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RESEARCH ARTICLE

Phytochemical screening, GC-MS analysis and antibacterial activity of *Acanthus ilicifolius* (L.) (Jeruju) fruit extract

Maizatul Akma Ibrahim[1,2*], Nurul Iffah Hannah Azrin[1], Nor Hafizah Zakaria[3], and Fadzilah Adibah Abdul Majid[3]

[1]Department of Plant Science, Kulliyyah of Science, International Islamic University Malaysia, 25200 Kuantan Pahang, Malaysia.
[2]Plant Productivity and Sustainable Resource Unit, Department of Plant Science, Kulliyyah of Science, International Islamic University Malaysia,25200 Kuantan, Pahang, Malaysia.
[3]Higher Institution Centre of Excellence (HICoE), Institute of Climate Adaptation and Marine Biotechnology (ICAMB), Universiti Malaysia Terengganu, 21030, Kuala Nerus, Terengganu, Malaysia.

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⊠* CORRESPONDING AUTHOR
Assoc. Prof. Dr. Maizatul Akma Ibrahim
Plant Productivity and Sustainable Resource Unit, Department of Plant Science, Kulliyyah of Science, International Islamic University Malaysia,Bandar Indera Mahkota. 25200 Kuantan, Pahang, Malaysia
Email: maizatulakma@iium.edu.my

ABSTRACT

Acanthus ilicifolius (L.) is a mangrove plant that contains various bioactive compounds contributing to its pharmacological activities. Although several studies have reported its antimicrobial efficacy, research specifically investigating the antibacterial properties of its fruit remains underexplored. This study was conducted to examine the phytochemical compounds in the methanolic extract of *A. ilicifolius* fruit using phytochemical screening assays and gas chromatography–mass spectrometry (GC-MS) analysis. The antibacterial activity was then determined using the agar well diffusion method, and minimum inhibitory concentration (MIC) was conducted using a 96-well resazurin assay. The presence of terpenoids, flavonoids, steroids, saponins and tannins were detected, while ten major metabolites were found in the non-polar fractions namely benzophenone, toluene, phenol-2,4-bis (1,1-dimethylethyl), n-hexadecanoic acid, 2(3H)-benzoxazolone, hexadecanoic acid, methyl ester, 9-octadecenoic acid, methyl ester, (E), 9,12-octadecadienoic acid (Z,Z)-, cis-vaccenic acid and 13-docosenamide, (Z). The agar well diffusion assay showed that the extract exhibited the highest inhibitory activity against *Escherichia coli* (15.73 ± 0.33 mm), followed by *Pseudomonas aeruginosa* (15.10 ± 0.70 mm) and *Staphylococcus aureus* (13.13 ± 0.57 mm). The lowest MIC values against *E. coli, P. aeruginosa* and *S. aureus* were found at 3.13 mg/mL, 12.50 mg/mL and 12.50 mg/mL, respectively. These findings suggest the potential efficacy of *A. ilicifolius* fruit as an antibacterial agent and further studies should focus on the assessments of the cytotoxicity of the fruit extract.

1. INTRODUCTION

Antimicrobial resistance poses a major global public health challenge and was estimated to have caused approximately 1.27 million deaths in 2019 (Kelly Tang et al., 2023). The 2022 Global Antimicrobial Resistance and Use Surveillance System report highlights worrying resistance levels in common bacterial infections (World Health Organization, 2021). Across 76 countries, the median resistance rates were 42% for third-generation cephalosporin-resistant *Escherichia coli* and 35% for methicillin-resistant *Staphylococcus aureus* (MRSA).

Moreover, in 2020, 20% of urinary tract infections caused by *Escherichia coli* showed reduced susceptibility to commonly used antibiotics, including co-trimoxazole, ampicillin, and fluoroquinolones (Bisseye et al., 2025). This growing resistance makes it increasingly difficult to treat common infections effectively, underscoring the urgent need for novel antibacterial drugs to combat resistant bacteria.

In this study, three species of bacteria were tested, including *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. *E. coli* is a Gram-negative, rod-shaped facultative anaerobe commonly found in the intestines, with some pathogenic strains causing foodborne illnesses and urinary tract infections (Ramos et al., 2020). *S. aureus* is a Gram-positive, cocci-shaped facultative anaerobe that commonly inhabits human skin and mucous membranes. It can cause a wide range of infections, from mild skin conditions to serious illnesses such as pneumonia, endocarditis, and toxic shock syndrome. Methicillin-resistant *S. aureus* (MRSA) remains a significant public health concern due to its resistance to multiple antibiotics (Bashabsheh et al., 2023). Meanwhile, *P. aeruginosa* is a Gram-negative, rod-shaped obligate aerobe found in soil, water, and hospital settings, known for its biofilm formation and role in opportunistic infections such as pneumonia, UTIs, and sepsis, particularly in immunocompromised patients (Sathe et al., 2023).

According to Nabi et al. (2022) medicinal plants are great sources of medicines to obtain a variety of drugs that

can benefit mankind. This is because medicinal plants offer many intricate and structurally diverse compounds that exert antibacterial properties through their secondary metabolites (Pothiraj et al., 2021). These biologically active constituents, isolated from plant extracts throughout the drug development process, could exert biological effects against various disease-causing pathogens. Additionally, Nasim et al. (2022) stated that medicinal plants are preferable to synthetic drugs, as they are more effective and have less side effects, less toxic, and more easily metabolized and absorbed by the body.

Throughout history, plant-derived compounds have been extensively used in clinical settings due to their superior patient tolerance and acceptance (Dehelean et al., 2021). Scientists have screened approximately 70,000 plant species as conventional remedies in Asia due to their potential medicinal benefits (Nasim et al., 2022). *Acanthus ilicifolius* (L.) has attracted attention due to its rich array of secondary metabolites and its long-standing use in traditional medicine. In Malaysia and Indonesia, it is commonly known as "Jeruju." The genus name *Acanthus* originates from the Greek word *acantha*, referring to the spiny or thorny nature of some species' leaves, while this species epithet *ilicifolius* is derived from Latin, meaning "holly-like leaves." This plant is also referred to by several common names, including holy mangrove, sea holly, and holly-leaved acanthus.

This plant has been used to treat various conditions such as paralysis, asthma, and snake bites. It also has applications as an analgesic and has been employed in preventing tumour growth and slowing the progression of cancer (Habib et al., 2018; Zakaria et al., 2024). Extracts of *A. ilicifolius* have been reported to exhibit diverse pharmacological activities, including antidiabetic, anticancer, antioxidant, anti-inflammatory, antiosteoporotic, and hepatoprotective effects (Widiastuti et al., 2020). These therapeutic properties are largely attributed to the presence of bioactive compounds such as flavonoids, phenolics, tannins, alkaloids, steroids, and saponins found throughout various parts of the plant (Nusaibah et al., 2021).

Although various parts of *A. ilicifolius* have demonstrated promising therapeutic properties, research has mostly concentrated on its leaves and roots, leaving the pharmacological potential of its fruit underexplored (Zakaria et al., 2024). For instance, leaf extracts have shown cytotoxic effects against MCF-7 human breast cancer cells (Vani & Manikandan, 2019), and root extracts have exhibited anti-diabetic activity in animal models (Venkataiah et al., 2013). However, the fruit remains poorly studied despite its potential as a source of bioactive compounds.

At the same time, antimicrobial resistance continues to rise globally, posing a serious public health threat and creating the urgent need for novel, effective antimicrobial agents, particularly those derived from natural sources. The absence of plant-based antimicrobials from *A. ilicifolius* fruit represents a critical gap in current pharmacognostic research. Addressing this gap is essential for diversifying the arsenal of therapeutic options available against resistant pathogens. Therefore, this study specifically aims to investigate the phytochemical composition and antibacterial activity of *A. ilicifolius* fruit extract, with the goal of identifying its potential as a novel plant-derived antimicrobial agent.

2. MATERIALS AND METHODS

2.1 Plant material

The fruits of *A. ilicifolius* were collected from the mangrove forest at Endau-Rompin National Park, Pahang (2°31'53.16"N, 103°24'51.07"E). After being washed with water, the fruits were cut into small pieces and dried in an oven at 50°C until fully dehydrated. Once dried, they were ground into a fine powder using a blender and kept an airtight container. The sample was stored in a dark and dry condition.

2.2.1 Extraction of *A. ilicifolius* fruit

Maceration technique was utilized for the extraction of *A. ilicifolius* fruit, with a ratio 1:10 of *A. ilicifolius* fruit to absolute methanol (99.8%) (Sigma-Aldrich, United States) (Cheong et al., 2022). A total of 25 g fine powdered fruit was mixed with 250 mL of methanol in the Erlenmeyer flask for 72 hours. The flask was sealed with aluminium foil, and the mixture was consistently agitated using an orbital shaker. Then, the extract was filtered using Whatman No. 1 filter paper and evaporated in a rotary evaporator at 60°C to obtain crude extract. The extract was further dried in the fume hood for a few days. Then, the crude extract was kept in chiller between 2 and 8°C for further used in phytochemical screening and antibacterial testing. The percentage yield of the crude extract was determined using the following formula:

$$Yield\ (\%) = \frac{Weight\ of\ dried\ crude\ extract\ (g)}{Dry\ weight\ of\ plant\ before\ extraction\ (g)} \text{x} 100$$

2.3. Phytochemical screening

2.3.1 Test for alkaloids

Mayer's test was performed to detect the presence of alkaloids. In this procedure, Mayer's reagent was slowly added into a test tube containing 1 mL of methanolic fruit extract. The appearance of creamy coloured precipitate would indicate the existence of alkaloids (Kancherla et al., 2019).

2.3.2 Test for terpenoids

The Salkowski assay was performed to detect the presence of terpenoids. In this assay, 0.5 mL of the methanolic

fruit extract was dissolved in 2 mL of chloroform, followed by gradual addition of 3 mL of concentrated sulphuric acid (H_2SO_4) (Sigma-Aldrich, United States). The formation of reddish-brown colour in the solution indicates the presence of terpenoid in the sample (Dubale et al., 2023).

2.3.3 Test for flavonoids

The presence of flavonoids was detected using the Shinoda test. About 1 ml of the methanolic fruit extract was added to a small amount of magnesium, followed by the addition of a few drops of concentrated hydrochloric acid (HCl) (Sigma-Aldrich, United States) along the wall of the test tube. The appearance of a pink to scarlet coloration indicates a positive result for flavonoids (Parbuntari et al., 2018).

2.3.4 Test for steroids

The presence of steroids was confirmed through the Salkowski assay. In 1 mL of the methanolic fruit extract, 2 mL of chloroform (Sigma-Aldrich, United States) and 1 mL of concentrated sulfuric acid (Sigma-Aldrich, United States) were slowly added until a double phase was formed. The presence of steroids was indicated by the formation of reddish-brown colour in the middle layer (Maria et al., 2018).

2.3.5 Test for saponins

A foam test was performed to investigate the presence of saponins. In this procedure, 1 mL of the methanolic fruit extract was introduced into a test tube and thoroughly mixed with 5 mL of water. The persistent formation of foam for more than 10 minutes indicates the existence of saponins (Dubale et al., 2023).

2.3.6 Test for tannins

Ferric chloride test was employed following the procedure by Alexandra et al. (2018) to examine the presence of tannins. 1 mL of the methanolic fruit extract was mixed with 2 mL of a 5% ferric chloride solution (Sigma-Aldrich, United States) in a test tube. The appearance of dark-blue or greenish-black colour shows the presence of tannins.

2.4. GC-MS analysis

The powdered plant material was extracted with methanol in a 1:10 (w/v) ratio and shaken at 120 rpm for 72 hours using an orbital shaker, following the method of Chirumamilla et al. (2022). After extraction, the mixture was filtered through Whatman No. 1 filter paper, and the solvent was evaporated to obtain the crude extract. GC–MS analysis was then conducted using a Clarus™ SQ 8 GC/MS system (PerkinElmer, Waltham, USA) equipped with an Elite-5MS capillary column. Helium was used as the carrier gas at a constant flow rate of 1.5 mL/min, and 1 µL of sample was injected. The ionization energy was set to 70 eV with a scanning mass range of m/z 25–700. The GC oven was initially held at 50 °C for 1 minute, then increased at a rate of 5 °C/min to 250 °C, followed by a 5-minute isothermal hold. The oven program run time was 45 minutes. The phytochemicals were determined based on the retention time and the generation of fragment ions. Then, the total peak area indicated the percentage of these bioactive compounds. The MS spectrum patterns were also compared with the standard mass spectra present in the NIST Mass Spectra Database (Bano et al., 2021).

2.5. Antibacterial assay

2.5.1 Preparation of inoculum

Two strains of Gram-negative bacteria (*Escherichia coli,* ATCC 25922 and *Pseudomonas aeruginosa*, ATCC 27853) and one strain of Gram-positive bacteria (*Staphylococcus aureus,* ATCC 25923) were used to determine the antibacterial properties of methanolic fruit extract *of A. ilicifolius*. The bacteria were obtained from Microbiology Laboratory, Kulliyyah of Science, International Islamic University Malaysia (IIUM). Before testing, all cultures were maintained in Mueller Hinton broth (MHB) (Sigma-Aldrich, United States) overnight at 37°C using an incubator shaker. The bacterial strains grown on MHB were adjusted to a turbidity of 0.5 McFarland Standard to ensure all bacterial broth concentrations were standardized for antibacterial assay (Hemeg et al., 2022). The measurement of the optical density (OD) was done using ultraviolet-visible (UV-VIS) spectrophotometer (Perkin Elmer, Singapore) at wavelength 625 nm. Each bacterial culture was placed into a cuvette, and their turbidity was quantified by using the spectrophotometer. The acceptable turbidity range was set at 0.08–0.13 OD at 625 nm wavelength, corresponding to the 0.5 McFarland Standard (Ambrosio et al., 2019). The OD reading was subjected to adjustment by adding bacterial culture or sterile broth until the desired final concentration of 1.5×10^8 CFU/mL was reached.

2.5.2 Preparation of test sample

The working stocks for the methanolic fruit extract of *A. ilicifolius* were prepared at concentrations of 20, 50 and 100 mg/mL. These concentrations were achieved by dissolving 40, 100 and 200 mg of the extracts into 2 mL of sterile distilled water. 10% of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, United States) was used as the negative control. Tetracycline (30 µg/mL) (Sigma-Aldrich, United States) was prepared using ethanol as the solvent and used as the positive control (Minarti et al., 2023).

2.5.3 Agar well diffusion assay

Antibacterial activity of methanolic fruit extract of *A. ilicifolius* was determined using agar well diffusion assay (Aisiah et al. 2022). The standardized bacterial culture (100 µL) was pipetted onto the centre of the agar surface and evenly spread using a sterile L-shaped glass cell spreader. After allowing the agar plates to dry for a few minutes, five wells (8 mm in diameter) were created in the inoculated agar using sterile 200 µL micropipette. A volume of 50 µL of the extract, prepared at concentrations of 20, 50, and 100, mg/mL, was added to each well. The same volume was also applied for both the positive and negative control treatments. After overnight incubation at 37 °C, antibacterial activity was evaluated by measuring the diameter of the inhibition zones. Each test was performed in triplicate, and the data are reported as mean values with standard deviation (SD).

2.6. Minimum inhibitory inhibition (MIC)

The resazurin broth microdilution assay was done using MIC assay (Kebede & Shibeshi, 2022). The crude extract was initially dissolved in 1% ethanol (Sigma-Aldrich, United States) and prepared at double the desired final concentration, then diluted 1:1 with sterile Mueller-Hinton Broth (MHB). In brief, 200 µL of the extract at a concentration of 100 mg/mL was added into the first well. Then, two-fold serial dilutions were done across decreasing concentrations (3.125, 6.25, 12.5, 25, 50, and 100 mg/mL) was carried out until well 8. From each row, 100 µL was transferred into the next row with 100 µL of MHB, mixed thoroughly, and this procedure was repeated up to achieve the desired concentration. Finally, 100 µL was discarded from the last well to ensure a final volume of 100 µL in all test wells.

3. RESULT AND DISCUSSION

3.1 Extraction and phytochemical screening

A. ilicifolius fruit was extracted using the maceration technique with methanol as the solvent at a 1:10 (w/v) ratio, resulting in a yield of 22.4% (w/v). This yield is slightly lower than that reported in a previous study, which documented a yield of 23% from 200 g of *A. ilicifolius* leaf powder extracted with 400 mL of methanol (Karim et al., 2021). However, Andriani et al. (2020) reported an even higher yield of 121.5% from 1000 g of *A. ilicifolius* leaf powder extracted with 96% methanol using the same technique.

Previous reports showed that the amount of plant extract production relies on the type of solvent used during the process. In a study reported by Arunita et al. (2023), methanol was employed as a solvent in the extraction of *A. ilicifolius* because methanol generates higher yields than other solvents such as ethyl acetate, ethanol, and acetone as those have lower polarity. Another study, Karim et al. (2021), reported that methanol is a promising solvent system for extraction process since various active compounds, namely flavonoids, phenols, tannins and saponins, were identified during the phytochemical screening of *A. ilicifolius* extracts. Raharjo et al. (2023), outlined that methanol possesses properties that facilitate the formation of hydrogen and water within plant tissue cells and can dissolve polar organic compounds.

Besides, methanol, which has a high polarity indexed solvents, has better capability to recover the compounds and constituents of plant fruits (Cheong et al., 2022). Phytochemical screening involves a qualitative analysis of plant extracts to detect the presence of secondary metabolites through observable colour and texture changes upon the addition of specific reagents (Fardiyah et al., 2020). In this study, the results revealed the presence of several phytochemical groups, including flavonoids, terpenoids, steroids, tannins, and saponins, as indicated by colour changes during the individual tests (Table 1). These findings are consistent with Karim et al. (2021), who also reported the presence of phenols, flavonoids, steroids, terpenoids, and saponins in *A. ilicifolius* extracts. Studies by Bora et al. (2017) and Andriani et al. (2020) similarly identified triterpenoids, saponins, flavonoids, steroids, phenols, and tannins in *A. ilicifolius* extracts. Pringgenies et al. (2020) confirmed similar findings, detecting steroids, flavonoids, and tannins. However, these studies focused only on the leaves, highlighting the lack of research on the fruit of this plant.

Table 1: Phytochemical screening results.

Compound	Test	Before	After	Observation	Result
Alkaloid	Mayer's test			No formation of creamy colour precipitate	(-)
Terpenoid	Liebermann-Burchard test			Formation of reddish-brown colour	(+)
Flavonoid	Shinoda test			Formation of pink, scarlet colour	(+)
Steroid	Salkowski test			Formation of brown ring at the middle layer	(+)
Saponin	Foam test			Formation of persistent foam	(+)
Tannin	Ferric chloride test			Formation of greenish-black colour	(+)

Alkaloids are primarily polar compounds and therefore require extraction using polar solvents. Methanol, being a polar solvent, is well-suited for dissolving and extracting such polar constituents (Rahayu et al., 2019). The absence of alkaloids in this study could be attributed to several factors. Alkaloid distribution within a plant is often tissue-specific. Although *A. ilicifolius* is known to produce alkaloids, their concentration in the fruit may be very low or undetectable

compared to other parts such as the leaves or roots (Wu et al., 2022).

Although methanol is a polar solvent commonly used in phytochemical extraction, it may not be optimal for isolating alkaloids, particularly in their free base form (Bitwell et al., 2023). Efficient extraction of such alkaloids typically requires acidified aqueous solvents, like dilute hydrochloric acid, to enhance their solubility. Without acidification, these compounds may remain insoluble or degrade, resulting in their non-detection during qualitative screening (Zhang et al., 2018). On the other hand, Islam et al. (2024) successfully detected alkaloids in the acetone extract of *A. ilicifolius*. These differences highlight the importance of solvent selection in alkaloid extraction. Thus, future studies should explore alternative solvents, such as acetone or ethanol, for more efficient alkaloid recovery.

3.2 GC-MS analysis

The GC-MS analysis is widely used for detecting functional groups and identifying diverse biologically active compounds in medicinal plants (Konappa et al., 2020). This analysis provides a chromatogram displaying the molecules extracted at various retention times, along with spectral data that corresponds to secondary metabolites. In this study, methanolic fruit extract of *A. ilicifolius* was subjected to GC-MS analysis, resulting in a chromatogram as in Figure 1. From this chromatogram, significant peaks were identified and interpreted.

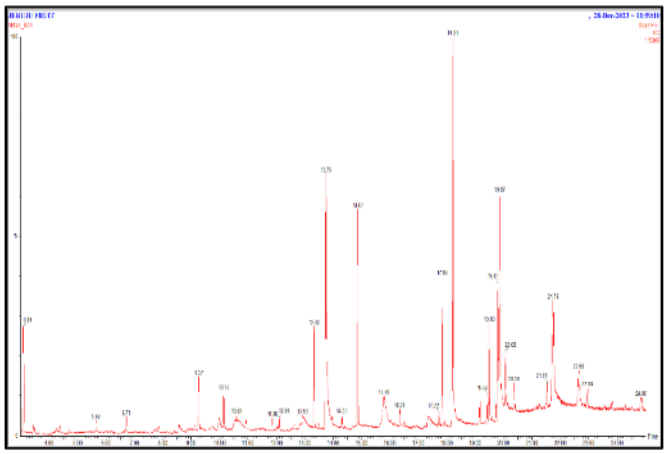

Figure 1: GC-MS chromatogram of methanolic fruit extract of *A. ilicifolius*.

The retention times, molecular formulas, molecular weights, and concentration percentages (%) of the identified compounds were recorded in Table 2. These results were obtained using the internal library search provided by the Perkin Elmer TurboMass software (PerkinElmer, Inc., Waltham, United States).

Table 2: The predicted compounds in methanolic fruit extract of *A. ilicifolius*.

RT	Compound	MF	MW	Conc. (%)
3.09	Toluene	C_7H_8	92	40.70
13.33	Phenol- 2,4-bis (1,1-dimethylethyl)	$C_{14}H_{22}O$	206	51.60
13.75	2(3H)-Benzoxazolone	$C_7H_5NO_2$	135	80.60
14.87	Benzophenone	$C_{13}H_{10}O$	182	83.50
17.84	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	69.40
18.21	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	83.00
19.50	9-Octadecenoic acid, methyl ester, (E)	$C_{19}H_{36}O_2$	296	8.00
19.81	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280	28.10
19.87	cis-Vaccenic acid	$C_{18}H_{34}O_2$	282	16.10
21.75	13-Docosenamide, (Z)	$C_{22}H_{43}NO$	337	50.10

* RT = Retention time, MF = Molecular formula, MW = Molecular weight

The GC-MS chromatogram of the methanolic fruit extract of *A. ilicifolius* showed 29 peaks, representing 29 compounds. The 10 most significant peaks were selected for further interpretation, revealing the presence of compounds such as benzophenone, toluene, phenol-2,4-bis (1,1-dimethylethyl), n-hexadecanoic acid, 2(3H)-benzoxazolone, hexadecanoic acid, methyl ester, 9-octadecenoic acid, methyl ester, (E), 9,12-Octadecadienoic acid (Z,Z)-, cis-vaccenic acid and 13-docosenamide, (Z), as detailed in Table 2. The top three compounds with the highest relative abundance were benzophenone, followed by n-hexadecanoic acid and hexadecanoic acid, methyl ester. According to Aranda et al. (2019), benzophenone which belongs to the ketone group, is an effective antimicrobial agent with activity against both Gram-positive and Gram-negative bacteria, while also being non-cytotoxic to mammalian cells.

Additionally, n-hexadecanoic acid, commonly known as palmitic acid, is a carboxylic acid with various bioactive properties. According to the previous study, n-hexadecanoic acid has anti-inflammatory, antioxidant, hypocholesterolemic, and antibacterial effects (Abubakar & Haque, 2020). Hexadecanoic acid, methyl ester, is another prominent compound in the studied plant extract, belongs to the ester-derived fatty acid group. According to Nabi et al. (2022), this compound possesses antibacterial, antioxidant, nematicide, and insecticidal properties, along with cholesterol-lowering effects. These findings highlight the potential therapeutic applications of the plant's bioactive compounds.

The current results align with a study reported by Warsinah et al. (2021) which identified the presence of n-hexadecanoic acid, hexanedioic acid, methyl ester, and

9,12-octadecadienoic acid (Z,Z) in *A. ilicifolius* using GC-MS analysis. Another study by Vani and Manikandan (2019) similarly found that n-hexadecanoic acid (2.143%) and 9,12-octadecadienoic acid (Z,Z) (0.815%) were present in the GC-MS profile of *A. ilicifolius*. However, other studies have shown different results. Rahayu et al. (2019) reported the presence of sphingosine, trienoic acid, topiramate, budesonide, and prostatetraenoic acid in the GC-MS analysis of *A. ilicifolius*. Additionally, Sofia and Merlee (2017) found compounds such as 2-octanone, phenol, 3,5-bis(1,1-dimethylethyl)-, ethanone, 1-(2-furanyl)-, 3-butyl indolizidine, 2-decen-1-ol, 5-decen-1-ol (Z)-1,2-benzenedicarboxylic acid, dioctyl ester, and lupeol in *A. ilicifolius* through their GC-MS analysis. The variability in GC-MS profiles for *A. ilicifolius* across different studies can be attributed to differences in extraction methods, solvents, plant parts, and analytical conditions (Islam et al., 2024).

3.3 Antibacterial screening

The methanolic fruit extract of *A. ilicifolius* was determined for its antibacterial activity using the agar well diffusion technique. In this study, the extract was tested against three bacterial strains: *E. coli, P. aeruginosa*, and *S. aureus*. The results of the antibacterial activity were presented in Table 3 and Figure 2, where the inhibition zone diameters were measured for each bacterial species in relation to the different concentrations of the extract, as well as the positive control (tetracycline) and the negative control (10% DMSO). At the highest concentration of 100 mg/mL, the extract exhibited the largest zone of inhibition against *E. coli* (15.73 ± 0.33 mm), suggesting that the extract is the most effective against this Gram-negative bacterium. This was followed by 15.10 ± 0.70 mm against *P. aeruginosa*, another Gram-negative pathogen known for its resistance to many antibiotics, and 13.13 ± 0.57 mm against *S. aureus*, a Gram-positive bacterium associated with a range of infections.

Table 3: Diameter of inhibition zone of the methanolic extract against the tested bacteria

Bacteria	Conc. (mg/mL)	Inhibition zone (mm)	Positive control	Negative control
E. coli	100	15.73 ± 0.33	13.93 ± 0.17	-
	50	14.23 ± 0.25		
	20	12.13 ± 0.57		
P. aeruginosa	100	15.10 ± 0.70	8.27 ± 0.25	-
	50	13.87 ± 0.73		
	20	11.67 ± 0.90		
S. aureus	100	13.13 ± 0.57	14.07 ± 0.12	-
	50	11.93 ± 0.57		
	20	10.80 ± 0.70		

Positive control = Tetracycline; negative control = DMSO; (-) indicates no inhibition zone observed. Values were the means ± standard deviations (SD) of triplicate samples.

When the extract concentration was reduced to 50 mg/mL, *E. coli* still exhibited the largest inhibition zone (14.23 ± 0.25 mm), followed by *P. aeruginosa* (13.87 ± 0.73 mm) and *S. aureus* (11.93 ± 0.57 mm). Even at this lower concentration, the extract continued to show significant antibacterial activity, particularly against *E. coli*. At the lowest tested concentration of 20 mg/mL, the extract's efficacy was reduced but remained effective, with *E. coli* again showing the largest inhibition zone (12.13 ± 0.57 mm), followed by *P. aeruginosa* (11.67 ± 0.90 mm) and *S. aureus* (10.80 ± 0.70 mm). These findings indicate that the methanolic fruit extract of *A. ilicifolius* possesses dose-dependent antibacterial activity, with *E. coli* being the most susceptible to the extract across all concentrations.

The varying degrees of antibacterial potency observed among the tested bacterial strains suggest that the bioactive compounds in the methanolic fruit extract of *A. ilicifolius* exhibit a stronger inhibitory effect against Gram-negative bacteria, particularly *E. coli*. This enhanced activity could be due to differences in bacterial cell wall structures, which could make Gram-negative strains more susceptible to certain bioactive components. This result is consistent with the findings of Pothiraj et al. (2021) who reported that plant crude extracts tend to be more potent against Gram-negative bacteria than Gram-positive ones. According to Cheong et al. (2022), Gram-negative bacteria are generally more resistant to antibiotics due to their complex outer membrane, composed mainly of lipopolysaccharides, proteins, and phospholipids. In contrast, Gram-positive bacteria possess a simpler outer structure, lacking lipopolysaccharide, and are mainly composed of peptidoglycan, teichoic acids, and lipoteichoic acids.

Despite this structural difference, the methanolic fruit extract of *A. ilicifolius* also demonstrated significant antibacterial activity against *S. aureus*, a Gram-positive bacterium, indicating its broad-spectrum potential. GC-MS analysis of the fruit extract identified several bioactive compounds, including benzophenone, n-hexadecanoic acid, and hexanedioic acid, which may be responsible for these antibacterial effects. These compounds likely act by disrupting bacterial cell membranes or inhibiting critical cellular processes, which may explain the extract's effectiveness against both Gram-positive and Gram-negative bacteria (Pothiraj et al., 2021).

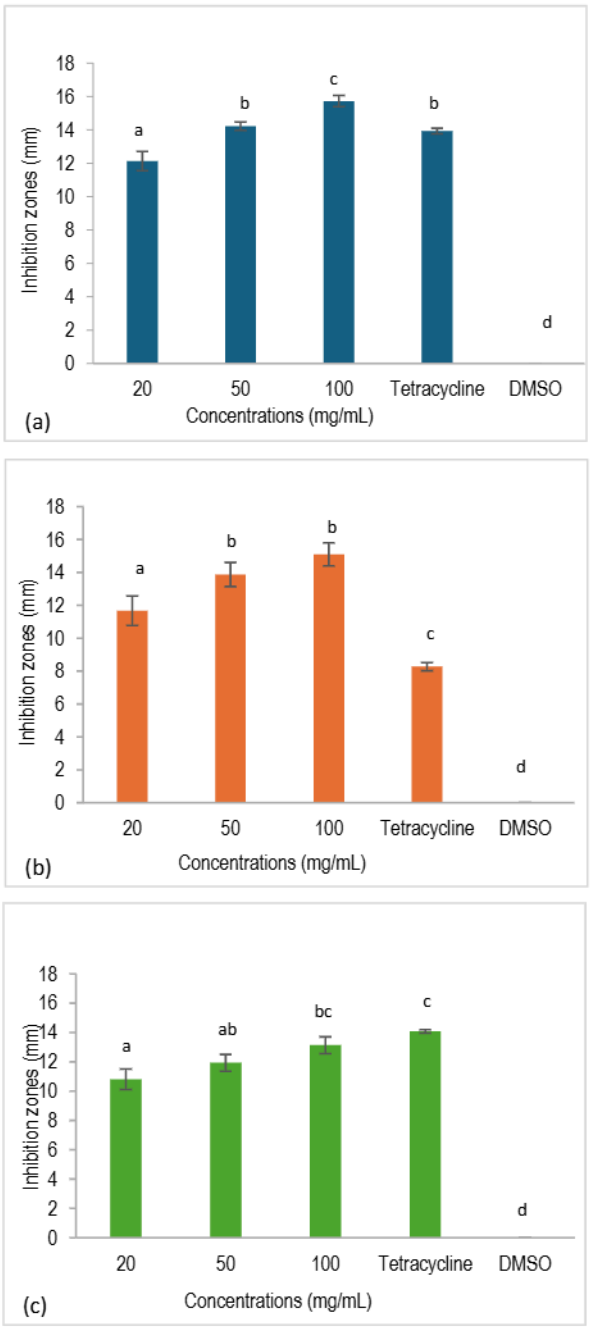

Figure 2: Inhibition zones of methanolic fruit extract *A. ilicifolius* with varied concentrations against (a) *E. coli*, (b) *P. aeruginosa*, and (c) *S. aureus*. Error bars indicate mean ± SD. Different superscripts (a, b, c, d) indicate statistically significant differences.

In comparison to previous studies, Bose and Bose (2008) reported that chloroform extracts of *A. ilicifolius* leaves showed strong inhibitory activity against *S. aureus* and moderate activity against *P. aeruginosa*. This consistent antibacterial activity of *A. ilicifolius*, regardless of the plant part used or the extraction solvent, suggests the presence of potent antimicrobial compounds. More recently, Pothiraj et al. (2021) also found that *S. aureus* was susceptible to 50 µg/mL of methanolic leaf extract of *A. ilicifolius*, further confirming the antibacterial potential of this plant.

One-way ANOVA confirmed a statistically significant difference in the mean diameters of inhibition zones among the different crude concentrations and controls in the antibacterial activity tests against *E. coli*, *P. aeruginosa*, and *S. aureus* ($p < 0.05$) (Figure 3). For *E. coli*, significant differences ($p < 0.05$) were found for all crude concentrations and controls, except between 50 mg/mL of crude and the positive control (tetracycline). For *P. aeruginosa*, significant differences ($p < 0.05$) were observed for all concentrations, except for 50 and 100 mg/mL of crude. In contrast, for *S. aureus*, significant differences ($p < 0.05$) were detected among all crude concentrations and controls.

3.4 Minimum inhibitory concentration (MIC) determination

The results in the broth microdilution method can be achieved by employing resazurin, a mildly fluorescent blue dye that is transformed into fluorescent resorufin (pink) through reduction by active bacteria, as shown in Figure 4. The changes of resazurin dye from blue to pink colour indicate that the bacterial growth cannot be inhibited by the crude extract. Meanwhile, no colour changes of resazurin dye indicate that bacterial growth can be suppressed by the crude extract (Krochmal & Wicher, 2021). The minimum inhibitory concentration (MIC) of the plant extract to test against the three bacterial strains was prepared in two-fold serial dilution at decreasing concentration range of 100, 50, 25, 12.5, 6.25 and 3.125 mg/mL.

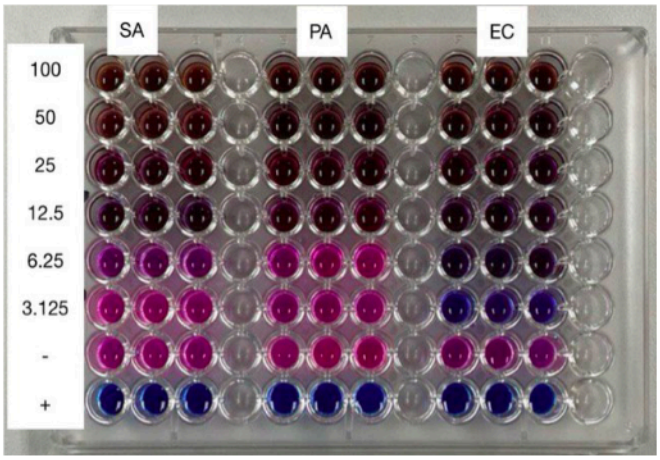

Figure 3: Observation of the minimum inhibitory concentration of methanolic fruit extract of *A. ilicifolius* against *E. coli* (EC), *P. aeruginosa* (PA) and *S. aureus* (SA) within the concentration range of 3.125–100 mg/mL. (-) is a negative control (Mueller-Hinton broth). (+) is a positive control (tetracycline).

The MIC of methanolic fruit extract of *A. ilicifolius* varied across the tested bacterial species. Among the three species, *E. coli* was the most susceptible, with the lowest MIC value of 3.125 mg/mL. No colour change was observed between concentrations of 3.125 mg/mL and 100 mg/mL, indicating effective inhibition. For *P. aeruginosa*, the lowest MIC value was 12.5 mg/mL, with colour changes observed

between 3.125 mg/mL and 6.25 mg/mL. Similarly, *S. aureus* exhibited an MIC of 12.5 mg/mL, with no colour changes observed from 12.5 mg/mL and above. These results suggest that a concentration of 12.5 mg/mL is sufficient to inhibit the growth of *P. aeruginosa* and *S. aureus*. In the negative control, the resazurin dye turned pink, indicating active bacterial growth. Conversely, in the positive control, no colour change was observed across all strains, confirming that tetracycline inhibited the bacterial growth.

These findings contradict the results reported by Sofia and Merlee (2017) who found the lowest MIC value of *A. ilicifolius* methanolic extract against *S. aureus* to be 0.469 mg/mL, and for ethanolic extracts, 1.042 mg/mL against *E. coli* and 0.521 mg/mL against *P. aeruginosa*. Similarly, Govindasamy and Arulpriya (2013) recorded a lower MIC of 1.0 mg/mL against *P. aeruginosa* and 3.0 mg/mL against *S. aureus* with methanolic extract. In contrast, Mondal et al. (2021) reported much higher MIC values of 750 mg/mL against *E. coli* and 187.50 mg/mL against *S. aureus* for ethanolic stem bark extract. Furthermore, Mohammad et al. (2017) found extremely low MIC values for the methanolic extract, with 0.004 mg/mL against *E. coli*, and 0.002 mg/mL against *S. aureus*.

4. CONCLUSION

In conclusion, the extraction of *A. ilicifolius* fruit employing the maceration technique with methanol as the solvent yielded a crude extract with a total yield of 22.4%. Phytochemical screening revealed the presence of terpenoids, flavonoids, steroids, saponins, and tannins, while alkaloids were absent. GC-MS analysis identified several bioactive compounds, including toluene, phenol 2,4-bis (1,1-dimethylethyl), 2(3H)-benzoxazolone, benzophenone, hexanedioic acid methyl ester, n-hexadecanoic acid, 9-octadecenoic acid methyl ester (E), 9,12-octadecadienoic acid (Z,Z), cis-vaccenic acid, and 13-docosenamide (Z). The antibacterial activity of the methanolic fruit extract showed the highest inhibitory effect against *E. coli*, with an inhibition zone of 15.73 ± 0.33 mm, followed by *P. aeruginosa* (15.10 ± 0.70 mm) and *S. aureus* (13.13 ± 0.57 mm). The MIC values further supported these findings, with *E. coli* being inhibited at 3.125 mg/mL, while both *P. aeruginosa* and *S. aureus* showed inhibition at 12.5 mg/mL. To expand on these findings, further efforts should focus on optimizing extraction methods, exploring alternative solvents, and improving both the yield and bioactivity of the extract. Additionally, comprehensive cytotoxicity assessments using human cell lines, along with *in vivo* studies in animal models, are essential to determine the extract's safety profile and therapeutic efficacy. Future research also should investigate the extract's efficacy against multidrug-resistant bacterial strains and consider fractionation to isolate and identify specific active compounds.

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