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Original Article

The Effect of Bacterial Enzymes on Reducing Chlorogenic Acid Levels in Cascara Robusta Coffee (*Coffea canephora* L.)

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ABSTRACT

Background: Coffee skin by-products, namely cascara, have several benefits, namely can ward off free radicals, with the ability of cascara to ward off free radicals so that cascara can potentially prevent the emergence of cancer cells. Cascara contains active compounds caffeine 1.3%, chlorogenic acid 2.6%, and caffeic acid 1.6%. **Objectivity:** This study aims to determine chlorogenic acid levels in decaffeinated robusta coffee *(Coffea canephora* L.) and see the influence of *Bacillus subtilis* bacteria on reducing chlorogenic acid levels. **Methods:** The experiment was conducted from June to August 2022 in the Pharmacy Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Pakuan, Indonesia. Cascara robusta coffee is fermented using *Bacillus subtilis* with a concentration of 6% and a time of 24 hours. After fermentation, the extraction is carried out using the UAE (Ultrasonic Assisted Extraction) method. The chlorogenic acid levels and zero control of cascara robusta coffee obtained were then analyzed using Statistical Package for the Social Science (SPSS) with the Paired sample t-test method previously carried out with normality test and homogeneity test first. **Results:** The study found chlorogenic acid levels produced from cascara robusta coffee that had undergone decaffeination. Quantitative analysis of chlorogenic acid levels in cascara robusta coffee was carried out using HPLC mobile phase methanol-water (adjust Orthoposphat pH 2.4), flow rate 0.7 mL/minute, with an isocratic system of an average of 14.8597%. **Conclusion:** Chlorogenic acid levels in robusta coffee cascara decaffeinated by microbial enzymes can affect chlorogenic acid levels.

Keywords: Cascara, Bacillus subtilis, chlorogenic acid, HPLC.

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BACKGROUND

Coffee used in Indonesia reached 276 thousand tons in 2016 and will be predicted to increase 8.22 percent to 370 thousand tons in the 2016-2021 period11. The abundant use of coffee produces a by-product, namely coffee skin waste (cascara). In Indonesia, coffee skin is used as animal feed and fertilizer[1]. However, the use of cascara in medicinal raw materials and beverages has not been widely used, while cascara can be used as a functional tea useful as an antioxidant[2,3].

Coffee skin by-products, namely cascara, have several benefits, namely, can ward off free radicals, with the ability of cascara to ward off free radicals so that cascara can potentially prevent the emergence of cancer cells. Cascara contains active compounds: caffeine 1.3%, chlorogenic acid 2.6%, and caffeic acid 1.6% [4,5]. Caffeine compounds found in robusta coffee cascara in addition to having a positive effect, but caffeine also has negative effects if consumed in large quantities, including accelerating heart rate, stimulating the central nervous system, and blood vasodilation[6,7], so that decaffeination is carried out so that there is a decrease in caffeine levels in robusta coffee cascara.

In addition to the bromelain enzyme from pineapple, microbes of the type Bacillus subtilis are also able to produce protease enzymes, where proteases are proteolytic enzymes that can catalyze the breaking of peptide bonds in proteins [8,9]. So, research updates are being carried out on decaffeination with protease enzymes from microbes of the type Bacillus subtilis. In the decaffeination process that has been carried out, not only the content of caffeine compounds will drop, but there are other dominant compounds found in cascara, namely chlorogenic acid, which is bound to the cell wall so that if decaffeination is carried out, it is likely that these compounds will go down along with the release of caffeine compounds in robusta coffee cascara. Chlorogenic acid is an antioxidant useful for reducing the effects of cell damage due to free radicals [3,9,10].

OBJECTIVE

Based on the description above, research will be conducted to determine chlorogenic acid levels with the HPLC (High-performance liquid chromatography) method found in robusta coffee cascara fermented using microbial enzymes of the type Bacillus subtilis.

MATERIALS AND METHODS

Materials

The materials used in this study were robusta coffee cascara derived from Bogor, Indonesia, Bacillus subtilis bacteria, 70% ethanol (Merck®), methanol, standard chlorogenic acid (Sigma-Aldrich®), silica gel, 2 N sulfuric acid, aquadest, mayer reagent, dragendroff, bouchardat, 5 M hydrochloric acid, Mg powder, Zn powder, iron (III) chloride solution, NaCl, gelatin, Nutrient Agar (NA), Nutrient Broth (NB), Aktivatot drops, measuring cups (Pyrex), goblet cups (Pyrex), measuring flasks (Pyrex®®), drip pipettes, micropipettes (Gilson®®), blenders, mesh-sifter 40 (CBN), spatulas, ovens (Memmert®), rotary

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evaporators (Buchi®) furnaces (Daihan® Scientific Furnace), filter paper (Whatman®), hermetically sealed containers, dark bottles, vials, analytical balances (Labpro-DT224C®), columns (Acquity®) C- 18 RP (5 µm, 4.6 µm × 150 mm), HPLC sample injector, 0.45 µm membrane filter (milipore no.2), desiccator, test tube (Pyrex®), funnel, iron spatel, Photo-Diode Array (PDA) detector (Jasco® MD-4010), pH meter (Ohaus®), vial, waterbath, aluminum foil.

Withdrawal of the total caffeine compound

Cascara powder that has been fermented using microbial enzymes. Furthermore, in extraction by UAE method, as much as 5 g of cascara dissolved with 70% ethanol and as much as 50 mL with a ratio (1: 10) was extracted with UAE at a temperature of 37°C with a time of 60 minutes. The filtrate obtained was then filtered using Whatman No.1 paper. Before being injected into HPLC, the sample was filtered again using a membrane filter, after which it was injected into HPLC[3].

Validation of HPLC method Determination

A chlorogenic acid solution was made by pipettes of 0.1 mL of raw solution from a 1000 ppm mother solution using a micropipette diluted with PA ethanol in a vial of 1 mL (100 ppm). The standard solution was filtered using a 0.45 µm (Millipore No.2) membrane filter, put into a vial, and degassed for 15 minutes. A 0.01 mL standard solution was injected and analyzed using the HPLC system. The maximum wavelength for chlorogenic acid compounds was analyzed using a Photodiode Array (PDA) Detector. The maximum wavelength search was performed using a Photodiode-array (PDA) detector, which was analyzed based on the sample absorption pattern. The mobile phase of methanol and water (adjust Orthoposphic acid pH 2.4) was 80:20, 60:40, 50:50, and 55:45. Then the sample was injected and detected using a UV-Vis detector at maximum wavelength, and the formed chromatogram was analyzed based on column coefficients[11,12].

Standard series determination and analysis

standard series of chlorogenic acid was made, pipettes of 100 ppm raw solutions as much as 0.01 mL, 0.02 mL, 0.04 mL, 0.08 mL, 0.16 mL, 0.32 mL, and 0.64 mL using micropipettes were then diluted with PA ethanol on a 1 mL flask. The concentrations obtained were 1, 2, 4, 8, 16, 32, and 64 ppm. The standard series was then filtered using a 0.45 µm (millipore no.2) filter membrane, fed into a vial, and degassed for 15 minutes. A 0.01 mL of standard solution was injected until a regression coefficient of > 0.9977 was obtained [11].

Verification method

Method verification was carried out with several parameters: linearity, accuracy and precision, and selectivity[13].

Sample Analysis

Determination of chlorogenic acid levels was carried out using the HPLC method. At room temperature, a total of 0.01 mL of sample solution and standard chlorogenic acid solution were injected into the C-18 RP column (5 µm, 4.6 µm × 150 mm). The chlorogenic acid content was detected at the wavelength obtained from the PDA detector results. The mobile phase used was methanol-orthophosphate (pH 2.4) with an eluent ratio based on the optimization results that have been obtained previously with a flow rate of 0.8 mL/minute and under isocratic conditions [14]. The chlorogenic acid content was calculated using the linear regression calibration curve method, where the standard concentration is as the x-axis (abscissa) and the standard area is as the y-axis (ordinate) so that the equation y = bx + a with a regression coefficient > 0.9977. Data on chlorogenic acid levels and obtained zero control of robusta coffee were then analyzed using SPSS 24 using the Paired sample t-test method.

Table 1. The results of % recovery.

Standard Solution (mg/L)	Test	% Recovery	Average ± SD
7.5	1	102.0440	102.1660 ± 1.8304
	2	104.0547	
	3	100.4000	
10	1	98.5400	97.0780±1.3368
	2	95.9180	
	3	96.7760	
12.5	1	104.7816	103.5730±1.1213
	2	103.3704	
	3	102.5672	

Table 2. The results of chlorogenic acid levels.

Run	Zero control (mg/5g)	Chlorogenic acid levels (mg/5g)	% Decline	Average ± SD
1	1.3632	1.2065	15.4150	14.8597 ± 2.2313
2	1.3660	1.2049	12.4032	
3	1.2980	1.1629	16.7608	

Table 3. ANOVA Test Results of Chlorogenic Acid.

Parameter	Result
Homogeneity test	< 0.298
Paired Sample T-test	0.003
Linearity (R^2)	0.9992

RESULTS

Results of determining the mobile phase and maximum wavelength

The results of optimizing the mobile phase can be seen in Figure 1. The determination of the maximum wavelength of chlorogenic acid was carried out using a PDA detector, and the aim was to obtain the maximum wavelength waves that could detect the chlorogenic acid compounds contained in the sample optimally. The results of measuring the wavelength of caffeine obtained were 324.4 nm, which can be seen in Figure 1.

Linearity, accuracy and precision

The linear regression equation was obtained, namely y = 143300x + 251059. In the accuracy and precision test, 3 different concentrations were used, namely 7.5, 10, and 12.5 µg/mL 3 times, 1,46282%, 1,12437%, and 0,88374%. The accuracy results were expressed as percent recovery (%recovery). The resulting %recovery range was 95.9180 – 104.7816%. The results of %recovery can be seen in Table 1.

Total chlorogenic acid

Data on chlorogenic acid levels using HPLC obtained an average decrease of 14.8597 %. The results are still beneficial because they can maintain chlorogenic acid of about 85% in decaffeinated cascara robusta coffee. The results of total chlorogenic acid can be seen in Table 2, and the results of the ANOVA test in Table 3.

DISCUSSION

Determination of chlorogenic acid levels using HPLC obtained an average decrease of 14.8597%. The results are still beneficial because they can maintain chlorogenic acid at about 85% in decaffeinated robusta coffee cascara. There is a decrease in chlorogenic acid due to an overhaul of the cell membrane where the cell membrane consists of 52% protein so that the protease enzyme produced by the microbe Bacillus subtilis can break down the components contained in the cell membrane, namely proteins so that chlorogenic acid and caffeine in the cell wall of robusta coffee cascara are also cut off and a decrease occurs[3,15]. This follows research conducted by Anh-Dao et al. (2024),

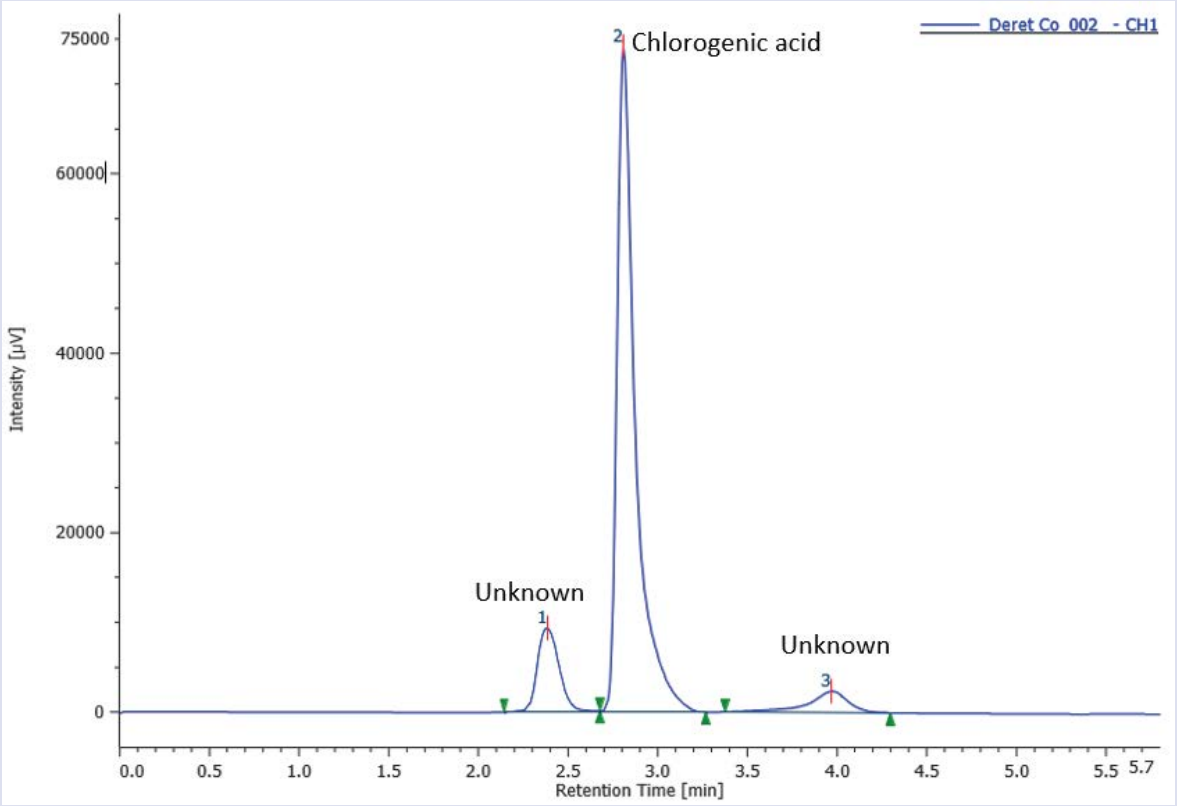

Figure 1. The chlorogenic acid chromatogram in optimizing of mobile phase.

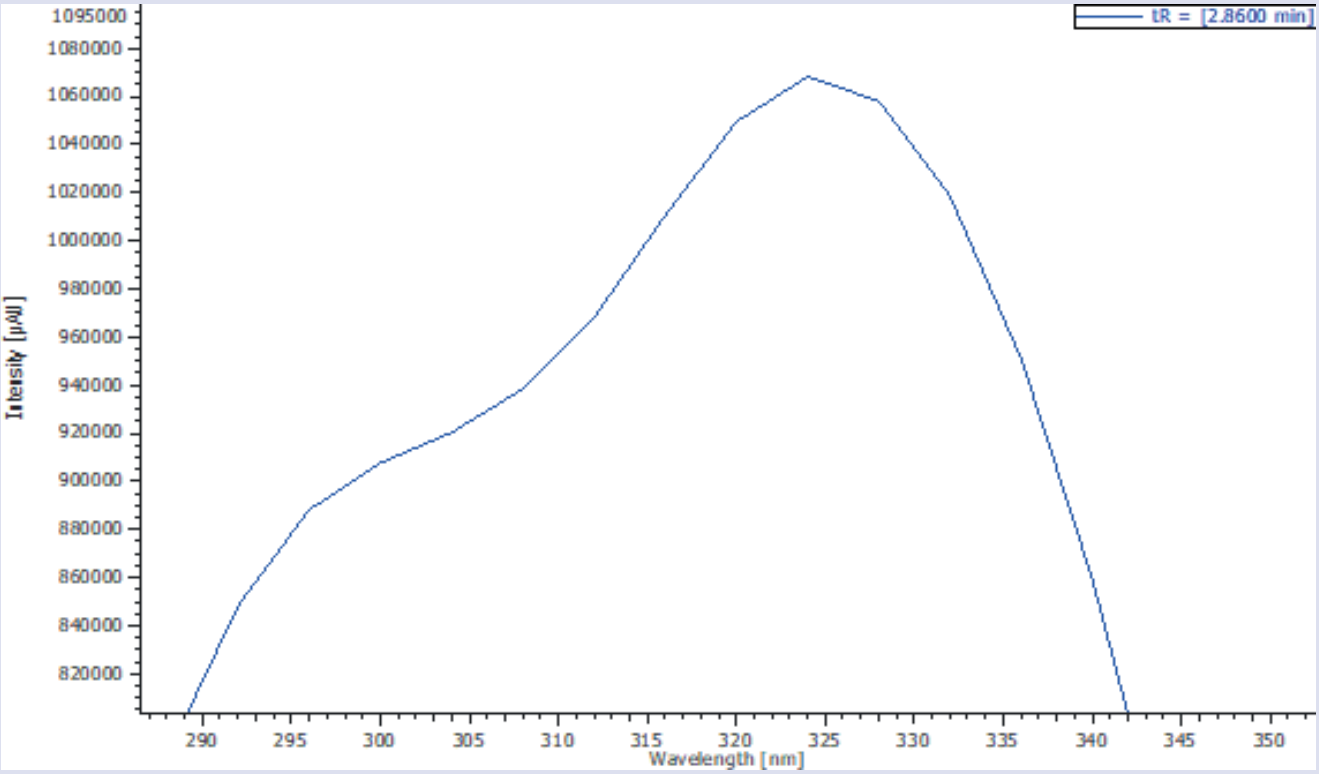

Figure 2. Maximum wavelength of Chlorogenic acid .

caffeine is in the cell wall of coffee beans in a state bound to chlorogenic acid, and caffeine levels decrease after fermentation with the enzyme bromelain. The reduction of chlorogenic acid occurs due to hydrolysis, resulting in a decrease in caffeine in coffee beans[16].

The mobile phase optimization is in Figure. One was carried out to determine the separated compounds in the liquid extract. The separation of compounds in the HPLC system was influenced by several factors, one of which is the bonding of the compound to the stationary phase.

The parameters for optimizing the mobile phase can be seen by separating caffeine compounds from peak shapes. Good results occurred in the mobile phase composition with a ratio of 45:55 and a peak detected at a wavelength of 324.4 nm (Figure 2). Determination of the maximum wavelength of chlorogenic acid is carried out using a PDA detector. The aim is to obtain the maximum wavelength waves that can optimally detect the caffeine compounds in the sample. The purpose of making a standard curve is to see the suitability between the measured response and the concentration of the analyte. The result coefficient value (R^2) of 0.9992 meets the requirements for a good linearity value ≥ 0.99 [3,13] .

In the accuracy and precision test, the result of %recovery meets the requirements, namely 85-110%. Precision was carried out to see the closeness between a series of analyses obtained from several measurements of the same sample. The results of precision test measurements are expressed in percent relative standard deviation (%RSD), resulting in a range of %RSD of 1,46282%, 1,12437% and 0,88374% have fulfilled the requirements, namely ≤ 2% [17] .

ANOVA test results in Table 2 were stated as significant. In this study, a statistical test was carried out using SPSS, which aimed to compare zero control robusta coffee cascara and decaffeinated robusta coffee cascara with *Bacillus subtilis*. The test was carried out using a normality test, and the normality test results obtained Shapiro Wilk sig results of 0.070 and 0.060 with the provision that the sig value > 0.05, and then the data is normally distributed. Furthermore, a homogeneity test was carried out. It was concluded that the treatment data was homogeneous if the sig value > 0.05 from the data entered and the sig value was obtained> 0.298, which means homogeneously distributed data. After the normally distributed and homogeneous data was carried out, further tests using the Paired Sample T-test Paired Sample T-test analysis compare the average of two variables. From the results obtained sig value. (2-tailed) of 0.003, the value obtained was> 0.05, so it can be concluded that there is a real difference between zero control chlorogenic acid levels and decaffeination chlorogenic acid levels.

CONCLUSIONS

Based on the results of the study, it can be concluded that chlorogenic acid levels in robusta coffee cascara that have been decaffeinated with microbial enzymes can affect chlorogenic acid levels by obtaining an average decrease in chlorogenic acid levels in robusta coffee cascara by 14.8597%. Chlorogenic acid levels in robusta coffee caskara that had been decaffeinated with microbial enzymes obtained significantly different results between zero control and decaffeination results.

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