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Original Research Article

Antibacterial Activity, TLC-Bioautography Analysis, and Determination of Bioactive Components in Ethyl Acetate Extract of Robusta Coffee Leaf (*Coffea canephora* L.) From Aceh, Indonesia

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ABSTRACT

Infectious diseases affect many people in the world, including Indonesia. One of the causes of infection is bacteria, including *Escherichia coli* and *Staphylococcus aureus*. Robusta coffee (*Coffea canephora* L.) is one of the plants that is widely encountered on Indonesian plantations. The leaves are usually discarded after being trimmed to maximize the growth of coffee beans. The results of several studies show that robusta coffee leaves demonstrate antibacterial and antioxidant activity due to the presence of flavonoids, alkaloids, tannins, saponins, polyphenols, and chlorogenic acids. The aim of this research to investigate antibacterial activities, total flavonoid content and inspect the phytochemicals of the bioactive compounds as antibacterial with TLC-Bioautography in the ethyl acetate extract of robusta coffee leaves. The identification of secondary metabolites was conducted by using the TLC-Bioautography method against *Escherichia coli* and *Staphylococcus aureus*. The determination of total flavonoid levels was carried out by using Spectrophotometry UV-Vis. Phytochemical testing showed that the extract contained alkaloids, steroids, phenolics, tannins, and flavonoids. The ethyl acetate extract inhibits the growth of *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 (P=0.00) with 100% extract as the most potent. The results of TLC-Bioautography showed the presence of polyphenolic compounds (R_f 0.36) which was confirmed by a clear zone in the disc diffusion test. The total flavonoid content in robusta coffee leaves was 57.255±0.0348 mg QE/g extract. Ethyl acetate extract from coffee leaves (*Coffea canephora* L). inhibits the growth of *S. aureus* and *E. coli* due to the presence of polyphenolic compounds

***Keywords*:** Antibacterial, *Coffea canephora* L, TLC-Bioautography, Total flavonoids, Spectrophotometry.

Introduction

Infectious diseases are a serious health concern in every country around the world, including Indonesia. One of the causes of infection is bacteria, including *Escherichia coli* (*E. coli*) and *Staphylococcus aureus (S. aureus)*. When *E. coli* and *S. aureus* are excessive in the body, they become harmful to human health, so it is necessary to prevent the growth of bacteria.[1] One of the plants that can be used as an antibacterial substance is coffee.[2,3] Indonesia is known as the largest producer of coffee (*Coffea* sp.) in the world and has managed to be in the fourth rank after Brazil, Vietnam, and Colombia. The coffee plant has been cultivated since the 15th century. To date, coffee has become one of the most consumed beverages and has even been considered a modern lifestyle.[4,5]

Robusta coffee (*Coffea canephora* L.) is one type of coffee plant that is often found in Indonesian plantations, but the use of robusta coffee leaves in natural medicines is still lacking. The coffee leaves are usually trimmed and discarded to maximize the growth of the coffee beans. It will be more beneficial when the leaves are used for therapeutic reasons. It is well known that the compounds contained in robusta coffee leaves show antibacterial and antioxidant activity. Compounds contained in coffee leaves are alkaloids, flavonoids, saponins, tannins, polyphenols, and chlorogenic acid.[6]

Muslim and Dephinto's research (2019) found that the ethyl acetate fraction of robusta coffee leaves had a higher inhibition zone compared to the water, ethanol, and n-hexane fractions against *S. aureus* and *E. coli*.[1] Robusta coffee leaves contain high levels of flavonoids.[2] Flavonoids exhibit antibacterial activity through the inhibition of nucleic acid synthesis, energy metabolism, and alterations in cytoplasmic membrane function.[7] One of the solvents that can extract flavonoid compounds in large concentrations is ethyl acetate.[8] Reviewing the current literature, no data was reported regarding the chemical and biological features of *Coffea canephora* L. grown in Aceh. The aim of this research was to investigate antibacterial activities, total flavonoid content and inspect the phytochemicals of the bioactive compounds as antibacterial with TLC-Bioautography in ethyl acetate extract of Robusta coffee leaf.

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Material and Methods

Plant material and bacteria

Robusta coffee leaves (*Coffea canephora* L.) used in this study were collected in May 2022 from Pante Cermin, Aceh Jaya, Indonesia. Robusta coffee leaves were identified and confirmed by Saida Rasnovi (261/UN11.1.8.4/TA.00.01/2022) at the *Laboratorium Biosistematika Tumbuhan*, Faculty of Math and Science, Syiah Kuala University, Aceh, Indonesia. *Staphylococcus aureus* ATCC 25923 and *Escherichia*

coli ATCC 25922 were sourced from the Fundament Lab Sains (*Laboratorium Pengujian Mikrobiologi*), Aceh Besar, Indonesia.

Equipment and chemicals
The material used were Aquadest (Brataco), ethyl acetate (Merck), n-hexane (Merck), disc paper, filter paper, silica gel TLC plate F254 (Merck), $FeCl_3$, $AlCl_3$, H_2SO_4, Dragendrof's solution, 0.9% NaCl solution, Mueller Hilton Agar (Oxoid) and DMSO (Merck), tweezers, autoclave (Sturdy SA-300VF-F-A500), laminary air flow (Scientific).

Preparation of Ethyl Acetate Extract of Robusta Coffee Leaves by Maceration
Robusta coffee leaf powder (300 grams) was soaked in 3000 mL of ethyl acetate for 6 hours, while being stirred occasionally, then it was allowed to sit for 18 hours. The macerate was then separated by filtration. The second maceration was carried out by adding 500 mL of ethyl acetate. All filtrate was then collected, and the solvents were evaporated using a vacuum rotary evaporator which produced a thick extract.[9]

Phytochemical test
The phytochemical screening was carried out using the standard method of flavonoid, saponin, tannin, steroid/triterpenoid and alkaloid test.[10]

Antibacterial test
Forty milliliters of Mueller Hilton Agar (MHA) was poured on to Petri dishes and was allowed to harden. *S. aureus* and *E. coli* bacteria were inoculated by using a cotton swab. Each media was divided into 5 areas, i.e. P0 was placed with discs that had been soaked with DMSO (negative control); P1 was placed with 25% concentration of Robusta coffee leaf ethyl acetate extract, P2 was placed with 50% Robusta coffee leaf ethyl acetate extract, P3 was placed with 75% Robusta coffee leaf ethyl acetate extract, P4 was placed with 100% Robusta coffee leaf ethyl acetate extract. All Petri dishes were incubated at 37°C for 2×24 hours with the petri dish inverted. Bacterial growth was observed in all of treatment. A caliper was used to measure the diameter of the inhibitory zone.[11]

TLC-Bioautography test
Thin Layer Chromatography (TLC) Setup
TLC Plate Preparation; The separation of compounds from the ethyl acetate extract of robusta coffee leaves was carried out using a silica gel plate F_{254} as the stationary phase with a size of 2 x 10 cm. Next, a line was marked on the top and bottom edges of the plate with a distance of 1 cm to indicate the initial position of the spots and the top edge as a boundary sign of the elution process. A total of eleven plates were prepared.
The ethyl acetate extract was smeared on the TLC plate as much as 5 times (in the same place) from the bottom edge of the plate above the TLC silica gel F254 plate using a capillary tube, and then the spots were allowed to dry.
Preparation for elution; The chamber was filled with the mobile phase, n-hexane-ethyl acetate (8:2, v/v). The filter paper was placed on the chamber wall to detect saturation which was often achieved in 10 minutes. The plate was then stained with the sample and placed into the chamber. The sample was allowed to elute to a swelling distance of 8.0 cm. The TLC plate was then removed and allowed to dry, which was indicated by the absence of the mobile phase smell. The R_f value of each spot was then recorded. The plate was observed first under a 254 nm and a 366 nm UV lamp. The plates were also sprayed with H_2SO_4, $FeCl_3$, Dragendrof, and lCl_3 reagents respectively. The other six plates were used for bioautographic tests against *S. aureus* and *E. coli*.[12]

Bioautography test
Three Petri dishes were used in this evaluation. Each dish was poured with 25 mL of MHA. After the media solidified, the bacterial suspension was inoculated with a cotton swab. Each Petri was placed with a chromatogram on the surface of the media and left in contact with the media for 30 minutes. The chromatogram was removed from the media and the Petri was tightly closed and incubated for 18-24 hours at 37°C. When the clear zone was formed, the R_f values were then measured. The chromatogram plates were then sprayed with H_2SO_4, $FeCl_3$, Dragendrof, and $AlCl_3$ reagents to identify the class of secondary metabolites which actively inhibit the bacteria.[13–15]

Determination of Total Flavonoid Level
Maximum wavelength determination.
Determination of the maximum wavelength of quercetin was carried out by running the quercetin solution in the wavelength range of 400-450 nm. The results of running showed that the maximum wavelength of the quercetin standard was at a wavelength of 435 nm. The maximum wavelength was used to measure the absorption of the sample of ethyl acetate extract of Robusta coffee leaves.
Quercetin standard curve generation
Ten milligrams of standard quercetin were dissolved in 50 mL of 70% ethanol to make a 200 ppm solution. This solution was diluted to make the 2, 4, 6, 8, and 10 ppm solution. From each concentration of the standard solution of quercetin, 1 mL was pipetted. Then 0.2 mL of 10% $AlCl_3$ and 0.2 mL of 1M potassium acetate were added. Samples were incubated for 30 minutes at room temperature. The absorbance was determined using the UV-Vis spectrophotometric method at a maximum wavelength of 435 nm.
Determination of the total flavonoid content of Robusta coffee leaf ethyl acetate extract.
Ten milligrams of the extract were dissolved in 50 mL of 70% ethanol. One mL of the solution was pipetted and then added with 0.2 mL of 10% AlCl3 solution and 0.2 mL of 1M potassium acetate. Samples were incubated for 30 minutes at room temperature. The UV-Vis spectrophotometric technique was employed to determine the absorbance at a maximum wavelength of 435 nm. Samples of the extract were prepared in triplicate.[16]

Data analysis
The data obtained from the bacterial inhibitory test was analyzed using One-Way ANOVA. If there were any significant differences, Post Hoc analysis was performed using Duncan. The result of the bioautography test was analyzed by comparing the spots which had a clear zone with the TLC profile to identify which compound is actively inhibiting the bacteria. The total flavonoid level was analyzed by the linear regression equation.

Results and Discussion

The sample used in this study was Robusta coffee leaves (*Coffea canephora* L.) that had been identified in Biology Herbarium, Faculty of Mathematics and Natural Sciences, Syiah Kuala University, Banda Aceh. The yield of ethyl acetate extract of robusta coffee leaves (EAE) was 4.96%. The extract was a blackish green, thick, and had a distinctive aroma. The phytochemical test results showed that EAE contains alkaloids, steroids, phenolics, tannins, and flavonoids (Table 1). Flavonoids are phenolic compounds because their colour will change when added with a base or ammonia. Flavonoids are also water-soluble compounds.[17]

Table 1: Phytochemical test results of robusta coffee leaf ethyl acetate extract

Metabolite Content	Test Results
Alkaloid	+
Steroid	+
Terpenoid	-
Saponin	-
Flavonoid	+
Phenolic	+
Tanin	+

(+) indicates contains the tested compound; (-) indicates contains no tested compound.

The results of antibacterial activity showed that the extract of Robusta coffee leaves shows inhibitory activity against *S. aureus*, which is indicated by the clear zone of 7.75; 8.27; 9.31; and 10.20 mm for concentrations of 25%, 50%, 75%, and 100% respectively. The evaluation against *E. coli* shows that the inhibition zone was only formed in the 75% and 100% extract, 6.68 mm and 7.31 mm, respectively (Figure 1 and Table 2). The ANOVA test indicated that the extract significantly inhibited (P=0.000) the growth of *S. aureus* and *E. coli*. The largest inhibition zone was observed at a concentration of 100% (10.20 mm). One of the reasons for this is due to the maximum concentration used in the test. The higher the concentration of the test material, the more active substances contained within it.[18] The clear zone resulted from the activity of the active compounds in inhibiting bacterial growth by various mechanisms. One of the compounds contained in EAE is a flavonoid. Flavonoids produce extracellular proteins which dissolve in the microbial cell wall and disrupt the bacteria cell cycle.[19]

The inhibitory activity is higher against *S. aureus* compared to *E. coli*. This is because the active compounds can easily penetrate the Gram-positive bacteria cells compared to Gram-negative. Gram-positive bacteria had thinner cell walls compared to Gram-negative. Gram-positive bacteria had more peptidoglycan, less lipid, and no lipopolysaccharide layer. Hydrophobic substances can easily pass through the cell wall, interfere in the cell walls, membrane, and internal parts of the cell lead to an increase in internal osmotic pressure resulting in cell lysis. The cell wall structure of Gram-negative bacteria is more complex, consisting of three layers including lipoprotein, peptidoglycan, and lipopolysaccharide. This relatively complex cell wall structure makes it more difficult for antibacterial compounds to enter the cells.[20] The ANOVA test showed that EAE inhibits bacterial growth significantly (P=0.000). Duncan's test results showed that the inhibitory activity of EAE was significantly different in each treatment against *S. aureus*. Tests conducted on *E. coli* showed that the EAE at concentrations of 75% and 100% were significantly different from the rest of the treatments. Based on the classification of Morales Inhibition, the EAE was able to inhibit the growth of *S aureus* in the strong category and *E. coli* in the moderate category (Table 2).

The results of the TLC-bioautography test against *S. aureus* and *E. coli* can be seen in Figure 2 and Table 3. Eight spots appeared on the TLC plate. The spots at R_f 0.07; 0.14; 0.19; 0.89 are flavonoids, at R_f 0.24 is an alkaloid, at R_f 0.29 is phenolic, at R_f 0.36 is a polyphenol and at R_f 0.50 is tannin (Figure 2 and Table 3). TLC-bioautography test combines TLC separation and biological activity detection.[12] By observing the clear zone formed in the test, it is possible to detect antimicrobial activity directly on the TLC plate.[12] TLC-bioautography is an advanced test that serves to determine the chemical compounds that provide antibacterial activity from the ethyl acetate extract of Robusta coffee leaves. This method is carried out by attaching a TLC plate on top of the agar media that has been inoculated with the test bacteria.[14,15] The results of TLC-bioautography showed that the EAE inhibits *S. aureus* and *E. coli* bacteria at R_f 0.36. Identification of chemical components was carried out to determine the class of chemical compounds contained in the extract using a TLC plate which then are sprayed with H_2SO_4, $AlCl_3$, $FeCl_3$, and Dragendorf reagents. This method is easy, safe, effective, and efficient.[21]

The R_f value of 0.14 showed the presence of flavonoid compounds. This spot was visible when it was sprayed with $AlCl_3$ and produced a yellow color. Reagent $AlCl_3$ reacts with flavonoid groups to form complexes between hydroxyl groups and neighboring ketones or with neighboring hydroxyl groups.

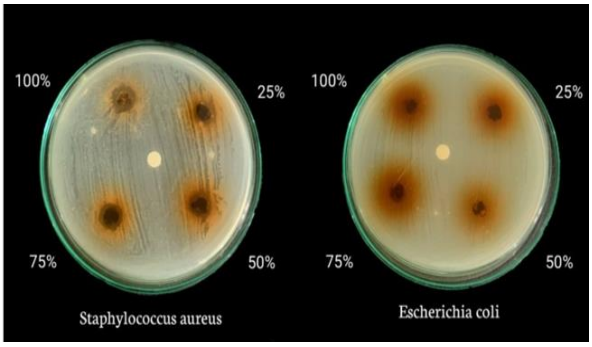

Figure 1: The results of the inhibition test of ethyl acetate extract of robusta coffee leaf against *S. aureus* and *E. coli*.

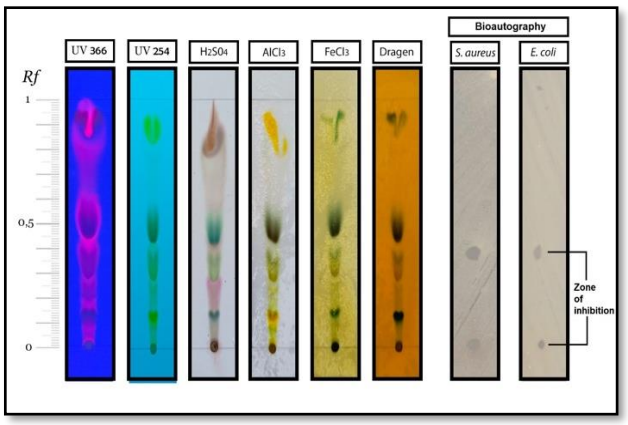

Figure 2. Results of TLC-Bioautography of ethyl acetate extract of robusta coffee leaves against *S. aureus* and *E. coli*.

Table 2. The results of the Duncan test and clasification of inhibition activity

Bacteria	Treatment	Average ± SD (mm)	Inhibition activity
S. aureus	DMSO	0 ± 0^a	None
	EAE 25%	7.75 ± 0.06^b	Moderate
	EAE 50%	8.27 ± 0.13^c	Moderate
	EAE 75%	9.31 ± 0.19^d	Moderate
	EAE 100%	10.20 ± 0.09^e	Strong
E. coli	DMSO	0 ± 0^a	None
	EAE 25%	0 ± 0^a	None
	EAE 50%	0 ± 0^a	None
	EAE 75%	6.68 ± 0.08^b	Moderate
	EAE 100%	7.31 ± 0.11^c	Moderate

EAE: Robusta coffee leaf ethyl acetate extract. Data represent means ± SD (n=5). Different letter superscripts indicate differences (P<0,05).

Table 3. TLC results of the ethyl acetate extract of robusta coffee leave in the bioautography test against *S. aureus*

R_f	Sighting of TLC Results Spots						TLC-Bioautography		Compound Group
	UV 366	UV 254	H_2SO_4	$Alcl_3$	$Fecl_3$	Dragendorf	*S. Aureus*	*E. Coli*	
0.07	Red Fluorescence	Yellow	-	Green	-	-	-	-	Flavonoid
0.14	Red fluorescence	Greenish yellow	Green	Yellow	Green	Blackish Green	-	-	Flavonoid
0.19	Black	Yellow	-	Green	-	-	-	-	Flavonoid
0.24	Red fluorescence	Yellow	Pink	-	-	Brown	-	-	Alkaloid
0.29	Red fluorescence	Greenish yellow	Green	-	Brown	-	-	-	Phenol
0.36	Red fluorescence	Yellow	Green	Blackish Green	Brownish Green	Green	+	+	Polyphenol
0.50	Black reddish fluorescence	Brownish Green	Turquoise	-	Blackish Green	Blackish Green	-	-	Tanin
0.89	Black reddish fluorescence	Yellow	Brownish Purple	Yellow	Green	Grey	-	-	Flavonoid

Bioautography (+): A clear zone is formed indicating the presence of antibacterial activity

Table 4: Total Flavonoid Content EAE

Absorbance	Flavonoid Content (mg QE/g extract)	Average Flavonoid Content ±SD (mg QE/g extract)
0.971	57.235	
0.972	57.295	57.255 ± 0.0348
0.971	57.235	

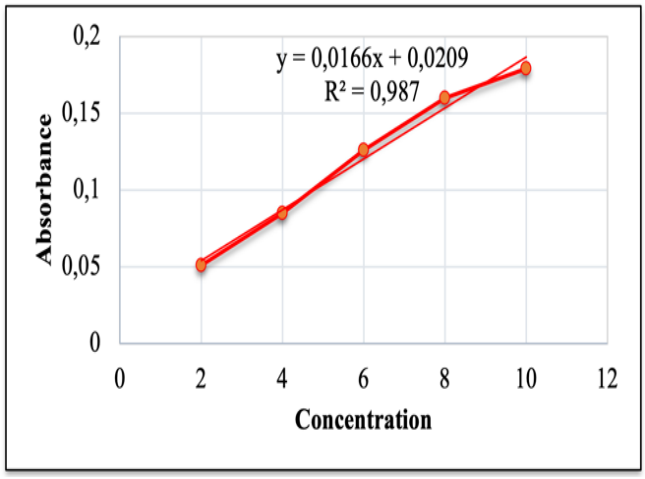

Figure 3: Quercetin Standard Regression Equation

Compound $AlCl_3$ reacts with the ketone group at C4 and the OH group at C3 or C5 on flavones or flavonol compounds to form a yellow stable complex compound.[22] On observation under UV 366 light, red glowing spots appear, the red color fluorescence results from UV 366 light, which possibly came from chlorophyll or other compounds covered by chlorophyll. Chlorophyll is non-polar and will give a red spot under 366 UV light.[23]

There is a clear zone observed at the R_f value of 0.36 in tests against *S. aureus* and *E. coli*. The presence of active compounds from the chromatogram spot, which diffused into the media and inhibited bacterial growth at the active compound diffusion point, generated the inhibition/clear zone.[13,14] The result of spraying $FeCl_3$ reagent produced a brownish-green spot, which indicated the existence of polyphenol. The brownish-green spot resulted from the reaction of phenol groups with Fe. The reaction is analogous to the reaction between the phenol group of flavonoids and $AlCl_3$ compounds because flavonoid compounds will form complexes with aluminium.[24] The antibacterial activity of polyphenolic compounds occurs due to the presence of hydroxyl groups that inhibit bacterial growth using protein denaturation.[2,25]

Determination of the flavonoid content of EAE was carried out by the UV-Vis spectrophotometry method. From the results of the study, it was found that the standard linear regression equation for quercetin y=0,0166x+0,0209 with R=0,987 (Figure 3). The flavonoid content of EAE was 57.255±0.0348 mg QE/g (Table 4). Ethyl acetate extract of Robusta coffee leaves also contains flavonoid compounds which are characterized by the formation of a red solution with the addition of concentrated Mg and HCl. This happened because the concentrated Mg and HCl could reduce the core of benzopyrone in the flavonoid structure so that the color changed from yellow/orange to red.[26] Total flavonoid levels were calculated using linear regression of the quercetin standard curve.[27] The results obtained from the standard curve measurement showed that the higher the concentration of the solution, the higher the absorbance value.[26,28] The linear regression obtained was Y= 0.0166x + 0.0209 with a correlation coefficient (R2) = 0.987 and The total flavonoid concentration of Robusta coffee leaves ethyl acetate extract was 57.2550.0348 mg QE/g.

Conclusion

Ethyl acetate extract of coffee leaves (*Coffea canephora* L.) inhibits the growth of *Staphylococcus aureus* in the strong category (10.20 mm) and *Escherichia coli* (7.31 mm) in the moderate category. The results of the TLC-Bioautography test at R_f 0.36 indicated the presence of polyphenolic compounds and produced a clear zone against both bacteria. The total flavonoid content of ethyl acetate extract of Robusta coffee leaves was 57.255±0.0348 mg QE/g extract.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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