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Original Research Article

Ultrastructural Study of *Elaeis guineensis* (Oil Palm) Leaf and Antimicrobial Activity of its Methanol Extract against *Staphylococcus Aureus*

Amala Rajoo[1], Sreenivasan Sasidharan[2*], Subramanion L Jothy[2], Surash Ramanathan[1] and Sharif M Mansor[1]
[1]Centre for Drug Research, [2]Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia.

For correspondence:* ***Email:* *srisasidharan@yahoo.com*

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Abstract

Purpose: *To evaluate the antimicrobial activity of the methanol extract of Elaeis guineensis leaf against Staphylococcus aureus and to determine the effect of extract treatment on the microstructure of the microbe*
Methods: *The antimicrobial activity of the methanol leaf extract of the plant against S. aureus was examined using disc diffusion and broth dilution methods. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were carried out to determine the major alterations in the microstructure of S. aureus after treatment with the extract.*
Results: *The extract showed a good antimicrobial activity against S. aureus with a minimum inhibition concentration (MIC) of 6.25 mg/mL and for Chloramphenicol was 30.00 µg/mL. The main changes observed under SEM and TEM were structural disorganization of the cell membrane which occurred after 12 h and total collapse of the cell 36 h after exposure to the extract.*
Conclusion: *We concluded that the methanolic extract of E. guineensis leaf exhibited good antimicrobial activity against S. aureus and this is supported by SEM and TEM.*

Keywords: *Antimicrobial activity, Elaeis guineensis, Staphylococcus aureus, Scanning electron microscopy, Transmission electron microscopy*

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INTRODUCTION

Nature has been the repository of medicinal plants for thousands of years and a huge number of modern drugs have been isolated from natural sources, notably of plant origin [1]. Resistance of microbes to commonly used antibiotics has enhanced morbidity and mortality and has triggered the search for new drugs [2]. The African oil palm (*Elaeis guineensis*) is one of the plants that are central to the lives of traditional societies in West Africa. It is a monocotyledon perennial tree crop of the order *Spodiciflorae* and family *Palmae*,, grouped under the *Cocoineae* tribe. All parts of this plant are useful. The leaf extract and juice from young petioles are applied to fresh wounds. The fruit mesocarp oil and palm kernel oil are administered as a poison antidote, and are also used externally with several other herbs as a lotion to treat skin diseases. Palm kernel oil is applied to convulsing children to regulate their body temperature. Oil palm is a folk remedy for cancer, headaches, and rheumatism,

and is considered an aphrodisiac, a diuretic, and a liniment [3].

Staphylococcus aureus a Gram positive bacterium is responsible for numerous infections from skin to soft tissue infections. It also causes endocarditis and septic arthritis, which can induce bacteremia (associated with 80 % mortality in the preantibiotic era). However, staphylococcal resistance developed as antibiotic use increased [4]. Hence, the present study was carried out to determine the antimicrobial activity of *E. guineensis* leaf extract against *S. aureus*. Furthermore, analysis by electron microscopy is required to obtain detailed understanding of the characteristics of cell structure. Light microscopy offers limited analysis and the resolution is often too broad to distinguish the intimate subcellular structures. Since the resolving power of light microscopes is often insufficient to analyze changes in topological and structural details, the studies of biomembranes and membranous organelles require transmission electron microscopy [5,6]. Scanning electron microscope, on the other hand, permits the study of the surface of the organism. Hence, these techniques were used to study the effect of *E. guineensis* leaf extract on the cell structure of *S. aureus.*

EXPERIMENTAL

Plant material

E. guineensis was collected in August 2009 from Pulau Pinang, Malaysia and authenticated by Mr Shanmugam of School of Biological Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia. A voucher specimen (no. 11037) has been kept for future reference at Herbarium of the School of Biological Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia. The leaves were washed with water to remove dirt and then dried in an oven at 40 °C for 7 days.

Extraction procedure

The leaf extract was prepared by maceration. Approximately, 100 g of dried powdered leaf sample was macerated in 400 ml methanol for 4 days. The macerated extract was then filtered with Whatman filter paper no. 1 and the filtrate further concentrated using a rotary evaporator (BUCHI Rotary Evaporator R-110). The crude extract was then freeze dried (Cole-Parmer, USA).

Evaluation of antimicrobial activity

S. aureus was used as the test organism and was obtained from School of Biological Sciences, Universiti Sains Malaysia. The bacterium was cultured on nutrient agar at 37 °C for 18 h and the stock culture maintained in the same medium at 4 °C.

The test microorganism was aseptically transferred with an inoculating loop and suspended in a universal bottle containing 10 ml of sterile distilled water. Sufficient inocula were added until the turbidity was equal to 0.5 McFarland (10^6 colony forming units per ml) standard (bioMerieux, Marcy Petoile, France). One milliliter of the suspension was inoculated onto nutrient agar and spread with an L shaped glass rod. Three sterile discs were used to screen the antimicrobial activity. Each sterile disc was impregnated with 20 µl of the extract (corresponding to 100 mg/ml of crude extract); Chloramphenicol (30 µg/ml, as positive control) and methanol (as negative control) before it was placed on the surface of the agar plate. The plates were incubated at 37 °C overnight and the zones of growth inhibition were observed. The mean of the three replicates were taken [7].

Determination of minimum inhibitory concentration (MIC)

A 16-h culture of *S. aureus* was suspended in sterile distilled water with reference to 0.5 McFarland standards to achieve inocula of approximately 10^6 colony forming unit per ml. The extract was serially diluted to give final concentrations between 0.195 and 100.00 mg crude extract per ml in a test tube containing 5 ml of nutrient broth. The tubes were then inoculated with 500 µl bacterial suspensions, homogenized and incubated at 37 °C. After incubation, 20 µl was withdrawn from each tube and incubated at 37 °C for 24 h. MIC value was determined as the lowest concentration of the extract in the broth medium that inhibited the visible growth of the test microorganism [7].

Scanning electron microscopy (SEM)

The effect of *E. guineensis* leaf extract on the morphology of *S. aureus* was determined by SEM. *S. aureus* cell suspension (100 µl, $1x10^6$ cells/ml) was inoculated on a nutrient agar plate and incubated at 37 °C for 6 h. The extract (300 µl, 6.25 mg/mL) was then dropped on the inoculated agar and further incubated for another 36 h at 37 °C. A methanol-treated culture was used as control. Segments (5 - 10 mm) were cut from cultures growing on the nutrient agar plates

at various time intervals (control, 12, 24 and 36 h) for SEM examination and placed on a planchette with double-stick adhesive tabs and then placed in a Petri plate. In a fume hood, 2 % osmium tetroxide in water was placed in an unoccupied quadrant of the plate. Once covered, the plate was then sealed with parafilm and vapor fixation of the sample lasted for 1 h. As soon as the sample was vapour-fixed, the planchette was plunged into slush nitrogen (-210 °C) and transferred to the 'peltier-cooled' stage of the freeze dryer (Emitech K750), where it was dried for 10 h. Finally, the freeze dried specimen was sputter coated with 5 - 10 nm gold before viewing in SEM (Fesem Leo Supra 50 VP, Carl Zeiss, Germany) operating at 15 kV at various levels of magnification (5000 to 10000x) [8].

Transmission electron microscopy (TEM)

TEM investigation was carried out to observe the effect of the extract on the morphology of *S. aureus*. *S. aureus* cell suspension (100 µl, 1 x 10^6 cells/ml) was inoculated on a nutrient agar plate and incubated at 37 °C for 6 h. The extract (1 ml, 6.25 mg/mL) was placed on the inoculated agar and further incubated for another 36 h at 37 °C. Methanol-treated culture was used as control. At various time intervals (0, 12, 24 and 36 h), the culture treated with extract was pooled from the plates into an Eppendorf tube by McDowell-Trump fixative prepared in 0.1M phosphate buffer (pH 7.2) and left for at least 2 h. The sample was centrifuged, washed in 0.1M phosphate buffer twice, centrifuged again and the pellet was re-suspended in 1 % osmium tetroxide prepared in phosphate buffer for 1 h.

Thereafter, the sample was re-suspended and washed in distilled water twice, centrifuged and the supernatant discarded. The tube containing the pellet of fixed cells was placed in a water bath at 45 °C for 15 - 30 min, depending on the amount of sample. A solution of agar (2 %) was prepared and warmed to 45 °C, a drop of it transferred to the tube containing the pellet of cells using a warm pipette, and stirred just enough to break the pellet into small blocks and to suspend the small blocks in the agar. Immediately, the agar containing the suspended pellet blocks was poured on a glass slide, allowed to set, cut into small cubes with a sharp blade and placed in a vial containing 50 % ethanol. The cubes were subjected to a series of alcohol dehydration with 50%, 75%, 95% and 100% ethanol respectively, infiltrated with resin using a mixture of acetone: Spurr's resin mix (1:1) in a rotator overnight. The blocks were then embedded in resin, cured at 60 °C for 12 - 48 h, cut using an ultramicrotome, and ultrathin sections collected in a grid. The ultrathin sections were stained with uranyl acetate and lead, and observed under a TEM (Leo Supra 50 VP Field Emission SEM, Carl Zeiss, Oberkochen, Germany) [9].

Statistical analysis

Data obtained from disc diffusion test were analysed using Student t-test and SPSS program, version 17. Differences were considered significant at $p < 0.05$.

RESULTS

Antimicrobial activity of extract

E. guineensis extract showed *in vitro* antimicrobial activities against *S. aureus* with inhibition zone diameters of 14.0 mm. In contrast, the inhibition zone of negative control (methanol) was zero, indicating that it had no activity. However, 30 µg/mL of chloramphenicol (positive control) was more effective than the leaf extract of *E. guineensis* with a inhibition zone diameters of 24.67 mm ($p < 0.05$). The extract showed a good antimicrobial activity against *S. aureus* with a MIC of 6.25 mg/mL, compared with 30.00 µg/mL for chloramphenicol.

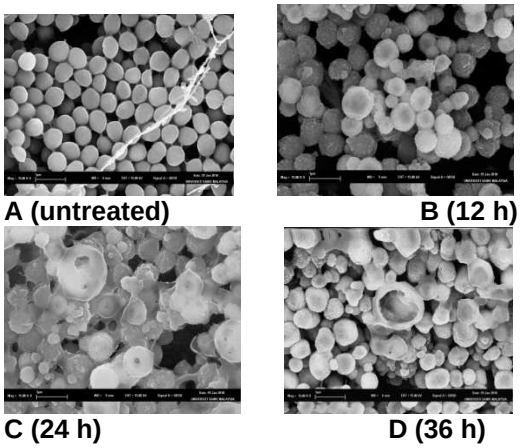

Fig 1: Scanning electron micrograph of (A) untreated and (B - D) reated cells of *Staphylococcus aureus*

Scanning electron micrographs

The effect of the extract on the morphology of *S. aureus* is shown in Figure 1. Changes can be in the morphology of the cells can be seen, with the degree of change varying with duration of exposure to the extract. Untreated cells (Fig 1A) showed normal coccus and smooth cell morphology. Mild effect after treatment with the extract was observed after 12 h of exposure. with the cells showing a rough appearance. Severe

alteration occurred after 24 h (Fig 1C) with pores present on the cells, showing a state of morphological disorganization; after 36 h of exposure to the extract, the cells completely collapsed, leaving huge pore on the cell surface (Fig 1D).

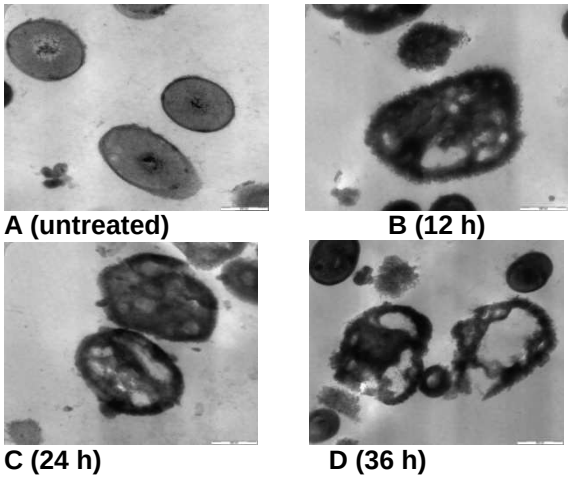

Fig 2: Transmission electron micrograph of (A) untreated and (B - D) treated cells of *Staphylococcus aureus*

Transmission electron micrographs

Further evidence of the alterations done to *S. aureus* by exposure to *E. guineensis* leaf extract is displayed in (Fig 2). Figure 2A (untreated *S. aureus* cells). shows a typically structured nucleus of *S. aureus* and a perfect cell wall. After 12 hours of exposure to the extract, a slight alteration can be observed in the cell cytoplasm (Fig 2B); 24 h later(Fig 2C), the cells exhibited notable alteration in cell cytoplasm. Figure 2D shows shows that the bacterial cells completely collapsed 36 h after treatment with the extract.

DISCUSSION

The extract exhibited good antimicrobial activity against the test microorganism. *E. guineensis* extract showed lower antibacterial activity compared to the positive control of chloramphenicol against the tested bacteria probably due to its low levels of bioactive compounds that exhibit antimicrobial activity in the crude extract of *E. guineensis.*

S. aureus was selected as the test microbe because it is a human pathogen of significant importance. It is responsible for numerous infections and syndromes ranging in severity from skin and soft tissue infections to endocarditis [10] and septic arthritis [11]. The extract caused complete growth inhibition of *S. aureus* at a strength of 10 0mg/ml. MIC was 6.25 mg/ml and the effect of the extract dose-dependent.

Both SEM and TEM showed that the extract damaged and ultimately caused complete collapse of the bacterial cell, and thus inhibited the growth of microorganism. The abnormal cell wall, which was indicated by the presence of pores and shrinkage of the cell wall in SEM, may be due to the disruption in synthesis of the cell wall. Cell death is ensured by the damage in the cell wall [12]. At this stage, it is believed that the cells had completely lost its metabolic functions. The TEM results indicate that the various bioactive compounds present in the extract may have acted on different targets in *S. aureus* cells. This hypothesis, however, needs to be further ionvestigated. It is noteworthy that the leaf extract and juice from young petioles are applied to fresh wounds by indigenous traditional healers [13]. Therefore, *E. guineensis* may find use as *S. aureus* infected wound healing agent in known dosages, especially in rural communities where conventional drugs are unaffordable and/or unavailable, and health facilities inaccessible.

CONCLUSION

This study confirms the antimicrobial activity of *E. guineensis* as described in literature [14]. However, the effect of *E. guineensis* on *S. aureus* with regards to its exact mode of action and morphological alterations needs to be further studied.

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