negative *E. coli* bacteria increased after 12 days of fermentation (Figure 1). Table 3 shows the diameter of the inhibitory zone on day 0 of fermentation, which ranged from 7.72 to 8.41 mm. While on day 12, the diameter of the inhibitory zone expanded from 8.12 to 9.27 mm. The fermentation can increase the antibacterial activity of kombucha. The formation of organic acids during fermentation by kombucha bacteria will lower the pH and make kombucha acidic. Acidic conditions can inhibit and prevent the growth of pathogenic bacteria (Ayed et al., 2017). As shown in Table 1, the pH of all samples decreased after fermentation. In addition, kombucha fermentation may increase ethanol yielded from fructose metabolism of by yeast and total phenol content (Table 1). Such happened through the breakdown of complex polyphenolic compounds and the formation of new phenolic compounds by kombucha microbes (Senthilkumar et al., 2011).

The statistical analysis of the inhibitory zone diameter after fermentation (D+12) against *E. coli* showed a significant difference in several concentrations of sappan wood kombucha. The largest diameter of the inhibitory zone was found at a 1.6% sappan wood kombucha of 9.27 mm, and the smallest diameter was at a concentration of 0.4% and 0.8% of 8.12 mm and 8.38 mm, respectively. This finding confirmed that increasing the concentration of sappan wood enhances the breakdown of compounds by microbes, thus increasing the total phenolic content and organic acids which have potential as antimicrobials.

Table 3 shows that a significant difference in the inhibitory zone was found in all treatments. The lowest inhibitory zone was observed at concentrations of 0.4% (0.40 mm), 0.8% (0.51 mm), and 1.2% (0.57 mm), while the highest change at concentrations of 1.6% (0.86 mm) and 2% (0.91 mm). Sappan wood contains various bioactive compounds that can act as antibacterial, especially from the homoisoflavonoids, phenols, flavonols, vitamins, minerals, and organic acids (Syamsunarno et al., 2021). These compounds act as antibacterial by disrupting cell membrane permeability, damaging cell walls, inhibiting nucleic acid and protein synthesis, and inhibiting metabolic enzymes (Ulma et al., 2018). Putri and Kiki (2018) added that the presence of hydroxyl groups (-OH) and aldehydes (-CHO) in these compounds can react with microbial proteins and enzymes, leading to protein lysis and enzyme deactivation.

Table 3. Antibacterial Activity of Kombucha with Variation in Sappan Wood Concentration during Fermentation against *E. coli* and *S. aureus* at 12 Hour Observation

Sappan Wood Concentration (%)	Antibacterial Activity against *E. coli* (mm)			Antibacterial Activity against *S. aureus* (mm)		
	D-0	D+12	Change	D-0	D+12	Change
0.4	7.72 ± 0.80	8.12 ± 0.75^{c}	0.40 ± 0.07^{b}	7.12 ± 1.10	7.80 ± 0.75	0.68 ± 0.83
0.8	7.87 ± 0.55	8.38 ± 0.57^{c}	0.51 ± 0.08^{b}	7.48 ± 0.40	8.10 ± 0.72	0.62 ± 0.45
1.2	8.00 ± 0.61	8.57 ± 0.44^{bc}	0.57 ± 1.19^{b}	7.33 ± 0.27	7.99 ± 0.62	0.66 ± 0.37
1.6	8.41 ± 1.35	9.27 ± 1.13^{a}	0.86 ± 0.34^{a}	7.30 ± 0.23	7.82 ± 0.56	0.52 ± 0.43
2	8.29 ± 1.19	9.20 ± 0.87^{ab}	0.91 ± 0.33^{a}	7.44 ± 0.38	8.10 ± 0.32	0.66 ± 0.20

Note: The data was the average result of three replications ± SD. Numbers followed by different letters showed a significant difference in Fisher's test (95% confidence).

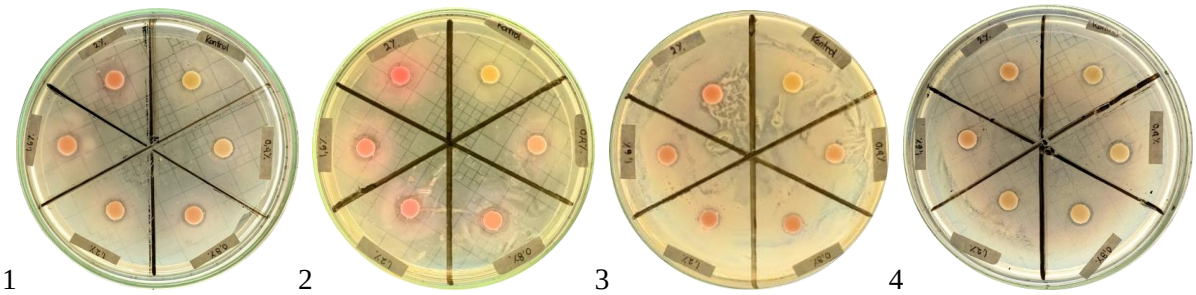

Figure 1. Antibacterial activity of kombucha with variation in sappan wood concentration before (left) and after (right) fermentation against *E. coli* and *S. aureus* at 12-hour observation. (Note: *E. coli* plates shown on the left (1, 2) and *S. aureus* shown on the right (3, 4))

Several studieson sappan wood's antibacterial activity shows a minimum inhibitory zone formed against Gram-negative bacteria (Bukke et al., 2015; Gaggia et al., 2019). However, this study obtained quite good antibacterial activity against *E. coli* species, which are Gram-negative bacteria, compared to *S. aureus* from Gram-positive. Gram-negative bacteria have a thinner cell wall structure of 5-10% peptidoglycan (Simpson, 2019). Therefore, the antibacterial compound in sappan wood kombucha can easily penetrate the cell wall of Gram-negative bacteria compared to Gram-positive bacteria, composed of a 90% peptidoglycan layer. Using sappan wood as a kombucha substrate allows the formation of new organic acid compounds and antibacterial compounds that are not found in pure extracts. Therefore, further in-depth research is needed.

b. *Antibacterial activity against staphylococcus aureus*

After 12 days of fermentation, sappan wood kombucha's antibacterial activity against *S. aureus* was increased (Figure 1). As shown in Table 3, on day 0, diameter of the inhibitory zones obtained were between 7.12 and 7.48 mm. On day 12, the diameter of the inhibitory zone formed ranged from 7.80-8.10 mm, giving the changes values ranged from 0.52-0.68 mm. The statistical analysis results also showed no significant difference ($p>0.05$). Fermentation can increase antibacterial activity due to organic substrate break down, causing a decrease in pH and an increase in total phenolic content, flavonoids, vitamins, and organic acids (Jayabalan et al., 2014). The acidic condition of the medium and the formation of bioactive compounds may act as antibacterial (Miranda et al., 2022).

However, compared to the existing literature, the antibacterial activity of sappan wood kombucha against *S. aureus* species is relatively low. Research by Hemthanon and Ungcharoenwiwat (2022) showed the antibacterial activity of 16.66% sappan wood extract in methanol and ethanol solvents against *S. aureus* bacteria ranging from 7.00-13.67 mm. This study used water solvents, leading to differences in the chemical compounds extracted. Therefore, some antibacterial compounds of sappan wood were not extracted during the boiling process, yet these compounds could inhibit the growth of *S. aureus*.

Determination of the best treatment

The criteria used in determining the best treatment were antioxidant activity and antibacterial activity against *E. coli* and *S. aureus* after fermentation (weight 5), change in total phenolic content (weight 4), change in pH (weight 3), and total sugar after fermentation (weight 2). Each criterion was weighted based on the purpose of this study. The alternatives involved were the concentration of sappan wood kombucha, which was 0.4, 0.8, 1.2, 1.6, and 2%.

A decrease in pH was chosen as a criterion because the data showed a significant difference between sappan wood kombucha concentrations. Meanwhile, the increase in total phenolic content was chosen as the determining criterion because at 0.8-2% of sappan wood, no significant difference was found. Therefore, increasing concentration did not effectively increase the total phenolic content in kombucha after fermentation.

Based on the preference calculation results in Figure 2, the best treatment of sappan wood kombucha was obtained at a concentration of 1.2%. Table 4 compares the chemical characteristics, antioxidant, and antibacterial properties of sappan wood kombucha with black tea kombucha. The paired t-test result showed no significant difference in all parameters except for total phenolic content.

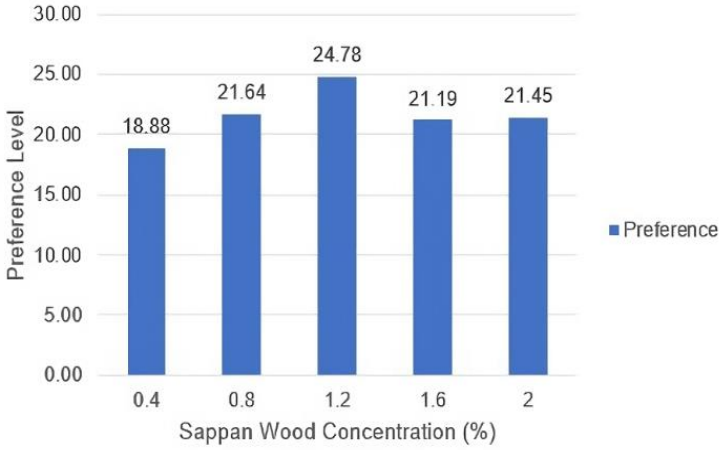

Figure 2. Preference level of sappan wood kombucha based on chemical characteristics and antibacterial activity

Table 4. Comparison of the best treatment of sappan wood kombucha with black tea kombucha based on chemical characteristics and antibacterial activity

Parameter	Sappan Wood Kombucha 1.2%	Black Tea Kombucha 0.4%
Antibacterial Activity against *E. coli* (mm)	8.57 ± 0.44	8.87 ± 0.70
Antibacterial Activity against *S. aureus* (mm)	7.99 ± 0.62	7.73 ± 0.60
Antioxidant Activity (IC50) (ppm)	85.43 ± 3.55	90.39± 7.77
Total Phenolic Content (mg GAE/mL)	874.44 ± 39.92	524.55 ± 3.46
pH	3.85 ± 0.37	3.88 ± 0.44
Total Sugar Content (%)	6.62 ± 0.91	6.50 ± 0.39

Conclusions

The use of sappan wood in various concentrations in making kombucha has an effect on decreasing pH, increasing total phenol, as well as increasing antioxidant activity and antibacterial activity against *E. coli*. Kombucha with a sappan wood concentration of 1.2% (w/v) was selected as the best treatment with a pH of 3.85, total phenol of 874.44 mg GAE/ml, total sugar of 6.62%, antioxidant activity of 85.43 ppm which is categorized as strong antioxidant, antibacterial activity against *E. coli* of 8.57 mm and against *S. aureus* of 7.99 mm. The best treatment of sappan wood kombucha showed a significant difference from the best treatment of black tea kombucha with a concentration of 0.4% on the parameters of total phenolic content.

Declarations

Conflict of interests The authors declare no competing interests.

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