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Anti-MRSA activity of *Penicillium minioluteum* ED24, an endophytic fungus isolated from *Orthosiphon stamineus* Benth

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Abstract. Nowadays, the medical concerns with Methicillin-resistant *Staphylococcus aereus* (MRSA) arised when in 2009, the proportion of *S. aureus* isolates that are resistant to methicilin has increased to 59.5%- 64.4% in South East Asia. Hence a new antibacterial agent from natural source is necessary to combat the infectious diseases. This study aimed to investigate the anti-MRSA activity of the endophytic fungus *Penicillium minioluteum* ED24, which was previously isolated from the leaf of the medicinal plant *Orthosiphon stamineus* Benth, in Penang, Malaysia. Methanol was used to extract the freeze-dried fungal biomass of the 14-days old fungal culture. The extract showed very significant anti-MRSA activity of disc diffusion assay with the minimal inhibitory concentration of 31.3 mg/mL and minimal lethality concentration of 250 mg/mL. Besides, 50% growth reduction of MRSA was observed at 33.2 h at the concentration of extract at MIC and 26.7 h at concentration of 2MIC. The structural degeneration of MRSA was observed by using scanning electron microscope (SEM). The SEM micrographs showed that the formation of cavities were observe on the extract treated cells and the cell wall structure of the MRSA was collapsed after treated with the fungal extract. The results suggesting that the bacterial cell wall is the target of the antibiotic compound(s) present in the extract. These results reveal that the endophytic fungus *P. minioluteum* ED24 a is potential source of anti-MRSA compounds.
Key words: Anti-MRSA activity, endophytes, *Penicillium minioluteum*, *Orthosiphon stamineus*.

Introduction

Nowadays, world health problems due to drug-resistant pathogens are increasing and *Staphylococcus aureus* has been identified as a major human pathogen that cause a wide range of infections from mild skin infection to bacteremia (Cosgrove *et al*., 2003; darah and Halim, 2005). However, the mis-usage of antibiotics in the past few decades have caused the emergence of methicilin-resistant *S. aureus* (MRSA) where the bacteria have developed resistance mechanisms to all antimicrobial agents that have been produced (Hardy *et al*., 2004). MRSA was first reported in Europe in 1960s, and the number of resistant isolates and outbreaks reported increased throughout the decades as the intensive care patients are at great risk of infection from MRSA (Cosgrove, 2006). Today, the main antibiotic used for treatment of MRSA is vancomycin but the recent studies of vancomycin resistant MRSA demonstrates how MRSA evolved constantly and suggests the future may not be bright (Bradley, 1997). Hence it is necessary to search for a new antibiotic compounds from natural source to combat the multi-drug resistant infectious agent.

Endophytes are a group of microorganisms which colonize the internal tissues of living plants, without overt negative effects to the host (Schulz *et al.*, 2002). The relationship between endophytes and their hosts is symbiotic: endophytes obtain nutrients from the plants and in return, contribute to an effective host defense against pathogenic microorganisms (Tan and Zou, 2001; Ding *et al*., 2008). Although the ability of endophytes to affect plant community diversity and structure is well characterized, the medical importance of endophytes has only recently become apparent (Schulz *et al.*, 2002; Huang *et al*., 2008). Hence it is wise to search for bioactive compounds from these group of unique microorganisms.

As our on going efforts to search for novel antibiotic compounds from endophytic fungi, an endophytic fungus *Penicillium minioluteum* ED24 that was previously isolated from medicinal herb *Orthosiphon stamineus* Benth was investigated. *P. minioluteum*, a biverticillate *Penicillium* species, is well known for its production of red pigmented compounds. This fungus was reported to produce dextranase that catalyzes the hydrolysis of α-1,6-glycosidic linkage in dextran polymers (Larsson *et al*., 2003). However, relatively little information is available on its biological activities. Larsson *et al*. (2003) reported a new antifungal antibiotic BE-31405 was isolated from from the culture broth of *P. minioluteum* and it showed significant anti-yeast activity on several pathogenic yeast strains. Besides, *P. minioluteum* was reported to be exhibited anticancer activity by producing a cell cycle inhibitor, HY558 and paclitaxel (Lee *et al*., 2001). No data was

available on its antibacterial activity. Hence, the present study was aimed to investigate the anti-MRSA activity of the endophytic fungus *P. minioluteum* ED24.

Materials and Methods

Storage of endophytic fungus

The endophytic fungus previously isolated by Tong *et al*. (2011) from the leaf of *O. stamineus* Benth was deposited at Industrial Biotechnology Research Laboratory, Universiti Sains Malaysia, Penang, Malaysia. The fungal isolate was cultivated on Potato Dextrose Agar (AES) slant and stored at 4^0C. The culture was subculturing on fresh medium every four weeks to ensure the viability of the isolate.

Culture media

Yeast extract sucrose broth (sucrose 40 g/L, yeast extract (AES) 20 g/L and magnesium sulfate 0.5 g/L) supplemented with the aqueous extract of *O. stamineus* was used to cultivate the endophytic isolates in the shake-flask system. The plant extract was prepared by boiling 10 g of the powdered plant materials in 500 mL distilled water for 30 minutes. The extract was filtered and mixed with freshly prepared culture media and autoclaved at 121 ℃ for 15 min. The aqueous extract of the host plant has been added in the culture media as previous study by Tong *et al*. (2011) showed that the addition of host plant extract in the culture media can significantly enhanced the antimicrobial activity of endophytic fungus.

Fermentation and extraction

The inoculum was prepared by introducing two mycelial agar plugs into 250 mL Erlenmeyer flasks containing 100 mL of the broth medium. Both agar plugs were 1 cm in diameter and excised from the 7-days-old fungal culture. The cultures were incubated at 30 ^{0}C with rotational speed of 120 rpm. After 14 days of incubation, the fermented broth and fungal biomass were separated out by centrifugation at 5311 g (Sigma, Model 4K15). Freeze-dried fungal biomass was extracted by soaking in methanol (1:50, w/v) overnight. The extract was filtered thrice with Whatman No1 filter paper and the extracts was evaporated under reduced pressure to obtain crude extract paste.

Test bacterial strain

The test bacteria used in the study was Methicilin-resistant *S. aureus* (MRSA) which previously isolated from clinical sample in Hospital Seberang Jaya, Penang, Malaysia. The culture was provided by Industrial Bitechnology Research Laboratory, Universiti Sains Malaysia, Penang, Malaysia. The bacterial culture was subcultured every two weeks on fresh nutrient agar (NA) slants (Hi-media) and incubated at 37 ^{0}C. All the cultures were then kept at 4 ^{0}C until further use. The inoculum was prepared by adding 4 mL of sterile physiological saline to the agar slant, and shake vigorously to get the cell or spore suspension. The turbidity of the bacterial suspensión was adjusted with sterile saline to match with Mc Farland 0.5 standard.

Disc diffusion assay

The crude extracts were dissolved in 5% dimethyl sulfoxide (DMSO) and sterilized by filtration with 0.2 µm Milipore filter. Mueller Hinton agar (Hi-media) plate was used as the culture médium. Disc diffusion assay was carried out by inoculating 100 µL of suspension containing 10^5 CFU/mL of bacteria on the surface of agar medium. Sterile Whatman antibiotic disc, impregnated with 20 µL of each extracts of 20 mg/mL concentration, were then placed on the surface of inoculated medium. Five percent DMSO was applied as a negative control to detect the solvent effects whereas 30 µg/mL chloramphenicol was used as the positive control. The plates were incubated at 37 ^{0}C for 24 hours for and the diameter of the clear zones surrounding the disc were measured after the incubation period.

Determination of MIC and MLC

The minimal inhibitory concentration (MIC) was determined by using broth microdilution assay in sterile 96-well microtiter plate. Sterile Muller Hinton broth was used to cultivate teh test bacteria. The fungal extract was dissolved in 5 % of DMSO to the concentration of 2 mg/mL, which then diluted to the highest concentration to be tested (1 mg/mL) after addition of 100 µL inoculum to achieve final volume of 200 µL. Then serial two fold dilution of the extract was carried out in a concentration range from 500 µg/mL to 15.63 µg/mL. The well containing only 5% DMSO and inoculum was used as the control. The plates were incubated at 37 ^{0}C for 24 hours.. After the incubation period, 40 µL of 0.2 mg/mL p-iodonitrotetrazolium violet salt (INT)

(Sigma) dissolved in 99.5% ethanol was added to each well as a growth indicator. The color of INT changed from yellow to purple indicates the microbial growth. The MIC value was recorded as the lowest concentration of extract that prevents any visible growth of teh test mucroorganism. To determine minimal lethality concentration (MLC) of the extract, the sample from each well was taken and viable cell count was conducted to judge the viability. The MLC was recorded as the lowest concentration of extract that resulted in 99.9% growth reduction relative to the control.

Time-kill curve study

A dilution of the inoculum was prepared by transferring 5 mL of bacterial inoculum into 45 mL of sterile Muller Hinton Broth. The dilution yielded a starting inoculum cell density of 10^5 CFU/mL of cells. Besides 1 mL of fungal extract was added into the flask to achieve the final concentration equals to MIC value and 1 mL of 5 % DMSO was added to another flask as negative control. The cultures were incubated at 37 ^{0}C in a rotary shaker at 150 rpm for 48 hours. A predetermined time points (0.48 hours with every 4 hourly intervals), 1 mL of simple was removed from each flask and diluted 10 fold with sterile saline. The aliquot was spreaded on Miller Hinton agar plates for viable cells determination. Inoculated plates were then incubated at 37 ^{0}C for 24 hours to obtain the cell counts.

Morphological changes on extract treated cells

To study the structural changes of extract treated cells, the bacterial cells were viewed under scanning electron microscope (SEM). One microliter of sample was withdrawn from each flask used for time-kill curve study at 24 hours. Then, the samples obtained were centrifuged at 5311 g (Sigma, model 4k15) to obtain the cell pellets. The pellets were then fixed with osmium tetroxide. After the freeze-drying process, the planchettes were coated with 5-10 nm of gold palladium alloy and viewed under scanning electron microscope (Leo supra 50VP field emission SEM).

Results and Discussion

On the disc diffusion assay, the methanolic extract showed a clear zone with the diameter of 17.3mm. In contrary, the clear zone of positive control chloramphenicol was 18.0mm. The presence of the clear zone indicates the inhibitory activity exhibited by the extract. The anti-MRSA activity of the extract was strong judging from the size of the clear zone. Positive control was necessary as only the clear zone that as clear as the zone produced by the control will be accepted as positive result. No data was available for comparison as this is the first report on antibacterial activity of *P. minioluteum.*

Based on the results, the MIC value obtained for methanolic extract was 31.3 µg/mL and the MLC value was 250 µg/mL on MRSA. On the other hand, the MIC value obtained for chloramphenicol was 1.95 µg/mL and MLC was 7.81 µg/mL. The result is in agree with the data obtained from disc diffusion assay, where the anti-MRSA activity of chloramphenicol was more significant than the methanolic extract. Besides the MLC value obtained was significant higher than MIC value for both chloramphenicol and methanolic extract, indicating that a higher concentration was needed to kill the bacterial cells, instead of inhibiting the growth. However the anti-MRSA activity of the methanolic extract was only bacteriostatic, as the MBC value obtained was higher than four-fold of its MIC value.

Table 1 shows the median time to achieve 25, 50, 90 and 99.9% growth reduction of MRSA from starting inoculum obtained from the time-kill curve. In general, the killing patterns obtained were similar to the MIC and MLC results. The growth of the control (5% DMSO) showed normal growth phases, and high colony count was obtained at the end of incubation period as the killing of the MRSA cells were not achieved. In contrary, 50% of killing of MRSA cells were observed for the methanolic extract, mirroring the susceptibility test result. However, the bactericidal effect of the extract was not observed, as the concentration used in this study was only the MIC, which is only sufficient to inhibit the growth of MRSA, a higher concentration of the extract was needed to kill the MRSA cells.

Figure 1(a) shows the SEM micrograph of MRSA cells without the treatment of methanolic extract. Based on the micrograph, the cells were cocci in shape, and the cell structures were intact. It is also clear that the surface of the cells were smooth. Figure 1(b) shows the MRSA cells treated with the methanolic extract. After 24 hours of exposure to the extract, most of the cells

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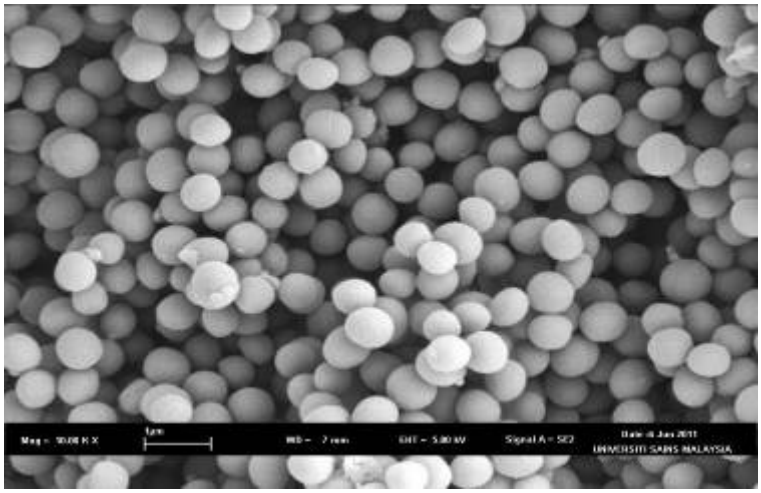
still showed intact cocci shape, meaning the extract at concentration of MIC do not sufficient in killing the bacterial cells, but only inhibiting the growth of the bacterial cells. However, it is clear that the bacterial cells started to clump together, as one of the stress response of the cells after treated with methanolic extract (Sreekanth *et al*., 2011). Besides, on some of the cells, the formation of the cavities can be observed. The formation of cavities increase the permeability of the cell wall and affect the physiological and metabolic activities of the cells (Mourad, 2010; Guo *et al*., 2011). This is mirroring that the Gram positive cell wall of MRSA is the target of antimicrobial compounds.

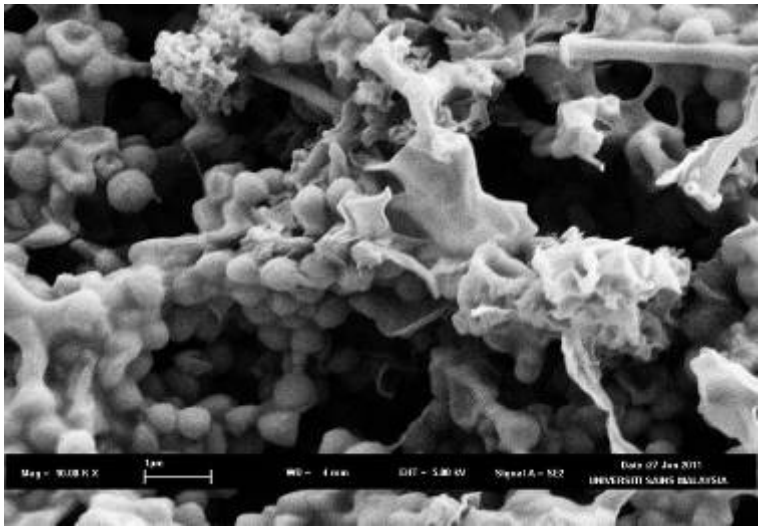
Table 1. Time to achieve 25, 50, 90 and 99.9% growth reductions in growth of MRSA from starting inoculum.

Growth reduction	Median time (hour)	
	Methanolic extract	Control
25%	13.7	NR
50%	22.4	NR
90%	NR	NR
99.9%	NR	NR

* NR- not reached



(a)



(b)

Figure 1. The SEM micrographs of MRSA cells treated with (a) 5% DMSO control and (b) methanolic extract at concentration MIC.

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Conclusions

The study showed that *P. minioluteum* ED24, an endophytic fungus from medicinal herb *O. stamineus* exhibited significant anti-MRSA activity. Hence further studies should be conducted to purify and identify the antibiotic compounds in the methanolic extract.

Acknowledgements

The authors are thankful to National Science Fellowship (NSF) and USM post-graduate research grand scheme for the research grant and financial support in this study.

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