Jurnal Pijar MIPA
https://jurnalfkip.unram.ac.id/index.php/JPM

ISSN 1907-1744 (Print)
ISSN 2460-1500 (Online)

Determination of Antioxidant Activity in Kombucha of Kecombrang Flower (*Etlingera elatior*) for the Development of Functional Beverages

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Received: January 4, 2024. Accepted: March 18, 2024. Published: March 30, 2024

Abstract: Researchers have widely reported that Kombucha is a probiotic drink with health benefits. Some researchers are innovating to find other kombucha media sources besides tea that contain high bioactive compounds. Kecombrang flowers contain high bioactive compounds and have physiological functions for health, but this plant has not been widely explored for other innovative beverage or food products. This study aimed to determine the optimal fermentation time of kecombrang flower kombucha (*Etlingera elatior*) to produce the highest antioxidant activity during fermentation. The experimental design used was a complete randomized design (CRD) with 7 variations of fermentation time (days 0, 3, 6, 9, 12, 15, and 18) with a sugar concentration of 10% b/v, and this treatment was repeated four times. In this research, pH, total acid, and total flavonoid tests were also carried out to see the characteristics of the kecombrang flower kombucha drink during fermentation. Data analysis was performed with an ANOVA at P < 0,05. The results showed that the length of fermentation time had a very significant effect (P < 0,01) on the antioxidant activity of Kombucha. These results indicate that the size of fermentation time affects the antioxidant activity of kecombrang flower kombucha. Optimal antioxidant activity was obtained after the kombucha drink was fermented for 6 days, as indicated by the low IC 50 value of 37,73 g/mL, with a pH of 3,7, total acid of 0,33%, and total flavonoids of 5,9 mg/L QE. Based on a research study, kecombrang flower kombucha has strong antioxidant activity (IC50 < 50 ppm), so kecombrang flower kombucha can be used as a choice for functional drinks.

Keywords: Antioxidant; Functional Drink; Kecombrang Flower; Kombucha.

Introduction

The food trend towards minimally processed, additive-free products with high nutritional value and health benefits is increasing along with consumer awareness. In this regard, the tradition of kombucha tea consumption in the community has recently attracted the attention of researchers and consumers due to its probiotic content. Various studies report the benefits of kombucha consumption, including boosting the immune system, improving gastrointestinal function [1], preventing cancer and cardiovascular disease, preventing pathogenic bacterial infections [2], having hypoglycemic [3], and having anti-lipidemic properties attributed to the antioxidant activity contained in Kombucha [4].

Kombucha is one of the fermented drinks made from fermented microorganisms called SCOBY (Symbiotic Culture of Bacteria and Yeast), known to have antioxidant effects. Antioxidants can slow or prevent cell oxidation by stabilizing, inactivating, or minimizing the harmful effects of free radicals caused by oxidative stress [5]. Several factors have been reported to correlate with the antioxidant activity of Kombucha, including the length of fermentation time [6] and the substrate or media used as kombucha raw material [7]. During fermentation, phenolic compounds such as acetic acid, glucuronic acid, phenolic acid, ethanol, and lactic acid contribute to the antioxidant activity of Kombucha [6]. Tea (*Camellia sinensis*) is usually used as a raw material for Kombucha. However, some researchers have begun to innovate by using ingredients rich in bioactive compounds as kombucha media, such as soursop leaves, guava leaves, bay leaves, betel leaves [8], rosella flowers [9], butterfly pea flowers [10] and others.

Kecombrag flower (*Etlingera elatior*), also known as bongkot in Bali, is a widespread plant in Indonesia. Balinese people generally process this plant into spices for various Balinese dishes or *sambal*. Several researchers have reported that kecombrang flowers contain multiple bioactive compounds, such as flavonoids, terpenoids, saponins, and tannins. Flavonoids from kecombrang flower extract show high antioxidant potential (IC50 = 21,14 mg/ml) [11] and show anticancer solid activity against MCF-7 and MDA-MB-231 tumour cells [12]. Exploration of kecombrang flowers to be processed into beverages or varied food products has not been widely done, even though the research results show that kecombrang flowers have potential as a functional food. This research was conducted to determine the best fermentation time to obtain optimal antioxidant activity from kecombrang flower kombucha. In addition, to develop Kombucha as a functional drink, it is necessary to test the pH, total acid, and total flavonoids during fermentation.

Research Methods

This research is an experimental study to determine the best antioxidant activity in Kombucha from the kecombrang flower (*Etlingera elatior*) by varying the

How to Cite:
Sintyadewi, P. R., & Fitriani, P. P. E. (2024). Determination of Antioxidant Activity in Kombucha of Kecombrang Flower (Etlingera elatior) for the Development of Functional Beverages. *Jurnal Pijar Mipa*, *19*(2), 343–347. https://doi.org/10.29303/jpm.v19i2.6616

fermentation time. Besides that, pH, total acid and total flavonoid tests were also carried out to see the characteristics of the kecombrang flower kombucha. Each treatment in this study was carried out as many as four repeats with seven variations in the length of fermentation time, precisely 0 days, 3 days, 6 days, 9 days, 12 days, 15 days, and 18 days.

The first stage was the preparation of kecombrang flower kombucha, which was fermented according to the treatment time. Each treatment was tested for pH, total acid, total flavonoid, and antioxidant activity. The research steps are described below.

Preparation of Kombucha Flower Kecombrang

A total of 1 liter of water was heated in a water bath until the temperature reached 90ºC and boiled for 15 minutes, then added 10% (b/v) sugar until dissolved, added 2,5% (b/v) of kecombrang flowers were allowed to stand for 5 minutes while stirring. The infusion was filtered into sterile jars and filled with boiled water up to 1 liter. The injection was cooled to 30ºC, then SCOBY sheets obtained from WikiKombucha Bali 10% (b/v) were poured into the jars and then covered with sterile napkins. Kombucha was fermented during treatment and harvested by separating the cellulose formed on the fermentation medium for further testing [13].

pH Test

The pH of Kombucha is measured using a pH meter by taking 10 ml of Kombucha with a dropper pipette, and then the pH is measured using a pH meter [14].

Total Acid Test

Several 1 mL samples were poured into an Erlemeyer flask and then diluted with 10 mL of distilled water, and then a 0,1 N NaOH solution was used to titrate the mixture. A phenolphthalein indicator is used to determine the endpoint of the titration. The titration was stopped when a pink color appeared.

Flavonoid Total Test

300 µL of sample was mixed, and 4.0 aquabides and 0,03 mL of 10% NaNO2 were added for 6 min. The mixture was added to 0,30 mL of $AlCl_3$ (10%) and allowed to stand for 5 minutes. Then, 4,0% of NaOH was added, and 10% and 1,1 mL of aquabides were added. Readings were taken using a UV-Vis spectrophotometer with an absorbance of 510 nm. Total flavonoids were expressed in mg RE g-1 of extract [12].

Antioxidant Activity

DPPH 0,1 mM solution was prepared by adding 0,004 g DPPH into a 100 ml volumetric flask, and then methanol was added until the line. Sample concentrations were varied between 0, 100, 200, 300, 400, and 500 mg/ml; a total of 0,5 ml was taken from each sample concentration that had been made, and 1 ml of 0,1 mM DPPH was added. 4 ml of methanol was added, vortexed until homogeneous, and then incubated for 30 minutes. Antioxidant activity readings were taken at a wavelength of 517 nm.

Data Analysis

The result of this study will be analyzed using an analysis of variance (ANOVA). If the result shows a difference ($p < 0,05$), run the Duncan's Multiple Range Test (DMRT) [15]

Results and Discussion

Total acid and pH

The average amount of total acid in kecombrang flower kombucha during fermentation varied from 0,02% to 0,13% on days 0 and 3 of observation, then 0,33% to 0,42% on the 6th and 9th days of observation, and 0,98% to 1,92% on the 12th to 18th of fermentation (Table 1). Based on these data, it is known that the total acidity increased with increasing fermentation time. The increase in total acid is thought to be due to bacteria converting alcohol into organic acids during the exponential growth phase and releasing them on the media during fermentation. The increase in organic acids is in line with the increase in fermentation time. The high content of organic acids causes an increase in the total acid content in the fermentation medium [16]. In addition, the release of H+ protons will also occur due to the dissolution of organic acids in the fermentation medium, which has an impact on reducing the pH of Kombucha [5].

pH measurement is essential to maintaining the optimal pH level during fermentation. pH is one of the environmental parameters that indicate the occurrence of the fermentation process in the medium. Table 1 shows a decrease in pH occurs in line with the length of fermentation time. Based on Table 1, the pH of the kecombrang flower kombucha tends to decrease during fermentation. This result follows the total acid test conducted previously, where the total acid increases with increasing fermentation time, so the increase in total acid causes the pH of the fermentation medium to decrease.

Table 1. Average Total Acid and pH of Kecombrang Flower (*Etlingera elatior*) Kombucha

Fermentation Day	Total acid (%)	pH
0	0.02± 0.01	5.2 ± 0.42
3rd	0.13 ± 0.07	4.9 ± 0.15
6th	0.33 ± 0.07	3.7 ± 0.31
9th	0.42 ±0.09	3.2 ±0.21
12th	0.98 + 0.21	2.9 + 0.12
15th	1.10 + 0.08	2.5 + 0.15
18th	1.92 ± 0.25	2.1 ± 0.15

Description: Data are presented as mean ± standard deviation (SD) of 4 replicates.

Table 1 shows that the average pH of the 6th until the 12th day of fermentation experienced a significant decrease from 4,9 to 2,9. In contrast, the pH value in other treatments experienced an insignificant decrease. The slow decline in pH at the beginning of fermentation is thought to be because the yeast is in the adaptation and logarithmic phases at this stage. The stage of sugar breakdown into simpler monomers as nutrients for bacterial growth occurs in the logarithmic phase so that acidic substances that cause a decrease in pH value have not yet formed and accumulated in the medium [17]. Similar results were reported in kombucha products from siam kintamani orange peel, where the pH of Kombucha decreased during the fermentation process [18]. In addition, Kombucha made from various types of leaves with high phenol content also showed a decrease in pH during the fermentation [19].

A significant decrease in pH is likely due to the microbes in Kombucha experiencing a stationary phase. The yeast in the stationary phase will produce ethanol, and its symbiont bacteria use ethanol to produce acetic acid, which causes a buildup of metabolites that can reduce the pH value of Kombucha. This decrease in pH can then inhibit bacterial growth due to inappropriate environmental conditions so that on the 15th until 18th days of fermentation, the reduction in pH does not occur significantly because the microbes enter the death phase due to alcohol compounds in the form of ethanol produced by yeast and acetic acid produced by bacteria starting to decrease. After all, food and nutrients run out. This then resulted in a decrease in acid production due to reduced ethanol levels [20].

The pH that changes during the fermentation process is influenced by the activity of yeast, which converts sugar substrates into alcohol. Alcohol in the form of ethanol is metabolized back by lactic acid and acetic acid bacteria into organic acids such as lactic acid, gluconic acid, glucuronic acid, citric acid, and acetic acid, which will decrease kombucha pH. The decrease in pH during fermentation will support the survival of *Acetobacter xylnum* bacteria in kombucha starter to carry out acetic acid metabolic activities to release free protons to reduce the pH of the solution [21]. Changes in pH are also closely related to changes in the structure of phytochemical compounds that can affect antioxidant activity [19]. Several studies show that the breakdown of phenolic compounds such as anthocyanins [22], flavonoids and catechins is optimal at low pH [21]. Based on this opinion, kecombrang flower kombucha has an optimal pH for microbial growth and the breakdown of phenolic compounds during fermentation.

Total Flavonoids and Antioxidant Activity

Flavonoids are secondary metabolites found in plant tissues. Flavonoids include phenolic compounds that work as antioxidants. Table 2 shows that the flavonoids contained in Kombucha made from kecombang flowers changed during fermentation. Based on the ANOVA statistical test, the length of fermentation time is known to significantly affect the total flavonoids of kecombrang flower kombucha (P<0,01). The average amount of total flavonoids increased until the 12th day of fermentation and decreased from the 15th to the 18th of fermentation. The highest amount of total flavonoids was obtained on the 9th day of fermentation, which amounted to 8.796 mg/L QE.

The total amount of flavonoids that increased in Kombucha from kecombrang flowers was thought to occur due to enzymes produced by bacteria and yeast during kombucha fermentation that degraded polyphenols contained in kecombrang flowers. This is supported by several studies showing that several species of lactic acid bacteria, such as *Lactobacillus plantarum* and *Lactobacillus acidophilus* in kefir [23] and *Lactobacillus hilgardii* found in wine, can release phenol compounds into polyphenols from the substrate. During fermentation, flavonoid compounds can be degraded or formed from the decomposition of other polyphenolic compounds [24].

Table 2. Total Flavonoids and Antioxidant Activity of Kesombrang Flower (*Etlingera elatior)* Kombucha During Fermentation

Fermentation Day	Total Flavonoid (mg/L QE)	Antioxidant Activity (IC 50) (µg/ml)
0	4.472± 0.01*	69.04 ± 0.48
3rd	4.511 ± 0.10	54.53 ± 0.55
6th	5.911 ± 0.08	37.73 ± 0.45**
9th	8.796 ±0.10**	47.87 ± 0.35
12th	8.143+ 0.10	52.87 ± 0.31
15th	6.664+ 0.10	60.67± 0.50
18th	5.292 ± 0.20	72.27 ± 0.50*

Description:
- Data are presented as mean ± standard deviation (SD) of 4 replicates.
- **) Highest total flavonoids
- *) Lowest IC50

Different letters on treatment showed very significantly different results (P<0,01) [15]

The capacity of Kombucha to absorb DPPH radicals is a sign of its antioxidant activity. The duration of fermentation was discovered to have a highly significant impact on the antioxidant activity of Kombucha (P<0,01), according to the results of the ANOVA statistical test. These results indicate that fermentation time affects the antioxidant activity of kecombrang flower kombucha. The Kombucha can scavenge free radicals with a low IC50 value of 37,73 µg/ml after fermentation for 6 days. The microbial species present during the fermentation of plant-based foods can affect the antioxidative activity. Fermentation positively influences the total phenolic content and antioxidant activity; However, the degree of influence depends on the species of microorganism. The ability of fermentation to improve antioxidant activity is primarily due to an increase in the amount of phenolic compounds and flavonoids during fermentation, resulting from a microbial hydrolysis reaction. Additionally,

fermentation causes the structural disintegration of plant cell walls, which releases or synthesizes a variety of antioxidant chemicals [21].

The decrease in antioxidant activity from the 9th until the 18th day of fermentation was due to increased organic acid content during fermentation caused by bacterial and yeast activities (Table 2). These results are similar to research on green tea kombucha, where it is known that the longer the fermentation time, the lower the antioxidant activity of green tea kombucha. The acidic atmosphere that increases during fermentation causes phenolic compounds to become increasingly stable, and it is challenging to release protons that can bind to DPPH so that their antioxidant activity decreases [25].

Based on Table 2, it can be seen that the pattern of antioxidant activity in kecombrang flower kombucha does not show a correlation with the results of testing the total flavonoid content. This result is different from research on Kombucha made from green tea. The increase in the antioxidant activity of green tea kombucha was also accompanied by an increase in total phenolic compounds [26]. The results of this study indicate that increasing total phenolic compounds does not always lead to optimal antioxidant activity. This result is supported by the opinion that phenolic compounds detected during the test result from the breakdown of polyphenolic complexes during fermentation, which have low activity. This causes the total flavonoid test results to remain high despite the low antioxidant activity [25]. Other Studies report that antioxidant activity is not only caused by the phenolic compounds and flavonoids but can also be caused by several types of proteins and peptides such as histidine, tyrosine, methionine and cysteine produced by the microbial consortium in Kombucha [27].

Specifically, a compound is said to be a powerful antioxidant if the IC50 value is < 50 ppm, medium if the IC50 value is >100ppm and <150ppm, and classified as weak if the IC50 value is >150 and <200ppm [28]. Based on this, the antioxidant activity of kecombrang flower kombucha has strong antioxidant activity on the 6th day of fermentation (IC50=37,73). These results are similar to research on kecombrang flower extract using methanol solvent, which has strong antioxidant activity with IC50 values of 21,14 mg/ml [11]. The results of this research indicate that kecombrang flower kombucha has the potential to be developed into a functional drink. Consuming kecombrang flower kombucha can be used as a new alternative to prevent diseases caused by radicals, although further research is needed to see its pharmacological effects.

Conclusion

The length of fermentation time had a very significant effect (P < 0,01) on the antioxidant activity of Kombucha. These results indicate that the size of fermentation time affects the antioxidant activity of kecombrang flower kombucha. Optimal antioxidant activity was obtained after the kombucha drink was fermented for 6 days, as indicated by the low IC 50 value of 37,73 g/mL, with a pH of 3,7, total acid of 0,33%, and total flavonoids of 5,9 mg/L QE. Based on research results, kecombrang flower kombucha has strong antioxidant activity (IC50<50 ppm), so kecombrang flower kombucha can be used as a choice for functional drinks. Further research on experimental animals must be conducted to see the pharmacological effects of kecombrang flower kombucha.

Acknowledgements

The authors would like to thank The Ministry of Education, Culture, Research, and Technology for supporting and funding this research.

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