

The Antibacterial Activity of *Acanthus ilicifolius* L. n-Hexane Fraction

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ABSTRACT

Background: Recently, the high prevalence of diarrhea caused by bacterial infection, the usage of antibiotics has increased. Antibiotic overuse might lead to several side effects and resistance, suggesting the development of an alternate antibacterial agent. A mangrove plant, *Acanthus Illinois*, contained triterpenoid, which has antibacterial properties.

Aim: This study aimed to evaluate the antibacterial effect of the *Acanthus Illinois* n-hexane fraction against *Escherichia coli* and *Shigella dysenteriae*.

Method: The *Acanthus Illinois* was fraction using n-Hexane, identification of secondary metabolite compound using GC-MS, and evaluation of antibacterial activity against *Escherichia coli* and *Shigella dysenteriae* under paper disc methods. This study was designed using a fully randomized design (CRD) with concentration of fraction 1%, 2%, and 4%. The GC-MS results were compared to the WILEY 9 library and analysis. Prism graph was used to measure inhibition zone of antibacterial activity.

Result: The n-hexane fraction yield is 3.3% and contains sesquiterpene compounds (trans (beta.)-caryophyllene, alpha humulene, naphthalene decahedron-4A-methyl), terpenoid alcohol (3,7,11,15-tetramethyl -2-hexadecane-1-ol), and fatty acids (hexadecanoic acid methyl ester. *Acanthus Illinois* n-hexane fraction have antibacterial activity against *Escherichia coli* and *Shigella dysenteriae* in doses-dependent manner.

Conclusion: The n-hexane fraction of leaves *Acanthus ilicifolius* contains sesquiterpene, alcohol terpenoids, and fatty, and has antibacterial activity against *Escherichia coli* and *Shigella dysenteriae*

Keywords: *Acanthus ilicifolius*, *Escherichia coli*, Fraction, GC-MS, *Shigella dysenteriae*

BACKGROUND

Medicinal plants were rich in active compounds in diarrhea therapy. Data shows that diarrhea is the highest cause of child mortality. The number of children dying reaches 760,000¹. The prevalence is 8.0% and children 11.0%, these number continues to increase every year, increasing from 2013, which is only 7.0% and 2.4%². Bacteria such as *Escherichia coli* and *Shigella dysenteriae* cause diarrhea. The treatment uses antibiotics but side effects of allergies and resistance³. some plants were used of antibacterial such as *Pyrrhosia* for itching, coughing⁴, dysentery⁴; *Microsorium* sp. as an antiulcer⁵; *Drynaria quercifolia* to treat diarrhea⁶, asthma, wound medicine and inflammation^{7,8}. and antibacterial. In this study, plants of *A. ilicifolius* were using. The selection of this plant was important for antibacterial. Jeruju (*Acanthus ilicifolius*) contains alkaloids, flavonoids, steroids, terpenoids⁹, with pharmacological activities such as analgesic, anti-inflammatory, antibacterial, and antioxidant.

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Acanthus ilicifolius empirically for therapy such as paralysis, asthma, ulcers, and wounds¹⁰, ethanol extract, and chloroform can inhibit *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Proteus vulgare bacteria*¹¹. Ethanol extracts effectively in *Candida albicans*, *Aspergillus fumigatus*, and *Aspergillus nige*¹¹. Chloroform and methanol extracts in roots, stems, and leaves have potential on bacteria and fungi^{12,13}. Gram-negative and positive potency¹⁴. Extracts of n-hexane contain triterpenoids¹⁵. Terpenoids have reduced the permeability of bacterial cell walls by damaging transmembrane proteins¹⁶. Inhibiting Methicillin Resistance *Staphylococcus aureus* (MRSA)¹⁷. Fraction, ethyl acetate, and n-butanol have inhibitory potential on *Vibrio harveyi* after incubation 12-48 hours¹⁸. Ethyl acetate extract can inhibit MDR (Multi-Drug Resistant); *Klebsiella sp*, *Enterobacter 10*, *E. Coli*, and *Pseudomonas sp*. Based on this information, it is necessary to explore the potential of compounds in *Acanthus ilicifolius* leaves as antibacterial recommended a traditional medicine for diarrhea.

METHODS

Extraction and Fractionation Process

The leaves of *Acanthus ilicifolius* were sorted, washed, chopped, and dried under sun light. Dried leaves made into powder. Five hundred grams of powder was extracted by the maceration method, using 96% ethanol with a ratio of dried leaves powder: ethanol (1:5) for 3 x 24 hours. The filtrate was filtered and evaporated using a rotary evaporator at 70°C. Furthermore, the fractionated process a liquid-liquid partition using n-Hexane. n-Hexane fractions were concentrated for further tests.

Phytochemicals of Acanthus ilicifolius

Tannin analysis, Analysis used was the method reported¹⁹. Each Extract/fraction sample (0.30 g) was weighed into a test tube and boiled for 10 minutes in a water bath containing 30 mL of water. Filtration was carried out after boiling using number 42 (125mm) Whatman filter paper. To 5 mL of the filtrate was added 3 drops of 0.1% ferric chloride. A brownish green or a blue black colouration showed positive test.

Saponin analysis. Methodology is as reported¹⁹. Distilled water (30 mL) was added to Extract/fraction sample (0.30 g) and boiled for 10 minutes in water bath and filtered using Whatman filter paper number 42 (125 mm). A mixture of distilled water (5 mL) and filtrate (10 mL) was agitated vigorously for a stable persistent froth. The formation of emulsion on addition of three drops of olive oil showed positive result.

Flavonoid analysis. The test for flavonoid adopted is as reported²⁰. Each sample (0.30 g) weighed into a beaker was extracted with 30 mL of distilled water for 2 hours and filtered with Whatman filter paper number 42 (125 mm). To 10 mL of the aqueous filtrate of each wood extract was added 5 mL of 1.0M dilute ammonia solution followed by the addition of 5 mL of concentrated tetraoxosulphate (VI) acid. Appearance of yellow colouration which disappeared on standing shows the presence of flavonoids.

Alkaloid analysis. Test for flavonoid used is as reported²¹. Extraction of component from 2 grams of each wood powder sample was carried out using 5% tetraoxosulphate (VI) acid (H₂SO₄) (20 mL) in 50% ethanol by boiling for 2 minutes and filtered through Whatman filter paper number 42 (125 mm). The filtrate was made alkaline using 5 mL of 28% ammonia solution (NH₃) in a separating funnel. Equal volume of chloroform (5 mL) was used in further solution extraction in which chloroform solution was extracted with two 5 mL portions of 1.0 M dilute tetraoxosulphate (VI) acid. This final acid extract was then used to carry out the following test: 0.5 mL of Dragendorff's reagent (Bismuth potassium iodide solution) was mixed with 2 mL of acid extract and precipitated orange colour infers the presence of alkaloid.

Steroid analysis. Analytical method used is according to¹⁹. Each sample (0.30 g) weighed into a beaker was mixed with 20 mL of ethanol; the component was extracted for 2 hours. To the ethanolic extract of each sample (5 mL) was added 2 mL acetic anhydride followed with 2 cm³ of concentrated tetraoxosulphate (VI) acid.

Terpenoids analysis. Methodology is as reported¹⁹. Each wood powder sample (0.30 g) was weighed into a beaker and extracted with 30 mL and component extracted for 2 hours. A mixture of chloroform (2 mL) and concentrated tetraoxosulphate (VI) acid (3 mL) was added to 5 cm³ of each extract to form a layer. The presence of a reddish brown colouration at the interface shows positive results for the presence of terpenoids.

Profiling analysis of *Acanthus ilicifolius* n-Hexane Fraction

A sample of 1 μ L inject of the GC-MS was operated using a glass column 25 m long, 0.25 mm in diameter, and 0.25 mm thick. CP-Sil 5CB stationary phase with programmed oven temperature between 60-270°C with a temperature rise rate of 10 °C/minute, Helium carrier gas pressure of 12 kPa, the total rate of 30 mL/minute and a split ratio of 1:50. This ionizing EI (Electron Impact) and ionization energy of 70 ev. The MS spectrum obtained is then matched with the reference standard mass spectrum in the tool library, then selected and identified the peak of the component that has a similarity index of more than or equal to 90%.

Antibacterial Activity

The method used is Kirby Bauer disc diffusion with a completely randomized design. The samples tested were negative control (DMSO 10%), positive control (ciprofloxacin), and fraction concentration treatment of 1%, 2%, and 4%. The treatment replicates three times. Parameters observed were the zone of inhibition. The present studies, *Escherichia coli* and *Shigella dysenteriae* suspensions on an agar medium under aseptic conditions. Disc paper is dipped into the sample and placed on the surface of the agar medium with tweezers. They incubated at 37°C for 24 hours. Then observe the zone of inhibition.

Statistical analysis

One way Analysis of Varian (Anova) was carried out to determine statistical significance and P value of less than 0.05 was considered as statistically significant. The phytochemistry analysis by description qualitative, GC-MS analysis of the n-hexane fraction was carried out in a qualitative description by the library. The antibacterial by graphad prism 8.

RESULTS

Acanthus ilicifolius was determined to the Biology Faculty, Jenderal Soedirman University with certificate number 867/UN.23.02.8/TA.00.01/2019. The plants were dried and then extracted with ethanol and fractionated with n-hexane. The ethanol extract of yield of 19.6%, and n-hexane fraction of 4.3%. These results fraction yield a larger of previous research that is 2.2%²².

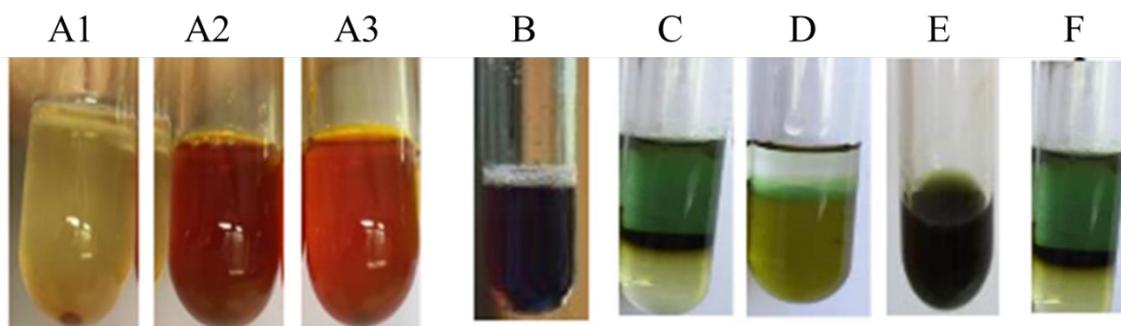


Figure 1. Phytochemical identification of *Acanthus ilicifolius*. (A1) Alkaloid test under mayer reagen. (A2) Alkaloid test under wagner reagen. (A3) Alkaloid test under dragendrof reagen.(B) Flavonoid test under Mg and HCl. (C) Terpenoid test under Liebermann-Buchard reagen. (D) Steroid test with Liebermann-Buchard reagen. (E) Tanin-fenolic test with FeCl₃. (F) Terpenoid test.

The results of phytochemical screening on ethanol extracts contain flavonoid compounds with red due to colors, tannins (blue), terpenoids (green), alkaloids (orange to red), and steroids (yellow) (Tab 1, fig 1). The color is due to a chemical reaction²³. Compounds have different characters from reactions for a class of compounds.

Table 1. Phytochemical screening of extract etanol and n-Hexane Fraction of *Acanthus ilicifolius* leaf

Phytochemical Compunds	Reagens	Ethanollic Extract	N-Hexane Fraction
Alkaloid	Mayer	+	-
	wagner	++	-
	dragendorf	++	-
Flavonoid	Mg powder+ HCl	++	-
Tannin	Ferri chloride	++	-
Terpenoid	Lieberman Burchard reagent	+++	+++
steroid	Lieberman Burchard reagent	+	-

+++ = appreciable amount (positive within 5 mins.); ++ = moderate amount (positive after 5 mins. but within 10 mins); + = trace amount (positive after 10 mins. but within 15 mins); - = negative

The results of the n-hexane fraction GC chromatogram analysis selected 15 peaks with high peaks, followed by MS analysis, then selected compounds with similarity index (SI) >90 and compared with the library on the tool (Figure 2 and Table 2).

Table 2. Compound Analysis on the mass spectrum of n-hexane fraction *Acanthus ilicifolius*

No	No peaks	Compunds	Simila rity index (SI)	Molekul formula	Retention time	Area (%)	Information
1	2.	Trans (.Beta.)-Caryophyllene	95	C15H24	5.883	7.28	Seskuiterpen
2	3.	Trans(.Beta.)-Caryophyllene	96	C15H24	5.969	8.02	Seskuiterpen
3	5.	Alpha.Humulene	92	C15H24	6.350	1.23	Seskuiterpen
4	6.	Naphtalene decahydro -4 -A-5-methyl	93	C15H24	6.69	2.01	Seskuiterpen
5	8.	9-6Octadecene	95	C18H36	7.554	1.34	Alkena
6	9.	Pen7tadecane	96	C15H32	7.604	1.87	Alkana
7	10.	9-Eic8osene	96	C20H40	8.915	1.33	Alkena
8	11.	Eicosa9ne	94	C20H42	8.956	1.03	Alkana
9	12	6,10,14-10-trimethyl-2-Pentadecanone	96	C18H36O	9.235	6.04	alkanon
10	13.	Hexadecanoic acid,methyl ester	97	C17H34O2	9.732	14.63	Alkana
11	14.	Hexadecanoic acid	95	C16H32	9.941	18.95	Alkana

12	17.	3,7,11,15-tetramethyl-2-Hexadecen-1-ol	94	$C_{20}H_{40}O_2$	10.845	4.83	Terpenoid Alcohol
13	18.	Octadecanoic acid,methyl ester (CAS)	91	$C_{19}H_{38}O_2$	10.897	2.66	
14	19.	9,12-Octadecadienoic acid (CAS)	94	$C_{18}H_{32}O_2$	10.968	15.41	alkana
15	20.	9-Octadecenoic acid	91	$C_{18}H_{34}O_2$	11.095	2.05	alkana

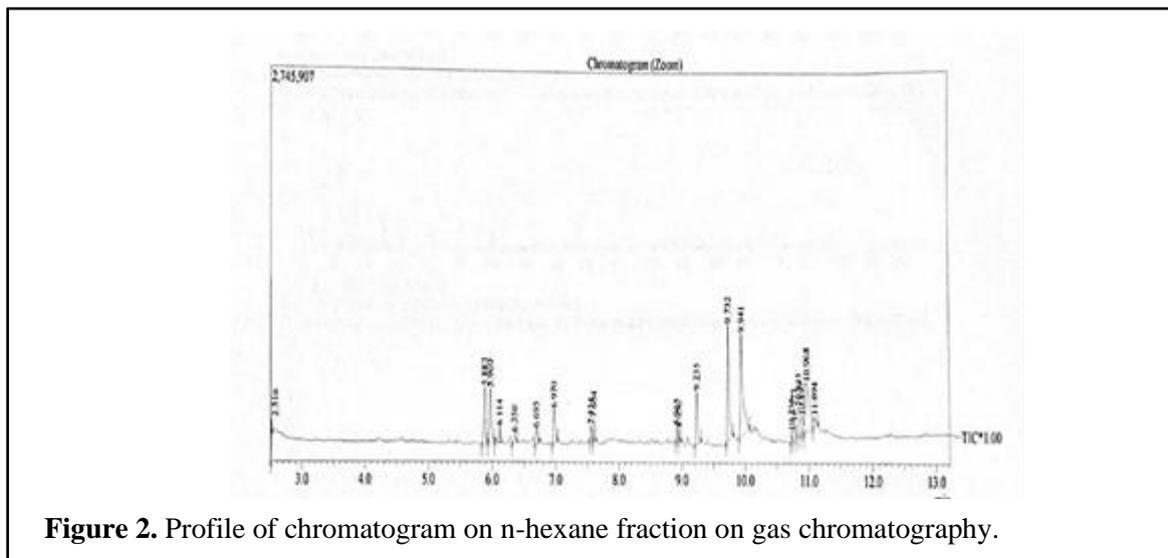


Figure 2. Profile of chromatogram on n-hexane fraction on gas chromatography.

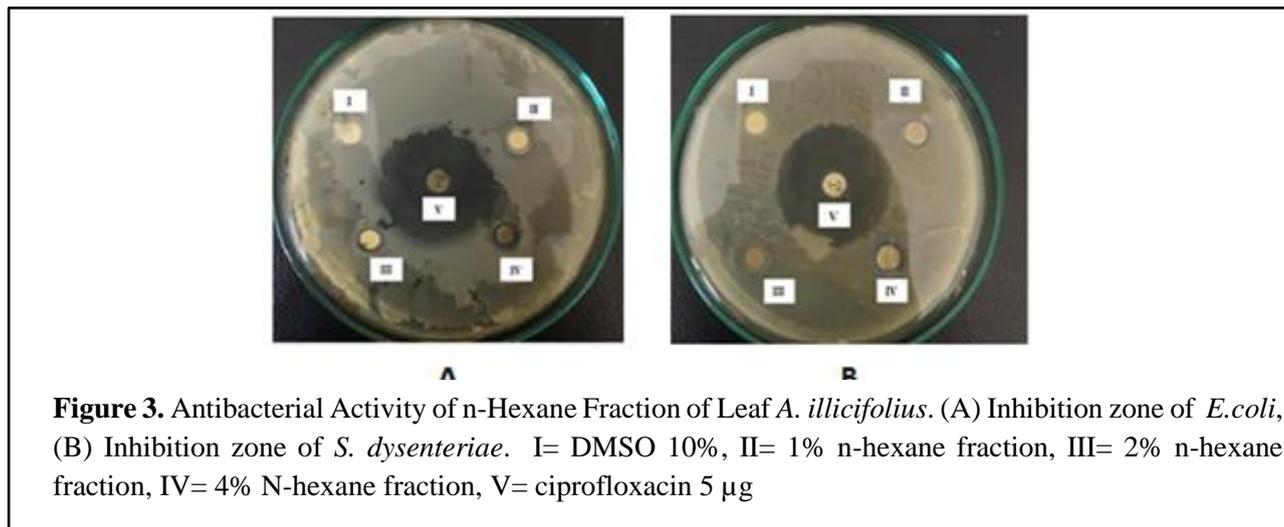


Figure 3. Antibacterial Activity of n-Hexane Fraction of Leaf *A. illicifolius*. (A) Inhibition zone of *E. coli*, (B) Inhibition zone of *S. dysenteriae*. I= DMSO 10%, II= 1% n-hexane fraction, III= 2% n-hexane fraction, IV= 4% N-hexane fraction, V= ciprofloxacin 5 µg

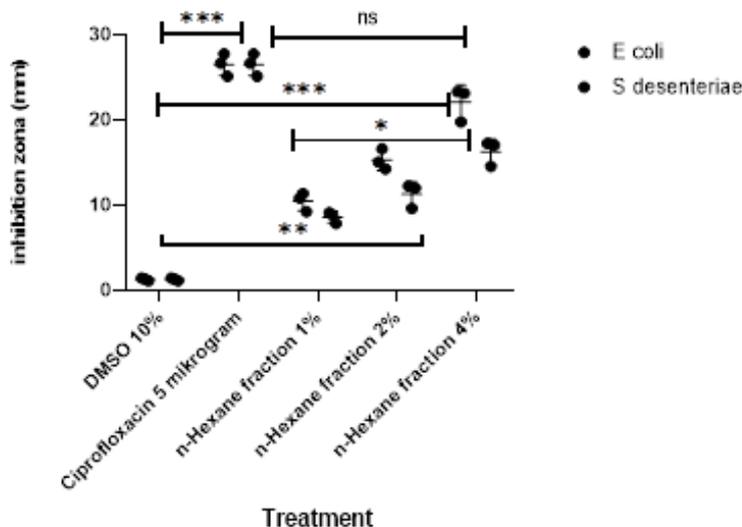


Figure 4. Antibacterial activity with inhibition zone in the treatment of n-hexane fraction, *** significantly ($p > 0.05$), ** significantly ($P > 0.05$). * Significant, NS= no Significant

The treatment in the antibacterial activity test was a negative control (DMSO 10%), positive control of ciprofloxacin, and three concentrations of the n-hexane fraction, namely 1%, 2%, and 4%), the present study. the fraction treatment could inhibit E coli and shigella bacteria (Figure 3 and Table 1).

DISCUSSION

The plant used was *A. ilifolius* leaf, which is from the Segara Anakan lagoon of Cilacap. The fraction yield of 4.3 percent, this result is greater than the previous researcher's 2.2% (suryati, 2018). GC-MS analysis showed 15 compounds with a similarity index (SI) > 90. These 15 compounds include terpenoids, fatty acids, alkanes, alkenes, and ketone compounds. The sesquiterpenes (terpenoid) on trans -beta-caryophyllene, alpha-humulene, Naphthalene decahydro-4 -A-5 methyl, which is strong in oral effect ^{24,25}. (Trans (beta)-caryophyllene (H15C24) has a base peak of m/e 41 and a molecular ion peak of m/e 204. Fragmentation is m/e 204 with cleavage of CH₃ (M+ -15) into fragment with a peak m/e of 189. Becomes with cleavage CH₂ = CH₂ (M+ -18) into fragment peak m/z 161, and leavage CH₂ (M+ -14) into fragment peak m/e 147. Becomes with the cleavage of CH₂ (M+ -14), CH (M+ -13), CH₃ (M+ -15), C₂H₃ (M+ -27), C₃H₂ (M+ -38) and tCH₂ (M+ -14) into a fragment ion with m/z peak of 41.

Fragmentation of m/e 204 with cleavage of CH₃ (M+ -15) into fragment ion m/e 189. CH₂ = CH₂ (M+ -18) becomes a fragment ion with a peak m/e of 161. Then cleavage of CH₂ (M+ -14) becomes a fragment ion with a peak of m/e of 147. Fragmentation of m/e 147 with termination of C₂H₃ (M+ -27). Hexadecanoic acid (C₁₆H₃₂O₂) with a molecular ion peak of m/e 256. There are three fragmentation pathways of hexadecanoic acid m/e 256. First, H₃C-CH₂ (M+ -29), H₃C-CH₂-CH₂ (M+ -43), H₂C= CH₂-(CH₂)₁₁CH₃ becomes fragment ion with a peak of m/e 60. In the second path, the fragment of m/e 227 occurs four times H₂C=H₂C cleavage. The H₃C-CH=CH₂ (M+ -42) becomes a fragment ion with a peak of m/e 73. The third pattern occurs Fragmentation at m/e 213 with H₂C=H₂C breaking three times and at fragment m/e 129 breaking CO₂ (M+ -44) and H₃C-CH₂-CH₃ (M+ -44) to fragment ion m/e 41. Fragmentation at m/e 60 with OH cleavage (M+ -

17) becomes fragment ion with peak m/e 43.

The n-hexane fraction of jeruju leaves contain terpenoids and fatty acids with antibacterial activity by transmembrane protein and reducing the permeability of bacterial cell walls¹⁶ and preventing the function of ATP synthase²⁶. sesquiterpenes (terpenoids) are hydrophobic. The compound is causing protein denaturation, cell membrane lysis²⁷. Ethyl acetate extract from *Grewia Pubescens* has a compound 3,7,11,15-tetramethyl-2-hexadecane-1-ol²⁸. These compounds inhibited *S. aureus*, *E. coli*, *B. subtilis*, and *P. aeruginosa* with high activity at 25-200 mg/ml.

CONCLUSION

The transplantation of MSCs ameliorated LF by reducing SGPT and SGOT concentration. The n-hexane fraction of jeruju leaves (*Acanthus ilicifolius* L) contains 3,7,11,15-tetramethyl-2-hexadecane-1-ol, and fatty acids have antibacterial activity. All concentrations of the tested fractions had activities, and the highest concentration of 4% activity was in both *E. coli* and *S. dysenteriae* with inhibition zones of 11.67 mm and 10.33 mm.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

AUTHORS' CONTRIBUTION

THW analyzes and interprets the GC-MS results. NE tested the activity of the extract against *E. coli* and *S. dysenteriae* bacteria. WA was performing the extraction, fractionation, phytochemical screening, and antibacterial analysis using GraphPad Prism 8, and the major contributor in the writing of the manuscript. All authors read and approved the final manuscript.

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