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Antimicrobial activity of *Aspergillus* sp. IBRL MP15 CCL, an endophytic fungus isolated from *Swietenia macrophylla* leaf against human pathogens

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ABSTRACT

Aims: Endophytes are microorganisms residing in the living tissues of the host plant and may contribute to their host plant by producing a plethora of bioactive compounds that provide survival value to the plant. This study aimed to evaluate the antimicrobial activity of *Aspergillus* sp. IBRL MP15 CCL, an endophytic fungus isolated from *Swietenia macrophylla* leaf.
Methodology and results: The antimicrobial activity was evaluated with disc diffusion and a colorimetric broth microdilution test against 15 organisms comprising of 4 Gram-positive bacteria and 4 Gram-negative bacteria, 4 fungi and 3 yeast. On disc diffusion assay, the fungal extract was shown to inhibit the growth of 7 test bacteria and 3 test yeast. The antibacterial activity was more pronounced with extract from fungal culture with host plant extract supplementation with significantly larger inhibition zones on all susceptible test microorganisms. The minimal inhibitory concentration of the extract ranged from 250 to 4000 µg/mL indicating different level of susceptibility of the tested pathogens against the fungal extract. The killing kinetic study shows that antimicrobial activity of the fungal extract is concentration dependent and it can act as bactericidal at higher concentration.
Conclusion, significance and impact of study: The findings of this study suggest that *Aspergillus* sp. IBRL MP15 CCL can be a promising source of antimicrobial agent to be further studied and developed.

Keywords: Antimicrobial activity, endophyte, *Swietenia macrophylla*, *Aspergillus*

INTRODUCTION

Extensive and often inappropriate use of antibiotics over the past three decades caused complacency about the threat of antibiotic resistance. The development of resistance by the existing pathogenic bacteria and fungi to the commercially established drugs not only reduces the effectiveness of treatments but also increases morbidity, mortality and the health care spending of the country (Friedman *et al*., 2016). As a result, intensive search for new and effective antimicrobial agents has been the center of spotlight among the pharmaceutical industry as well as the academic institutions (Xing *et al*., 2011; Zhao *et al*., 2011).

Nevertheless, drug discovery has been suffering from a lack of exploitable sources leading to the slow emergence of truly novel agents in recent years. Thus, researchers have shifted their focus to microorganisms isolated from special and unusual environments for more novel discoveries. Endophytes are a range of microbial species that reside in the plant tissues without causing apparent damage to the host plant (Tan and Zou, 2001; Strobel and Daisy, 2003). They occupy a unique biotope with global estimation up to one million species and appear to be associated with plants by producing compounds that promote vegetative growth, competitiveness and protection of the host against pathogens (Porras-Alfaro and Bayman, 2011). Therefore, endophytic fungi is a great source of study for new bioactive secondary metabolites such as the alkaloids, terpenoids, steroids, quinones, isocoumarins, lignans, phenylpropanoids, phenols, and lactones for medical

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applications (Rodriguez *et al*., 2009; Radic and Strukelj, 2012). It has been reported that 51% of all biologically active substances have been isolated from endophytic fungi. One of the renowned discovery of bioactive compounds from endophytic fungi is taxol, a powerful anticancer agent discovered from the endophytic fungi isolated from the bark of *Taxus brevifolia* (Stierle *et al*., 1993).

In the current study, we focus on the endophytic fungi isolated from *Swietenia macrophylla* for new inhibitory agents that are effective against human pathogens. *Swietenia macrophylla* is a member of the family *Meliaceae* which located in more than 40 countries including Brazil, Bolivia, Mexico, Guatemala, Peru and other Central American countries (Kadir, 2011). It was traditionally used to treat various diseases as well as for the production of swietenolide, which exhibited broad spectrum antimicrobial activity (Rahman *et al*., 2009). However, reports on endophytic strains of *S. macrophylla* are hardly available. In this study, by using bioassays with a panel of laboratory standard pathogenic control strains including fungi, yeast and bacteria, an endophytic fungus *Aspergillus* sp. IBRL MP15 CCL isolated from *S. macrophylla* leaves was shown possessed antimicrobial activity compared to authentic standards. This endophytic fungus has significant scientific and industrial potential as a promising producer of new antimicrobial agents.

MATERIALS AND METHODS

Endophytic fungal isolate

The endophytic fungus *Aspergillus* sp. IBRL MP15 CCL used in this study was previously isolated from *S. macrophylla* leaf. The fungal cultures were maintained on Malt extract agar 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 and 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 Czapek-Dox Broth [30 g/L sucrose, 3 g/L sodium nitrate, 1 g/L di-potassium hydrogen phosphate, 0.5 g/L potassium chloride, 0.5 g/L magnesium sulphateheptahydrate, 0.01 g/L ferrous sulphate heptahydrate] with and without host plant extract. The inoculated flasks were incubated in the dark at 25 °C in static condition for 20 days.

Extraction

After the incubation, an equal volume of 99.5% acetone was added into the fermentative broth culture at 1:1 ratio (v/v). The mixture was then centrifuged at 5000 rpm for 15 min. The acetone was removed by using a rotary evaporator (EYELA) at 56 °C. Then the fermentative broth was extracted with equal volume of ethyl acetate (v/v) in a separatory funnel. The organic layer was then collected as the ethyl acetate extract. The crude extracts were again dissolved in methanol during the bioassay for antimicrobial activity screening. A control was also prepared by using sterile medium following the same procedure used for fermentative broth.

Test microorganisms

The test microorganisms used in this study were clinical isolates obtained from hospitalized patients in Hospital Seberang Jaya, Penang and maintained at Industrial Biotechnology Research Laboratory, Universiti Sains Malaysia. A total of eight bacteria (*Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Acinetobacter anitratus*, *Streptococcus faecalis, Klebsiella pneumoniae and Shigella boydii*), four fungi (*Trichophyton rubrum, Microsporum fulvum, Rhizopus stolonifer and Aspergillus fumigatus*) and three yeasts *(Candida albicans, C. utilis and Cryptococcus neoformans*) were used in this study. These pathogens were selected in this study as they are among the most common causing agents of infection. The inoculum was prepared by adjusting the turbidity of bacterial and yeast suspensions optically using 0.5 McFarland standard. The density of the fungal suspensions was adjusted to approximately 4×10^5 spores/mL using haemacytometer (Neubauer) under a light microscope.

Disc diffusion assay

The assay was carried out as previously reported by Tong *et al.* (2014) to screen the extracts for antimicrobial activity. Mueller Hinton agar (Hi-media) was used to for bacteria, Mueller Hinton agar containing 2% dextrose and 0.5 µg/mL methylene blue for test yeasts, and RPMI 1640 (Sigma) agar was used for test fungi. 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 inoculum was spread on the agar plate. Standard 6 mm 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. Methanol was applied as a negative control whereas 30 µg/mL chloramphenicol was used as the positive controls for bacteria, 30 µg/mL

amphotericin B for fungi and yeasts, respectively. The plates were incubated at 30 °C for 48 to 96 h for fungi, and at 37 °C for 24 h for bacteria and yeasts. The zone of inhibition diameter was measured and the results were expressed as mean values ± standard deviation as the experiment was done in triplicate.

Microbial susceptibility testing

The test microorganisms that exhibited susceptibility to the extract during disc diffusion assay were subjected to the broth microdillution assay to determine the Minimal inhibitory concentration (MIC). The assay was conducted in sterile 96 wells, U-shaped microtiter plates as according to Tong *et al.* (2014). Nutrient broth and Sabouraud Dextrose broth were used as growth medium for the test bacteria and yeast respectively. 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 concentration 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 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. Minimal inhibitory concentration (MIC) is defined as the lowest concentration of antimicrobial agent that able to inhibit the growth of microorganisms. 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.

Killing kinetic study

The assay was conducted as reported previously by Ibrahim *et al.* (2014). Briefly, the fungal extracts were tested at 3 final concentrations: half MIC, MIC (500, 1000 and 250 µg/mL respectively for *S. faecalis*, *K. pneumoniae* and *C. utilis*) and double the MIC. Methanol was included as negative control. One milliliter of crude extract was added into flask containing a final volume of 25 mL of inoculum diluted with sterile nutrient broth. 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.

Micro-morphology observation

The agar plug containing the fungal mycelium was fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4 °C and post-fixed in 2% aqueous osmium tetraoxide for 4 h in the same buffer. After the post-fixation, the samples were dehydrated in series of graded alcohols. The dried samples were mounted over the stubs with double sided carbon tape. A thin layer of gold was coated over the samples by using an automated sputter coater. The specimens were observed using scanning electron microscope (FESEM LEO Supra 50VP, Carl Zeiss, Germany).

RESULTS AND DISCUSSION

Figure 1 shows the microscopic characteristics of isolate IBRL MP15 CCL grown on PDA (Figure 1A). Both micrographs show typical features of *Aspergillus* sp. with smooth walled conidiophores about 200-250 µm in length. The stipe width was about 6-7 µm. Conidial heads were radiate to slightly globose and biseriate. Its apical vesicle appears to be globose to subglobose. Conidia was globose to ellipsoidal with diameter around 3-4 µm which are the typical morphology of *Aspergillus* sp.

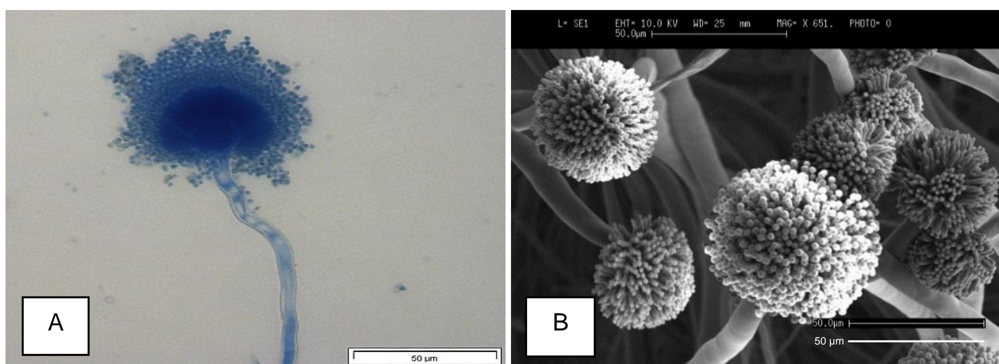

Figure 1: Micro-morphology of the isolate IBRL MP15 CCL. (A) Observed under a light microscope (400× magnification) (B) Scanning Electron Micrograph.

Table 1 shows the antimicrobial activity of *Aspergillus* sp. IBRL MP15 CCL on disc diffusion assay. Our results demonstrated that *Aspergillus* sp. IBRL MP15 CCL showed a broad spectrum antimicrobial activity with the

size of inhibition zones ranged from 8 to 16 mm. Both of the fungal ethyl acetate extract, from the cultivation media with or without host plant extract, showed inhibitory activity on all the 4 Gram positive bacteria, 3 Gram negative bacteria and 3 yeasts. The two extracts showed similar inhibitory activity on the Gram positive bacteria tested but not on the Gram negative bacteria. In general, the extract from culture with host plant extract showed significantly larger inhibition zones on the susceptible test microorganisms. This could be resulted from the increased of bioactive metabolites production by the endophytic fungi triggered by the presence of the host extract culture medium. As suggested by Strobel and Daisy (2003) that the endophytes and their biotopes are subjected to constant metabolic and environmental interaction which in turn will promote production of even more secondary metabolites. In this study, none of the extracts exhibited antimicrobial effect on filamentous fungi. Similarities in the eukaryotic structural characteristic could contribute to the resistant of test fungi to the extracts.

Table 1: Antimicrobial activity of *Aspergillus* sp. IBRL MP15 CCL extract on disc diffusion assay.

Test microorganism	Plant extract	Without plant extract	Positive control	Negative control
S. faecalis	++	++	+++	-
B. cereus	+++	++	+++	-
B. subtilis	++	+	+++	-
MRSA	++	++	+++	-
A. anitratus	++	++	+++	-
E. coli	-	-	+++	-
S. boydii	+++	-	+++	-
K. pneumoniae	+++	+	+++	-
Rhizopus sp.	-	-	+	-
A. fumigatus	-	-	+	-
T. rubrum	-	-	+	-
M. fulvum	-	-	++	-
C. albicans	+	+	++	-
C. utilis	+	+	++	-
C. neoformans	++	+	+++	-

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.

Table 2: MIC and MLC values of the extract of *Aspergillus* sp. IBRL MP15 CCL.

Microbes species	MIC value (µg/mL)	MLC value (µg/mL)
Gram-positive bacteria		
Streptococcus faecalis	500	4000
Bacillus subtillis	2000	2000
Bacillus cereus	1000	4000
Methicillin-resistant *Staphylococcus aureus*	4000	4000
Gram-negative bacteria		
Acinetobacter anitratus	2000	4000
Shigella boydii	2000	4000
Klebsiella pneumoniae	1000	4000
Yeast		
Candida albicans	250	1000
Candida utilis	250	4000
Cryptococcus neoformans	1000	4000

Table 2 shows the results of the antimicrobial susceptibility test using the fungal extract prepared with host extract. Minimal inhibitory concentration (MIC) is defined as the lowest concentration of antimicrobial agent that able to inhibit the growth of microorganisms. The fungal extract shows different susceptibility levels against the test microorganisms as indicated by the wide range values of MIC from 250 µg/mL to 4000 µg/mL. The difference in the effectiveness of the fungal extract to control and inhibit the growth of the microorganisms could be due to the complexity of chemical structure and concentration of active compounds in the extract. The variation could also stem from the difference in diffusion rate through the cell membrane particularly among the Gram positive and Gram negative bacteria. The extract however possessed significant fungicidal activity on the test yeasts. Parallel with the finding of Levison (2009) that the fungicidal drugs is usually not more than 4-fold higher

than the MIC. In general, the MLC of the extract is significantly higher than the MIC as the antimicrobial activity of the fungal extract is concentration dependent. Nevertheless, the extract of *Aspergillus* sp. IBRL MP15 CCL has only bacteriostatic effect on the test bacteria as higher concentration of extract was needed to kill the test microorganisms, than to inhibit the growth. The finding is significant since *C. albicans* is medically important opportunistic pathogens.

Result of time-kill kinetic studies at three different MIC of the fungal extract on *S. faecalis, K. pneunomiae* and *C. utilis* are shown in Figures 2 to 4. All the test microorganisms exhibit 4 distinct growth phases of lag phase, log phase, stationary and death phase. It is also shown that that the killing kinetic is concentration dependent whereby the higher concentration of the extract, the greater reduction of microbial growth in term of colony forming units. However, log growth phase was absent when concentration of the extract increased to double MIC indicating that the extract can as well act as bactericidal as higher concentration, where 99.9% killing of the microbial cells were achieved. However, such post-antibiotic effects were only observed in *S. faecalis* and *K. pneunomiae,* but not *C. utilis*. Echoing the results of the antimicrobial susceptibility test, *S. faecalis* was most susceptible to the extract as 50% of the growth reduction relative to control was observed during the log phase (4 – 20 h) at concentration of 500 µg/mL or MIC. This might be contributed by the typical structural characteristic of Gram positive bacteria with the absence of LPS outer membrane that in turn facilitates access of cell-wall active antibiotics to their site of action as reported in many studies previously. It is commonly accepted that Gram-negative bacteria exhibit high-level resistance to most classes of antibiotics, due to rapid expulsion of the impermeability of the bacteria's outer membrane (Poole, 2002).

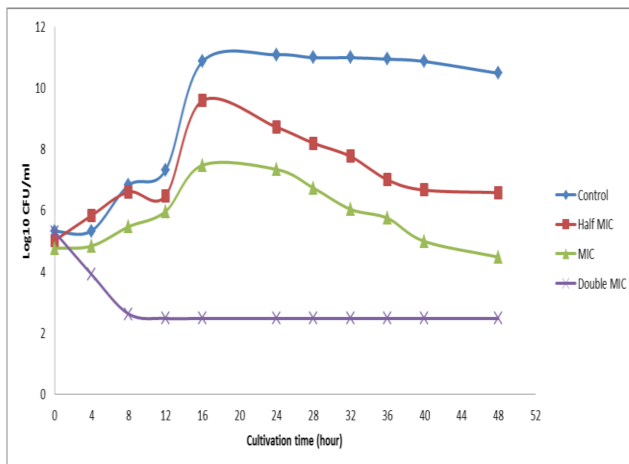

Figure 2: Growth profiles of *S. faecalis* after addition of various concentration of extract into its growth medium. 250 µg/mL (half MIC), 500 µg/mL (MIC) and 1000 µg/mL (double MIC).

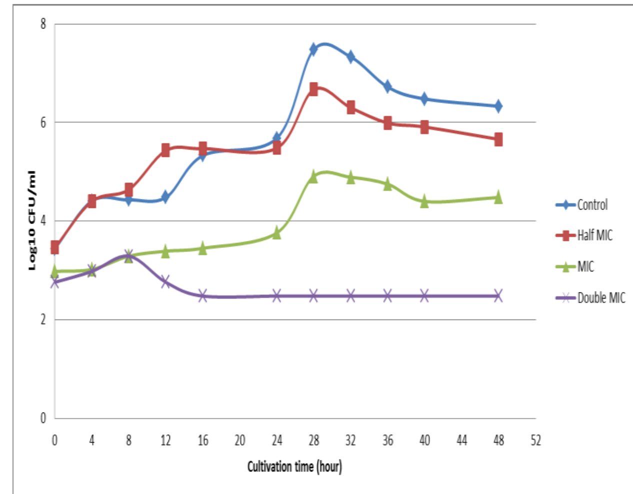

Figure 3: Growth profiles of *K. pneumoniae* after addition of various concentration of extract into its growth medium. 500 µg/mL (half MIC), 1000 µg/mL (MIC) and 2000 µg/mL (double MIC).

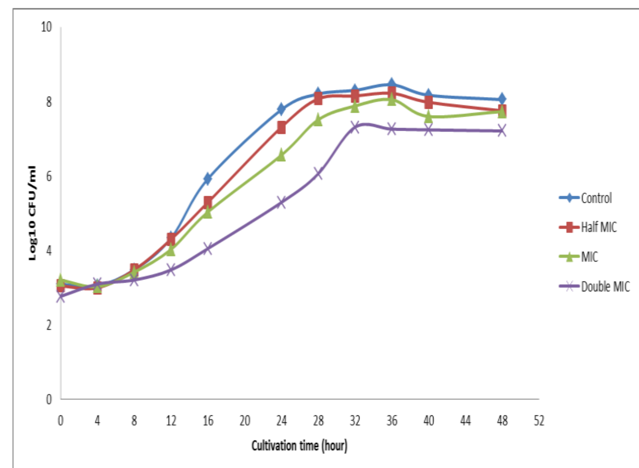

Figure 4: Growth profiles of *C. utilis* after addition of various concentration of extract into its growth medium. 125 µg/mL (half MIC), 250 µg/mL (MIC) and 500 µg/mL (double MIC).

Aspergillus is a genius comprised of filamentous fungi with both beneficial and pathogenic species. The diversity of secondary metabolites found in *Aspergillus* still largely remain unknown, this is mostly because many of the biosynthetic pathways remain silent or inactive normal conditions (Nützmann *et al.*, 2011). Nevertheless, metabolites produced are molecules essential to pharmaceutical, industrial, and agricultural interests and applications. The finding of this study have proven that the *Aspergillus* sp. IBRL MP15 CCL, an endophytic fungi isolated from *S. macrophylla* leaves was able to produce secondary metabolites that inhibit the growth of both

bacteria and yeasts, which is a promising source of new and effective antibiotic. However, further analysis and identification of the bioactive compound is necessary to understand the mechanisms and mode of its action in killing or prohibiting growth of microorganisms.

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