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In Vitro Evaluation of Antioxidant and Lipase Inhibition Properties of Rosella (*Hibiscus sabdariffa* L.) Flower Kombucha

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ABSTRACT

Kombucha, a traditional fermented beverage made from tea, sugar, and a kombucha culture, is known for its unique flavor, aroma, and health benefits. The fermentation process, typically lasting between 8 and 12 days, influences the beverage's physical and chemical properties. While traditionally brewed from tea, alternative substrates like rosella flowers (*Hibiscus sabdariffa* L.) are also used. Rosella flowers, rich in vitamins and bioactive compounds, have demonstrated potential in combating obesity and exhibiting antioxidant properties. This study aimed to evaluate the chemical and biological characteristics of rosella flower kombucha, particularly its antioxidant and lipase inhibitor activities. The fermentation process led to significant biochemical changes, including a decrease in pH and reducing sugar content, an increase in microbial biomass, and a reduction in ascorbic acid content. The results revealed that rosella flower kombucha had a higher total phenol content and better DPPH radical scavenging activity compared to rosella flower infusion, indicating enhanced antioxidant potential. Additionally, rosella kombucha demonstrated more effective lipase inhibition, suggesting its potential benefits for weight management and metabolic health. These findings emphasize rosella kombucha's potential as a healthful functional beverage, recommending further research in these areas.

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1. Introduction

Kombucha is a traditional fermented beverage known for its unique flavor and aroma, created from a blend of tea, sugar, and a kombucha culture. Originally introduced by the Chinese, it gained recognition in Indonesia as "kombu tea" during the 1930s. Kombucha, also referred to as Manchurian tea pellicle, is valued for its health benefits due to its diverse beneficial compounds (Naland, 2008). The fermentation process of kombucha typically lasts between 8 and 12 days at temperatures ranging from 18°C to 20°C. Variations in fermentation time from 7 to 14 days can significantly alter its physical and chemical properties, including total acidity, pH, alcohol content, and antioxidant levels. Optimal results are achieved with fermentation periods of 5 to 14 days (Sari, 2014). Research indicates that kombucha offers several health benefits, including the ability to inhibit lipid absorption in cases of obesity (Kim et al., 2020).

Roselle flowers (*Hibiscus sabdariffa* L.) have been selected as an alternative substrate for kombucha production due to their rich nutritional profile and bioactive compound content. Unlike traditional kombucha, which is typically brewed using tea, roselle flowers provide a unique source of essential nutrients such as vitamin C, vitamin A, calcium, and a variety of amino acids (Windyaswari, 2018). Additionally, they contain significant levels of anthocyanins, the flavonoid compounds responsible for the vibrant red color of the flowers, which are known for their strong antioxidant properties (Gruenwald et al., 2004). These compounds contribute to the potential health benefits of roselle, particularly in promoting antioxidant activity and protecting against oxidative stress. The use of alternative substrates like roselle in kombucha fermentation not only introduces a broader range of bioactive compounds but also expands the potential health benefits of the beverage. According to Susilowati (2013), the type of substrate used can influence the fermentation process, affecting the final product's nutritional value and bioactivity. Given roselle's well-documented benefits, including its content of anthocyanins and flavonoids, its inclusion in kombucha fermentation presents an opportunity to create a functional beverage with enhanced health-promoting properties. This aligns with the growing interest in using diverse ingredients in kombucha to optimize its bioactive content.

Rosella flowers have demonstrated potential in combating obesity due to their content of flavonoids, anthocyanins, phenolic acids, and organic acids, which are beneficial for pharmacological applications (Nerdy, 2014). Antioxidant activity studies on rosella petals have shown promising results. For example, a study by Nopiyanti and Harjanti (2016) found that ethanol extracts of rosella flowers exhibited an IC_{50} of 8.416% in free radical measurements using the DPPH method. Additionally, Ariani et al., (2019) reported that rosella flower ethanol extracts demonstrated a lipase inhibitor activity of 83.65%±0.003. Despite the extensive research on various teas as lipase inhibitors and antioxidants, there is a gap in the exploration of rosella kombucha's potential in these areas. Further research is needed to evaluate the effectiveness of rosella kombucha as a lipase inhibitor and antioxidant.

2. Methods

2.1. Tools and Material

The tools used include analytical scales, test tubes, pipettes, micropipettes, pH meter, beakers, cuvettes, a UV-Vis spectrophotometer, centrifuge tubes, and a vortex mixer. The materials required for the study are dried rosella flowers (*H. sabdariffa* L.), kombucha starter culture, SCOBY (Symbiotic Culture of Bacteria and Yeast), gallic acid, DPPH (*1,1-Diphenyl-2-picrylhydrazyl*) solution, pancreatic lipase, buffer Tris-HCl, pH 8, pNPP (*p-Nitrophenyl Palmitate*), acetonitrile, and DMSO.

2.2. Kombucha Starter Preparation

Kombucha starter culture known as "SCOBY", (generally consisting of *Acetobacter xylinum*, *Gluconobacter* and *Saccharomyces cerevisiae*) obtained from Laboratory of Microbiology Universitas Ahmad Dahlan, Yogyakarta. The starter culture used in this study was stored in a refrigerator (4°C) and consisted of broth acid and a layer of cellulose ("tea mold" floating on the surface).

2.3. Rosella Flower Kombucha Preparation

The process of making rosella flower kombucha began with the creation of a rosella flower infusion. This involved steeping 1 gram of dried rosella flowers in 200 mL of boiling water for 15 minutes. Following the brewing process, the resulting mixture was strained into a sterile glass container or jar to extract the liquid filtrate. Subsequently, 20 grams of powdered sugar, constituting a 10% weight/volume ratio, were added to the filtrate and stirred until the sugar had completely dissolved. The sweetened infusion was then covered and allowed to cool to approximately 30°C. At this lower temperature, 10% of the starter culture, consisting of 12 mL of liquid starter and 8 grams of SCOBY starter material, was introduced. The jar was securely covered with sterile gauze and the mixture was left to ferment at room temperature (28-30°C). The kombucha was fermented for various durations (0, 3, 6, 9, and 12 days).

2.4. Chemical Characteristics of Kombucha

The chemical characteristics of kombucha were assessed through pH measurement, reducing sugar content, total phenol content, and vitamin C (ascorbic acid) content. The pH acidity of the product was measured using a pH meter with three replicates. Reducing sugar content was determined using the Somogyi-Nelson method. The total phenolic content was evaluated following the Folin-Ciocalteu method described by Agbor et al., (2014). For the vitamin C content test, the iodimetric titration method was employed. Twenty-five mL of rosella flower kombucha was pipetted and diluted with 100 mL of distilled water, and the initial weight was recorded. Ten mL of this diluted sample was transferred into a 250 mL Erlenmeyer flask, and 2 mL of 1% amylum indicator solution was added. Titration was then performed using 0.01 N iodine until a blue color developed. The concentration of vitamin C was calculated using the formula: (A×0.88×FP)/W, where A represents the volume of iodine used for titration (mL), 0.88 mg is the amount of ascorbic acid equivalent to 1 mL of 0.01 N iodine solution, FP is the dilution factor, and W is the sample weight (mg).

2.5. Biological Characteristics of Kombucha

The biological characteristics of kombucha were assessed by measuring the pellicle dry weight and optical density. The optical density (OD) of the kombucha was measured using a spectrophotometer at a wavelength of 600 nm. The dry weight of the SCOBY, also referred to as pellicle dry weight, was determined by drying it in an oven at 80°C until a constant weight was reached, after which it was weighed.

2.6. Antioxidant Activity Assay Using the DPPH Method

The sample tested for antioxidant activity was an infusion of rosella flowers and kombucha that had been fermented for 12 days. The antioxidant activity of rosella flower kombucha was evaluated using the DPPH method. Initially, a DPPH solution was prepared by dissolving 0.5 mg of DPPH in 50 mL of methanol p.a to achieve a 10 mg/L concentration. The control solution was made by homogenizing this mother solution and incubating it for 30 minutes at 37°C. For the antioxidant activity test, 1 mL of the 10 mg/L DPPH solution was combined with 50 µL of the sample liquid, and the mixture was diluted with methanol p.a to a final volume of 5 mL. This mixture was then incubated for 30 minutes at 37°C, after which the absorbance was measured at 515 nm. Antioxidant activity was assessed based on the inhibition of DPPH radical uptake. The percentage inhibition was calculated using the formula (Aji et al., 2023a):

$$\%\ \text{DPPH Inhibition} = \frac{\text{Abs}_{\text{Blanko}} - \text{Abs}_{\text{sampel}}}{Abs_{Blanko}} x\ 100\%$$

where $\text{Abs}_{\text{Blank}}$ denotes the absorbance of the DPPH radicals at 515 nm, and $\text{Abs}_{\text{Sample}}$ represents the absorbance of the sample at the same wavelength.

2.7. *In Vitro* Lipase Inhibitor Activity Assay

The samples tested for *in vitro* lipase inhibitor activity assay included rosella flower infusion and kombucha, which had been fermented for 12 days. The lipase inhibitor activity test was conducted as follows: A stock solution of rosella flower infusion was first prepared by taking 100 mL of the pure infusion. Kombucha samples that had undergone fermentation for 3, 6, 9, and 12 days were then centrifuged to obtain clear solutions, which were subsequently stored at 4°C. For the enzyme preparation, a 1 mg/mL stock solution of pancreatic lipase was prepared by dissolving 10 mg of the enzyme in 10 mL of a 50 mM Tris-HCl buffer with a pH of 8. Additionally, a 50 mM stock solution of pNPP in acetonitrile was prepared by dissolving 0.19 g of pNPP in 9.81 mL of acetonitrile, making up a final volume of 10 mL. To test the inhibitor activity, a mixture was prepared containing 0.1 mL of the 1 mg/mL lipase solution, 0.2 mL of either the rosella flower infusion or kombucha (at various concentrations), and 0.7 mL of the 50 mM Tris-HCl buffer (pH 8). This mixture was incubated for 15 minutes at room temperature (37°C), after which 0.1 mL of the 50 mM pNPP solution was added. The solution was then incubated for an additional 30 minutes at room temperature, and the absorbance was measured at 410 nm using a spectrophotometer. The test was conducted in duplicate. For the positive control test, a stock solution of orlistat was prepared by dissolving 20 mL of orlistat in 20 mL of distilled water. The enzyme stock solution and the pNPP substrate were prepared in the same manner as previously described. The activity test involved mixing 0.1 mL of the 1 mg/mL lipase solution with 0.2 mL of orlistat (at various concentrations) and 0.7 mL of the 50 mM Tris-HCl buffer (pH 8). This mixture was incubated for

15 minutes at 37°C, followed by the addition of 0.1 mL of the 50 mM pNPP solution. After another 30 minutes of incubation at room temperature, the absorbance was measured at 410 nm using a spectrophotometer. This test was also performed in duplicate. The percentage of inhibition was calculated using the formula (Aji et al., 2023b):

$$\%\ \text{Inhibition} = \frac{A_0 - A}{A_0} x\ 100\%$$

where A_0 represented the lipase activity without an inhibitor, and A represented the activity in the presence of an inhibitor.

2.8 Data Analysis

The experiment was conducted with three repetitions to ensure the reliability of the results. A statistical t-test was performed on the data to compare the samples of roselle flower infusion and kombucha made from roselle flowers. This analysis was used to determine if significant differences existed between the two samples, with a p-value of less than 0.05 indicating statistical significance.

3. Results and Discussion

During the 12-day fermentation process of rosella flower kombucha, significant biochemical changes occur that affect the final product's quality and nutritional value. As showed in Figure 1, the pH of the kombucha decreases from 3.79 to 1.97, indicating a rise in acidity. This acidification, driven by microbial activity, aligns with findings from previous studies by Wang et al., (2023) demonstrated that the pH in kombucha typically drops as fermentation progresses due to the production of acetic acid, gluconic acid, and glucuronic acid by the kombucha symbiotic culture of bacteria and yeast (SCOBY). Concurrent with the decrease in pH, the reducing sugar content drops from 3.01 mg/mL to 1.97 mg/mL. This gradual decline in sugar levels, in line with observations by Kushargina et al., (2024), suggests that sugars are being metabolized by the microbes, leading to acid production and contributing to the pH reduction. The study by Cohen et al., (2023) noted a similar trend, where a decrease in reducing sugars was closely associated with the formation of organic acids throughout the fermentation process.

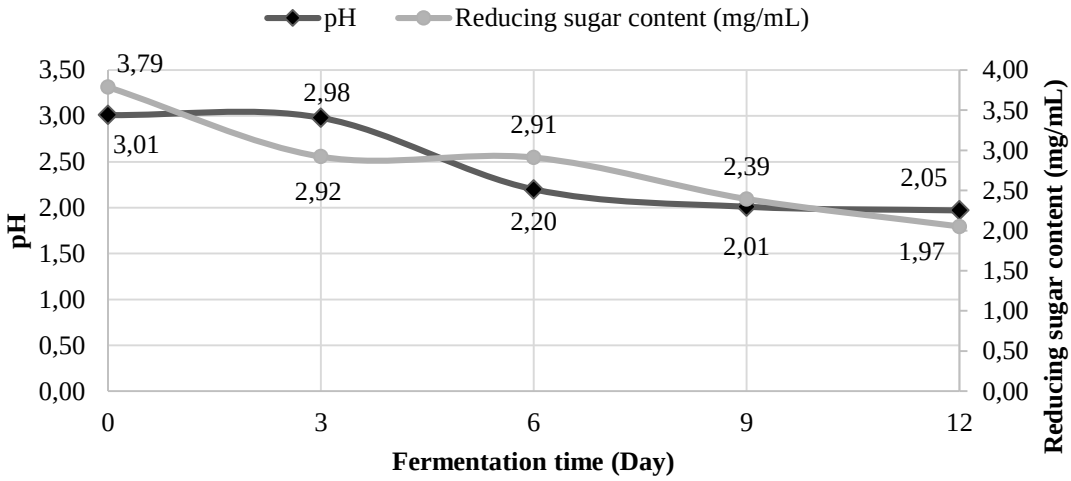

Figure 1. Changes in pH and reducing sugar content during rosella flower kombucha fermentation

Changes in OD_{600} and dry weight of the pellicle (cellulose pellicle) during 12 days of roselle flower kombucha fermentation are depicted in Figure 2. The optical density (OD) at 600 nm, a measure of microbial growth, increases from 0.12 to 0.28, indicating a steady accumulation of microbial biomass. This finding is consistent with previous studies, such as those by Ahmed et al., (2020), which reported an increase in OD_{600} corresponding with the growth of the kombucha SCOBY throughout fermentation. This microbial proliferation is correlated with a substantial increase in the pellicle dry weight, from 1.49 grams to 5.07 grams, with the most significant growth occurring after day 6. This growth pattern supports the findings of Charoenrak et al., (2023), who observed similar trends in cellulose pellicle development during kombucha fermentation. The term "pellicle" here refers to the cellulose pellicle produced by a symbiotic culture of bacteria and yeast (SCOBY),

including species such as *A. xylinum*, known for its cellulose-synthesizing abilities. This suggests that the conditions created during the early stages of fermentation are favorable for microbial growth, which in turn supports the accumulation of pellicle biomass, likely through the activities of these bacteria and yeast in breaking down substrates that facilitate pellicle development.

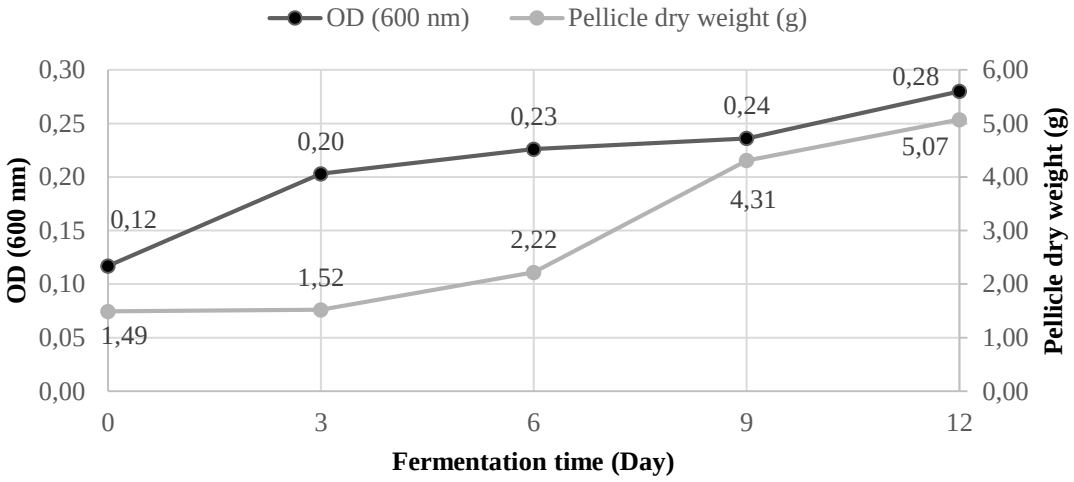

Figure 2. Changes in optical density (OD) and pellicle dry weight during rosella flower kombucha fermentation

In relation to the ascorbic acid content, Figure 3 shows a marked decline from 15.22 mg/100g to 7.70 mg/100g over the fermentation period. The most rapid reduction occurs in the early stages (days 0 to 3), after which the decline slows and approaches a more stable value by day 12. This reduction in ascorbic acid could be attributed to its degradation under acidic conditions or microbial utilization. Vitamin C, an essential vitamin but unstable molecule, is prone to degradation due to its sensitivity to light, alkaline pH, high temperatures, oxygen, heavy metals, UV rays, and extended storage times. During fermentation, this degradation is exacerbated by the acidic conditions and the microbial activities involved. Specifically, the oxidation process and the presence of ascorbate oxidase enzymes produced by the fermenting microorganisms contribute significantly to the loss of ascorbic acid (Jafarpour & Hashemi, 2023).

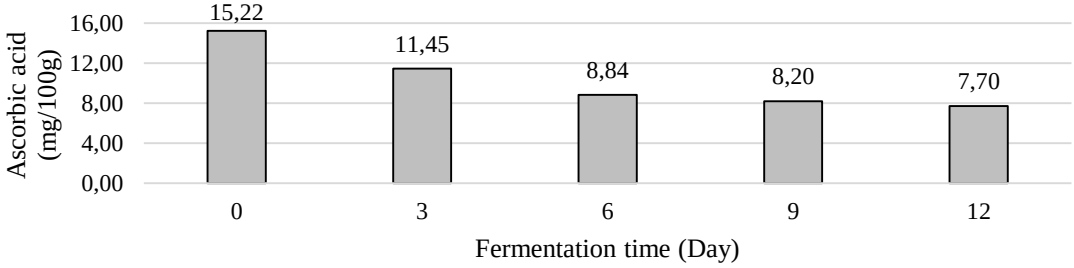

Figure 3. Ascorbic acid content over time during rosella flower kombucha fermentation

The comparison between Roselle flower infusion and Roselle kombucha reveals several notable differences in their chemical and antioxidant properties. Roselle kombucha contains a higher total phenol content (0.02 µL/mL) compared to Roselle flower infusion (0.01 µL/mL), suggesting a potential for greater antioxidant activity due to the higher concentration of phenolic compounds. Although the statistical analysis shows no significant difference in total phenol content (p=0.26), other key parameters reveal significant enhancements in kombucha's bioactivity. Roselle kombucha exhibited a significantly higher % inhibition of DPPH at 25% (v/v) (62.50±0.43) compared to Roselle flower infusion (55.88±0.67), with a p-value of 0.00, indicating its superior free radical scavenging ability. Additionally, the IC50 value for antioxidant activity, which measures the concentration needed to inhibit 50% of free radicals, was significantly lower in kombucha (16.25±0.02) than in the infusion (16.85±0.14), demonstrating kombucha's greater potency as an antioxidant (p=0.00). When assessing lipase inhibition, Roselle kombucha also

outperformed the infusion. The IC50 for lipase inhibition was significantly lower in kombucha (0.15±0.02) compared to the infusion (0.25±0.03), with a p-value of 0.01, indicating stronger inhibition of lipase activity in kombucha.

The higher bioactivity in kombucha can be attributed to the fermentation process. During fermentation, microbial metabolism breaks down complex compounds into simpler bioactive molecules, such as organic acids, flavonoids, and additional phenolic compounds. This process can increase the availability and concentration of these bioactive compounds, enhancing the antioxidant and enzyme-inhibitory effects of the beverage. Moreover, fermentation can lead to the formation of new compounds with improved biological activity, which are not present in the original infusion. This explains why kombucha consistently exhibits stronger antioxidant and lipase inhibitory activity compared to the non-fermented Roselle infusion. These findings align with previous research, such as Aji et al., (2023b), which found that butterfly pea flower kombucha was more effective in inhibiting pancreatic lipase than the tea infusion. Moreover, studies by Cardoso et al., (2020) and Jayabalan and Waisundara (2019) have demonstrated that kombucha fermentation can enhance bioactive compound concentration and antioxidant capabilities, further supporting the enhanced properties of Roselle kombucha.

Table 1. Comparison of total phenol content, antioxidant activity, and in vitro lipase inhibition between roselle flower infusion and roselle flower kombucha

	Roselle flower infusion	Roselle flower kombucha (12^{th} day fermentation)	p-value
Total phenol content (GAE µL/mL)	0.01±0.00	0.02±0.01	0.26
% inhibition of DPPH at 25% (v/v)	55.88±0.67	62.50±0.43	0.00
IC_{50} antioxidant activity (% v/v)	16.85±0.14	16.25±0.02	0.00
IC_{50} inhibition of lipase activity (% v/v)	0.25±0.03	0.15±0.02	0.01

Note: T-test comparison of roselle flower infusion and kombucha samples, showing p-values for significance ($p<0.05$).

The study comparing Roselle flower infusion and Roselle kombucha highlights several significant implications for health and functional beverage development. Roselle kombucha's superior antioxidant properties, evidenced by its higher total phenol content and better DPPH inhibition, suggest it could offer more effective protection against oxidative stress compared to Roselle flower infusion. This enhanced antioxidant activity may contribute to reduced risk of chronic diseases and support overall health. Additionally, kombucha's greater lipase inhibition suggests potential benefits for managing fat digestion and absorption, making it a promising option for weight management and metabolic health.

4. Conclusion

The study demonstrates that rosella flower kombucha possesses superior antioxidant and in vitro lipase inhibitory properties compared to rosella flower infusion, likely due to the fermentation process that increases the concentration of bioactive compounds. Based on the data, a 12-day fermentation period is recommended for optimal Roselle flower kombucha production. However, further research is needed to optimize the ideal fermentation duration. These findings suggest that kombucha, particularly when fermented with functional ingredients like rosella, could serve as a potent health-promoting beverage. The enhanced antioxidant activity and lipase inhibition indicate potential benefits in managing oxidative stress and fat metabolism, making rosella kombucha a promising candidate for functional food development.

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