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Antibacterial activity of an endophytic fungus *Lasiodiplodia pseudotheobromae* IBRL OS-64 residing in leaves of a medicinal herb, *Ocimum sanctum* Linn.

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ABSTRACT

The study was carried out to investigate the antibacterial activity of an endophytic fungal isolate, *Lasiodiplodia pseudotheobromae* IBRL OS-64 residing in leaves of a medicinal herb, *Ocimum sanctum* Linn. Qualitative screening of the antimicrobial activity was done using an agar plug assay, and the results showed that the fungal isolate was able to inhibit all the 13 test bacteria. Three Gram-positive bacteria (methicillin-resistant *Staphylococcus aureus* [MRSA] ATCC 33591, *Staphylococcus aureus,* and *Streptococcus mutans*) were the most susceptible species with the inhibition zones of ≥21 mm. The other three (*Bacillus cereus* ATCC 10876*, Bacillus subtilis* IBRL A3, and *Streptococcus agalactiae*) showed the inhibition zones of 11–≤20 mm of diameter. As for Gram-negative bacteria, *Yersinia enterocolitica* was the most susceptible to the fungal isolate with the size of inhibition zone of ≥21 mm, followed by *Klebsiella pneumoniae* ATCC 13883, *Salmonella typhimurium,* and *Shigella boydii* ATCC 9207 with the inhibition zones of 11–≤20 mm, whereas *Escherichia coli* IBRL 0157, *Proteus mirabilis,* and *Pseudomonas aeruginosa* ATCC 27883 were the least susceptible with the inhibition zones of ≤10 mm. Quantitative screening using disc diffusion assay showed that the fungal ethyl acetate extract prepared from the fermentative broth (extracellular) exhibited better antibacterial activity compared to the methanolic extract prepared from the fungal biomass (intracellular). The results showed that the ethyl acetate extract exhibited antibacterial activity against all the 13 test bacteria with the inhibition zone sizes of 20.0 ± 0.3–31.3 ± 1.2 mm in diameter for Gram-positive bacteria and 10.31 ± 0.6–20.1 ± 0.6 mm in diameter for Gram-negative bacteria. On the other hand, the methanolic extract only inhibited three Gram-positive bacteria (MRSA ATCC 33591, *S. aureus,* and *S. mutans*) with the inhibition zones of 9.0 ± 0.6–11.0 ± 0.3 mm in diameter, whereas only one Gram-negative (*S. typhimurium*) with the inhibition zone size of 13.3 ± 1.5 mm diameter. The minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the ethyl acetate extract on Gram-positive bacteria were in the range of 62.50–125.00 and 62.50–500.00 µg/mL, respectively, whereas for the Gram-negative bacteria, the MIC and MBC values were 125.00–250.00 and 250.0–1000.00 µg/mL, respectively. On the other hand, the MIC and MBC values for methanolic extract against Gram-positive bacteria were 250.00–500.00 µg/mL and against Gram-negative bacteria were 1000.00 µg/mL, respectively. Both of the extracts exhibited bactericidal effects on test bacteria with the MBC/MIC ratio ≤4. Further, detail of the effects of the ethyl acetate extract on the bacterial cells was observed from the scanning electron microscopy photomicrographs which revealed the severity of the morphological deterioration experienced by the extract-treated cells were beyond repair, and the most possible mode of actions were by interrupting the cell wall biosynthesis and cell membrane permeability.

1. INTRODUCTION

The problems of bacterial resistance strains are increasing, and the outlook for the use of the existing antibiotics against human pathogenic microbes has prompted an intensive search for newer and more effective antibiotics [1]. Therefore, actions must be taken to reduce this problem by controlling the overuse of antibiotics and to continue searching for new save drugs, either synthetic or natural. The ultimate goal is to offer appropriate and efficient antibiotics to the patients. Infections caused by bacterial pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci, and many more [2,3] remain a growing health risk globally [4], and identification of new molecules active against these pathogenic bacteria is highly desired.

Endophytic fungi have been known to be the reservoir of many useful bioactive compounds [5]. They are special group of microorganisms residing within the plant tissues, which play an important role

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in defending the host plant against stresses such as pathogenic invasions and drought. Furthermore, they are colonizing the internal or external parts of healthy plants tissue asymptotically and most of the plants that have been previously studied become host at least for one endophyte [6]. In fact, endophytic fungi are generally non-pathogenic in nature but may produce secondary metabolites that enable them to survive in the competitive world of plant interstitial space without harming their host. Endophytic fungi have evolved tremendously with their host plants, and they are able to produce arrays of diverse secondary metabolites with various functions [5] such as antimicrobial [7], antibacterial [8,9], antifungal [10,11], anticancer [12,13], anti-inflammatory [14], antiviral [15], and antioxidant activities [16]. Thus, studies on endophytic fungi become more popular amongst researchers.

Lasiodiplodia pseudotheobromae is better known as a common fungal pathogen that causes diseases on large number of hosts plants in the tropics and subtropics such as on mango which causes dieback disease [17,18], which can cause trunk cankers in *Acacia mangium* in Venezuela [19], postharvest fruit rot of lemon in Turkey [20], and persimmon rot in Brazil [21]. However, recently, it has been known as an endophytic fungus which residing in various parts of host plants and has been reported to exhibit various pharmaceutical activities such antibacterial [22,23], xanthine oxidase inhibitory [24], fibrinolytic [25], and anticancer [26] activities. In fact, Qian *et al.* [27] reported the ability of *Lasiodiplodia* sp. ME4-2, an endophytic fungus from the floral parts of *Viscum coloratum*, to produce indole-3-carboxylic acid and other aromatic metabolites.

In general, the study on antimicrobial activity of *L. pseudotheobromae* is scarce and thus the current study is planning to investigate the antibacterial activity of *L. pseudotheobromae* IBRL OS-64 against various test bacteria. The study is very important and significant since this is the first report of *L. pseudotheobromae* isolated from *Ocimum sanctum* Linn. leaves.

2. MATERIALS AND METHODS

2.1. Endophytic Fungus, Culture, and Maintenance

The endophytic fungus previously isolated from leaf of *O. sanctum* Linn. was provided by the culture collection of the Industrial Biotechnology Research Laboratory (IBRL), Universiti Sains Malaysia, Penang, Malaysia. The fungal isolate was cultured and maintained on potato dextrose agar (PDA) supplemented with powdered host plant (2 g/L) and incubated at 30°C aerobically for 7 days before storing it at 4°C before use. The isolate was subcultured on sterile fresh medium once a month to ensure its purity and viability.

2.2. Culture Medium

Yeast extract sucrose broth (YES; Merck, Germany) [9] which contained (g/L) yeast extract, 20; sucrose, 40; and magnesium sulfate, 0.5 was added into 1000 mL distilled water supplemented with *O. sanctum* leaf water extract. The host plant extract was prepared by boiling 1.0 g of the powdered leaf in 500 mL distilled water for 30 min. The mixture was then filtered through Whatman filter paper, and the filtrate was mixed with freshly prepared culture medium, followed by autoclaving at 121°C for 15 min. The medium was used to cultivate the endophytic fungal isolate in a shake-flask system. The pH of the culture medium was adjusted to 5.8.

2.3. Cultivation and Extraction

The inoculum was prepared by introducing two mycelial agar plugs approximately 1.0 cm in diameter and 4.0 mm thickness, which were excised from the periphery of 7-day-old endophytic fungal cultures into 250 ml Erlenmeyer flasks containing 100 mL of YES broth medium. The cultures were cultivated at 30°C with agitation speed of 120 rpm for 20 days in the dark. After the incubation period, the fermented broth and fungal biomass were separated out using Whatman No. 1 filter papers. The fungal biomass was washing twice using sterile distilled water and freeze-dried before macerating in methanol (1:50, w/v) overnight. The macerated mixture was filtered using muslin cloth and followed by Whatman no. 1 filter paper to separate the biomass filtrate from the biomass residue. The fungal biomass filtrate was then concentrated to dryness under reduced pressure in a rotary evaporator to obtain a methanolic crude extract paste. On the other hand, the filtered fermented broth was extracted thrice with equal volume of ethyl acetate (1:1, v/v). The upper organic phase was collected and subsequently was concentrated to dryness under reduced pressure in a rotary evaporator to obtain an ethyl acetate crude extract paste. A control was included by extracting the sterile medium following exactly the same steps as that for fungal cultures due to antimicrobial activities exhibited by *O. sanctum* Linn. extract.

2.4. Test Microorganisms and Culture Maintenance

A total of 13 test bacteria provided by IBRL, Universiti Sains Malaysia, Penang, Malaysia, were used in this study including six Gram-positive bacteria (*Bacillus cereus* ATCC 10876, *Bacillus subtilis* IBRL A3, MRSA ATCC 33591, *S. aureus*, *Streptococcus mutans,* and *Streptococcus agalactiae*) and seven Gram-negative bacteria (*Klebsiella pneumoniae* ATCC 13883, *Shigella boydii* ATCC 9207, *Escherichia coli* IBRL 0157, *Salmonella typhimurium, Pseudomonas aeruginosa* ATCC 27844, *Yersinia enterocolitica,* and *Proteus mirabilis*). The inoculum of bacteria was prepared by transferring 3–4 single pure colonies into 5.0 mL of 0.85% sterile physiological saline (w/v) and mixing well to obtain cell suspension. The turbidity of the bacterial suspension was adjusted to match 0.5 McFarland standard (approximately 1×10^8 CFU/mL). To obtain the desirable inoculum size as required by CSLI [28], further, dilution with 0.85% (w/v) sterile physiological saline was conducted. The cultures were maintained on nutrient agar and the inoculated plates were incubated at 37°C, 24 h before storing them at 4°C until used. The cultures were subculture every month on fresh nutrient agar slants to ensure its viability.

2.5. Agar Plug Diffusion Assay

Primary screening of antimicrobial activity of the endophytic fungal isolates was carried out by adopting the modified agar plug method described previously by Mohanraj *et al.* [29]. The isolated endophytic fungal cultures were inoculated onto PDA plates containing host plant powder (2 g/L) and incubated at 25°C for 20 days before cutting them using a sterile cork borer into agar plugs of 1.0 cm diameter and 4.0 mm thickness. The agar plugs were then placed on the Mueller-Hinton agar (MHA) seeded with test bacteria, and the plates were initially kept overnight at 4°C to allow diffusion of bioactive compounds and subsequently incubated at 37°C for 24 h. Chloramphenicol (30 µg/mL) was used as a positive control and the inhibition zone formed around the endophyte agar plugs were measured. The experiments were carried out in triplicate and the results were expressed as mean value ± standard error of the inhibition zone obtained from three separated experiments. Endophytic fungal isolates exhibiting significant antibacterial activity against test bacteria were subjected to secondary screening.

2.6. Disc Diffusion Susceptibility Assay

The assay was carried out according to the method as previously described by Clinical and Laboratory Standard Institute (CLSI) standard M100-S16 (CLSI, 2006) [30] and M100-S22 [31]. The inocula were prepared by transferring 3–4 single colonies into 5.0 mL of sterile physiological saline and mixing well to obtain cell suspensions of approximately 1×10^8 CFU/mL, by comparison with 0.5 McFarland standards. The test bacteria were seeded on MHA using a sterile swab with spread plate technique.

The fungal crude extracts were prepared by dissolving 10.0 mg of extract in 0.2 mL of 5% dimethyl sulfoxide (DMSO) and subsequently added with 0.8 mL of sterile distilled water to get a concentration of 1 mg/mL. The sterile 6.0 mm diameter of antibiotic discs was impregnated with 20 µL of the extract and air-dried before placing them on the Mueller-Hinton seeded agar surface. Chloramphenicol at the concentration of 30 µg/mL was used as a positive control and 1.0% of DMSO as a negative control. The inhibition zone formed around the endophyte agar plugs were measured after incubation for 24 h. The experiments were carried out in triplicate, and the results were expressed as mean value ± standard error of the inhibition zone obtained from three separated experiments.

2.7. MIC and MBC Determinations

The MIC of the fungal ethyl acetate crude extract against test bacteria was determined by broth microdilution assay as described by Jorgensen and Ferraro [32] with some modifications. Only test microorganisms that showed significant inhibitory activity on disc diffusion assay were proceeded and evaluated using sterile 96 wells, U-shaped microtiter plate. A single-fold dilution of the fungal extract was prepared in sterile Mueller-Hinton broth (MHB) medium and 100 µL of the extract was dispensed into each well of the microtiter plate. Then, 100 µL of test microorganisms' inocula at approximately 1×10^7 CFU/mL was added into each well for a final volume of 200 µL, and the final concentration of microbial inocula in each well was 1×10^7 CFU/mL. Chloramphenicol was used as a reference drug. The 5% methanol and bacterial inoculum were included as control. After 24 h of incubation period at 37°C, 40 µL of 0.2 mg/ml p-iodonitrotetrazolium violet (INT) dissolved in 99.5% ethanol was loaded to each well as a growth indicator. The MIC value was determined and recorded as the lowest concentration of the ethyl acetate crude extract that capable to inhibit the visible growth of test microorganisms after the incubation period [33].

The MBC of the fungal ethyl acetate crude extract was subsequently determined on reading of the MIC values. The viable cells from wells that showed no microbial growth were enumerated on MHA by a standard viable plate count after 24 h of incubation period at 37°C for overnight. The MBC was observed and recorded as the lowest concentration of fungal ethyl acetate crude extract that resulted in reduction of 99.9% bacterial growth relative to the growth control [34].

2.8. Scanning Electron Microscopy (SEM)

For sample preparation, 50 µL of bacterial inoculum (approximately 1×10^8 CFU/mL) was transferred into a 25 mL Erlenmeyer flask containing 945 µL of MHB and incubated in an orbital shaker at 37°C, 150 rpm for 18–20 h. A volume of 0.5 mL of ethyl acetate crude extract (5 mg/mL) was added into the bacterial culture after incubation period to yield a volume of 10 mL mixture with extract concentration of 0.5 mg/mL. At the meantime, 0.5 mL of 20% DMSO (v/v) was added into bacterial culture as a replacement to the extract and served as control. The mixture was then incubated at 37°C, 150 rpm for 36 h. The cultures were harvested at 0 and 36 h of incubation period and proceed for SEM works as described previously by Borges *et al*. [35]. The prepared samples were then viewed under SEM (Leica Cambridge, S-360, UK).

2.9. Statistical Analysis

The data obtained in this study were analyzed by Students' *t*-test for comparing the effect of ethyl acetate extract on test microorganism cells against control using the SPSS Version 12.0. All tests were independently performed in triplicates. The results obtained were analyzed using a one-way ANOVA test and reported as the mean ± standard deviation.

3. RESULTS

3.1. Qualitative Screening for Antibacterial Activity

The endophytic fungus, *L. pseudotheobromae* IBRL OS-64, was primarily screened using agar plug diffusion assay to detect the existing of antibacterial activity against test microorganisms [Table 1]. The extract exhibited antibacterial activity against all the 13 bacterial species tested. Three Gram-positive bacteria (MRSA ATCC 33591, *S. aureus,* and *S. mutans*) were the most susceptible species with the inhibition zones of ≥21 mm. The other three (*B. cereus* ATCC 10876*, B. subtilis* IBRL A3, and *S. agalactiae*) showed the inhibition zones of 11–≤20 mm of diameter. As for Gram-negative bacteria, *Y. enterocolitica* was the most susceptible to the fungal endophytes isolate with the size of inhibition zone of ≥ 21 mm, followed by *K. pneumoniae* ATCC 13883, *S. typhimurium,* and *S. boydii* ATCC 9207 with the inhibition zones of 11–≤20 mm, whereas *E. coli* IBRL 0157, *P. mirabilis,* and *P. aeruginosa* ATCC 27844 were the least susceptible with the inhibition zones of ≤10 mm. The results obtained showed that *L. pseudotheobromae* IBRL OS-64 possessed prominent antibacterial

Table 1: Primary screening of antibacterial activity of *Lasiodiplodia pseudotheobromae* IBRL OS-64 against test microorganisms using agar plug diffusion assay

Test bacteria	Inhibition zones
Gram-positive bacteria	
Bacillus cereus ATCC 10876	++
Bacillus subtilis IBRL A3	++
Methicillin-resistant *Staphylococcus aureus* ATCC 33591	+++
Staphylococcus aureus	+++
Streptococcus mutans	+++
Streptococcus agalactiae	++
Gram-negative bacteria	
Escherichia coli IBRL 0157	+
Klebsiella pneumoniae ATCC 13883	++
Proteus mirabilis	+
Pseudomonas aeruginosa ATCC 27844	+
Salmonella typhimurium	++
Shigella boydii ATCC 9207	++
Yersinia enterocolitica	+++

+=Inhibition zone≤10 mm, ++=Inhibition zone 11 to≤20 mm, +++=Inhibition zone≥21 mm

activity against test bacteria which comprised pathogenic bacteria to human. The results also revealed that Gram-positive bacteria were more susceptible to the extract compared to Gram-negative bacteria.

3.2. Disc Diffusion Assay

The antibacterial activity of *L. pseudotheobromae* IBRL OS-64 extracts was determined using disc diffusion assays and the results are shown in Table 2. In general, the ethyl acetate extract prepared from the fermentative broth (extracellular) exhibited better antimicrobial activity compared to the methanolic extract prepared from the fungal biomass (intracellular). The results showed that the ethyl acetate extract exhibited antibacterial activity against all the 13 test bacteria with the inhibition zone sizes of 20.0 ± 0.3–31.3 ± 1.2 mm diameter for Gram-positive bacteria and 10.31 ± 0.6–20.1 ± 0.6 mm diameter for Gram-negative bacteria. On the other hand, the methanolic extract only inhibited three Gram-positive bacteria (MRSA ATCC 33591, *S. aureus,* and *S. mutans*) with the inhibition zones of 9.0 ± 0.6–11.0 ± 0.3 mm diameter, whereas only one Gram-negative (*S. typhimurium*) with the inhibition zone size of 13.3 ± 1.5 mm diameter.

The results obtained from this study indicated that the antibacterial compounds were mainly not associated with the fungal biomass but extracellularly released by the fungus into the fermentative broth. The extracellular compound might be an advantage to obtain higher extraction yield since the intracellular compound needs a proper disruption of the fungal mycelial to release and gain higher yield of the compounds [9]. Table 2 also revealed that the ethyl acetate extract of the fermentative broth showed a good antimicrobial activity against Gram-positive compared to Gram-negative bacteria, indicating that Gram-positive bacteria were more susceptible to the extract. These could be due to the differences in the cell morphology and structures between the two groups of bacteria.

3.3. MIC and MBC Determinations

In MIC and MBC determinations, it is not appropriate to use the disk diffusion method, as it is impossible to quantify the amount of the antimicrobial agent diffused into the agar medium. Therefore, microdilution method is the most appropriate ones for the determination of MIC values since they offer the possibility to estimate the concentration of the tested antimicrobial agent in the broth medium (microdilution). Either broth or agar dilution method may be used to quantitatively measure the *in vitro* antimicrobial activity against bacteria and fungi. MIC value recorded is defined as the lowest concentration of the assayed antimicrobial agent that inhibits the visible growth of the microorganism tested, and it is usually expressed in mg/mL.

The results showed that the Gram-positive bacteria were more susceptible to both of the extracts, ethyl acetate and methanolic extracts, with the MIC values ranged between 31.25 and 125.00 µg/mL and 250.00 and 1000.00 µg/mL, respectively [Table 3]. However, Gram-negative bacteria were slightly resisted to both of the extracts, ethyl acetate with the MIC ranged between 125.00 and 250.00 µg/mL and methanolic extract with the MIC value of 1000.00 µg/mL. Again, the ethyl acetate extract showed superior antibacterial activity compared to the fungal biomass extract (methanolic extract). The MBC values were also determined and the MBC values of the ethyl acetate extract against the six Gram-positive bacteria were in the range of 62.50–500.00 µg/mL. In the meantime, the MBC values of the ethyl acetate extract against the seven Gram-negative bacteria were in the range of 250.00–1000.00 µg/mL. The MBC values of the methanolic extract against Gram-positive and Gram-negative bacteria were 250.00–1000.00 µg/mL and 1000.00 µg/mL, respectively. Both of the extracts exhibited bactericidal effects on test bacteria with the MBC/MIC ratio ≤4.

3.4. Structural Degeneration and Morphological Changes of the Extract-treated Cells

Further, detail of the effects of the ethyl acetate extract on the bacterial cells was observed from the SEM photomicrographs which revealed the severity of the morphological deterioration experienced by the extract-treated cells was beyond repair, Figure 1 shows the photomicrographs of the ethyl acetate extract on the untreated (control) and extract-treated MRSA ATCC 33591 cells, a Gram-positive bacteria. Figure 1a

Table 2: Antibacterial activity of *Lasiodiplodia pseudotheobromae* IBRL OS-64 ethyl acetate (extracellular) and methanolic (intracellular) extracts against test microorganisms using disc diffusion assay

Test microorganisms	Ethyl acetate extract (fermented broth; mm)	Methanolic extract (fungal biomass; mm)	Chloramphenicol (30 µg/mL; mm)
Gram-positive bacteria			
Bacillus cereus ATCC 10876	21.0±1.0	-	29.3±1.0
Bacillus subtilis IBRL A3	26.0±1.0	-	28.7±1.2
Methicillin-resistant *Staphylococcus aureus* ATCC 33591	31.3±1.2	10.0±0.3	32.3±0.6
Staphylococcus aureus	30.0±1.0	9.0±0.6	29.3±1.2
Streptococcus mutans	24.0±0.6	11.0±0.3	29.4±0.6
Streptococcus agalactiae	20.0±0.3	-	30.4±1.0
Gram-negative bacteria			
Escherichia coli IBRL 0157	15.7±0.6	-	31.3±0.6
Klebsiella pneumoniae ATCC 13883	15.3±1.5	-	29.3±1.2
Proteus mirabilis	15.1±0.6	-	30.3±1.0
Pseudomonas aeruginosa ATCC 27844	10.3±0.6	-	22.3±0.6
Salmonella typhimurium	16.7±1.0	13.3±1.5	29.7±1.0
Shigella boydii ATCC 9207	14.3±0.6	-	30.7±0.6
Yersinia enterocolitica	20.1±0.6	-	28.3±1.2

is the untreated cell of MRSA ATCC 33591, which shows the cells with the presence of regular Gram-positive bacterial cells, undamaged and smooth surface with intact spherical shape. The cells were also observed growing actively. However, after 36 h of treatment with the extract (Figure-1b), the cells become irregular in shape and some of the cells were shrunken cavitized and crumpled losing their coccal or spherical shape. In fact, some of the cells undergone binary fission but cannot completely separate from their mother cells. Severe damage of the cell morphologies were observed as the cells envelope (cell wall) was broken and resulted in leakage of cytoplasm. The lysed cell walls were extremely devoid of cytoplasmic contents which led to completely collapsed and left only the homologous cell masses (or cells debris). Eventually, the cell death completely occurred.

Figure 2 revealed the effects of the ethyl acetate extract on the *P. aeruginosa* ATCC 27844, a Gram-negative bacterial cells. Figure 2a shows the control or untreated cells with the rod shape bacteria with smooth surface. Some of the cells undergo binary fission. On the other hand, after 36 h of exposure to the extract (Figure 2b), remarkable morphological and distinct feature changes of the cells occurred where there were invaginations and cavitation formed on the cells surfaces and shrunken cells were also observed. The most possible mode of actions of the ethyl acetate extract were by interrupting the cell wall biosynthesis and cell membrane permeability. The results showed that the cells had undergone severe morphological changed and cytological alterations. These damaged cells were lost their metabolic functions and the damage was beyond repair.

4. DISCUSSION

The development of resistance by the existing pathogenic bacteria to commercial drugs is a serious problem faced by health services and has become the main concern around the world. This scenario involves many factors such as extensive and often inappropriate use of antibiotics, poor hygienic conditions, continuous movement of travelers, increased numbers of immune-compromised patients, and delay in diagnosis of infections [36]. As a result, an intensive search for new, effective antimicrobial agents is necessary, which is facilitated by exploring new resources, especially the endophytic fungi which are underexplored. Endophytic fungi are considered as promising sources for producing large variety of bioactive molecules that can protect the plant against pathogens [37,38]. Therefore, numerous attempts have been made to isolate and identify bioactive metabolites from endophytic fungi.

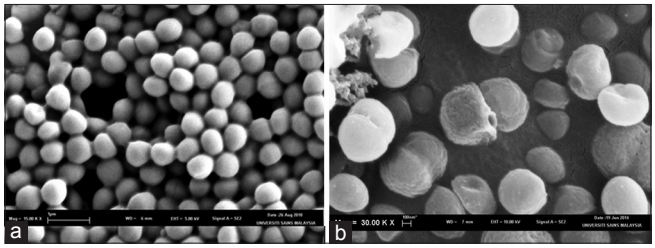

Figure 1: Scanning electron microscopy photomicrographs showing the effects of *Lasiodiplodia pseudotheobromae* IBRL OS-64 ethyl acetate extract at the concentration of 1.0 mg/mL on methicillin-resistant *Staphylococcus aureus* ATCC 33591 cells, (a) untreated cell (control) and (b) extract-treated cell (×5000)

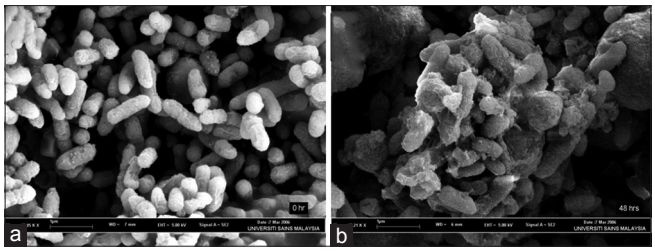

Figure 2: Scanning electron microscopy photomicrographs showing the effects of *Lasiodiplodia pseudotheobromae* IBRL OS-64 ethyl acetate extract at the concentration of 1.0 mg/mL on *Pseudomonas aeruginosa* ATCC 27844 cells, (a) untreated cell (control) and (b) extract-treated cell (×5000)

In the present study, *L. pseudotheobromae* IBRL OS-64 was subjected for preliminary antibacterial screening by agar plug method. This qualitative assay only determines the present of antibacterial substance secreted by the fungal agar plugs. The results showed that the fungus produced a broad-spectrum substance that can inhibit all the 13 test bacteria which consisted of six Gram-positive and seven Gram-negative bacteria.

To quantify the antibacterial activity from fermentative broth and fungal biomass, disc diffusion assay is the best of choice and recommended by many researchers [30]. Usually, endophytic fungi secreted their secondary metabolites extracellularly into the fermentative medium and seldom keeping them intracellularly. Since secondary metabolite substances are mean to protect the endophytic fungi from predators or pathogens, thus they are more suitable to be secreted extracellularly [11]. The results demonstrated the sizes of the inhibition zones which reflected the susceptibility level of the test bacteria.

The difference in bacteria response to the ethyl acetate extract was possibly due to the nature of the bacterial species. The extract, however, showed greater antibacterial activity against Gram-positive than Gram-negative bacteria. These results are in agreement to other researcher's findings who reported that majority of the fungal extract to be more active against Gram-positive bacteria than the Gram-negative bacterial strains [8,39,40]. We suspected this condition is caused by the differences in the cell envelopes composition of Gram-positive and Gram-negative bacteria which are complex dynamic structures that play a variety of protective and adaptive roles [41].

Theoretically, the cell envelope of Gram-negative bacteria are surrounded by a thin peptidoglycan cell wall, which itself is surrounded by an outer membrane containing four layer cell walls consisted of asymmetric distribution of the peptidoglycan, lipoprotein, phospholipids outer membrane, and lipopolysaccharide. These conditions make them more resistant to the penetration of antibacterial agents and consequently protect their cell membrane permeability [42]. Besides, it also hinders the movement of foreign substance into the cell. This characteristic is absent in the cell envelope of Gram-positive bacteria where there is no outer membrane but only consisted of one layer of peptidoglycan, which make them, are more susceptible to antibacterial agent [43]. Furthermore, the cell wall of Gram-positive bacteria contains lipoteichoic acids that represent unique and essential structural components to the cells and should be good drug targets to the bioactive compounds of *L. pseudotheobromae* IBRL OS-64.

Many studies attempted to investigate the effects of fungal extracts on different bacteria to support the antibacterial activity after exposure them to the extracts. In this study, the SEM photomicrograph's observations exhibited clearly the antibacterial effects of the ethyl acetate extract

Table 3: Antibacterial activity of *Lasiodiplodia pseudotheobromae* IBRL OS-64 ethyl acetate (extracellular) and methanolic (intracellular) extracts against test microorganisms

Test microorganisms	Ethyl acetate extract (fermentative broth)			Methanolic extract (fungal biomass)		
	MIC (µg/ml)	MBC (µg/ml)	MBC/MIC	MIC (µg/ml)	MBC (µg/ml)	MBC/MIC
Gram-positive bacteria						
Bacillus cereus ATCC 10876	62.5	125.0	2	-	-	-
Bacillus subtilis IBRL A3	62.5	125.0	2	-	-	-
Methicillin-resistant *Staphylococcus aureus* ATCC 33591	125.0	250.0	2	250.0	500.0	2
Staphylococcus aureus	62.5	125.0	2	250.0	500.0	2
Streptococcus mutans	31.25	62.5	2	500.0	1000.0	2
Streptococcus agalactiae	125.0	500.0	4	-	-	-
Gram-negative bacteria						
Escherichia coli IBRL 0157	250.0	1000.0	4	-	-	-
Klebsiella pneumoniae ATCC 13883	250.0	500.0	2	-	-	-
Proteus mirabilis	250.0	500.0	2	-	-	-
Pseudomonas aeruginosa ATCC 27844	250.0	500.0	2	-	-	-
Salmonella typhimurium	125.0	500.0	4	1000.0	1000.0	1
Shigella boydii ATCC 9207	250.0	500.0	2	-	-	-
Yersinia enterocolitica	125.0	250.0	2	-	-	-

MIC: Minimal inhibitory concentration, MBC: Minimum bactericidal concentration

on both Gram-positive and Gram-negative bacterial cells. There were clear morphological changes with collapsed cells, and this could be due to the leak in the cell wall or maybe some alterations occurred in the cell membrane permeability. These findings augmented the antibacterial prospects of the ethyl acetate extract is a promising broad-spectrum antibacterial agent. It was suggested that the morphological changes of the antibiotic-treated bacteria occur when the antibacterial agent attacked the cell membrane. In this case, the bioactive compound of the ethyl acetate extract of *L. pseudotheobromae* IBRL OS-64 that locked on the cell surface structure had permeabilized the bacterial cell membrane. Any disruption in cell wall integrity would have a great influence in bacterial growth. This prediction was coincided well with the findings of Darah *et al*. [44] who reported the extract of *Urtica dioica* exerted its inhibitory effect on the cell wall of the bacterial cells which led to the complete damage of the cells. Various studies were reported to investigate the mechanism of actions involved in bacterial killing process. The most possible mode of action involved could be the interaction of antibacterial compound with the cell membrane. As shown by the SEM micrographs where the cells became crumpled and exhibited the formation of holes, these damages may indicate the loss of cellular materials and organelles from the cell cytoplasm. These unstable and altered cells were collapsed beyond repair and finally led to cell death.

The results from this study revealed that the *L. pseudotheobromae* IBRL OS-64 possessed antibacterial activity against both Gram-positive and Gram-negative bacteria. Wei *et al*. [22] also reported the ability of extract from *L. pseudotheobromae* F2 which residing in the flower of *Illigera rhodantha* showed antibacterial activity against Gram-positive (*Streptococcus* sp. and *Peptostreptococcus* sp.) and Gram-negative (*Bacteroides vulgates* and *Veillonella parvula*) bacteria. In fact, they managed to isolate six compounds, lasiodiplines A-F and found lasiodipline E demonstrated significant antibacterial activity. Therefore, the compound lasiodipline could be the bioactive compound with antibacterial activity in the *L. pseudotheobromae* IBRL OS-64 ethyl acetate extract.

CONCLUSION

The findings of the current study unveiled a novel, powerful, and broad-spectrum antibacterial activity of the *L. pseudotheobromae* IBRL OS-64 ethyl acetate extract against a number of Gram-positive and Gram-negative pathogenic bacteria by exerting bactericidal effects and causing severe morphological changes beyond repair. This study suggests that the possibly mode of action of the ethyl acetate extract against bacteria is through their cell membranes and cell walls. The isolation and identification of the bioactive compounds are in progress and will be reported elsewhere.

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