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Original Research Article

Effect of the Extract of Endophytic fungus, Nigrospora sphaerica CL-OP 30, Against the Growth of Methicillin-Resistant Staphylococcus aureus (MRSA) and Klebsiella pneumonia cells

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Abstract

Purpose: *To investigate the effect of the ethyl acetate extract of endophytic fungus, Nigrospora sphaerica CL-OP 30 against the growth of methicillin-resistant Staphylococcus aureus (MRSA) and Klebsiella pneumonia cells.*
Methods: *Time-kill assay was used to examine the effect of the extract on the bacterial growth profile. The effects of extract on ultrastructure of MRSA and K. pneumoniae cells were analyzed by scanning electron microscope (SEM) and transmission electron microscope (TEM).*
Results: *The time-kill test revealed that the bacteriocidal activity of the extract was both concentration- and time-dependent. After 12 h treatment, interaction of extract with MRSA cells resulted in the formation of pit, disintegration of cell wall and membrane, and ultimately cell death, while K. pneumoniae cells became crumpled, and the cell walls and membranes disintegrated, resulting in leakage of their cytoplasmic contents.*
Conclusion: *These data suggest that the Nigrospora sphaerica CL-OP 30 extract principally affects the cell wall in growing MRSA and K. pneumonia cells.*

Keywords: *Endophytic fungus, Nigrospora sphaerica, Antimicrobial activity, Cellular structure degeneration*

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INTRODUCTION

Infections caused by bacteria have always inflicted high mortality rate and great loss to human population, veterinary and aquaculture industries [1]. Besides, the evolution of resistant pathogenic bacteria to antibiotic has become another challenging problem for treating disease caused by resistant pathogenic bacteria [2]. Therefore, searching a new drug to combat the pathogenic bacteria is now a growing need for scientists.

Endophytic fungi have been shown to be a promising source of new natural products [3]. This fungus usually colonizes plants tissues internally without causing any apparent disease in plants. They produce a number of compounds which can inhibit pathogens. However, they are relatively not much explored as sources of novel

natural products for pharmaceutical industry. Hence, the endophytic fungus from medicinal plant can be a good source of antimicrobial compounds.

Swietenia macrophylla King is a medicinal plant or known as big leaf mahogany in Malaysia. Traditionally, this plant has been used to treat infectious diseases such as diarrhoea, skin ailments, and wound infection [4]. Some researchers have proved that various parts of this plant exhibited antimicrobial, antioxidant and anti-inflammatory properties [5,6].

Nigrospora sphaerica has been reported as an endophytic fungi or a pathogen in plants [7,8]. This species of fungus is known to possess an antimicrobial activity [9]. Thus, in this study the effect of the *N. sphaerica* extract on morphology and structure of growing bacterial cells was evaluated.

EXPERIMENTAL

Endophytic fungus and storage

An endophytic fungal isolate CL-OP30 was provided by Industrial Biotechnology Research Laboratory, Universiti Sains Malaysia, Penang, Malaysia. This fungus was previously isolated from a healthy old leaf of *Swietenia macrophylla* [4]. It was cultivated on Malt Extract Agar (MEA, AES) supplemented with powdered host plant materials (5 g/L) at 25 ^{o}C for three weeks. The stock was sub-cultured on fresh media every two months to ensure the viability of the isolates.

Test microorganisms

Test microorganisms used in this study were methicillin-resistant *Staphylococcus aureus* (MRSA, Gram positive) ATCC 33591 and *Klebsiella pneumoniae* ATCC 13883 (Gram negative). The cultures were provided by Industrial Biotechnology Research Laboratory and Plant Pathology Research Laboratory, Universiti Sains Malaysia, Penang, Malaysia.

Time-kill study

Previous study showed that the minimum inhibitory concentration (MIC0 of ethyl acetate extract of endophytic fungus, *Nigrospora sphaerica* CL-OP 30, against the MRSA and K. pneumonia was 0.4 mg mL^{-1} and 1.6 mg mL^{-1} respectively [10]. In time-kill study, the extract was tested at concentration of 0.5, 1.0 and 2 times the MIC for each bacteria. A volume of 0.1 ml bacterial inoculum (approximately 1 x 10^{8} CFU mL^{-1}) was transferred into flask containing 19.9 ml Mueller Hinton Broth (MHB) (Merck, Germany), with different concentrations of extract. This mixture yielded an initial inoculum cell density of approx 5 × 10^{5} CFU mL^{-1}. Control culture composed of bacteria and 1 % DMSO in MHB was included for comparison. The cultures were then incubated at 37 °C in a rotary shaker at 150 rpm for 48 h. At 4 h time interval from 0 – 48 h, a 0.1 mL aliquot was taken for viable cell counts. The samples were diluted and spread onto nutrient agar (Merck, Germany) plates followed by incubating at 37 °C for 24 h and the bacteria colonies were counted. Number of colonies ranging from 30-300 was used to calculate colony forming unit per milliliter (CFU mL^{-1}). A time-kill curve (log CFU mL^{-1} vs. time) was drawn for each concentration of extract and control culture and the time to achieve 50 (T50), 90 (T90), 99 (T99) and 99.9 % (T99.9) reduction was calculated as equation 1. The experiment was conducted in triplicate on separate occasions.

$$\text{Reduction (\%)} = V_O - V_Z/V_O \times 100 \quad \ldots\ldots\ldots \quad (1)$$

Where V_O is the initial viable cell count and V_Z is the viable cell count at time z

Scanning electron microscopy (SEM)

The bacterial inoculum (0.1 mL) was inoculated in a flask containing 18.9 mL MHB and incubated in a shaker at 37 °C, 150 rpm for 18 h. After incubation period, 1 mL of extract (8 mg mL^{-1}) was added to the bacterial culture to yield a volume of 20 mL mixture composed of 0.4 mg mL^{-1} extract. At the same time, a 1 ml aliquot of 20 % dimethylsulphoxide (DMSO) was added into similar volume of the bacterial culture without extract to serve as a control. The mixtures were then incubated at 37 °C with agitation rate of 150 rpm for 12 h. After incubation time, the pellets of the treated bacterial culture were suspended with McDowell-Trump fixative solution in 0.1 M phosphate buffer (pH 7.2) for at least 2 h to fix the cell's original condition [11]. For the post-fixation step, the sample was suspended in 1 % Osmium tetroxide in 0.1 M phosphate buffer (pH 7.2) for one hour, centrifuged, and the supernatant was discarded [12]. Then, the pellet was dehydrated using 50, 75, 95, 100 % (repeated twice) ethanol and HMDS for 10 min [13,14]. Dehydration steps were performed by suspending cell pellet in each solution consecutively, centrifuged at 1500 g for 10 min, and discarded the supernatant. The dehydrated pellets were then dried in a desiccator at room

temperature for at least 24 h and the dried cells were mounted on specimen holders using conductive tape. Samples were coated with 5 - 10 nm of gold palladium alloy using sputter coater machine (Fison SC-515, UK) and viewed under a scanning electron microscope (Leica Cambridge, S-360, UK).

Transmission electron microscopy (TEM)

The treated bacterial sample and McDowell-Trump fixation were performed using standard procedure. Pellets of fixed cells were embedded with agar solution and the solidified agar was then cut into 10 × 1 × 1 mm^3 stripes and placed in a vial containing 50 % ethanol. The stripes were dehydrated successively with 75, 95 and 100 % ethanol for 15 min and lastly suspended with 100 % acetone for 10 min [12,15]. A mixture of acetone: Spurr's resin [1:1 (v/v)] was added to the vials and rotated 15 - 30 min for infiltration. The resin blocks were cut according to cross section method accompanied by razor blades, glass knifes and a microtome instrument (Sorvall Ultra Microtome MT500, USA) to obtain ultrathin section (< 0.1 µm). The ultrathin sections were placed on copper grids, stained with uranyl acetate, followed by lead citrate solutions [15] and the cells on copper grid were then viewed under TEM machine (LIBRA 120 EFTEM, Germany).

RESULTS

Time-kill study

The killing growth profile of *N. sphaerica* CL-OP30 ethyl acetate extract against MRSA are shown in Fig 1 and 2. Bacteria cells treated with extract concentration half the MIC value (MRSA:0.2 mg mL^{-1} ; *K. pneumonia*: 0.8 mg mL^{-1}) showed lag phase within 0 - 4 h, exponential phase at time interval 4 - 24 h, and lastly reached stationary phase (24 - 48 h). Higher concentration of extract (twice the MIC) was required to exert inhibiting or killing effects on MRSA and *K. pneumonia* cells as well; for both bacteria, reduction in cell viability was only observed in the extract twice the concentration of the MIC (Table 1).

Table 1: Reduction in viable MRSA and *K. pneumoniae* cells counts in the presence of extract twice the concentration of reference MIC

Percentage of reduction (%)	Time (h)	
	MRSA	***K. pneumoniae***
50	16-20	0-4
90	20-24	4-8
99	24-28	8-12
99.9	24-28	20-24

Structural degeneration of the bacteria cells

Figure 3 showed the morphological changes in MRSA cells after treated with the ethyl acetate extract of *N. sphaerica* CL-OP30 at MIC concentration. The untreated cells appeared as typical coca shape with smooth surface and maintained rigidity (Fig. 3ai). After 12 h exposure to the extract, there were formations of cavities and cell debris on the bacterial cells (Fig. 3aii). Some of the treated cells lysed, shrunk abruptly and collapsed completely. The coca shape of MRSA cell was no longer seen, but left only crumpled cell residues.

TEM micrographs shown the thin section of untreated cell presented typical features of MRSA which appeared as round shape and surrounded by cell wall and membrane (Fig. 3bi). Cell wall was homogeneous and rigid while the cell membrane was smooth and intact. The untreated cell was found undergoing the process of cell division with apparent septa cross wall and contrasted septal midline.

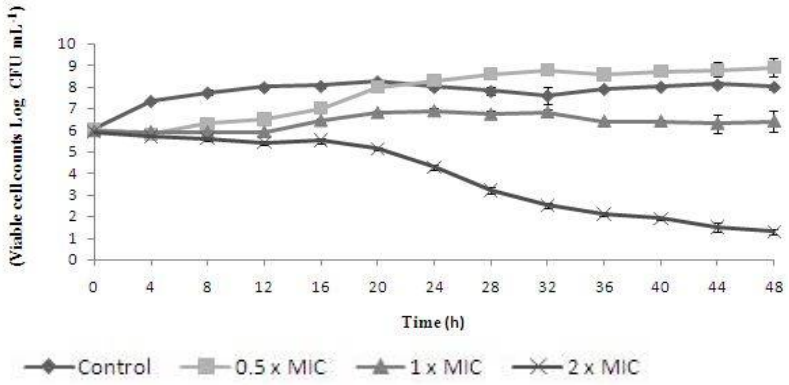

Fig. 1: Viable cell count of MRSA exposed to *N. sphaerica* CL-OP 30 ethyl acetate extract at different concentrations

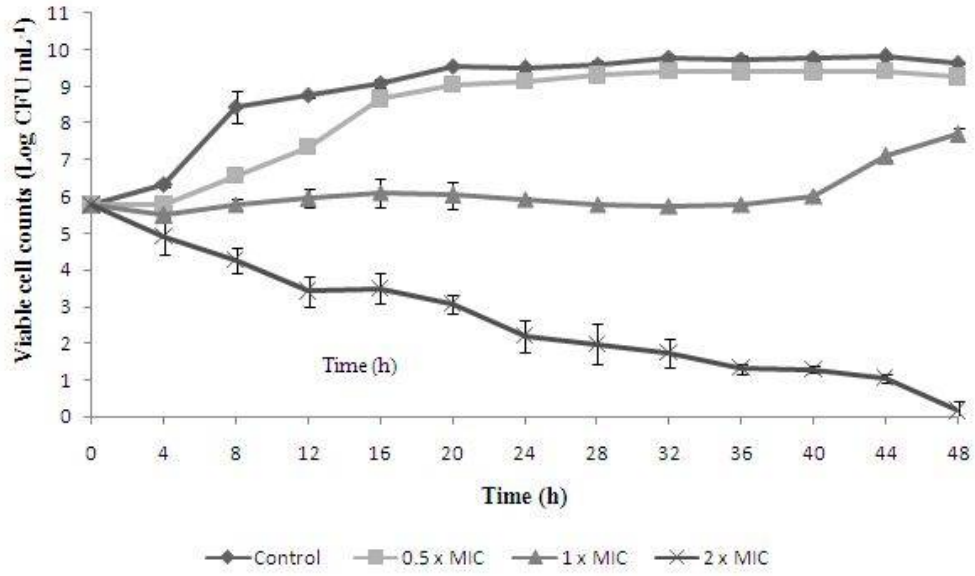

Fig. 2: Viable cell count of *K. pneumoniae* exposed to *N. sphaerica* CL-OP 30 ethyl acetate extract at different concentrations

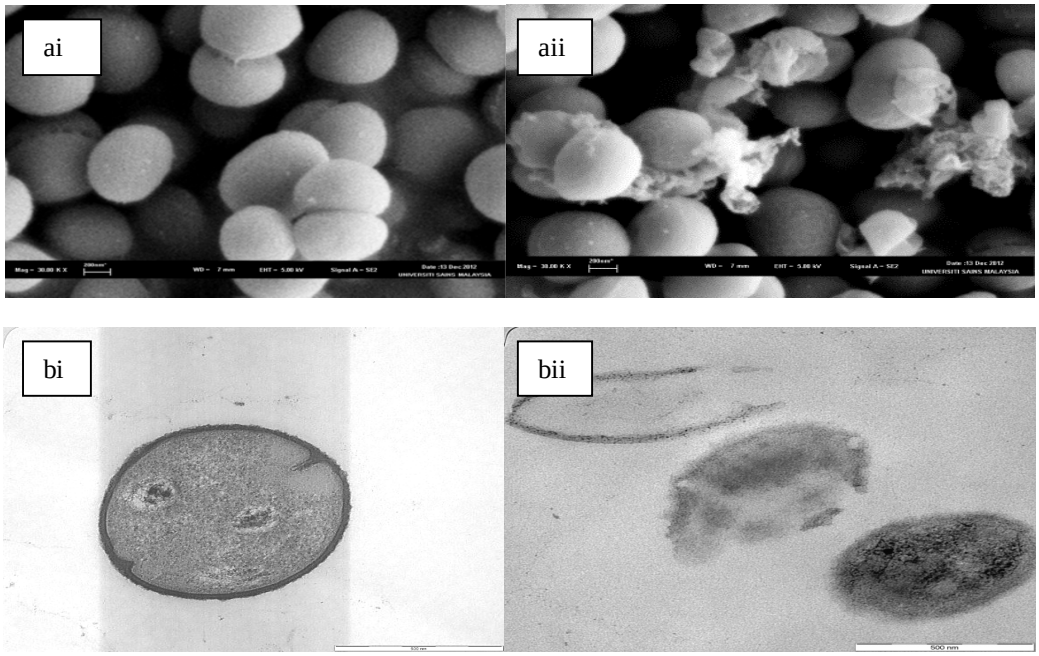

Fig. 3: SEM (a) and TEM (b) micrographs of MRSA. i: control (without treatment), ii: treated with 0.4 mg mL^{-1} of *N. sphaerica* CL-OP 30 ethyl acetate extract for 12 h. Bars: 200 nm

Severe damage of cells was observed (Fig. 3bii) as the cell envelope was broken and there was leakage of cytoplasm. Lysed cells were devoid of cytoplasmic contents, with emptied and broken cell envelope and subsequently, the cell collapsed completely.

Untreated cells of *K. pneumoniae* had normal cell condition with rugose surface, rod shape and rigid (Fig. 4ai). Longer rod was observed in dividing cell. After treatment for 12 h, the cell surface became crumpled and shrunken (Fig. 4aii). The cells also showed irregular shape where the cells shrunk. Some of them opened outwards and leaving cleavage in cell surface.

The alterations within cells were portrayed in the micrographs of cellular cross sections. Based on the Fig. 4bi, the untreated cell (control) appeared as typical ellipsoidal shape with cytoplasm

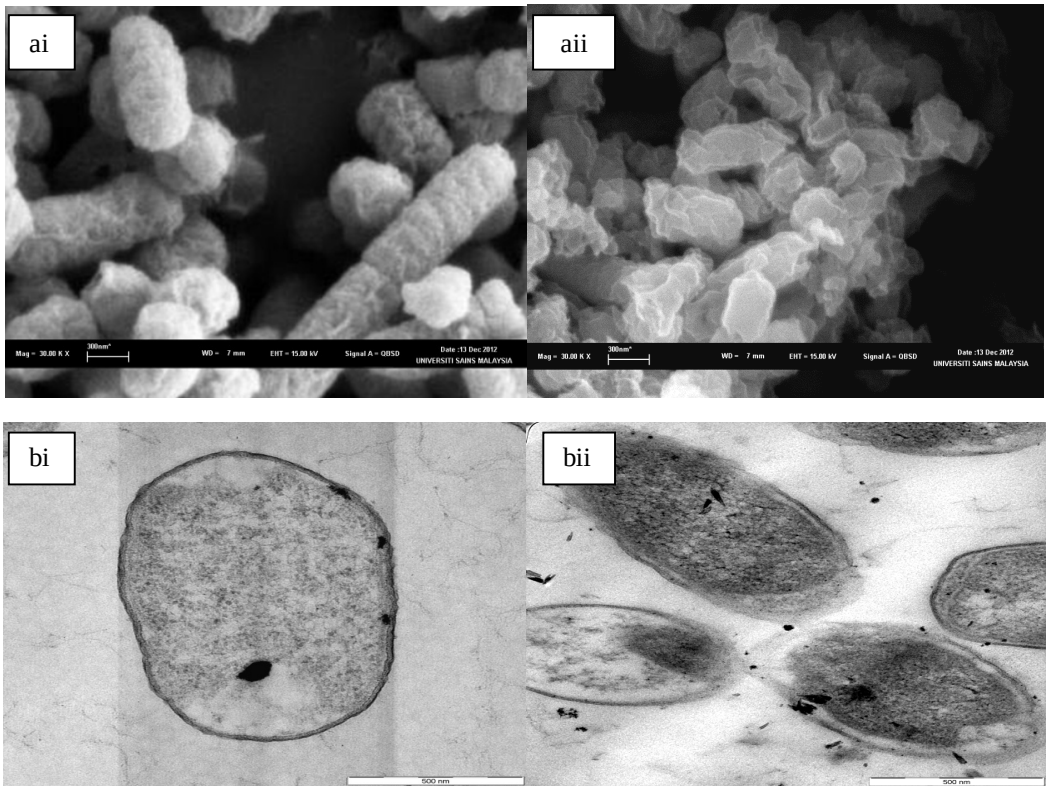

Fig. 4: SEM (a) and TEM (b) micrographs of *K. pneumoniae*. i: control (without treatment), ii: treated with 1.6 mg mL^{-1} of *N. sphaerica* CL-OP 30 ethyl acetate extract for 12 h. Bars: 200 nm

surrounded by cytoplasmic membrane and cell wall. The outer membrane of the cell wall had a typical wavy appearance and the cytoplasmic membrane was continuously in close contact with the cell wall. Cellular damages induced by the extract were noticed after treatment with extract for 12 h. As illustrated in Fig. 4bii, larger periplasmic region between the outer membrane and cytoplasmic membrane was observed in the cells. Severe damage was observed in the lysed cells with disintegrated cell wall, broken cytoplasmic membrane and leakage of cytoplasmic contents.

DISCUSSION

This study has revealed a time-dependent killing of MRSA and *K. pneumoniae* treated with the extract twice the MIC. Morphological evaluation of the bacteria cells indicated that the extract effect was on the cell wall. SEM study on MRSA cells showed that the extract induced formation of cavities and appearance of cell debris on the surface of cells. The formation of cavities (pores) indicated the cell envelope was destructed or disintegrated, possibly indicated the rupture of cell membrane and cell wall [16]. The cell debris on the surface could be the residue of cell envelop where disintegration occurred. Cell envelop of gram positive bacteria is constituted by cell wall and cell membrane [17]. The collapsed cells with shrunken cell residues may represent the leakage or loss of cytoplasmic contents. In TEM study, the micrograph of the thin sections portrayed the alteration happened during the cell's dying process. The extract caused formation of pit, irregular shape, deformed septa, cell wall disintegration, and loss of cytoplasm contents. Similar effects of cell wall had been described for *oritavancin, rhodomyrtone* and marine bacteria derived antibiotic [18-20].

K. pneumoniae cells treated with the ethyl acetate extract demonstrated unusual morphology compared to normal cells. The result of the studies by SEM showed that the extract caused the shrinkage of cells, cell surface crumpled, irregular shape, and cleavage of cell envelope. These features may indicate the damage of cell envelope and loss of cellular contents from cytoplasm of cells. Hence, *K. pneumoniae* cellular damage could have like resulted from cell leakages or lysis. *K. pneumoniae* is a Gram negative bacteria with cell envelope composed of outer cell membrane, thin peptidoglycan layer and cytoplasmic membrane

[17]. The cleavage of cell envelope could have resulted from the action of extract on either the peptidoglycan or cytoplasmic membrane or both. The use of TEM had exposed the changes within the cell when treated with the extract. The unusual morphology of the cells included outer cell membrane and periplasmic thickening, disintegration of cell wall, disintegration of cytoplasmic membrane, and mass leakage of cytoplasmic contents. Similar response was also found in *K. pneumoniae* treated with the hexane extract of *H. discoidea* [21]. The thickening of periplasm was also reported in *K. pneumoniae* treated with membrane active agent [22]. As proposed in the report, the thickening of this region could be due to the intake of water from medium into the cell. It suggested that the penetration rate of the agent maybe retarded by retention in the lipid bilayer. The possible factor that contributed to the cell lysis was disintegration of cell wall. The loss of protection of cell wall could cause influx of water due to osmosis and subsequent cell lysis [17]. In this study, the lysed cells have disintegrated cell envelope and loss of cytoplasmic contents. The loss of cytoplasmic contents can then caused the shrinkage, crumpling, and irregular shape of the cells, as described in SEM study.

CONCLUSION

The results of this study proved that ethyl acetate extract of *N. sphaerica* CL-OP30 exhibited antibacterial activity by disrupting the normal cell wall formation of MRSA and *K. pneumoniae*, which caused cell lysis and ultimate death.

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