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Production of herbal kombucha with agarwood (*Aquilaria crassna* Pierre ex Lec.) leaves and its biological properties

Suwanposri, A.[1], Khamphinit, W.[1], Sonthichai, P.[1], Maliyam, R., Charoensook, K.[2] and Boonna, S.[2*]

[1]Department of Plant Production and Landscape Technology, Faculty of Agro-industrial Technology, Rajamangala University of Technology Tawan-ok, Chanthaburi campus, Chanthaburi, Thailand; [2]Department of Food Innovation and Business, Faculty of Agro-industrial Technology, Rajamangala University of Technology Tawan-ok, Chanthaburi campus, Chanthaburi, Thailand.

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Abstract Herbal kombucha from roselle (*Hibiscus sabdariffa* L.) and peppermint (Mentha × piperita) mixed with agarwood leaves (*Aquilaria crassna* Pierre ex Lec.) was produced and its biological activity was analyzed. After 14 days of static fermentation, it was found that all kombucha formulations had acetic acid levels ranging from 0.28±0.01% to 0.41±0.00%, alcohol levels from 1.07±0.15% to 1.32±0.08%, and total phenolic content from 3,577.78±96.86 to 8,066.67±905.13 µg/mL. DPPH scavenging activity ranged from 56.02±0.11% to 84.91±0.84%. The population of acetic acid bacteria, lactic acid bacteria, and yeast ranged from 7.75±0.05 to 7.97±0.01 log CFU/mL, 7.63±0.07 to 7.98±0.02 log CFU/mL, and 7.59±0.04 to 7.89±0.017 log CFU/mL, respectively, which meet the standards of the Food and Drug Administration. Kombucha made from roselle and roselle mixed with agarwood leaves at concentrations of 0.01% and 0.05% inhibited *Staphylococcus aureus* and *Escherichia coli*. Additionally, sensory evaluation scores for overall acceptability were in the range of like slightly to like moderately. It indicated that the production of herbal kombucha with agarwood leaves couble be suitable for commercial development.

Keywords: Agarwood leaves, Biological properties, Herbal, Kombucha tea

Introduction

Due to the Covid-19 pandemic, along with societal, cultural, and economic changes, as well as advancements in technology, people are increasingly focusing on health, particularly in regard to the food they consume. The International Food Information Council (IFIC) has gathered data from teams of experts in food, nutrition, and health, combined with insights from surveys of U.S. consumers, and found that food trends in 2024 are leaning towards items

[*]**Corresponding Author:** Boonna, S.; **Email:** sureeporn_bo@rmutto.ac.th

that help balance the body and provide refreshment. IFIC predicts that beverages that aid in balancing and refreshing the body will continue to play a significant role, particularly those with prebiotics and probiotics for gut health (The International Food Information Council, 2024), for examples fermented drinks like kombucha, which are popular among consumers concerned with gut health. Reports indicate that kombucha is experiencing rapid growth in the functional beverage market and is one of the most popular low-alcohol fermented drinks worldwide (Troitino, 2017; Baschali, 2017). In 2023, the global market value of kombucha was $2.0 billion, and it is expected to grow from $2.4 billion in 2024 to $5.9 billion by 2029, with a compound annual growth rate (CAGR) of 19.4% (MarketsandMarkets, 2024).

Kombucha, also known as Cha-mug in Thailand, is a fermented beverage with a tangy flavor and fizzy. It is made by fermenting tea, sugar, and symbiotic culture of bacteria and yeast (SCOBY) together for at least 7-14 days. The result is a beverage with an acidic effect, primarily composed of acetic acid, with a slightly fizzy and a mild alcoholic smell (less than 0.5%). The SCOBY consists of three main groups of microorganisms that live in a symbiotic relationship, including acetic acid bacteria (*Acetobacter*, *Gluconacetobacter*, and *Komagataibacter*) (Sutthiphatkul *et al.,* 2023). They produce acetic acid and cellulose film on the top of fermentation liquid. The second microorganism is yeasts (*Saccharomyces*, *Zygosaccharomyces*, *Pichia*, *Dekkera*, and *Brettanomyces* (Greenwalt *et al.,* 2000), which adhere to the bottom of the cellulose film. The yeasts produce carbondioxide and ethanol which promotes acetic acid production by acetic acid bacteria (Liu *et al.,* 1996). Lastly of microorganism is lactic acid bacteria including *Lactobacillus negelii* and *Oenococcus oeni* (Ludwig *et al.,* 2009). After the fermentation process, kombucha contains various beneficial compounds such as tea polyphenols, organic acids (acetic acid, glucuronic acid, lactic acid, and oxalic acid), fiber, amino acids (lysine), trace elements (Cu, Fe, Mn, Ni, and Zn), vitamins (C and B), hydrolytic enzymes, and antibiotic substances (Bortolomedi *et al.,* 2022). These compounds come from the raw materials and the interaction between bacteria and yeasts during fermentation. Additionally, kombucha contains probiotic microorganisms, making it a functional food with health benefits including anti-inflammatory effects, cancer spread reduction, antioxidant properties, cholesterol and blood pressure reduction, inhibition of pathogenic microorganisms, diabetes prevention, cardiovascular and neurodegenerative disease prevention, liver function improvement, immune system support, and digestive health enhancement (Sena *et al.,* 2020; Kitwetcharoen *et al.,* 2023).

Kombucha is typically produced using black tea and green tea, or sometimes oolong tea. Recently, fruits, coconut water, coffee, vegetables, and herbs have been incorporated into kombucha production (Anantachoke *et al.,* 2023) to enhance its properties. Using different ingredients to make kombucha results in variations in its physicochemical properties, functional properties, and sensory characteristics. Among the kombuchas gaining significant interest are those made with herbs, which are expected to improve the efficacy of the fermented beverage as a low-cost, easily accessible traditional medicine made by communities using locally available herbs.

Roselle (*Hibiscus sabdariffa* L.) is a plant with a red color and sour taste, belonging to the Malvaceae family, found in tropical and subtropical regions. Roselle contains bioactive compounds such as anthocyanins, flavonoids, organic acids, polysaccharides, and antioxidants (Da-Costa-Rocha *et al.,* 2014). It has been studied and used as an ingredient in various foods, beverages, and medicinal products (Monteiro *et al.,* 2017). Peppermint (*Mentha* × *piperita*) is an aromatic herb in the Lamiaceae family, found in the Mediterranean, Europe, Asia, and North America. Peppermint leaves contain flavonoids (eriocitrin, luteolin, and hesperidin), rosmarinic acid, and phenolic compounds, which give peppermint its antiviral, antimicrobial, antitumor, antioxidant, immune-boosting, and appetite-stimulating properties (McKay and Blumberg, 2006). Peppermint is used in food, confectionery, perfumes, health products, medicines, and cosmetics (Maslowski *et al.,* 2021). Agarwood (*Aquilaria crassna* Pierre ex Lec.) is a plant in the Thymelaeaceae family, commonly found in eastern Thailand. Extracts from agarwood leaves have antioxidant, antimicrobial, antidiabetic, anti-inflammatory, and laxative effects. The main compounds in agarwood leaves include phenolic compounds (iriflophenone and its glycosides), xanthonoids (mangiferin), and flavonoids (genkwanin) (Ito and Ito, 2022). Agarwood leaves are used in traditional medicine for treating fractures and bruises and are also made into tea (Zhou *et al.,* 2008; Adam *et al.,* 2017). Surjanto *et al.* (2019) studied the toxicity of agarwood leaf tea in albino rabbits and found no skin changes after 72 hours of exposure, indicating that agarwood leaf tea is safe for consumption and does not cause skin irritation. However, despite its safety and numerous benefits, agarwood leaf tea has a bitter taste, making it less popular in Thailand and limiting its widespread use. Therefore, exploring new products using agarwood leaves is of interest.

The study focused on the production of a new kombucha beverage using local herbs by incorporating roselle and peppermint into kombucha, and enhancing it with agarwood leaves at various concentrations. The physicochemical characteristics, total phenolic content, antioxidant activity, antimicrobial properties, and sensory evaluation were also determined to provide guidance on using other herbs as substitutes for tea leaves, diversifying the product, improving kombucha's properties, and utilizing local raw materials effectively.

Materials and methods

Sample, kombucha culture, pathogenic microorganisms and culture media

Agarwood leaves were obtained from Trat Province, and peppermint was sourced from farmers in Chanthaburi Province. The leaves were cleaned thoroughly, then cut into small pieces and dried at 50°C for 8 h. Dried roselle was purchased from a local market in Chanthaburi Province, Thailand. Oolong tea used was Three Horses brand, number 1. The commercial kombucha starter culture was purchased from Nature Kombucha shop.

Three pathogenic bacterial strains used in this study were *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus* were collected from the Department of Plant Production and Landscape Technology, Faculty of Agro-industrial Technology, Rajamangala University of Technology Tawan-ok, Chanthaburi campus. These strains were maintained on nutrient agar slants at 4°C.

Microbiological culture media including Glucose Yeast Extract Calcium Carbonate (GYC) agar, De Man Rogosa and Sharpe (MRS) agar, Yeast Extract Peptone Dextrose (YEPD) agar and Nutrient (NA) agar were obtained from Himedia (India). Folin-Ciocalteu reagent and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma (USA).

Preparation of kombucha starter culture

One liter of water was boiled until warm for 5 minutes, then 100 grams of sugar was added and stirred until dissolved. Next, five grams of oolong tea was immersed for 10 minutes and tea leaves were strained out. The mixer was poured into a sterilized jar and the jar was covered with cheesecloth. After, the mixer was cooled, 100 mL of kombucha culture with 2-3 cm piece of cellulose (SCOBY) was added. The jar was fermented at room temperature for 14 days and used as starter culture.

Production process of herbal kombucha with agarwood

The production process of herbal kombucha mixed with agarwood leaves was examined. The two variables namely, type of herbals (roselle and peppermint) and the amount of agarwood leaves (0, 0.01 and 0.05 g/L) were studied using a complete randomized factorial design (Factorial 2x3 in CRD), resulting in a total of 6 experimental treatments (Table 1).

Table 1. Experimental treatments for determining the production process of herbal kombucha with agarwood leaves

Formula number	Factor	
	Type of herbal (0.5 g/L)	**Amount of agarwood leaves (0.5 g/L)**
1	roselle	0.00
2	roselle	0.01
3	roselle	0.05
4	peppermint	0.00
5	peppermint	0.01
6	peppermint	0.05

The production of herbal kombucha with agarwood according to Table 1 was started by adding various herbs to 2 L of water. Then, dried agarwood leaves were added and adjusted the initial total dissolved solids (TSS) to 15°Brix using sugar cane. The mixer was boiled for 15 minutes followed by 0.5% oolong tea was added, immersed for 10 min, and then strained out the tea leaves. The filtered liquid was poured into a sterilized jar and the jar was covered with cheesecloth. After, the filtrated liquid was cooled, 10% of starter culture was added. The fermentation of herbal kombucha with agarwood leaves was performed at room temperature for 14 days. At the end of the fermentation process, the produced kombuchas were filtered through a pre-boiled cheesecloth. The physical properties, biological activity and sensory evaluation of the filtrated kombuchas were examined.

Analysis of pH, acetic acid, TSS and alcohol contents

pH value and were examined using a pH meter (ExStik PH100, Extech, USA.). For acetic acid content was determined using titration method by adding 2 mL of kombucha sample into a 250 mL Erlenmeyer flask with 100 mL of distilled water. One drop of phenolphthalein was added and the mixer was titrated with 0.1N sodium hydroxide standard solution until the endpoint was reached (the solution turned pink). Acetic acid content was calculated using as following equation:

$$\text{Acetic acid content} = \frac{(\text{N of NaOH } \times \text{volume of NaoH } \times \text{M.W. of acetic acid } \times 100}{\text{Volume of sample } \times 1{,}000}$$

The TSS and alcohol content were analysed using Brix reflectometer (MASTER-URC/NM, Atago, Japan) and Ebulliometer (No. 99283, DuJardin-Salleron, France), respectively.

Determination of total phenolic content

Total phenolic content (TPC) was determined using a method described by Wong *et al.* (2006) with a slightly modification. Briefly, 50 µL of kombucha sample was transferred into a test tube. The 2.0 mL of 10% Folin-Ciocalteu reagent was added, following by 2.0 mL of 7% sodium carbonate solution, and 2.0 mL of distilled water. The mixer solution was kept in the dark for an hour. The absorbance was measured at 765 nm using a spectrophotometer (Lambda 365, Perkin Elmer, USA) and compared with a standard curve of gallic acid solution. The TPC result was reported as µg gallic acid equivalent per mililiter of sample (µg GAE/mL sample).

Analysis of antioxidant activity

Antioxidant activity assay was analyzed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay according to a modified method of Shimada *et al.* (1992). The 100 µL of kombucha sample was transferred into a test tube, following by 2.9 mL of 0.1 mM DPPH solution. The mixer solution was mixed and kept in the dark for 30 minutes. The absorbance of DPPH solution (A) and kombucha sample with DPPH solution (B) was measured at 517 nm using a spectrophotometer (Lambda 365, Perkin Elmer, USA). The DPPH scavenging activity was calculated using the following equation:

$$\text{DPPH scavenging activity (\%)} = [(A - B)/A] \times 100$$

Microbiological analysis

The total viable cells of acetic acid bacteria, lactic acid bacteria and yeast were examined using the spread plate technique on GYC agar, MRS agar and YEPD agar, respectively. All plates were incubated at 30°C for 24-48 h. and the results were expressed as Log colony-forming units (CFU)/ml.

Evaluation of antimicrobial activity

The antimicrobial activity was monitored using the agar disc diffusion method. The Gram-positive bacteria (*B. subtilis* and *S. aureus*) and Gram-negative bacteria (*E. coli)* were used as the test pathogenic microorgamsims. All strains were grown on NA slants at 30°C for 48 h. Then, the microbial sample was diluted with sterilized distilled water to achieve an absorbance of 0.2 at 600 nm. The microbial suspension was spreaded on the NA plates using the swab technique. Next, sterililed discs were placed on the surface of NA and 15 µL of each kombucha samples were dropped onto the discs. All plates were incubated at 30°C for 48 h. Following that, the sensitivity and resistance of each tested microorganisms was examined by measuring the inhibition zone around the disc (clear zone) in milimeter.

Sensory evaluation

For consumer acceptance, the sensory evaluation of all kombucha samples was conducted by 30 untrained panelists (students, instructors, staffs and ordinary citizens) from Rajamangala University of Technology Tawan-ok, Chanthaburi campus with an age of 20-70 years. For testing, the kombucha sample were randomized and the five parameters including color, odor, flavor and overall acceptability were scored using a 9-point hedonic scale ranging from 1 (extremely dislike) to 9 (extremely like).

Statistical analysis

All experimental treatments were done in three replications (n = 3), and the results were indicted as mean ± standard deviation (SD). The data were statistical analysed by Analyze of variance (ANOVA) and compared mean difference using Duncan's New Multiple Range Test (DMRT) at a 95% confidence level (p<0.05).

Results

In this study, six formulations of herbal kombucha were produced as follows: F1 (roselle without agarwood leaves), F2 (roselle with 0.01% agarwood leaves), F3 (roselle with 0.05% agarwood leaves), F4 (peppermint without agarwood leaves), F5 (peppermint with 0.01% agarwood leaves), and F6 (peppermint with 0.05% agarwood leaves) with 14 days of fermentation. After 2 days of fermentation, a thin white film was observed floating on the surface of the kombucha liquid in all formulations. After 6-7 days of fermentation, a sour smell was generated which intensified over the fermentation period. At the end of the fermentation process F1, F2 and F3 had a reddish-pink color, while F4, F5 and F6 had a yellow color (Figure 1).

pH value and acetic acid content

After the fermentation process, the produced kombucha had pH values ranging from 3.02±0.02 to 3.20±0.01, which decreased from the initial range of

5.5-6.0. The pH values of the F1, F2 and F3 were similar and lower than those of the F4, F5 and F6. Meanwhile, the pH values of the peppermint kombucha varied depending on the amount of agarwood leaves, increasing the amount of agarwood leaves resulted in a greater decrease in pH. This correlates with the study findings that acetic acid was produced in all kombucha formulations, with acetic acid levels ranging from 0.28±0.01% to 0.41±0.00%. The F2 had the lowest acetic acid content compared to F1 and F3. Conversely, the acetic acid content in peppermint kombucha increased with the amount of agarwood leaves, though it remained lower than that of roselle kombucha (Figure 2).

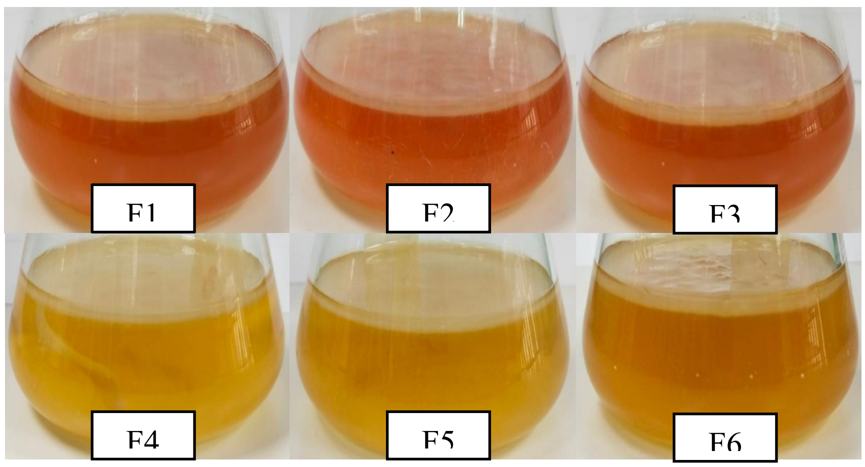

Figure 1. Characteristics of the produced herbal kombucha teas

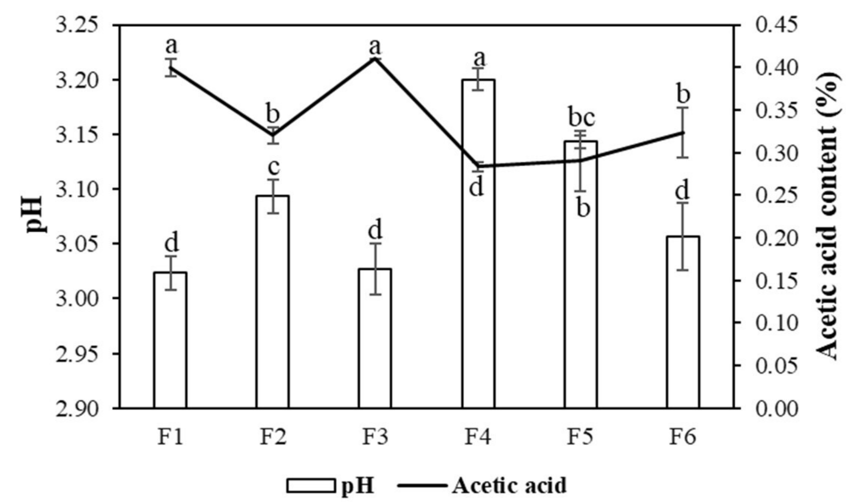

Figure 2. pH value and acetic acid content of the produced herbal kombucha teas

TSS and alcohol content

The study on producing herbal kombucha with agarwood leaves started with an initial TSS content of 15.0 ºBrix. At the end of the fermentation process, all kombucha formulations had a reduced TSS content, ranging from 12.0±0.00 to 13.0±0.00 ºBrix. Increasing the amount of agarwood leaves resulted in a decrease in TSS in the roselle kombucha. Conversely, adding 0.01% agarwood leaves caused a greater reduction of TSS in peppermint kombucha compared to roselle kombucha. However, increasing the agarwood leaves to 0.05% resulted in a smaller reduction in TSS. The roselle and peppermint kombucha had similar TSS content. The alcohol content ranged from 1.07±0.15% to 1.32±0.08%. Adding agarwood leaves increased the alcohol content in both roselle and peppermint kombuchas, the F5 gave the highest alcohol content (Figure 3).

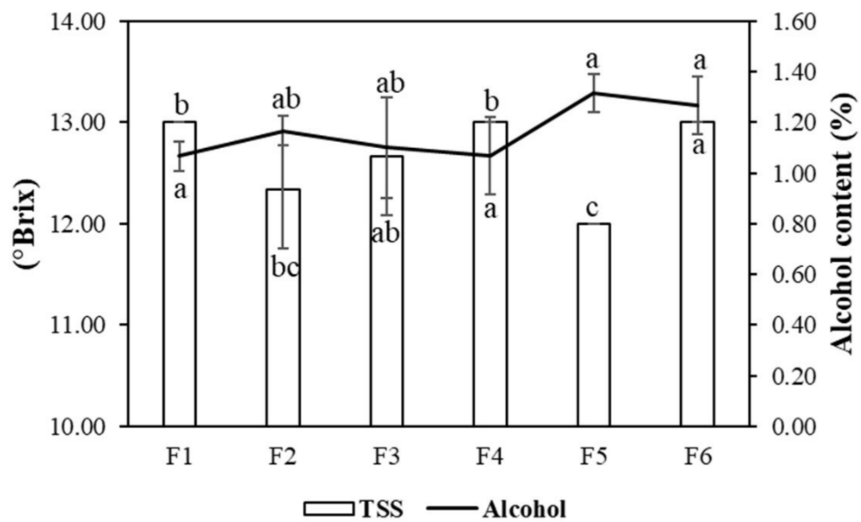

Figure 3. TSS and alcohol content of the produced herbal kombucha teas

TPC and antioxidant activity

The TPC and antioxidant activity of the six kombucha formulations is shown in Table 2. The TPC ranged from 3,577.78±96.86 to 8,066.67±905.13 µg/mL. The F1, F2 and F3 had higher TPC than the F4, F5 and F6. The F3 had the highest TPC, significantly higher than the other formulations. The DPPH scavenging activity of the produced kombuchas ranged from 56.02±0.11% to

84.91±0.84%. The F1, F2 and F3 had higher antioxidant activity compared to the F4, F5 and F6. Additionally, the F1 exhibited higher DPPH antioxidant activity compared to F4, but no statistically different from F3.

Table 2. Total phenolic content and antioxidant activity of the produced herbal kombucha teas

Formulation Number	Total phenolic content (µg/mL)	DPPH radical scavenging activity (%)
F1	6,962.96±530.24^{b}	84.91±0.85^{a}
F2	6,548.15±351.60^{b}	77.03±1.78^{b}
F3	8,066.67±905.13^{a}	83.26±4.36^{a}
F4	3,577.78±96.86^{c}	56.02±1.11^{d}
F5	6,414.81±533.49^{b}	67.65±1.74^{c}
F6	4,318.52±233.42^{c}	66.24±1.57^{c}

[1/a-d] Mean values with different letters in each column are significantly different (p<0.05)

Acetic acid bacteria, lactic acid bacteria, and yeast contents

The acetic acid bacteria, lactic acid bacteria, and yeast content in the six kombucha formulations are shown in Table 3. The acetic acid bacteria and lactic acid bacteria content ranged from 7.75±0.05 to 7.97±0.01 log CFU/mL and 7.63±0.07 to 7.98±0.02 log CFU/mL, respectively. The F1, F2 and F3 had significantly higher acetic acid bacteria and lactic acid bacteria content compared to the F4, F5 and F6. The yeast content ranged from 7.59±0.04 to 7.89±0.017 log CFU/mL. In F1, F2 and F3, the yeast content showed some variation, while in peppermint kombucha, adding agarwood leaves increased the yeast content, which varied according to the amount of agarwood leaves added.

Table 3. Acetic acid bacteria, lactic acid bacteria and yeast content of the produced herbal kombucha teas

Formulation number	Viable cell counts (log CFU/mL)		
	Acetic acid bacteria	Lactic acid bacteria	Yeast
F1	7.97±0.01^{a}	7.96±0.02^{a}	7.76±0.06^{b}
F2	7.96±0.02^{a}	7.92±0.05^{a}	7.89±0.05^{a}
F3	7.93±0.03^{a}	7.98±0.02^{a}	7.62±0.07^{c}
F4	7.77±0.06^{c}	7.63±0.07^{b}	7.59±0.04^{c}
F5	7.77±0.05^{b}	7.70±0.13^{b}	7.75±0.04^{b}
F6	7.75±0.05^{c}	7.73±0.05^{b}	7.89±0.01^{a}

[1/a-c] Mean values with different letters in each column are significantly different (p<0.05).

Antimicrobial activity

The antimicrobial activity tests of the produced kombuchas revealed that the F1, F2 and F3 did not inhibit *B. subtilis* but did inhibit *E. coli* and *S. aureus*. Increasing the concentration of agarwood leaves in the hibiscus kombucha resulted in a decreased inhibition of *E. coli*. The F1 showed the highest inhibition of *E. coli*, but it was not statistically different from F2. Conversely, the roselle kombucha exhibited increased inhibition of *S. aureus* with higher concentrations of agarwood leaves. The F3 showed the highest inhibition of *S. aureus*, significantly higher than the other formulations. No antimicrobial activity was observed against any tested microorganisms in the F4, F5 and F6 (Table 4).

Table 4. Antimicrobial activity of the produced herbal kombucha teas

Formulation number	Inhibition zone (mm)		
	B. subtilis	***E. coli***	***S. aureus***
F1	-	10.67±1.15[a]	7.67±0.58[c]
F2	-	9.67±0.58[a]	8.67±0.58[b]
F3	-	7.33±0.58[b]	11.00±1.00[a]
F4	-	-	-
F5	-	-	-
F6	-	-	-

[1]/ - no inhibition zone
[2]/ [a-b] Mean values with different letters in each column are significantly different (p<0.05).

Sensory evaluation

The sensory evaluation of the herbal kombucha with agarwood leaves are shown in Table 5. In terms of color, the six kombucha formulations received scores ranging from 6.30±1.21 to 7.13±1.60, with the F1 receiving the highest score, though it was not statistically different from the F2. For odor, the scores ranged from 5.33±1.67 to 6.80±1.35, with the F6 receiving the highest score, but not statistically different from the F5. The F1 received the lowest odor score. In terms of flavor, the scores ranged from 5.27±1.74 to 6.93±1.55, with F4, F5 and F6 receiving higher scores than the F1, F2 and F3. The F5 received the highest flavor score, but it was not statistically different from the F6. For overall acceptability, the scores ranged from 5.77±1.57 to 7.17±1.40, with the F4, F5 and F6 receiving higher scores than the F1, F2 and F3. The F5 received the highest overall acceptability score, significantly higher than the other formulations (Table 5).

Table 5. Sensory scores of the produced herbal kombucha teas

Formula number	Sensory score			
	Color	Odor	Flavor	Overall acceptability
F1	7.13 ± 1.60^{a}	5.33 ± 1.67^{c}	5.57 ± 1.80^{b}	6.00 ± 1.89^{bc}
F2	7.10 ± 1.37^{a}	5.87 ± 1.90^{bc}	5.37 ± 2.04^{b}	5.77 ± 1.57^{c}
F3	7.00 ± 1.29^{ab}	5.90 ± 1.45^{bc}	5.27 ± 1.74^{b}	5.83 ± 1.56^{c}
F4	6.60 ± 1.57^{ab}	5.73 ± 1.68^{bc}	5.73 ± 1.91^{b}	6.00 ± 1.78^{bc}
F5	6.30 ± 1.21^{b}	6.60 ± 1.59^{ab}	6.93 ± 1.55^{a}	7.17 ± 1.40^{a}
F6	6.60 ± 1.35^{ab}	6.80 ± 1.35^{a}	6.83 ± 1.53^{a}	6.83 ± 1.49^{ab}

$^{1}/^{a\text{-}c}$ Mean values with different letters in each column are significantly different (p<0.05).

Discussion

In general, kombucha is fermented using black or green tea and sometimes oolong tea is ulitilized. This study focuses on developing kombucha production using locally available and safe herbs, roselle, and peppermint, which are affordable and widely used in various food applications. Additionally, agarwood leaves, commonly used for essential oil extraction from the trunk, are incorporated. Recently, agarwood leaves have been used to produce tea for consumption, though the resulting tea is bitter and not widely popular.

The pH values of the various herbal kombucha formulations decreased due to the production of organic acids, such as acetic acid, during fermentation. A lower pH promotes the growth of acetic acid bacteria, lactic acid bacteria, and yeast (Aung and Eun, 2021) and inhibits the contamination of pathogenic microorganisms (Greenwalt *et al.,* 1998). Kayisoglu and Coskun (2020) studied the physical and chemical properties of kombucha made from different herbal teas, including black tea, green tea, sage, linden, and mint. They found that the pH of all kombucha types decreased after fermentation, with linden tea kombucha showing the highest reduction of up to 60%. The pH reduction variability in kombucha may result from the composition of the tea and other raw materials used, as different teas contain varying levels of polyphenols and catechins, which influence the activity of bacteria and yeast in breaking down sucrose into glucose for ethanol and carbon dioxide production. The ethanol is then oxidized to form acids, affecting the kombucha's pH. Acetic acid bacteria, including *Acetobacter*, *Komagataeibacter*, and *Gluconobacter*, are primary microorganisms in kombucha that convert glucose to gluconic and glucuronic acids and utilize ethanol to produce acetic acid (Leonarski *et al.,* 2021). As a result, acetic acid was detected in all kombucha formulations, with roselle kombucha producing more acetic acid due to its natural acidity, malic acid, antioxidants, and various vitamins. The acids in the raw materials help reduce pH, creating an optimal fermentation environment more quickly. The optimal pH for *Acetobacter* and *Komagataeibacter* is 4.00-5.00, and for *Saccharomyces* sp. is 4.50-6.20, leading to higher acetic acid content and lower pH in roselle kombucha compared to peppermint kombucha. Excessive acetic acid can slow microbial growth and reduce alcohol conversion to acetic acid, increasing alcohol levels, which, if above 12%, can inhibit microbial growth (Lin *et al.,* 2012). The study found that roselle kombucha and roselle kombucha with agarwood leaves had similar pH values, lower than peppermint kombucha, with comparable and higher acetic acid levels due to roselle's acidic and nutrient-rich properties. The agarwood leaves concentration did not affect the pH and acetic acid content of roselle kombucha, while these parameters in peppermint kombucha varied with the amount of agarwood leaves. Increasing agarwood leaves content reduced pH and increased acetic acid production, indicating that adding up to 0.05% agarwood leaves enhances microbial acetic acid production, lowering pH.

The total soluble solids in all kombucha formulations decreased because yeast utilizes the added sugar (sucrose) as a carbon source, breaking it down with the enzyme invertase into glucose and fructose. These sugars are then converted into ethanol through glycolysis (Vargas *et al.,* 2021). Additionally, Chakravorty *et al.* (2016) studied the relationship between biomolecules and biochemistry during kombucha fermentation. They found that in the first 7 days of fermentation, the initial sugar concentration was 8.2±0.7 grams per liter. After 21 days of fermentation, the sugar concentration decreased to 2.25±0.3 grams per liter, due to the acetic acid bacteria breaking down sugar to produce acetic acid. The study also observed that alcohol production occurred in all kombucha formulations, with the highest concentration being 1.32±0.08%. This result was due to increased yeast fermentation activity. Kombucha with 0.01% agarwood leaves showed the highest alcohol content, which aligns with the findings of Talebi *et al.* (2017). Their study on 18 commercial kombucha samples showed significant alcohol increases after 7 and 14 days of fermentation, with alcohol concentrations of 1.57% and 1.7%, respectively. After 21 days, the alcohol level slightly decreased and stabilized because carbon dioxide produced during fermentation can inhibit the conversion of alcohol to acetic acid. Furthermore, the addition of agarwood leaves resulted in a decrease in total soluble solids and higher alcohol production compared to kombucha without agarwood leaves. This effect may be due to agarwood leaves enhancing yeast's ability to utilize sugar and convert it to ethanol more effectively. The alcohol levels found in the herbal kombucha produced in this study were not very high, as the optimal kombucha for consumption should have a low alcohol content.

Phenolic compounds are widely found in plants and have various health benefits, including antioxidant properties, cancer prevention, lowering cholesterol and triglyceride levels in the blood, and boosting the immune system. These compounds are produced by plants to aid in their growth and reproduction. The amount of phenolic compounds is related to antioxidant activity. Higher phenolic content generally corresponds to greater antioxidant activity. The total phenolic content in kombucha ranges from 3,577.78±96.86 to 8,066.67±905.13 µg/mL. According to Coelho *et al.* (2020) report that the most prevalent polyphenols in kombucha are epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG). The total phenolic content in kombucha increases with the duration of fermentation. However, it has been reported that during fermentation, enzymes from yeast and bacteria, such as phytase, tannase, and α-galactosidase, break down complex phenolic compounds into smaller molecules, resulting in a reduction of total phenolic content (Jayabalan *et al.,* 2007). Additionally, the use of phenolic compounds during kombucha fermentation also contributes to the decrease in total phenolic content (Amarasinghe *et al.,* 2018). Kombucha with 0.05% agarwood leaves exhibited the highest statistically significant total phenolic content. This may be due to the high phenolic content in red roselle, combined with the phenolic compounds in agarwood leaves and those produced by microorganisms. Consequently, kombucha with roselle has a higher total phenolic content compared to kombucha with peppermint. Moreover, adding agarwood to peppermint kombucha promotes an increase in the total phenolic content of the resulting kombucha.

The DPPH method assesses the antioxidant activity of kombucha, which contains various bioactive compounds, including antioxidants like ascorbic acid, D-saccharic acid-1,4-lactone (DSL), total phenolics, and total flavonoids. In this study, kombucha made with roselle exhibited the highest DPPH antioxidant activity, at 84.91±0.84%. This high activity is attributed to roselle's rich content of antioxidants, including vitamin C, anthocyanins, beta-carotene, and lycopene. Additionally, changes in the number of primary microorganisms in kombucha lead to increased antioxidant activity, consistent with research by Chu and Chen (2006), which found that the DPPH antioxidant activity of kombucha increased with fermentation time, peaking around 1.7 times higher on day 15 of fermentation. However, other kombucha samples showed a slow increase in DPPH antioxidant activity, with an initial decrease potentially related to the high antioxidant content that may inhibit microbial growth. In roselle kombucha, increasing the concentration of agarwood leaves led to higher DPPH antioxidant activity. Conversely, adding agarwood leaves to peppermint kombucha decreased the DPPH antioxidant activity, though this decrease was not statistically significant compared to peppermint kombucha without agarwood leaves. This indicates that adding agarwood in appropriate amounts can enhance DPPH antioxidant activity. Moreover, the increase in bioactive compounds, total phenolics, and secondary metabolites with antioxidant properties in herbal kombucha is due to the growth of acetic acid bacteria, lactic acid bacteria, and yeast in SCOPY (Zhang *et al.,* 2012).

The measurement of acetic acid bacteria, lactic acid bacteria, and yeast in kombucha showed results similar to the study by Tran *et al.* (2020), which investigated changes in yeast and acetic acid bacteria during kombucha fermentation. The study found that the highest counts of bacteria and yeast during fermentation were 4.93±4.11 log CFU/ml and 5.54±4.66 CFU/ml, respectively. These counts peaked on day 7, slightly decreased by day 14, and remained stable until the end of fermentation. The levels of acetic acid bacteria corresponded with the measured levels of acetic acid. The addition of agarwood leaves did not affect the counts of acetic acid bacteria and lactic acid bacteria but did influence yeast counts. Adding 0.01% agarwood leaves resulted in the highest yeast count in roselle kombucha, though this was not statistically different from peppermint kombucha with 0.05% agarwood leaves. The addition of agarwood leaves also affected acetic acid bacteria and yeast counts, with 0.01% agarwood leading to higher acetic acid bacteria counts. Increasing agarwood leaf concentration promoted yeast growth. Furthermore, the microbial counts in the produced kombucha met the standards of the Food and Drug Administration, with counts not lower than 6.00 log CFU/g, in accordance with the FDA Notification No. 346.

Testing the antimicrobial activity against three pathogenic microorganisms in kombucha with herbal extracts and agarwood leaves revealed the following results: 1) None of the kombucha samples showed any inhibition against *B. subtilis* and 2) Only roselle kombucha and roselle kombucha with agarwood leaves demonstrated inhibition against *E. coli* and *S. aureus*. These findings are consistent with the study by Yang *et al.* (2009), which investigated kombucha fermentation and its ability to inhibit microorganisms. Their research confirmed that kombucha could inhibit various pathogens, including *S. aureus*, *S. epidermidis*, *Shigella sonnei*, *E. coli*, *Aeromonas hydrophila*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Campylobacter jejuni*, *Salmonella enteritidis*, *S. typhimurium*, *B. cereus*, *Helicobacter pylori*, and *Listeria monocytogenes*, all of which are sensitive to acetic acid. However, peppermint kombucha and peppermint kombucha with agarwood leaves did not exhibit inhibition against any of the tested microorganisms. This may be due to the lower levels of acetic acid in these samples compared to others, potentially resulting in lower or no antimicrobial activity. The antimicrobial effects are attributed to polyphenols and tannins from the tea, which can inhibit both gram-positive and gram-negative bacteria. Additionally, the presence of acetic acid, ethanol, and polyphenolic compounds produced by the bacteria and yeast in kombucha may contribute to its antimicrobial activity (Sabel *et al.*, 2017).

In the sensory evaluation, the results showed that all tested formulas were rated between moderately liked to highly liked. In term of color**,** roselle kombucha and roselle kombucha with agarwood leaves received higher color preference scores than peppermint kombucha and peppermint kombucha with agarwood leaves. This is because the roselle kombucha had a more attractive reddish-pink color compared to the pale-yellow color of peppermint kombucha. The addition of agarwood leaves did not affect the color significantly due to the small amount used. For the odor, peppermint kombucha and peppermint kombucha with agarwood leaves were preferred over roselle kombucha and roselle kombucha with agarwood leaves. This is due to the refreshing aroma of peppermint, which masks the acidic smell of acetic acid. The addition of agarwood leaves had little impact on the aroma because its scent was subtle and overshadowed by the stronger aromas of roselle and peppermint. For the flavor, peppermint kombucha and peppermint kombucha with agarwood leaves received higher taste preference scores than roselle kombucha and roselle kombucha with agarwood leaves. Roselle kombucha was quite tart. Peppermint kombucha with 0.01% agarwood leaves received the highest taste preference score, significantly higher than peppermint kombucha with 0.05% agarwood leaves. Both formulations had a pleasant odor and a flavor that was not overly sour or sweet, although the 0.05% agarwood leaves formula had a slight bitterness. The overall acceptability shown that peppermint kombucha with 0.01% agarwood leaves received the highest overall preference score, though not statistically different from peppermint kombucha with 0.05% agarwood leaves. The formula that consumers preferred most was peppermint kombucha with 0.01% agarwood leaves. This finding aligns with the study by Kayisoglu and Coskun (2020), which produced kombucha from five types of tea extracts, black tea, green tea, sake tea, linden tea, and mint tea. Mint tea received the highest sensory scores, while linden tea received the lowest due to increased acidity after fermentation. As fermentation progresses, the taste of kombucha evolves from a fruity, slightly sour flavor with pleasant bubbles to a more vinegar-like taste, which is better accepted by consumers (Marsh *et al.*, 2014).

In conclusion, the study found that producing herbal kombucha with agarwood leaves is a promising option for commercial development. This is due to its phenol content, DPPH antioxidant activity, and the levels of acetic acid bacteria, lactic acid bacteria, and yeast meeting the standards set by the Food and Drug Administration. Additionally, it has shown effectiveness in inhibiting *S.*

aureus and *E. coli*. The overall acceptability scores for this product were between like slightly to like moderately. However, it is important to control the alcohol content to be less than 0.5% by weight. This can be achieved by extending the fermentation time or reducing the initial total dissolved solids.

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