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The Effect of Fermentation Time Variation on Antioxidant Activity of Fermented Robusta Coffee Leaves (*Coffea canephora* Pierre ex A.Froehner) Brew

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ABSTRACT
Antioxidants are electron-donor compounds that can delay, slow down and prevent free radical reactions. Fermentation is a chemical change that occurs due to the activity of microorganisms which can increase the amount of phenols in tea thereby increasing its antioxidant activity. Fermentation is carried out with the help of a Symbiotic Culture of Bacteria and Yeast (SCOBY) culture, which is commonly used in the production of kombucha. Kombucha is a fermented tea drink produced from leaves that contain high phenols such as robusta coffee leaves (*Coffea canephora* Pierre ex A.Froehner). Factors that affect the fermentation process are pH and fermentation time. This study aimed to explain the effect of fermentation time on the antioxidant activity of fermented robusta coffee leaves brew. Brewed robusta coffee leaves were made by brewing robusta coffee leaves simplicia powder, adding kombucha liquid starter, then fermented for 0; 3; 7; 14; and 21 days, then pH and antioxidant activity was measured using UV-Visible spectrophotometer using the 2,2-dipheny-1,1-picrylhydrazyl (DPPH) at a wavelength of 516 nm. This study showed that 0; 3; 7; 14; and 21 days fermented robusta coffee leaves brew have pH of 6.07; 4.62; 4.07; 3.21; and 2.74, and IC_{50} values is 285.1645 µg/ml; 219.7012 µg/ml; 192.2325 µg/ml; 187.0434 µg/ml; and 173.9249 µg/ml. There is an effect of fermentation time variation on the antioxidant activity of fermented robusta coffee leaves brew.
Keyword: Antioxidant, Fermentation, pH, Robusta coffee leaves, Time variation

ABSTRAK
Antioksidan adalah senyawa pendonor elektron yang dapat menunda, memperlambat dan mencegah reaksi radikal bebas. Fermentasi merupakan perubahan kimia yang terjadi akibat aktivitas mikroorganisme yang dapat meningkatkan jumlah fenol di dalam teh sehingga meningkatkan aktivitas antioksidannya. Fermentasi dilakukan dengan bantuan kultur *Symbiotic Culture of Bacteria and Yeast* (SCOBY) yang biasa digunakan untuk pembuatan kombucha. Kombucha merupakan minuman hasil fermentasi dari teh yang diproduksi dari daun-daun yang mengandung fenol seperti daun kopi robusta (*Coffea canephora* Pierre ex A.Froehner). Faktor yang mempengaruhi proses fermentasi adalah pH dan waktu fermentasi. Penelitian ini bertujuan untuk menjelaskan pengaruh waktu fermentasi terhadap aktivitas antioksidan seduhan daun kopi robusta. Seduhan daun kopi robusta dibuat dengan menyeduh serbuk simplisia daun kopi robusta, ditambahkan starter cair kombucha, kemudian difermentasi selama 0; 3; 7; 14; dan 21 hari, lalu diukur pH dan aktivitas antioksidan seduhan dengan menggunakan spektrofotometer UV-Visibel dengan metode uji *2,2-diphenyl-1-picrylhydrazyl* (DPPH) pada panjang gelombang 516 nm. Hasil penelitian menunjukkan bahwa seduhan daun kopi robusta dengan waktu fermentasi 0; 3; 7; 14; dan 21 hari memiliki pH 6,07; 4,62; 4,07; 3,21; dan 2,74, serta nilai IC_{50} yaitu 285,1645 µg/ml; 219,7012 µg/ml; 192,2325 µg/ml; 187,0434 µg/ml; dan 173,9249 µg/ml. Terdapat pengaruh variasi waktu fermentasi terhadap aktivitas antioksidan seduhan daun kopi robusta.
Keyword: Antioksidan, Daun kopi robusta, Fermentasi, pH, Waktu

1. Introduction

Free radicals are atoms or molecules that are highly unstable due to having one or more unpaired electrons. These single atoms or molecules strive to have paired electrons, making them highly reactive. In humans, the most common form of free radicals is singlet oxygen molecules. When these singlet oxygen molecules act as radicals, they steal electrons from normal molecules, causing damage to those normal molecules. Over time, this damage can become irreversible and lead to the development of diseases like cancer [1].

Antioxidants are electron-donor compounds, and in biological terms, the term "antioxidant" refers to all compounds that can neutralize the negative effects of free radicals [2]. Endogenous antioxidants that the human body already possesses are not sufficient to counter excessive free radicals, so exogenous antioxidants are needed. Exogenous antioxidants can be obtained synthetically, such as BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), TBHQ (tertiary butyl hydroquinone), and PG (propyl gallate), or naturally from plants that have polyphenols, flavonoids, vitamin C, vitamin E, and β-carotene [3].

One of the high antioxidant plants is coffee. Coffee is one of the most common agricultural commodities found worldwide, including in Indonesia. One of the coffee-producing regions in Indonesia is Sumatera Island, known for its robusta coffee (*Coffea canephora*). Robusta coffee contains various chemical compounds such as caffeine, trigonelline, glucose, protein, theophylline, chlorogenic acid, tannins, minerals, and fats. The robusta coffee leaves contain compounds like tannins, steroids, monoterpenes, sesquiterpenes, triterpenes, flavonoids, alkaloids, saponins, and polyphenols. The phenolic compounds, flavonoids, alkaloids, and saponins found in robusta coffee play a role in its antioxidant activity [4].

The antioxidant activity of a plant can be enhanced through the fermentation process (Nurhidayah, 2018). Fermentation is a process of chemical change in an organic substrate through the activity of enzymes produced by microorganisms [5]. Kombucha is a fermented beverage made from tea and sugar by SCOBY (Symbiotic Culture of Bacteria and Yeast), a consortium of microorganisms, including acetic acid bacteria like *Acetobacter xylinum* and yeast like *Saccharomyces cerevisiae*. Kombucha can be produced from high-phenolic leaves and can be used as a functional beverage. Kombucha has functions such as boosting the body's immunity, acting as an antioxidant, and having antimicrobial properties against certain infectious diseases [6].

During kombucha fermentation, the SCOBY culture converts glucose into alcohol and carbon dioxide (CO_2), with CO_2 reacting with water to form carbonic acid. Alcohol is further oxidized to acetic acid. Gluconic acid is formed from the oxidation of glucose by bacteria of the genus Acetobacter. The culture simultaneously produces organic acids such as acetic acid, lactic acid, glucuronic acid, folic acid, and vitamin C. The fermentation process that produces these organic acids can enhance the antioxidant properties of tea [4].

2. Methods

2.1. Materials

Aquadest, *2,2-diphenyl-1-picrylhydrazyl* (DPPH), sugar, quercetin, methanol p.a, and *Symbiotic Culture of Bacteria and Yeast* (SCOBY).

2.2. Plant material

Coffea canephora Pierre ex A.Froehner was obtained from Gajah Village, Karo Regency, North Sumatera.

2.3. Preparation of Robusta Coffee Leaves

The sample processing is carried out by taking robusta coffee leaves and separating them from the stems (dry sorting), then thoroughly washed with running water to clean off any attached dirt (wet sorting). Subsequently, the leaves are dried at room temperature (20-25ºC) for approximately 1 week, and then they are

further dried using a drying cabinet at a temperature of 40-45ºC for 4 hours. Once dried, the leaves are ground into a fine powder.

2.4. Preparation of Fermented Robusta Coffee Leaves Brew

The preparation of fermented robusta coffee leaves brew is carried out by sterilizing a glass jar in a pot filled with boiling water for 10 to 15 minutes. Afterward, it is removed and left to stand for 3-5 minutes before use. The robusta coffee leaves are brewed at 1% (w/v) in water at a temperature of 70°C for 10 minutes. Then, 10% (w/v) sugar is added, and the mixture is filtered into the glass jar. The solution is then left to cool down to 36-37°C, and 10% (v/v) liquid starter of SCOBY is added. The top of the jar is covered with a tissue, and it is placed away from direct sunlight and without being moved. The robusta coffee leaf brew is fermented for 0, 3, 7, 14, and 21 days. At the end of the fermentation period, the kombucha is filtered, pasteurized at a temperature of 85°C for 15 minutes.

2.5. pH Analyzation of Fermented Robusta Coffee Leaves Brew

The pH analyzation of the fermented robusta coffee leaves brew is measured by using a pH meter that has been previously calibrated with buffer solutions. The electrode of the pH meter is rinsed with distilled water and dried. After that, the calibrated pH meter is dipped into the solution to measure its pH level.

2.6. Preparation of Antioxidant Activity Testing of Fermented Robusta Coffee Leaves Brew using DPPH Method

2.6.1. Preparation of the Standard Solution I, Standard Solution II, and Blank Solution with Concentration of 16 µg/mL of DPPH

Dissolved 5 mg of DPPH with methanol p.a in 25 mL volumetric flask to made standard solution I with a concentration of 200 µg/mL. Then, diluted 2 mL of the standard solution I with methanol p.a in 10 mL volumetric flask to made standard solution II with a concentration of 40 µg/mL. Then, diluted 2 mL of the standard solution II with methanol p.a in 5 mL volumetric flask to made blank solution with a concentration of 16 µg/mL.

2.6.2. Measurement of the Maximum Absorption Wavelength and Operating Time of DPPH

The blank solution with concentration of 16 µg/mL poured into a cuvette and placed in a UV-Visible spectrophotometer. Its absorbance is measured at wavelengths ranging from 400 to 800 nm. Subsequently, its absorption is measured at a wavelength of 516 nm every 1 minute for a total duration of 60 minutes.

2.6.3. Preparation of the Standard Solution of Fermented Robusta Coffee Leaves Brew

Dissolved 100 µl of fermented robusta coffee leaves brew with methanol p.a in 10 mL volumetric flask to made standard solution with a concentration of 1000 µg/mL.

2.6.4. Antioxidant Activity Testing of Fermented Robusta Coffee Leaves Brew

Dissolved 0; 0.5; 0.75; 1; 1.25; and 1.5 mL of standard solution of fermented robusta coffee leaves brew with concentration 1000 µg/mL with methanol p.a in 5 mL volumetric flask to made variation of concentration 0; 100; 150; 200; 250; and 300 µg/ml, then added 2 ml of standard solution II of DPPH with a concentration of 40 µg/mL. The sample incubated for 26 minutes and measured by spectrophotometer UV-visible at a wavelength of 516 nm. The percentage of inhibition was calculated by the formula below:

$$Percentage\ of\ inhibition\ (\%) = \frac{(A\ control - A\ sample)}{A\ control} \times 100\%$$

A control = control absorbance
A sample = sample absorbance
Then determined the inhibitory concentration 50 (IC_{50}) value by the equation below:

$$y = ax + b$$

y = 50
x = concentration
a = *slope*
b = intercept

2.6.5. Preparation of the Standard Solution of Quercetin Calibration Curve

Dissolved 10 mg of quercetin with methanol p.a in 10 mL volumetric flask to made quercetin solution with a concentration of 1000 µg/mL. Diluted 1 ml of 1000 µg/mL solution with methanol p.a in 10 mL volumetric flask to made 100 µg/mL. Dissolved 0; 0.05; 0.1; 0.15; 0.2; dan 0.25 mL of 100 µg/mL solution with methanol p.a in 5 mL volumetric flask to made variation of concentration 0; 1; 2; 3; 4; dan 5 µg/mL, then added 1 ml of standard solution I of DPPH with a concentration of 200 µg/mL. The sample incubated for 26 minutes and measured by spectrophotometer UV-visible at a wavelength of 516 nm. Then the percentage of inhibition and inhibitory concentration 50 (IC_{50}) value was determined by the formula and equation above.

2.7. Statistical Analysis

The values of inhibitory concentration 50 (IC_{50}) were analyzed using ANOVA method with Statistical Product and Service Solution (SPSS) version 22 program to determine differences in antioxidant activity on the fermentation time of robusta coffee leaves (*Coffea canephora* Pierre ex A.Froehner).

3. Result and Discussion

3.1. Maximum Absorption Wavelength and Operating Time of DPPH

The study showed that the DPPH solution produces a maximum wavelength at 516 nm with an absorbance of 0.4362. The theoretical wavelength for DPPH measurement ranges from 515 to 520 nm [7], and indicates an absorbance with the smallest photometric error of ±0.4343 [8]. The study also showed the DPPH solution, which was measured every minute for 60 minutes, indicating stability from the 26 to 30 minutes. Operating time is used to determine the measurement time of a compound obtained when the absorbance is most stable. Determining the operating time is performed to minimize measurement errors during the process. The recommended DPPH measurement time is 30 minutes [7].

3.2. pH of Fermented Robusta Coffee Leaves Brew

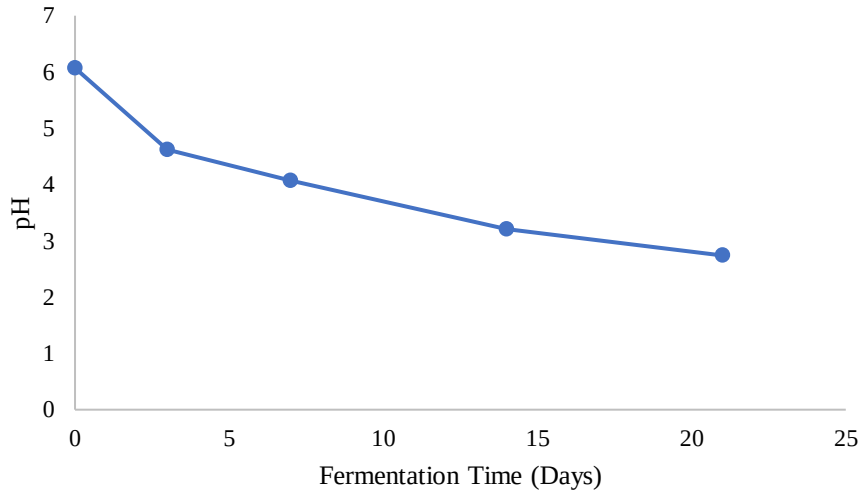

Figure 1. pH versus Fermentation Time Curve

Based on Figure 1, it can be observed that the pH decreases as the fermentation time progresses. The average pH of the test samples on days 0, 3, 7, 14, and 21 are 6.07, 4.62, 4.07, 3.21, and 2.74, respectively. This aligns with the literature where the decrease in pH during fermentation is attributed to the activity of microorganisms present in fermented robusta coffee leaves brew. During the fermentation process, sugar (disaccharide) is hydrolyzed into monosaccharides, fructose and glucose, by the invertase enzyme produced by yeast. Fructose is then converted into alcohol through glycolysis, while glucose is transformed into gluconic acid and alcohol to produce acetic acid by acetic acid bacteria. Consequently, as the fermentation process progresses, the pH decreases due to the high concentration of acetic acid [9].

3.3. Antioxidant Activity of Fermented Robusta Coffee Leaves Brew using DPPH Method

The results of the DPPH absorbance measurement can be calculated as a percentage of inhibition (%). The DPPH inhibition percentage by the fermented robusta coffee leaf brew and quercetin regression equation and linear coefficient can be seen in Table 1. The percentage of DPPH inhibition by the fermented robusta coffee leaf brew with fermentation time and quercetin curve can be seen in Figure 2.

Table 1. Regression Equation and Linier Coefficient

Sample	n	Regression Equation	Linier Coefficient (R^2)
0 Day Fermented	1	y=0.1796x-1.5298	0.9965
Robusta Coffee Leaves	2	y=0.1818x-1.7408	0.9952
Brew (FCLB 1)	3	y=0.1817x-1.6263	0.9958
3 Days Fermented	1	y=0.2165x+2.2242	0.9933
Robusta Coffee Leaves	2	y=0.2183x+2.1036	0.9942
Brew (FCLB 2)	3	y=0.2175x+2.3231	0.9936
7 Days Fermented	1	y=0.2470x+2.5603	0.9932
Robusta Coffee Leaves	2	y=0.2455x+2.4676	0.9944
Brew (FCLB 3)	3	y=0.2504x+2.1425	0.9954
14 Days Fermented	1	y=0.2508x+3.0408	0.9905
Robusta Coffee Leaves	2	y=0.2541x+2.3107	0.9940
Brew (FCLB 4)	3	y=0.2528x+2.8803	0.9923
21 Days Fermented	1	y=0.2734x+2.6877	0.9911
Robusta Coffee Leaves	2	y=0.2728x+2.3921	0.9924
Brew (FCLB 5)	3	y=0.2746x+2.1242	0.9929
	1	y=11.5959x+0.6415	0.9973
Quercetin	2	y=11.6738x+0.6840	0.9977
	3	y=11.7151x+0.5192	0.9980

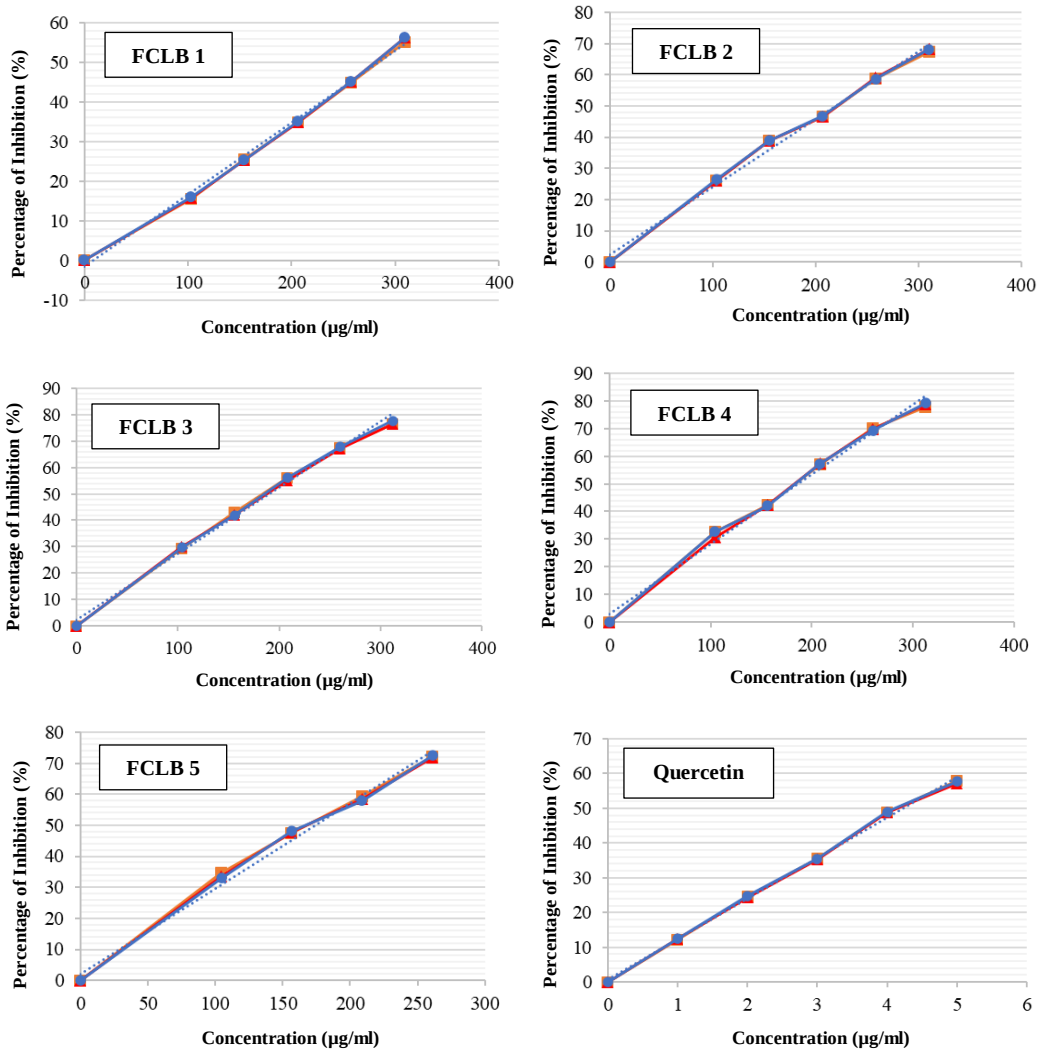

Figure 2. The Percentage of DPPH Inhibition by the Fermented Robusta Coffee Leaf Brew with Fermentation Time FCLB 1; FCLB 2; FCLB 3; FCLB 4; FCLB 5; and Quercetin Curve

The study showed that the increase in DPPH percentage of inhibition by the fermented robusta coffee leaves brew and quercetin is proportional to the increase in concentration and decrease in absorbance value. DPPH is reduced through hydrogen or electron donation processes by fermented robusta coffee leaves

brew and quercetin, resulting in a color change from violet to yellowish with a color intensity change proportional to the amount of electron donation and decrease in DPPH absorbance [10].

The IC_{50} value represents the concentration of the test compound (fermented and non-fermented robusta coffee leaf brew) needed to scavenge 50% of DPPH radicals. The smaller the IC_{50} value, the higher the antioxidant activity [7]. The IC_{50} value of fermented robusta coffee leaves brew and quercetin can be seen in Table 2.

Table 2. IC_{50} Value

No	Sample	IC_{50} Value (µg/ml)				Category
		n1	n2	n3	Average	
1	FCLB 1	286.8665	284.5663	284.0607	285.1645	Weak
2	FCLB 2	220.6085	219.3073	219.1878	219.7012	Medium
3	FCLB 3	192.0620	193.5601	191.0756	192.2325	Medium
4	FCLB 4	187.1838	187.6243	186.3221	187.0434	Medium
5	FCLB 5	172.9979	174.4786	174.2984	173.9249	Medium
6	Quercetin	4.2565	4.2244	4.2236	4.2348	Very strong

The change in antioxidant activity is caused by fermentation, where bacteria and yeast increase the amount of organic acids in the tea, thus enhancing its antioxidant activity. Organic acids contain hydroxyl groups (OH^-) that can donate electrons to the DPPH free radicals. Fermented robusta coffee leaf brew will produce more organic acids and more hydroxyl groups as well. Therefore, the longer the fermentation time, the higher the antioxidant activity of the tested sample [11].

The antioxidant properties of the fermented robusta coffee leaf brew also come from the abundant presence of phenolic compounds, such as chlorogenic acid and quercetin. Besides phenolic compounds, robusta coffee leaves also contain alkaloids, tannins, steroids, monoterpenes, sesquiterpenes, triterpenes, and saponins. Flavonoids are one of the phenolic groups known for their strong antioxidant properties [3].

As the fermentation time increases, the antioxidant activity becomes stronger. However, this antioxidant activity will stop at a certain point when the acidity level also becomes higher. This is because Acetobacter bacteria, found in the Symbiotic Culture of Bacteria and Yeast (SCOBY), live in a pH range of 3-5, while Saccharomyces yeast in SCOBY live in a pH range of 4-5. Thus, at a very acidic pH (below 3), both types of microorganisms cease to survive, meaning that fermentation does not occur, and the antioxidant activity no longer increases significantly [12; 13].

The fermented robusta coffee leaf brew exhibits different antioxidant activities based on the fermentation time. Considering the pH of the brew, the suitable day for consumption is the seventh day, as this study obtained a pH of 4.07. This aligns with the literature, which suggests that kombucha (fermented tea brew) for consumption should have a pH between 2,5-4,6. Lower pH indicates a more acidic fermentation result. Hence, the choice of fermentation day can be adjusted according to consumers' preference for the taste of the fermented robusta coffee leaf brew [9].

Based on the statistical analysis using the Statistical Product and Service Solution (SPSS) program, the results showed a significance value (Sig.) <0,05, which means that H_0 is rejected, and H_1 is accepted. This indicates a significant difference in antioxidant activity concerning the fermentation time of the fermented robusta coffee leaf brew.

4. Conclusion

There is an effect of fermentation time variation on the antioxidant activity of the fermented robusta coffee leaves (Coffea canephora Pierre ex A.Froehner) brew. The IC_{50} values for fermentation on day 0, 3, 7, 14, and 21 are 285,1645 µg/ml, 219,7012 µg/ml, 192,2325 µg/ml, 187,0434 µg/ml, and 173,9249 µg/ml, respectively.

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