

Effect of Different Extraction Methods on Epigallocatechin Gallate (EGCG) content and Antibacterial Activity (*Pseudomonas aeruginosa*) of Green Tea Leaves (*Camellia sinensis* L.)

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Abstract: *Pseudomonas aeruginosa* is an opportunistic gram-negative bacterium and is often the most common cause in people with immunocompromised problems. Infections by this bacterium, especially in patients with impaired immunity, are difficult to treat due to a number of antibiotic resistance mechanisms. The polyphenol compound Epigallocatechin Gallate (EGCG) contained in *Camellia sinensis* L. is known to have antibacterial activity against several bacteria so that it has the potential as an antibiotic to overcome the problem of antibiotic resistance in *Pseudomonas aeruginosa*. To obtain this compound, extraction with the right method is needed. In addition, the EGCG was dominant extracted at the ethyl acetate fraction. This study aims to analyze the effect of extraction methods on EGCG content and antibacterial activity (*Pseudomonas aeruginosa*) of ethyl acetate fractions of green tea leaves (EAFGL). The research design is an experimental laboratory using a true experimental posttest only control group design. Green tea leaf powder was extracted with 96% ethanol using the sonication and maceration methods, then fractionated evenly with n-hexane, chloroform and ethyl acetate solvents. The EGCG content of EAFGL was determined using High Performance Liquid Chromatography (HPLC). Antibacterial activity of EAFGL at the concentration 12.5; 50; 100; and 200 mg/mL were carried out using the disc diffusion method. Positive and negative control groups used Aztreonam and 10% DMSO, respectively. The antibacterial activity of EAFGL was analyzed using the Kruskal-Wallis and Mann-Whitney test. The results showed that EAFGL extracted using maceration revealed significantly higher EGCG content ($170,97 \pm 17,51 \mu\text{g EGCG/mg fraction}$). The EAFGL at a concentration of 200 mg/mL, obtained through maceration, exhibited the significantly ($p < 0.05$) highest antibacterial activity against *P. aeruginosa* with the inhibition zone of 16.7 mm. Therefore, maceration is an appropriate method for extracting green tea leaves, as it can produce optimal EGCG content and antibacterial activity.

Keywords: Antibacteri, Extraction, *Pseudomonas aeruginosa*, *Camellia sinensis* L.

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INTRODUCTION

Indonesia is one of the largest tea producing countries in the world, with large tea plantations spread across various provinces in Java and Sumatra. In 2021, West Java Province is the region with the largest tea plantation in Indonesia with a total plantation area of 34,138 hectares (68%) of the total tea plantation area in Indonesia. National consumption accounts for nearly two-thirds of Indonesia's tea production. The rest is exported in the form of black tea and green tea.

Tea comes from the genus *Camellia* and species *Camellia sinensis L.*, which is processed by utilizing its young leaf shoots [1]. Green tea leaves contain several phenolic compounds such as flavanols (1-2.5%), flavanones (1.5-3%), phenolic acids (2-5%), caffeine (1.5-2.5%), amino acids (1-5.5%), and organic acids (1-1.8%). In addition, green tea leaves are also the main source of catechin compounds with varying contents ranging from 25% to 35% [2]. Catechin compounds known to be present in green tea are epigallocatechin gallate (EGCG), epicatechin (EC), epicatechin-3-gallate (ECG) and epigallocatechin (EGC) [3]. The EGCG contained in *Camellia sinensis L.* is known to have antibacterial activity against several pathogenic bacteria such as: *Staphylococcus aureus*, *Streptococcus mutant*, *Escherichia coli*. In *Staphylococcus aureus* bacteria [4]. The mechanism of EGCG as an antibacterial is to work by damaging the bacterial cell membrane, inhibiting fatty acid synthesis and inhibiting enzyme activity in bacteria. The ethyl acetate fraction had greater inhibition compared to the n-hexane fraction and the water fraction on *Pseudomonas aeruginosa* bacteria.

Pseudomonas aeruginosa is a gram-negative bacterium that is opportunistic and often the most common cause in immunocompromised people [5]. Infections by these bacteria, especially in immunocompromised patients, are difficult to treat due to a number of antibiotic resistance mechanisms [6]. Fabian (2020) in his research on the resistance of *Pseudomonas aeruginosa* bacteria at Dr. Soetomo Hospital found that resistance occurred in ceftazidime antibiotics (27.4%), gentamicin (24.1%), imipenem (14%), meropenem (11.7%) and amikacin (4.9%) [7]. The antibacterial effectiveness of EGCG contained in green tea leaves has the potential as an antibiotic to overcome the problem of antibiotic resistance. To get this compound, it is necessary to do the right extraction process.

Referring to these problems, researchers

are interested in conducting research on the effect of different extraction methods of green tea leaves (*Camellia sinensis L.*) on antibacterial activity (*Pseudomonas aeruginosa*). The purpose of this study was to determine the effect of different extraction methods of green tea leaves (*Camellia sinensis L.*) on EGCG content and antibacterial activity (*Pseudomonas aeruginosa*). This research is expected to be useful for knowing the different effects of the ethyl acetate fraction of green tea leaf (EAFGL) extracted by sonication and maceration methods as an inhibitor of the growth of *Pseudomonas aeruginosa* bacteria and knowledge of natural materials as natural antimicrobials.

MATERIALS AND METHODS

Materials and Tools

The tools used in this study were sieve number 60, analytical balance, stative separator funnel, glass jar, Whatman No.1 filter paper, Erlenmeyer, water bath, ruler, autoclave, inoculation loop, needle, petri dish, septic box, spirit lamp, micropipette, incubator, tweezers, spuid, glassware, rotary evaporator, sonicator, HPLC (Waters), BSC (Thermo Scientific 1300 Series A2). The materials used in this study were green tea leaf simplisia, the technical solvent (96% ethanol, n-hexane, chloroform, ethyl acetate), the HPLC grade solvent (methanol, acetonitrile, and water), Mueller Hinton agar, MacConkey agar, *Pseudomonas aeruginosa* ATCC 27853 bacterial culture, sterile NaCl, paper disk, positive control (aztreonam), 10% DMSO, *McFarlan* 0.5 and phosphate buffer pH 7.4.

Sample Preparation

Sampling was conducted in the Pangalengan area, West Java. Samples were taken in the morning on May 14, 2024. Plant determination was carried out at the Biosystematics and Molecular Laboratory of the Department of Biology, Faculty of Mathematics and Natural Sciences, Padjadjaran University with the collection number 519. The samples received were dried green tea leaves.

Preparation of Green Tea Leaf Simplisia

The dried samples of green tea leaves were then weighed and the dried samples were powdered using a blender. The powdered simplisia was then sieved using a sieve with a mesh number

of 50. After that, the powdered simplisia was stored in an airtight container, protected from light and given silica gel (food grade).

Preparation of Green Tea Leaf Ethanol Extract

The extraction methods used in this study were maceration and sonication methods. For extraction by maceration method, 400 grams of simplisia powder was dissolved with 4 L of 96% ethanol (1:10 b/v) and allowed to stand for 3 days while occasionally stirring. On the 3rd day the solution was filtered with filter paper and then remacerated. Remaceration was carried out using 2 L of 96% ethanol for 1 day. The solution was then filtered with filter paper and the macerate from maceration and remaceration was combined. The macerate was then concentrated using a Rotary Evaporator at 40°C and a speed of 75 rpm [8]. For extraction by sonication method, 100 grams of green tea leaf simplisia were weighed and then dissolved with 1 L of 96% ethanol (1:10 b/v). Extraction was carried out using a sonicator with a frequency of 20 kHz at 30°C for 45 minutes [9]. After 45 minutes, the solution was filtered with filter paper and then remacerated with 500 mL of 96% ethanol under the same sonicator conditions. The remacerated solution was then filtered with filter paper. The entire extracted macerate was evaporated using a rotary evaporator at 40°C with a speed of 75 rpm [10].

Fractionation of Green Tea Extract

Fractionation was carried out using 4 solvents based on differences in their level of polarity, namely water (polar), ethyl acetate (semi-polar), chloroform (semi-polar), and n-hexane (non-polar). The sample used was 20 grams of green tea extract extracted by sonication and maceration methods. Separation was carried out using a 500 mL separatory funnel. In the process, the mixture was shaken gently by rotating horizontally and occasionally the cover of the separatory funnel was opened, so that the trapped air could escape so that the air pressure decreased.

Determination of EGCG levels using HPLC

Chromatogram system

The stationary phase used was an XBridge RP C-18 column (4.6 X 150 mm, 5 µm). The mobile phase consisted of 0.1% phosphoric

acid: methanol: acetonitrile: water in a ratio of 11:3:5:6 (v/v/v/v). The mobile phase mixture was then added to a pH 7.4 phosphate buffer until a pH of mobile phase was 4. The flow rate was 0.5 mL/minute.

Analysis of sample

A series of standard EGCG solutions of 20, 40, 60, 80, and 100 ppm were prepared by diluting a 1000 ppm EGCG solution using the mobile phase. Ten mg of the ethyl acetate fraction of green tea leaves extracted using the maceration and sonication methods was dissolved in the mobile phase. The sample solution was diluted to a concentration of 200 ppm. Each standard and sample solution was filtered using a 0.45 µl filter membrane. The solution was placed in an HPLC auto-sampling device. The solution was run using an appropriate chromatogram system. The concentration of EGCG was calculate using the followed formula.

$$\text{EGCG concentration (ppm)} = \frac{C \times V \times df}{W}$$

C= Concentration regression (x) (ppm), obtained from the standard curve (Y= bx + a)

V= Volume to dilute the sample (mL)

df= dilution factor

W= weight of sample (mg)

Rejuvenation of Bacteria

Bacterial isolates that have been tested at the West Nusa Tenggara Provincial Health Laboratory Testing and Calibration Center are then rejuvenated at the Medical Education Study Program Laboratory, Faculty of Medicine and Health Sciences, Mataram University. Bacterial rejuvenation was carried out on *MacConckey* media and then incubated at 37°C for 24 hours.

Preparation of Antibacterial Test Media

Weighed 19 grams of MHA media powder and dissolved in 500 mL Erlenmeyer using distilled water. The mouth of the Erlenmeyer was then plugged with cotton and covered with aluminum foil. The media was sterilized with an autoclave at 121°C for 20 minutes along with petri dishes that would be used to make the media. Each media was put in a Petri dish as much as 20 mL aseptically and after the media solidified, it was stored in a refrigerator or refrigerator.

Antibacterial Test

Bacterial suspension was made first by taking isolate of *Pseudomonas auregonosa* MDR bacteria using an inoculation loop, then put into sterile NaCl solution. The solution was then compared with 0.5 mL *McFarland* standard solution. The bacterial suspension was taken using a sterile micropipette as much as 100 microliter and a smoothing swab was made on MHA media and then allowed to stand for 10 minutes. While waiting for the swab results, the paper disks were immersed in a container that contained ethyl acetate fractions extracted by maceration and sonication methods of several concentrations, as well as negative controls. Then the disk that has been soaked in the fraction is placed in a Petri dish that already contains a bacterial suspension and incubated in an incubator at 37°C for 24 hours. The diameter of the inhibition zone was then measured using a ruler and compared with the inhibition zone in the control.

Data Analysis

The statistical analysis was carried out using SPSS v.25. Quantitative data analysis of inhibition zones from antibacterial tests was performed statistically using the Kruskal-Wallis and Mann-Whitney test. The EGCG content of both EAFGL fractions was analyzed using independent t-test.

RESULTS AND DISCUSSION

The Yield of Extract

The yields of thick green tea leaf extract by maceration and sonication methods were 16.55% and 11.7%, respectively. The yield of thick extracts from the two extraction methods obtained is stated to have met the standard of >10%. The greater the yield produced, the more efficient the treatment given and it is assumed that the compound components in the extract produced are higher. The yield results of green tea leaf extract can be seen in Table 1.

Table 1. The yield of *Camellia sinensis* leaves extract

Extraction method	Time extraction	Yield of extract (%)
Maceration	3x24 hours	16.5
Sonication	2x45 minutes	11.7

The extracts obtained from both extraction methods were then fractionated. The purpose of fractionation is to separate the target compounds (EGCG). The fractionation process is carried out in stages using several types of solvents with different polarity properties. The solvents used were distilled water, n-hexane, chloroform and ethyl acetate.. Multilevel fractionation is carried out with the aim that the fraction obtained is a specific fraction based on its polarity so that with this multilevel fractionation, secondary metabolite compounds can be maximally attracted by the solvent [11]. The n-hexane solvent extracted the non-polar compound, such as steroid [12]. Meanwhile, the chloroform and ethyl acetate was the semi-polar solvent, but ethyl acetate has the higher polarity than chloroform. The chloroform was reported can extraction the alkaloid [13]. In addition, the EGCG was dominant extracted at the ethyl acetate fraction [14]. The yield of ethyl acetate fraction of maceration and sonication extracts was 0.793 % and 0.9521 %, respectively.

EGCG content

The standard solution calibration curve was created using five concentration series, namely 20, 40, 60, 80, and 100 ppm. These five concentrations series were then analyzed using HPLC. The results of the analysis will yield the AUC value. The AUC value can be seen in Table 2. The standard regression curve for EGCG was then created by plotting the concentration and AUC values. From the standard curve shown in Figure 4.6, the equation $y = 14265x - 24554$ was obtained with an R^2 value of 0.9915. The two variables, concentration and AUC value, can be said to have linear values because they have a correlation coefficient (R^2) close to 1. The R^2 value ranges from 0 to 1, where the closer it is to 1, the better the correlation level shown by the regression curve.

Table 2. AUC value of EGCG standard

Concentration (ppm)	AUC
20	144267
40	274127
60	511386
80	1049328
100	1464368

Table 3. EGCG content of ethyl acetate fraction of green tea leaves

Method	Area Under Curve (AUC)	EGCG content (μg EGCG/mg)	$\bar{x} \pm \text{SD}$ (μg EGCG/mg)
Maceration	223868	174,1	
	192520	152,1	$170,97 \pm 17,51^a$
	241839	186,7	
Sonication	89940	80,2	
	66796	64	$73 \pm 8,24^b$
	82263	74,8	

The different superscript letter (^{a,b,ect}) at the one column showed the significantly different antibacterial activity ($p < 0.05$). The statistical analysis was determined using independent t-test.

The EGCG content measurements showed differences in content between each extraction method. Table 3 showed that the EAFGL from maceration extraction method has a significantly higher average EGCG content compared to the sonication method. The result may be due to several factors, the first of which is extraction time. Short extraction times may result in the bioactive compounds in green tea leaves, especially EGCG, not being extracted to their full potential. According to Kristina et al. (2022), extraction time that is too short can result in not all bioactive compounds being extracted from the sample [15]. Meanwhile, in the maceration method, the extraction process is carried out for a longer period, so that the longer contact time between the sample and the solvent allows the bioactive compounds to be extracted more effectively.

Another factor that affects EGCG levels is temperature. Extraction using the maceration method does not involve any heat treatment. Maceration is extraction carried out at room temperature without any increase in temperature or heating. Therefore, with this method, heat-sensitive compounds such as EGCG are not easily damaged or decomposed [16]. Based on research conducted by Widyaningrum et al. (2019), it was found that EGCG is stable at 2°C, and Fanguerio et al. (2014) stated that EGCG is stable at 2°C [14]. Additionally, in the extraction process using the sonication method, temperature treatment is applied. In this study, the temperature used was 30°C with a time of 2x45 minutes. Temperature is one of the factors that influence the extraction results using the sonication method. Extracting green tea at low temperatures for a

short time or high temperatures for a long time using the sonication method is not effective in obtaining sufficient amounts of EGCG from green tea [17]. Therefore, optimal conditions are essential for extraction using the sonication method.

Antibacterial activity

The results of rejuvenation on *MacConckey* media are shown in Figure 1. The results showed that the bacterial colonies that grew on *MacConckey* media were yellow in color. In addition, *Pseudomonas aeruginosa* bacteria do not ferment lactose so they are colorless on *MacConckey* media [18].



Figure 1. Macroscopic observation results on *MacConckey* medium

After rejuvenating the bacteria, identification is then carried out by gram staining. The purpose of gram staining is to ensure that the bacteria used during testing are pure *Pseudomonas aeruginosa* bacteria without being contaminated with other bacteria. The results of gram staining are shown in Figure 2. From the results of gram staining, it was found that there were bacterial colonies that showed a red color with bacillus-shaped morphology and solitary arrangement.

The test media used for antibacterial testing is MHA media. From 500 mL of MHA media solution, 25 media were obtained in Petri dishes. For antibacterial testing, 12 Petri dishes were used for 4 series of test concentrations with 3 replications for each concentration.

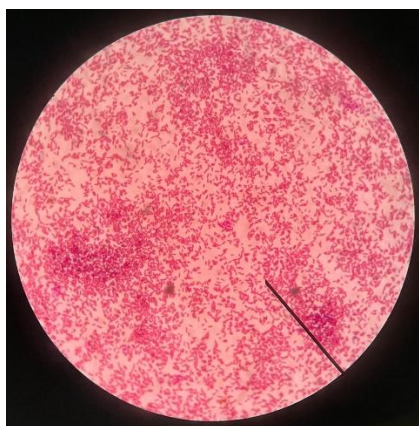


Figure 2. Microscopic observation of *Pseudomonas aeruginosa* on Gram staining medium

Table 4. Antibacterial activity of ethyl acetate fraction of *Camellia sinensis* leaves

Method	Concentration (mg/mL)	Inhibition zone (mm) $\bar{x} \pm SD$	Categorized
Maceration	12.5	9 ± 2.29^a	Moderate
	50	10.16 ± 0.28^a	Strong
	100	15 ± 1.32^{ab}	Strong
	200	16.7 ± 0.57^b	Strong
Sonication	12.5	2.23 ± 0.38^c	Weak
	50	2.37 ± 1.15^c	Weak
	100	2.63 ± 0.57^c	Weak
	200	3.33 ± 1.52^c	Weak
Control	Aztreonam	17.3 ± 0.91^{bd}	Strong
	DMSO	0.00^e	-

The different superscript letter (^{a,b,ect}) at the one column showed the significantly different antibacterial activity ($p < 0.05$). The statistical analysis was determined using Kruskal-Wallis and followed with Mann-Whitney test

Antibacterial tests were carried out using the disc diffusion method through 4 concentrations, namely 12.5 mg/mL, 50 mg/mL, 100 mg/mL and 200 mg/mL. Ethyl acetate fraction can inhibit the growth of *Pseudomonas aeruginosa* bacteria in all concentration series with 200 mg/mL concentration series of macerated extracts most effective in inhibiting the growth of *Pseudomonas aeruginosa* compared to other concentrations and sonication method. The presence of inhibition on the growth of *Pseudomonas aeruginosa* bacteria is characterized by the appearance of a clear zone around the disc [9]. The results of the antibacterial activity test are presented in table 4.

The entire concentration series showed the presence of an inhibition zone against bacteria. The classification of antibacterial is divided into several classes, namely weak (diameter ≤ 5 mm), moderate (diameter 5-10 mm), strong (diameter 10-20 mm), and very strong (diameter ≥ 20 mm) [19]. The inhibition zone at all concentrations of sonication extracts is in the weak category, as shown in Table 3. The EAFGL extracted using maceration method at a concentration of 12.5 mg/mL showed a moderate inhibition category. While the concentrations of 50 mg/mL, 100 mg/mL and 200 mg/mL are included in the strong inhibition category.

From the results obtained, the inhibition zone of EAFGL extracted using maceration was significantly ($p < 0.05$) better than the EAFGL extracted using the sonication (Table 3). This can be caused because in the maceration process, the contact time between the sample and the solvent is longer so that the compound which has antibacterial activity, especially EGCG, can be extracted maximally. Meanwhile, the sonication method, the extraction time is only carried out for 2x45 minutes so that the sonication method at that time is likely to be less than optimal in extracting the target compound, EGCG. The extraction more extraction time, the more EGCG content that was obtained [20]. Extraction time is one of the factors that affect the extraction results with the sonication method. A short extraction time can cause not all bioactive compounds to be extracted from the sample [15]. In addition, the maceration method was the cool extraction method. Thus, the antibacterial compound that was sensitive to temperature was better protected. The EGCG at green tea leaves was higher at the temperature extraction 10°C than the 40°C [20].

In the antibacterial test performed, the positive control used was aztreonam with the test results showing a strong inhibition category. The reason for using aztreonam as a positive control in this study is because aztreonam is only active

against gram-negative organisms such as *Pseudomonas aeruginosa*, and does not retain any activity against gram-positive or anaerobic organism. In the antibacterial test carried out, a negative control was also used, namely DMSO 10%. The use of 10% DMSO as a negative control and fraction solvent is based on its nature which does not have antibacterial activity and generally only functions as a solvent [21]

CONCLUSION

The results revealed the ethyl acetate fraction of green tea leaves (EAFGL) from macerated extract has significantly higher EGCG content ($170,97 \pm 17,51 \mu\text{g EGCG/mg}$ fraction). In addition, the EAFGL from sonication and maceration extracts had antibacterial activity against *Pseudomonas aeruginosa* bacteria. The EAFGL of the macerated extract with a concentration of 200 mg/mL is the most optimal concentration in inhibiting the growth of *Pseudomonas aeruginosa* bacteria with an inhibition diameter of 16.7 mm. The ability of the ethyl acetate fraction from the maceration extraction method is greater than the sonication method because in the maceration process, the contact time between the sample and the solvent is longer so that the target compound (EGCG) which has antibacterial activity can be extracted maximally. Thus, maceration is a suitable technique for extracting green tea leaves, yielding optimal EGCG levels and strong antibacterial activity.

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