CHEESA: Chemical Engineering Research Articles
ISSN 2614-8757 (Print), ISSN 2615-2347 (Online)
Available online at: http://e-journal.unipma.ac.id/index.php/cheesa

Research Article

The Antibacterial Potential of Ethyl Acetate Fraction from *Plectranthus amboinicus* Leaves and Identification of Active Compounds Using LC-MS

Potensi Antibakteri Fraksi Etil Asetat Daun Jinten (Plectranthus amboinicus) dan Identifikasi Senyawa Aktif Menggunakan LC-MS

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Article History
Submitted: 08th September 2022; Revised: 10th May 2024; Accepted: 15th May 2024;
Available online: 06th June 2024; Published Regularly: June 2024

doi: 10.25273/cheesa.v7i1.13826.15-23

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Abstract

The Indian Borage plant (*Plectranthus amboinicus*) is a traditional medicinal ingredient in Indonesia, containing flavonoids, alkaloids, polyphenols, saponins, and essential oils. Therefore, this study aimed to determine the antibacterial activity of *P. amboinicus* leaves ethyl acetate fraction using the disc diffusion method and identify the composition with LC-MS. The antibacterial activity test was conducted with concentration variations of 15%, 20%, and 25% with Chloramphenicol 500 mg and 5% DMSO as a positive and negative control, respectively. The results showed that the ethyl acetate fraction at a concentration of 20% had the largest inhibition zone diameter, with an average of 30 mm. The LC-MS results identified 115 compounds, and the highest concentration values were found in kaempferol 3-(6"-caffeoylglucoside) and kaempferol 3-glucosyl-(1→2) galactosyl-(1→2)-glucoside, with a composition of 3.26109% and 3.26141%, respectively.

Keywords: Antibacterial activity; Ethyl acetate fraction; LC-MS; *Plectranthus Amboinicus*

Abstrak

Tanaman jinten (Plectranthus amboinicus) merupakan bahan obat tradisional di Indonesia yang mengandung senyawa flavonoid, alkaloid, polifenol, saponin, dan minyak atsiri. Penelitian ini bertujuan untuk mengetahui aktivitas antibakteri fraksi etil asetat daun jinten dengan metode difusi cakram serta komposisi senyawa yang terkandung menggunakan LC-MS. Uji aktivitas antibakteri dilakukan dengan menggunakan variasi konsentrasi 15%, 20%, dan 25%, Kloramfenikol 500 mg sebagai kontrol positif, dan DMSO 5% sebagai kontrol negatif. Hasil dan kesimpulan yang diperoleh pada uji antibakteri metode difusi cakram yakni fraksi etil asetat daun jinten dengan konsentrasi 20% memiliki diameter zona hambat terbesar dengan rata-rata sebesar 30 mm. Hasil dari LC-MS terdapat sebanyak 115 senyawa yang ada di daun jinten. Hasil identifikasi LC-MS senyawa dengan nilai konsentrasi komposisi tertinggi yaitu senyawa kaempferol 3-(6"-caffeoylglucoside) dengan komposisi senyawa 3,26109%, dan kaempferol 3-glucosyl-(1→2) galactosyl-(1→2)-glucoside dengan kompisisi senyawa 3,26141%.

***Kata kunci**: antibakteri; daun jinten; fraksi etil asetat; LC-MS*

1. Introduction

Indonesia is renowned for having the global greatest biodiversity, comprising fauna and flora with great potential as medicines. In general, traditional medicine has various advantages, including affordability, simpler treatment techniques, relative safety without strict supervision, ease of administration without needing medical personnel, and relatively low side effects [1]. With these various advantages, traditional medicine is expected to become an alternative to chemical medicines in the future.

Among the rich flora in Indonesia, Indian Borage (*Plectranthus amboinicus*) has great potential as a traditional herbal medicine. This plant, commonly found in India, Ceylon, and South Africa, is characterized by thick, upright, and oval-shaped leaves [2], known to contain various secondary metabolites, including flavonoids, alkaloids, polyphenols, saponins, and essential oils [3]. The leaves have antibacterial activity capable of killing or inhibiting the growth of bacteria, including *Escherichia coli*. This is due to the flavonoids and polyphenol content which have antioxidant and antibacterial properties [4].

E. coli is a gram-negative bacteria responsible for 85% of urinary tract infection (UTI) cases and 50% of nosocomial infections. The bacteria is also a major cause of diarrhea due to the production of enterotoxins that bind to the mucosa of the small intestine [5]. Effective antibacterial agents are needed to target infectious and non-infectious organisms [6]. An effective traditional medicinal material that can be used is *P. amboinicus* leaves.

Agustianasari et al. [3] reported that the ethyl acetate fraction of *P. amboinicus* seeds had better and more effective antibacterial properties compared to water and n-hexane against *Bacillus* and *E. coli* bacteria. Therefore, this study aimed to determine the antibacterial activity of *P. amboinicus* leaves ethyl acetate fraction using the disc diffusion method and analyze the compound composition with LC-MS.

2. Research Methods

2.1 Tools and Materials

The tools used include Ultra-Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) from Shimadzu, a set of maceration tools, micropipettes, a rotary evaporator, and an autoclave. The materials used include fresh *P. amboinicus* leaves, distilled water, 70% ethanol, n-hexane, ethyl acetate, Nutrient Broth (NB), Nutrient Agar (NA), *E. coli* bacteria, DMSO (Dimethyl sulfoxide), Chloramphenicol, $FeCl_3$, Dragendorff reagent, Mayer's reagent, NaCl, Mg, and HCl.

2.2 Preparation of Simplisia

The simplisia from *P. amboinicus* leaves was prepared by thoroughly washing the leaves and cutting them to uniform thickness. The leaves were then air-dried away from direct sunlight. Depending on the weather, this drying process typically took 48 hours. After drying, the leaves were sorted to remove any dirt or foreign particles. The next step entailed grinding the simplisia into a powder, which was then sieved using an 80-mesh sieve to ensure uniform consistency. [7].

2.3 Preparation of *P. amboinicus* Leaves Extract

The *P. amboinicus* leaves extract was made using the maceration method with 70% ethanol as the solvent. A total of 500 grams leaves powder was placed in a

maceration vessel, and 1000 mL of 70% ethanol was added. The maceration process lasted 4 to 24 hours with occasional stirring. The obtained extract was filtered using flannel cloth until the solution was clear. Subsequently, the leaves extract was concentrated using an evaporator until a thick extract was obtained [8].

2.4 Phytochemical Screening

2.4.1 Alkaloid Compounds

Tests for alkaloid compounds could be performed using the Dragendorff and the Mayer methods. In the Dragendorff test, 2 mL of extract was added to 1 mL of the reagent, while in the Mayer test, 1 mL of extract was added to 2 N HCl and the reagent. The Mayer test results were indicated by the formation of a white precipitate in the extract, and a positive result for the Dragendorff test was demonstrated by an orange precipitate [9].

2.4.2 Flavonoid Compounds

To test for flavonoid compounds, 2 mL of extract was added to 2 mL of 70% ethanol, stirred, heated, and filtered. The filtrate was then added with Mg and concentrated HCl. A positive result was indicated by the formation of an orange or red precipitate [10].

2.4.3 Saponin Compounds

Saponin compounds were tested by adding 2 mL of extract to hot water and shaking vigorously. The presence of stable foam for 5 minutes after adding 1 drop of 2 N HCl indicated the presence of saponins [10].

2.4.4 Tannin Compounds

P. amboinicus leaves were added to 3 mL of hot distilled water and stirred until homogeneous. After cooling, 5 drops of a 10% NaCl solution were added. Two test tubes were used, one as a blank and one with the filtrate to which 3 drops of $FeCl_3$ reagent were added. The remaining filtrate was added with gelatin. The presence of tannin compounds in the extract was indicated by a color change to blue-black or brownish-gree [11].

2.5 Fractionation

About 5 grams of the extract were dissolved in 25 mL of distilled water, and 25 mL of n-hexane were added. The solution was shaken, and the two fractions were separated. The filtered aqueous phase was further divided by adding 25 mL of ethyl acetate. This process was repeated three times with the same steps for several fraction solutions. The filtrate from the filtration was evaporated using a rotary evaporator.

2.6 Preparation of Media

2.6.1 Nutrient Agar (NA)

The 4 grams of Nutrient Agar (NA) were dissolved in 200 mL of distilled water in an Erlenmeyer flask. The solution was stirred and homogenized using a stirrer while being heated. Subsequently, the solution was sterilized using an autoclave at 121°C for 15 minutes. The NA media was then poured into Petri dishes, incubated at 37°C for 24 hours and the bacteria used were *E. coli.*

2.6.2 Nutrient Broth (NB)

The 0.3 grams of Nutrient Broth were dissolved in 30 mL of distilled water and homogenized. Subsequently, 6 mL of the media solution was poured into each reaction tube. Using an autoclave, the media was sterilized at 121°C for +60 minutes [12].

2.7 Preparation of Test Solutions

2.7.1 Test Solution of *P. amboinicus* Leaves Fraction

Test solutions of the ethyl acetate fraction at concentrations of 15%, 20%, and 25% were prepared by weighing 1.5 grams, 2 grams, and 2.5 grams, respectively, and dissolving in 10 mL of 5% DMSO.

2.7.2 Positive Control Solution

The positive control used was Chloramphenicol 500 mg tablet, which was ground into a fine powder. Subsequently, the powder was weighed and dissolved in 50 mL of 5% DMSO in a volumetric flask. The resulting Chloramphenicol solution had a concentration of 50 µg/50 µL.

2.7.3 Negative Control Solution

5% DMSO was used as the negative control. It was prepared by dissolving 5 mL of DMSO in distilled water up to 100 mL.

2.7.4 Bacterial Suspension

Bacteria were cultured in NA media for 24 hours at 37°C. The suspension was prepared by transferring the culture into a 0.9% NaCl solution, and the turbidity level was visually standardized using a McFarland 0.5 standard. About 100 µL of the suspension was pipetted and added to 10 mL of NB to obtain a colony count of 1-2 x 10^6 CFU/mL.

2.8 Antibacterial Activity Test

2.8.1 Disc Diffusion Method

A volume of 10 mL of NA media was poured into Petri dishes/plates and allowed to solidify. *E. coli* bacteria cultures were taken using a pipette from NB media into previously sterilized plates, adding 200 µL. The culture was then spread evenly using a sterile cotton swab. Discs soaked in test solutions were transferred onto the NA media containing *E. coli* in an aseptic manner and then incubated at 37 °C for 24 hours. [12].

2.8.2 Measurement of Inhibition Zone

The clear zones formed around the discs, commonly referred to as inhibition zones, were measured using calipers. The strength of the bacterial inhibition zones was determined based on the classification in Table 1.

Table 1. Classification of Bacterial Inhibition Zones

Diameter (mm)	Strength of Inhibition Zone
≤ 5	Weak
6-10	Moderate
11-20	Strong
≥ 21	Very Strong

2.9 Compound Identification with LC-MS

The process of identifying compounds with LC-MS started with sample preparation. A total of 1 L precipitated sample was injected into the Shimadzu LC-MS-8040 instrument. The analysis was performed using UPLC-MS equipped with a binary pump. LC was connected to a Quadrupole Time of Flight (QTOF) mass spectrometer using Electrospray Ionization (ESI) as the ionization source. Furthermore, the mass spectrometry (MS) system used was the QTOF system in positive ionization mode. The ESI parameters included a capillary temperature of 350 degrees Celsius and an atomizer gas flow rate of 60 mL/HR, with a voltage source of 5.0V. Full scanning was performed in the m/z range of 100-5000 with a source temperature of 100 degrees Celsius. For the UPLC column, a Shimadzu Shim Pack FC-ODS with dimensions of 2mm x 150mm and 3µm particles was used. The eluent consisted of 90% methanol and water, with a 0.5 mL/minute flow rate. The

identification results showed all types of compounds contained in the *P. amboinicus* leaves extract, along with the percentage of each.

2.10 Statistical Analysis

Data from the antibacterial activity test of *P. amboinicus* leaves fractions against *E. coli* bacteria were analyzed using One-Way ANOVA to observe the differences in the average diameter of inhibition zones for each test sample using Statistical Product and Service Solution (SPSS 23) software. A p-value <0.05 indicated that variation in the concentration of *P. amboinicus* leaves ethyl acetate fraction affected the growth of *E. coli* bacteria.

3. Results and Discussion

3.1 Phytochemical Screening

The purpose of phytochemical screening was to provide an overview of the compounds contained in *P. amboinicus* leaves. Table 2 shows the results of compounds including alkaloids, flavonoids, saponins, and tannins. Flavonoids damage bacteria cell membranes by forming protein complex compounds. Saponins decrease the surface tension of bacteria cell walls, leading to cell damage and death, while tannins coagulate proteins, having a similar effect to phenolic compounds [13].

3.2 Antibacterial Activity and Measurement of Inhibition Zones

Antibacterial agents are substances that can kill or inhibit bacterial growth. The clear zones around the growing bacteria indicate that the active compounds in the test substance can inhibit growth.

Chloramphenicol was used as a positive control due to its potentially strong antibacterial activity. This study compared the effectiveness of the test compounds in inhibiting bacteria bacterial growth with a widely tested standard. The 5% DMSO was used as a negative control due to its ability to dissolve almost all compounds, whether polar or non-polar and because it was not bactericidal. This ensures that any antibacterial activity observed can be attributed solely to the *P. amboinicus* leaves ethyl acetate fraction, free from solvent interference. Consequently, the results obtained from this study can be considered pure and accurate [14].

Table 2. Results of Phytochemical Screening

Compound	Reagent	Test Result	
Alkaloid	Extract + Dragendorff reagent	Orange	(+)
Flavonoid	Extract + DMSO + Dragendorff reagent	Orange	(+)
Saponin	Extract + HCl 2N	Presence of foam	(+)
Tannin	Extract + distilled water + 10% sodium chloride + $FeCl_3$	Brownish-green	(+)

Description: (+) indicates the presence of the compound, and (-) indicates the absence of the compound.

Table 3. Diameter of Antibacterial Inhibition Zones of *P. amboinicus* Leaves Ethyl Acetate Fraction

Treatment	Diameter of Inhibition Zone (mm)				Description	p-value
	I	II	III	Mean		
15%	25	20	31	25,33	Very Strong	
20%	25	45	20	30	Very Strong	
25%	20	23	25	22,67	Very Strong	0,000
K +	45	50	43	46	Very Strong	
K -	00	00	00	00	-	

Figure 1 shows the formation of clear zones in all 3 variations of the ethyl acetate fraction from *P. amboinicus* leaves. Table 3 presents the average diameter of the three concentration variations in inhibition zones. Based on the results, all three variations of the ethyl acetate fraction had strong inhibitory activity, suggesting antibacterial activity. Several factors can influence antibacterial activity testing, including the content of antibacterial compounds, the number and type of bacteria inhibited, as well as the concentration of leaves extract [15].

The One-Way ANOVA test results showed a p-value <0.05, indicating that variations in the ethyl acetate fraction from *P. amboinicus* leaves significantly affected the growth of *E. coli* bacteria. A total of 5 data groups were compared namely concentrations of 20%, 15%, and 25%, as well as positive and negative controls. Each group has an average value for *E. coli* bacteria growth. As shown in Table 4, subsets indicate which groups have average values without significant differences. Different subsets imply significant differences between these groups.

Based on the result, the ethyl acetate fraction with a concentration of 20% had an average value close to the positive control and was in the same subset, indicating a fairly good antibacterial effect. Meanwhile, fractions with concentrations of 15% and 25%, as well as the negative control, were in different subsets, indicating significant differences in antibacterial activity.

Table 4. Tukey Test Subset Data

Concentration (%)	Average bacterial growth value	Subset
15	25.3333	2
20	30.0000	3
25	22.6667	2
+	46.0000	3
-	0.0000	1

3.3 LC-MS Identification

LC-MS identification was conducted at the Muhammadiyah University of Malang. The purpose of conducting LC-MS is to analyze organic, inorganic, and biological compounds in a complex sample commonly found in the environmental origin. The principle is based on the separation of analytes in line with the polarity level of compounds. LC-MS consists of a stationary phase column and a specific solution for the mobile phase. Compounds are separated according to polarity levels and respective speeds to reach the detector, leading to different retention times and observed spectra of separated peaks [13].

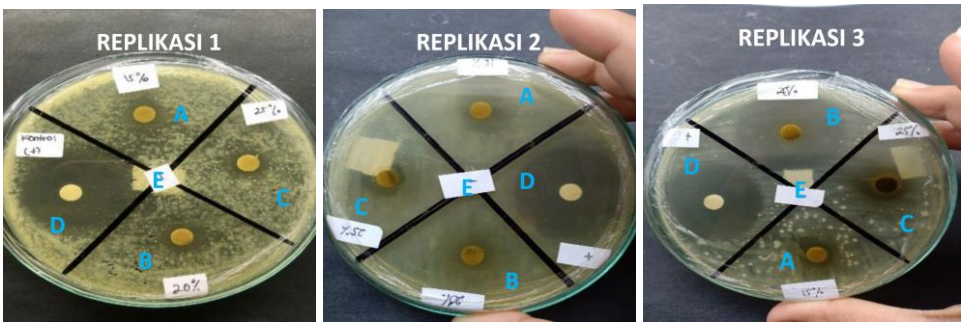

Figure 1. Results of Antibacterial Activity Test (Note: A = Ethyl Acetate Fraction 15%; B = Ethyl Acetate Fraction 20%; C = Ethyl Acetate Fraction 25%; D = Positive Control; E = Negative Control)

Figure 2 shows the chromatogram results of the LC-MS test for *P. amboinicus* leaves ethyl acetate fraction. Based on the LC-MS results, peaks ranged from low to high, with 115 compounds. The highest peak was number 101, identified with the largest composition of 3.26109%, and named kaempferol 3-(6”-caffeoylglucoside) (Figure 3.). Meanwhile, the compound identified at peak number 113 had a composition of 3.26141% and was named kaempferol 3-glucosyl-(1→2) galactosyl-(1→2)-glucoside (Figure 4.). Kaempferol 3-(6”-caffeoyl glucoside) compound has a molecular weight (m/z) of 610.1323, with the chemical formula $C_{30}H_{26}O_{14}$, and kaempferol 3-glucosyl-(1→2) galactosyl-(1→2)-glucoside has a molecular weight (m/z) of 772.2062 with the chemical formula $C_{33}H_{40}O_{21}$.

Both compounds, belonging to the flavonoid group, are suspected to be the most important in the ethyl acetate fraction of *P. amboinicus* leaves regarding antibacterial activity [16]. Flavonoids, as antibacterial agents, inhibit the function of the cytoplasmic membrane, nucleic acid function, and energy metabolism of bacteria. These compounds also inhibit RNA synthesis within the DNA cell, disrupt metabolism within bacteria, and damage cell membranes, releasing intracellular compounds from the bacteria [13].

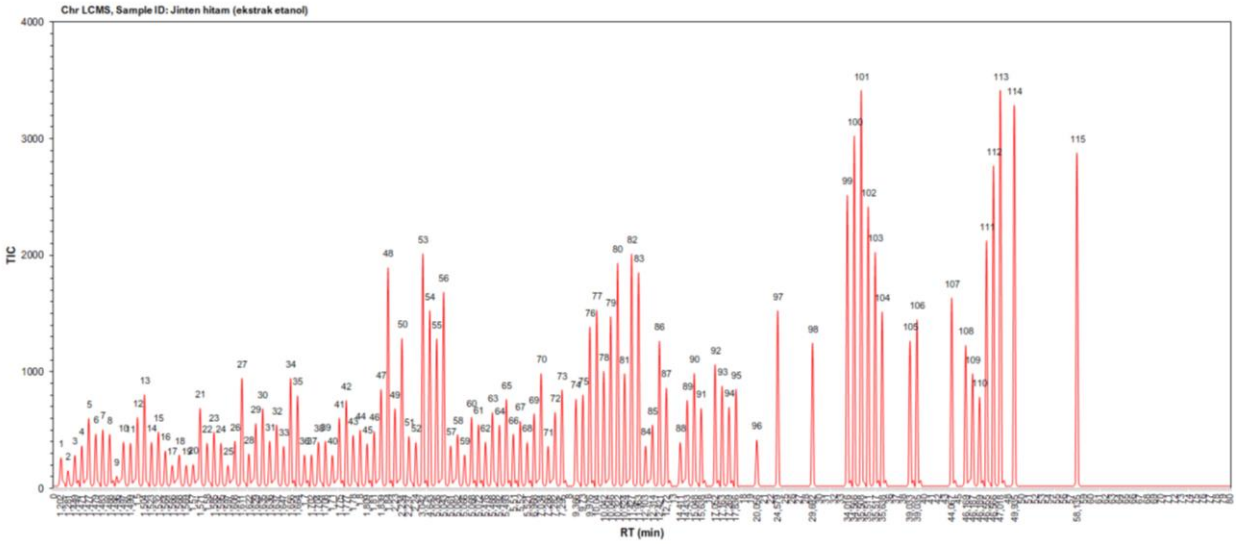

Figure 2. Chromatogram results from LC-MS (Liquid Chromatography-Mass Spectrometry)

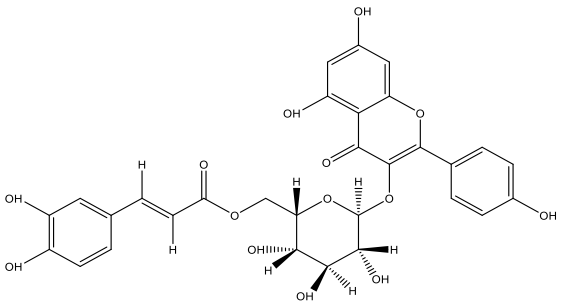

Figure 3. Structure of compound kaempferol 3-(6”-caffeoylglucoside)

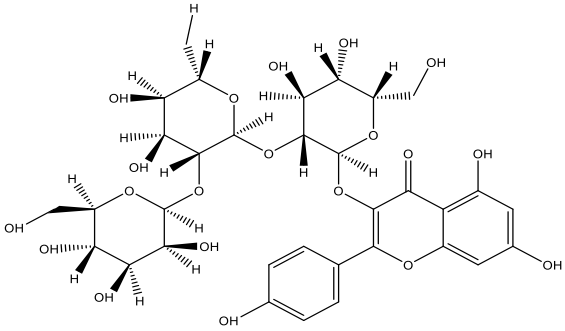

Figure 4. Structure of compound kaempferol 3-glucosyl-(1→2) galactosyl-(1→2)-glucoside

4. Conclusion

The ethyl acetate fraction of *P. amboinicus* leaves showed antibacterial activity against *E. coli*. The 20% fraction concentration demonstrated had optimum inhibitory zone against *E. coli* with a value of 30 mm. A total of 115 compounds were identified in *P. amboinicus* leaves, with the largest composition being 3.26109% for the compound kaempferol 3-(6''-caffeoylglucoside) and 3.26141% for kaempferol 3-glucosyl-(1→2) galactosyl-(1→2)-glucoside. Based on the results, further studies are needed using other methods, such as Soxhlet extraction and antibacterial testing against gram-positive bacteria. Additionally, the isolation of secondary metabolites using Thin Layer Chromatography (TLC) could provide rapid and accessible information about the quantity and types of compounds present in the sample without requiring specialized instruments for detection.

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