Antioxidant activity optimisation of young Robusta coffee leaf kombucha by modifying fermentation time and withering pre-treatment

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Abstract. Kombucha is a fermented drink made from liquid tea. The fermentation is involving bacteria and yeast called Symbiotic Culture of Bacteria and Yeast (SCOBY) The current study aims to investigate the possibility of young Robusta coffee leaf tea as a raw material for kombucha, by optimizing its antioxidant activity and applying withering pre-treatment. The Response Surface Methodology (RSM) was applied to design the observation up to 21 days. Within the duration of fermentation, the optimum IC₅₀ was observed on the third days of fermentation. The results showed that withering treatment significantly affect the pH values, total sugar, total phenolic content, and IC₅₀ of young coffee leaf kombucha. The sensory quality also shows that 5 out of 19 attributes, including brown color, sour taste, bitter taste, alcoholic aroma, sour aroma were also affected by the treatment (p-value<0.05). Regardless the chemical and sensory qualities, the kombucha was still significantly accepted (p-value<0.05) by the respondents.

1. Introduction

Coffee is one of the most consumed plants in the world. Indonesia is among the 8-world coffee producing countries which 75% of coffee production in Indonesia is Robusta coffee [1]. The commercial use of coffee plants has only focused on processing coffee beans as brewed drinks and food additives. Coffee leaf is a part of the coffee plant that has not been widely used as a food product, especially functional food products. Coffee leaves can be proceeded in to tea. Tea made from coffee leaves is known as "kawa daun" or "kawa kopi" originating from the Payakumbuh [2]. Coffee leaves contain high antioxidant compounds (90-697 μg/mL) and anti-inflammatory [3]. The content of phenolic compounds in Robusta coffee leaves is higher than arabica, 13.4-67.6 mg gallic acid/ g leaf [4]. The previous research [4] reported that Robusta coffee leaves are more potentially to be processed into functional food product, because of the high content of phenolic compounds which have high antioxidant activities that are beneficial to the health.

Most research about coffee leaves focused on the process to making of tea or herbal drinks [4], but only very limited studies were reported to make it as kombucha. Kombucha is a fermented tea drink that
known have prophylactic and therapeutic benefits [5]. Kombucha is generally made from liquid tea fermented by symbiotic bacteria and yeast. It has been reported kombucha can be made of both *Camellia sinensis* tea leaves and non-tea leaves with high phenolic content such as coffee leaves [6]. It also has been found further that kombucha made from coffee leaves has higher total phenolic content than that of tea leaves kombucha [6].

Kombucha is a fermented tea known for its various health benefits. Fermentation is known to increase antioxidant activity [7]. The increasing of antioxidant activity is caused by the presence of free phenolic produced during the fermentation process, so that the higher the phenolic content produced, the higher the antioxidant activity [8]. During kombucha fermentation, microorganisms also produce organic acids that may affect to the taste and aroma of kombucha [8]. This shows that the fermentation time affected the antioxidant activity of kombucha, so it is necessary to do research on the duration of kombucha fermentation which produces optimum antioxidant activity, and organoleptic testing to determine the sensory response to kombucha with optimum fermentation time. In the market, the kombucha is mostly made from both green tea and black tea of *Camellia sinensis* leaves. Thus, processing the young leaves of Robusta coffee with and without withering were designed to resemble the processing of green tea and black tea. Therefore, this mimicking process may provide sufficient understanding on the influence of leaves oxidation in conjunction to kombucha fermentation for obtaining optimum antioxidant activity.

2. Materials and Method
2.1 Materials
The samples used in this study were young Robusta coffee leaves which were pre-treated with withering and non-withering process. The leaves were obtained from the coffee plantation maintained by "Tani Harapan" Farmer Group in Dampit District, Malang Regency taken in the dry season. The young *Camellia sinensis* tea leaves were obtained from Wonosari Tea Plantation, Lawang District, Malang Regency and pre-treated with withering and non-withering as well. Young leaves were picked from the bud up to the third layer. Materials used in this study is kombucha culture in the form of nata (SCOBY) and baby kombucha in the form of liquid kombucha. Other materials used for analysis were sucrose, distilled water, folin ciocalteau reagent (Merck), Na$_2$CO$_3$ (Merck), gallic acid (Merck), tannic acid (Merck), Ca$_3$ (Sigma Aldrich), Pb-acetate (Sigma Aldrich), anthrone, H$_2$SO$_4$ (Sigma Aldrich), 70% alcohol, methanol, DPPH 0.2 mM in methanol, PP indicator, oxalic acid (Sigma Aldrich), 0.1 N NaOH, peptone, agar count plate, methylated, pH 4 buffer and pH 7 buffer.

2.2 Method
This study designed by Response Surface Method (RSM) one factor design with 2 samples using Design Expert 10 software. Each sample is optimized with the independent variable of the fermentation time (X1) and the optimized response is antioxidant activity (Y1). The kombucha with optimum antioxidant activity was then analyzed for product characteristics (pH using pH meter method, total acid, total sugar using anthrone method, total phenol, antioxidant activity using IC$_{50}$ method, color using color reader method, microbial total) and organoleptic test (Rate All That Apply/RATA method). The RATA method was used due to its applicability over any other descriptive tests such as Quantitative Descriptive Analysis and Visual Analogue Scale [9]. In this current study 115 untrained panelists were involved. Except for the RSM data, the experimental data collected was statistically evaluated by General Linear Model on MiniTab ver.16.0.

3. Results and Discussion
3.1 Raw materials
The parameters of raw materials analysis included water content, total sugar, total acid, total phenolic content, color and antioxidant activity. Data analysis of raw material characteristics can be seen in Table 1.
The young coffee and tea leaf powder samples have met the SNI quality requirements for tea. Water content in food ingredients determines the freshness and durability of the food. High water content induces bacteria, molds, and yeast to multiply, so that changes will occur in food ingredients. Water content greatly affects the quality and shelf life of tea powder [10].

Based on Table 1, the total sugar, total phenolic content, and all color parameters of non-withering pre-treatments tended to be higher than those of withering one (p-value<0.05). Meanwhile, the antioxidant activity IC$_{50}$ of withering processed tea are higher than that of non-withering for both tea and coffee leaves.

The phenolic compounds affect the activity of catching free radicals of a material [11]. The amount of phenolic content in a material is influenced by processing methods such as withering. The longer the withering time, the phenolic content in the material tended to decrease because it was oxidized by oxygen from the air that mediated by oxidase enzymes [12]. Another factor is temperature, phenolic compounds will decrease by the increasing temperature [13]. Antioxidant activity increases with increasing levels of total phenols and flavonoids which are bioactive compounds that act as antioxidants [14].

Antioxidant activity decreases due to the treatment of differences in enzymatic oxidation and withering processes. The value of lightness shows the brightness of a color [10]. The notation L represents a brightness parameter that has a value of 0 (black) up to 100 (white) [13]. Based on Table 1, the lightness value of the young coffee leaf tea powder sample with withering is lower than that of non-withering. This may be related to the enzymatic oxidation treatment, as enzymatic oxidation plays a role in changing the content of the tannin compound to the aflavin and the arubigin that may change the color of brewed tea.

### 3.2 Optimisation of kombucha fermentation time to antioxidant activity

IC$_{50}$ values of kombucha coffee tended to increase during fermentation up to 21 days. Thus, it suggests that the antioxidant activity of coffee leaves kombucha tended to decrease during fermentation. During the fermentation, there is an increase of organic acids amount due to bacterial and yeast activity in kombucha. Acidic condition causes phenolic compounds to become more stable and difficult to release protons that can bind to DPPH and thus antioxidant activity decreases [14].

The recommended optimization point is in the range of day 0 to day 5. The magnitude of antioxidant activity in the 0 to 5-day fermentation period is likely due to the phenolic compounds contained in the substrate not yet fully degraded, and the pH which is still in the range for the stability of phenolic compounds. Not only phenolic compound, Tannin is also expected to have antioxidant activity [15].

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### Table 1. Characteristics of raw material

| Parameter                      | Young coffee leaf powder | Young tea leaf powder | References |
|--------------------------------|--------------------------|-----------------------|------------|
|                                | With withering | Non withering | With withering | Non withering | Black Tea$^{(a)}$ | Green Tea$^{(b)}$ |
| Water content (%)              | 2.06±0.18       | 2.15±0.14       | 2.22±0.11   | 2.62±0.72   | Max. 10          | Max. 8          |
| Total sugar (%)                | 7.01±0.62a      | 9.66±0.27b      | 6.95±0.81a  | 8.66±0.44b  |                   |                 |
| Total acid (%)                 | 0.17±0.04       | 0.21±0.03       | 0.15±0.03   | 0.19±0.06   |                   |                 |
| Total phenolic content (%)*    | 4.23±0.08a      | 6.17±0.04b      | 4.95±0.11a  | 10.41±0.16b | Min. 9           | Min. 15         |
| Antioxidant activity IC$_{50}$ (ppm)* | 34.66±1.35a   | 29.20±2.14b     | 70.49±0.81a | 35.56±1.25b |                   |                 |
| Lightness*                     | 49.17±0.47a     | 52.60±0.35b     | 40.77±0.18a | 45.97±0.97b |                   |                 |
| a (greenish)*                  | -3.73±0.95a     | -4.97±0.55b     | -1.07±0.15a | -2.17±0.61b |                   |                 |
| b (yellowish)*                 | 16.03±0.32a     | 18.20±0.53b     | 9.73±0.40a  | 12.93±0.49b |                   |                 |

Source: ¹⁰SNI 3753-2014, ¹⁰SNI 3945-2016
Notes: Symbol * indicates a significant different (p-value<0.05) between coffee and tea leaves parameters. Different letter notation indicates a significant different (p-value<0.05) among pre-treatments within the same type of leaves. The data is presented as mean ± standard deviation for 3 replicates.
It was found that the optimum IC50 for coffee leaves kombucha treated by withering and non-withering process are 62.14 ppm and 51.90 ppm consecutively. Those optimum points were obtained after 3 days of fermentation. Verification and prediction results were carried out T test with Minitab 17 to determine suitability of prediction results. The p-value in the response of the two samples > 0.05 (0.1360 and 0.1841), which means that the predictive value and the value of the study are not significantly different (not significant). This indicates that the model is appropriate and the solution provided by the Design Expert 10 program is acceptable.

3.3 Characteristics of kombucha after three days of fermentation

Leaf types show differences in brewed tea and kombucha pH values (Table 2.). Decreasing pH after 3 days fermentation of kombucha occurs due to the increasing of concentration of acetic acid and the other organic acids during the fermentation [16]. The decrease in pH during fermentation is also due to the activity of microorganisms. During the fermentation, sugar is hydrolyzed by yeast and invertase enzyme. Those resulted fructose that further converted into alcohol through the glycolysis pathway.

Meanwhile glucose converted into gluconate acid and alcohol to produce acetic acid by acetic acid bacteria. The longer the fermentation takes place, the higher the concentration of acetic acid, this causes the pH value of Kombucha drinks decrease [16]. Brewed tea and kombucha pH from young coffee leaves processing with withering are higher than pH in non-withering. This shows that leaves treated with withering have lower acid content than leaves which is processed without withering. This is in accordance with states that at the end of the fermentation process green kombucha tea has higher acid levels than black kombucha tea [17].

| Parameters                  | Brewed Coffee Leaves Tea | Brewed Tea | Coffee Leaves Tea Kombucha | Tea Kombucha |
|-----------------------------|--------------------------|------------|-----------------------------|--------------|
|                           | Withering | Non Withering | Withering | Non Withering | Withering | Non Withering | Withering | Non Withering | Withering | Non Withering | Withering | Non Withering |
| pH*                        | 10±0.06a   | 9.9±0.06a   | 6.6±0.10a   | 6.4±0.06b   | 3.6±0.06a | 3.4±0.06b   | 3.7±0.06a | 3.6±0.06b   |           |              |
| Total Sugar (%)*           | 60.8±0.41a | 63±0.97a    | 54.7±0.18b  | 56.9±0.18a  | 36.1±0.77b | 39.8±0.44  | 23.6±0.77b | 29.7±0.44a  |           |              |
| Total Phenolic (%)*        | 28.6±0.71b | 63.1±0.54a  | 192.5±0.35b | 202.4±0.35a | 95.2±0.94b | 125.3±0.71a| 269±0.71b  | 291.3±0.35a |           |              |
| IC50 (ppm)*                | 57.4±0.36a | 48.2±1.46b  | 28.5±0.61a  | 23±1.41b    | 63.4±1.33a | 53.3±1.04b | 23.4±1.73a | 20.4±0.76b  |           |              |

Notes: Different letter notation at the same leaf type indicates significant different at 95% confidence level. Symbol * on the attributes indicate indicates significant different between samples types at 95% confidence level. The data is presented as mean ± standard deviation for 3 replicates.

After 3 days of fermentation, there was a decrease in the total sugar value in all kombucha samples. Based on the analysis, there was a decrease in total sugar between brewed tea and the kombucha sample, where the total sugar in the kombucha sample was smaller than the total sugar in the brewed tea sample. During the fermentation process, the added sugar (sucrose) in the kombucha is hydrolyzed to form monosaccharide by invertase yeast enzymes [10]. Therefore, the sugar content of kombucha tended to be lower than the brewed tea.

Based on the type of tea processing, green tea kombucha had a higher reducing sugar level compared to reducing sugar levels in black tea kombucha [18]. Green tea is the result of processed tea leaves without going through a fermentation process (without withering) or known as unfermented tea. This happens because black tea undergoes perfect fermentation (with withering), causing polyphenol compounds and various other compounds (polysaccharides, starches, proteins) to become oxidized.

While the processing of green tea does not undergo the fermentation stage, so there are still many...
substances contained in green tea. During the processing of green tea, it causes enzymes to be inactive, reduced starch and gum content accompanied by increased sugar (glucose) content [19].

After 3 days of fermentation, there was an increase in the total phenolic content in all kombucha samples. The results showed that the total phenol increase in each kombucha sample was different. This is presumably because the phenolic compounds contained in the leaves are also different. Phenol compounds can be improved by fermentation. In the fermentation process, the arubigine depolymerization is possible and this can explain the phenomenon of increasing the total phenol content that occurs during fermentation [11].

In the sample test of kombucha young coffee leaves, IC50 values increased during kombucha fermentation, while in the tea leaf kombucha decreased IC50 value during kombucha fermentation. The lower the IC50 value of antioxidants, the higher the antioxidant activity. This is because the use of lower concentrations can inhibit DPPH by 50% [20]. Antioxidant activity increases along with the increase in total levels of phenol compounds. Polyphenols can be antioxidants because of their ability to donate hydrogen atoms, capture free radicals, and bind metals [21].

In this study, especially in the test samples, namely young coffee kombucha leaves, antioxidant activity was not directly proportional to total phenol. In the sample of young coffee kombucha leaves there was a decrease in antioxidant activity but the total phenol in young coffee leaves kombucha increased. The pattern of antioxidant activity of kombucha did not show a correlation with the results of testing total phenolic levels. This might be caused during the fermentation process, the degradation of the polyphenol complex into a simple molecule with low activity, but still detected as a phenol compound so that the measurement results remain high even though the antioxidant activity [8].

3.4 Rate-All-That-Apply (RATA)
RATA method is a quantitative method for sensory profiling which aims to profile and rate consumer perceptions of a product [20]. The ANOVA results show that there were 5 significant attributes (p-value <0.05) including brown color, sourness, bitterness, alcoholic aroma and acid aroma (Table 3). This shows that the interactions between leaf types and leaf processing methods lead to 5 distinctly rated attributes and panelists can feel the difference in intensity of sensory attributes between the samples presented.

It was also observed that 4 out of 5 significant attributes were affected by different leaf type. The coffee leaves kombucha tended to be less brown and less bitter than that of tea kombucha (p-value<0.05) but tended to be more intense in terms of alcoholic and acid aroma (p-value<0.05). As refer to Table 1 and 2, it may be attributed by the different composition of coffee leaves and tea leaves.
Table 3. Sensory profiles of coffee leaves kombucha

| Attributes      | Leaf type                              | Tea processing | Mean    |
|-----------------|----------------------------------------|----------------|---------|
| Brown color*    | Young coffee leaves kombucha           | With withering | 3.0 ± 0.12a |
|                 |                                        | Non withering  | 2.9 ± 0.12a |
|                 | Tea kombucha                           | With withering | 3.7 ± 0.11a |
|                 |                                        | Non withering  | 3.3 ± 0.12b |
| Soursness       | Young coffee leaves kombucha           | With withering | 2.1 ± 0.14a |
|                 |                                        | Non withering  | 1.5 ± 0.12b |
|                 | Tea kombucha                           | With withering | 1.5 ± 0.15a |
|                 |                                        | Non withering  | 1.7 ± 0.15a |
| Bitterness*     | Young coffee leaves kombucha           | With withering | 0.6 ± 0.10a |
|                 |                                        | Non withering  | 0.7 ± 0.09a |
|                 | Tea kombucha                           | With withering | 3.2 ± 0.14a |
|                 |                                        | Non withering  | 2.8 ± 0.16b |
| Alcoholic aroma*| Young coffee leaves kombucha           | With withering | 1.7 ± 0.15a |
|                 |                                        | Non withering  | 1.3 ± 0.12b |
|                 | Tea kombucha                           | With withering | 0.95 ± 0.11a |
|                 |                                        | Non withering  | 0.95 ± 0.11a |
| Acid aroma*     | Young coffee leaves kombucha           | With withering | 2.83 ± 0.15a |
|                 |                                        | Non withering  | 2.42 ± 0.14b |
|                 | Tea kombucha                           | With withering | 1.79 ± 0.15b |
|                 |                                        | Non withering  | 2.12± 0.15a |

Notes: Different letter notation at the same leaf type indicates significant different at 95% confidence level. Symbol * on the attributes indicate indicates significant different between samples types at 95% confidence level. The data is presented as mean ± standard deviation for 3 replicates

4. Conclusions

The optimization results of the fermentation time of young coffee leaf kombucha with withering and non-withering showed that the duration of fermentation produced optimum IC_{50} antioxidant activity was fermented for 3 days. Comparison between young coffee leaf kombucha samples and kombucha tea leaf samples fermented according to the optimum fermentation time for 3 days showed that antioxidant activity in young coffee leaf kombucha was lower than kombucha tea leaf. Withering process results in lower antioxidant activity than non-withering processing. The results of the testing of product characteristics showed that the young coffee leaf kombucha with withering showed significant differences to non-withering in pH value, total sugar, total phenol, and IC_{50}. The results of organoleptic kombucha testing on 19 attributes using the RATA method showed significant differences between samples on 5 attributes, namely brown color, sour taste, bitter taste, alcoholic aroma, and sour aroma.

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