Malaysian Journal of Microbiology, Vol 16(3) 2020, pp. 219-228
DOI: http://dx.doi.org/10.21161/mjm.190605

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

# Time-kill study and morphological changes of *Proteus mirabilis* cells exposed to ethyl acetate crude extract of *Lasiodiplodia pseudotheobromae* IBRL OS-64

**Mohd Taufiq Mat Jalil*, Darah Ibrahim and Nurul Syakirah Mohamad Suhaimi**

Industrial Biotechnology Research Laboratory, School of Biological Sciences,
Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia.
Email: fiqz04@yahoo.com.sg

Received 3 October 2019; Received in revised form 6 November 2019; Accepted 23 December 2019

## ABSTRACT

**Aims:** To investigate time-kill curve and morphological changes of *Proteus mirabilis* cells exposed to ethyl acetate crude extract of endophytic fungus, *Lasiodiplodia pseudotheobromae* IBRL OS-64, isolated from *Ocimum sanctum*.
**Methodology and results:** Inhibitory effect of the fungal extract against the test bacteria via disc diffusion assay showed a fair antibacterial activity with diameter of inhibition zone was 12.0 ± 0.4 mm. The Minimal Inhibition Concentration (MIC) and Minimal Bactericidal Concentration (MBC) values of the ethyl acetate extract against *P. mirabilis* was 250 and 500 µg/mL, respectively. The value of MBC which is two-fold higher than MIC value indicated that the fungal extract exerted bactericidal effect on bacterial cells of *P. mirabilis*. Time-kill curve study revealed that the bactericidal effect of the crude extract towards test bacteria was both dose and time dependent. Scanning electron microscope (SEM) observation revealed that the bacterial cells of *P. mirabilis* exposed to fungal crude extract resulted in formation of pits, irregular shape of the bacterial cell and ultimately cell death beyond repair.
**Conclusion, significance and impact of the study:** The time-kill curve study, and cell morphological changes suggested the potential of ethyl acetate extract of *L. pseudotheobromae* IBRL OS-64 against *P. mirabilis* infection by formation of cavities, irregular bacterial cell that leads to ultimate cell death and the extract may have pharmaceutical potential to be develop as antibacterial agent.

*Keywords: Proteus mirabilis*, minimal inhibition concentration (MIC), minimal bactericidal concentration (MBC), time-kill study, scanning electron microscopy

## INTRODUCTION

*Proteus mirabilis* is one of human intestinal flora that has been known to cause serious infections in humans. It is a Gram-negative bacterium with rod shaped that is able to produce hydrogen sulfide (O'Hara *et al.*, 2000). This organism is one of the most common pathogens found in clinical specimens and could cause a variety of community acquired infections such as wound, urinary tract and blood stream infections (Endimiani *et al.*, 2005). *Proteus mirabilis* is also a well-known bacterium that possess ability to secrete urease which could generates ammonia and increases the pH of the urine up to 7.2 (Broomfield *et al.*, 2009). The alkaline urine induces calcium and magnesium crystallization which are able to block the catheter lumen resulting acute urinary retention. This phenomenon causes the development of bacteriuria and other infections that finally lead to shock, bacteremia and pyelonephritis (Kunin, 1989). Besides that, the strain can form biofilm on medical devices including catheter surface which resulting several complications (Scavone *et al.*, 2016). The emergence of biofilm-related infection as a result of the widespread use of medical devices in healthcare settings elevate polymer-association infections which mainly infected the immunocompromised patients with implanted medical devices (Sadovskaya *et al*., 2005; Percival *et al.*, 2015).

In recent years, an increasing trend of antimicrobial resistance by *Enterobacteriaceae* species, including *P. mirabilis* has been observed and this leads to a change of antimicrobial therapies, poor prognoses and thus elevates the mortality of rate of hospitalized patients (Chen *et al.*, 2012). According to Yu *et al.* (2015), this strain is found to be resistant to ampicillin, amikacin, chloramphenicol, norfloxacin, gentamicin, sulfamethoxazole, and sulfamethylisoxazole but susceptible to ofloxacin, cefotaxime, and ceftriaxone sodium. Due to its resistance to several antibiotic, there is a need for discovery of new antibiotic with broad spectrum and mode of actions.

Nowadays, the discovery of endophytic fungi isolated from medicinal plants has become popular among the researchers due to their capability to produce secondary

***Corresponding author**


ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 16(3) 2020, pp. 219-228
DOI: http://dx.doi.org/10.21161/mjm.190605

metabolites with pharmaceutical potentials (Strobel and Daisy, 2003; Zhang *et al.*, 2006). In the present study, the Malaysian ethnomedicinal plant, *Ocimum sanctum* Linn. or 'selasih' (local name) was selected as a host to harbor fungal endophytes due to its traditional use and ethnobotanical history. The plant has been reported to possess several bioactive activities including antibacterial (Rathnayaka, 2013), antifungal (Balakumar *et al.*, 2011), antioxidant (Selvam *et al.*, 2013), antiparasitic (Garcia *et al.*, 2010), antidiabetic (Somasundaram *et al.*, 2012), anticancer (Sridevi *et al.*, 2016) and so on. Interestingly, fungal endophytes isolated from medical plants are able to produce almost similar bioactive compounds or secondary metabolites as their hosts (Alvin *et al.*, 2014). Some of compounds are co-produced by endophytes with their respective hosts including azadirachtin, the natural insecticide (Kusari *et al.*, 2012) and anticancer drug, camptothecin (Puri *et al.*, 2005). The overlapping production of secondary metabolites by endophytes and their hosts may be due to their close relationship. Moreover, endophytes may have combined and shared genetic information from their hosts which elevates their defense mechanism against insects and pathogens and also stimulates their adaptability towards unfavourable conditions (Abdalla and McGaw, 2018).

*Lasiodiplodia pseudotheobromae* is one of the members of Botryosphaeriaceae that are known as plant pathogens which cause several infections including dieback (Kwon *et al.*, 2017), canker (Castro-Medina *et al.*, 2014), fruit rot (Nogueira Junior *et al.*, 2017) and pedicel/peduncle discoloration (Dissanayake *et al.*, 2015). This strain was reported to exert several pharmaceutical potentials including antibacteria (Wei *et al.*, 2014), antioxidant (Zhou *et al*., 2018), xanthine oxidase inhibitory (Kapoor and Saxena, 2014) and fibrinolytic activity (Meshram and Saxena, 2016). Several compounds have been reported from *L. pseudotheobromae* such as indole-3-carboxylic acid (Qian *et al.*, 2014), dihydroisocoumarin (Adetunji *et al.*, 2018) and palmarumycins (Lue *et al.*, 2014). However, the information regarding bioactive compounds and pharmaceutical potentials of *L. pseudotheobromae* are very scarce. Thus, the present study was designed to evaluate and observe the antibacterial activity of ethyl acetate extract of *L. pseudotheobromae* IBRL OS-64 against *P. mirabilis* and its effect to the bacterial cells. The study is very significant since the best of our knowledge, this is first report of *L. pseudotheobromae* IBRL OS-64 isolated from medicinal plant, *Ocimum sanctum* Linn.

## MATERIALS AND METHODS

### Endophytic fungus culture and maintenance

The endophytic fungus, *L. pseudotheobromae* IBRL OS-64 was provided by the Industrial Biotechnology Research Laboratory (IBRL), School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. The fungal isolate was cultured and maintained on potato dextrose agar (PDA) nourished with powdered host plant (2 g/L) and stored at 4 °C prior to use. The isolate was subcultured on sterile fresh media once a month to ensure purity and viability.

### Test microorganisms

The *P. mirabilis* culture from clinical sample was provided by the Industrial Biotechnology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. The bacterial culture was grown on nutrient agar (NA) and incubated at 37 °C for 24 h. The bacterial inoculum was prepared by picking five single colonies from 24 h old culture and transferred them into 5 mL of 0.85% (w/v) sterile physiological saline. The turbidity of the bacterial suspension was adjusted to match 0.5 McFarland standards (approximately $1 \times 10^8$ CFU/mL).

### Culture medium

Yeast extract sucrose (YES) broth (Merck, Germany) nourished with host plant water extract was used as a fermentative medium. Two grams of host plant powder was added into 1000 mL distilled water, boiled for 30 min and the mixture was filtered with muslin cloth. The filtered host plant water extract was added with 20 g/L yeast extract, 40 g/L sucrose and 0.5 g/L magnesium sulfate. The pH of the culture medium was adjusted to 6.0 and the medium was autoclaved at 121 °C for 15 min.

### Fermentation and extraction

Two mycelial plugs of 3 days old of endophytic fungal culture was introduced into 250 mL Erlenmeyer flasks containing 100 mL YES broth. The cultures were then incubated at 30 °C under static condition in the dark for 16 days. The fermentative broth and fungal biomass were separated out using filter papers (Whatman, No.1). The filtered broth was extracted thrice with an equal volume of ethyl acetate (1:1; v/v). The upper organic phase was collected and concentrated using a rotary evaporator. The concentrated mixture was left to dryness in a fume hood to obtain ethyl acetate fungal crude paste.

### Disc diffusion assay

The assay was carried out according to the method described by Jorgensen and Ferrero (2007); the fungal crude extracts were dissolved in 50% (v/v) ethyl acetate from fermentative broth to obtain 1 mg/mL extract. Microbial inoculum was swabbed on the surface of the Mueller Hinton agar (MHA) using sterile cotton swabs. An amount of 20 µL of the fungal extract was impregnated to the sterile Whatman antibiotic disc and then placed on the surface of previously seeded medium. Five percent of ethyl acetate was used as a negative control whilst chloramphenicol (30 µg/mL) was set as a positive control. The plate was then inversely incubated at 37 °C for 24 h. The diameter of inhibition zone formed surrounding the

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

disc was measured and recorded. The experiments were carried out in triplicates at different occasions.

**MIC and MBC determination**

The minimum inhibition concentration (MIC) of the fungal crude extract was determined in broth microdilution assay as described by Jorgensen and Ferraro (2009) in a sterile, 96-wells, U-shaped microtiter plate. The fungal extract was prepared in sterile Mueller Hinton Broth (MHB) medium and 100 µL of the extract was dispensed into each wells of microtiter plate. On the other hand, 100 µL of bacterial inoculum at approximately $1 \times 10^6$ CFU/mL was added into each of the wells for a final volume of 200 µL and the final concentration of bacterial in each well was $5 \times 10^5$ CFU/mL. Chloramphenicol was used as a positive control and 5% methanol was set as a negative control. An amount of 40 µL of 0.2 mg/mL p-iodonitrotetrazolium violet (INT) dissolved in 99.5% ethanol solution was loaded to each well as a growth indicator after a 24 h of incubation at 37 °C. The MIC value was determined and recorded as the lowest concentration of the crude extract that capable to inhibit the visible growth of test bacteria after 24 h of incubation period.

Minimum bactericidal concentration (MBC) of the fungal crude extract was subsequently determined from the MIC value according procedures described by CLSI (1999). An amount of 0.1 mL of sample in the wells that not show any bacterial growth at concentrations greater than the defined MIC was streaked on a fresh MHA and the procedures were repeated for the next wells in ascending extract concentrations. The inoculated plate was incubated at 37 °C for overnight. The MBC was observed and recorded as the lowest concentration of fungal crude extract that resulted in reduction of 99.9% bacterial growth relative to the growth control.

**Time-kill study**

The efficacy of the fungal extract against test bacteria was tested via time-kill study at concentration of 0.5× MIC, 1× MIC, and 2× MIC. The MIC value of *P. mirabilis* was 250 µg/mL as previously obtained. A volume of 0.1 mL of bacterial inoculum (approximately $1 \times 10^8$ CFU/mL) was introduced into 50 mL Erlenmeyer flask containing 19.9 mL MHB with different concentrations of extract (0.5 × MIC; 125 µg/mL, 1× MIC; 250 µg/mL and 2× MIC; 500 µg/mL) yielded the initial bacterial inoculum of approximately $5 \times 10^5$ CFU/mL. The culture consist of bacterial inoculum and 1% DMSO (v/v) in MHB was set as a growth control. Chloramphenicol (30 µg/mL) was used as the drug reference. The cultures were subsequently incubated in a rotary orbital shaker at 37 °C with agitation rate of 150 rpm for 48 h. A volume of 0.1 mL of aliquot sample was taken every 4 h during time interval of 0 to 48 h for viable cell count. The samples were diluted and spread plate onto fresh MHA followed by incubating at 37 °C for 24 h and the viable bacteria colonies were then counted. To determine the colony unit per millilitre (CFU/mL), the plates only with the number of colonies ranging from 30-300 was counted. A time-kill curve ($\log_{10}$ CFU/mL vs. time) was plotted for each extract concentrations and control. The growth reduction in which the time to achieve 50%, 90% 99% and 99.9% of bacterial cells reduction was calculated.

**Scanning Electron Microscopy (SEM)**

Bacterial sample was prepared according to method described by Taufiq and Darah (2019). The mixture was then incubated at 37 °C, 150 rpm for 36 h. At every 12 h of time interval, the mixture was harvested and centrifuged to obtain the bacterial cell pellet. Primary fixation, post fixation and dehydration process of bacterial pallet was carried out according to procedures described by Borgers *et al.* (1989). Prior to SEM viewing, the dried bacterial cells were mounted on specimen stub using conductive tape and coated with 5-10 nm gold using sputter coater machine, Fison SC-515, UK. The specimen was then viewed under a scanning electron microscope (SEM) (Leica Cambridge, S-360, UK).

**Statistical analysis**

All the experiments were performed in triplicates (n=3) and the experimental data were expressed as mean ± standard deviation (SD). The data were analysed by means of the One-Way ANOVA using SPSS 15.0 and Duncan test was used to access the differences between means. The results were considered statistically significant if $p < 0.05$.

## RESULTS

**Disc diffusion assay**

Figure 1 shows the inhibitory effect of the fungal extract against *P. mirabilis* indicating by clear zone surrounding the impregnated disc. Meanwhile, Table 1 exhibits the diameter of inhibition zone of the extract towards *P. mirabilis* with the value of 12.8 mm. Result showed that ethyl acetate extract of *L. pseudotheobromae* IBRL OS-64 exerted a fair antimicrobial activity against the test Gram-negative bacteria.

**MIC and MBC determination**

Table 2 shows the MIC and MBC values of the extract against *P. mirabilis* culture. The MIC and MBC values for *P. mirabilis* were 250 µg/mL and 500 µg/mL, respectively. The results revealed that MBC value was two-fold higher than the MIC value, indicating that the concentration of the fungal extract would have to be significantly increased to kill bacterial cells, instead of inhibiting their growth. On the other hand, the ethyl acetate extract of *L. pseudotheobromae* IBRL OS-64 exerted bactericidal effect against test bacteria since MBC/MIC ratio was less than 4 (Keepers *et al.*, 2014).

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 16(3) 2020, pp. 219-228
DOI: http://dx.doi.org/10.21161/mjm.190605

**Time-kill study**

The killing growth profile of ethyl acetate crude extract of *L. pseudotheobromae* IBRL OS-64 against *P. mirabilis* cells are shown in Figure 2. The growth of *P. mirabilis* cells without exposed to the fungal extract (control) showed a normal bacterial growth pattern with lag (0– 8 h), exponential (12-24 h), stationary (28-40 h) and death (44-48 h) phases. At ½× MIC value, the ethyl acetate crude extract of *L. pseudotheobromae* IBRL OS-64 exhibited a drop in growth profile of *P. mirabilis* after 8 h of exposure time. The bacterial growth was slightly increased thereafter until it achieved the stationary phase similarly to the control. It is noteworthy that in this stage, the bacterial growth was higher than the control indicating the antibiotic-induced phenomenon. On the other hand, the MIC samples showed the reduction of viable bacterial cells within 20 h of exposure time but a stagnant rate was observed thereafter from the 20 h to 48 h of incubation time. At the concentration of 2× MIC, the growth curve exhibited a significant reduction in cells growth throughout the incubation period. Bacterial cells of *P. mirabilis* was treated with the fungal extract showed a dose-dependent pattern in its growth. Time-kill curve clearly showed that the increment in the extract concentration had resulted in the enormous reduction of viable cells number. Table 3 shows the reduction in viable of *P. mirabilis* cell counts in the presence of extract at different concentrations viz. ½× MIC, MIC and 2× MIC. The result reflects that the higher concentration of extract and longer incubation period were required to exert inhibiting or killing effects *on P. mirabilis*. On the other hand, higher concentration of extract such as 2× MIC and MIC capable to kill bacterial cell up to 99.9% but the highest concentration of extract (2× MIC) need shorter time to reduce and kill the bacterial cells (20-24 h) compared to MIC (32-36 h). Overall, the time-kill curve and growth reduction analysis described that ethyl acetate extract of L. pseudotheobromae IBRL OS-64 has significant potency to kill bacterial cells (bactericidal effect) at higher extract concentration (MIC and 2× MIC) and the effect was depending on dose as well as exposure time.

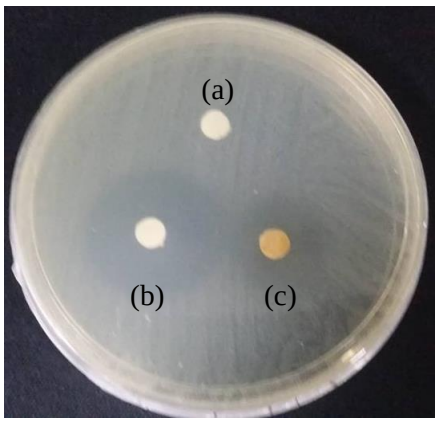


**Figure 1:** Inhibitory effect of ethyl acetate extract of *L. pseudotheobromae* IBRL OS-64 against *P. mirabilis*. (a) negative control, (b) positive control and (c) ethyl acetate extract.

**Table 1:** Diameter of inhibition zone of ethyl acetate extract and control drug against *P. mirabilis*.

| Test bacterium | Diameter of inhibition zone (mm ± SD) | | |
|---|---|---|---|
| | Ethyl acetate extract (1 mg/mL) | Chloramphenicol (30 µg/mL) | Negative control (5% ethyl acetate) |
| *Proteus mirabilis* | 12.0 ± 0.4 | 22.0 ± 0.8 | - |

**Table 2:** Minimal inhibition concentration (MIC) and minimal bactericidal concentration (MBC) values of fungal crude extract towards *P. mirabilis*.

| Test bacterium | MIC (µg/mL) | MBC (µg/mL) | Ratio (MBC/MIC) | Conclusion |
|---|---|---|---|---|
| *Proteus mirabilis* | 250 | 500 | 2 | Bactericidal |

**Table 3:** Growth reduction of *P. mirabilis* cells against incubation time at different level of extract concentrations.

| Percentage of reduction (%) | Time (h) | | | |
|---|---|---|---|---|
| | Control | ½× MIC | MIC | 2× MIC |
| 50.0 | NR | NR | 4–8 | 4–8 |
| 90.0 | NR | NR | 8–12 | 8–12 |
| 99.0 | NR | NR | 16–20 | 16–20 |
| 99.9 | NR | NR | 32–36 | 20–24 |

NR, not reached

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 16(3) 2020, pp. 219-228
DOI: http://dx.doi.org/10.21161/mjm.190605

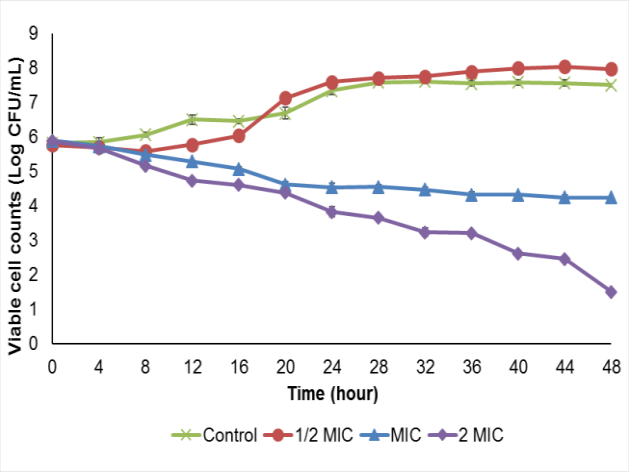


**Figure 2:** Time-kill curve of ethyl acetate crude extract of *L. pseudotheobromae* IBRL OS-64 against *P. mirabilis* at different extract concentrations.

### Morphological changes of the bacterial cells

MIC values of ethyl acetate extract of *L. pseudotheobromae* IBRL OS-64 was employed to qualitatively observe the morphological changes and differences between untreated *P. mirabilis* cells compared to the treated bacterial cells at different exposure time via SEM analysis. The results for untreated *P. mirabilis* cells were presented in Figure 3a whereas the treated bacterial cells at 12 h, 24 h and 36 h of exposure time were illustrated in Figure 3b, 3c and 3d, respectively. The untreated cells of *P. mirabilis* had normal Gram-negative cell conditions with rugose surface, rigid and rod shape. After 12 hours exposed to the fungal extract, formation of hole and pit (circle) were observed on the surface of the bacterial cells. Figure 3c shows the 24 h of exposure to the fungal extract where the cells started to undergo some changes in their cell morphology. There were cavities formed on the surface of bacterial cells (circle) and this condition seems to be resulted from the leakage of the cell cytoplasm. Some debris or small particles of the bacterial cells were also observed indicating the bursting on the cells (circle). The bacterial cells exposed to the ethyl acetate extract at 36 h exhibited physical damages including irregular shape of cells and ruptured cells. This phenomenon suggests that some cells undergone lysis and became completely disrupted. The cells were might be burst beyond the repair indicated by small cell debris. However, some of the cells were still in intact shape and this may be due to efficacy of the extract and the concentration of the extract might has to be increased in order to completely killed all the cells.

## DISCUSSION

*Ocimum sanctum* had been extensively studied for the pharmaceutical potentials of the fungal endophytes associated with the medicinal plant. Several studies were reported the capability of endophytic fungi isolated from *O. sanctum* to exert significant antimicrobial activity against pathogenic bacteria, fungi and yeast including *Nigrospora oryzae* (Desale Monali and Bodhankar, 2014) and *Penicillium* sp. (Jain and Gupta, 2012). In disc diffusion assay, ethyl acetate extract of *L. pseudotheobromae* IBRL OS-64 exhibited inhibitory effect on test bacteria, *P. mirabilis* indicating by formation of inhibition zones surrounding the impregnated disc. This finding demonstrated that antibacterial compounds presence in the extract when using ethyl acetate as extraction solvent. The result was in agreement with Kumari *et al.* (2018) who reported the maximum biological activity of fungal crude was observed in the ethyl acetate extract demonstrating the semi-polar nature of bioactive compounds.

In present study, the MIC value of the extract against test bacteria were 250 µg/mL. The MIC value defined as the lowest amount of extract need to completely inhibit the growth of bacterial cells and the effectiveness of antibacterial activity of the fungal extract against *P. mirabilis* correlated with its MIC value. The extract with the MIC values below 8 mg/mL was considered to have significant antimicrobial activity (Fabry *et al.*, 1998). The capability of antibacterial activity of fungal extract might be due to ability of the antimicrobial agent to penetrate into cell wall. According to Hyldgaard *et al.* (2012), there are specific interactions between the bioactive compounds with the cell wall layers. This interaction might be aiding the penetration of bioactive compounds into bacterial cell and thus, interfering in the cell biosynthesis which resulted in cell death.

On the other hand, the MBC value of the extract was 500 µg/mL and the result exhibited the MBC value was two-fold higher than MIC value. According to Levison (2000), good antibacterial drugs should have MBC values not more four-fold higher than their MIC values. Moreover, the MBC value that equals or eight-fold higher than its MIC value will have tendency as bacteriostatic agents (Rajabi *et al.*, 2005). The present finding revealed that the fungal extract exerted bactericidal effect at higher concentrations and bacteriostatic effect at lower concentrations. The result was in agreement with Lim *et al.* (2006). According to Novais *et al.* (2017), it is possible to identify and predict an antibacterial compounds profile either bactericidal or bacteriostatic by determine the ratio of MBC/MIC. The extract had bactericidal effect on the bacterial growth if the ratio of MBC/MIC less than four whilst if the value more or equal to four, it was concluded to exert bacteriostatic effect (Rakholiya *et al.*, 2014). Therefore, the MIC and MBC values of the extract could be a useful guideline in order to choose appropriate and effective dose of therapeutic substances in pharmaceutical and medical practices.

Time-kill curves are alternative approaches which provide more details and dynamic information to evaluate the relationship between antibiotic concentrations over the exposure time (Nunart *et al.*, 2017). In the present study, the bacterial cells were reduced within 8 h of

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 16(3) 2020, pp. 219-228
DOI: http://dx.doi.org/10.21161/mjm.190605

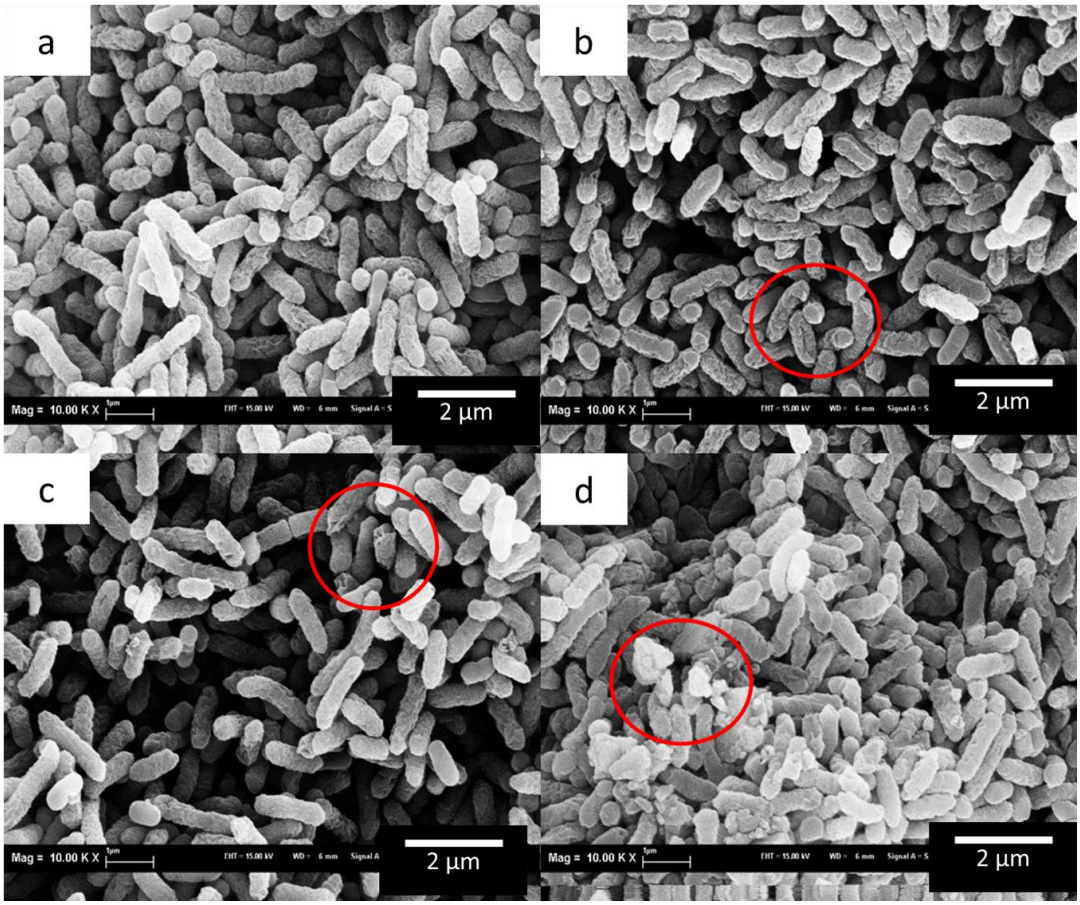


**Figure 3:** SEM micrographs of *Proteus mirabilis* treated with 0.5 mg/mL of *L. pseudotheobromae* IBRL OS-64 ethyl acetate extract different time exposure. (a) 0 hour (control), (b) 12 hours, (c) 24 hours, (d) 36 hours. Scale bars: 2 µm; magnification: 10000×

exposure to ½× MIC extract but, regrowth was occurred thereafter. Similar observation was reported by Idris *et al.* (2013) and they postulated that this phenomenon might be due to insufficient extract to combat the bacterial cells. The regrowth phenomenon may be attributed by two distinct subpopulations with different susceptibility whereby the resistant sub-population take over the preferential killing of the susceptible sub-population at a specified interaction time (Tam *et al.*, 2005). Antibiotic persistence may also induced the regrowth of bacterial cells which is defined as the ability of a bacterial subpopulation to survive upon antibiotic exposure due to non-heritable phenotype variation that is distinct from the mechanisms that generate resistance and these persister cells even though represent a small portion of total cells but, their survival allow the population to survive even in high antibiotic dose exposure (Cabral *et al.*, 2018). The persisters could revert to an antibiotic-sensitive state after stress subsides, reinitiate their cells growth and repopulate the local environment. Current result also revealed the antibiotic-induced phenomenon indicated by the regrowth of bacterial cells in sub-MIC value beyond the control. According to Kaplan (2011), the low dose antibiotic trigger to the induction of bacterial biofilm formation which is one of antibiotic resistant mechanism in several bacterial strains that might be due to inefficient diffusion antimicrobial agent within biofilm matrix, presence of persister cells and slow growth rate in biofilm. This occurrence might result in the presence of resistance bacterial cells that are able to resume their growth upon low antibiotic dose. Furthermore, the post antibiotic effect (PAE) might be occurred at low extract concentration in which the remaining bacteria cells will resume their growth (Ingerman *et al.*, 1986). PAE is define as the potential of antibiotic agent to delay regrowth of bacterial population after short-term exposure and removal of a drug (Mirjani *et al.*, 2010). The present study also revealed that the fungal extract is able to kill bacterial cells at higher concentration such as 2× MIC rather than to inhibit their growth. Similar observation was reported by Ibrahim *et al.* (2015). Darah *et al.* (2013) suggested that the extract possessed bactericidal properties at higher concentration whereby the bacterial cell growth will be eradicated. According to Pankey and Sabath (2004), the

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

efficacy of antibacterial agents may be attributed by the saturation of the protein binding site for the agents to attach and react with the bacterial cells.

SEM studies were carried out to have a clearer view of what happening in the time-kill studies and the results revealed that the fungal extract produced considerable morphological changes in the bacterial cells. There are several main targets of antibiotic drugs in bacterial cells including cell wall synthesis, plasma membrane integrity, nucleic acid synthesis, ribosomal function and folate synthesis (Harold and Thomas, 1996). However, the cell wall synthesis and cell membrane integrity are the main target of the antibiotic drugs. According to Taufiq and Darah (2018), the crude extracts of natural products either plants or microbial extracts would affect the cell membrane permeability and the cell wall biosynthesis. Bugg *et al.* (2011) reported that the biosynthesis of the peptidoglycan layer in bacterial cell walls is a well-proven target for antibiotic action since it is the action site of several clinically important classes of antibiotics such as ß-lactam (penicillin) and glycopeptides (vancomycin). For instance, beta lactam antibiotic interfere in penicillin binding proteins (PBPs) by producing ß-lactam ring that mimicking the D-alanyl-alanine portion of peptide chain that are normally bound by PBP. The PBP interaction with ß-lactam ring resulted in blocking biosynthesis of new peptidoglycans and the disruption of peptidoglycan layers lead to the bacterial cell lysis (Kapoor *et al.*, 2017).

Darah *et al.* (2013) reported the bioactive compound of the methanolic extract of *W. chinensis* leaves that attached on the cell surface structure had permeabilized the bacterial membranes and any disruption the cell wall integrity will have a great impact in bacterial growth. As shown by the SEM micrographs, the bacterial cells became crumpled and exhibited formation of the pits that lead to the cell's leakage. According to Al-Adham *et al.* (1998), these damages may indicate the loss of cellular materials and organelles from the cell cytoplasm. In addition to that, Hartmann *et al.* (2010) suggested the formation of holes, dents and craters on the surface of bacterial cells indicated the failure or mechanism rupture of the cell wall and membrane. These altered and unstable cells were collapsed and finally led to the cell death.

## CONCLUSION

The result of this study revealed that the ethyl acetate crude extract of *L. pseudotheobromae* IBRL OS-64 exhibited bactericidal activity towards *P. mirabilis* with dose and time dependent. The fungal extract also showed antibacterial activity by disrupting the normal cell envelope of *P. mirabilis* which cause cell lysis and ultimate death.

## ACKNOWLEDGEMENTS

The authors are grateful to Universiti Sains Malaysia for awarding the RUI research grant scheme (ac: 1001/PBIOLOGI/811326) to support this study.

## REFERENCES

**Abdalla, M. A. and McGaw, L. J. (2018).** Bioprospecting of South African plants as a unique resource for bioactive endophytic microbes. *Frontiers in Pharmacology* **9, 456.**

**Adetunji, C. O., Oloke, J. K., Mishra, P., Oluyori, A. P., Jolly, R. S. and Bello, O. M. (2018).** Mellein, a dihydroisocoumarin with bioherbicidal activity from a new strain of *Lasiodiplodia pseudotheobromae* C1136. *Beni-Suef University Journal of Basic and Applied Sciences* **7(4), 505-510.**

**Al-Adham, I. S. I., Dinning, A. J., Eastwood, I. M., Austin, P. and Collier, P. J. (1998).** Cell membrane effects of some common biocides. *Journal of Industrial Microbiology and Biotechnology* **21, 6-10.**

**Alvin, A., Miller, K. I. and Neilan, B. A. (2014).** Exploring the potential of endophytes from medicinal plants as sources of antimycobacterial compounds. *Microbiological Research* **169(7–8), 483-495.**

**Balakumar, S., Rajan, S., Thirunalasundari, T. and Jeeva, S. (2011).** Antifungal activity of *Ocimum sanctum* Linn. (Lamiaceae) on clinically isolated dermatophytic fungi. *Asian Pacific Journal of Tropical Medicine* **2011, 654-657.**

**Borgers, M., Van de Ven, M. A. and Van Cutsem, J. (1989).** Structural degeneration of *Aspergillus fumigatus* after exposure to saperconazole. *Journal of Medical and Veterinary Mycology* **27, 381-389.**

**Broomfield, R. J., Morgan, S. D., Khan, A. and Stickler, D. J. (2009).** Crystalline bacterial biofilm formation on urinary catheters by urease-producing urinary tract pathogens: A simple method of control. *Journal of Medical Microbiology* **58, 1367-1375.**

**Bugg, T. D. H., Braddick, D., Dowson, C. G. and Roper, D. I. (2011).** Bacterial cell wall assembly: Still an attractive antimicrobial target. *Trends in Biotechnology* **29(4), 167-173.**

**Cabral, D. J., Wurster, J. I. and Belenky, P. (2018).** Antibiotic persistence as a metabolic adaptation: Stress, metabolism, the host, and new directions. *Pharmaceuticals* **11, 14.**

**Castro-Medina, F., Mohali, S. R., Urbez-Torres, J. R. and Gubler, W. D. (2014).** First report of *Lasiodiplodia pseudotheobromae* causing trunk cankers in *Acacia mangium* in Venezuela. *Plant Disease* **98(5), 686.**

**Chen, C.-Y., Chen, Y.-H., Lu, P.-L., Lin, W.-R., Chen, T.-C. and Lin, C.-Y. (2012).** *Proteus mirabilis* urinary tract infection and bacteremia: Risk factors, clinical presentation, and outcomes. *Journal of Microbiology, Immunology and Infection* **45(3), 228-236.**

**CLSI, Clinical and Laboratory Standards Institute (1999).** Methods for determining bactericidal activity of antimicrobial agents; approved guideline. NCCLS document M26-A. Clinical and Laboratory Standards Institute, Wayne, PA. **pp. 1-29.**

**Darah, I., Lim, S. H. and Nithianantham, K. (2013).** Effects of methanol extract of *Wedelia chinensis* Osbeck (Ast.eraceae) leaves against pathogenic

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

bacteria with emphasise on *Bacillus cereus*. *Indian Journal of Pharmaceutical Sciences* **75(5), 533-539.**

**Desale Monali, G. and Bodhankar, M. G. (2014).** Antimicrobial activity of endophytic fungi isolated from *Ocimum sanctum* Linn. *American International Journal of Contemporary Scientific Research* **1(3), 17-22.**

**Dissanayake, A. J., Zhang, W., Mei, L., Chukeatirote, E., Yan, J. Y., Li, X. H. and Hyde, K. D. (2015).** *Lasiodiplodia pseudotheobromae* causes pedicel and peduncle discolouration of grapes in China. *Australasian Plant Disease Notes* **10, 21.**

**Endimiani, A., Luzzaro, F., Brigante, G., Perilli, M., Lombardi, G., Amicosante, G., Rossolini, G. M. and Toniolo, A. (2005).** *Proteus mirabilis* bloodstream infections: Risk factors and treatment outcome related to the expression of extended-spectrum ß-lactamases. *Antimicrobial Agents and Chemotherapy* **9(7), 2589-2605.**

**Fabry, W., Okemo, P. O. and Ansorg, R. (1998).** Antibacterial activity of East African medicinal plants. *Journal of Ethnopharmacology* **60, 79-84.**

**Garcia, M., Monzote, L., Montalvo, A. M. and Scull, R. (2010).** Screening of medicinal plants against *Leishmania amazonensis*. *Pharmaceutical Biology* **48(9), 1053-1058.**

**Harold, C. N. and Thomas, D. G. (1996).** Antimicrobial chemotherapy (Chapter 11). *In:* Medical Microbiology, 4th edn. Baron, S. (ed). University of Texas Medical Branch, Galveston. **pp. 1-18.**

**Hartmann, M., Berditsch, M., Hawecker, J., Ardakani, M. F., Gerthsen, D. and Ulrich, A. S. (2010).** Damage of the bacterial cell envelope by antimicrobial peptides gramicidin S and PGLa as revealed by transmission and scanning electron microscopy. *Antimicrobial Agents and Chemotherapy* **54(8), 3132-3142.**

**Hyldgaard, M., Mygind, T. and Meyer, R. L. (2012).** Essential oils in food preservation: Mode of action, synergies, and interactions with food matrix components. *Frontiers in Microbiology* **3, 12.**

**Ibrahim, D., Lee, C. C., Yenn, T. W., Zakaria, L. and Sheh-Hong, L. (2015).** 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. *Tropical Journal of Pharmaceutical Research* **14(11), 2091-2097.**

**Idris, M. A., Muyibi, S. A., Karim, M. I. A., Jamal, P. and Jami, M. S. (2013).** *In-vitro* antibacterial activity of crude defatted *Moringa oleifera* seed extract: Kill time study. *Australian Journal of Basic and Applied Sciences* **7(6), 149-153.**

**Ingerman, M. J., Pitsakis, P. G., Rosenberg, A. F. and Levison, M. E. (1986).** The importance of pharmacodynamics in determining the dosing interval in therapy for experimental Pseudomonas endocarditis in the rat. *The Journal of Infectious Diseases* **153, 707-714.**

**Jain, P. and Gupta, S. (2012).** Effect of carbon and nitrogen sources on antimicrobial metabolite production by endophytic fungus *Penicillium* sp. against human pathogens. *Journal of Pharmacy Research* **5(8), 4325-4328.**

**Jorgensen, J. H. and Ferrero, M. J. (2007).** Susceptibility test methods: Dilution and disk diffusion methods: *In*: Manual of Clinical Microbiology. Murray, P. R., Baron, E. J., Jorgensen, J. H., Landry M. L. and Pfaller, M. A. (eds.). ASM press, Washington, **pp. 1152-1172.**

**Jorgensen, J. H. and Ferrero, M. J. (2009).** Antimicrobial susceptibility testing: A review of general principles and contemporary practices. *Clinical Infectious Diseases* **49(11), 1749-1755.**

**Kaplan, J. B. (2011).** Antibiotic-induced biofilm formation. *International Journal of Artificial Organs* **(9), 737-751.**

**Kapoor, G., Saigal, S. and Elongavan, A. (2017).** Action and resistance mechanisms of antibiotics: A guide for clinicians. *Journal of Anaesthesiology, Clinical Pharmacology* **33(3), 300-305.**

**Kapoor, N. and Saxena, S. (2014).** Potential xanthine oxidase inhibitory activity of endophytic *Lasiodiplodia pseudotheobromae*. *Applied Biochemistry and Biotechnology* **173(6), 1360-1374.**

**Keepers, T. R., Gomez, M., Celeri, C., Nichols, W. W. and Krause, K. M. (2014).** Bactericidal activity, absence of serum effect, and time-kill kinetics of ceftazidime-avibactam against β-lactamase-producing enterobacteriaceae and *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy* **58(9), 5297-5305.**

**Kumari, M., Taritla, S., Sharma, A. and Jayabaskaran, C. (2018).** Antiproliferative and antioxidative bioactive compounds in extracts of marine-derived endophytic fungus *Talaromyces purpureogenus*. *Frontiers in Microbiology* **9, 1777.**

**Kunin, C. M. (1989).** Blockage of urinary catheters: Role of microorganisms and constituents of the urine on formation of encrustations. *Journal of Clinical Epidemiology* **42(9), 835-842.**

**Kusari, S., Verma, V. C., Lamshoeft, M. and Spiteller, M. (2012).** An endophytic fungus from *Azadirachta indica* A. Juss. that produces azadirachtin. World *Journal of Microbiology and Biotechnology* **28, 1287-1294.**

**Kwon, J.-H., Choi, O., Kang, B., Lee, Y., Park, J., Kang, D.-W., Han, I. and Kim, J. (2017).** Identification of *Lasiodiplodia pseudotheobromae* causing mango dieback in Korea. *Canadian Journal of Plant Pathology* **39(2), 241-245.**

**Levison, M. E. (2000).** Pharmacodynamics of antibacterial drugs. *Infectious Disease Clinics of North America* **14, 281-291.**

**Lim, S. H., Darah, I. and Jain, K. (2006).** Antimicrobial activities of tannins extracted from *Rhizophora apiculata* barks. *Journal of Tropical Forest Science* **18(1), 59-65.**

**Lue, X., Chen, G., Li, Z., Zhang, Y., Wang, Z., Rong, W. and Bai, J. (2014).** Palmarumycins from the endophytic fungus *Lasiodiplodia pseudotheobromae* XSZ-3 *Helvetica Chimica Acta* **97, 1289-1294.**

**Meshram, V. and Saxena, S. (2016).** Potential fibrinolytic activity of an endophytic *Lasiodiplodia pseudotheobromae* species. *3 Biotechnology* **6(1), 114.**

**Mirjani, R., Rafii, F., Sharifzadeh, M., Amanlou, M. and Shahverdi, A. R. (2010).** Post-antibiotic and post-antibiotic sub-MIC effects of gentamicin, sophoraflavanone G, and their combination against a clinical isolate of *Staphylococcus aureus*. *Asian Biomedicine* **4(5), 821-826.**

**Nogueira Junior, A. F., Santos, R. F., Pagenotto, A. C. V. and Sposito, M. B. (2017).** First report of *Lasiodiplodia pseudotheobromae* causing fruit rot of persimmon in Brazil. *New Disease Reports* **36, 1.**

**Novais, J. S., Campos, V. R., Silva, A. C. J. A., de Souza, M. C. B. V., Ferreira, V. F., Keller, V. G. L., Ferreira, M. O., Dias, F. R. F., Vitorino, M. I., Sathler, P. C., Santana, M. V., Resende, J. A. L. C., Castro, H. C. and Cunha, A. C. (2017).** Synthesis and antimicrobial evaluation of promising 7-arylamino-5,8-dioxo-5,8-dihydroisoquinoline-4-carboxylates and their halogenated amino compounds for treating Gram-negative bacterial infections. *Royal Society of Chemistry Advances* **7, 18311-18320.**

**Nunart, T., Chatsuwan, T. and Treyaprasert, W. (2017).** Time-kill study of the *in vitro* antimicrobial activity of tedizolid against methicillin-resistant *Staphylococcus aureus*. *Thai Journal of Pharmaceutical Sciences* **4(1), 25-29.**

**O'Hara, C. M., Brenner, F. W. and Miller, J. M. (2000).** Classification, identification, and clinical significance of *Proteus*, *Providencia*, and *Morganella*. *Clinical Microbiology Reviews* **13(4), 534-546.**

**Pankey, G. A. and Sabath, L. D. (2004).** Clinical relevance off bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections. *Clinical Infectious Diseases* **38, 864-870.**

**Percival, S. L., Suleman, L., Vuotto, C. and Donelli, G. (2015).** Healthcare-associated infections, medical devices and biofilms: risk, tolerance and control. *Journal of Medical Microbiology* **64, 323-334.**

**Puri, S. C., Verma, V., Amna, T., Qazi, G. N. and Spiteller, M. (2005).** An endophytic fungus from *Nothapodytes foetida* that produces camptothecin. *Journal of Natural Products* **68, 1717-1719.**

**Qian, C. -D., Fu, Y. -H., Jiang, F. -S., Xu, Z. -H., Cheng, D.-Q., Ding, B., Gao, C.-X. and Ding, Z.-S. (2014).** *Lasiodiplodia* sp. ME4-2, an endophytic fungus from the floral parts of *Viscum coloratum*, produces indole-3-carboxylic acid and other aromatic metabolites. *BMC Microbiology* **14, 297.**

**Rajabi, L., Courreges, C., Montoya, J., Aguilera, R. J. and Primm, T. P. (2005).** Acetophenones with selective antimycobacterial activity. *Letters in Applied Microbiology* **40, 212-217.**

**Rakholiya, K., Vaghela, P., Rathod, T. and Chanda, S. (2014).** Comparative study of hydroalcoholic extracts of *Momordica charantia* L. against foodborne pathogens. *Indian Journal of Pharmaceutical Science* **76(2), 148-156.**

**Rathnayaka, R. M. U. S. K. (2013).** Antibacterial activity of *Ocimum sanctum* extracts against four food-borne microbial pathogens. *Scholars Journal of Applied Medical Sciences* **1(6), 774-777.**

**Sadovskaya, I., Vinogradov, E., Flahaut, S., Kogan, G. and Jabbouri, S. (2005).** Extracellular carbohydrate-containing polymers of a model biofilm-producing strain, *Staphylococcus epidermidis* RP62A. *Infection and Immunity* **73(5), 3007-3017.**

**Scavone, P., Iribarnegaray, V., Caetano, A. L., Schlapp, G., Haaortel, S. and Zunino, P. (2016).** Fimbriae have distinguishable roles in *Proteus mirabilis* biofilm formation. *Pathogens and Disease* **74(5), ftw033.**

**Selvam, K., Rajinikanth, R., Govarthanan, M., Paul, A., Selvankumar, T. and Sengottaiyan, A. (2013).** Antioxidant potential and secondary metabolites in *Ocimum sanctum* L. at various habitats. *Journal of Medicinal Plants Research* **7(12), 706-712.**

**Somasundaram, G., Manimekalai, K., Salwe, K. J. and Pandiamunian, J. (2012).** Evaluation of the antidiabetic effect of *Ocimum sanctum* in type 2 diabetic patients. *International Journal of Life Science and Pharmaceutical Research* **2(3), 75-81.**

**Sridevi, M., John, B. and Yamini, K. (2016).** Anti-cancer effect of *Ocimum sanctum* ethanolic extract in non-small cell lung carcinoma cell line. *International Journal of Pharmacy and Pharmaceutical Sciences* **8(4), 242-246.**

**Strobel, G. and Daisy, B. (2003).** Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular Biology Reviews* **4, 491-502.**

**Tam, V. H., Schilling, A. N. and Nikolaou, M. (2005).** Modelling time–kill studies to discern the pharmacodynamics of meropenem**.** *Journal of Antimicrobial Chemotherapy* **55, 699-706.**

**Taufiq, M. M. J. and Darah, I. (2018).** Anti-MRSA of the ethyl acetate crude extract from *Lasiodiplodia pseudotheobromae* IBRL OS-64, and endophytic fungus isolated from leaf of *Ocimum sanctum* Linn. *International Journal of Pharmacy and Pharmaceutical Sciences* **10(8), 50-55.**

**Taufiq, M. M. J. and Darah, I. (2019).** Antibacterial activity of an endophytic fungus *Lasiodiplodia pseudotheobromae* IBRL OS-64 residing in leaves of a medicinal herb, *Ocimum sanctum* Linn. *Journal of Applied Biology and Biotechnology* **7(02), 35-41.**

**Wei, W., Jiang, N., Mei, Y. N., Chu, Y. L., Ge, H. M., Song, Y. C., Ng, S. W. and Tan, R. X. (2014).** An antibacterial metabolite from *Lasiodiplodia pseudotheobromae* F2. *Phytochemistry* **100, 103-109.**

**Yu, W., He, Z. and Huang, F. (2015).** Multi-drug resistant *Proteus mirabilis* isolated from newly weaned infant rhesus monkeys and ferrets. *Jundishapur Journal of Microbiology* **8(6), e16822.**

Malays. J. Microbiol. Vol 16(3) 2020, pp. 219-228
DOI: http://dx.doi.org/10.21161/mjm.190605

**Zhang, H. W., Song, Y. C. and Tan, R. X. (2006).** Biology and chemistry of endophytes. *Natural Product Reports* **23(5), 753-771.**

**Zhou, J., Diao, X., Wang, T., Chen, G., Lin, Q., Yang, X. and Xu, J. (2018).** Phylogenetic diversity and antioxidant activities of culturable fungal endophytes associated with the mangrove species *Rhizophora stylosa* and *R. mucronata* in the South China Sea. *PLoS ONE* **13(6), e0197359.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538