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Antibacterial And Antioxidant Potential of Three Kinds of Kombucha

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ABSTRACT

Introduction: Kombucha is a type of drink produced by fermented sweet tea with culture of yeast and bacterial. This drink have the potential to be a probiotic drink. Soursop leaves and butterfly pea flowers are known to contain phenolic compounds which are useful as antibacterials and antioxidants, that have possibility as raw material of Kombucha.

Objective : The aim of the research is to prove the probiotic potential of 3 kinds of kombucha, namely soursop leaves (*Annona muricata*) kombucha, butterfly pea flowers (*Clitoridea ternatea*) kombucha and a mixed both of plants kombucha.

Methods : The research was conducted in the laboratory with a post test control group design. Each of infusion of soursop leaves (12g), butterfly pea flowers (12g) and a mixture both of plants (@6 g) in 1L of boiling water with 100g sugar added and 30 ml (Symbiotic Culture of Bacterial and Yeast) SCOBY added. Fermentation was carried out for 14 days. Total levels of phenols, flavonoids, and tannins were measured calorimetrically using a spectrophotometer. Antibacterial potential was determined by measuring the inhibition of zone (ZOI) for the growth of *E coli* and *S. aureus* bacteria. Antioxidant potential was determined by calculating the inhibition concentration (IC) 50 for of 2,2- diphenyl-1-picrylhydrazyl (DPPH) inhibition. Analysis of results used One Way Analysis of Variant (ANOVA) with value $p<0.05$

Results : The best of total phenolic is mixed kombucha (155.97 mg GAE/g) while the best total flavonoid and tannin is sorsoup leaves kombucha (132.57mg QE/g and 138,15 mg GAE/g)). The three kinds of kombuchas have weak antibacterial potential against *E.coli* and *S.aureus* bacteria (9 mm). The best antioxidant potential is possessed by soursop leaves kombucha (IC50 DPPH 13991,44).

Conclusion : Soursop leaf kombucha is the best potential as probiotic drink among three kinds of kombucha.

INTRODUCTION

Probiotics are normal flora that live in the intestine, which play a role in metabolic processes and human immunity. Probiotics modulate innate and adaptive immune responses, maintain the integrity of the intestinal epithelial barrier and produce antibacterials (Tegegne & Kebede, 2022). The types of bacteria most commonly used in products containing probiotics are *Lactic acid bacteria* (LAB) and *Bifidobacteria* strains (Ranjha, et al., 2021). Kombucha is a fermented beverage product from tea leaf infusion by bacteria and yeast or Symbiotic Culture of Bacteria and Yeast (SCOBY). Fermentation results from bacteria and yeast will produce organic acids,

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phenolic compounds, vitamins, minerals and alcohol also contains probiotics (Jakubczyk, et al., 2020). Kombucha is widely consumed because it modulates the normal bacterial flora in the intestine, has antibacterial and antioxidant effects so that it can reduce imbalance in the intestinal microbiota (Al-Mohammadi AR, 2021) , control obesity and prevent diabetes and malignancies (Miranda, et al., 2022).

Soursop leaves contain the main compound Acetogennin and several other active compounds such as flavonoids, phenolic compounds, alkaloids and terpenoids. Soursop leaves are widely used in traditional medicine as anti-diarrheal, anti-diabetic, anti-cancer, antioxidant, anti-inflammatory, anti-microbial, anti-viral and anti-parasitic (Nolasco-González, et al., 2022). Meanwhile, butterfly pea flowers are also known to contain the active compounds flavonoids, anthocyanin glycosides, terpenoids and phytosterols. Butterfly pea flowers are known to have health effects as analgesic, antipyretic, anti-inflammatory, antioxidant, antidiabetes, antimicrobial, antihelminthic, hepatoprotective and antiasthmatic (Jeyaraj, et al., 2020). These two plants contain phenol compounds so they can be used as basic ingredients for making kombucha to replace tea leaves. Until now, there has been no research comparing the antibacterial and antioxidant potential of kombucha made from soursop leaves, butterfly pea flowers and a mixture of soursop leaves and butterfly pea flowers.

This study aims to compare the probiotic potential of soursop leaves kombucha, butterfly pea flower kombucha, and a mixture both of plants kombucha. Indicators as probiotics are assessed from their potential as antibacterials, antioxidants and levels of active compounds.

METHODS & MATERIALS

This type of research is experimental research carried out at the Herbal Laboratory of Medical Faculty of Universitas Islam Malang (UNISMA), Malang, the Materia Medika Laboratory, Batu, and the Microbiology Laboratory of Universitas Muhammadiyah Malang, Malang. The research was carried out in May -July 2023.

Kombucha Making Process

The tools used in the process of making kombucha are a stainless steel pan, glass jar, cloth covering the jar, rubber band, filter, beaker glass, measuring cup, 100 ml measuring flask, Erlenmeyer 250, oven, digital scale, and blender. The selected soursop leaves are washed thoroughly, then drained. The leaves were oven-dried at 50°C until the weight was around 10% of the initial weight. The dried leaves were blended to obtain a sample in powder form. The same method is used for butterfly pea flowers (Wahyuni, et al., 2014). Making kombucha is a modification of research conducted by Suhardini and Zubaidah, (2016). Soursop leaf powder is weighed as much as 12 g and put into a tea bag, dipped in 1 L of boiling water for 5 minutes. Next, the water is transferred into a glass jar and 100 g sugar is added, stirred until evenly distributed. Once cool, add 30 ml of liquid SCOBY then cover the jar with a clean cloth and ferment for 14 days. The same method was used for 12 g of dried butterfly pea flowers and the mixture both of plant (6 g of soursop leaf powder + 6 g of butterfly pea flowers). After 14 days, measure the pH of the three types of kombucha using a pH meter (Suhardini & Zubaidah, 2016). The fermentation results showed that the pH of soursop leaf kombucha was 3.8, butterfly pea flower 2.88 and the mixture of both was 2.84.

Determination of total Phenolic, flavonoid, and tannin

The process of measuring total levels of phenols, flavonoids, and tannins uses the colourimetric method which is carried out in accordance with the procedures applicable at the Balai Materia Medika Laboratory, Batu. The equipment used consisted of a 25 ml volumetric flask, analytical balance and UV-VIS spectrophotometer. The raw materials used consist of 3 kinds of fermented drink samples of soursop leaves, butterfly pea flowers and a mixture of both, raw Galic Acid (Sigma- Aldrich S7768449017), raw Quercetin (Sigma-Aldrich Q4951), and ethanol pro analysis (PA).

Total phenol concent (TPC) determination was using the modified Folin-Ciolcalteu reagen. 25 g of each sample was weighed and put into a 25 ml volumetric flask, PA ethanol was added to the mark and sonicated for 10 minutes. 0.2 ml of solution was put into a vial and 1 ml of Folin ciocalteu LP (7.5%) was added, incubated at room temperature for 8 minutes. Next, 0.8 ml of 1% NaOH was added and incubated for 1 hour. Gallic acid was using as standart. The absorption of each sample and blank solution was measured using a spectrophotometer with a maximum absorption wavelength (756 nm), then a calibration curve was made and the concentration of the test solution was calculated. TPC was expressed as a percentage of total gallic acid equivalent per gram kombucha (mg GAE/g).

Total flavonoid content (TFC) determination was using colorimetry assay. 25 g of each sample was weighed and put into a 25ml volumetric flask, PA ethanol was added to the mark and sonicated for 10 minutes. 0.5 ml of solution was put into a vial and 1.5 ml of PA ethanol, 0.1 ml of 10% concentrated aluminum chloride ($AlCl_3$), 0.1 ml of 1M sodium acetate (CH_3COONa) and 2.8 ml of distilled water were added. at room temperature for 30 minutes. Quersetin (Sigma Q4951) was using as standart. The absorption of each sample and blank solution was measured using a spectrophotometer with the maximum absorption wavelength, then a calibration curve was made and the concentration of the test solution was calculated. The TFC was stated as percentage of total quercetin equivalent per gram kombucha (mg QE/ g).

Total Tannin content (TTC) Determination was using colorimetry assay. 25 g of each sample was weighed and put into a 25 ml volumetric flask, PA ethanol was added to the mark and sonicated for 10 minutes. 0.2 ml of solution was put into a vial and 1 ml of Folin ciocalteu LP (7.5%) was added, incubated at room temperature for 8 minutes. Next, 0.8 ml of 1% NaOH was added and incubated for 1 hour. Gallic acid is used as standart. The absorption of each sample and blank solution was measured using a spectrophotometer with a maximum absorption wavelength (500 nm), then a calibration curve was made and the concentration of the test solution was calculated. The percentage of total tannin content in the kombucha drink is equal to the tannic acid per gram kombucha (mg GAE/g).

Antioxidant Activity Test

The preparation of DPPH solution and determination of DPPH scavenging activity was performed by using modification method from Blois (1958) (Molyneux, 2004) . Standard solution was made by weighing 120 mg of the extract into a measuring cup and dissolving it with 70% ethanol to 5 ml to obtain a stock standard solution concentration of 24.000 ppm. Next, graded dilutions were carried out to obtain test doses of 12000 ppm, 16000 ppm, 20000 ppm, and 24000 ppm. A total of 4 mg DPPH was weighed and dissolved in 100 ml ethanol P.A to obtain a DPPH concentration of 0.1 mM. A blank solution or negative control is a solution that does not contain the test material. The blank solution used was 2 ml of ethanol plus 2 ml of DPPH 0.1 mM (ratio 1: 1).

The positive control used is Vitamin C. To make a standart curve of Vitamin C, the first thing to do is make a standard solution by weighing 25 mg of Vitamin C into a measuring cup and dissolving it with 70% ethanol to 250 ml to obtain the concentration of the Vitamin C standard solution of 100 ppm. Next, graded dilutions were carried out to obtain Vitamin C doses of 2.3 ppm, 3 ppm, 3.4 ppm, and 3.6 ppm.

Activity of antioxidant test was carried out 2 ml of each test solution was pipetted into a test tube, added 2 ml of DPPH 0.1 mM, and shaken until homogeneous. Incubated at a temperature of 37 degrees Celsius for 30 minutes and the absorbance was measured using a spectrophotometry device at a wavelength of λ 517 nm. The same thing was also done for the positive control solution. This was repeated 3 times for each test solution, positive control solution and negative control solution. Calculation of the IC50 value is based on the percentage of inhibition of DPPH radicals from each sample solution concentration using the formula:

$$\% \text{ Inhibition} = \frac{(\text{A blank} - \text{A sample})}{\text{A blank}} \times 100\%$$

The sample and control vitamin C concentration values and their percent inhibition are plotted respectively on the x-axis and Y-axis in the linear regression equation so that the equation y = a+bc is obtained. Next, the IC50 value is calculated by means of a Y value of 50 and the x value will be obtained which is the IC50 value. The IC50 value shows the concentration of the extract and comparison control needed to reduce 50% DPPH.

Antibacterial Activity Test

The antibacterial testing method is a modification of research conducted by Nurhayati et al. To make Muller Hinton Agar (MHA) media, weigh 19 g of MHA powder (CM0337B), put it in an Erlenmeyer flask and dissolve it in distilled water until it reaches a volume of 500 ml ~~cc~~. Next, it is heated until the solution is homogeneous. The media was sterilized using an autoclave at 121°C for 15 minutes. 25 ml of media was poured into a petri dish and left to solidify.

Preparation of bacterial suspension was carry out by taken one loop of *E.coli* bacterial colonies from solid NA media, then put into a reaction tube containing 5 mL of physiological NaCl. Turbidity in the test colony suspension was standardized to the 0.5 McFarland standard (approximately 1.5 x 10^8 CFU/mL). The suspension should be used as an inoculum within 15 minutes. The same method is used to make *S.aureus* bacterial suspension.

The *E.coli* bacterial suspension was applied to the solid MHA medium using a cotton swab, and waited for 10 minutes. 3 paper discs soaked in 3 types of kombucha with a concentration of 100% for 15 minutes. Ciprofloxacin was used as a positive control antibiotic. The disc paper is placed on the surface of the MHA media which has previously been treated with a suspension of *E.coli* bacteria. The incubation process was for 24 hours at 37° C. . The number of repetitions was carried out 9 times on the same day with the same bacterial suspention. The clear zone formed which represents the inhibition of bacterial growth was measured using OptiLab version 22 software. The same procedure was carried out an antibacterial test against *S.aureus* (Nurhayati, et al., 2020).

Data analysis

Data from measurements of total levels of flavonoids, phenols, tannins and IC50 of DPPH inhibition are the average of 3 measurement results and analyzed descriptively analytically. The

ZOI measurement data were displayed as mean ± SD and analyzed using One Way ANOVA with a significance of $p<0.05$ and followed by the Tukey test.

RESULT

The results of determination of the total levels of phenols, flavonoids, and tannins from the three kinds of kombucha, can be seen in table 1.

Table 1. TPC, TFC, and TTC of three kinds of kombucha

Sample	TPC (mg E GA/g)	TFC (mg E Q/g)	TTC (mg E GA/g)
Soursop leaves	130.03±0.04	132.7 ±0.07	138.15 ±0.04
Butterfly pea flowers	123.42±0.03	122.3 ± 0.2	77.56 ±0.04
Mixture both of plants	155.97± 0.04	93.7 ± 0.07	118.27 ± 0.03

Note: Data is the average result of 2 measurements. TPC: Total Phenol Content, TFC: Total Flavonoid Content, TTC (Total Tannic Acid Content).

The research results showed that soursop leaf kombucha had higher levels of flavonoids and tannins than butterfly pea flower kombucha and mixture both of plant The mixture both of plants kombucha has the highest phenol content among the three kinds of kombucha.

Standart curve of Vitamin C can be seen in figure 1 and the IC50 DPPH inhibition from the three kinds of kombucha can be seen in table 2.

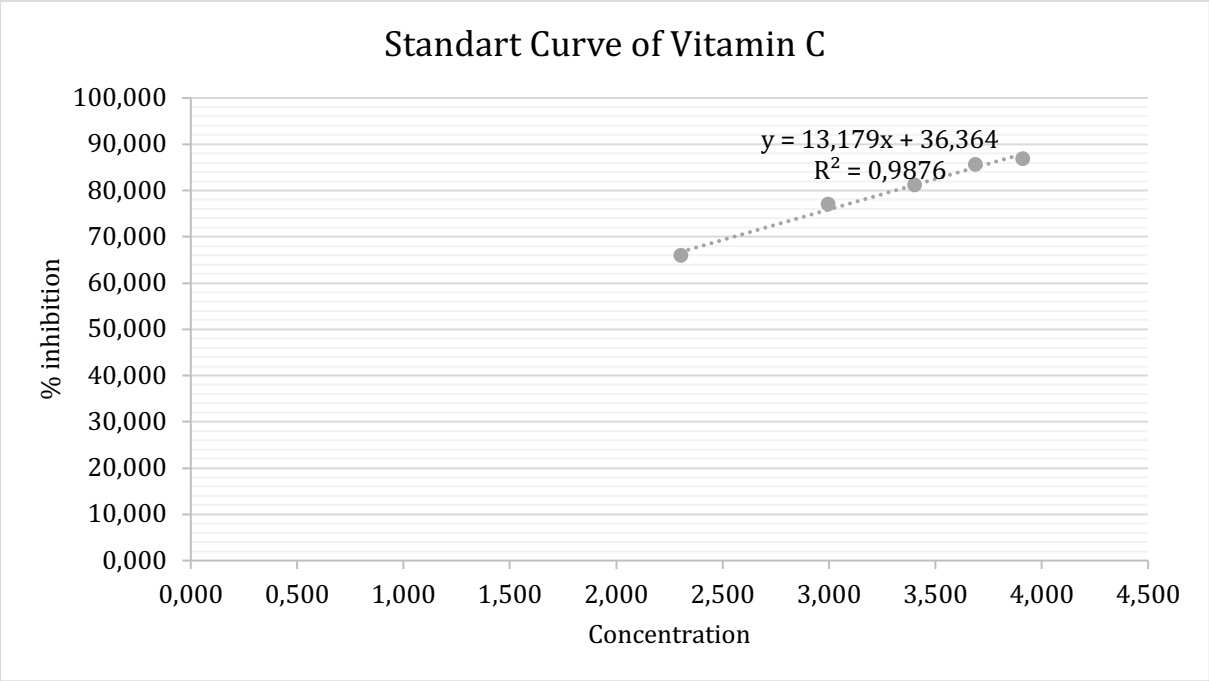

Figure 1. Standart Curve of Vitamin C

Table 2. IC50 of DPPH from the Three kinds of Kombucha

	Consentration (ppm)	% Inhibition	IC50 DPPH
Soursop leaves	12000	45,455	13991,44
	16000	56,186	
	20000	60,902	
	24000	81,34	
Butterfly pea flowers	12000	8,544	23549,01
	16000	24,949	
	20000	40,602	
	24000	50,923	
Mixture both of plants	12000	0,068	32284,33
	16000	12,645	
	20000	33,356	
	24000	39,918	
Vitamin C	2,30	68,01	2,184
	3,00	77,03	
	3,40	79,84	
	3,69	85,51	

Note: % inhibition data is the average result of 3 repetitions. Control of antioxidant test used Vitamin C. IC50 DPPH was analyzed using descriptive analitic.

Based on the IC50 DPPH, the three kinds of kombucha have weak potential as antioxidants when compared to the Vitamin C. The higher the IC50 value, the lower the ability as an antioxidant. However, soursop leaves kombucha has higher potential as an antioxidant than others.

The potential for inhibiting the growth of *E.coli* and *S.aureus* bacteria from the three kinds of kombucha can be seen in table3.

Table 3. ZOI of *E. coli* and *S.aureus* of three kinds Kombucha

Sample	ZOI (mm)	
	E. coli	S.aureus
Positif Control	29,38±0,52[a]	29,722±0,12[a]
Sorsoup leave	9,75±0,81[b]	9,63±0,51[b]
Butterfly pea flower	9,43±0,41[b]	9,85±0,91[b]
Mixture	9,88±0,57[b]	9,21±0,6[b]

Note : ZOI data is the average result of 9 repetitions. Positif control used Ciprofloxacin. Data were analyzed using ONEWAY ANOVA with $p<0.05$, post hog test using Tukey test with $p<0.05$. ([a]) indicates a significant difference between positif control and three kinds of kombucha $p<0.001$ ([b]) indicates no significant different between the three kinds of kombucha

The results of the study showed that the three kinds of kombucha haven't different potential as antibacterial, but have significant different compared to the positive control Ciprofloxacin. All types of kombucha have weak antibacterial potential against *E.coli* and *S.aureus* bacteria.

DISCUSSION

The research results showed that all kombuchas contain active compounds in the flavonoids, phenols, and tannins. Soursop leaves kombucha has higher levels of flavonoids and tannins than

others. Mixture kombucha has the highest phenol content among the three types of kombucha. The research results also prove that all of kombucha have not significant potential as weak antioxidants. However, among the three, soursop leaf kombucha has the potential to be the best antioxidant.

The potential as an antioxidant is thought to be due to the content of polyphenolic compounds, especially in the form of flavonoids, because 2/3 of phenolic compounds are in the form of flavonoids. Du et al. stated that the combination of polyphenolic compounds, especially in the form of flavonoids from *Crataegus monogyna* extract with Vitamin C, will increase the antioxidant effect to prevent hypertension in experimental animals (Du, et al., 2022). The results of research by Jakubczyk et al. found that the total flavonoid content of green tea leaf kombucha was 181.3 ± 4.8 mg/L and black tea leaf kombucha contained 126.7 ± 5.2 mg/L of flavonoids. The levels of phenolic compounds in green leaf kombucha were 320.1 ± 3.5 mg/L and 206 ± 1.2 mg/L in black tea leaf kombucha (Jakubczyk, et al., 2020). This result is greater than the levels of flavonoids and phenols in soursop leaf kombucha, butterfly pea flower and a mixture of both.

Phenolic compounds are generally known as secondary plant metabolites that have an aromatic ring containing at least one hydroxyl group. Phenolic compounds are secondary metabolites, which are often found in fruit, vegetables and fermented drinks (Rahman, et al., 2022). Phenolic compounds consist of 2 types, namely flavonoids and non-flavonoids. Flavonoid compounds consist of flavonols, flavones, anthocyanins and isoflavones. The non-flavonoid compounds consist of benzoic acid and phenolic acid. Stilbeston, tannin and lignin are derivatives of phenolic acids (Tungmunnithum, et al., 2018).

Phenolic compounds have pharmacological effects as antioxidants, antibacterials, anti-cancer, cardioprotective, immunomodulatory and anti-inflammatory (Tungmunnithum, et al., 2018). Phenolic and flavonoid compounds are the greatest antioxidants from plants. Antioxidant potential is determined by the number of hydroxyl groups. As antioxidants, phenolic and flavonoid compounds act as free radical scavengers and chelates Fe2+ ions (Rahman, et al., 2022).

The fermentation process can increase the antioxidant activity of phenolic compounds. This is due to bacterial activity in the fermentation process breaking down phenol glycoside compounds into aglycone or free form phenol compounds. Previous research stated that the length of the fermentation process influences antioxidant activity. Antioxidant activity began to increase 24 hours after the fermentation process and decreased after the 12th day of the fermentation process (Zhao, et al., 2021).

The research results prove that the three kinds of kombuchas have a weak antibacterial effect to inhibit the growth of *E.coli* and *S.aureus* bacteria in vitro. The diameter of the growth inhibition zone for the three kinds of kombucha against *E. coli* and *S. aureus* bacteria is around 9 mm. This shows weak antibacterial properties.

E. coli is a colony of bacteria found in the human gastrointestinal system. These microbes are commensal, but in some conditions they can turn into pathogens that cause several diseases that can cause death. The change in the nature of *E. coli* to become a pathogenic bacteria is caused by a decrease in host resistance and damage to the intestinal barrier epithelium. *E.coli* can cause diarrhea, infections in the urinary system and meningitis (Pokharel, et al., 2023).

S.aureus is a gram-positive coccus bacteria, a colony of pathogenic bacteria that can be found on the skin, respiratory tract and gastrointestinal system (Howden, et al., 2023) . These bacteria have the ability to invade the circulatory system, depending on the resistance of the host, the balance of microflora, and the defense of the barrier system in the mucosal epithelium. Apart from that, this bacteria has the ability to produce biofilm and has the property of easily undergoing

DNA mutation. This causes *S.aureus* bacteria to often cause antibiotic resistance (Cheung, et al., 2021). S. aureus is one of the main bacteria that causes bacteriemic infections in humans such as endocaditis, infections of skin tissue, osteomyelitis, septic arthritis, pneumonia, gastroenteritis, meningitis, urinary tract infections and toxic shock syndrome (Tong, et al., 2015).

Probiotics are digestive tract bacteria that have the ability to attach to intestinal cells and compete with pathogenic bacteria. Probiotics can produce antibacterial components such as lactic acid and bacteriocins. Several studies have proven that probiotics will stimulate the growth of normal bacterial flora in the intestine, thereby inhibiting the growth of pathogenic bacteria (Ranjha, et al., 2021). Acharjee et al. proves that several probiotic products have a synergistic effect when combined with antibiotics against several bacteria such as (*Escherichia coli, Klebsiella spp., Pseudomonas spp., Staphylococcus spp., Salmonella spp., Shigella spp., Vibrio spp*. and *Bacillus spp*. The antibacterial effect measured from the inhibition zone formed against the growth of bacteria from probiotics - these probiotics range from 10-30mm (Acharjee, et al., 2022).

Kombucha is a probiotic drink. SCOBY is needed in the fermentation process to make kombucha containing yeast and bacteria. The most common types of bacteria and yeast are the *Brettanomyces* and *Komagataeibacte*r groups. Both play a role in producing organic acids such as lactic acid, acetic acid, glucuronic acid, thus giving kombucha a sweet and sour taste (Harrison & Curtin, 2021). Other research states that the organic acid content such as acetic acid, citric acid, and glucuronic acid in kombucha causes the pH of kombucha to range from 2.5-5, which is thought to have an antibacterial effect. Low pH causes damage to bacterial cell membranes (Al-Mohammadi, et al., 2021).

Other active compounds contained in kombucha which act as antibacterials are phenolic compounds, flavonoids, and tannins. The mechanism of flavonoids as antibacterials has been mentioned by several researchers through inhibiting nucleic acid synthesis, increasing membrane permeability, forming porins in cell membranes, disrupting membrane function. cells and energy formation, reducing the expression of adhesion molecules and preventing the formation of bacterial biofilms (Shamsudin, et al., 2022). Ogawa & Yazaki, stated that tannins increase the antibacterial effect of antibiotics. Tannin compounds from the bark of *Acacia mearnsii De Wild* have been proven to have a synergistic effect with antibiotics to inhibit the growth of *E.coli, Bacteriodes* and *Clostridium bacteria*, so that tannin is thought to be able to maintain the balance of the microbiotome in the digestive tract (Ogawa & Yazaki, 2018).

The weakness of this research was that it did not measure the ethanol content in the three kinds of kombucha. Kombucha contains ethanol as a result of the sugar fermentation process by bacteria and yeast. The presence of ethanol in kombucha is still controversial in studies of Indonesian halal products. Fatwa of the Indonesian Ulama Council (MUI) no.10 of 2018 states that food and beverage products containing alcohol/ethanol are limited to the percentage of ethanol in the drink must be less than 0.5% and not be dangerous to human health. The alcohol content in kombucha varies depending on the fermentation time. The results of other research state that the ethnaol content of kombucha tea with variations in fermentation time states that fermentation for 5-8 days produces ethanol levels of between 0.2-0.3% (Ihsani, et al., 2021). Thus, it is necessary to develop future research on these three kinds of kombucha to measure alcohol levels and test acute and chronic toxicity on experimental animals.

CONCLUSION

Based on the facts above, it can be concluded that the three kinds of kombucha contain the active compounds phenol, flavonoids, and tannins. The best type of kombucha as a probiotic based on its potential as an antibacterial and antioxidant is soursop leaf kombucha. From the results of this study, researchers recommend conducting further research to identify the probiotic bacterial content in soursop leaves kombucha and determine the antibacterial properties of kombucha against probiotics bacteria (such as lactobacillus).

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