ISSN: 0974-2115
www.jchps.com Journal of Chemical and Pharmaceutical Sciences

Anti-MRSA Activity of *Penicillium minioluteum* ED24

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ABSTRACT

This study was aimed to ascertain the *in vitro* anti-MRSA effect of *Penicillium minioluteum*, an endophytic fungus previously isolated from medicinal herb *Orthosiphon stamineus* Benth. The anti-MRSA activity of the dichloromethane extract was determined by disc diffusion and broth microdilution assay. The size of the inhibition zone was comparable to chloramphenicol control. The extract also exhibited bactericidal effect on the MRSA cells based on its low minimal lethality concentration value obtained. The structures of the extract-treated bacterial cells were observed by using scanning electron microscope. After 48 hours exposure to the fungal extract, the bacterial cells lost their intact cocci shape and the structures were totally collapsed. Besides, the dichloromethane extract did not exert any acute and chronic toxicity effect on brine shrimp lethality assay. The dichromethane extract of *P. minioluteum* ED24 demonstrated significant inhibitory effect on MRSA cells. To our best knowledge, this is the first report of the antibacterial activity of *P. minioluteum*.

KEY WORDS: Anti-MRSA, Endophyte, *Penicillium minioluteum*.

1. INTRODUCTION

Methicilin-resistant *Staphylococcus aureus* (MRSA) can be defined as a strain of *S. aureus* that gained resistant to a large group of beta-lactam antibiotics, which include penicillin and cephalosporin (Harris, 2001). In Malaysia, MRSA was introduced in the early of 1970s and today, MRSA is the causative agent of 21% *S. aureus*-infected individuals and it is affecting every state hospital in this country (Ehsanollah, 2009). In 2010, Hospital Kuala Lumpur located at the capital city records an annual MRSA prevalence of over 40%, particularly in orthopedic clinic (Ramli, 2012; Mariana, 2008) reported the first community-acquired MRSA in Malaysia from healthy university student. The finding also demonstrated the spread of ecologically successful European clone to Asian continent and hence continued surveillance was necessary. In recent years, Rashid (2013) reported five cases of community acquired-MRSA from the clinical samples in Universiti Kebangsaan Malaysia Medical Centre. All cases were skin and soft-tissue infection, and all these strains were resistant to penicillin, oxacillin and methicilin. Hence the emergence of MRSA in Malaysia with epidemic and pathogenic potential should be taken seriously.

This study was aimed to ascertain the *in vitro* anti-MRSA effect of *Penicillium minioluteum*, an endophytic fungus previously isolated from medicinal herb *Orthosiphon stamineus* Benth. Besides, the toxicity effect of the extract was also determined by brine shrimp lethality assay.

2. MATERIALS AND METHODS

2.1. Endophytic fungus and storage: The endophytic fungus *P. minioluteum* ED24 previously isolated from *O. stamineus* Benth was deposited at Industrial Biotechnology Research Laboratory, Universiti Sains Malaysia, Penang, Malaysia. The fungal isolate was cultivated on Potato Dextrose Agar (PDA; AES) supplemented with powdered host plant materials (5 g/L) and stored at 4 ^{0}C prior to use. The isolate was subcultured on fresh medium every four weeks to ensure purity and viability.

2.2. Culture media: Yeast extract sucrose (YES) broth (yeast extract 20 g/L, sucrose 40 g/L, magnesium sulfate 0.5 g/L) supplemented with water extract of *O. stamineus* was used to cultivate the fungus in the shake-flask system. The plant extract was prepared by boiling 10g of dried plant material in 500 mL distilled water for 30 min. The extract was filtered and mixed with freshly prepared culture medium and autoclaved at 121^{0}C for 15 min.

2.3. Fermentation and extraction: The inoculum was prepared by introducing two mycelial agar plugs into 250 mL Erlenmeyer flasks containing 100 mL of YES medium. The cultures were grown at 30 ^{0}C in a shaker at 120 rpm. After 20 days of incubation, the fermentative broth and fungal biomass were separated by centrifugation at 5311g (Sigma; Model 4K15).The freeze-dried fungal biomass was then extracted thrice with dichloromethane (1:50, w/v). The extract was concentrated to dryness using a rotary evaporator under reduced pressure to a crude extract paste.

2.4. Disc diffusion assay: The assay was conducted as per the procedure defined by Espinel-Ingroff (2007) and Jorgensen and Turnidge (2007). Mueller Hinton agar (Hi-media) plate was used. The assay was carried out by inoculating 100 µL of suspension containing 1×10^6 cfu/mL of MRSA. Sterile Whatman antibiotic disc, impregnated with 20 µL of each extracts of 50 mg/mL concentration, were then placed on the surface of inoculated medium. Ten 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 bacteria. The diameter of the inhibition zone was measured after the incubation period.

2.5. Broth Microdilution Assay: The minimal inhibitory concentration (MIC) was determined by using sterile 96-well microtiter plate (Espinel-Ingroff, 2007; Jorgensen and Turnidge, 2007). The prepared inoculum was diluted by sterile Muller Hinton broth medium to the inoculum size 5×10^3 cfu/mL of MRSA cells after the addition of the extract. The fungal extract was prepared at 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. 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 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 where the microbial growth occurred. To determine minimal lethality concentration (MLC) of the extract, 100 µL of the sample from each well was taken and suitably diluted before spreading on agar plates to judge the viability. The viable cell count method was performed. The MLC was recorded as the lowest concentration of extract that resulted in 99.9% growth reduction relative to the control.

2.6. Time kill curve study: The experiment was conducted according to recommendations by the CLSI with modifications (Swenson, 2007). The fungal extracts were tested at four concentrations: the MIC susceptibility breakpoint concentration, twice the MIC, MLC and also twice the MLC. The ethyl acetate extract of the fungal biomass was prepared at concentration ten times higher than the desired concentration. To achieve the final volume 50 mL in each flask, 1: 10 dilution of the extract was performed by addition of 25 mL inoculum and 20 mL of sterile broth medium. The flasks were incubated in rotatory shaker for 48 hours at 37 ^{0}C with rotational speed of 150 rpm. At predetermined time points (0, 4, 8, ... 44, 48 hours), 1 mL of sample was removed from the flask and diluted suitably with sterile saline. Viable cell count was performed by inoculating the diluted sample on nutrient agar plates.

2.7. Structural degeneration of the fraction-treated MRSA cells: Firstly 1.0 mL of bacterial inoculum was added into an 100 mL Erlenmeyer flask containing 23 mL of sterile nutrient broth. To obtain the seed culture of MRSA, the flask was incubated at 37 ^{0}C for overnight with agitation speed of 150 rpm. The bacterial growth was indicated by the cloudy appearance of the broth medium. After the incubation period, 0.1 mL of fraction Ma10 was added into the seed culture to achieve the final concentration of MIC (32 µg/mL). Besides, 1.0 mL of methanol was added into the seed culture as the negative control to detect the solvent effect. The mixtures were then incubated again at 37 ^{0}C for 12 hours, 24 hours and 48 hours respectively. After the pre-determined incubation period, the pellet of the bacterial cells was obtained by centrifugation at 10 g. The bacterial cell pellet was collected and fixed in Mc Dowell-Trump fixative.

The fixed cell pellets were suspended in 1% osmium tetroxide in phosphate buffer (pH 7.2) for one hour. The pellets were then re-suspended in sterile distilled water and centrifuged at 5 g for 15 minutes. The pellets were then dehydrated by immersion in 50%-70%-95%-100% ethanol series and also hexamethyldisilazene (HMDS) for 15 minutes consecutively. After that, the cell pellets were dried in a dessicator and the dried cells were glued on a sample holder using double-sided tape before covering with gold by Sputter Coater (Fison SC-515, UK). The sample was viewed under scanning electron microscope (SEM) to observe the morphology of the bacterial cells.

***2.8. In vivo* toxicity:** The brine shrimp lethality assay was employed in this study (Carballo, 2002). Firstly, artificial seawater with salinity of 25 ppt was prepared by dissolving 38 g of sea salt in 1.0 L of distilled water. After that, 1.0 g of dried *Artemia salina* cysts from Great Sea Lake (Sander, USA) was added into the freshly prepared seawater at 28 ^{0}C, under continuous light regime and aeration for 48 hours. Under this condition, the nauplii were hatched.

Then, 200 mg/mL of fraction Ma10 was prepared by dissolving 0.2 g of the dried paste in 1 mL of DMSO. Then 5.0 mL of the fractions with final concentration ranged from 0.2 to 2.0 mg/mL were prepared by diluting the fraction with artificial seawater. The mixture was shaken gently to ensure complete diffusion of the fraction. On the other hand, 1% DMSO diluted with artificial seawater was used as negative control to detect the solvent effect.

Approximately 48 hours after hatching, the active phototrophic nauplii were collected with a Pasteur pipette from the lighted side and concentrated in a small vial. About 20 active nauplii were transferred to each universal bottle containing the fraction Ma10 diluted with artificial seawater. All of the universal bottles were shaken from time to time to increase the aeration. The toxicity was determined after 6 hours (acute toxicity) and 24 hours (chronic toxicity) respectively. The number of survivors was counted under light microscope. The larvae were considered as dead if they did not exhibit any internal or external movement. The percentage of lethality was calculated.

$$\text{Percentage of lethality (\%)} = \frac{\text{Number of dead nauplii}}{\text{Initial number of live nauplii}} \times 100$$

3. RESULTS AND DISCUSSION

The dichloromethane extract exhibited a clear zone with a diameter of 18.7 ± 0.5 mm on disc diffusion assay. The size of the zone is comparable to the antibiotic control (18.0 ± 1.0 mm). To the best of our knowledge, this is the first report of antibacterial activity of *P. minioluteum*. Besides, the extract also showed a MIC value of

31.25 µg/mL and MLC value of 125 µg/mL on MRSA. The low MIC value indicates the significant inhibitory activity of MRSA. It is also note that the MIC value was significantly higher than the MLC value, indicating that a higher concentration of extract was needed to kill the bacterial cells, instead of inhibiting the bacterial growth. The results again showed that endophytic fungi can be a potential source of antimicrobial compounds to combat multi-drug resistant pathogenic strains. The activity was bactericidal as the MLC value of the extract was not more than fourfold of its MIC value.

Figure 1 shows the time kill curve of the dichloromethane extract on MRSA. Based on the kill curve study, the control growth curve showed four distinct growth phases, they are lag phase, log phase, stationary phase and death phase. However, with the addition of the fungal extract, the time kill curves of the test bacteria showed different growth pattern. With the increase of the extract concentration, the growth of bacteria in term of colony forming units was reduced, and the time to achieve 99.9% reduction in the colony forming unit in contrary to initial inoculums was also reduced. No post-antibiotic effect was observed in any of the extract concentration tested. At the concentration of MIC, 99.9% killing of initial inoculums was not achieved, but no exponential growth was observed even after 24 hours of incubation. This implies that the concentration was only sufficient to inhibit the growth of test bacteria, but not killing the bacterial cells. At the concentration of MLC, 99.9% killing was observed, mirroring the results obtained via broth microdilution assay. The bactericidal activity of the extract can be detected by the reduction of viable cells that more than 3 $\log_{10}$ in the kinetic kill curves. At the concentration of 2MLC, the kill curve did not show any significant growth. The time to achieve 99.9% killing was significantly shorter than the MLC and the viable cells obtained at the end of the incubation period were lower than the MLC.

Figure 2 shows the MRSA cells treated with methanol as the negative control to detect the solvent effect. The bacterial cells were in intact coccal shape (Figure 2A). The surfaces of the bacterial cells were smooth. After 48 hours of treatment with methanol, the bacterial cells still remained in its intact structures (Figure 2B). The bacterial cells were grown actively which indicated by the cell underwent binary fission (indicated by arrow). The SEM micrographs showed that the methanol did not exhibit any inhibitory activity on the bacterial cells.

Figure 3 shows the structural degeneration of MRSA cells after treated with the fraction Ma10. In general, the degree of alteration of the bacterial cells was correlated with the time of exposure, where severity of alterations increased as the exposure time increased. At the beginning of the experiment, the bacterial cells showed intact coccal structure (Figure 3A). After 12 hours exposure to fraction Ma10, the bacterial cells started to clump together as the stress response to the fraction (Figure 3B). Under stressed condition, the secretion of sticky mucus caused the bacterial cells clumped together and their normal cellular function was affected (Ron, 2006).However, no significant change was noted in the structure of the cells. Only very small numbers of the cells were lysed. The SEM micrograph of the bacterial cells after 24 hours of exposure to fraction Ma 10 was mirroring the results of broth microdilution assay. At 24 hours, most of the cells still intact in shape, it is marking that this concentration was not effective in killing the bacterial cells, but only inhibiting the growth (Figure 3C). The bacterial cells were covered by a thick mucoid layer, this is one of the response of bacterial cells under stress condition.

After 48 hours of exposure, the structures of the cells were totally collapse, meaning that most of the cells lost their intact cocci shape (Figure 3D). The invaginations of the cell walls were observed for the bacterial cells. The complete invagination of the cell wall indicates the lost of the cellular materials and organelle from the cytoplasm of the cells (Sasidharan, 2008). The degeneration of the cell wall structure causes the lysis of the cells when subjected to low osmotic pressure. The cells were totally damaged and its structures were totally collapsed. At this stage, the bacterial cells lost their metabolic activities and the growth of the bacteria was completely inhibited by the fraction Ma10.

Figure 4 shows the graph of concentration versus percentage of lethality for both acute and chronic toxicity of the dichloromethane extract. By definition, 50% lethal concentration (LC_{50}) is the concentration that kills half of the sample population in a specific period of exposure (Simionatto, 2005). The acute toxicity describes the adverse effects of a substance in a short period of time which usually less than 10 hours, where the chronic toxicity describes the adverse effects of a substance over a long period of time, usually 24 hours (Sasidharan, 2008). In general, the mortality of *A. salina* increased as the concentration of the fraction tested increased. Based on the results, the LC_{50} values for acute and chronic toxicity were significantly different, where the LC_{50} value for acute toxicity was 1.48 mg/mL and chronic toxicity was 1.13 mg/mL. According to Simionatto (2005), the extract with LC_{50} value more than 1.0 mg/mL indicated that the extract is not toxic to *A. salina*. In this study, the fraction Ma10 was not toxic to *A. salina* based on the LC_{50} value obtained.

Galvalisi (2012) reported the toxicity effect of the culture filtrate of two different strains of *P. minioluteum*, one of them was nontoxic and another strain was highly toxic to *A. salina*. To date, no report available was on mycotoxin production by *P. minioluteum*. Based on the results, the toxicity effect of *P. minioluteum* was strain-dependent. Besides, the toxic substance produced by the fungus may be eliminated through the fractionation process, which contributed to the high LC_{50} value of the fraction Ma10 in this study.

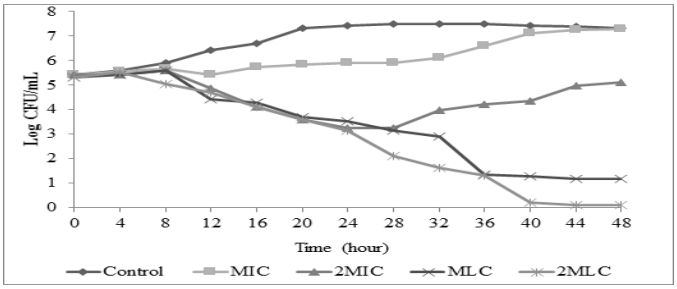
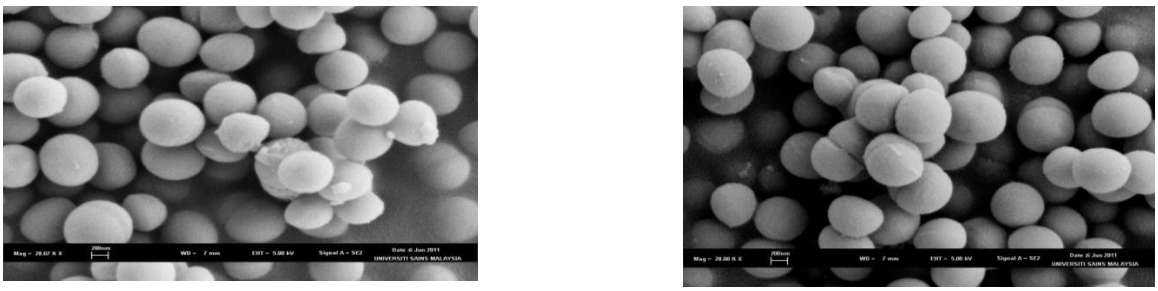
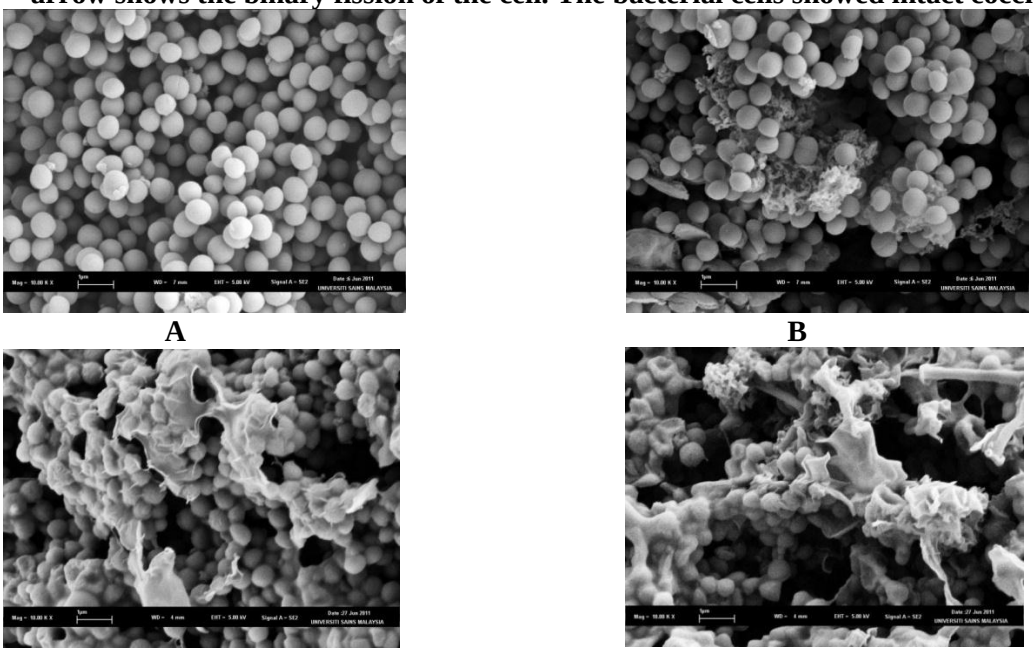


Figure.1.Time kill curve of dichloromethane extract of *P. minioluteum* ED24 on MRSA



A **B**

Figure.2.The structures of the MRSA cells treated with methanol for (A) 0 hour and (B) 48 hours. The arrow shows the binary fission of the cell. The bacterial cells showed intact cocci shape



A **B**

C **D**

Figure 3: The structures of the MRSA cells treated with fraction Ma10 at concentration of MIC for (A) 0 hour (B) 12 hours (C) 24 hours and (D) 48 hours.The degree of alteration of the bacterial cells was correlated with the time of exposure, where severity of alterations increased as the exposure time increased

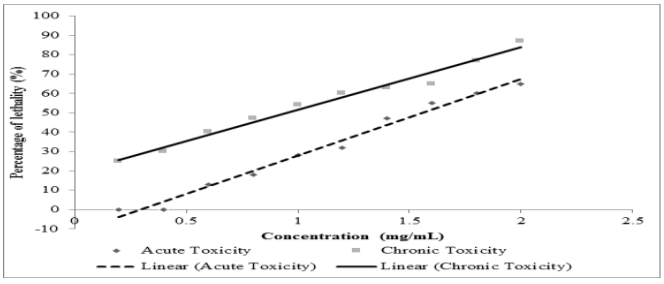


Figure.4.The acute and chronic toxicity study of dichloromethane on brine shrimp lethality assay. The mortality of *A. salina* increased as the concentration of the extract tested increased

4. CONCLUSION

The dichloromethane extract of *P. minioluteum* exhibited significant *in vitro* anti-MRSA activity. It exhibited bacteriocidal effect on the bacterial cells and the cell structures were totally collapsed after 48 hours of exposure of the extract. Further investigation is necessary to isolate and identify the bioactive constituents of the extract.

5. ACKNOWLEDGEMENT

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

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