Changes in chlorophyll and polyphenols content in *Camellia sinensis var. sinensis* at different stage of leaf maturity

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Abstract. Chlorophyll and polyphenols are chemical compounds related to parameter quality of green tea. We studied the variation of chlorophyll and polyphenol in the development stage of tea leaves (bud, 1st, 2nd, 3rd, and 4th). Five clones of tea (*Camelia sinensis var. sinensis*) from Indonesia and a clone from Japan were used in this study. The results showed that total chlorophyll and total polyphenol content in bud between 1.59-2.15 mg/g (db) and 12.24-14.59%, respectively. The concentration of chlorophyll increased significantly with developments stage of leaf while total polyphenol tended to decrease with leaf maturity. Pearson Correlation analysis showed that chlorophyll content was negatively correlated (r = -0.83; p =0.05) with total polyphenol during developmental stage of tea leaves. Results suggests that five clones of tea from Indonesia have similar quality with tea clone from Japan in chlorophyll and polyphenol content. The present study also provides guidelines on application plucking standard to produce high quality of green tea.

1. Introduction

Tea is a beverage that contains many health benefits. According to the nomenclature, tea is divided into two varieties namely *Camelia sinensis var. assamica* from Assam and *Camelia sinensis var. sinensis* originating from China. Both types of varieties have different morphological characteristics. Sinensis varieties of tea plant from Indonesia has been officially released by Minister of Agriculture in 2009 which consisted of 5 clones: GMBS 1, GMBS 2, GMBS 3, GMBS 4, and GMBS 5 [1].

Chlorophyll and polyphenol are chemical compounds related to parameter quality of green tea. Chlorophyll, natural green pigment, has function in photosynthesis metabolism. Chlorophyll a content in fresh tea leaf varies from 1.39 to 5.39 mg.g⁻¹ and chlorophyll b 0.77 to 2.06 mg.g⁻¹ [2,3]. Chlorophyll content in tea increase gradually as the leaves matured and significantly increase the photosynthesis rate [4]. Polyphenols in green tea were based on the polyphenols content from the fresh leaves. Leaf positions represent a specific stage that affect in primary and secondary metabolites. Generally, young leaves, positioned at apical, considered rich in polyphenols compared with the old
leaves. Young leaves contain more tea polyphenols, caffeine, amino acids, and their concentration decrease significantly from the 1\textsuperscript{st} leaf to the 3\textsuperscript{rd} leaf [4]. Therefore, a metabolic balance between chlorophyll and polyphenols contents might present in tea leaves at different stages [5].

The present study aimed to determine the total chlorophyll content and polyphenols at various leaf positions. This research focused on RITC (Research Institute for Tea and Cinchona) clones, GMBS 1 to GMBS 5, compared with Yabukita clone originated from Japan. The leaf position used includes apical bud, first, second, third, and fourth leaves where the leaves indicate leaf maturity level. This study also observed correlation among chlorophyll and polyphenols in development of tea leaf maturity level. The results of this study might serve as a useful guideline on application of plucking standard to produce high quality green tea.

2. Materials and Methods

2.1. Plants material and experimental location

Apical bud and four leaves of \textit{Camellia sinensis} \textit{var.} sinensis from Research Institute for Tea and Cinchona (RITC) clones (GMBS 1, GMBS 2, GMBS 3, GMBS 4 and GMBS 5) and from Japan (Yabukita) were precisely plucked by hand and immediately separated based on specified criteria (a bud, 1\textsuperscript{st} leaf, 2\textsuperscript{nd} leaf, 3\textsuperscript{rd} leaf, and 4\textsuperscript{th} leaf). All leaves were immediately brought to laboratory to determine the moisture content (ISO1572) and for further analysis. The research was carried out in Gambung tea plantation (Latitude: 7° 08' 37" N, longitude: 107° 30' 54" R, altitude 1200 m) which belongs to Research Institute for Tea and Cinchona (RITC) in West Java. All samples were plucked in January- March 2017 with average rainfall of 218 mm and average minimum and maximum temperature of 16.5 °C and 24.2 °C, respectively.

2.2. Determination of chlorophyll

Chlorophyll was determined according to the method of Ni et.al. [6] with minor modification. Fresh leaves were placed in mortar and grinded finely. Four hundreds (400) mg sample was added with 5 ml of acetone 80\% in 15 ml falcon tube, mixed for 5 minutes, and was kept in the dark in a temperature of 4 °C for 15 minutes (chlorophylls degrade under light). Then the mixture was centrifuged for 15 min (at 3,000 rpm), the supernatant was transferred to a new centrifuge tube, and kept in the dark. This treatment was repeated twice and the supernatant was combined so that the total volume was made up to 25 ml using acetone 80\%. Shake the mixture thoroughly and measure the absorbance of chlorophyll content using spectrophotometry (Varian carry win UV) with acetone 80\% as blank control. Chlorophyll content was calculated using formula:

\[
\text{Chlorophyll } a_{(mg/g)} = \frac{[12.7 \times A_{663} - 2.69 \times A_{645}] \times V}{1000 \times W}
\]

\[
\text{Chlorophyll } b_{(mg/g)} = \frac{[22.9 \times A_{645} - 4.86 \times A_{663}] \times V}{1000 \times W}
\]

\[
\text{Chlorophyll } a + b_{(mg/g)} = \frac{[8.02 \times A_{663} + 20.20 \times A_{645}] \times V}{1000 \times W}
\]

Where V = volume of the extract (ml); W = Weights of fresh leaves (g); A645: absorbance at 645 nm; A663: absorbance at 663 nm.

2.3. Determination of total polyphenols

2.3.1. Preparation and extraction of fresh leaf

Fresh leaves were placed in a mortar and grinded with a pastel finely. Added 2 g of sample with 30 ml boiled methanol 70\% heated up for 10 minutes and followed by maceration in oven 60 °C for 2 hours.
The mixture was then sonicated for 30 min using a Sonicator (Branson-220 Inst., New York, USA). The extraction mixture was constantly kept cold by adding ice water into the sonicator vessel. The mixture was then passed through a filter with a Whatman No. 1 filter paper to obtain a clear extract. The residues and all glassware were then washed with 70% aqueous methanol and the total volume of the extract was made up to 50 ml in a volumetric flask. One millilitre of the extract was pipetted into a 25 mL volumetric flask and diluted with distilled water. The dilution extract was further used in determining total polyphenol.

2.3.2. Determination of total polyphenol
Folin-Ciocalteau method was used to determine the total polyphenol according to ISO 14502-1:2005 standardization [7]. One millilitre of dilution extract was pipetted into tube flask. The extract was further reacted with 5 mL of Folin-Ciocalteau’s reagent (10%) for 5 min to form a blue-colored solution. Then, 4 mL of sodium carbonate 7.5% (37.5 g was diluted with 500 ml distilled water) solution to stabilize the colour formed. The blue colour was allowed to develop for at least 2 h and its absorbance was then measured at 740 nm using UV-vis Spectro (Varian carry win UV). The amount of total polyphenols was obtained from the standard curve equation of gallic acid solution with a concentration range of 1-100 mg / L (ppm).

2.4. Statistical analysis
The data obtained in the study were analyzed by using one-way ANOVA at a significance level of 95% followed by Duncan's multiple range test (DMRT). Person’s correlation analysis was used to assess a correlation between chlorophyll and total phenol. All data analysis was performed using XLSTAT 2014.

3. Results and Discussion
3.1. Profile of chlorophyll in various clones at different stage of leaf maturity
Chlorophyll is natural pigment with green color in leaf. The function of chlorophyll in leaf is for photosynthesis metabolism. Leaf position represents a specific developmental stage that influences both photosynthesis and respiration [8]. Table 1 and 2 show the chlorophyll a and b content of apical bud, the first, second, third, and fourth leaf from various tea clones. Apical bud from various clones has the lowest chlorophyll content ranging from 1.14-1.46 mg/g and 0.44-0.77 mg/g on chlorophyll a and chlorophyll b respectively. Apical bud from GMBS 4 clone has the lowest chlorophyll a and b while those from GMBS 3 and GMBS 5 clones have the highest chlorophyll a and chlorophyll b respectively. Furthermore, fourth leaves from various tea clones show the highest chlorophyll content ranging from 3.40-4.72 mg/g and 1.31-1.68 on chlorophyll a and b respectively. The highest content of chlorophyll a and b are found in Yabukita clones and GMBS 5, respectively.

| Leaf position | GMBS1 | GMBS2 | GMBS3 | GMBS4 | GMBS5 | Yabukita |
|---------------|-------|-------|-------|-------|-------|----------|
| Bud           | 1.24±0.13* | 1.38±0.45* | 1.47±0.02* | 1.14±0.31* | 1.37±0.21* | 1.46±0.06* |
| 1st leaf      | 1.88±0.42a  | 1.83±0.65a  | 2.47±0.27b  | 1.46±0.36a  | 2.23±0.37bc | 2.38±0.03a  |
| 2nd leaf      | 2.77±0.53b  | 2.77±0.85db | 3.29±0.29bc | 2.33±0.38b  | 2.42±0.65db | 3.58±0.28b  |
| 3rd leaf      | 3.46±0.50c  | 3.71±1.03b  | 3.50±0.52e  | 3.21±0.52c  | 3.29±1.05bc | 3.59±1.26b  |
| 4th leaf      | 3.58±0.24c  | 4.10±1.15b  | 3.98±0.84c  | 3.40±0.52e  | 3.87±0.59c  | 4.72±0.24c  |

Data are represented as mean ± SD (standard deviation) of three replications. Different letters indicate significant differences between leaf position at P < 0.05 according to a Duncan test.

Total chlorophyll of apical bud of GMBS 1 was 1.74 mg/g increased significantly in first, second, third and fourth leaf with 2.57, 3.74, 4.66, and 4.91 mg/g (Figure 1), respectively. Visual observations on the fourth leaves of various clones showing dark green color while apical bud that have low chlorophyll content, has a yellowish green color. Tea leaf positions from various clones significantly (P <0.05) affect chlorophyll a and b and total chlorophyll content. Increased chlorophyll in tea leaves correlates with increasing maturity of tea leaves [1,9]. Increased chlorophyll in the fourth leaf due to
an increase in photosynthetic capacity in maturity leaves which contain more starch than in younger leaf (apical bud) [8]. The mean increase in total chlorophyll of all clones in fourth leaf was more than 65% from total chlorophyll of apical bud. The highest increase, 69%, was in the Yabukita clone where the total chlorophyll in the fourth leaf was 6.42 mg/g (Figure 1).

Table 2. Profile of Chlorophyll b content (mg/g db) in various clones of Camellia sinensis var. sinensis.

| Leaf position | GMBS1 | GMBS 2 | GMBS 3 | GMBS 4 | GMBS 5 | Yabukita |
|---------------|-------|--------|--------|--------|--------|----------|
| Bud           | 0.49±0.07bc | 0.57±0.18a | 0.50±0.04a | 0.44±0.12c | 0.72±0.31a | 0.52±0.01a |
| 1st leaf      | 0.65±0.15a  | 0.63±0.26a | 0.80±0.16b | 0.52±0.14ab | 1.02±0.61b | 0.76±0.04a |
| 2nd leaf      | 0.92±0.19b  | 1.02±0.38ac | 1.08±0.05bc | 0.80±0.12bc | 1.18±0.77bc | 1.12±0.12bc |
| 3rd leaf      | 1.18±0.12bc | 1.37±0.37bc | 1.17±0.22bc | 1.12±0.16bc | 1.46±0.85bc | 1.23±0.38bc |
| 4th leaf      | 1.31±0.03bc | 1.52±0.49bc | 1.48±0.46bc | 1.33±0.29bc | 1.68±0.37bc | 1.63±0.07bc |

Data are represented as mean ± SD (standard deviation) of three replications. Different letters indicate significant differences between leaf position at P < 0.05 according to a Duncan test.

Chlorophyll a (C55H72O5N4Mg) and chlorophyll b (C55H70O6N4Mg) have different functional groups of their chemical structure. In addition, the color of Chlorophyll a is dark green and chlorophyll b is light green. Chlorophyll-a is the main pigment which converts light energy into chemical energy. Chlorophyll-b as accessory pigment acts indirectly in photosynthesis by transferring the light energy into chemical energy through the catalysis of chlorophyllase (Figure 1).

Figure 1. Total chlorophyll in various clones of Camellia sinensis var. sinensis at different stage of leaf position

3.2. Total polyphenol in various clones at different stage maturity of leaf

The results of total polyphenols from bud to fourth leaf in different types of clones are presented in Figure 2. Apical bud from GMBS 2 contains the highest total polyphenols (14.59%) than GMBS 1, GMBS 3, GMBS 4, GMBS 5 and Yabukita with total polyphenols 12.12; 12.98; 13.24; 13.80; and 12.93% respectively. The percentage of total polyphenols in various tea leaf positions tended to decrease significantly of about 34% from first to fourth leaf. Total polyphenols in the fourth leaf ranging from 7.48-9.40% where GMBS 3 has the smallest total polyphenol content. Overall, apical bud contains the highest polyphenols and the concentration decrease significantly with the maturity of
leaf. The results found in this study, whereby polyphenols decreased at development stage of leaf is similar with previous study reported by Li et al.[8] and Samanta et al. [5].

**Figure 2.** Total polyphenol content in various clones of *Camellia sinensis var. sinensis* at different stages of leaf position.

Fresh tea leaves contain various chemical compounds that play a role in determining the quality parameters of tea products. Total polyphenols in tea leaves predominantly flavan-3-ols (catechins) and flavonol glycosides and phenolic acids and these constitute 20-30% to total component on dry weight basis [16–18]. Phenols have responsibility for characteristics, color and astringent taste of green tea. Polyphenol content of fresh tea leaves is also affected by seasonal variation, leaf handling and harvest methods, leaf maturity, and variety of tea clones [19]. Polyphenols have been reported to have antioxidant activity and may contribute to a reduced risk of cardiovascular disease, cancer, and has many other beneficial effects on health [20,21].

3.3. **Correlation analysis between chlorophyll and total polyphenol as influenced by leaf position.**

Table 3 shows Pearson correlation coefficient (r) analysis between chlorophyll and Total polyphenol affected by leaf position. Total polyphenol correlated negatively with chlorophyll content (P <0.05). This study showed that the maturity level of leaves contributes to changes in chlorophyll and polyphenol contents in tea leaves. Increasing of chlorophyll content in tea leaves is associated with leaf maturity level. During maturity leaves physiological functions are reduced, even though the photosynthetic capacity increased with the degree of leaf maturity [8]. The old leaves provide nutrients to tea plants and play important roles in the storage of nutrients that are required for the germination and growth of new shoots [22]. Starch is a product generated from photosynthesis in which increased starch correlates with increased chlorophyll in tea leaves [8,23].

The decrease of polyphenolic content in mature tea leaves is probably due to low enzyme activity of Leucocyanidin reductase (LAR) and anthocyanidin synthase (ANS) [15]. Tea catechins, group of flavanol, are considered to be synthesized through phenylpropanoid and flavonoid pathway and stored in the vacuole [24]. The formation of catechins in tea leaves might be affected by the chlorophyll accumulation. MEP (methylerthyritol-4-phosphate) pathway that is responsible for the synthesis of precursors of chlorophyll might suppress the catechin formation pathway during tea leaf maturity as both these pathways use common precursor phosphoenol pyruvate [10].
Table 3. Correlation between total polyphenol, chlorophyll a, chlorophyll b, and total chlorophyll parameters as influenced by leaf position.

| Variables | Total phenol | Chl a | Chl b | Total Chl |
|-----------|--------------|-------|-------|-----------|
| Total Phenol | 1            |       |       |           |
| Chl a      | -0.8324*     | 1     |       |           |
| chl b      | -0.7901*     | 0.9405* | 1     |
| Total Chl  | -0.8294*     | 0.9957* | 0.9679* | 1        |

* Significantly different at P < 0.05

4. Conclusions

The results of this study showed that chlorophyll content and total polyphenols from various GMBS clones were not significantly different from clone from Japan. Leaf maturity level of all clones influenced the chlorophyll and polyphenol content. Chlorophyll content in tea leaves increase significantly with leaf maturity but negatively correlated with polyphenol contents. Processing of tea using old leaves will affect the decrease of tea quality. The present study also provides guideline on application of plucking standard to produce high quality of green tea.

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