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Effect of Fermentation Duration on the Chemical Characteristics and Antioxidant Activity of Sambiloto Leaf (*Andrographis paniculata*) Kombucha

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Abstract: Fermentation is a biotechnological process that can modify chemical characteristics and increase antioxidant activity. The effect of fermentation time is very important for the quality of Andrographis paniculata (*Andrographis paniculata*) kombucha. This study aims to investigate the potential of sambiloto leaves (*Andrographis paniculata*) as a base ingredient in kombucha production and to analyze the effect of varying fermentation durations (0, 3, 6, 9, and 12 days) on the chemical characteristics and antioxidant activity of the resulting kombucha. The parameters evaluated include pH, total titratable acidity, total reducing sugars, total flavonoid content, and antioxidant activity. The results showed that the pH of kombucha decreased with increasing fermentation time, from 3,92 ± 0,02 on day 0 to 3.08 ± 0.01 on day 12, indicating enhanced activity of acetic acid-producing microorganisms during fermentation. Titratable acidity increased from 0.11 ± 0.01% to 0.93 ± 0.02%, reflecting the accumulation of organic acids. Total reducing sugar levels initially increased on day 3 (655.72 ± 0.68 mg/mL), then declined to 309.94 ± 0.83 mg/mL by day 12, possibly due to microbial consumption. The total flavonoid content showed a consistent increase from 0.008 mg/mL QE to 0.019 mg/mL QE, indicating the gradual release of bioactive compounds throughout the fermentation process. The highest antioxidant activity was recorded on day 9 (74 ± 0.47%), followed by a slight decrease on day 12 (72 ± 0.00%). These findings suggest that the inclusion of sambiloto leaves does not inhibit fermentation but rather enhances the production of beneficial bioactive compounds. Therefore, sambiloto holds strong potential as a functional ingredient in kombucha rich in natural antioxidants.

Keywords: Antioxidant Activity; Fermentation; Flavonoids; Kombucha; Sambiloto Leaves.

Introduction

Kombucha is a traditional fermented beverage made from a tea and sugar solution, fermented by a community of microorganisms that form a cellulose biofilm known as the *Symbiotic Culture of Bacteria and Yeast* (SCOBY) [1]. The SCOBY consists of *Acetobacter xylinum* and various types of yeast such as *Brettanomyces*, *Zygosaccharomyces*, and *Saccharomyces* [2]. During the fermentation process, this culture produces various bioactive compounds, including organic acids (lactic, acetic, malic, oxalic, gluconic, butyric, and nucleic acids), vitamins, amino acids, and enzymes [3],[4], all of which contribute to health benefits such as improved digestion, detoxification, and cell regeneration.

The duration of fermentation affects the chemical characteristics, flavonoid content, and antioxidant activity of kombucha. Generally, longer fermentation leads to higher levels of phenolic and flavonoid compounds; however, antioxidant activity may decline after reaching a certain point [5]–[7]. In addition to being a probiotic beverage, kombucha also exhibits antidiabetic properties and has been shown to reduce cholesterol and triglyceride levels [8].

One of the key contributors to kombucha's health benefits is its antioxidant content. Antioxidant compounds such as phenolics, flavonoids, and vitamins play a vital role in neutralizing free radicals that can cause degenerative diseases including cancer, diabetes, and cardiovascular disorders [9]–[14]. Since the body's endogenous antioxidants are often insufficient, the intake of external antioxidants is essential [15].

One herbal plant with high antioxidant potential is sambiloto (*Andrographis paniculata*). Studies have shown that sambiloto extract exhibits strong antioxidant activity, with IC_{50} values ranging from 15.55 to 16.63 µg/mL [16],[17]. Sambiloto contains several active compounds, including andrographolide, flavonoids, tannins, and saponins, which are beneficial for treating various degenerative diseases [18]–[21]. Notably, the water extract of sambiloto has been found to have higher antioxidant activity than its ethanol extract, due to its higher flavonoid content [22],[23].

Compared to other medicinal plants such as basil leaves, kejibeling leaves, or mengkudu leaves, sambiloto demonstrates the highest antioxidant activity [24]–[27]. However, to date, there has been no research exploring the development of kombucha beverages made from sambiloto leaves. Therefore, this study aims to investigate the potential of sambiloto leaves as a main ingredient in the production of kombucha.

Research Methods

Preparation of Sambiloto Leaf Kombucha

A total of 500 grams of fresh sambiloto leaves were washed, air-dried at room temperature, chopped into approximately 0.5 cm pieces, and packed into tea bags,

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each containing 0.5 grams of leaves. To prepare the base solution, 1000 mL of sterile distilled water was brought to a boil, after which four tea bags of sambiloto leaves were added and steeped for 30 minutes. The tea bags were then removed, and 100 grams of granulated sugar was added to the solution, stirred until completely dissolved, and transferred into a sterile glass jar. Once the solution had cooled to room temperature, 100 mL of kombucha liquid starter and one SCOBY were added. The jar was covered with a sterile cloth and allowed to ferment at room temperature for 0, 3, 6, 9, and 12 days [19].

pH Value Test

The pH analysis of sambiloto leaf kombucha was conducted using a pH meter calibrated with standard buffer solutions of pH 4.01 and 6.86. A total of 30 mL of sample from each fermentation period (0, 3, 6, 9, and 12 days) was transferred into a beaker, and the pH was measured using the calibrated pH meter until a stable reading was obtained [20].

Total Titratable Acid Test (TTA)

The analysis of total titratable acidity in sambiloto leaf kombucha began with the titration of a blank solution consisting of 20 mL of distilled water and three drops of 1% phenolphthalein (PP) indicator using 0.1 N NaOH to determine the correction volume. Subsequently, 10 mL of samples from fermentation periods of 0, 3, 6, 9, and 12 days were diluted in a 100 mL volumetric flask, homogenized, and filtered. Then, 20 mL of the filtrate was titrated using 1% PP indicator and 0.1 N NaOH, performed in triplicate [20].

Total Reducing Sugar Test

The total reducing sugar test began with the determination of the maximum wavelength (λ max) of the glucose compound. This was done by reacting 1 mL of a 0.05 mg/mL glucose solution with 1 mL of Nelson's reagent, followed by heating in a water bath for 30 minutes and then cooling to room temperature. After cooling, 1 mL of arsenomolybdate reagent was added and mixed until the precipitate completely dissolved. Subsequently, 7 mL of distilled water was added, and the absorbance was measured in the wavelength range of 400–800 nm. A standard curve was prepared using various glucose concentrations (0.02–0.18 mg/mL) treated with the same procedure, in order to obtain a linear regression equation. Kombucha samples from fermentation days 0, 3, 6, 9, and 12 were filtered, diluted as needed, and reacted using the Nelson-Somogyi method. Absorbance was measured at a wavelength of 745.5 nm to determine the total reducing sugar content in sambiloto leaf kombucha. The absorbance values obtained were used to calculate the total reducing sugar concentration based on the standard curve [21].

Flavonoid Test

The determination of total flavonoid content in sambiloto leaf kombucha began with identifying the maximum wavelength of quercetin compounds. This was done by reacting 0.5 mL of a 0.01 mg/mL quercetin equivalent (QE) solution in a 10 mL volumetric flask with 2 mL of ethanol p.a., 0.2 mL of 10% $AlCl_3$, 0.2 mL of 1 M potassium acetate, and adding distilled water up to the mark. The solution was incubated for one hour at room temperature, and absorbance was measured in the range of 400–450 nm using a UV-Vis spectrophotometer. A standard curve was then prepared using quercetin solutions with concentrations ranging from 0.001 to 0.009 mg/mL QE, treated in the same manner to obtain a linear regression equation. Kombucha samples from fermentation days 0, 3, 6, 9, and 12 were reacted with the same reagents, incubated for one hour, and measured for absorbance at a wavelength of 437.4 nm. The absorbance values were used to calculate the total flavonoid content of the sambiloto leaf kombucha by referencing the standard curve [22].

Antioxidant Activity Test

The antioxidant activity analysis was conducted by first determining the maximum wavelength (λ max) of the DPPH solution. The solution was prepared by mixing 1 mL of sterile distilled water, 3 mL of 96% ethanol, and 1 mL of 0.2 mM DPPH solution. It was then incubated for 30 minutes in the dark at room temperature, and the absorbance was measured in the range of 400–600 nm using a UV-Vis spectrophotometer. Analysis of the sambiloto leaf kombucha samples (fermentation days 0, 3, 6, 9, and 12) was carried out by adding DPPH and ethanol solutions, followed by incubation and measurement of absorbance at a wavelength of 518.5 nm. A positive control in the form of a black tea solution was also analyzed using the same procedure for comparison [20].

Results and Discussion

The analysis of pH values at various fermentation times aimed to evaluate the extent to which the fermentation process progressed optimally and how the acidity level changed over time. Based on the pH results, the data were found to be not normally distributed ($p < 0.05$); therefore, the Kruskal–Wallis test was applied. The results indicated that fermentation time had a significant effect on the decrease in the pH of sambiloto leaf kombucha ($p < 0.05$). Further analysis using the Mann–Whitney test was conducted to identify significant differences between treatments. The following are the data obtained from the pH analysis of sambiloto leaf kombucha:

Table 1. Results of pH Value Analysis of Sambiloto Leaf Kombucha

No.	Fermentation Time (days)	pH Value
1	0	3.92 ± 0.02^{a}
2	3	3.59 ± 0.01^{b}
3	6	3.44 ± 0.03^{c}
4	9	3.28 ± 0.01^{d}
5	12	3.08 ± 0.01^{e}

Note: Values followed by the same letter indicate no significant difference (>0.05)

Table 1 shows that the fermentation period from 0 to 12 days significantly affected the decrease in the pH of sambiloto kombucha. The pH value of sambiloto leaf

kombucha consistently decreased with increasing fermentation time, from 3.92 ± 0.02 on day 0 to 3.08 ± 0.01 on day 12. This value remains within acceptable consumption standards, as it falls below the maximum limit of 4.5 and above the minimum limit of 2.5 [23]. This downward trend in pH is consistent with findings from previous studies: black tea kombucha showed a pH decrease from 6.60 to 2.30 [1], faloak bark kombucha from 3.86 to 2.78 [20], and red dragon fruit peel kombucha from 4.00 to 3.00 [24]. The decrease in pH in sambiloto leaf kombucha is closely related to the increase in Total Titratable Acidity (TTA). This test aims to determine the effect of fermentation duration on TTA using the acid–base titration method. Based on the TTA results, the data were not normally distributed ($p < 0.05$); therefore, the *Kruskal–Wallis* test was applied and revealed that fermentation time had a significant effect on the increase in TTA ($p < 0.05$). Further analysis using the *Mann–Whitney* test was conducted to identify significant differences between treatments. The following graph illustrates the relationship between fermentation time and the TTA of sambiloto leaf kombucha:

Table 2. Results of TTA analysis of sambiloto leaf kombucha

No.	Fermentation Time (days)	TTA (%)
1	0	0.11 ± 0.01[a]
2	3	0.37 ± 0.02[b]
3	6	0.51 ± 0.04[c]
4	9	0.72 ± 0.05[d]
5	12	0.93 ± 0.02[e]

Note: Values followed by the same letter indicate no significant difference (>0.05)

Table 2 shows that the fermentation period from 0 to 12 days significantly affected the increase in the Total Titratable Acidity (TTA) of sambiloto leaf kombucha, from 0.11 ± 0.01% to 0.93 ± 0.02%. This increase is consistent with findings from previous studies: breadfruit and lemon leaf kombucha showed an increase in TTA from 0.038% to 0.348% [19], faloak bark kombucha from 0.19% to 0.62% [20], and cascara Arabica coffee kombucha from 0.06% to 1.51% [25]. The decrease in pH and increase in TTA in sambiloto leaf kombucha occurred as a result of the fermentation process, during which sucrose is converted by bacteria and yeast into various organic acids such as lactic acid, acetic acid, gluconic acid, and glucuronic acid [1, 26]. Sucrose is hydrolyzed into glucose and fructose by the enzyme invertase, which serves as the primary energy source for the microorganisms involved in fermentation [25]. In line with this process, the total reducing sugar content in the kombucha also undergoes changes. Based on the results, the total reducing sugar data were not normally distributed ($p < 0.05$), and thus the Kruskal–Wallis test was applied. The test indicated that fermentation time had a significant effect on the total reducing sugar content of sambiloto leaf kombucha ($p < 0.05$). Further analysis using the Mann–Whitney test was conducted to identify significant differences between treatments. The following are the data obtained from the analysis of total reducing sugar in sambiloto leaf kombucha:

Table 3. Total reducing sugars of sambiloto leaf kombucha

No.	Fermentation Time (days)	Concentration (mg/mL)
1	0	46.38 ± 1.37[a]
2	3	655.72 ± 0.68[b]
3	6	581.27 ± 0.69[c]
4	9	434.64 ± 1.04[d]
5	12	309.94 ± 0.83[e]

Note: Values followed by the same letter indicate no significant difference (>0.05)

Table 3 shows that the fermentation period from 0 to 12 days significantly affected the changes in total reducing sugar content of sambiloto leaf kombucha, with an increase from 46.38 ± 1.37 mg/mL to 655.72 ± 0.68 mg/mL on the 3rd day, followed by a decrease to 309.94 ± 0.83 mg/mL on the 12th day. This trend is consistent with findings from other studies: black tea kombucha showed a reduction in total reducing sugars from 122 mg/L to 7 mg/L [5], cascara Arabica coffee kombucha maintained a level of 7.46% [25], and faloak bark kombucha decreased from 9.09% to 7.15% [20]. At the early stage of fermentation, sucrose hydrolysis is minimal due to low yeast activity. Over time, the enzyme invertase begins to break down sucrose into glucose and fructose, resulting in an increase in reducing sugar levels [27]. As fermentation progresses further, reducing sugars are utilized by microorganisms as an energy source, leading to a decline in their concentration [25], [28]. Changes in total reducing sugar during fermentation not only reflect microbial substrate consumption but are also associated with the transformation of other bioactive compounds, including flavonoids. Based on the total flavonoid content results, the data were not normally distributed ($p < 0.05$); therefore, the Kruskal–Wallis test was applied and revealed that the fermentation period had a significant effect on increasing the total flavonoid content of sambiloto leaf kombucha ($p < 0.05$). Further analysis using the Mann–Whitney test was conducted to identify significant differences between treatments. The following are the results of the total flavonoid content analysis of sambiloto leaf kombucha:

Table 4. Total flavonoids of sambiloto leaf kombucha

No.	Fermentation Time (days)	Concentration (mg/mL QE)
1	0	0.008[a]
2	3	0.010[b]
3	6	0.012[c]
4	9	0.015[d]
5	12	0.019[e]

Note: Values followed by the same letter indicate no significant difference (>0.05)

Table 4 shows that the fermentation period from 0 to 12 days significantly affected the increase in total flavonoid content of sambiloto leaf kombucha, from 0.008 mg/mL QE to 0.019 mg/mL QE. This increase aligns with findings from other studies, such as faloak bark kombucha, which showed an increase from 3494.4 mg/L QE to 4135.14 mg/L QE [20], and black tea kombucha infused with butterfly pea flower, which increased from 35.7 mg QE/g to 68.4 mg QE/g [29]. Flavonoids are phenolic compounds found in sambiloto leaves that possess high antioxidant activity.

During fermentation, microbial enzymes can enhance their bioavailability by converting complex compounds into more biologically active flavonoids [30, 20]. The total flavonoid content in sambiloto leaf kombucha is closely associated with its antioxidant activity, as flavonoids act as natural antioxidants that neutralize free radicals through electron or hydrogen atom donation [31]. Based on the antioxidant activity results, the data were not normally distributed ($p < 0.05$), prompting the use of the Kruskal–Wallis test, which showed that fermentation time had a significant effect on increasing the antioxidant activity of sambiloto leaf kombucha ($p < 0.05$). Further analysis using the Mann–Whitney test was conducted to determine the differences between treatments. The following are the results of the antioxidant activity analysis of sambiloto leaf kombucha:

Table 5. % inhibition values of samples

No.	Sample	% inhibition
1	Kombucha 0 days	69 ± 0.47^{a}
2	Kombucha 0 days	70 ± 0.47^{b}
3	Kombucha 0 days	73 ± 0.47^{c}
4	Kombucha 0 days	74 ± 0.47^{d}
5	Kombucha 0 days	72 ± 0.00^{c}
6	Black tea	62 ± 0.00^{e}

Note: Values followed by the same letter indicate no significant difference (>0.05)

Table 5 shows that the fermentation period from 0 to 12 days had a significant effect on increasing the antioxidant activity of sambiloto leaf kombucha, from 69 ± 0.47% on day 0 to a peak of 74 ± 0.47% on day 9, followed by a slight decrease to 72 ± 0.00% on day 12. This pattern of increase and subsequent decrease is consistent with findings from previous studies: red galangal (*Alpinia purpurata*) kombucha showed an increase in antioxidant activity from 80.52% on day 0 to 89.75% on day 8, before slightly decreasing to 79.46% on day 10 [32]; green tea kombucha increased to 93.79% on day 7, then slightly declined to 93.21% on day 11 [5]; and another study on green tea kombucha showed an increase from 90,83% on day 1 to 92.05% on day 10, then a slight decrease to 91.24% on day 15 [33]. The increase in antioxidant activity of sambiloto leaf kombucha is correlated with the accumulation of flavonoids during fermentation. However, the decline after day 9 is likely due to a drop in pH to a level that may inhibit or damage microbial activity and lead to the degradation of antioxidant compounds [6]. The results also showed that the antioxidant activity of sambiloto leaf kombucha was higher than that of fermented black tea, which was used as a positive control. This enhanced activity is attributed to the high flavonoid content of sambiloto leaves, which contributes significantly to the antioxidant capacity. Although black tea also contains phenolic compounds, the fermentation of sambiloto leaves results in a more active combination of compounds capable of neutralizing free radicals [34]. Therefore, sambiloto leaf kombucha shows strong potential as a natural functional beverage with powerful antioxidant effects.

Conclusion

This study shows that the use of sambiloto leaves in kombucha production does not inhibit the fermentation process. Over the 12-day fermentation period, a decrease in pH, an increase in total titratable acidity, a reduction in total reducing sugars, and an increase in total flavonoid content were observed, indicating that fermentation occurred effectively. The antioxidant activity of sambiloto leaf kombucha increased until day 9, followed by a slight decline on day 12, possibly due to a drop in pH that inhibited microbial activity and led to the degradation of antioxidant compounds. Overall, sambiloto leaf kombucha has strong potential as a functional beverage with high antioxidant activity, which may contribute to the prevention of oxidative damage.

Author's Contribution

Tri Diansy Yana Purwono: The author of the article, responsible for preparing the content, analyzing the data, and presenting the information as part of academic activities. Rudiana Agustini: The supervisor who provided direction, guidance, and valuable input throughout the writing process of this article.

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