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EVALUATION OF ANTI-METHICILLIN RESISTANT *Staphylococcus aureus* PROPERTY OF *Clausena excavata* LEAVES BY USING ATOMIC FORCE MICROSCOPY AND FLOWCYTOMETRY TECHNIQUES

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Clausena excavata is a famous folklore medicinal plant in tropical and subtropical Asian region that is being used for the treatment of different disorders. This study was conducted to investigate the antibacterial activity of leaves extract of *C. excavata* against methicillin-resistance *Staphylococcus aureus* (MRSA), using atomic force microscopy (AFM) and flowcytometry techniques. Cell viability assay was performed by using microplate AlamarBlue assay. Among different extracts, ethyl acetate (EA) extract was found to have the lowest minimum inhibitory concentration (MIC) of 375 µg/mL followed by chloroform (CH) and methanol (MOH) extracts with MIC values of 750 and 1500 µg/mL, respectively. EA and CH extracts were selected for further mechanistic study by using AFM, and propidium iodide (PI) flowcytometry techniques. AFM results of treated cells showed highly damaged cells scattered all over due to loss of the potential to form clusters of cocci. Flowcytometry results showed that there were increases in PI fluorescence due to increase in the number of dead cells. This is the first report that highlights the antimicrobial activity of *C. excavata* against MRSA by the using above mentioned techniques. Further investigations are required to identify the pure compounds responsible for anti-MRSA activity, and its mechanistic study.

Keywords: *Clausena excavata*, *Staphylococcus aureus*, atomic force microscopy, antimicrobial activity.

INTRODUCTION

Antibiotic-resistant bacterial infections have widely spread around the world. The number of cases for antibiotic resistance is constantly increasing, and mortality rate caused by infectious diseases are highly increasing in intensive care unit (ICU) (Haddadin *et al*., 2002; Al-Bahrani *et al*., 2020).

Consequently, there is an urgent need of new antibacterial agents in order to fight these multi-drug-resistant (MDR) strain infections (Al-naddawi *et al*., 2019). Hence, there is an increased interest to obtain natural and safe antibacterial agents from various natural plants, especially due to lack of adverse side-effects which are often associated with conventional antimicrobial agents, such as hypersensitivity, allergic reaction, and immunosuppression (Li *et al*., 2017; Albaayit and Maharjan, 2018; Albaayit *et al*., 2020; Albaayit *et al*., 2020; Albaayit *et al*., 2020).

Atomic force microscopy (AFM) is a recently developed scanning probe microscopy, widely used in different fields of physics, chemistry, biochemistry, microbiology for imaging the fine surface structures of various types of specimens at high-resolution (Farhan Li *et al*., 2016). Similarly, propidium iodide (PI) flowcytometry has been employed to find the percentage of dead cells, and membrane integrity after exposure to compounds (Sánchez *et al*., 2010).

Clausena excavata (Burm. F., Rutaceae) is a wild shrub widely distributed in tropical and subtropical Asian regions, and famous in folk medicine due to its high antioxidant properties (Albaayit *et al*., 2016, Albaayit *et al*., 2020). It is commonly used to treat cough, colic, rhinitis, headache, fever, and sores. Its leaves, stems, rhizomes, flowers, and roots constitute many potent compounds which were proven to have various biological properties, including immunomodulatory, antifungal, antimycobacterial, antibacterial, anti-HIV-1, anti-malaria, anti-inflammatory, wound healing, and detoxifying agent (Albaayit *et al*., 2015). Among the many coumarins isolated from *C. excavata*, nordentatin showed the best antibacterial activity against *Bordetella brochiseptica*, *Bacillus subtilis*, *Pneumococcus*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Wu *et al*., 1982). Xanthoxyletin, a coumarin isolated from the leaves extract of *C. excavata*, was active against *Bacillus megaterium*, *Escherichia coli*, *Chlorella fusca*, and *Microbotryum violaceum* (Sunthitikawinsakul *et al*., 2003; Tsassi *et al*., 2010).

Most of the previous studies reported the biological properties of the bark and stem of this plant. Thus, this study was undertaken to evaluate the leaves of *C. excavata* for its anti-MRSA activity using AFM and propidium iodide flowcytometry technique.

MATERIALS AND METHODS

The *C. excavata* leaves used in the preparation of the extract was identified by Dr Shamsul Khamis (Resident Botanist) at the Biodiversity unit, Institute of Bioscience, Universiti Putra Malaysia, voucher specimen (TI-013201- CE). Fresh leaves collection, processing, and extraction were followed as previously described by Albaayit *et al*. (2014), and four different extracts of petroleum ether (PT), chloroform (CH), ethyl acetate (EA), and methanol (MOH) were prepared, and then stored at 4 °C until further use.

***Assessment of Clausena excavata against MRSA*:** Stock solutions of 60 mg/mL of MOH, EA, CH, and PT extracts of *C. excavata* were individually prepared in pure DMSO. A broth dilution method, mentioned by CLSI guidelines was used to make concentration range (3000, 1500, 750, 375, 187.5, 93.75, 46.18 µg/mL) of extracts by diluting in Mueller Hinton Broth (MHB) media such that their total volume became 100 µL. Fully grown *S. aureus* cultures (NCTC 13277, and NCTC 13143) were 1000 times diluted in MHB media, and 100 µL of this suspension was dispensed to all wells containing 100 µL of extract and bacteria such that the final cell concentration would be in the range of (0.5-1.0)×$10^{\wedge 6}$ CFU/mL. Untreated control was wells with cells only without any treatment. All samples were in triplicates. The plate was sealed, and incubated at 37 °C for 18-20 h. The next day, all wells were visually checked to confirm the clear and turbid wells, and then 20 µL of AlamarBlue dye was added, and the plate was incubated in the dark for 2 h in a shaking incubator at 37 °C. The change in color of the dye from blue to pink indicated the reduction of dye due to reducing environment of viable cells, whereas blue color indicated non-viable cells. Absorbance were recorded at 570 and 600 nm using a spectrophotometer (Thermo Scientific), and the percent of inhibition of bacteria due to compound or extract was calculated by putting the absorbance values in the formula mentioned by Lancaster *et al*. (1996).

***Investigation of surface morphology by the atomic force microscopy (AFM)*:** The EA and CH fractions were used for the AFM study. Bacterial sample *S. aureus* (NCTC 13143) was prepared using inoculum of 2-3×10^7 CFU/mL, and treated with the EA and CH extracts at a concentration of 750µg/mL. Untreated control contained only media and bacteria. After incubation for 2 h, these eppendorf tubes were centrifuged at 5000 rpm for 5 min, and washed twice with double distilled water. Pellets formed were dispersed in a little quantity of remaining water in the tube and 10 µL of this suspension (treated and non-treated bacteria) were placed in poly-L-lysine (0.01%) pre-coated silicon wafer slides, and left for drying (Eaton *et al*., 2008). Sample scanning was carried out with AFM (Agilent Technologies-5500, AZ, USA) in tapping mode. All topographical images were analyzed using PicoView 1.2 imaging analysis software to get 2D, and 3D images of bacteria.

***Determination of cells viability by using Propidium Iodide (PI) dye*:** The cell viability of *S. aureus* (NCTC 13143) treated with EA and CH extracts was determined by using BD FACSCELESTA™ flow cytometer (Becton, Dickinson and Company) (Eaton *et al*., 2008). The bacterial suspension of 5x10^7 CFU/mL in MHB was treated with EA and CH extracts at 750 µg/mL, and incubated at 37 °C for 2h. Untreated control was bacteria only in media. The 100 °C treated cells was used as positive control. The sample was then centrifuged twice at 5000 rpm for 5 min with phosphate buffer saline (PBS), and 10 µL of PI dye (Sigma Aldrich) from 1 mg/mL stock solution was added and incubated at 37 °C in dark for 30 min to allow uptake of dye. Samples were analyzed immediately after incubation using PI-A filter (EX-535 nm, EM-617 nm) at X-axis, and side scattered count at Y-axis. Gating was done in dot plot (FSC vs SSC) to sort out only bacterial cells, and these gated cells were finally plotted in PI-A vs SSC dot plot; quadrants were assigned as Q1, Q2, Q3, and Q4. The percentage of each quadrant was noted to find the viability of cells. Q4 quadrant shows the percentage of dead cells, as the dead cells with compromised cell membrane stain PI dye, shifting the fluorescence on the right side. For each sample, 10,000 cells were counted in triplicates. The BD CellQuestpro software was used for data evaluation.

RESULTS

Anti-MRSA Activity of C. excavata: The decrease in Alamar Blue dye reduction suggested that the MOH, EA, and CH extracts have antibacterial properties. The results of the minimum inhibitory concentration (MIC) of different extracts on different *S. aureus* strains (NCTC 13277, and NCTC 13143) are shown in Table 1. Among the different extracts, EA extract was found to have the lowest concentration of 375µg/mL, whereas CH and MOH extracts had MIC of 750 µg/mL. PT extract was found to be inactive on both strains. For further mechanistic studies, EA and CH extracts were evaluated for their antibacterial activity by treating *S. aureus* (NCTC 13143) at 750 µg/mL concentrations. MOH extract was active, but it was difficult to separate bacteria from its turbid suspension; therefore, it was not evaluated for further activity. AFM and flowcytometry techniques were used for further study. Ofloxacin, a standard drug was found to be active at 50µg/mL.

Table 1. Minimum inhibitory concentration (MIC) (µg/mL) of different extracts against *S. aureus* (NCTC 13277 and NCTC 13143).

	***S. aureus* (NCTC 13277)**	***S. aureus* (NCTC 13143)**
Chloroform (CH) extract	750	750
Ethyl acetate (EA) extract	375	375
Methanol (MOH) extract	1500	1500
Petroleum ether (PT) extract	Not active	Not active

Ofloxacin	50	50

***Effect of C. excavata leaves extract on the surface morphology of MRSA*:** The morphological damages on MRSA (NCTC 13143) due to antibacterial effect of both EA and CH extracts were visualized by AFM technique (Figure 1). The images of the untreated *S. aureus* cells showed clusters of compact cocci with typical rounded shape, and smooth surfaces with a mean diameter of 1 µm (Figure 1a). Structural integrity of these untreated cells was well preserved with no detectable pore, or rupture in the cell surfaces. However, distinct morphological changes were observed in the bacterial cells treated with EA and CH extracts (Figure 1b, and 1c, respectively). These treated bacteria were irregular and had very rough surface, with individual cocci showing a tendency to lose cluster formation. Cell surface roughness due to irregular lines of rupture showed the alteration in the structural integrity. These treated cells showed fragments of cells scattered due to the spillage of cytoplasmic content, thus confirming the potent anti-MRSA activity of these extracts.

***Determination of cells viability by using PI dye*:** PI dye was used for flowcytometry analysis of cell viability of *S. aureus* after treatment with EA and CH extracts at 750µg/mL for 2 h (Figure 2). Gating was done in FSC vs SSC dot plot, and these gated cells were then plotted in PI-A vs SSC dot plot.

As untreated control cells (Figure 2a) were live with intact cell membranes, they did not stain PI, because of which, Q3 quadrant showed the highest percentage of (94.8%) and dead cells (Q4 = 5.1%), whereas, cells treated at 100 °C for 30 min

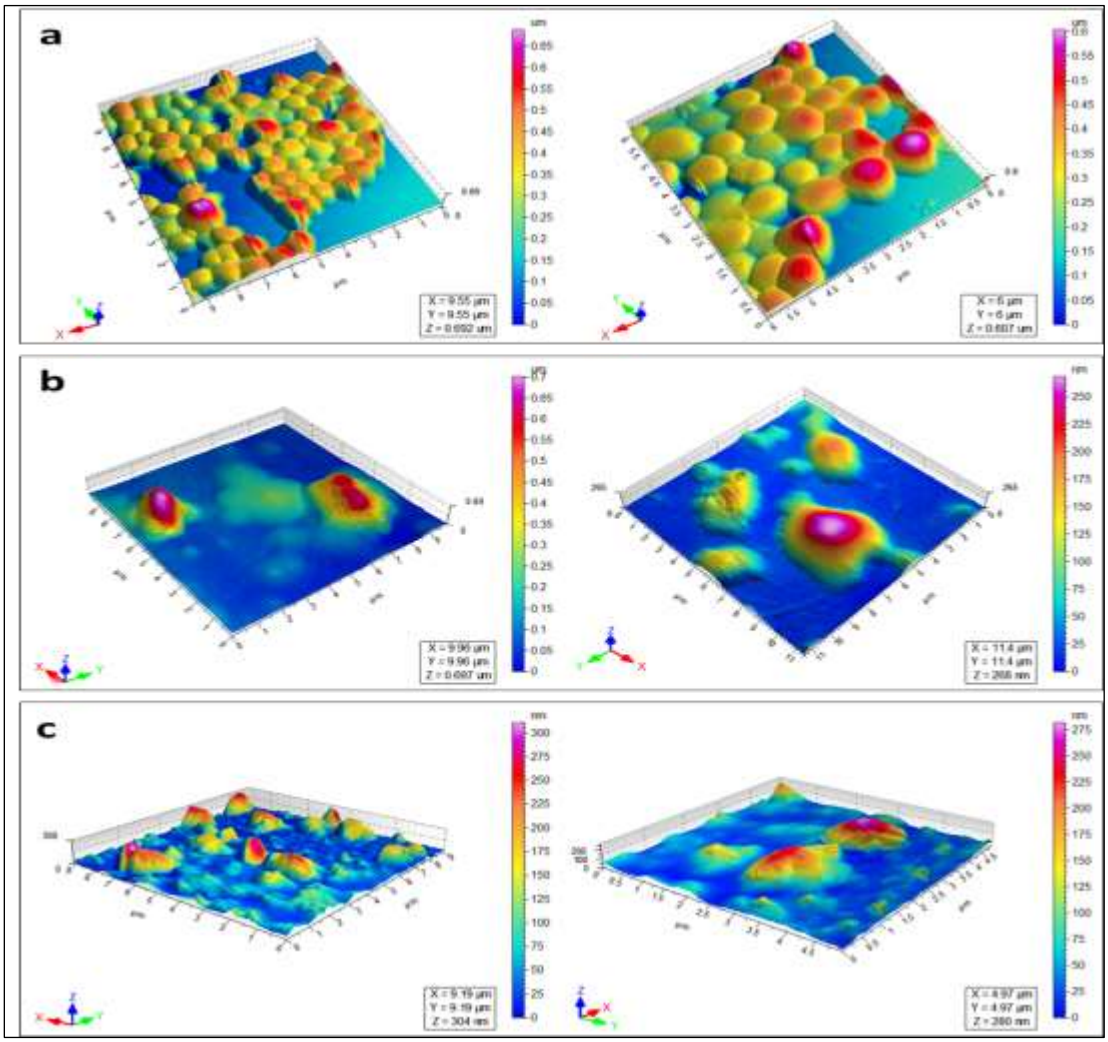

Figure 1. Atomic force microscopy images of *S. aureus* (NCTC 13143), (a) untreated control, (b) ethyl acetate (EA) treated cells at 750µg/ml, and (c) chloroform (CH) treated cells at 750µg/mL

(Fig. 2b) showed very high percentage of dead cells (Q4 = 90.2%) with a high significant difference ($p< 0.001$). EA (Figure 2c), and CH (Fig. 2d) extract treated cells also showed PI fluorescence (Q4= 41% and 30.6%, respectively), which had significant difference ($p< 0.05$) as compared to untreated cells. This result showed that EA extract was more potent than CH extract, indicating the potent antibacterial compounds present in the EA extract which needed to be isolated and explored.

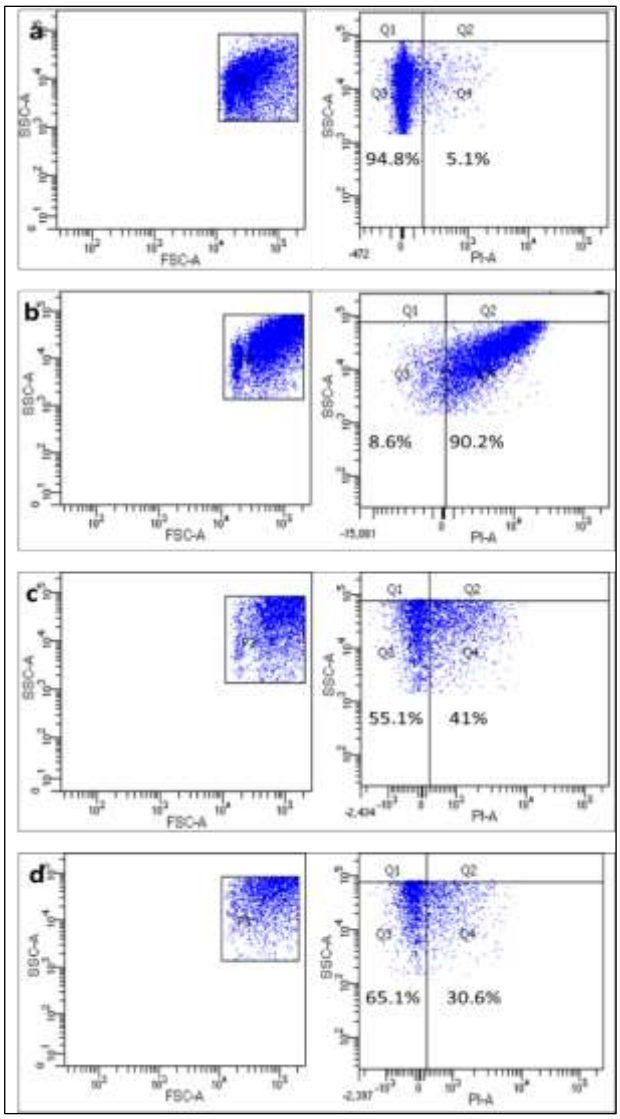

Figure 2. Propidium Iodide (PI) dye flowcytometry of *S. aureus* (NCTC 13143) cells. (a) Untreated control cells, (b) 100 ˚C treated cells, (c) EA extract (750µg/ml) treated cells, and (d) CH extract (750µg/ml) treated cells.

DISCUSSION

The rapid escalation of multidrug resistant strains of clinically relevant bacterial pathogens has posed a great challenge to the scientists. To cope with these challenges, many researchers focused their research on the ethno pharmacology, especially on natural products from plants with potent antimicrobial properties (Albaayit and Ozaslan 2019; Albaayit *et al*., 2019). *C. excavata* leaves have been widely used in folklore medicine, but only a few pharmacological studies showed its therapeutic efficacy and mechanism of action (Albaayit *et al*., 2016; Albaayit *et al*., 2020). As a follow-up to those studies, the present study examined the antibacterial properties of *C. excavata* leaves extracts by using AFM and PI dye flowcytometry.

In the extraction process, polarity of solvents are gradually increased such that compounds will separate out with respect to difference in polarity, and thus obtained results will have difference in the biological activity, and in the phytochemical profiles of different extracts (Panda *et al*., 2011; Altemimi *et al*., 2017). The polar solvent (MOH) extract was active at higher MIC whereas the nonpolar solvent (CH and EA) extracts were active at lesser MIC values. Thus, it indicates that the nonpolar active constituents present in nonpolar solvent extract are responsible for the strong anti-MRSA activity, as previously reported in other plants (Panda *et al*., 2011; Elisha *et al*., 2017). Coumarin and carbazole derivatives, isolated from roots and leaves of *C. excavata* have been reported for their antimicrobial properties (Albaayit *et al*., 2015).

Previous studies showed that the mechanism of action of natural product was focused to explore their effects on cellular membranes (Skandamis *et al*., 2001; Ultee *et al*., 2002). In this study, the AFM analysis of the bacteria treated with EA and CH extracts showed scattered cytoplasmic content, and these images confirmed their lost cellular integrity. Propidium iodide staining, a fluorescence dye is used in detecting the integrity of cell membranes (Sánchez *et al*., 2010). This simple and rapid method showed that the EA extract treated cells showed a higher PI fluorescence, as more PI dye penetrated inside these cells due to compromised cell membrane integrity to bind with the DNA. In the current work, leaves extracts of *C. excavata* was found to have potent antibacterial properties against MRSA bacterial strains, which could be attributed to its rich coumarins (nordentatin), and carbazole derivative (xanthoxyletin).

***Conclusion*:** *C. excavata* has a great potential to be developed into a highly effective drug in the treatment of MRSA-related conditions. For this, more investigations have to be done to identify the potent antibacterial compound, and its detailed mechanistic study.

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***Conflict of interest*:** None.

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