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Article

Antimicrobial efficacy of endophytic *Penicillium purpurogenum* ED76 against clinical pathogens and its possible mode of action

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This study was aimed to evaluate the antimicrobial activity of *Penicillium purpurogenum* ED76 on several clinically important microorganisms. The endophytic fungus *P. purpurogenum* ED76 was previously isolated from *Swietenia macrophylla* leaf. The antimicrobial efficacy of *P. purpurogenum* ED76 dichloromethane extract was determined via disc diffusion and broth microdilution assay. A kill curve study was conducted and the morphology of extract treated bacterial cells were viewed under scanning electron microscope. The dichloromethane extract showed significant inhibitory activity on 4 test bacteria and 2 test yeasts. The minimal inhibitory concentration of the extract ranged from 125 to 1,000 µg/ml, which indicates the different susceptibility levels of the test microorganisms to the fungal extract. The kill curve study has revealed a concentration-dependent inhibition for all test microorganisms. With the increase of the extract concentration, the microbial growth was significantly reduced. The scanning electron micrograph of dichloromethane extract-treated *Staphylococcus aureus* cells showed the total damage of the cells. The cell wall invagination of the bacterial cells also indicates the loss of cellular materials and metabolic activity. The gas chromatography mass spectrometry analysis of the extract also showed that the major compound was stigmasterol, which constitutes 45.30% of the total area. The dichloromethane extract of *P. purpurogenum* ED76 exhibited significant inhibitory activity on several clinically important bacteria and yeasts. The study proposed a possible mode of action that the extract cause significant damage to the morphology of *S. aureus* cells.

Keywords: *Penicillium purpurogenum*, *Swietenia macrophylla*, antimicrobial activity, endophyte

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The synthesis of large numbers of antibiotics over the past three decades caused complacency about the threat of antibiotic resistance. Drug resistance is frequently encountered in hospital-acquired pathogens that usually in critically ill or immune-suppressed patients (Andhale *et al*., 2016). The resistance of microorganisms to low cost antibiotics also significantly increase the health care spending of the country (Friedman *et al*., 2016). Thus, the pharmaceutical industry and academic institutions are investing vast resources to produce safe and effective antimicrobial drugs (Pandey *et al*., 2015; Pandey and Shweta, 2016).

The possibility of getting organisms containing new metabolites are reducing rapidly. Endophytes, which occupy a unique biotope with global estimation up to one million species, are a great choice to avoid replication in the study of natural com-

pounds (Strobel and Daisy, 2003). By definition, endophytes are microorganisms which reside a part of its life cycle in the living plant tissues, without causing any diseases symptoms to the host plant (Tan and Zou, 2001). They are potential sources of new natural bioactive compounds and also plant metabolites. Since endophytes can be found in nearly all living plant species, a scientific basis in plant selection is crucial to isolate microorganisms with pharmaceutical potential. In this study, the endophytic fungus was previously isolated by our group from a medicinal plant, *Swietenia macrophylla*. The plant is originated from Central and South America. The plant was traditionally used for pain relief and tonic. Besides, it is known for the production of swietenolide, which exhibited broad spectrum antimicrobial activity (Rahman *et al*., 2009).

The members of genus *Penicillium* are well known for the production of antibiotics. *Penicillium purpurogenum* has been isolated from wide ranges of substrates, particularly soil and wood. They are known for the production of polyketide red pigments, which are natural colorants used in food industry (Padmapriya *et al*., 2015). They are also reported as a good producers of enzymes, such as xylosidases, xylanases, phytase, glucosidases, and glucoronisadases (Naverrete *et al*., 2012). However, their antimicrobial activity is not well characterized. The reports on endophytic strains of *P. purpurogenum* also hardly available. Thus, this study was aimed to evaluate the antimicrobial activity of *P. purpurogenum* ED76, an endophytic fungus isolated from *S. macrophylla*. Besides, the morphology of the extract-treated bacterial cells were observed. The bioactive constituents of the dichloromethane extract was characterized via gas chromatography-mass spectrometry (GCMS) analysis.

Materials and Methods

Endophytic fungal isolate

The endophytic fungus *P. purpurogenum* ED76 used in this study was previously isolated from *S. macrophylla* leaf by Darah *et al*. (2014). The fungal cultures were maintained on Malt extract agar (MEA) slants at 4°C prior to use.

Culture medium

Yeast extract sucrose (YES) broth [20 g/L yeast extract (AES), 40 g/L sucrose, 0.5 g/L Magnesium sulphate] was used to cultivate the fungus, with and without aqueous extract of host plant *S. macrophylla*. To prepare the aqueous extract, fresh *S. macrophylla* leaves were collected in Universiti Sains Malaysia main campus, Pulau Pinang (5° 21′ 15.494, 100° 18′ 10.507) by hand picking method. The samples were washed from the extraneous matter with running tap water and rinse repeatedly. The leaf samples were cut into smaller pieces and dried in an oven at 60°C until constant weight were obtained. After that, the leaves were ground into powder form using a food grinder. The aqueous extract was prepared by boiling 5 g of dried plant material in 1 L of distilled water for 30 min. The pH of the medium was adjusted to pH 6.0 prior to autoclave at 121°C for 15 min.

Fermentation

The inoculum was prepared by introducing two fungal plugs of 1.0 cm in diameter into 250 ml Erlenmeyer flasks containing 100 ml of YES medium. The inoculated flasks were incubated in the dark at 25°C in static condition for 20 days.

Extraction

After the incubation period, the cultures were harvested by mixing with acetone at 1:1 ratio (v/v). The mixtures were then centrifuged at 5,300 × *g* for 15 min. The acetone was then removed from the supernatant by evaporation under reduced pressure at 56°C. An equal volume of dichloromethane (1:1, v/v) was added into a separation funnel to extract the bioactive compounds. The organic layer was collected as dichloromethane extract. The extract was dried with rotatory evaporator to obtain the crude extract paste. A control was also prepared by using sterile medium following the same procedure used for fermentative broth.

Test microorganisms

The test microorganisms were clinical isolates from hospitalized patients obtained from the microbial cultures collection of Industrial Biotechnology Research Laboratory, Universiti Sains Malaysia. The microorganisms used in this study were 9 bacteria (*Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*,

Acinetobacter anitratus, *Streptococcus faecalis*, *Klebsiella pneumoniae*, and *Shigella boydii*), 3 yeasts (*Candida albicans*, *Candida utilis*, and *Cryptococcus neoformans*) and 4 fungi (*Trichophyton rubrum*, *Microsporum fulvum*, *Rhizopus stolonifera*, and *Aspergillus fumigatus*). The turbidity of bacterial and yeast suspensions was optically adjusted to match the 0.5 McFarland standard. The density of the fungal suspensions was counted with haemacytometer (Neubauer) under a light microscope. The density of suspension was adjusted to approximately 4×10^5 spores/ml.

Disc diffusion assay

The assay was performed to screen the antimicrobial activity of the extracts according to protocols defined by Tong *et al*. (2014). Nutrient agar (NA) was used for test bacteria and yeasts whereas the test fungi were inoculated on potato dextrose agar (PDA). Each plate was poured with 20 ml of molten agar onto 9 cm Petri dish to get a uniform thickness. 0.1 ml of microbial suspension was spread on agar plate. Crude extracts were dissolved in methanol. Sterile paper discs impregnated with 20 µl of crude extracts at a concentration of 50 mg/ml were placed on the surface of the inoculated medium. For positive control, 30 µg/ml of chloramphenicol was used for test bacteria and 30 µg/ml ketoconazole was used for test yeasts and fungi. Methanol was included as negative control to detect the solvent effect. Within 15 min after the discs were applied, the plates were incubated in an inverted position. All plates containing bacteria and yeast were incubated at 37°C for 24 h, whereas fungi were incubated 30°C for 72 h. The formation of inhibition zones around the discs indicated the antimicrobial activities. The diameter of the inhibition zones and was measured.

Broth microdilution assay

The test microorganisms that exhibited susceptibility to dichloromethane extract of the culture supplemented with host plant extract were selected for further study. The assay was performed in sterile, 96-wells, U-shaped, microtiter plates according to Tong *et al.* (2014) & Thambi and Shafi (2015). Nutrient broth was used for test bacteria and Sabouraud Dextrose broth was used for test yeasts. A serial two-fold dilution of the extract was carried out with double strength sterile broth medium. For a final volume of 200 µl in each well, 100 µl of extract was added into 100 µl microbial suspension. The final concentrations of the extracts were ranged from 8000.00 µg/ml to 15.63 µg/ml. The sterility and negative control was also included. After 24 h of incubation at 37°C, 40 µl of *p*-Iodonitrotetrazolium Violet salt (Sigma) at the concentration of 0.2 mg/ml was added into each well to detect microbial growth. The color change from yellow to purple indicates the microbial growth. The minimal inhibitory concentration (MIC) was recorded as the lowest concentration to inhibit the growth of test microorganisms (Zakaria *et al*., 2011). After the MIC was recorded at 24 h, the viability of the test microorganisms in each well were determined by streaking a loopful of the mixture on NA plates. Minimal lethality concentration (MLC) was determined as the lowest concentration of extract to kill the test microorganisms.

Kill curve study

The assay was conducted as per defined by Darah *et al.* (2015). A dilution of inoculum was prepared by transferring 1 ml of inoculum into 23 ml of sterile nutrient broth. To achieve a final volume of 25 ml in each flask, 1 ml crude extract was added into the flask respectively. The fungal extracts were tested at 3 final concentrations: half MIC, MIC, and twice the MIC. Methanol was included as negative control. All the flasks were incubated at 37°C in a shaker with agitation speed of 150 rpm. Every 4 h, 500 µl of the samples was taken out within two consecutive days. The viable cell counts of the samples were determined by spreading the diluted inoculum on NA plates. The assay was repeated thrice at separate occasions. The kill curves were plotted as logarithm of the number of the viable cells versus incubation times.

Structural degeneration of the extract-treated *Staphylococcus aureus*

The standard inoculum of *S. aureus* was spread on NA plates. After the incubation at 37°C for 48 h, 1.0 ml of 50 mg/ml dichloromethane extract was pipetted on the agar surface. The extract was spread by swirling the plate. The plate was then further incubate at 37°C for 24 h. Methanol was used as negative control. After the incubation period, the agar cubes with approximately 5×5 mm^2 were excised from the plates.

The agar cubes were placed on a planchette with Tissue-Tek. The vapor fixation of the sample was done for 2 h by adding a few drops of 2% Osmium tetroxide. After the freeze drying process, the planchette was coated with 10 nm of gold palladium alloy. The samples were then viewed under scanning electron microscope (Leo Supra 50VP) to observe the morphology of the bacterial cells.

GCMS analysis

The analysis was performed by using dichloromethane extract of the culture supplemented with host plant extract by using gas chromatography instrument (Hewlett-Packard 6890N) with mass spectrometer (Hewlett-Packard 5973 inert mass selective detector). The column HP-5MS (length 30.0 m, internal diameter 0.25 mm) (Agilent) was used. The instrument was calibrated using absolute methanol (blank sample). The oven temperature was fixed at 70 to 285°C at 30°C/min and a hold for 2 min. Helium was used as carrier gas with a flow rate of 1.2 ml/min. The injector temperature was 280°C, injection volume of 1 µl with a split ratio of 1:5. The mass spectra were taken at 70 eV with a mass scan range of 35–650 amu. The identification of compounds was based on the comparison of their mass spectra with NIST02 Library.

Results and Discussion

Table 1 shows the antimicrobial activity of *P. purpurogenum* ED76 on disc diffusion assay. Our results demonstrated that *P. purpurogenum* ED76 showed a broad spectrum antimicrobial activity with the size of inhibition zones ranged from 8 to 17 mm. Both of the fungal dichloromethane extracts, from the cultivation media with or without host plant extract, showed inhibitory activity on 2 Gram-positive bacteria, 2 Gram-negative bacteria, and 2 test yeasts. Similarly, Geweely and Neveen (2011) reported that *P. purpurogenum* inhibits the growth of

Table 1. Antimicrobial activity of *P. purpurogenum* ED76 dichloromethane extract on disc diffusion assay

Test microorganisms	Plant extract	Without plant extract	Positive control	Negative control
BACTERIA				
Bacillus subtilis	-	-	+++	-
Bacillus cereus	++	+	+++	-
Staphylococcus aureus	+++	++	+++	-
Salmonella typhimurium	-	-	++	-
Klebsiella pneumoniae	-	-	++	-
Pseudomonas aeruginosa	-	-	+++	-
Acinetobacter anitratus	++	+	+++	-
Streptococcus faecalis	-	-	++	-
Shigella boydii	++	+	+++	-
YEASTS				
Candida albicans	+	+	++	-
Candida utilis	-	-	+	-
Crytococcus neoformans	++	+	+	-
FUNGI				
Trichophyton rubrum	-	-	+	-
Microsporum fulvum	-	-	+	-
Rhizophus stolonifer	-	-	+++	-
Aspergillus fumigatus	-	-	++	-

Notes: "Plant extract" refers to the dichloromethane extract of the fungal culture supplemented with host plant extract, "Without plant extract" refers to dichloromethane extract of the fungal culture without host plant extract supplementation, "Positive control" refers to drug control (chloramphenicol for test bacteria and yeasts, ketoconazole for test fungi), "Negative control" refers to methanol solvent control.
The antimicrobial activity was determined based on the diameter of inhibition zone measured in mm. The result of screening test was recorded according to scale: +++= ≥ 15 mm, ++= 10-14 mm, += ≤ 9 mm, - = no inhibition zone observed.

all bacterial species tested. The two extracts showed inhibitory activity on the same microbial species, however the extract from culture with host plant extract showed significantly larger inhibition zones on all susceptible test microorganisms ($P \leq 0.05$). The increase in zone size can be resulted from the increased bioactive metabolites production by the isolate triggered by the presence of host extract in the culture medium. The observation is in contrast with the results reported by Tong *et al*. (2012) where they observed the anticandidal compound production by endophytic fungus when the host extract was supplemented in the culture medium. In this study, none of the extracts exhibited antimicrobial effect on filamentous fungi. The resistant of test fungi to the extracts can be due to the similarities in eukaryotic structural characteristics.

The antimicrobial susceptibility test results for dichloromethane extract, prepared from fungal culture with host extract, are presented in Table 2. The MIC of the extract ranged from 125 to 1,000 µg/ml. The wide range of MIC values indicates the different susceptibility levels of the test microorganisms to the fungal extract. The variation in the susceptibility can be caused by the bioactive compounds present in the extract, where some of the microorganisms are more sensitive to certain type of compounds, which leads to low MIC (Ciusa *et al*., 2012). This contributes to the low MIC value. The MLC of the extract is significantly higher than the MIC, indicates that the antimicrobial activity of the extract was concentration-dependent. A higher concentration of extract was needed to kill the test microorganisms, than to inhibit the growth. *C. albicans* and *C. neoformans* were susceptible to the extract. The extract exhibited significant fungicidal activity on *C. neoformans*, as Levison (2000) reported that the MLC for fungicidal drugs is usually not more than four-fold higher than their MIC. The finding is significant as *C. albicans* and *C. neoformans* are medically important opportunistic pathogens. However, the extract showed bacteriostatic effect only on all test bacteria.

Table 2. MIC and MLC of *P. purpurogenum* ED76 dichloromethane extract on broth microdilution assay

Test microorganisms	MIC (µg/ml)	MLC (µg/ml)
Staphylococcus aureus	125	2,000
Bacillus cereus	125	4,000
Shigella boydii	1,000	4,000
Acinetobacter anitratus	500	4,000
Candida albicans	250	2,000
Cryptococcus neoformans	500	1,000

The standard plate count method was employed to enumerate the bacterial cells in the sample. This method was used because it enumerates only the living cells present in the sample. This is crucial to provide information about the effect of fungal extract on the growth of microbial cells. The kill curve study of *P. purpurogenum* ED76 dichloromethane extract against *S. aureus*, *A. anitratus*, and *C. albicans* are shown in Fig. 1. For all the test microorganisms, no post-antibiotic effects were observed in any of the extract concentration tested. All the growth curves exhibited 4 distinct growth phases: lag phase, log phase, stationary phase and death phase. The study has revealed a concentration-dependent kill curve for all test microorganisms. With the increase of the extract concentration, the microbial

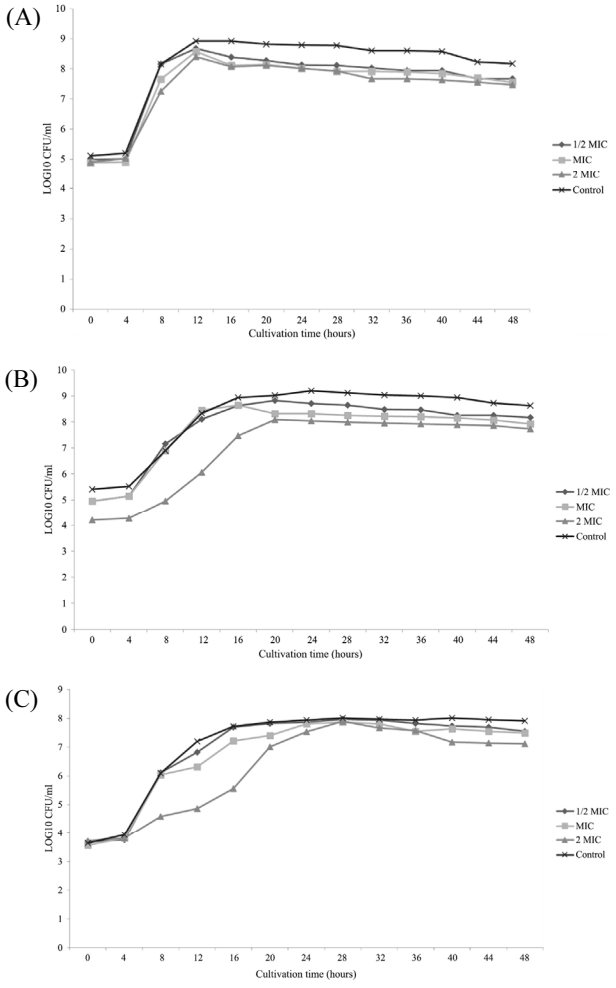

Fig. 1. The kill curve of *P. purpurogenum* ED76 dichloromethane extract on (A) *S. aureus*, (B) *A. anitratus*, and (C) *C. albicans*.

growth in term of colony forming units was significantly reduced. The results are in agreement with the data obtained from microdilution assay, where 99.9% killing of the microbial cells were not achieved for all the concentrations lower than MLC, implying that the concentration was not sufficient to kill any of the test microorganisms. Mirroring the results of antimicrobial susceptibility test, *C. albicans* was susceptible to the extract as 50% of growth reduction relative control was observed during log phase (8–20 h), at extract concentration of 2MIC.

Figure 2 shows the morphological changes of *S. aureus* cells after treated with *P. purpurogenum* ED76 dichloromethane extract at concentration of MIC. The bacterial cells treated with methanol that's served as negative control showed intact cocci shape. The surfaces of the bacterial cells were smooth. The SEM micrographs showed that methanol did not cause any changes on the cell morphology. The active growth of the bacterial cells can be indicated by the cells underwent binary fission. However, with the treatment of dichloromethane extract, the structures of the bacterial cells were damaged. Most of the bacterial cells lost their intact cocci shape. Besides, the invaginations of cell wall were observed for most of the *S. aureus* cells. The invaginated cell wall resulting in the release of cellular materials. At this stage, the metabolic activities of the bacterial were affected (Darah *et al*., 2015).

The retention time, area, chemical formula and the molecular weight of the compounds detected in GCMS analysis were depicted in Table 3. A total of 6 compounds were detected on the GCMS chromatogram with the matching factor ≥ 90%. In general, the major constituent of this fraction was sterol, where 2 sterols were detected based on the GCMS analysis. The major compound in the extract demonstrated 98% of similarity with stigmasterol, which constitutes 45.30% of the total area. Stigmasterol is a type of phytosterol that exhibited significant antibacterial and antifungal activity (Sanchez *et al*., 2007). Stigmasterol was previously isolated from *Penicillium crustosum*, *Penicillium chrysogenum*, and *Penicillium notatum* (Geweely and Neveen, 2011). To date, no report was available on stigmasterol production by *P. purpurogenum*. The antimicrobial activity of *P. purpurogenum* is mainly contributed by its polyketide pigments (Ghanem *et al*., 1990; Patil *et al*., 2015).

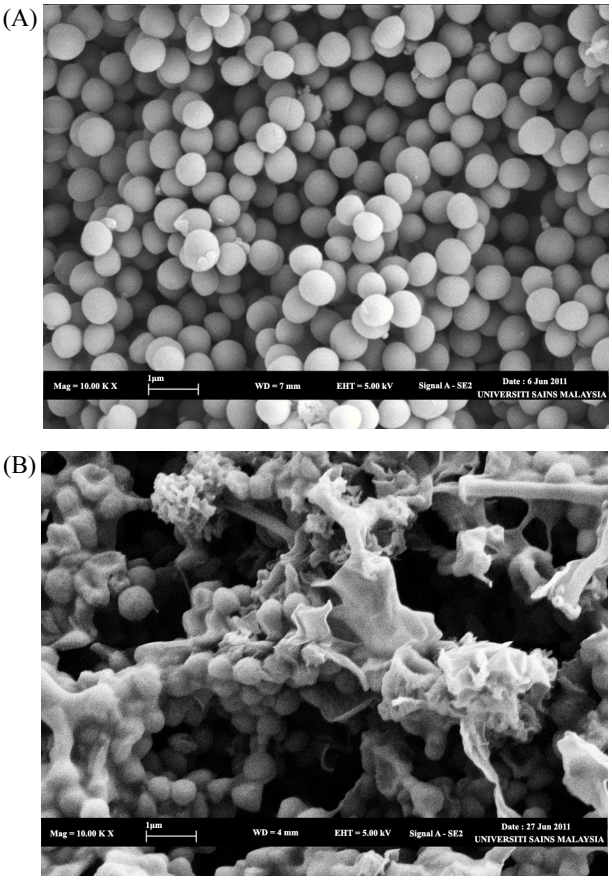

Fig. 2. The SEM of *S. aureus* cells treated with (A) methanol (B) dichloromethane extract of *P. purpurogenum* ED76 for 24 h.

Table 3. Characteristics of the compounds from GCMS analysis of the dichloromethane extract of *P. purpurogenum* ED76

No	Putative compound	Retention time (min)	Area (%)	Molecular formula	Molecular weight	Similarity (%)
1	7-Methyl-7H-dibenzo[b,g]carbozole	4.12	4.25	$C_{21}H_{15}N$	281	91
2	Dodecamethylcyclohexasiloxane	7.08	13.90	$C_{12}H_{36}O_6Si_6$	444	83
3	Tetradecamethylcycloheptasiloxane	8.22	3.89	$C_{14}H_{42}O_7Si_7$	519	75
4	Stigmastan-3,5,22-trien	17.98	10.65	$C_{29}H_{46}$	395	99
5	Stigmasterol	21.47	45.30	$C_{29}H_{48}O$	413	99
6	Cyclocholest-22-en-6-one	24.51	22.01	$C_{29}H_{46}O$	411	93

Conclusion

The dichloromethane extract of *P. purpurogenum* ED76 exhibited significant inhibitory activity on several clinically important bacteria and yeasts. The study proposed a possible mode of action that the extract cause significant damage to the morphology of *S. aureus* cells.

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