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Antibacterial Activity And Tlc-Biotography Profile Of The Ethyl Acetat Fraction Of Asian Pigeonwings Flower (*Clitoria ternatea*) Against *Escherichia coli*

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Abstract

One of the infectious diseases is diarrhea caused by *Escherichia coli*. There are many potential herbal plants as alternative antibacterial antibiotics, one of which comes from the Asian pigeonwings flower (*Clitoria ternatea*). This study aims to determine the antibacterial activity of the ethyl acetate fraction of pigeonwings flower, phytochemical compounds, and its TLC-bioautography profile. The antibacterial mixture of pigeonwings flower was extracted by maceration using ethanol, then fractionated by ethyl acetate, and made solutions with concentrations of 5%, 10%, 20%, and 40%, respectively. The experiment was carried out with three replications using the suitable diffusion method followed by TLC-bioautography to determine the TLC-bioautography profile. Phytochemical compounds of pigeonwings flower were determined by thin layer chromatography (TLC). Antibacterial activity data were analyzed using the ANOVA and continued with the Tukey HSD 5% through SPSS. The ethyl acetate fraction of the pigeonwings flower had the best antibacterial activity at a concentration of 40% with an inhibition zone diameter of 11.54 mm. The diameter of the inhibition zone showed significantly different in each concentration, and it can be said that the ethyl acetate fraction of the pigeonwings flower could inhibit the growth of *E. coli*. The phytochemical compounds in the ethyl acetate fraction of pigeonwings flower are flavonoids and alkaloids, with flavonoids being the most active compounds in inhibiting the growth of *E. coli.* The implication of this research is the use of plant extracts to inhibit the growth of *E. coli* bacteria that may cause diarrhea.

Keywords: antimicrobial; phytochemical compounds; ethyl acetate fraction; thin layer chromatography (TLC), flavonoid

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Introduction

Infectious disease is a type of disease that mainly affects developing countries, including Indonesia. One of the most common infectious diseases suffered by the people of Indonesia is diarrhea. One of the bacteria that causes diarrhea is *Escherichia coli* (1). Diarrhea is caused by contamination with *E. coli* in food or drinks. In general, *E. coli* is essential to the human intestinal tract, but some *E. coli* can be pathogenic and cause disease (2). One of the species of *E. coli* that causes diarrhea in many places in the world is Enterotoxigenic *Escherichia coli* (ETEC).

Herbal plants such as the Asian Pigeonwings flower (*Clitoria ternatea*) are widely used as traditional medicine. Some of the active substances contained in the pigeonwings flower make it have the ability as an antibacterial compound. The active substances are alkaloids, phenols, saponins, tannins, and flavonoids. Each of these active compounds has a different mechanism for inhibiting bacterial growth (3). This study aimed to determine the activity of the ethyl acetate fraction of pigeonwings flower in inhibiting the growth of *E. coli*. To determine the inhibitory concentration of the ethyl acetate fraction of pigeonwings flower in the growth of *E.*

coli and to determine the profile of TLC Bioautography After doing the research, it is expected that traditional medicine plants can be used as antibacterial with lower prices and fewer side effects.

Material and Methods

This study was conducted in April-July 2022. A sampling of the pigeonwings flower was carried out around the city of Surakarta. Extract and fractionation were made at the Sebelas Maret University Integrated Laboratory. Preparation and antibacterial activity were carried out at the Laboratory of the Faculty of Medicine, UNS. TLC and Bioautography were carried out at the Biology Laboratory, Faculty of Mathematics and Natural Science, Sebelas Maret University.

Preparation of Pigeonwings Flower

The pigeonwing flowers were collected and washed, then air-dried overnight and continued in an oven at 40ºC. The dried pigeonwing flower samples were blended and sieved.

Extraction

The fine powder of the pigeonwings flower was extracted using the maceration method. The first maceration extraction was done using 150 grams of fine powder of pigeonwings flower with 70% ethanol solvent, as much as 1125 ml, for five days. The extraction process was continued using 70% ethanol, as much as 375 ml, for two days. The sample was filtered a second time to obtain the second filtrate. The two filtrates obtained were mixed and concentrated using a rotary evaporator at a temperature of 60ºC (4).

Fractionation

The concentrated ethanol extract was fractionated using the Can-ake method (2004) (5); fractionation was carried out using 96% ethanol and water in a ratio of 2:3 and ethyl acetate. Ten grams of the thick extract was dissolved in 96% ethanol solvent and water and then stirred in a separating funnel. The next step is partitioning using 100 ml of ethyl acetate. The sample was allowed to stand for 30-60 minutes to determine the occurrence of separation between ethanol water and ethyl acetate (5). The ethanol-water layer is at the bottom, while the ethyl acetate layer is at the top. The ethyl acetate fraction obtained was put in a separating funnel, then shaken and left for another 30 minutes. The same steps were carried out to ensure the fraction obtained was 100% ethyl acetate fraction. The obtained fraction was concentrated using a rotary evaporator at a temperature of 50ºC (6).

Antibacterial Activity Test

Media preparation. 38 g of Mueller Hinton Agar (MHA) media was dissolved in 1 liter of distilled water. The solution was homogenized and heated until completely dissolved. The media solution was sterilized using an autoclave at 121oC for 15 minutes. The sterile media was poured into a petri dish and waited for it to solidify (7). Physiological Solutions NaCl was prepared with as much as 0.09 grams dissolved in 10 ml of distilled water. The solution was homogenized until wholly dissolved, poured into a test tube, and then sterilized in an autoclave at 121ºC for 15 minutes. The viscous fraction of ethyl acetate was dissolved using 10% DMSO. The extract was made with 40, 20, 10, and 5% concentrations. A 40% extract concentration was prepared by taking a thick fraction of 800 mg and then dissolved using 10% DMSO to 2 ml. A 20% test solution was made by taking 1 ml of stock solution and adding 1 ml of 10% DMSO. The 10% and 5% test solutions were prepared using the same dilution method (8).

Bacterial Suspension. The culture of *Escherichia coli* that has been inoculated on nutrient agar media for one night is taken approximately one inoculating lope. The bacteria carried were suspended in a test tube containing 10 ml of 0.9% NaCl. The suspension was homogenized, and the turbidity compared with the standard Mc Farland 0.5 or equivalent to 1.5 x 108 bacterial cells (4).

Ethyl Acetate Fraction Antibacterial Activity Test of Pigeonwings Flower

Antibacterial activity testing was carried out using an excellent method. Petri dishes containing sterile MHA media were planted with bacterial suspension by swab using a clean cotton swab until evenly distributed. The media containing bacteria is then made into a well using a cylindrical drill. The holes were then filled with ethyl acetate fraction with concentrations of 40, 20, 10, and 5%. Positive control with the antibiotic cotrimoxazole and 20 L of negative control using 10% DMSO. They were then incubated for 24 hours at 37ºC. The experiment was carried out in 3 triplicates. The diameter of the inhibition zone was observed and measured using a caliper.

Thin Layer Chromatography

Plate Preparation. A silica gel 60 F254 plates were prepared with a size of 1x10 cm, then made a mark with a distance of 1 cm on the top and bottom edges as marking marks. The plates were washed using methanol for 15 minutes and then activated using an oven at 110oC for 30 minutes (9). The flavonoid test was carried out using eluent toluene: ethyl acetate: ethanol in a ratio of 3:3:0.5 (10). Alkaloids were tested using chloroform: ethyl acetate eluent with a ratio of 1:8.11

TLC test. The ethyl acetate fraction of the pigeonwings flower was spotted using a capillary tube on an activated TLC plate. Spotting is done more than once so the results can be seen clearly. The plate is inserted into the chamber and waits for the results to form. The results obtained were observed at 254 nm and 366 nm UV lamps. Based on the stains created, the Rf value can be determined (11). After knowing the Rf value, it was sprayed with Dragendorff's reagent, citronic acid, FeCl3, and anisaldehyde, then waited to dry and observed the color change.

TLC Bioautography

The chromatogram was placed on Mueller Hinton media planted with *E. coli* bacteria. The chromatogram plate was left for 30 minutes and then removed. After being released, they were incubated for 24 hours at 37ºC. The inhibition zone formed is a bright area that is not overgrown with bacteria (12).

Data analysis

The data obtained were analyzed using the One Way Anova test (Analysis of Variance) with a 95% confidence level using SPSS after the normality and homogeneity tests. Data analysis was continued with the Tukey HSD 5% test to determine the significant difference in the effects of each treatment as an antibacterial against *E. coli* (13).

Results and Discussion

Pigeonwings Flower Extract and Fractionation

In the maceration extraction process, sample immersion is carried out; this immersion can cause cell wall rupture due to differences in concentration inside and outside the cell so that compounds in the cytoplasm in the sample can be attracted by the solvent (4). Yield is the ratio between the extract obtained and the weight of the simplicial obtained. From 190 grams of simplicial powder, the yield was 7.9%. Compounds derived from plants have different polarities due to differences in their structure and chemical bonds. Polar compounds can be bound by polar and semi-polar solvents such as methanol and ethyl acetate, while nonpolar compounds can be secured by solvents such as n-hexane. The ethyl acetate solvent can dissolve semi-polar compounds such as flavonoids (1). The ethyl acetate fraction obtained was then thickened using a rotary evaporator at a temperature of 60ºC to get a thick brownish-black ethyl acetate fraction with a pungent odor of 2 g.

Antibacterial Activity

The antibacterial activity of the ethyl acetate fraction of pigeonwings flower is shown in Figure 1 in the presence of a clear zone around the well.

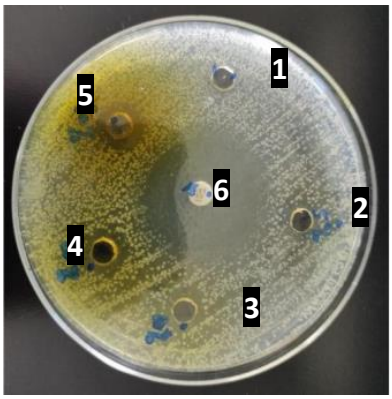

Figure 1. Antibacterial Activity Test

Note: 1. Negative control (10% DMSO), No. 2-5 ethyl acetate fraction of pigeonwings flower with certain concentration (5%, 10%, 20%, 40% respectively), 6. Positive control (antibiotic cotrimoxazole).

The inhibition zone or clear zone is a zone that is not overgrown with *E. coli*. Bioactive compounds from the ethyl acetate fraction are thought to inhibit the growth of *E. coli*. The diameter of the inhibition zone indicates the strength of antibacterial activity. Based on the calculation of the inhibition zone diameter, the results are shown in Table 1.

Table 1. Inhibitory zone of the pigeonwings flowers ethyl acetate fraction against *Echerichia coli*

Treatment	Inhibition Zone Diameter (mm) ± sd
P-	0^{a}
P1	1.76 ± 0.45^{a}
P2	8.25 ± 0.49^{b}
P3	9.62 ± 1.05^{bc}
P4	11.54 ± 2.78^{c}
P+	28.70 ± 0.59^{d}

Superscript letters (a-c) show significant differences in each treatment based on Tukey HSD post hoc ($P<0.05$).

P- : negative control (DMSO 10%)
P1 : ethyl acetate fraction of pea flower concentration 5%
P2 : ethyl acetate fraction of pea flower concentration 10%
P3 : ethyl acetate fraction of pea flower with a concentration of 20%
P4 : ethyl acetate fraction of pea flower with a concentration of 40%
P+ : positive control (cotrimoxazole antibiotics)

The ethyl acetate fraction of pigeonwings flowers with a concentration of 40% were able to inhibit the growth of *Escherichia coli* with an inhibition zone diameter of 11.54 mm. Based on the statement of Purwanto (2015) (1), the response to the inhibition of the ethyl acetate fraction of pigeonwings at a concentration of 40% is included in the strong category so that it is effective for use as an antibacterial (1). DMSO 10%, which acts as a solvent for the ethyl acetate fraction of pigeonwings flower, did not show a clear zone formed. DMSO 10% could not inhibit the growth of *E. coli*, so the pure *E. coli* growth inhibitory activity came from the fraction used.

Thin Layer Chromatography Profile of Ethyl Acetate Fraction of Pigeonwings Flower

TLC is a method of separating compounds based on the distribution of two phases: the mobile phase and the stationary phase. TLC showed that the ethyl acetate fraction of the pigeonwings flower contained phytochemical compounds, alkaloids, and flavonoids. Flavonoids can be appropriately detected using eluent toluene: ethyl acetate: ethanol 3:3:0.5. Proof of flavonoid compounds was carried out using standard quercetin and spraying citronic acid. A change in the color of the spots to yellow indicates the presence of flavonoids. Based on the color change, it is suspected that a Retention factor (Rf) of 0.49 detects the presence of flavonoids. The chromatogram showing flavonoids can be seen in Figure 3.

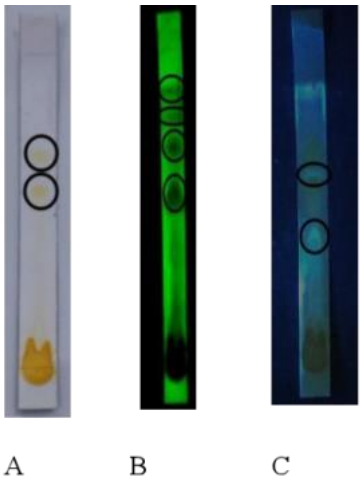

Figure 3. Flavonoid Chromatogram
Description: A. visible light, B. UV light 254 nm, C. UV light 366 nm.

Alkaloids can be detected well-using chloroform eluent: ethyl acetate 1:8. Proof of alkaloid compounds was carried out using standard quinine and spraying Dragendorff's reagent. A change in the color of the spots to brownish-yellow indicates the presence of Alkaloids. Based on the color change, it is suspected that Rf 0.88 detect alkaloids' presence. The chromatogram showing the alkaloids can be seen in Figure 4.

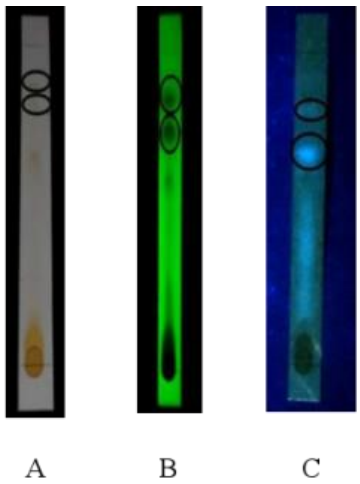

Figure 4. Alkaloid Chromatogram
Description: A. visible light, B. UV light 254 nm, C. UV light 366 nm.

This study's qualitative tests on the ethyl acetate fraction of butterfly pea flowers showed the presence of metabolites, namely alkaloids, in TLC (Figure 4). TLC is useful for isolating pharmacologically active compounds present in plants. The presence of phytochemical compounds such as alkaloids makes these plants capable of being used as an alternative to treat certain bacterial infections. Internal and external factors influence the content of secondary metabolites of a plant. Internal factors that influence include genes, while external factors include light, temperature, humidity, pH, nutrient content in the soil, and altitude. The altitude of the place affects the ambient temperature. The site's temperature can affect a plant's growth, especially in its metabolic process. If the metabolic process of a plant is disturbed, the compounds produced will also be different (14).

Inhibitory Activity of Phytochemical Compounds of Pigeonwings Flower

TLC bioautography conducted in this study showed an inhibition zone in one of the spots in the flavonoid group at Rf 0.49. This indicates that the flavonoid compounds in the ethyl acetate fraction of the pigeonwings flower have a more active ability to inhibit the growth of Escherichia coli. On the other hand, alkaloids have less antibacterial ability than flavonoids. The results of the bioautography TLC can be seen in Figure 5 and Figure 6.

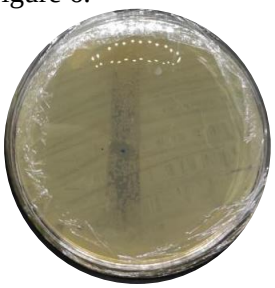

Figure 5. TLC Bioautographic

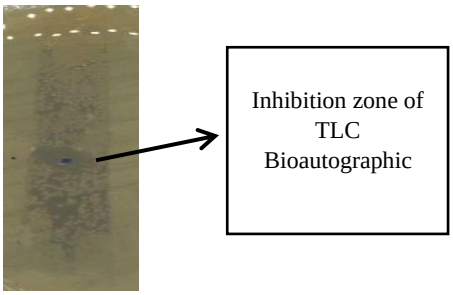

Figure 6. Inhibitory Zone of TLC Bioautography

Through TLC bioautography, flavonoids showed a better ability and were more active in inhibiting the growth of *E. coli*. This is known based on the presence of an inhibitory zone on the chromatogram of the TLC on flavonoids. Flavonoids are polar compounds. Flavonoids generally dissolve in polar solvents such as ethanol, methanol, butanol, acetone, ethyl acetate, and water. Flavonoids are readily soluble in polar solvents because flavonoids contain bound sugars. In flowers, flavonoids contribute to flower color and self-protection from insects and microbes and are also beneficial for humans (15). Flavonoids have a role as antibacterial compounds by disrupting the integrity of cell membranes. It was proven in this study that flavonoids were able to inhibit the growth of *E. coli* (4). Flavonoids have an A ring and a B ring that play a role in inter-classification or hydrogen bonding by accumulating nucleic acid bases that can inhibit the formation of DNA and RNA. The 2',4' or 2',6' hydroxyl groups are hydroxylated in ring B while 5,7 are hydroxylated in ring A, which plays an essential role in the antibacterial activity of flavonoids. The interaction process between flavonoids and bacterial DNA can cause damage to the permeability of bacterial cell walls, microsomes, and lysosomes. Flavonoids can form complex compounds and extracellular proteins, which are then dissolved, damaging the bacterial cell membrane and causing the release of intracellular compounds. Flavonoids can inhibit the binding of enzymes such as ATPase and phospholipase, which causes inhibition of cell membrane function. Flavonoids can inhibit cytochrome C reductase so that bacterial energy metabolism will be inhibited (16).

Conclusion

The ethyl acetate fraction of Asian pigeonwings flower has a solid and effective antibacterial activity for *Escherichia coli* bacteria at a concentration of 40%. The minimum inhibitory concentration of the ethyl acetate fraction of the pigeonwings flower was at a concentration of 5%. Based on the TLC test showed that the ethyl acetate fraction of the pigeonwings flower contained phytochemical alkaloids and flavonoids. The TLC-bioautography profile revealed the presence of an inhibitory zone formed on the Rf chromatogram, which detected flavonoids. The bioactive compounds that play an active role as antibacterial in the ethyl acetate fraction of pigeonwings flower are flavonoids.

Conflict of interest

All authors declare no conflicts of interest in this paper

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