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Production of kombucha from *Muntingia calabura* L. leaves and evaluation of its antibacterial activity and total flavonoid content

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ARTICLE INFO

Received on: 28/01/2022
Accepted on: 18/05/2022
Available Online: 04/08/2022

Key words:

Muntingia calabura L.,
kombucha, fermentation,
healthy beverage, flavonoid,
antimicrobial.

ABSTRACT

Kombucha is a supplement beverage from the fermentation of tea leaves by a symbiotic culture of acidic bacteria and yeast. In this study, we aim to produce kombucha from *Muntingia calabura* L. leaves and evaluate its characteristics, antibacterial effect, and total flavonoid content. The characteristics of kombucha were evaluated by the established method for the kombucha product. The antibacterial activity was determined by agar diffusion, and the total flavonoid content was assessed by UV-Vis spectrophotometry. Kombucha from *M. calabura* leaves was produced with acceptable characteristics. The pH and acidity of kombucha were 4.78 and 0.22%, respectively. The alcohol content of kombucha was 0.726%. The value of total plate count (TPC) bacteria and fungi were zero colony forming unit (CFU)/ml and 2×100 CFU/ml, respectively. The value of the most probable number of coliform and pathogenic bacteria exhibited the absence of bacterial growth on lactose broth medium and eosin methylene blue agar medium. Kombucha from *M. calabura* leaves showed a remarkable potency of antimicrobial activity against a pathogenic bacterium, demonstrating the inhibition of the growth of *Escherichia coli*, *Shigella dysenteriae*, *Salmonella typhi*, *Bacillus subtilis*, and *Vibrio cholerae*. The total flavonoid content of kombucha was 35 mg/ml quercetin equivalent. The kombucha from *M. calabura* leaves was produced with good characteristics and safety for a daily beverage. Moreover, it has an antibacterial activity toward some pathogenic bacteria and can be a source of natural flavonoids.

INTRODUCTION

Kombucha is a traditional drink from fermented black tea or sometimes green tea and sugar using a symbiotic culture of bacteria and yeast (Martinez-Leal *et al.*, 2018). Generally, the manufacture of kombucha involves a symbiosis between yeast such as *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Schizosaccharomyces pombe*, and *Torulasporea delbrueckii* and acetic acid bacteria such as *Acetobacter xylinum* or *Bacterium gluconicum* (Martinez-Leal *et al.*, 2018). After the fermentation, kombucha becomes a drink that contains various chemical components that produce acids and vitamins (Miranda *et al.*, 2016). In the process of fermentation, there will be changes in

physical and chemical properties such as sugar, alcohol, pH, and total flavonoid levels (Havas *et al.*, 2015).

The acidity level of kombucha is formed during the fermentation due to the production of organic acids. The organic acids in kombucha are acetic acid, gluconic acid, glucuronic acid, and lactic acid. The vitamins contained in kombucha are vitamin B1, B2, B6, B12, and C (Villarreal-Soto *et al.*, 2018). In addition, kombucha from *Camelia sinensis* contains phenolic content of around 30% of the dry mass of tea leaves (Dufresne and Farnworth, 2000). The phenolic components in kombucha contain epicatechin gallate, epigallocatechin, catechin, epicatechin, and epigallocatechin gallate (Kaewkod *et al.*, 2019). The use of kombucha drinks has become quite popular around the world due to their benefits and several pharmacological effects, including antidiabetic (Bhattacharya *et al.*, 2016) and antioxidant and antimicrobial (Battikh *et al.*, 2013; Bhattacharya *et al.*, 2011) effects.

Muntingia calabura L. leaves have been used in traditional medicine in several Southeast Asian countries and

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tropical America. A previous study exhibited that *M. calabura* was used for the treatment of ulcers, headaches, colds, stomach aches, measles, and acne (Mahmood *et al.*, 2014). Moreover, some studies reported that *M. calabura* leaves were used as antioxidant (Zakaria *et al.*, 2014) and antimicrobial and anticancer (Sufian *et al.*, 2013) agents. Making kombucha from *M. calabura* leaves is expected to increase the potential of the chemical components in *M. calabura* leaves on health. At the same time, we hope that this product can be consumed as a daily beverage. Therefore, in this study, we aim to produce kombucha from the leaves of *M. calabura* and evaluate its characteristics, its total flavonoid content, and its antimicrobial activity.

The kombucha of *M. calabura* leaves was produced and evaluated. The characteristics of the kombucha drinks were measured using some established parameters. The total flavonoid content was determined, which was expressed in quercetin equivalent (QE). The antimicrobial activity was carried out using the agar diffusion method.

MATERIALS AND METHODS

Plant material

Muntingia calabura leaves were provided commercially and authenticated by the Division of Botany, Laboratory of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Muslim Indonesia.

Starter culture

The culture of kombucha was obtained from the Laboratory of Microbiology, Faculty of Pharmacy, Universitas Muslim Indonesia. The production of kombucha consisted of 1% of *M. calabura* leaves and 10% of sucrose. The starter culture of kombucha was used as a symbiotic culture of acidic bacteria and yeast (Kaewkod *et al.*, 2019).

Preparation of kombucha

The *M. calabura* leaves were obtained in Makassar city. The leaves were picked at 10:00 a.m. and sorted. The leaves were dried in an oven at 105°C for 60 minutes to reduce the moisture content. The leaves were then mashed to facilitate the extraction process. As much as 5 g of *M. calabura* leaves was added to 500 ml of distilled water and boiled for 15 minutes. Into the hot tea, sucrose 10% was added. The mixture was cooled at room temperature. The starter culture 10% was inoculated into a mixture and left at fermentation temperature for 12 days (Kaewkod *et al.*, 2019). After the fermentation process, the kombucha layer was removed and then washed with clean water. The kombucha product was sterilized.

Determination of pH and total acidity

The pH of kombucha was determined using a digital pH meter. The pH meter was calibrated with a phosphate buffer pH 4 and pH 7 before being used. The total acidity of kombucha was evaluated according to a previous study (Kaewkod *et al.*, 2019). Kombucha was titrated with 0.1 M NaOH. Phenolphthalein 1% was used as an indicator of the reaction. The volume of the NaOH

solution was calculated in terms of grams of acetic acid per liter of the sample.

Determination of alcohol content

Analysis of alcohol content was carried out using an ultraviolet-visible (UV-Vis) spectrophotometer (Thermo Scientific Evolution 201). The kombucha was prepared in various concentrations, namely, 1%, 4%, 7%, 10%, and 13%, and then analyzed at a maximum wavelength of 263 nm. In this analysis, the absorbance value of the sample whose concentration is known will be tested in percent. The linearity of the standard series was carried out through analysis. The results of the linearity equation were used to determine the amount of alcohol in the sample (Rahmi *et al.*, 2013).

To support the data of the UV-Vis spectrophotometer, the alcohol content was determined also by high-performance liquid chromatography (HPLC). The HPLC analysis was performed by the standard addition method. A standard solution of ethanol was made with the concentration series. The sample of 2.5 ml of kombucha was diluted in 10 ml of distilled water. The standard ethanol addition is carried out by adding a standard solution of 10%, 15%, and 20% ethanol of as much as 1.5 ml to a volumetric flask containing 2.5 ml of the sample. The samples were injected into the HPLC system using column C18 reverse phase. A mixture of acetonitrile and phosphate buffer at a ratio of 5:9 at pH buffer 7 was used as the mobile phase. The HPLC data were analyzed to determine the amount of ethanol in the sample (Fawwaz and Baits, 2016; Rahmi *et al.*, 2013).

Determination of microbiological contaminants

Total microbial analysis (bacteria and fungi) was carried out by taking 1 ml of each of the dilution samples and putting it in a sterile Petri dish. Furthermore, 15 ml of NA and potato dextrose agar was poured into the Petri dish, respectively. The Petri dishes were carefully rotated and moved horizontally or in parallel to homogenize the mixture and then allowed to solidify. The Petri dishes were incubated at a temperature of 36°C for 24 hours and 25°C for 48–72 hours for bacteria and fungi, respectively.

In the most probable number (MPN) test, nine test tubes each contained Durham tubes and lactose broth (LB) medium. A sample of 1 ml was inserted into each test tube based on the sample concentration series, namely, 100, 101, and 102, in three series of tubes and then incubated at 37°C for 24 hours.

Determination of antibacterial activity

The antibacterial activity was carried out using the agar diffusion method as in a previous study described with slight modification (Kaewkod *et al.*, 2019). A single colony of bacteria *Escherichia coli*, *Shigella dysenteriae*, *Salmonella typhi*, *Bacillus subtilis*, and *Vibrio cholerae* was prepared in the form of a suspension in a 0.85% NaCl solution. The bacteria suspension was inserted of as much as 1 ml into a test tube of semisolid nutrient agar (NA) media, which was still liquid and homogenized, and then poured into a Petri dish that had been placed with a buffer. After the semisolid medium solidified, the kombucha with various concentrations of 0.1%, 1%, 10%, and 100% was put into the reservoir according to the concentration of each sample. The

plates were further incubated at 37°C for 24 hours. Vernier calipers measured the zones of bacterial growth inhibition.

Determination of total flavonoid content

The total flavonoid content of kombucha was assessed by the aluminum chloride colorimetric method (Baba and Malik, 2015). Kombucha 100 µg/ml was prepared for 1 ml, and then 0.3 ml of a 5% NaNO₂ solution and 0.3 ml of a 10% AlCl₃ solution were added after 5 minutes of incubation. After the mixture was allowed to stand for 6 minutes, 2 ml of a 1 mol/L NaOH solution was added. The final volume of the mixture was brought to 10 ml with distilled water. The mixture was allowed to stand for 15 minutes, and the absorbance was measured at 417 nm by a UV-Vis spectrophotometer. The total flavonoid content was calculated from a calibration curve, and the result was expressed as mg quercetin equivalent per g dry weight (mgQE/g).

RESULTS AND DISCUSSION

Exploration of natural sources is a trend in current research, in addition to increasing added value as well as reducing waste from natural materials (Fawwaz *et al.*, 2018, 2019). *Muntingia calabura* is a natural source that has not been widely used but is widely grown in Makassar, Indonesia. Therefore, the use of this plant needs to be continued so that this plant not only grows as an ornamental plant but also can be used for health, such as in the production of kombucha.

Kombucha from *M. calabura* leaves was produced with acceptable characteristics. The starter culture of kombucha was a symbiotic culture of acidic bacteria and yeast. The organoleptic evaluation showed that the color of kombucha was light brown with a sour smell and sweet taste.

pH and total acidity

The pH of kombucha (4.78) is safe for consumption because the acidity is higher than 2.5. As is known, the pH level of kombucha will drop dramatically during the fermentation process, from 6.71 to pH 2.91, and the lowest it can reach is 2.5 (Zhao *et al.*, 2018). The acidic level, 0.22% as acetic acid, is in the range of the acidic level requirement from the Indonesia National Standard, which is 0.2–0.9%. During fermentation, there is a breakdown of sucrose into alcohol and also the formation of other organic acids by bacteria. The acid formed will release protons so that the pH value becomes low (Ganigué *et al.*, 2016).

The fermentation time of kombucha affects its acidity. The longer fermentation time correlates to the total acid increases. This is because during the fermentation process yeast and bacteria metabolize sucrose and produce a number of organic acids such as acetic acid, gluconic acid, and glucuronic acid; therefore, an increase in the levels of organic acids occurs. The higher organic acids contained in kombucha contribute to the higher total acid (Martinez-Leal *et al.*, 2018). It is known that the sour taste that occurs in kombucha is largely a contribution of the emergence of organic acids, antioxidants, and vitamin C in kombucha (Martinez-Leal *et al.*, 2018).

Alcohol content

The quantitative analysis of alcohol was performed by UV-Vis spectrophotometry at a wavelength of 263 nm as the maximum wavelength of ethanol. The concentration series of the sample was analyzed by a UV-Vis spectrophotometer at the maximum wavelength. The absorbance of the sample can be seen in Table 1, and from these data, a linear curve was obtained with the R² value 0.996, as shown in Figure 1. Linear regression analysis was performed to prove the existence of a relationship between actual concentration and method response. The determination of the alcohol content in the kombucha was analyzed based on the calculation of the obtained linear regression equation $y = 0.0658x + 0.1118$. The alcohol content determined by the UV-Vis spectrophotometry method exhibited that the higher concentration of kombucha was in line with the absorbance, as shown in Table 1.

The chromatogram data of kombucha based on the ethanol addition standard using sample concentrations of 10%, 15%, and 20% were shown in Table 2. The determination of the alcohol content in the fermented kombucha leaves was performed by the HPLC method (Fig. 2). The analysis was carried out by the measurement of the maximum wavelength of pure alcohol using a UV-Vis spectrophotometer because the HPLC for beverage analysis uses a UV-Vis detector. The maximum wavelength (λ_{max}) of ethanol was 263 nm. Determination of alcohol content using HPLC using a phosphate buffer pH 7 has provided the maximum peak area. The pH 7 of the buffer solution was the optimum condition for analyzing the ethanol content of beverages using a mobile phase mixture of acetonitrile: phosphate buffer (5:9). The linear regression, $y = 0.13x + 1.6167$, was obtained by a linear curve equation based on the area of the peak with the concentration of the sample. The results of measuring the alcohol content by HPLC showed that the alcohol content of the sample with a concentration

Table 1. Alcohol content of kombucha from *M. calabura* leaves by UV-Vis spectrophotometry.

Kombucha concentration (%)	Absorbance			Average	Alcohol concentration (%)
	R.1	R.2	R.3		
Blank	0.000	0.000	0.000	0.000	0.000
1	0.177	0.175	0.181	0.177	0.997
4	0.393	0.391	0.395	0.393	2.087
7	0.535	0.539	0.544	0.539	2.824
10	0.769	0.804	0.779	0.784	4.063
13	0.969	0.965	0.971	0.968	4.993

R = Replication.

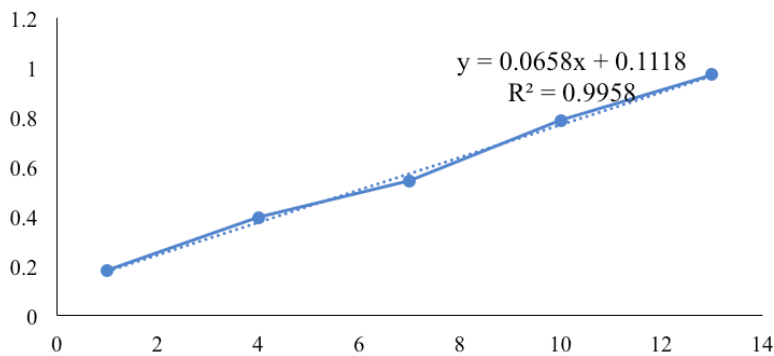


Figure 1. Linear curve of kombucha from *M. calabura* leaves by a UV-Vis spectrophotometer.

Table 2. Alcohol content of kombucha from *M. calabura* leaves by the HPLC method.

No.	Standard addition concentration (%)	Peak height (mAU)	Peak width (minutes)	Peak area (mAU × minutes)
1.	10	53.97	0.15	4.2
2.	15	78.43	0.20	3.6
3.	20	102.86	0.25	2.9

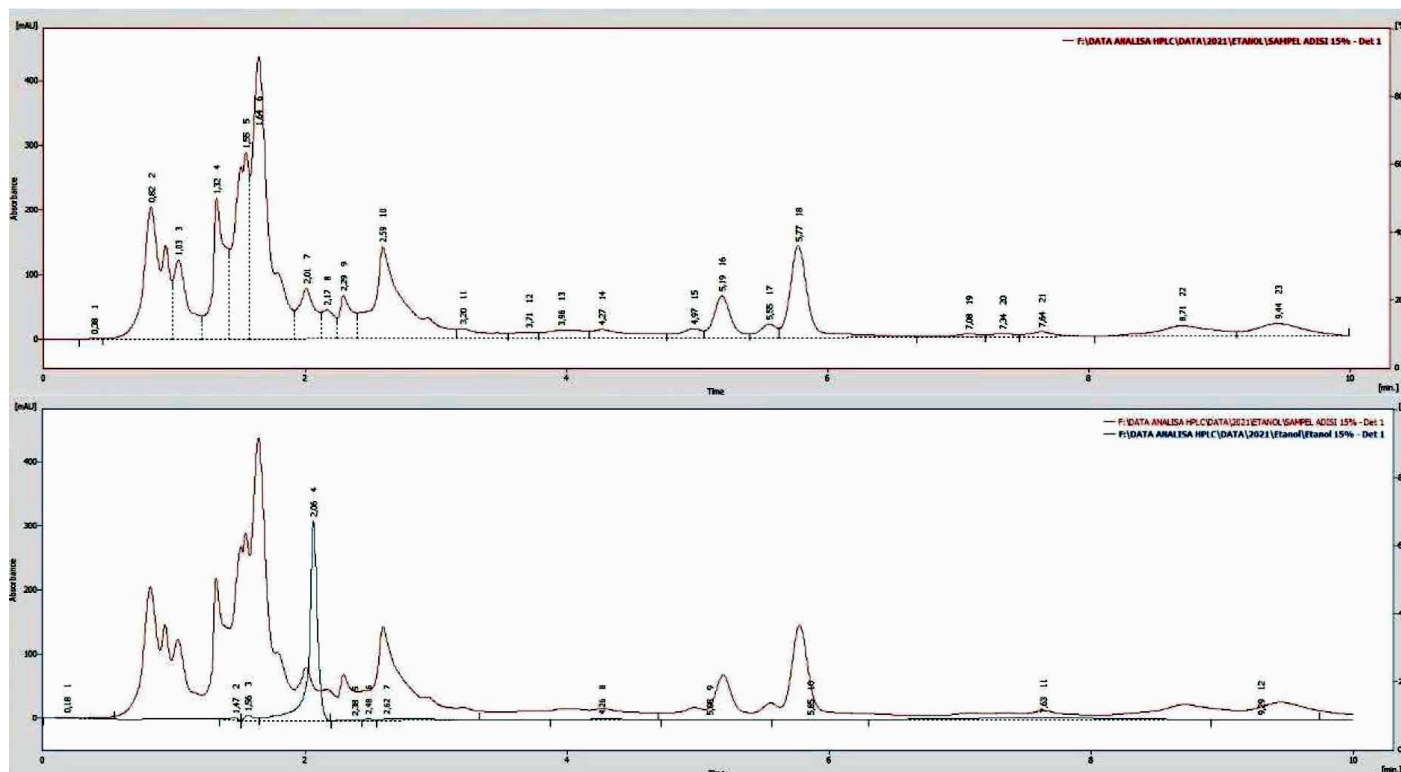


Figure 2. The chromatogram of kombucha from *M. calabura* leaves.

of 10% kombucha drink was 0.726%. The formation of alcohol in kombucha was the result of the fermentation process (Kaewkod *et al.*, 2019).

Microbiological contaminants

Microbiological analysis based on the total plate count (TPC) value of bacteria and fungi exhibited that the kombucha

from *M. calabura* has no microbial contamination. The values of TPC-bacteria and fungi were zero colony forming unit (CFU)/ml and 2 × 100 CFU/ml, respectively. The value of the MPN of coliform and pathogenic bacteria exhibited the absence of bacterial growth on the LB medium and eosin methylene blue agar medium. Both microbiological contaminant assessments conclude that kombucha is safe as a daily beverage because the values were

Table 3. Antibacterial activity of kombucha from *M. calabura* leaves by agar diffusion.

Sample	K (%)	Average diameter of inhibition zone (mm)				
		EC	SD	ST	BS	VC
Kombucha	0.1	0	0	0	0	0
	1	0	0	0	0	0
	10	0	0	12	10	9
	100	22	19	32	30	13

K = Sample concentration, EC = *Escherichia coli*, SD = *Shigella dysenteriae*, ST = *Salmonella typhi*, BS = *Bacillus subtilis*, and VC = *Vibrio cholera*.

in the range of the standard of the Indonesian Food and Drug Authority (BPOM).

Antibacterial activity

Kombucha showed a remarkable potency of antimicrobial activity against a pathogenic bacterium, which has demonstrated the inhibition of the growth of *E. coli*, *S. dysenteriae*, *S. typhi*, *B. subtilis*, and *V. cholerae*. The largest inhibition zone diameter was obtained at a concentration of 100% (32 mm) against *S. typhi*, as shown in Table 3.

These results showed that the fermented kombucha has antibacterial activity based on the formation of an inhibition zone. The activity was due to the presence of organic acids in the *M. calabura* leaves kombucha which can inhibit the growth of bacteria such as gluconic acid, glucuronic acid, ascorbic acid, and acetic acid (Kaewkod *et al.*, 2019).

The diameter of the inhibition zone of kombucha with concentrations of 0.1%, 1%, and 10% indicates activity in the weak category while at a concentration of 100% the diameter of the inhibition zone toward bacteria *E. coli* (22 mm), *S. typhi* (32 mm), and *B. subtilis* (30 mm) was classified as strong activity, *S. dysenteriae* (19 mm) was classified as moderate activity, and *V. cholerae* (13 mm) was classified as weak activity. The interpretation of the antibacterial activity of an agent based on the magnitude of the clear zone is divided into three categories, namely, weak if the resulting clear zone diameter is ≤ 11 mm, moderate if the resulting inhibitory zone diameter ranges from 12 to 21 mm, and strong if the resulting clear zone diameter is ≥ 22 mm (Harmita and Radji, 2008).

The organic acids formed in the kombucha of *M. calabura* leaves during the fermentation process provide antibacterial activity. One of these compounds is acetic acid, and the formation of acetic acid in kombucha can inhibit the growth of Gram-negative bacteria (Sreeramulu *et al.*, 2000). Undissociated acetic acid can damage the lipid bilayer structure of bacteria and introduce protons into the cytoplasm. The large number of intracellular protons makes the cytoplasm in acidic conditions, thus causing protein denaturation and energy loss. Therefore, the high acetic acid content can inhibit bacterial growth (Park *et al.*, 2021). In addition, the organic acids that are formed in the kombucha will form the pH of the drink at an acidic level. The low pH of kombucha affects bacterial growth.

Total flavonoid content

The determination of total flavonoid content was performed by UV-Vis spectrophotometry at a wavelength of 417 nm as the maximum wavelength of quercetin. The concentration series of quercetin was measured by a UV-Vis spectrophotometer.

The absorbances of the quercetin series were used to obtain the linear regression. The R^2 value was 0.996. Linear regression was performed to prove the existence of a relationship between the actual concentration and method response. The determination of the total flavonoid content in the kombucha was analyzed based on the calculation of the obtained linear regression equation $y = 0.0081x - 0.0725$.

The absorbance of the sample was measured in triplicate. The total flavonoid content in kombucha was expressed in QE, which is the amount of quercetin milligram equivalence in milliliters of samples. The calculation of total flavonoid levels based on the absorbance data exhibited that the kombucha contains 35 mg/ml QE. This result indicates that the kombucha has a potential to be an antioxidant due to its total flavonoid content. The polyphenol and the carotenoid compound contribute to the antioxidant activity of natural resources (Fawwaz, 2021). In addition, the total flavonoid content was positively correlated with the antibacterial activities. Although in this study we did not prove that, a previous study exhibited that the level of total phenolic and flavonoid content in the extract contributed to the antibacterial activities against *E. coli* and *S. aureus* (Mahboubi *et al.*, 2015).

The total flavonoid content was determined using aluminum chloride reagents. Aluminum chloride will form a stable complex with the carbonyl groups in C4 and hydroxyl in C3 (flavanols) and C5 in flavanols and flavones. The principle of the aluminum chloride method is the formation of a stable complex with the C4 keto group, as well as in the C3 or C5 hydroxyl groups of flavones and flavanols. In addition, aluminum chloride forms a stable acidic complex with the orthohydroxyl groups in the A- or B- rings of flavonoid compounds. Quercetin was chosen as a comparison solution because it is one of the flavonoid group compounds that can react with aluminum chloride to form complexes (Pełkal and Pyrzyńska, 2014).

CONCLUSION

The kombucha from *M. calabura* leaves was produced with good characteristics and safety for a daily beverage. It has an antibacterial activity toward some pathogenic bacteria and can be a source of natural flavonoids. Therefore, the development of kombucha from *M. calabura* leaves can be developed as a healthy drink consumed by the public.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and

agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

FUNDING

There is no funding to report.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

PUBLISHER'S NOTE

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How to cite this article:

Herwin H, Fitriana F, Nuryanti S. Production of kombucha from *Muntingia calabura* L. leaves and evaluation of its antibacterial activity and total flavonoid content. *J Appl Pharm Sci*, 2022; 12(08):187–192.

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