

Research

Antibiofilm Efficacy and Mode of Action of *Etilingera elatior* Extracts Against *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus represents a major bacterial human pathogen that causes a wide variety of clinical manifestations. Various medicinal plants have been used to control its infection, however, the effect of *Etilingera elatior* on *S. aureus* biofilm is still uncertain. Thus, the objective of this study was to evaluate the antibacterial and antibiofilm efficacy of *E. elatior* extracts against *S. aureus*. Phytochemical screening was carried out to determine the presence of phenols, tannins, saponins, and alkaloids in different extracts (acetone, methanol, ethanol, and aqueous) of *E. elatior*. Antibacterial activities were determined by disk diffusion assay, minimum inhibitory concentration assay (MIC), and minimum bactericidal concentration assay (MBC) while antibiofilm activities were determined by crystal violet assay and Fourier-transform infrared (FTIR) spectroscopy. All the extracts were found to contain phenols, tannins, saponins, and alkaloids. Only acetone extract showed a high amount of saponins. Among all the extracts, acetone extract showed the widest inhibition zone (21.23±0.2 mm), lowest MIC (20 mg/mL), and lowest MBC (50 mg/mL) values. The acetone extract also showed the highest antibiofilm activities at all biofilm stages (6 hr: 12%-31%; 12 hr: 20%-36%; 18 hr: 27%-32%; 24 hr: 5%-46%). Further analysis with FTIR spectroscopy revealed spectral changes associated with proteins (1700–1400 cm⁻¹), phospholipids, and polysaccharides (1300–700 cm⁻¹) in *S. aureus* biofilm following the treatment with 200 mg/mL of *E. elatior* extracts. In conclusion, *E. elatior* is a potential source of antibacterial and antibiofilm agents to control *S. aureus* infections. Changes in the composition of proteins, phospholipids, and polysaccharides may mediate the biofilm inhibition by *E. elatior* extracts. The acetone extract of *E. elatior* may be useful for various applications such as antimicrobial topical cream and wound dressing.

Key words: Antibiofilm, biofilm, *Etilingera elatior*, *Staphylococcus aureus*

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INTRODUCTION

Nosocomial infections are hospital-acquired infections commonly caused by staphylococci, pseudomonads, and *Escherichia coli*. In the United States, these infections cost more than \$4 billion and contribute to more than 88,000 deaths. The major factors causing nosocomial infections include indiscriminate antimicrobial use in hospitals, antimicrobial resistance, and improper prevention procedures. In general, nosocomial infections can be categorized into central line-associated bloodstream infections (CLABSI), catheter-associated urinary tract infections (CAUTI), surgical site infections (SSI), and ventilator-associated pneumonia (VAP) (Sikora & Zahra, 2023). One of the most common Gram-positive pathogens causing nosocomial infections is *Staphylococcus aureus*.

Staphylococcus aureus is a Gram-positive bacterium that tends to form a grape-like cluster. Typically, this bacterium can grow aerobically or anaerobically (facultative) and at temperatures between 18 °C and 40 °C. It causes a range of clinical manifestations including food poisoning, endocarditis, septic arthritis, and cellulitis. *S. aureus* is known

to stimulate Toll-like receptor-2 (TLR-2) signaling and Lipoteichoic acid (LTA) which assist the bacterial recognition by the host cells (Bhattarai *et al.*, 2018). The stimulation of LTA results in the activation of proinflammatory nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) cascade in the host cells (Bougarn *et al.*, 2010) leading to the production of pro-inflammatory cytokines, interferons, and chemokines which are required for the initiation of the immune response. Recent work has revealed a high number of potential antigenic proteins in *S. aureus* which are useful for the development of drugs and vaccines (Zulkipli *et al.*, 2022). *S. aureus* is also one of the common biofilm producers. In general, bacterial biofilm refers to a colony of microorganisms that attach themselves to surfaces, differentially express essential proteins (Othman & Yahya 2019; Isa *et al.*, 2022), and produce a protective matrix made up of an extracellular matrix containing various organic molecules (Yahya *et al.*, 2018; Yaacob *et al.*, 2021; Kamaruzzaman *et al.*, 2022). The use of natural plant resources for possible therapeutic interventions against biofilm infections has offered a promising advantage (Johari *et al.*, 2020; Zawawi *et al.*, 2020; Hamdan *et al.*, 2023).

Etlintera is a plant family of Zingiberaceae that is usually found in tropical regions and lowland forests. *Etlintera elatior* is the most known species of the Etlintera genus recognized with different local names such as "Torch Ginger" and "Bunga Kantan". The torch ginger is a perennial plant that can reach a height of up to 15 feet (4.5 meters). Its foliage consists of large, glossy, lance-shaped leaves that can grow up to 3 feet (1 meter) in length. The leaves emerge from the base of the plant and form an attractive, lush green crown. In Malaysia, particularly in Borneo, *E. elatior* has widely been used as a traditional remedy for stomachache, food poisoning, and gastric problems (Guzzo *et al.*, 2020). The inhibitory activities of *E. elatior* extracts against Gram-positive bacteria including *S. aureus* have previously been reported (Chan *et al.*, 2011; Anzian *et al.*, 2020). However, the efficacy of *E. elatior* against the biofilm formation by *S. aureus* remains uncertain. Therefore, the objective of this study was to determine the antibacterial and antibiofilm activities of *E. elatior* extracts against *S. aureus*.

MATERIALS AND METHODS

Preparation of test microorganism

Staphylococcus aureus ATCC 25923 was obtained from the Microbiology Laboratory, Faculty of Applied Sciences, UiTM Shah Alam, Selangor, Malaysia, and was grown in Muller Hinton Broth (MHB). For antibacterial and antibiofilm assays, the concentration of bacterial suspension was adjusted to approximately 10^6 CFU/mL by diluting bacterial suspension 1:100 with fresh sterile broth.

Preparation of plant extracts

Inflorescences of the torch ginger plant were collected from the local farm at Semenyih, Selangor, Malaysia (Coordinate: 2.961307, 101.836019). The inflorescences were fresh, unopened, and had equal maturity with no apparent physical defects. The inflorescences were surface cleaned cautiously to remove all the adhering debris. Further, the inflorescence samples were freeze-dried for 48 hr. Then, the samples were ground into a fine powder using a commercial kitchen blender and stored at 4 °C in amber-colored glass bottles, covered with aluminum foil to prevent direct exposure to light until further analysis. For solvent extraction, one gram of freeze-dried samples was mixed with 40 mL of desired solvent (ethanol, methanol, acetone, or aqueous), stirred using a magnetic stirrer at 1200 r.p.m., and heated on a hotplate for 1 hr. Following that, the extracts were filtered using Whatman No. 1 filter paper and placed in a reagent bottle covered with aluminum foil (Wijekoon *et al.*, 2013).

Phytochemical screening

The phytochemical screening of the extracts was conducted using standard procedures as previously described (Rao *et al.*, 2012). The qualitative tests performed herein were phenols, tannins, saponins, and alkaloids assays.

Disk diffusion assay

Six different extract concentrations (200 mg/mL, 100 mg/mL, 50 mg/mL, 20 mg/mL, 10 mg/mL & 5 mg/mL) were applied in the paper disc (6 mm diameter) and air-dried for 15 min. Vancomycin (1 mg/mL) served as positive control while 0.5% DMSO served as negative control. A sterile cotton swab was dipped into the bacterial inoculum and the excess was removed. Then, the surface area of the plate was swabbed completely by rotating the plate (Kurumisawa *et al.*, 2021). The plates were air-dried for 5 min. Then, using sterilized forceps, each disc was placed on the plate. Each disc was lightly touched with forceps to ensure that it was in good contact to avoid misplacement. The plates were incubated for

24 hr at 37 °C and inhibition zones were measured.

Determination of minimum inhibitory concentration (MIC)

One hundred μL of bacterial inoculum was added into each well of a sterile 96-well microplate. Then, 100 μL of plant extracts at different concentrations were added into the wells. Vancomycin (1 mg/mL) and 0.5% DMSO were used as positive and negative controls, respectively. Further, the microplate was wrapped loosely with parafilm and incubated at 37 °C for 24 hr. Following incubation, turbidity of microbial cultures was observed and the wells showing no visible growth were referred to as the MIC value (Yahya *et al.*, 2014).

Determination of minimum bactericidal concentration (MBC)

After the MIC determination, 50 μL from all the wells showing no visible bacterial growth was placed on brain heart infusion (BHI) agar plates and incubated for 24 hr at 37 °C. MBC was determined when 99.9% of the bacterial population was killed at the lowest concentration of plant extracts.

Biofilm formation assay

One hundred μL each of bacterial inoculum and plant extracts were added into individual flat-bottomed 96-well microtiter plates and incubated further at 37 °C for 24 hr. Vancomycin (1 mg/mL) served as a positive control while 0.5% DMSO served as a negative control. The biofilm in a 96-well microplate was quantified using a crystal violet assay. The same experimental design was repeated for the biofilm formation assay within 24 hr, 36 hr, and 48 hr. For FTIR spectroscopy, the biofilm was grown on glass coverslips in 6-well microplates at 37 °C for 24 hr whereby two mL of bacterial inoculum was mixed with two mL of plant extracts at different concentrations.

Crystal violet assay

Briefly, the 96-well microtiter plates were washed twice with sterile distilled water, air dried for 45 min. The wells were stained with 200 μL of 1% crystal violet and were washed thrice with sterile distilled water to remove unabsorbed stains and incubated at room temperature for 15 min. At this point, biofilms were observed as purple rings at the side of the wells. The semi-quantitative assessment of biofilm formation was done by adding 125 μL of absolute ethanol and the absorbance was measured at 590 nm using a microplate reader. The mean absorbance value of the samples was used to calculate the percentage of biofilm inhibition using the following equation (Famuyide *et al.*, 2019).

$$\text{Percentage of inhibition (\%)} = \frac{\text{OD Value of Negative Control} - \text{OD Value Sample}}{\text{OD Value Negative Control}} \times 100$$

Fourier transform infrared (FTIR) spectroscopy

Biofilms grown on the glass coverslips were harvested, rinsed with distilled water twice, and dried for 40 min at 40 °C. FTIR spectra were acquired using an FTIR Tensor 27 spectrometer coupled to the microplate adapter HTS-XT (Bruker Optics GmbH). The infrared spectra were recorded in transmission mode in the spectral range between 2000 and 600 cm^{-1} .

RESULTS AND DISCUSSION

Phytochemicals in *E. elatior* extracts

Phytochemical screening was carried out to determine the presence of phenols, tannins, saponins, and alkaloids in *E. elatior* extracts. All *E. elatior* extracts were found to contain phenols, tannins, saponins, and alkaloids (Table 1). Compared to other extracts, acetone and methanol extracts showed higher amounts of phenols and tannins. Only acetone extract showed a higher amount of saponins. The presence of secondary metabolites such as flavonoids, phenols, saponins, tannins, and terpenoids in the flower extract of *E. elatior* has previously been reported (Lachumy *et al.*, 2010; Saudah *et al.*, 2022).

Antibacterial properties of *E. elatior* plant extracts

The inhibition zones produced by the extracts are shown in Table 2 while the MIC and MBC values are shown in Table 3. Acetone extract showed the highest antibacterial activity against *S. aureus* due to its widest inhibition zone (21.23 ± 0.2 mm) at the highest concentration (200 mg/mL), whereas MIC and MBC values for acetone extract were 20 mg/mL and 50 mg/mL, respectively. Methanol extract also

showed inhibition zones at 200 mg/mL (12.07 ± 0.2 mm) and 100 mg/mL (8.40 ± 0.49 mm). MIC and MBC for methanol extract were found to be 50 mg/mL and 100 mg/mL, respectively. Furthermore, ethanol and aqueous extracts showed inhibition zone only at the highest concentration (200 mg/mL) with 9.01 ± 0.12 mm and 10.03 ± 0.09 mm. Both extracts were observed to have MIC at 100 mg/mL and MBC at 200 mg/mL.

Table 1. Phytochemical analysis of *Etilingera elatior* extracts

Compound	Extracts			
	Acetone	Methanol	Ethanol	Aqueous
Phenols	++	++	+	+
Tannins	++	++	+	+
Saponins	++	+	+	+
Alkaloids	+	+	+	+

++ : high amount

+ : low amount

Table 2. Inhibition zones produced by *Etilingera elatior* extracts against *Staphylococcus aureus*

Bacteria	Concentrations	Zone of inhibition (mm)				Positive Control	Negative Control
		Acetone	Methanol	Ethanol	Aqueous		
<i>S. aureus</i>	5 mg/mL	0	0	0	0		
	10 mg/mL	0	0	0	0		
	20 mg/mL	6.83 ± 0.43	0	0	0	20.01 ± 0.21	0
	50 mg/mL	14.10 ± 0.41	0	0	0		
	100 mg/mL	18.40 ± 0.25	8.40 ± 0.49	0	0		
	200 mg/mL	21.23 ± 0.2	12.07 ± 0.2	9.01 ± 0.12	10.03 ± 0.09		

The experiment was performed in triplicates

Positive control: Vancomycin

Negative control: 0.5% DMSO

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Etilingera elatior* extracts against *Staphylococcus aureus*

Extracts	<i>Staphylococcus aureus</i>	
	MIC (mg/mL)	MBC (mg/mL)
Acetone	20	50
Methanol	50	100
Ethanol	100	200
Aqueous	100	200

In this study, the acetone extract of *E. elatior* showed strong antibacterial activity against *S. aureus* which may be due to a high number of phenols, tannins, and saponins. The presence of phenolic hydroxyl groups with a high affinity for protein binding may inhibit microbial enzymes while concurrently increasing the affinity for cytoplasmic membranes, promoting antibacterial activity (Miklasińska-Majdanik *et al.*, 2018). Since acetone has a lower polarity than aqueous and ethanol, it contains more terpenoids and tannins and has a more potent antibacterial action. This result is in line with Karupiah and Rajaram (2012) reporting that extracts from Zingiberaceae species are effective on Gram-positive bacteria, especially *S. aureus*.

Antibiofilm activities of *Etilingera elatior* extracts

The result of the antibiofilm activity of *E. elatior* extracts is shown in Figure 1. In general, *E. elatior* extracts showed differential antibiofilm activity towards *S. aureus*. At 6 hr, all extracts at 200 mg/mL showed acceptable antibiofilm activity (>20%) except for aqueous extract (14.21%). Famuyide *et al.*, (2019) reported that interference with forces (such as sedimentation, electrostatic contact forces, and Lifshitz–Van der Waals) that favor the deposition and adherence of bacteria to surfaces, may explain the plant extracts' capacity to disrupt the initial stage of biofilm formation. Plant extracts may also

reduce the nutrient availability at the early biofilm stage hence resulting in a higher percentage of inhibition (Yu et al., 2018).

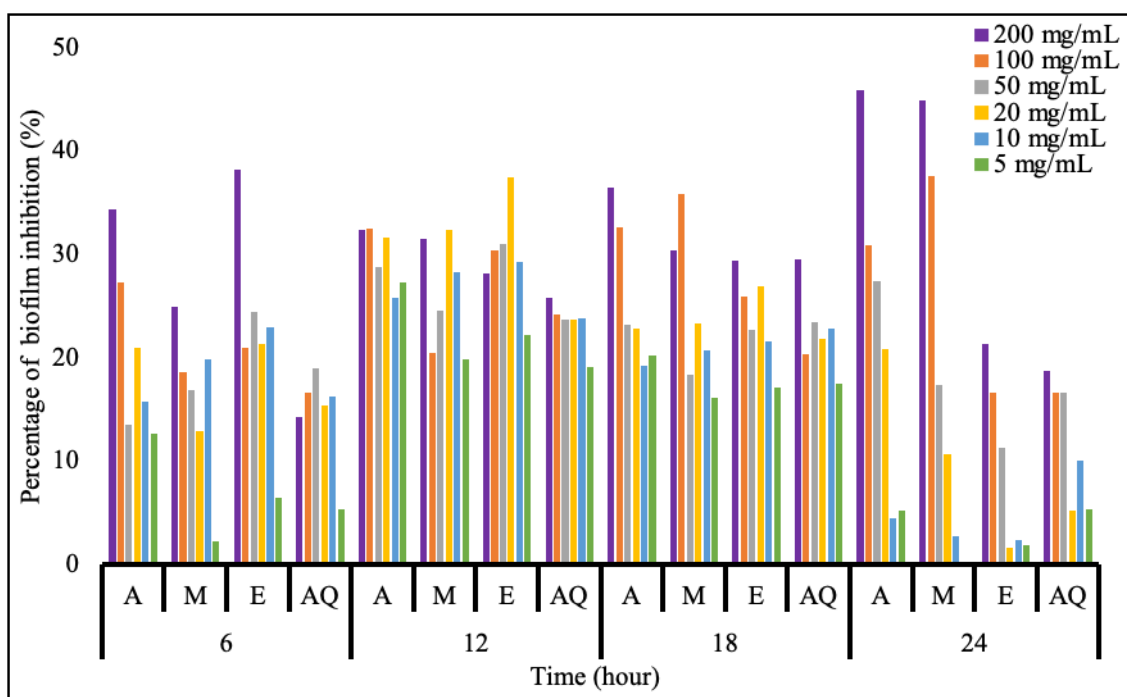


Fig. 1. Percentage of biofilm inhibition by *E. elatior* extracts at different time intervals. A: acetone extract; M: methanol extract; E: ethanol extract; AQ: aqueous.

Furthermore, the antibiofilm activities of all *E. elatior* extracts at 12 hr and 18 hr were found to be higher than those at 6 hr. At 24 hr, acetone extract at the highest concentration (200 mg/mL) showed the highest antibiofilm activity (45.8%) against *S. aureus* while the second highest was shown by the methanol extract (44.9%). The ethanol (21.3%) and aqueous (18.7%) extracts showed lower antibiofilm activity at 200 mg/mL. The result of the present study is in line with Sheikhi et al. (2019) demonstrating that acetone and methanol extracts of *Urginea maritima* exhibited greater antibiofilm effect on *S. aureus*. They emphasized that by increasing the concentration of acetone and methanol extracts, the biofilm inhibition activity would also increase.

The result obtained herein is also consistent with Bazargani et al. (2016) where ethanol plant extracts demonstrated limited or low activity potential against *S. aureus* biofilm formation and growth. Mature *S. aureus* biofilm (24 hr) was found to be more resistant to *E. elatior* extracts. Inhibiting the later developmental stages of biofilms is more difficult than inhibiting the cell attachment (Bazargani et al., 2016). Furthermore, according to the result, the antibiofilm potential of extracts may be related to the presence of tannins which disrupt the cell wall formation and cytoplasm (Dong et al., 2017).

FTIR spectral changes in *S. aureus* biofilm following the treatment with *E. elatior* extracts

In the present study, 200 mg/mL of *E. elatior* extracts were further tested against 24 hr *S. aureus* biofilm as they showed the highest biofilm inhibition in most assays. The 24 hr biofilm was used for the FTIR spectroscopy because it represents the mature developmental stage which is suitable for the investigation of the effect of *E. elatior* extracts on the biofilm structure and biochemical composition. The treatment with 200 mg/mL of *E. elatior* extracts caused changes in the spectral regions associated with proteins (1700–1400 cm^{-1}), and polysaccharides (1300–700 cm^{-1}) in *S. aureus* biofilm (Figure 2, Table 4). A considerable change in protein and fatty acid regions was also observed in the 1450–1200 cm^{-1} region.

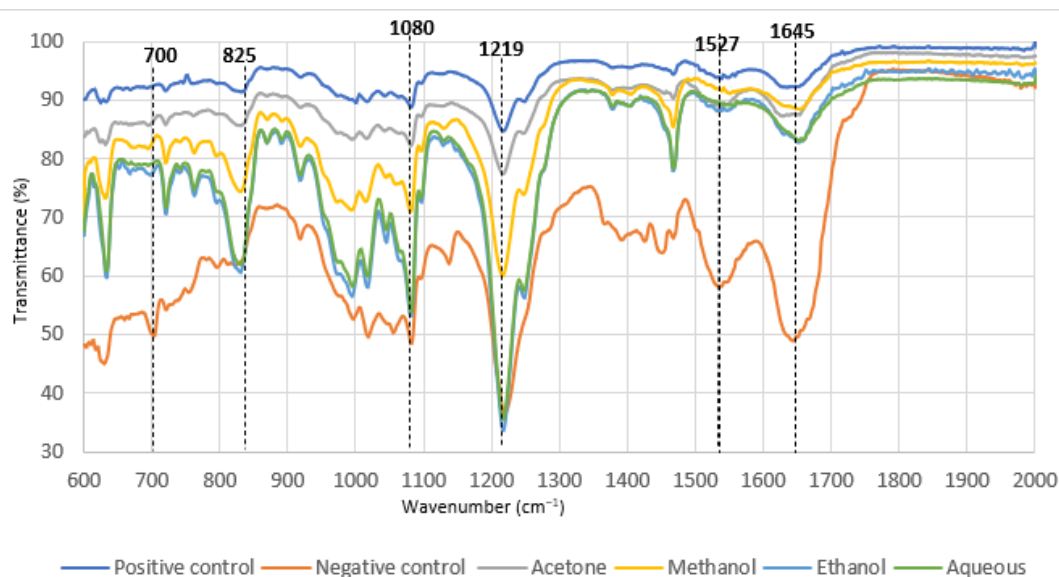


Fig. 2. FTIR spectra of *S. aureus* biofilm treated with 200 mg/mL of *E. elatior* extracts.

Table 4. Assignment of FTIR spectral peaks (Davis & Mauer, 2010)

Wavelength (cm ⁻¹)	IR Assignment	Classification
1645	Amide I	Protein
1527	Amide II	Protein
1485	C=C Stretching	Protein
1219, 1080	P=O symmetric stretching	Nucleic acid, phospholipid
700,825	C-O-C, C-O-H Stretching	Polysaccharide

The breakdown of the bacterial membrane phospholipid may be shown by a decrease in the strength of the peak 1219 cm⁻¹ (Davis & Mauer, 2010). The disappearance of spectral peaks at 1527 cm⁻¹ (amide II) may indicate altered protein structures upon treatment with *E. elatior* extracts (Derbel *et al.*, 2023). There was a spectral peak shifting at 700 cm⁻¹ indicating the stretching vibration of C–O–C and C–O–H from glycosidic bonds. In general, the FTIR spectral peak shifting in *S. aureus* biofilm following treatment with *E. elatior* extracts corroborates previous FTIR spectroscopic studies of biofilms (Yahya *et al.*, 2014; Kamaruzzaman *et al.*, 2022; Johari *et al.*, 2023). It has been established that FTIR spectroscopy provides real-time data and is sensitive to the structure, thus, any changes in the structure of the molecules are reflected in their spectroscopic fingerprint, peak position, and peak intensity. FTIR spectroscopy can elucidate the interaction of antibiofilm agents with bacterial cell components such as lipids, proteins, and nucleic acids. By monitoring changes in the infrared spectra of these biomolecules in the presence of antibiofilm agents, researchers can identify the specific targets and mode of action (Yahya *et al.*, 2018). The present study may provide the first evidence of FTIR spectroscopy-based identification of the mode of action of *E. elatior* extracts against *S. aureus* biofilm.

CONCLUSION

In this study, the phytochemicals, antibacterial, and antibiofilm activities of *Etilingera elatior* extracts were determined. Acetone extract displayed the best antibacterial and antibiofilm activities against *S. aureus*. Meanwhile, FTIR spectroscopy revealed the possible mode of action of *E. elatior* extracts against *S. aureus* biofilm. Therefore, *E. elatior* represents a potential source of antibiofilm agents for the control of *S. aureus* infections.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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