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The effect of altitude against total phenolic and *Epigallocatechin Gallate* (EGCG) content in green tea leaves

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Abstract

Introduction The amount of total phenolic and *epigallocatechin gallate* (EGCG) contained in green tea simplicia is an important factor for determining the product quality. Some factors can influence the amount of active ingredients produced by green tea plant. One of the factor is the altitude of plant source that has never been reported until now.

Objective This study aimed to determine the difference of total phenolic and EGCG content on 3 regions of green tea sources that have different altitudes in central Java, Indonesia namely PT Sari Medini with altitude about 2050 m asl, PT Sari Kemuning with altitude about 800-1540 m asl, and PT. Tambi with altitude about 250-2250 m asl which most dominated by 500-1000 m asl.

Methods The simplicia which gathered from the three sources, each was extracted using infundation method, the extract was treated under extreme decrease temperature and pH decrease with pH 4 buffer solution then diffracted by ethyl acetate until it turned into powder. Total phenolic content measurement was obtained using Follin-ciocalteu method, while EGCG measurement was obtained using HPLC.

Result The result showed that the highest total phenolic content was PT. Sari Medini, followed by PT. Sari Kemuning, then PT.Tambi namely 900,87 mg/g GAE, 847,85 mg/g GAE and 818,51 mg/g GAE. Furthermore, the highest EGCG was from PT.Sari kemuning, followed by PT. Tambi, then PT. Sari Medini namely 26,18 % w/w, 25,85 % w/w and 22,35 % w/w.

Conclusion The highest amount of total phenolic green tea was produced by PT. Sari Medini, while the highest EGCG was PT. Sari Kemuning. It can be concluded that a higher location of tea plant growth will produce a higher amount of total phenolic content but specific factors such as temperature, humidity, amount of solar radiation, amount of carbondiaoxide and seasons for influencing the total phenolic and EGCG content have to be clarified in further studies.

Keywords Altitude place to grow, total phenolic content, *epigallocatechin gallate* (EGCG)

1. Introduction

Green tea leaves contain catechin as the main active ingredient. Besides that, several types of polyphenol inside green tea leaves are 51,88% *epigallocatechin gallate* (EGCG), 12,24% *epicatechin* (EC), 6,12% *epicatechin gallate* (ECG) and 5,5% *epigallocatechin* (EGC) (Nagle et al. 2006; Butt & Sultan, 2009). EGCG is a phenol derivates that included in flavan-3-ol, the amount of EGCG in green tea leaves makes EGCG as the marker substance for green tea leaves which responsible as alternative medicine. The greater phenol content in green tea leaves then cause the greater EGCG contained therein, but some studies stated diverse amount of EGCG. According to previous study Sugihartini (2013), EGCG concentration was

6,7%. EGCG content inside various green tea product ranged between 0,21-9,63% (Prayong et al. 2007) and EGCG concentration on green tea bags ranged between 2,08-3,96% (Martono, 2012). The different concentration from each study might be caused of the various type of tea plants sources.

The previous study Monschein et al. (2015) evaluates the influence of altitudinal variation on the content of flavonoids in E. angustifolium in two consecutive growing periods, the result was rising concentrations of flavonol 3-O-glycosides could be detected with increasing altitude. This was suitable with the research by Yao et al. (2012) and Bernal et al. (2013) that stated the higher azuki bean (Vigna angularis) plants source and Buxus sempervirens leaves will produce higher phenolic content.

According to the altitude location of tea plant growth, the researcher divided those into 3 altitudes namely under 1000 m asl, 1000-2000 m asl, and over 2000 asl. However, most of highlands in Indonesia have different altitude in each region so the most similar one to altitude range is chosen in this study. Green tea from PT.Tambi Wonosobo was chosen for growth location under 1000 m asl because the altitude in Tambi's tea yard was around 250-2250 m asl but dominated by 500-1000 m asl (Web 1). Furthermore, green tea from PT. Sari Kemuning Karang Anyar with 1540 m asl altitude was chosen for growth location 1000-2000 m asl (Web 2). Last, green tea from PT. Sari Medini Kendal with 2000-2050 m asl altitude was chosen (Web 3). Total phenolic and EGCG content from those three locations then will be observed. This study hope will be the direction as initial study for the further study that using tea plants related to the needs of substance used.

2. Materials and methods

2.1 Material

The green tea is gathered from PT.Tambi Wonosobo, PT. Medini Kendal, and PT.Sari Kemuning Karang Anyar. The materials for total phenolic testing are gallic acid, Follin-Ciocalteu reagent, aquadest, phosphoric acid (PA), ethyl acetate, ice, methanol, acetonitrile, and triethylamine

2.2 Equipment

The equipments for this study are stainlesteel pan, thermometer, pH meter brands Hanna HI 98121, rotary evaporator Heildolph Germany, Thermostat water bath HH-6, Moisture balance brand Shimadzu EP-90 models Moc63u, water bath, electric scales, tools glass (pyrex), HPLC column C 18, and High-performance Liquid Chromatography brand Shimatsu.

2.3 Working steps

1) Making green tea leaves simplicia

   Green tea leaves from PT.Sari Medini and PT.Sari Kemuning were withered on 100^{o}C temperature for 5 minutes, then dried with 100^{o}C temperature for 60 minutes. Green tea leaves from PT. Tambi were withered on 100^{o}C temperature for 5 minutes, then drying process was applied on 100^{o}C temperature for 60 minutes.

2) The making of ethyl acetate fraction of green tea extract

   Epigallocatechin gallate (EGCG) in green tea leaves simplicia was extracted based on the modified previous study (Row & Jin, 2006; Hirun & Roach, 2011). First, 3 grams

simplicia were infudated on 90^{0}C temperature for 30 minutes with 60 mL distilled water. The infusa then filtered and dip into ice soon with 0^{o}C for 30 minutes until the extract reached 5^{o}C temperature, after that a few drops of phosphoric acid added until the pH became 4. The filtrate was fractionated with 60 ml of ethyl acetate. The ethyl acetate fraction then thickened by a rotary evaporator. After viscous, then evaporated over a water bath until the ethyl acetate run out in order to obtain a dry powder ethyl acetate fraction of green tea extract with a moisture content <2%.

3) Ethyl acetate fraction of green tea leaves specification

Ethyl acetate fraction of green tea leaves specification was obtained from 3 sources tea producing region. According to the altitude location of tea plant growth, the researcher divided those into 3 altitudes namely under 1000 m asl, 1000-2000 m asl, and over 2000 asl. PT.Tambi Wonosobo was chosen for growth location under 1000 m asl, PT. Sari Kemuning Karang Anyar was chosen for growth location between 1000-2000 m asl, and PT. Rumpun Sari Medini Kendal was chosen for over 2000 m altitude category.

The specification that obtained in this study is total phenolic content testing that measured based on gallic acid and EGCG content test. The highest total phenolic and EGCG content were chosen from the three sources.

1) Total phenolic content specification using Follin ciocalteu method

Total phenolic content was measured by folin-ciocalteu reagent using spectrophotometry with gallic acid as the reference standard. Gallic acid is the acid that has three phenolic hydroxyl groups. Total phenolic content was expressed by gallic acid equivalent.

The amount of polyphenol was determined using the modiefied Folin Ciocalteu method (Erol et al. 2009) The modified (Quesille-Villalobos et al. 2013). The result stated as gallic acid equivalent (GAE).

Gallic acid standard processing

Gallic acid stock solution with 100 ppm (mg/L) concentration was made as series standard with various concentration.

The making of gallic acid standard curve

Gallic acid stock solution with various concentration was added by folin reagent respectively and then added by Na2CO3 5%. Each solution was rested for 60 minutes, the absorbance was measured on the maximum wave length. Calibration curve was obtained using regression equation $y = bx + a$.

Total Phenolic Content Testing

1 mL sample / extract was added into a tube then added by 2 mL folin 10% and 2,5 mL Na2CO3 7,5% and rested for 60 minutes in room temperature and dark place. The absorbance from each solution was measured using UV-VIS spectrophotometry on 725 nm wave length.

2) EGCG Specification using HPLC

Ethyl acetate fraction of green tea leaves extract specification was obtained based on *Epigallocatechin gallate* (EGCG) using reversed phase HPLC with isocrathic elution

system (Martono, 2012).

HPLC suitability test

i) The making of HPLC mobile phase system

Mobile phase that used in this study was the combination of 0.1% phosphoric acid: methanol: acetonitrile: aquabidest with a ratio of 14: 1: 3: 7 (v / v / v / v). The distilled water had been filtired previously. After those four solvents mixed, then tri etilamine was added until the pH solution became 4 (Martono, 2012).

ii) The making of *epigallocatechin gallate* (EGCG) mother solution

Epigallocatechin gallate (EGCG) weighed 25,0 mg then dissolved in the mobile phase. This mother solution then used for linearity, repeatability, precision, and the system suitability test (Sugihartini, 2013).

iii) HPLC condition for epigallocatechin gallate (EGCG) assay

The method was obtained based on the study result (Martono, 2012). The condition for *epigallocatechin gallate* (EGCG) assay was the sample injected with 20 µl injection volume then eluted using C18 stationary phase and the combination of 0.1% phosphoric acid: methanol: acetonitrile: aquabidest with a ratio of 14: 1: 3: 7 as the mobile phase on flow rate 1,2 ml/minutes and detected using spectrophotometry UV detector on λ 280 nm.

3. Results and discussion

According to table 1. It showed that the amount of total phenolic content was 900,87 mg/g GAE from PT.Sari Medini, 847,85 mg/g GAE from PT. Sari Kemuning, and 818,51 mg/g GAE from PT.Tambi. It can be concluded that a higher location of tea plant growth will produce a higher amount of total phenolic content. This is because the higher altitude causing the tea plant form more secondary metabolites. If compared with the previous study (Turkmen Erol et al. 2009), the amount of total phenolic content from ethyl acetate fraction of green tea extract was 580,96 mg/g GAE, another study (Quesille-Villalobos, et al. 2013) stated that the highest phenolic content on green tea leaves was 128,7 mg/g. It can be concluded that from the three regions tested in Central Java (PT.Sari Medini, PT.Sari Kemuning, and PT.Tambi) had higher amount of total phenolic content than previous studies.

On the other hand, the highest EGCG content was 26,18 % w/w from PT.Sari Kemuning, 25,85 % w/w from PT.Tambi, and 22,35 % w/w from PT.Sari Medini. If compared with previous studies, EGCG concentration was only 6,7% (Sugihartini, 2013). EGCG content in various green tea product ranged between 0,21-9,63% (Prayong et al. 2007) and EGCG concentration in tea bags ranged between 2,08-3,96% (Martono, 2012). It showed that the three tested green tea sources (Sari Medini, Sari Kemuning dan Tambi) had higher amount of EGCG. It may be caused of ethyl acetate fraction making process that the researcher used the method (Hirun & Roach, 2011) which was treated with an extreme decrease in temperature after infundation process and lowered the pH with phosphoric acid until the pH became 4. The treatment could increase EGCG concentration 16% higher than methanol solvent, while the green tea after infudation process which lowered the temperature gradually (without any treatment) until 22^{o}C temperature showed that EGCG concentration was 30% lower than methanol solvent. The green tea that treated by infudation process 90^{o}C during 30 minutes without any treatment, showed the same result with methanol solvent (Hirun & Roach, 2011).

Table 1. Total phenolic content and EGCG concentration on 3 regions with different altitudes.

Green tea sources	Approximate altitude of the area (m dpl)	Total phenolic content (mg/g GAE)	EGCG concentration (% w/w)
PT. Sari Medini	≥ 2000	900,87	22,35
PT. Sari Kemuning	1540	847,85	26,18
PT. Tambi	500-1000 (250-2250)	818,51	25,85

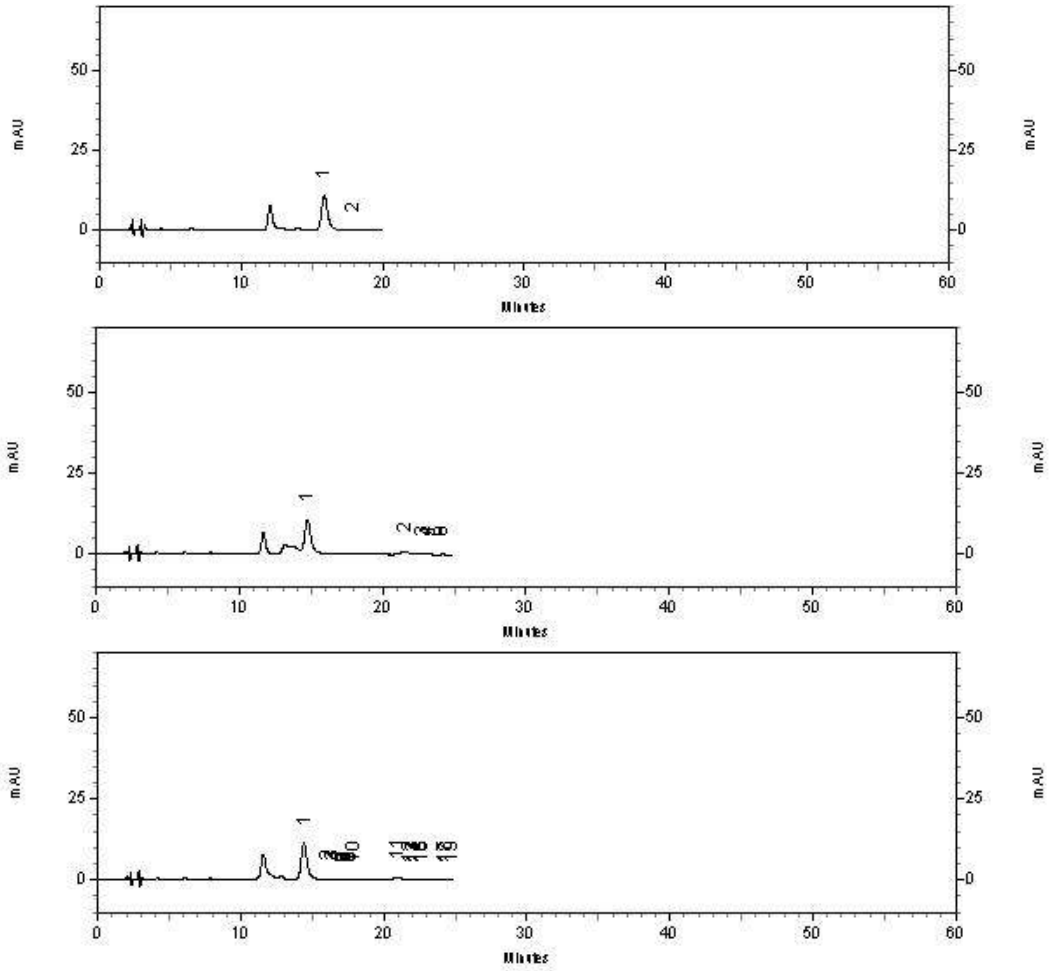

Figure 1. EGCG from PT.Tambi (top), PT.Sari Medini (middle), and PT. Sari kemuning (bottom).

The higher total phenolic content actually will cause the higher of EGCG content obtained, but on table 1. PT. Sari Medini had the lowest EGCG content than PT.Sari Kemuning and PT.Tambi. It may be caused of simplicia making process had not been adjusted to EGCG characteristics. EGCG is an unstable substance. EGCG is easily oxidated, unstable to heat, and well disolved at pH 4-6. EGCG is very unstable at alkali pH (pH >8), EGCG stability is influenced by PH which an acidic pH will make EGCG more unstable (Lestari and Trisusilawati, 2010). Moreover, EGCG is unstable to light because it will be degredated up to 85% after expossed by radiation for 1 hour (Bianchi et al. 2011). Another thing that might occur is a decrease of EGCG concentration at certain time. The previous study (Friedman et.al 2009) stated that

EGCG in 8 green tea products circulating in the USA, Japan, and Korea showed a decrease of EGCG concentration after being stored in a dry temperature (20°C) for 6 months. It was because EGCG as an antioxidant could experience oxidation due to the antioxidant activity of green tea itself was not enough to cope with the excessive oxidative stress.

4. Conclusion

The highest amount of total phenolic content was PT.Sari Medini, while the highest EGCG content was PT.Sari kemuning. The altitude is the factor that influenced the amount of total phenolic and EGCG content in green tea leaves. However, the making process and another factors such as humidity, air polution, land condition, and sun light exposure also affected the amount of total phenolic and EGCG content.

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