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# Fermentation Effect of Cacao Beans Originate from Jember on Polyphenol-Flavonoid Content and Radical Scavenging Activity

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radical scavenging activity.

**ABSTRACT.** Cacao is attractive for its flavor and taste and its beneficial effects. Therefore, this commodity is promising to be developed as a functional food. Many studies reported the bioactive compounds in cacao beans and their health benefits. However, to attain desirable flavor and taste, the curing process of cacao beans is a common practice, in which fermentation is one of the processes. Despite its significance, fermentation also alters the bioactive components in cacao beans. To develop a functional food rich in bioactive compounds, measuring the bioactive contents of treated and untreated cacao beans is desired. For that reason, in this study, the analysis of total polyphenol, flavonoid, catechin, and epicatechin, as well as the radical scavenging activity of fermented cacao beans, was performed. The study revealed that fermentation negatively affected all parameters. Cacao beans fermentation up to 96 h resulted in the loss of 54% of total polyphenol, 77% of total flavonoid, and 56% of radical scavenging activity. Determination of the flavan-3-ol components, catechin, and epicatechin, revealed that they were reduced as the fermentation occurred.

## INTRODUCTION

Cacao beans, derived from cacao (*Theobroma cacao* L.), originated from rainforest regions of tropical America (de Souza *et al*., 2018). This commodity has been used to produce a variety of chocolate products. In Indonesia, cacao is a significant agricultural commodity, contributing to foreign exchange along with other commodities such as palm and rubber trees (Abdoellah, 2021). Along with Ghana and Ivory Coast, Indonesia is the third largest cacao producer (Direktorat Statistik Tanaman Pangan Hortikultura dan Perkebunan, 2020).

Recently, the interest in this commodity is not limited to its flavor and taste. The possibility of beneficial health effects upon consuming cacao-based products is another reason that makes this plant attractive to be further studied. Cacao has been reported to have high polyphenolic content. The study of the total phenolic and flavonoid content and antioxidant capacity of cocoa powder compared with black tea, green tea, and wine revealed that cocoa powder surpassed the others (Lee *et al*., 2003). In addition, comprehensive studies are available that report the bioactive component of this plant. Ali *et al*. (2015) characterized five phenolic compounds from cocoa powder using HPLC-UV-ESI-MS/MS, in which the highest concentration was protocatechuic acid of 33113.7 µg/g. Other authors have successfully described and quantified phenolic compounds from cocoa extracts and fractions using HPLC-MSESI-QTOF. They characterized 33 flavan-3-ol derivatives (including procyanidins), flavonols (including quercetin), and N-phenylpropenoyl-L-amino acids (Cádiz-Gurrea *et al*., 2014). Many articles have reported the potential health benefit of cacao, such as acts as antioxidant, increases cell apoptosis in human lung carcinoma (Bauer *et al*., 2016), diminishes the risk of cardiovascular diseases, increases the immune system, anti-inflammation, and anti-cancer (Andújar *et al*., 2012), and helps to protect skin from UV damage (anti-aging effects) (Scapagnini *et al*., 2014). Moreover, in 2012, EFSA published a report permitting a claim that cocoa

flavanols positively contribute to maintaining normal endothelium-dependent vasodilation. Such characteristics may benefit normal blood flow (EFSA, 2012).

Considering those factors, developing cacao-based products highlighting the bioactive compounds contained in this plant is of great interest. However, despite the potential, it has been understood that the bioactive compounds in cacao beans are vulnerable to alteration and deterioration due to the processing steps. One of the processes is the fermentation of cacao beans. In this process, fruit pulp enclosing the cacao bean is degraded by yeasts and bacteria. The fermentation process would then result in heat and organic acid formation, which are essential in flavor formation (Kadow *et al*., 2015). Moreover, fermentation could promote the loss of astringency and bitter taste of fresh cacao seeds (Albertini *et al*., 2015).

To get insight into the effect of fermentation on the bioactive compounds of cacao bean extract, especially those related to antioxidant activity, measurements of total polyphenols and flavonoids were performed. This information is essential to understand how fermentation alters the bioactive content that will be valuable, especially in developing cacao-based products with beneficial effects on health. Studies on Indonesian cacao bean varieties have been reported but are still limited (Fahrurrozi *et al*., 2021; Septianti *et al*., 2020). For that reason, an investigation of the total polyphenol and flavonoids was performed in the present study. Moreover, the concentration of the flavan-3-ol compounds, namely catechin and epicatechin, as well as the radical scavenging activity of fermented cacao bean extracts were conducted. The cocoa beans in this study originated from the Jember variety. This study is intended as a preliminary study and will be important to help direct the next step in performing cacao bean fermentation.

## RESEARCH METHODS

### Materials

All reagents used were analytical grade and obtained from Merck and Sigma Aldrich. Extraction was conducted using thermoblock rotator SN 06BN (Ni-Nissin). The total polyphenol and total flavonoid were determined using UV-Vis Spectrophotometer UVmini-1240 (Shimadzu). The concentration of catechin and epicatechin was determined using High-Performance Liquid Chromatography (HPLC) 1260 Infinity Agilent Technology.

### Fermentation of Cacao Beans

Cacao fermentation was performed at the Indonesian Coffee and Cacao Research Institute, East Java. Briefly, the cacao seeds were removed from the pods, kept in a wooden box, and covered. The fermentation process was conducted for 96 hours. During the fermentation process, cacao seeds were sampled at 0 hours and then every 24 hours. Cacao seeds were then dried under the sunlight and ground. The cacao powder obtained was then subjected to extraction.

### Cacao Beans Extraction

The extraction of cacao was conducted according to Suazo *et al*. (2014) with minor modifications. Fermented cacao seeds (3 g) were mixed with 15 mL of n-hexane. The mixture was mixed using a vortex for 3 minutes and subjected to centrifugation at 4 °C and 3000 rpm for 15 minutes. This process was conducted four times, and the obtained pellet was dried at room temperature in the fuming hood. Subsequently, 1 g of dried defatted cacao was mixed with 25 mL methanol (80% v/v). The mixture was subjected to mixing in a thermoblock rotator for 2 hours and then filtered. The obtained methanol extract was then kept at 80°C prior to use.

### Total Polyphenol Assay

The total polyphenol content of the cacao extract was determined using a method adapted from Batista *et al*. (2016). For this purpose, 2.5 mL Folin Ciocalteu 10% (v/v) and 2 mL $Na_2CO_3$ 4% (w/v) were added to 0.5 mL cacao extract. The mixture was mixed well and incubated for 2 hours in the dark condition. After incubation, the absorbance of the mixture was determined using a spectrophotometer UV-Vis at 750 nm. Gallic acid was the standard used in this study. A series of standard solutions (25 – 200 mg $L^{-1}$) was prepared to construct a calibration curve. The calibration curve equation and R-squared obtained are $y = 0.0061x - 0.1175$ and $R^2 = 0.9921$, respectively. The total polyphenol content of the extract was expressed as the gallic acid equivalent (GAE, mg $g^{-1}$ defatted sample). This experiment was performed in duplicate.

**Total Flavonoid Assay**

The Flavonoid content of the cacao extract was determined in duplicate employing aluminum chloride in the presence of sodium nitrite, according to Maleyki *and* Ismail (2010), with minor modifications. For this purpose, 0.5 mL cacao extract (50 times dilution) was mixed with 0.15 mL of $NaNO_2$ 5% (w/v) for 5 minutes. 0.15 mL $AlCl_3$ 10% (w/v) was added and mixed into the mixture. After 6 minutes, 1 mL of 1 M NaOH was added to the mixture. Deionized water was then added to up to 5 mL of the final volume. The absorbance of the solution was measured at a wavelength of 510 nm. Catechin was employed as standard, and a calibration curve was constructed from a series of catechin solutions (5-200 mg $L^{-1}$). The calibration curve equation and R-squared obtained are y = 0.0025x + 0.0083 and $R^2$ = 0.9999, respectively. Total flavonoid content was expressed as catechin equivalent (CAE, mg $g^{-1}$ defatted sample).

**Analysis of Catechin and Epicatechin**

The cacao extract's catechin and epicatechin were analyzed using high-performance liquid chromatography (Shumow and Bodor, 2011). 20 µL of cacao extract was injected and analyzed using a reverse-phase C-18 column (Zorbax SB C-18, Agilent) at a flow rate of 0.65 mL $min^{-1}$, 40 $^{\circ}$C, for 50 minutes. Diode array detector (DAD) was used to detect the polyphenol compounds at a wavelength of 200 nm. Catechin and epicatechin were used to construct the calibration curve (catechin: y = 97.121x + 2621.5, $R^2$= 0.9598; epicatechin: y = 76.217x + 3047, $R^2$ = 0.9485). The analysis was conducted using a gradient of two eluent systems, 0.2% acetic acid (A) and 0.2% acetic acid in acetonitrile (B), as follows: 5 – 30% B (30 minutes), 30 – 80% B (5 minutes), 80–5% B (5 minutes). Meanwhile, integration was performed to the respective peaks for the quantitative analysis of catechin and epicatechin in cacao bean extract, and the concentration was calculated using the linear equation obtained from the calibration curve of the standards.

**DPPH (2,2-diphenyl-1-picrylhydrazyl) Assay**

Cacao extract was subjected to DPPH assay according to Abramovic *et al*. (2018) with minor modifications. DPPH solution being used was prepared according to Shimamura *et al*. (2014). For the assay, 0.2 mL of cacao extract (50 times dilution) was mixed well with 0.8 mL of 0.1 M sodium acetate buffer pH 5 and 1 mL of 0.2 mM DPPH. The resulting solution was incubated in the dark condition for 30 minutes. Subsequently, the absorbance of the solution was measured at 515 nm. Ascorbic acid was used as standard (5 – 75 mg $L^{-1}$) (y = -0.0122x + 0.9713; $R^2$ = 0.9962). The antioxidant activity was expressed as ascorbic acid equivalent (AAE, mg $g^{-1}$ of sample) and conducted in duplicate.

**RESULTS AND DISCUSSION**

Qualitative analysis to detect catechin and epicatechin was performed by comparing the retention time of the standard with the retention time of respected peaks in the sample (Figure 1). Catechin and epicatechin were observed at 19.49 min and 22.77 min (Figure 1c), respectively. The analysis of catechin and epicatechin concentration was performed along the fermentation process (0 – 96 h) and further discuss in the following sections. Moreover, the analysis of total polyphenol, flavonoid, concentrations of catechin and epicatechin, as well as radical scavenging activity of cacao extract was conducted.

Based on the assay, the total polyphenol of the cacao extract, as measured in a gallic acid equivalent unit (GAE) per mass of the sample, ebbed as the fermentation progressed (Figure 2). At 24 hours of fermentation, there was only a slight change in polyphenol content. However, the total polyphenol decreased after more than 48 hours of fermentation. The most significant decrease was detected at 72 hours of fermentation. Total polyphenol was recorded to decrease by 45% after 72 hours of fermentation. The further declining occurred after 96 hours of fermentation with a 72 mg GAE $g^{-1}$ defatted sample. Compared to the initial polyphenol content (154.5 mg GAE $g^{-1}$ defatted sample), on day 4 of fermentation, a 53.5% reduction of total polyphenol content in cacao extract was observed.

Similar cases were also reported in previous studies. Fermentation had a negative impact on polyphenol content. This had been the case for Lampung cacao bean varieties (Fahrurrozi *et al*., 2021), cacao (Forastero hybrid cultivar) derived from Brazil (Brito *et al*., 2017), Arriba Nacional (Ecuador) variety (Albertini *et al*., 2015), and cacao beans derived from Nicaragua (Suazo *et al*., 2014). The reduction of the total polyphenol content reported by those studies ranges from 31%–66.6%.

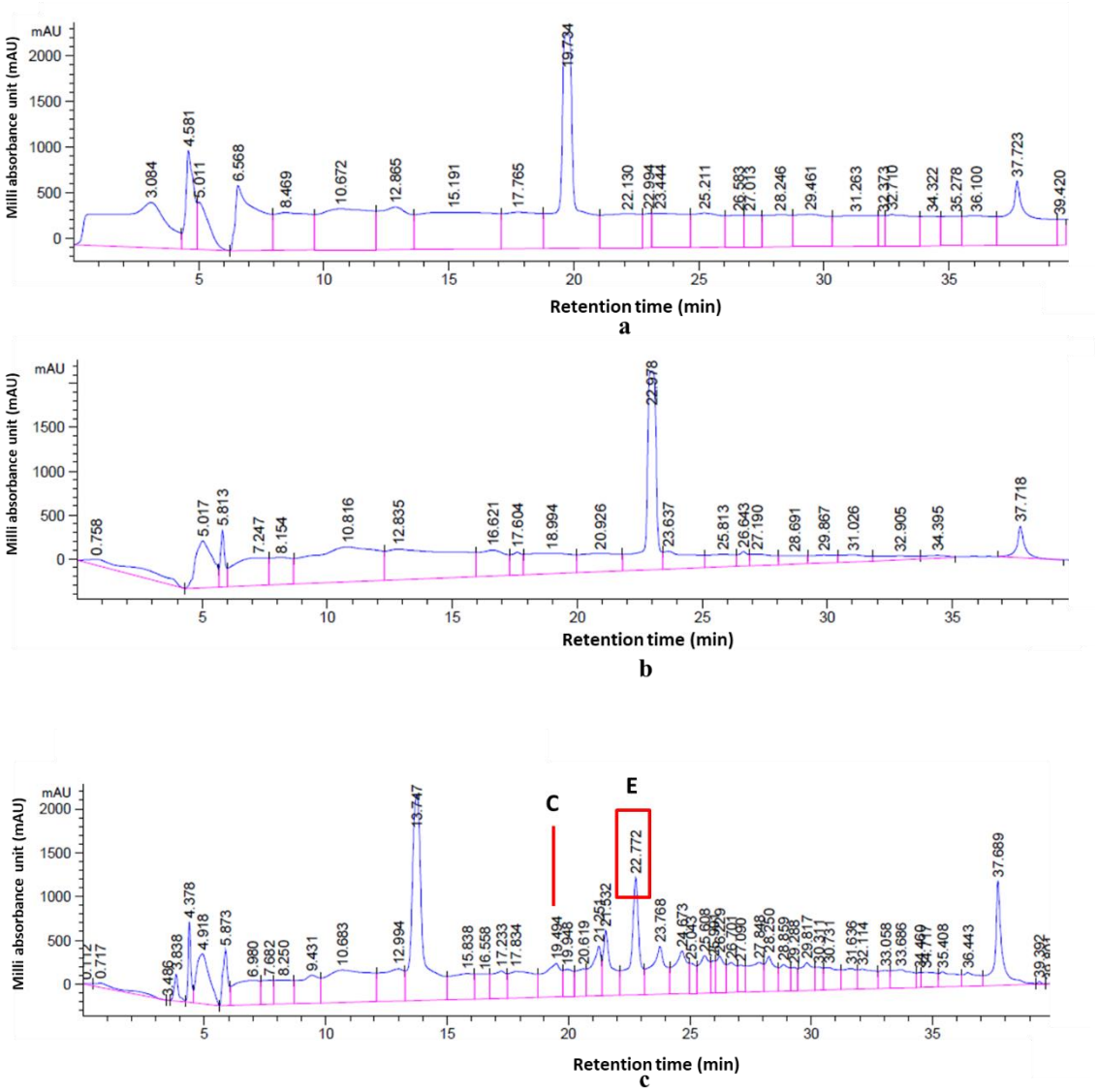


**Figure 1.** Chromatogram of the HPLC analysis of catechin (a), epicatechin (b), and a sample of methanol extract of cacao from 96 h of fermentation (c). Peak C belongs to the catechin, and E belongs to the epicatechin.

In addition to total polyphenols, the analysis of total flavonoids was also conducted. Flavonoid belongs to the group of polyphenol compounds. They belong to the largest and the most varied of group phenolic compounds in cacao beans (Oracz *and* Nebesny, 2016). In accordance with the decrease of polyphenols, the total flavonoid content of the cacao extract was also observed to decline along the fermentation process (Figure 3). The reduction of flavonoids had been clearly observed at 24 h of fermentation. At 96 h of fermentation, there was a 77% reduction in total flavonoid content with a 12 mg CAE $g^{-1}$ defatted sample, compared to the initial flavonoid content (53 mg CAE $g^{-1}$ defatted sample). This result complies with previous studies (Fahrurrozi *et al*., 2021; Melo *et al*., 2021).

The flavonoid component from the class of flavan-3-ol in cacao extract, namely catechin and epicatechin, was investigated using HPLC analysis. Catechin and epicatechin concentrations along the fermentation process are presented in Table 1. As predicted from the decrease in total flavonoid content, both compounds decreased significantly in a similar trend during the fermentation process. However, the proportion of catechin was at a lower level compared to epicatechin. As a result, the final concentration at the end of fermentation was much lower than its isomer epicatechin. At 96 hours of fermentation, concentrations of catechin and epicatechin were 0.042 mg $g^{-1}$

defatted sample and 3.84 mg $g^{-1}$ defatted sample, respectively. These values were equal to the reduction of 93.2% for catechin and 63.8% for epicatechin.

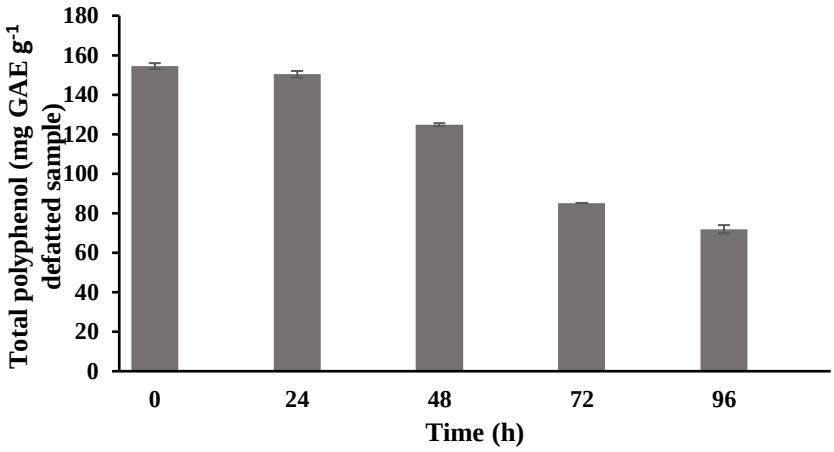


**Figure 2.** The profile of total polyphenol content of the cacao extract during 96 h of fermentation.

The decrease in catechin and epicatechin concentration during fermentation was also reported by previous studies (Fahrurrozi *et al*., 2021; Melo *et al*., 2021; Payne *et al*., 2010). Payne *et al*. (2010) reported relatively similar catechin and epicatechin concentrations for the Ivory Coast cocoa beans after 4 – 5 days of fermentation (0.08±0.0 and 1.69±0.1 mg/g, respectively). However, cocoa beans from Papua New Guinea subjected to long (up to 10 days) fermentation displayed lower catechin and epicatechin (Payne *et al*., 2010). Nevertheless, Fahrurrozi *et al*. (2021) and Melo *et al*. (2021) reported higher catechin and epicatechin concentration. The discrepancies in the concentration of catechin and epicatechin throughout the studies were understood for several reasons, such as the composition of polyphenolic constituents among cocoa trees might vary according to the location of cultivation, the maturity of the beans, climate, harvest season, and post-harvest storage time (Oracz *et al*., 2015), as well as the length of fermentation (Melo *et al*., 2021; Payne *et al*., 2010).

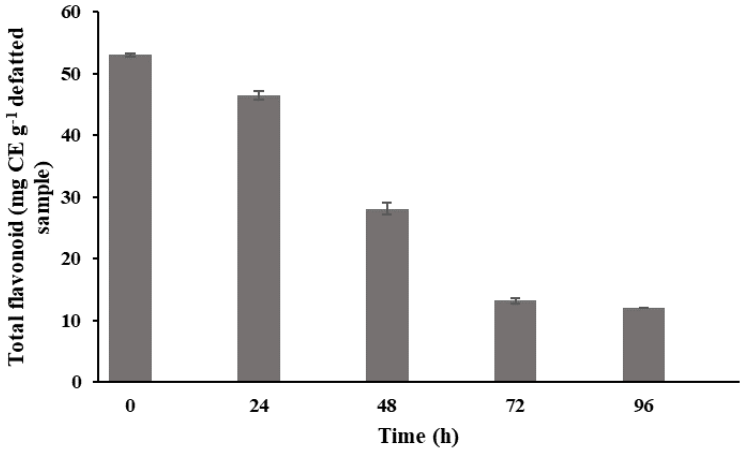


**Figure 3.** Total flavonoid content of methanol extract of cacao for 96 h of fermentation.

Despite the variation in the concentration of polyphenolic compounds, those studies reported the reduction of polyphenol compounds. The decrease of polyphenols is due to several factors, such as diffusion of soluble polyphenols into fermentation, sweating, oxidation, and the use of an enzyme (enzymatically process by polyphenol oxidase and non-enzymatically process such as the sun-drying process) (Albertini *et al*., 2015). The diffusion of metabolites, both into and out of the cotyledons, would enable polymerization and reaction of polyphenols with other compounds, yielding complexes, such as catechins, form complex tannins and the hydrolyze of anthocyanins to form anthocyanidins (Di Mattia *et al*., 2017; Melo *et al*., 2021). Microscopic analysis revealed that phenolic bodies were observed in a high number in the non-fermented seed until 24 hours of

fermentation. However, as the fermentation occurred, the phenolic bodies diffused throughout the cotyledon for up to 48 hours (De Brito *et al*., 2001).

**Table 1.** The concentration of catechin and epicatechin in the cacao extract along the fermentation process

| Time (h) | Concentration (mg $g^{-1}$ defatted sample) | |
|---|---|---|
| | Catechin | Epicatechin |
| 0 | 0.62 | 10.62 |
| 24 | 0.59 | 10.52 |
| 48 | 0.27 | 9.49 |
| 72 | 0.05 | 7.87 |
| 96 | 0.04 | 3.84 |

Subsequently, the radical scavenging capacity of cacao extract was determined by employing DPPH radical. The data in Figure 4 shows the radical scavenging activity gradually decreased as the fermentation occurred. The reduction trend and percentage resembled the decrease in total polyphenols. The radical scavenging capacity was barely reduced at 24 hours. However, at the end of fermentation, the activity was significantly reduced by 56% of its initial capacity. Suazo *et al*. (2014) also reported a similar case in which fermented cocoa had lower antioxidant activity using the same method. It was reported that fermented cocoa had an 82.5% reduction in antioxidant activity, measured using Trolox as a standard compound (Suazo *et al*., 2014). Another study reported a lower reduction of scavenging capacity (39%) as measured using ABTS radical (Brito *et al*., 2017).

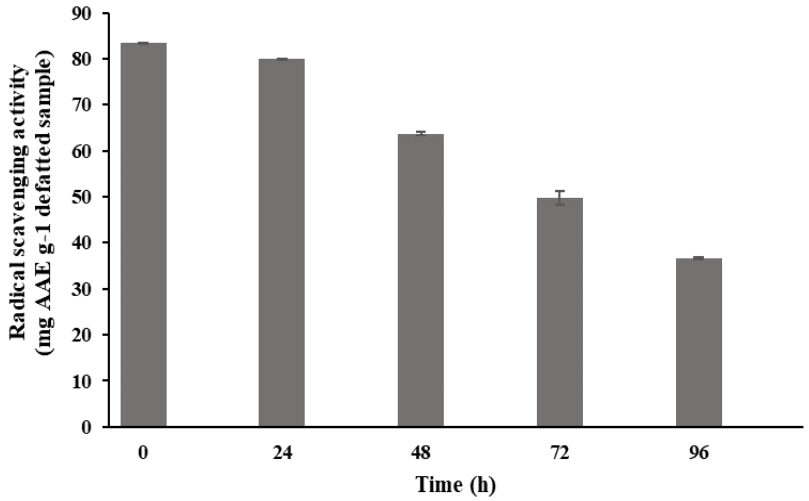


**Figure 4.** Radical scavenging capacity of methanol extract of cacao according to DPPH assay.

Based on the above data, this study presents an insight into the effect of fermentation on the cocoa beans originating from Jember. This study showed that the fermentation process negatively impacted all parameters determined in this study (Figure 5). The most significant decrease was observed in total flavonoid content (77%). This reduction agreed with the concentration of simple flavan-3-ol, catechin, and epicatechin (Table 1). Despite the decrease in total flavonoid, the reduction of radical scavenging activity was more similar to the reduction of total polyphenol. Both parameters showed 56% and 54% of reductions, respectively. This indicates that other components might contribute to the radical scavenging activity since the study only determined the simple flavan-3-ols. Further investigation on bioactive compounds originating from fermented cacao beans is required to reveal which components might contribute to the radical scavenging activity and other bioactivities.

Considering the reduction of total polyphenol, flavonoid, catechin, and epicatechin, the approach should be directed to enable cacao bean fermentation while maintaining the bioactive components that may be beneficial for human health. The shorter length of fermentation while maintaining the perfection of flavor and taste formation would be demanded. Other studies applied several approaches to retain the bioactive compounds of cacao beans. Fahrurrozi *et al.* (2021) reported that using a starter culture could impose on fermented cacao beans with desirable total polyphenol and flavonoid content. Another study revealed that water blanching at 95 °C for 5 minutes in

unfermented cacao beans successfully inactivated the polyphenol oxidase enzyme, thus increasing the total polyphenol content (Indiarto *et al*., 2019).

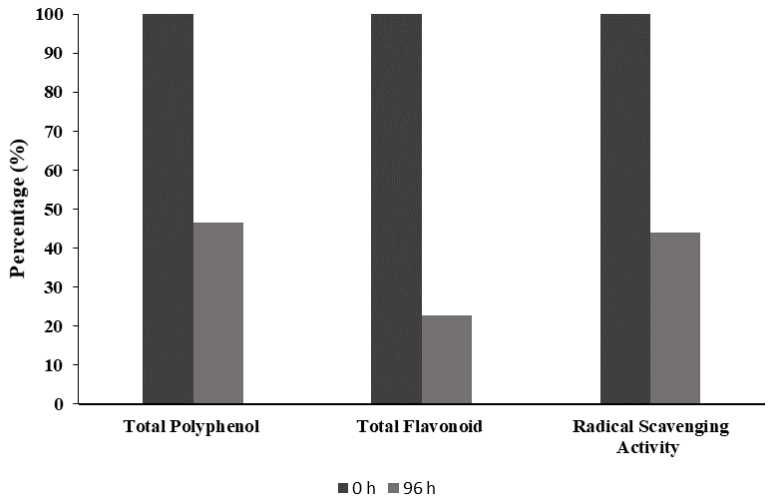


**Figure 5.** Comparison of total polyphenol, flavonoid, and radical scavenging activity values of the cacao extract at 0 hours and 96 hours after fermentation.

Nevertheless, fermentation is one part of the curing process for cacao beans before their derivatization into multiple products. Studies on various cacao bean processing and the effect on its polyphenolic as well as antioxidant properties have been reported (Lieberei *et al*., 2013; Oracz *and* Nebesny, 2016; Payne *et al*., 2010) and may likely be still further studied. In addition, there is still much to explore, especially on the bioactive component which contributes to the antioxidant capacity of the cacao bean. Bioactive components reported in this study are parts of the polyphenolic components. Other polyphenolics or even other classes of bioactive components may also be beneficial and contribute to the antioxidant capacity and other biological activities.

## CONCLUSION

This research presents an insight into the effect of fermentation on polyphenolic, flavonoid, catechin, and epicatechin, as well as the radical scavenging activity of cacao beans originating from Jember. The decreases of total polyphenol, flavonoid, catechin, epicatechin, and radical scavenging activity after fermentation treatment were 54%, 77%, 93,2%, 63,8%, and 56%, respectively. Therefore, the approach to providing fermented cacao beans with high polyphenolic content should be intended to perform fermentation with the least possible loss of the components. Despite the limitation of this study, the data obtained gave valuable information to guide us in taking the following strategy in fermenting cacao beans.

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