

# *In vitro* Antibacterial Properties of *Etlingera elatior* Flower Extracts against Acne-Inducing Bacteria: *Propionibacterium acnes* and *Staphylococcus aureus*

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## ABSTRACT

**Introduction:** Acne is a common skin disorder and is generally caused by *Propionibacterium acnes* and *Staphylococcus aureus*. *Etlingera elatior* flower extract is known to have antibacterial properties however, the properties against these bacteria have not been extensively reported. Thus, this study aimed to investigate the antibacterial properties of the flower extract against these bacteria. **Materials and Methods:** The flower extract was subjected to sequential extraction using three different solvent polarities; n-Hexane, dichloromethane (DCM) and ethanol. The antibacterial properties were evaluated using the disc diffusion and broth dilution assays techniques by determining the inhibition zone diameter, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Total phenolic acids (TPC) and flavonoids contents (TFC) were estimated using Folin-Ciocalteu and aluminium-chloride colorimetric assay respectively. Morphological changes of the treated bacteria were studied using scanning electron microscope (SEM). **Results:** DCM flower extract showed the highest antibacterial properties against *P. acnes*; at 25 mg/ml it had the widest inhibition zone ( $11.39 \pm 0.45$  mm) and the lowest MIC (6.25 mg/mL) and MBC (12.5 mg/mL). The ethanolic flower extract had the highest antibacterial properties against *S. aureus*; at 50 mg/ml- the inhibition zone was  $6.21 \pm 0.25$  mm and the MIC and MBC were both 12.5 mg/mL. Ethanolic extracts had the highest TPC ( $966.304 \pm 114.08$  mg GAE/g) and TFC ( $796.33 \pm 65.78$  mg QE/g). There was significant morphological changes of the treated bacteria observed under SEM. **Conclusion:** *E. elatior* flower extracts exhibited antibacterial properties against acne-inducing bacteria.

**KEYWORDS:** Antibacterial properties, *Etlingera elatior* flower, Acne-inducing bacteria.

## INTRODUCTION

Acne is one of the most common skin disorder and primarily appears in areas rich in pilosebaceous units. It represents a significant challenge to dermatologist due to its range of clinical presentation, complexity and prevalence. The various clinical appearances of acne include inflammatory lesions, non-inflammatory lesions, seborrhoea, post inflammatory hyperpigmentation, and variable degrees of disfiguring scars.<sup>1</sup> Acne is a multifactorial disease and one of the acne

pathogenesis is colonization of bacteria. According to Lee *et al.*, *Propionibacterium acnes* and *Staphylococcus aureus* are the major pathogens and most abundant bacteria on the surface of acne-affected skin.<sup>2</sup>

Acne rarely causes death but the psychological stress due to scars and pustule lesions especially on the face may lead to high emotional impact on a patient's life.<sup>3</sup> The long term use of antibiotics such as erythromycin and clindamycin against acne may lead to increase the prevalence of antimicrobial resistant organisms, while other treatments for acne such as hormones and isotretinoin may cause many unwanted side effects.

*Etlingera elatior* or torch ginger is a medicinal plant under the botanical family of Zingiberaceae. It is highly prevalent in South East Asia and is a native

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herbaceous perennial plant to Malaysia. *E. elatior* flowers also known locally as 'bunga kantan' are available as an ingredient for local products such as soap, shampoo and perfume and known for its antimicrobial and antioxidant properties.<sup>4</sup> Previous study by Lachumy *et al.* reported the pharmacological properties of *E. elatior* flower extracts and its potential to be developed as a natural-product based remedy.<sup>5</sup>

Herbal and botanically derived treatment are becoming increasingly popular as an alternative treatment for acne because they are readily available over-the-counter and generally considered as safe remedies. Daud *et al.* believed that medicinal plant extracts have fewer side effects than synthetic agents do in topical acne treatment.<sup>6</sup>

The antibacterial properties of *E. elatior* flower extracts have been widely reported by previous studies. Lachumy *et al.* reported that methanol extract of *E. elatior* flower has antimicrobial properties against common human pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Bacillus thuringiensis*, *Bacillus subtilis*, *Micrococcus* spp., *Salmonella* spp, *Proteus mirabilis*, *Aspergillus niger* and *Candida albicans*.<sup>5</sup> According to Wijekoon *et al.* the extracted essential oil and the crude solvent extracts of *E. elatior* flower using distilled water, absolute ethanol, and 50% ethanol as solvents exhibited antibacterial properties against *Bacillus subtilis*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Klebsiella pneumoniae*.<sup>7</sup> Ghasemzadeh *et al.* revealed that aqueous and ethanolic extracts of *E. elatior* flower have antibacterial properties against *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Escherichia coli* and *Salmonella* Typhimurium.<sup>8</sup> All these studies however were conducted using a single solvent extraction method. To date, there are no extensive studies reported on the antibacterial properties of the *E. elatior* flower extracts using three different polarities of solvents namely n-Hexane (nonpolar), DCM (semipolar) and ethanol (polar) against acne-inducing bacteria: *P. acnes* and *S. aureus*. Thus, this study aimed to investigate the antibacterial properties of *E. elatior* flower extract using three different polarities of solvents namely n-Hexane (nonpolar), dichloromethane (DCM) (semi-polar) and ethanol (polar) against acne inducing bacteria, *S. aureus* and *P. acnes*.

## MATERIALS AND METHODS

### Preparation of *Etilingera elatior* flower

Fresh and young light pink flowers of *Etilingera elatior* were collected from Kelantan, Malaysia. The collected flowers were verified as *E. elatior* at the Faculty of Bioresources and Food Industry, UniSZA with the voucher number 000346. The flowers were washed under running tap water, dried in the oven at 50°C. The dried flowers were blended into coarse powder and later stored at room temperature.

### Preparation of *Etilingera elatior* flowers extracts

The flower in powdered form were subjected to sequential extraction starting with n-hexane (non-polar), dichloromethane (DCM) (semi-polar) and ethanol (polar).<sup>9</sup> The chemical solvents used were analytical grade chemicals from Sigma-Aldrich®. The respective extracts were collected and dried under reduced pressure at 50°C using rotary vacuum evaporator. In this study, the solvents were removed based on the setting pressure programed by Eyela vacuum controller NVC-2300 at 160, 480 and 67 hPa respectively. The final weight of each extract were taken three times until a constant weight of each extract was achieved. It was to ensure that all of the respective solvents used (n-hexane, DCM and ethanol) had completely evaporated. The crude extracts were stored at 4°C until further use. The stock solution for each *E.elatior* flower extract was prepared by dissolving 0.1 g of extract in 1 mL of 100 % dimethyl sulphate oxide (DMSO) to produce a final concentration of 100 mg/mL.

### Inoculum preparation

The two test bacteria were obtained from the American Type Culture Collection (ATCC)®. *Propionibacterium acnes* (ATCC 6919) and *Staphylococcus aureus* (ATCC 9144) were used in this study. *P. acnes* was verified using Gram-staining method, catalase and indole tests; while Gram-staining, catalase and coagulase tests were used to verify *S. aureus*. The inoculum of each bacterium was prepared by subculturing the *P. acnes* in brain heart infusion (BHI) broth with 1% glucose for 72 hours while *S. aureus* was subcultured in Muller Hinton (MH) broth for 24 hours. All of the culture media used in this study were purchased from

Thermo Scientific™ Oxoid. The optical density (OD) of each inoculum suspensions was adjusted to reach 0.5 McFarland ( $1.5 \times 10^8$  CFU/ml).<sup>10</sup>

### Susceptibility testing

Two fold serial dilution of each extract was prepared to reach 100, 50, 25, 12.5, 6.25, 1.25, 0.75 and 0.39 mg/mL concentration by dissolving with DMSO.

### Disk diffusion assay

Disk diffusion assay was performed according to Aziman *et al.*<sup>11</sup> Sterile paper disks (6 mm in diameter) were impregnated with 100, 50, 25 and 12.5 mg/mL concentration of each extract. The impregnated, negative and positive control disks were then placed onto the BHI and MH agar. The negative control disk was prepared by impregnating 100 % DMSO. Clindamycin (2 µg/disk) and oxacillin (1 µg/disk) were used as positive control disks for *P. acnes* and *S. aureus*, respectively. The plates were then incubated anaerobically for 72 hours at 37°C for *P. acnes* while aerobically for 24 hours at 37°C for *S. aureus*. All disk diffusion tests were performed in triplicate and the antibacterial activity was expressed as the mean of inhibition zone diameters.

### Broth dilution assay

#### *Determination of Minimum Inhibitory Concentration (MIC)*

The MIC was determined by using broth microdilution assay.<sup>12</sup> Two fold serial dilution of each extract was prepared to reach 100, 50, 25, 12.5, 6.25, 1.25, 0.75 and 0.39 mg/mL concentration in a 96-well enzyme-linked immunosorbent assay (ELISA) plate. Fifty (50) µL of inoculum suspension of each bacteria was added to each well. The 96-well ELISA plates were incubated for 72 hours at 37°C anaerobically for *P. acnes* and at 37°C for 24 hours for *S. aureus*. After incubation, 10 µL of resazurin solution (R&D System® AR002) was added to each well and was further incubated for 4 hours for the observation of colour change. The living cells will reduce the blue dye of resazurin to pink while the dead cells will maintain the blue colour.<sup>13</sup> MIC value of the extract was taken as the lowest concentration of the

extract that managed to maintain the blue colour of resazurin.

#### *Determination of Minimum Bactericidal Concentration (MBC)*

The MBC was determined according to Witkowska *et al.*<sup>14</sup> Five (5) µl of the suspension from the MIC wells was subcultured on BHI agar and MH agar plates for *P. acnes* and *S. aureus* respectively. The plates were incubated anaerobically at 37°C for 72 hours for *P. acnes* and aerobically at 37 °C for 24 hours for *S. aureus*. The MBC was defined as the lowest concentration of the extract, which killed 99% of the bacteria.<sup>15</sup>

#### *Estimation of Total Phenolic Acids Compound (TPC)*

TPC of the extracts was estimated using Folin-Ciocalteu method.<sup>16</sup> All the chemicals and reagents used were analytical grade chemicals. One (1) ml of the individual extract was diluted in 10 ml distilled water. The solution then was mixed with 1.5 ml of Folin-Ciocalteu's phenol reagent and was allowed to stand at room temperature for 5 minutes. Four (4) ml of 20% sodium carbonate solution was added to the mixture and the volume was adjusted by adding distilled water to reach 25 ml. The reaction mixtures were then incubated at room temperature for 30 minutes. The absorbance of the mixture at 765 nm was measured by a spectrophotometer (Thermo Genesys™ 20 Visible Spectrophotometer). The blue coloration produced is proportionate to the total amount of phenolic acids compounds originally present in the extracts.<sup>17</sup> A dose response linear regression curve was generated by using the Gallic acid standard absorbance and the results were expressed as 'mg of Gallic acid equivalent (GAE) per gram of extract'.

#### *Estimation of Total Flavonoids Compound (TFC)*

TFC was estimated by aluminium chloride method.<sup>16</sup> All the chemicals and reagents used were analytical grade chemicals. 0.5 ml of each extract was mixed with 1.5 ml 95% methanol, 0.1 ml of 10% aluminium chloride, 0.1 ml of 1 M potassium acetate and 2.8 mL of distilled water. The solution was mixed well and kept for 30 minutes at room temperature. The absorbance for each sample was measured at 415 nm by a spectrophotometer. The TPC was calculated using a standard Quercetin calibration curve. The

results were expressed as 'mg of Quercetin equivalent (QE) per gram of extract'.

#### *Morphological study under scanning electron microscope (SEM)*

The prepared inocula of *P. acnes* and *S. aureus* were treated with one mL of n-hexane, dichloromethane and ethanolic extracts of *E. elatior* flower at their respective MIC value and were incubated at 37°C anaerobically for 72 hours for *P. acnes* and aerobically for 24 hours for *S. aureus*. Untreated *P. acnes* and *S. aureus* were also prepared as control samples. After incubation, all the test and control samples were centrifuged at 1500 G for 10 minutes to obtain cell pellet. The samples were washed five times with fresh media and then fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4°C for 1 hour before rinsing three times with 0.1 M sodium cacodylate buffer, pH 7.2 for 10 minutes and fixed with 1% osmium tetroxide, pH 7.2 for 2 hours. The obtained cell pellets were rinsed three times with 0.1 M sodium cacodylate buffer (pH 7.2) and dehydrated with 25%, 50%, 75%, 90%, and 100% ethanol for 10 minutes each. All the chemicals and reagents used were analytical grade chemicals. The samples were dried using critical point dryer (CPD) before being mounted on carbon stub and sputter-coated with gold in an ion coater. The mounted samples were observed by microscopic examination using SEM (JEOL 6360 LA).

## RESULTS

### **Antibacterial properties of *Etilingera elatior* flower extracts**

The inhibition zone diameter of the extracts against *P. acnes* and *S. aureus* is shown in Table 1 and the MIC and MBC values are shown in the Table 2. All types of extracts exhibited antibacterial properties against the bacteria. DCM extract showed the highest antibacterial properties against *P. acnes*, at 25 mg/ml it has the widest inhibition zone (11.39 ± 0.45 mm) and the lowest MIC (6.25 mg/mL) and MBC (12.5 mg/mL). The ethanolic extract of the flower has the highest antibacterial properties against *S. aureus*; at 50 mg/ml the inhibition zone was 6.21 ± 0.25 mm and the MIC and MBC were both 12.5 mg/mL. The negative control (DMSO) demonstrated no inhibition zones.

### **Total phenolic acids and flavonoid contents of *Etilingera elatior* flower extracts**

The total phenolic content (TPC) and total flavonoid content (TFC) of the *E. elatior* flower extracts are shown in Table 3. TPC is expressed as Gallic acid equivalent (GAE) (the standard curve equation:  $y = 0.9614x + 0.0605$ ;  $R^2=0.9955$ ) while TFC is expressed as Quercetin equivalent (the standard curve equation:  $y = 0.6447x - 0.0259$ ;  $R^2 = 0.9909$ ). The highest TPC value was detected in the ethanolic extract (966.304 ± 114.08 mg GAE/g), followed by DCM extract (110 ± 26.75 mg GAE/g) and n-Hexane extract (79.398 ± 38.49 mg GAE/g). For TFC, the highest value was demonstrated in the ethanolic extract (796.33 ± 65.78 mg QE/g), followed by DCM extract (444.34 ± 85.09 mg QE/g) and n-Hexane extract (107.23 ± 11.75 mg QE/g).

### **Morphological study under scanning electron microscope (SEM)**

The morphological changes of the treated *P. acnes* and *S. aureus* were viewed under SEM at 15 000 times magnification. All of the treated *P. acnes* demonstrated significant morphological changes compared to the untreated *P. acnes* that displayed smooth branched pleomorphic rod-shaped bacteria (Figures 1 A, B, C, and D). The n-Hexane extract treated *P. acnes* bacteria were found to have distorted shape with formation of multiple pores noted as shown in Figure 1B. DCM extract treated *P. acnes* demonstrated distorted cells with holes formation as shown in the Figure 1C while the ethanolic extract treated *P. acnes* cells appeared to be disfigured, shorten and flatten (Figure 1D).

Regarding *S. aureus*, all of the treated *S. aureus* also demonstrated significant morphological changes compared to the untreated *S. aureus* that appeared as cocci shaped bacteria arranged in clusters (Figures 2 A, B, C and D). The n-Hexane treated *S. aureus* demonstrated irregular, rough surface cells with pores formation as shown in the Figure 2B. The DCM extract treated *S. aureus* were observed to have the same features as n-Hexane extract treated *S. aureus* plus the presence of ruptured cells (Figure 2C) while in Figure 2D, majority of the cells of the ethanolic extract treated *S. aureus* were ruptured and appeared clumping to each other.



**Table 1** Inhibition zone diameter of *Etingera elatior* flower extracts against *Propionibacterium acnes* and *Staphylococcus aureus*

Bacteria	Concentration (mg/mL)	Zone of inhibition (mm)				
		n-Hexane extract	DCM extract	Ethanol extract	Positive control	Negative control
<i>P. acnes</i>	12.5	0	0	0		
	25	6.33±0.00	7.89±0.20	7.04±0.00	54.29±1.39	0
	50	6.57±0.11	8.88±0.42	7.40±0.16		
	100	7.55±0.50	11.39±0.45	7.61±0.25		
<i>S. aureus</i>	12.5	0	0	0		
	25	0	0	0	30.75±0.69	0
	50	0	0	6.21±0.25		
	100	0	7.71±0.07	6.43±0.43		

## DISCUSSION

MIC is defined as the least concentration of the plant extracts that can inhibit the growth of bacteria (bacteriostatic) while MBC is the lowest concentration of plant extract that can killed 99% of the bacteria (bactericidal).<sup>18</sup> In this study, all types of *E. elatior* flower extract were found to have both bacteriostatic and bactericidal effect against acne inducing bacteria namely *P. acnes* and *S. aureus*. As shown by the individual MIC and MBC values (Table 2).

**Table 2** Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Etingera elatior* flower extracts against *Propionibacterium acnes* and *Staphylococcus aureus*

Types of Extraction	<i>Propionibacterium acnes</i>		<i>Staphylococcus aureus</i>	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
n-Hexane	6.25	25	12.5	50
Dichloro-methane	1.56	6.25	6.25	25
Ethanol	12.5	12.5	12.5	12.5

Based on the results, DCM and ethanol were found to be the most effective solvents for extracting the antibacterial agents from *E. elatior* flowers against *P. acnes* and *S. aureus*. Study by Sharifi-Rad *et al.* revealed that antibacterial activities of plants were contributed by various classes of secondary metabolites, including fatty acids, sterols, alkaloids, flavonoids and phenolic acids compounds.<sup>19</sup> Less polar flavonoids for examples isoflavones and flavonols are generally extracted by semi-polar solvents while polar flavonoids such as anthocyanidins are generally extracted with polar solvents.<sup>20</sup> This evidence support the highest

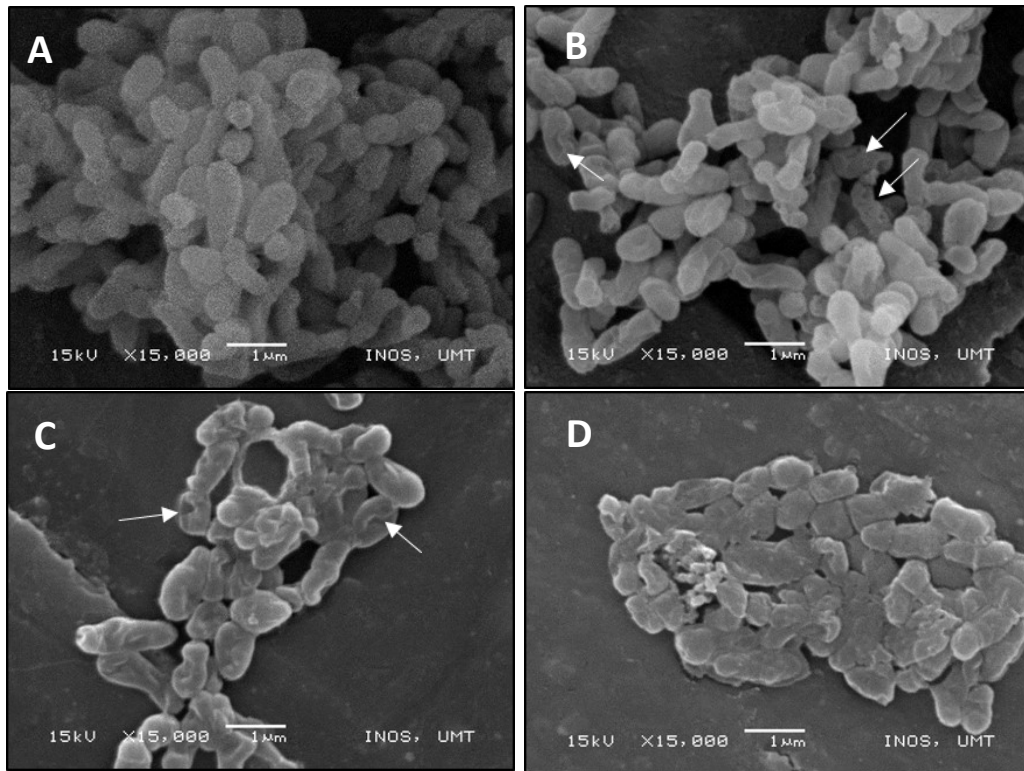
antibacterial properties demonstrated by the ethanolic and DCM extracts of *E. elatior* flower.

**Table 3** Total phenolic content (TPC) and total flavonoid content (TFC) of *Etingera elatior* flower extracts

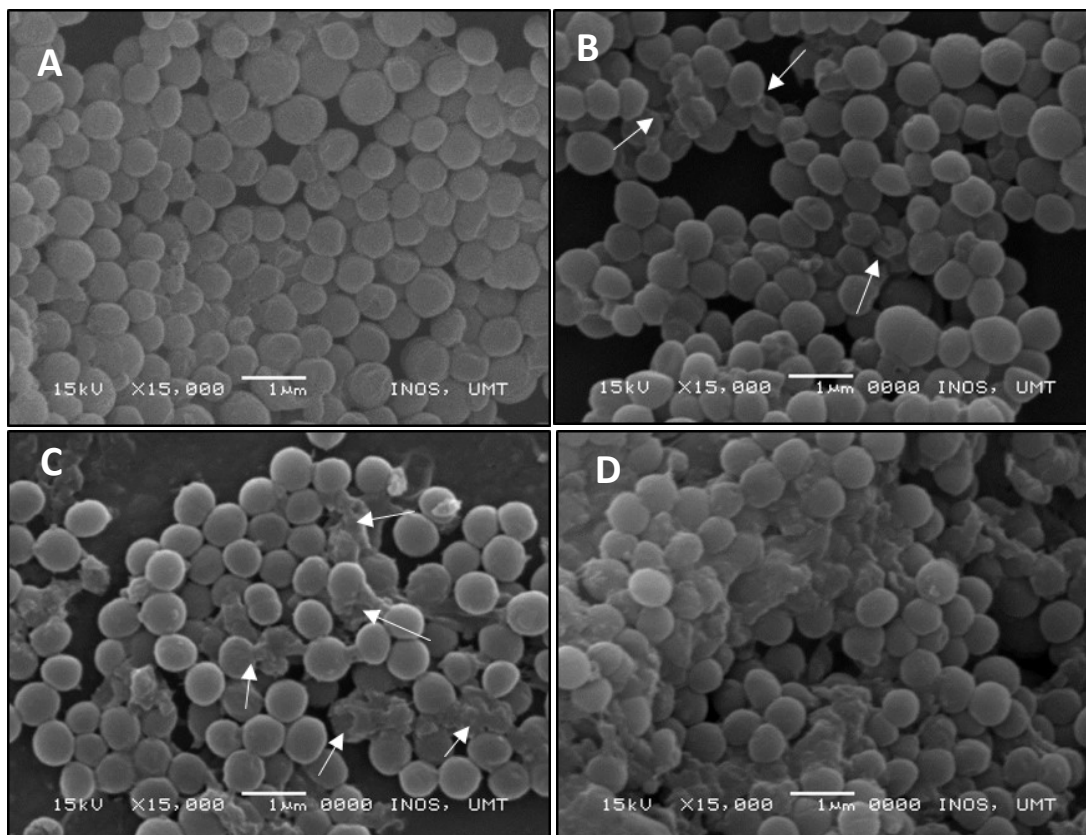
Extracts	Total phenolic acids compound (mg of GAE/g of extract)	Total flavonoids compound (mg QE/g of extract)
n-Hexane	79.398 ± 38.49	107.23 ± 11.75
Dichloro-methane	110 ± 26.75	444.34 ± 85.09
Ethanol	966.304 ± 114.08	796.33 ± 65.78

In this study, all different polarities of *E. elatior* flower extracts contained phenolic acids and flavonoids compounds which is in agreement with previous study on phytochemical screening of *E. elatior* flower extracts.<sup>21</sup> The degree of plant bioactive constituents solubility depends on the solvent used and different solvents have diverse solubility capacities for different phytochemicals.<sup>22</sup> Ethanolic extract was found to have the highest TPC and TFC compared to DCM and n-Hexane extracts. This is due to the characteristic of phenolic acids and flavonoids that are more extractable in polar solvent such as ethanol.<sup>23</sup> According to Abdul Mutalib *et al.* ethanol was found easier to alter polyphenol oxidases and cause degradation to the cell wall, thus extracting more endocellular constituents from the plant extract.<sup>24</sup>

Based on SEM analysis, the morphological changes of the bacteria are believed to be due to the binding of *E. elatior* active component to the membrane-bound enzyme and the phospholipid bilayer of the cytoplasmic membrane of the bacteria.<sup>25</sup>



**Figure 1** Scanning electron micrograph (SEM) of *Propionibacterium acnes* (A) Untreated *P. acnes* displayed smooth branched pleomorphic rod-shaped bacteria; (B) n-Hexane extract-treated *P. acnes* demonstrated distorted shape with formation of multiple pores as shown with arrows; (C) DCM extract-treated *P. acnes* demonstrated distorted cells with holes formation as shown with arrows; (D) ethanolic extract-treated *P. acnes* appeared to be disfigured, shortened and flatten.



**Figure 2:** Scanning electron micrograph (SEM) of *Staphylococcus aureus* (A) Untreated *S. aureus* appeared as cocci shaped bacteria arranged in clusters; (B) n-Hexane-treated *S. aureus* appeared irregular, rough surface with pores formation as shown with arrows; (C) DCM extract-treated *S. aureus* demonstrated irregular, rough surface, pores formation with the presence of ruptured cells as shown with arrows; (D) ethanolic extract-treated *S. aureus* were ruptured and appeared clumping to each other.

The damaged of the cytoplasmic membrane and cell wall of the bacteria led to the loss of structural integrity and the ability of the membrane to act as a permeability barrier.<sup>26</sup> The extensive loss of cell contents, the initiation of autolytic processes and the leakage of critical cell components resulted to bacterial cell death.<sup>27,28</sup>

## CONCLUSION

*Etilingera elatior* flower extracts exhibited significant antibacterial properties against acne inducing bacteria namely *Propionibacterium acnes* and *Staphylococcus aureus*. This result suggests that *E. elatior* flower extracts could potentially be used as alternative treatment agents against acne inducing bacteria.

## CONFLICT OF INTEREST

We (authors) would like to declare that there is no conflict of interests in this study.

## ACKNOWLEDGEMENTS

The authors are grateful to the authorities of Faculty of Medicine and Faculty of Bioresources and Food Industry of Universiti Sultan Zainal Abidin (UniSZA) and Institute of Oceanography and Environment (INOS) of Universiti Malaysia Terengganu (UMT) for the laboratory facilities.

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