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Research Article

Muscodor sp. IBRL OS-94, A Promising Endophytic Fungus of *Ocimum sanctum* with Antimicrobial Activity

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Abstract

Background: An endophytic fungus, *Muscodor* sp. IBRL OS-94 isolated from the leaf of *Ocimum sanctum* was believed to possess significant antimicrobial activity and several assays were carried out to evaluate its pharmaceutical potential.

Methods: Agar plug diffusion and the disk diffusion assays were performed to evaluate the antimicrobial activity of the fungal extract. Also, the broth microdilution assay was done to investigate the minimum inhibitory concentration (MIC) of the fungal extract. Meanwhile, the scanning electron microscope (SEM) was employed to observe the structural degeneration of the microbial cells treated to the extract.

Results: The results revealed that fungal isolate showed favorable antimicrobial activity through agar plug diffusion assay and the disk diffusion assay demonstrated that most of the test microorganisms were susceptible to extracellular extract compared to extracellular extract. As for the MIC and MLC values, the extracellular fungal extract exerted a bactericidal/fungicidal effect against all five Gram-positive bacteria, four Gram-negative bacteria, one yeast, and none of the test fungi. Meanwhile, the intracellular fungal extract exhibited bactericidal/fungicidal activity against three Gram-positive bacteria, one Gram-negative bacterium, and one yeast. The structural degeneration study via SEM revealed that various cell abnormalities including severe damage to the cell wall which led to microbial cell death.

Conclusion: The present study suggests the fungal extract from *Muscodor* sp. IBRL OS-94 as an antimicrobial agent.

Introduction

The emergence and outbreak of superbugs due to the miss-usage of antibiotics have grabbed attention to various cartels especially a member of medical and scientific communities. Globally, infections caused by pathogenic bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci remain a growing health threat.[1] MRSA is responsible for most global *S. aureus* bacteremia cases which exhibit high rates of morbidity and mortality and can lead to complicated infections such as sepsis.[2] Moreover, a fungal infection caused by *Candida* sp. can cause a life-treating disease called candidiasis.[3] According to Chapman,[4] candidiasis is one of the main candidal infections that can cause lethal to immunocompromised patients and HIV-treated patients. The drug-resistant bacteria, yeast, and fungi could lead to a serious problem that demands immediate alternative efficient strategies for the discovery of novel compounds with pharmaceutical properties to overcome this problem. Therefore, endophytes are depicted as outstanding strategies since they are known as a treasure house of microbial metabolites with pharmaceutical properties.[5] Endophytes are microorganisms that colonize and reside within plant tissues without causing any disease symptoms to their host and they interact via a chemical signal which is important for host survival fitness and in turns, the plants modulate the metabolic process of these endophytes to produce compounds with protective functions towards the microbes and their hosts.[6] Endophytic fungi belong to mitosporic and meiosporic ascomycetes that live in the internal tissues of healthy plants especially the epidermal cell layer and they are found naturally in the temperate regions and tropical rainforests and associated with more than 300,000 terrestrial host-plant species.[7] For decades, the fungal endophytes have been recognized as a reservoir of many useful bioactive substances with pharmaceutical properties.[8] Endophytes have evolved tremendously with their hosts and they can formulate diverse secondary compounds with various functions including antibacterial,

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anticancer, antifungal, anti-inflammatory, antioxidant, and antiviral activities. Therefore, studies on fungal endophytes from medicinal plants have become more popular among researchers.

Muscodor sp. is one of the endophytic fungi that have been extensively studied for their biological activity. They are classified in family *Xylariaceae*, which are the dominant endophytic fungal group that can produce volatile organic compounds (VOCs) with prominent antimicrobial activity, possessed intertwined rope-like mycelia without producing any spores.[9] Besides that, Banerjee[10] reported that volatile compounds produced by this genus are lethal to a broad range of pathogenic bacteria and fungi. Meshram[11] reported several new volatile produced by *Muscodor kashayum* with antimicrobial properties including 1,6-dioxacyclododecane-7,12-dione; 2,6-bis(1,1-dimethylethyl)-4-(1-oxopropyl) phenol, and 4-octadecylmorpholine. Moreover, *Muscodor sutura* was reported to produce volatile compounds with wide-spectrum antifungal activity such as butanoic acid, 2-methyl, chamigrene, thujopsene, and isocaryophyllene.[12] Thus, this study was aimed to evaluate the potency of endophytic fungus, *Muscodor* sp. that has previously isolated from the leaf of *Ocimum sanctum* as an antimicrobial agent. Furthermore, the study has also described the effect of the extract on the morphologies of bacteria and yeast cells after being exposed to it.

Materials and Methods

Culture and maintenance of endophytic fungus

The endophytic fungus, *Muscodor* sp. IBRL OS-94 was cultivated and maintained according to the method described by Taufiq & Darah.[13] Sub-culture was done routinely to ensure its purity and survivability.

Test microorganisms

A total of 20 microbial strains (ATCC and clinical isolates) which were provided by the Industrial Biotechnology Research Laboratory (IBRL), School of Biological Sciences, Universiti Sains Malaysia, Penang was used in the present study. The bacterial strains consisted of six Gram-positive bacteria (*Bacillus cereus* ATCC 10876, *Bacillus subtilis* IBRL A1, MRSA ATCC 33591, *Staphylococcus aureus*, *Streptococcus agalactiae* ATCC 13813, and *Streptococcus mutans* ATCC 25175), seven Gram-negative bacteria (*Escherichia coli* IBRL 0157, *Klebsiella pneumoniae* ATCC 13883, *Proteus mirabilis*, *Pseudomonas aeruginosa* ATCC 27844, *Salmonella Typhimurium*, *Shigella boydii* ATCC 9207, and *Yersinia enterocolitica*), yeasts (*Candida albicans*, *Candida utilis*, and *Cryptococcus* sp.) and fungi (*Aspergillus niger*, *Microsporum fulvum*, *Rhizopus oryzae*, and *Trichophyton rubrum*). All the microbial strains were maintained on nutrient agar (NA) and potato dextrose agar (PDA) slants for bacteria and yeast as well as fungi, respectively. The cultures were incubated for 24 h at 37°C for bacteria, 48 h (37°C) for yeasts, and 7 d for fungi (37°C) and kept in a chiller at 4°C until further use.

Culture medium, fungal cultivation, and extraction

The culture medium, cultivation, and extraction of endophytic fungus were carried out according to procedures described by Taufiq and Darah.[13] A sterile control medium was prepared using a similar process as in endophytes culture preparation due to the biological activity of *O. sanctum* extract.

Morphology observation of fungal endophyte isolate

The fungal endophyte isolate was observed under a light microscope to study its microscopic structures and characteristics. The isolate was cultured on the PDA plate and incubated for 14 d at 30°C. Then, a small portion of the fungal hyphae was transferred out onto a clean glass slide containing lactophenol cotton blue. A coverslip was then slowly placed on to the specimen and the slide was subsequently observed under a light microscope (Olympus BX41-CCD). For scanning electron microscope (SEM) viewing, the 7 d old fungal hyphae on the PDA was cut in the size of 5.0 mm x 5.0 mm and then, placed on the Tissue-Tek planchette. Two percent of Osmium tetroxide (OsO_4) was subsequently dropped on the planchette that was previously placed in the petri dish and the dish was immediately closed and left for 1 – 2 h. After the sample was fixed, the planchette was placed into slushy nitrogen -210°C, followed by transferring into the freeze dryer (Emitech K750) and left for another 10 h. After that, the planchette was kept in a desiccator before the viewing process. The sample was coated with 5-10 nm of gold and subsequently observed under the SEM microscope (Leo Supra 50VP Field Emission SEM).

Agar plug diffusion assay

Screening for antimicrobial activity of the endophytic fungal isolates was determined according to the methods described by Taufiq and Darah.[13]

Disc diffusion assay

Disc diffusion assay for test bacteria was performed according to the procedures described by Taufiq & Darah[1]. Meanwhile, for test fungi, the methods described by Mu'azzam & Darah[3] was employed. Chloramphenicol (30 µg/ml) and Ketoconazole (30 µg/ml) were used as positive controls for bacteria and yeast/fungi, respectively whereas 1.0 % of DMSO was set as a negative control. The inhibition zone formed was measured after incubation for 24 h. The study was done in triplicate and the results were expressed as mean value ± standard error of the mean.

Evaluation of minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) values

Broth microdilution assay was performed to determine the MIC values of the fungal extract against test microorganisms in a sterile 96-well microtiter plate according to the method described by Taufiq & Darah[1] and Mu'azzam & Darah[3] The lowest concentration of extract that inhibits any visible of test microbial growth was recognized as MIC, whereas the

MLC was recorded as the lowest concentration of extract that resulted in 99.9% growth reduction relative to the control.

Structural degeneration of microbial cells

The method described by Taufiq & Darah[1] was employed to prepare microbial samples and the specimen preparations for SEM were done according to the method described by Borgers.[14] The prepared specimens were then viewed under a scanning electron microscope (Leica Cambridge, S-360, United Kingdom).

Statistical analysis

The SPSS Version 12.0 (t-test) was employed to compare the effect of the extract against different microbial strains and control. Statistically significance was assumed at the 0.05 significance level ($p < 0.05$). All the experiments were carried out in triplicate and the data were expressed as mean ± standard deviation.

Results

Morphology of Muscodor sp.

Figure 1 shows the macromorphology of *Muscodor* sp. on different agar media. Figure 1a exhibits the growth of the fungal endophytes isolate on potato dextrose agar (PDA). The white fungal hyphae were observed to densely colonize only at the center of agar. On the other hand, the fungal growth on PDA agar nourished with host plant extract is shown in Figure 1b. The white fungal mycelium densely colonized the plate. The cultures of this fungus on both PDA and PDA supplemented with plant extract from a whitish, flocculose colony with uncolored reverse and a mycelium grows slowly. Figure 2 demonstrates the micromorphology of *Muscodor* sp. observed under a light microscope and SEM microscope. Under the light microscope, the septate hyphae showing variation in width and intertwining hyphae was observed (Figure 2a). Besides, a rope-like strand of rugulose hyphae, hyphal coil formation, and the swollen cell could be observed under SEM microscopy (Figure 2b).

Figure 1. Morphology of *Muscodor* sp. on different media PDA agar (left) PDA agar nourished with host plant extract, 2 g/L (right).

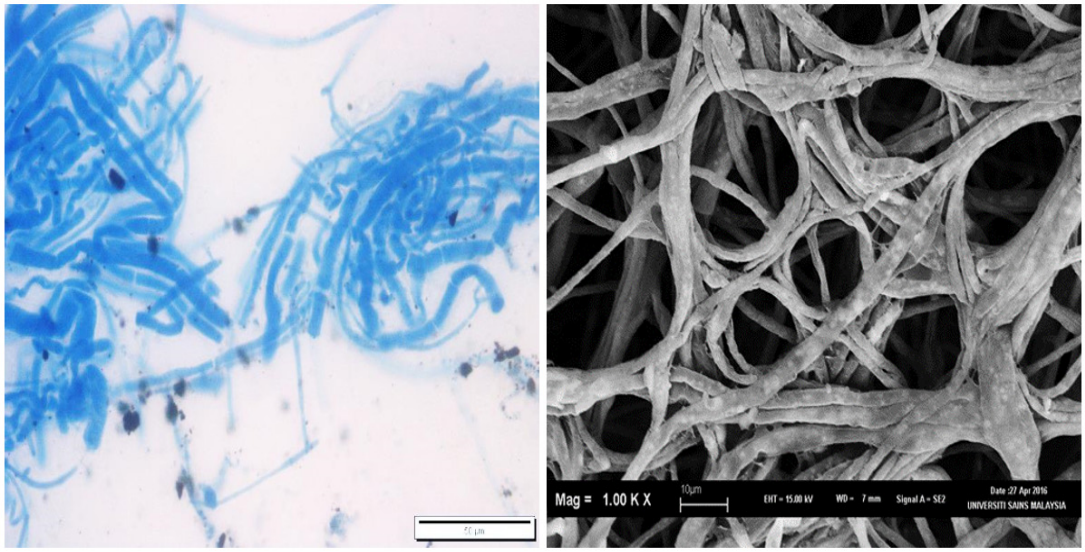

Figure 2. Morphology of *Muscodor* sp. observed under light microscope (left) scanning electron microscope (right).

Preliminary antimicrobial assay of endophytic fungus

The endophytic fungus isolate, *Muscodor* sp. was screened for antimicrobial activity through agar plug diffusion assay. The fungal isolate showed favorable antimicrobial activity against 15 out of 20 microorganisms tested (Table 1). Gram-positive bacteria were susceptible to the fungal isolate since all five species were inhibited with *Staphylococcus aureus* and *Streptococcus mutans* ATCC 25175 were the most susceptible species with the inhibition zones beyond 21 mm. Besides, the oral cavity bacterium, *Streptococcus agalactiae* ATCC 13813 was the least susceptible Gram-positive bacteria with an inhibition zone of less than 10 mm. The other three test bacteria (*Bacillus cereus* ATCC 10876, *Bacillus subtilis* IBRL A1, and Methicillin-resistant *Staphylococcus aureus* ATCC 33591) exhibited the diameter of inhibition zones in the range of 11 to 20 mm.

Table 1. Primary screening via agar diffusion assay.

Test microorganism	Inhibition zones
Gram-positive bacteria	
Bacillus cereus ATCC 10876	++
Bacillus subtilis IBRL A1	++
Methicillin-resistant *Staphylococcus aureus* ATCC 33591	++
Staphylococcus aureus	+++
Streptococcus agalactiae ATCC 13813	+
Streptococcus mutans ATCC 25175	+++
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	+++
Yeasts	
Candida albicans	++
Candida utilis	+
Cryptococcus sp.	-
Fungi	
Aspergillus niger	+
Microsporum fulvum	+
Rhizopus oryzae	-
Trichophyton rubrum	-

Notes: (+) = Inhibition zone ≤10 mm, (++) = Inhibition zone 11 to ≤ 20 mm, (+++) = Inhibition zone ≥ 21 mm

As for Gram-negative bacteria, *Yersinia enterocolitica* was the most susceptible to the fungal isolate with the diameter of inhibition zone more than 21 mm followed by *Proteus mirabilis* with the size of inhibition in the range of 11 to 20 mm. On the other hand, the other three test bacteria such as *Escherichia coli* IBRL 0157, *Klebsiella pneumoniae* ATCC 13883, and *Shigella boydii* ATCC 9207 were the least susceptible with the inhibition zone of < 10 mm, whereas two species (*Pseudomonas aeruginosa* ATCC 27844 and *Salmonella* Typhimurium) were not inhibited by the fungal isolate. For yeasts, *Candida albicans* was the most susceptible to the fungal endophyte isolate with a diameter of inhibition zone between 11 to 20 mm followed by *Candida utilis* with the size of inhibition less than 10 mm, whereas the other test strain (*Cryptococcus* sp.) was not inhibited by the isolate. *Aspergillus niger* and *Microsporum fulvum* were the test fungi inhibited by the fungal isolate with a diameter of inhibition zone less than 10 mm, whereas *Rhizopus oryzae* and *Trichophyton rubrum* were resistant to the isolate. The results obtained demonstrated that *Induratia* sp. possessed prominent antimicrobial activity against pathogenic microbes. The present finding also revealed that Gram-positive bacteria were more susceptible to the fungal isolate compared to Gram-negative bacteria, yeast, and fungi.

Evaluation of the antimicrobial activity of crude extracts

The antimicrobial activity of extracellular and intracellular extracts of *Muscodor* sp. was shown in Table 2. For extracellular extract, the diameter of the inhibition zone for Gram-positive bacteria was ranged between 16.2±0.5 and 27.4±0.7 mm with *Streptococcus agalactiae* ATCC 13813 and *Streptococcus mutans* ATCC 25175 were the least and most susceptible species against the fungal extract, respectively. As for Gram-negative bacteria, the size of the inhibition zone was in the range of 26.3±0.5 to 15.7±0.1 mm with *Yersinia enterocolitica* and *Salmonella* Typhimurium were the most and least susceptible strains, respectively. For yeast species, the fungal extract was inhibited *Candida albicans* and *Candida utilis* with a diameter of inhibition zone of 20.2±0.7 and 14.4±0.5 mm, respectively. However, the other yeast strains, *Cryptococcus* sp. was found to be resistant to the extract due no inhibition zone was observed. For test fungi, *Trichophyton rubrum* and *Aspergillus niger* were the most and least susceptible to the fungal extract with the size of the inhibition zone of 16.5±0.7 and 13.2±0.3 mm, respectively. Another strain, *Microsporum fulvum* demonstrated a significant diameter of the inhibition zone with a value of 15.4±0.5 mm, whereas *Rhizopus oryzae* was resistant to the fungal endophyte extract. As for intracellular fungal extract, the size of the inhibition zone for Gram-positive bacteria was ranged between 12.6±0.7 to 15.6±0.3 mm with *Bacillus cereus* ATCC 10876 and *Staphylococcus aureus* were the least and most susceptible strains, respectively. An almost similar size of inhibition zone was observed for *Bacillus subtilis* IBRL A1 and *Streptococcus mutans* ATCC 25175 with a value

Table 2. Antimicrobial activity of extracellular and intracellular extracts of *Muscodor* sp. against test microorganism through disk diffusion assay.

Test microorganism	Diameter of inhibition zones (mm)		
	Extracellular (ethyl acetate)	Intracellular (methanol)	Control* (30 µg/ml)
Gram-positive bacteria			
Bacillus cereus ATCC 10876	24.6±0.5	12.6±0.7	32.1±0.7
Bacillus subtilis IBRL A1	26.3±0.5	14.2±0.5	29.4±0.3
MRSA ATCC 33591	23.0±0.7	-	30.6±0.1
Staphylococcus aureus	21.0±0.3	15.6±0.3	31.6±0.9
Streptococcus agalactiae ATCC 13813	16.2±0.5	-	24.3±0.3
Streptococcus mutans ATCC 25175	27.4±0.7	14.3±0.5	34.4±0.5
Gram-negative bacteria			
Escherichia coli IBRL 0157	15.7±0.3	-	28.7±0.3
Klebsiella pneumoniae ATCC 13883	18.4±0.3	-	29.9±0.7
Proteus mirabilis	21.7±0.1	8.4±0.7	32.4±0.9
Pseudomonas aeruginosa ATCC 27844	17.6±0.1	-	29.9±0.5
Salmonella Typhimurium	15.7±0.1	-	31.2±0.3
Shigella boydii ATCC 9207	21.0±0.7	10.6±0.5	31.9±0.7
Yersinia enterocolitica	26.3±0.5	12.3±0.7	33.5±0.7
Vibrio parahaemolyticus ATCC 17802	22.6±0.7	-	34.6±0.3
Yeasts			
Candida albicans	20.2±0.7	8.7±0.3	32.9±0.7
Candida utilis	14.4±0.5	-	28.5±0.3
Cryptococcus sp.	-	-	29.7±0.5
Fungi			
Aspergillus niger	13.2±0.3	-	27.6±0.7
Microsporum fulvum	15.4±0.5	-	30.4±0.3
Rhizopus oryzae	-	-	29.9±0.9
Trichophyton rubrum	16.5±0.7	-	31.8±0.5

*Chloramphenicol was used as a standard control for bacteria and ketoconazole was used a positive control for yeasts and fungi

of 14.2±0.5 and 14.3±0.5 mm, respectively. However, two bacterial strains (MRSA ATCC 33591 and *Streptococcus agalactiae* ATCC 13813) were found to be resistant to the intracellular fungal extract. As for Gram-negative bacteria, only three species (*Shigella boydii* ATCC 9207, *Yersinia enterocolitica,* and *Proteus mirabilis*) were susceptible to the fungal extract with the inhibition zone ranged between 8.4±0.7 and 12.3±0.7 mm. However, the other five species were resistant to the extract. For yeasts, the fungal extract was only inhibited *Candida albicans* with the value of the inhibition zone of 8.7±0.3 mm and the extract was inactive against the other two strains (*Candida utilis* and *Cryptococcus* sp.). Ironically, none of the test fungi were inhibited by the intracellular fungal extract since no inhibition zones were observed.

Evaluation of minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) values

The MIC and MLC values of the fungal endophyte's extracts on various test microorganisms were presented in Table 3. For Gram-positive bacteria, the MIC and MLC values for extracellular extract were in the range of 62.5 – 500.0 µg/ml and 125.0 – 4000.0 µg/ml, respectively. The fungal extract exerted a bactericidal effect towards all test Gram-positive bacteria since MLC/MIC ratio less than or equal to four except *Streptococcus agalactiae* ATCC 13813. For extracellular extract, the MIC and MLC values were ranged in between 250.0 – 500.0 µg/ml and 1000.0

Table 3. Determination of MIC and MLC values of the extract via broth microdilution assay.

Test microorganism	Extracellular			Intracellular		
	MIC (µg/mL)	MLC (µg/mL)	Ratio	MIC (µg/mL)	MLC (µg/mL)	Ratio
Gram-positive bacteria						
Bacillus cereus ATCC 10876	62.5	125	2	500	2000	4
Bacillus subtilis IBRL A1	250	1000	4	250	2000	8
MRSA ATCC 33591	125	250	2	-	-	-
Staphylococcus aureus	125	125	1	250	1000	4
Streptococcus agalactiae ATCC 13813	500	4000	8	-	-	-
Streptococcus mutans ATCC 25175	250	250	1	500	2000	4
Gram-negative bacteria						
Escherichia coli IBRL 0157	250	2000	8	-	-	-
Klebsiella pneumoniae ATCC 13883	500	2000	4	-	-	-
Proteus mirabilis	250	500	2	250	2000	8
Pseudomonas aeruginosa ATCC 27844	500	4000	8	-	-	-
Salmonella Typhimurium	250	2000	8	-	-	-
Shigella boydii ATCC 9207	500	4000	8	500	4000	8
Yersinia enterocolitica	250	1000	4	500	2000	4
Vibrio parahaemolyticus ATCC 17802	250	1000	4	-	-	-
Yeasts						
Candida albicans	250	1000	4	1000	4000	4
Candida utilis	500	2000	8	-	-	-
Fungi						
Aspergillus niger	500	4000	8	-	-	-
Microsporum fulvum	250	2000	8	-	-	-
Trichophyton rubrum	500	4000	8	-	-	-

– 2000.0 µg/ml, respectively. The bactericidal effect of the extract was observed against *Bacillus cereus* ATCC 10876, *Staphylococcus aureus*, and *Streptococcus mutans* ATCC 25175, whereas a bacteriostatic effect was observed on *Bacillus subtilis* IBRL A1.

The MIC and MLC values of extracellular fungal extract for Gram-negative bacteria were found to be in the range of 250.0 – 500.0 µg/ml, respectively. The extract exhibited bactericidal activity towards several strains such as *Klebsiella pneumoniae*, *Proteus mirabilis*, *Yersinia enterocolitica*, and *Vibrio parahaemolyticus* ATCC 17802, but bacteriostatic effect towards *Escherichia coli* IBRL 0157, *Pseudomonas aeruginosa* ATCC 27844, *Salmonella* Typhimurium, and *Shigella boydii* ATCC 9207. On the other hand, the MIC and MLC values of the intracellular extract against Gram-negative bacteria were in the range of 250.0 – 500.0 µg/ml and 2000.0 – 4000.0 µg/ml. The MLC/MIC ratio of the fungal extract against *Yersinia enterocolitica* was 4 (bactericidal effect) and for *Shigella boydii* ATCC 9207 and *Proteus mirabilis* were 8 which is a bacteriostatic effect.

For yeasts, the MIC and MLC values of extracellular extract were observed in the range of 250.0 – 500.0 µg/ml and 1000.0 – 2000.0 µg/ml, respectively. The extract exhibited yeastocidal and yeastostatic activities against *Candida albicans* (MLC/MIC ratio ≤ 4) and *Candida utilis* (MLC/MIC ratio > 4), respectively. Besides, the intracellular extract exerted a yeastocidal effect towards *Candida albicans* since MIC and MLC values were 1000.0 – 4000.0 µg/ml, respectively. As for test fungi, the MIC and MLC values of the extracellular fungal extract in the range of 250.0 – 500.0 µg/ml and 2000.0 – 4000.0 µg/ml, respectively. The extract was observed to exert a fungistatic effect towards all three test fungi (*Aspergillus niger*, *Microsporum fulvum*, and *Trichophyton rubrum*) with the MLC/MIC ratio equal to eight.

Structural degeneration of microbial cells exposed to the extract

Figure 3 demonstrated the morphological changes in *Streptococcus mutans* after treated with the ethyl acetate crude extract of *Muscodor* sp. IBRL OS-94 at MLC concentration. The untreated cells appeared as a typical smooth surface with the normal coccal chains and maintained rigidity (Figure 3a). However, there was the formation of cavities and some cell debris was observed on the bacterial cells after 48 hours of exposure to the fungal extract (Figure 3b). The disruption of bacterial cells has occurred whereby most of them were lysed, shrunk, and completely collapsed. In fact, the typical normal smooth coccal chains shape of *S. mutans* was no longer seen with only the crumpled cell residues were left.

The changes in structure and morphology of the Gram-negative bacterium, *Yersinia enterocolitica* treated with 1000 µg/mL of the ethyl acetate extract were illustrated in Figure 4. Untreated cells of *Y. enterocolitica* had a typical normal condition with a rigid rod shape and rugose surface (Figure 4a). Some longer rod was observed in a dividing cell which is typical cell replication by bacteria. Figure 4b shows the bacterial cells after 48 hours exposed to the ethyl acetate extract of *Muscodor* sp. Some alterations occurred on the bacterial cell surface followed by irregular shape. Cell surface becomes crumpled, flaccid, and shrunk with some of them opened outwards, thus leaving cleavage in their surface.

The SEM photomicrographs showing the effect of ethyl acetate extract produced by an endophytic fungus, *Muscodor* sp. IBRL OS-94 on *Candida albicans* were illustrated in Figure 5. Figure 5a demonstrates the untreated cells with an oval and smooth surface. Some budding was also observed on the cell's surface. On the other hand, remarkable morphological changes of the cells occurred after 48 hours treated with the fungal extract whereby some invaginations and cavitation formed on the cells' surfaces (Figure 5b). Some of the cells were crumpled and

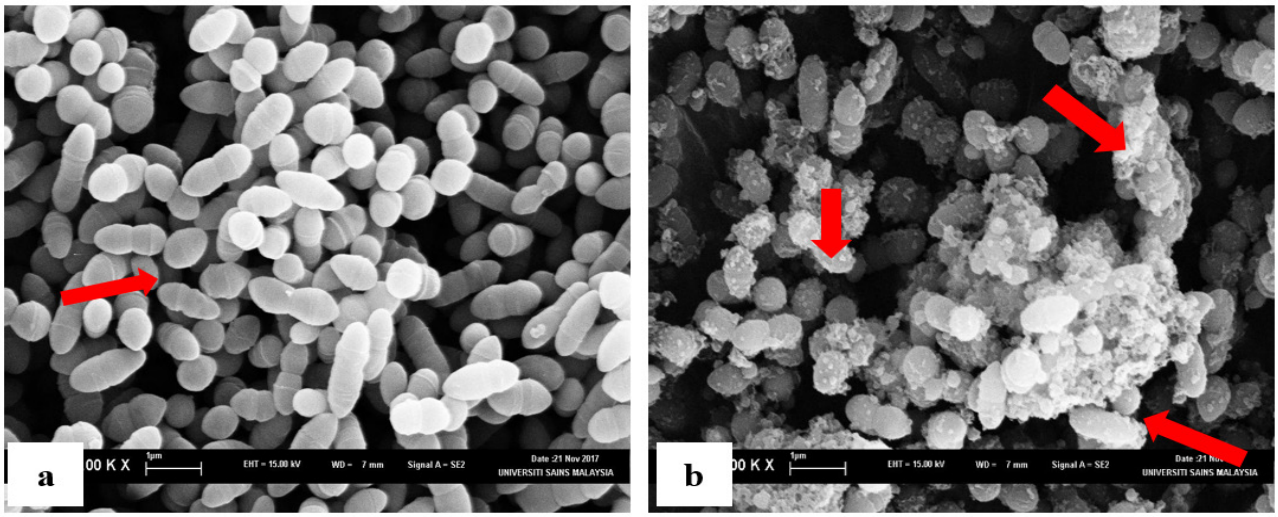

Figure 3. SEM photomicrographs of Gram-positive bacterium, *Streptococcus mutans* treated with 250 µg/mL of ethyl acetate crude extract. (a) 0 hour [control] (b) 48 hours. Scale bars: 100 nm.

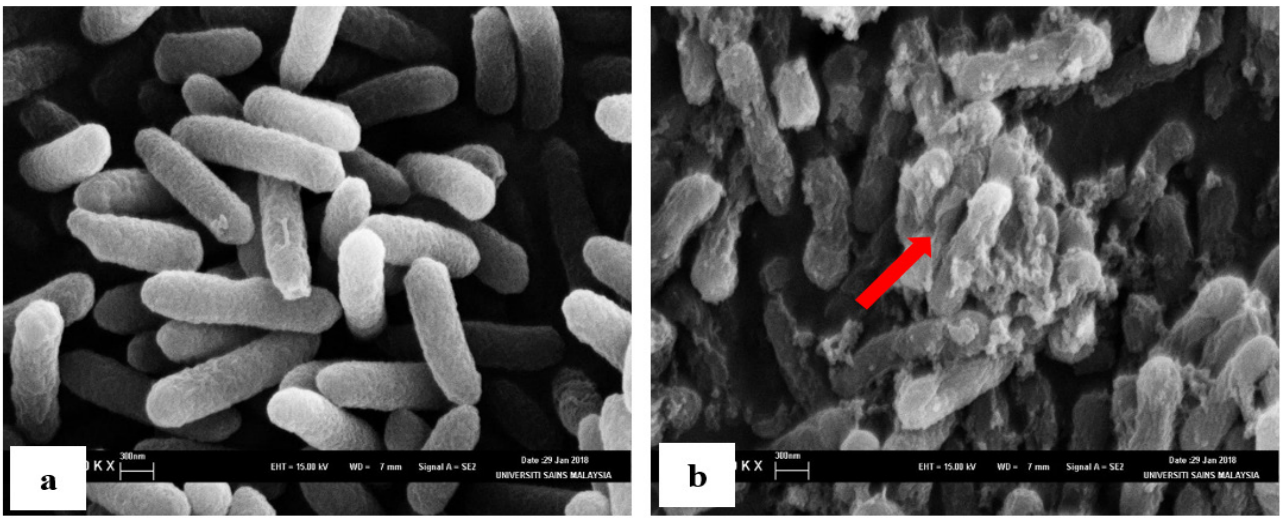

Figure 4. SEM photomicrographs of Gram-negative bacterium, *Yersinia enterocolitica* treated with 500 µg/mL of ethyl acetate crude extract. (a) 0 hour [control] (b) 48 hours. Scale bars: 100nm.

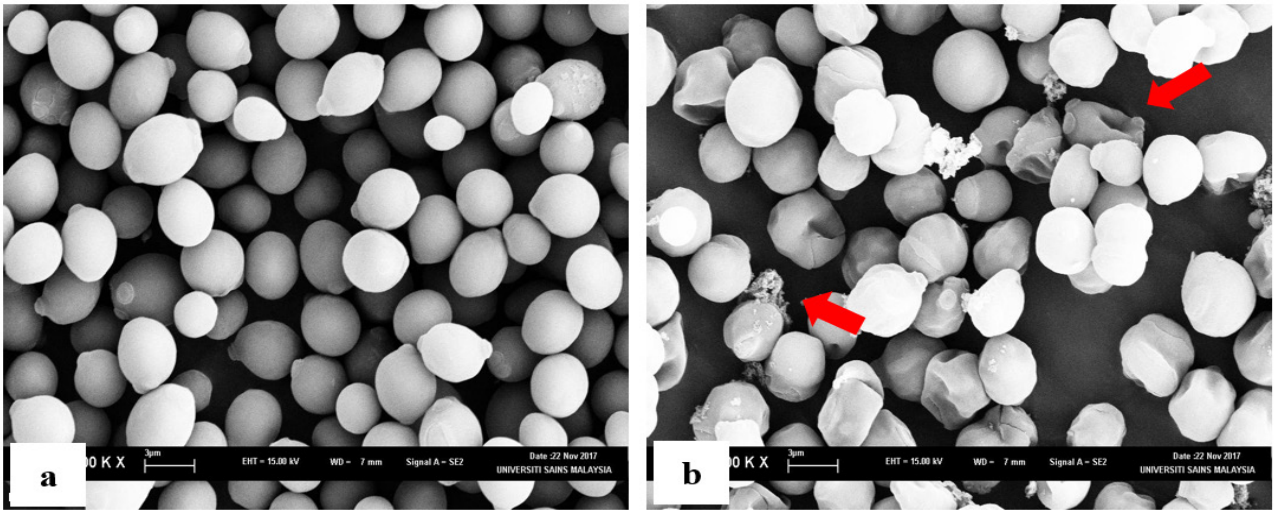

Figure 5. SEM photomicrographs of yeast, Candida albicans treated with 500 µg/mL of ethyl acetate crude extract. (a) 0 hour [control] (b) 48 hours. Scale bars: 200nm.

shrunken. This phenomenon indicated that the candidal cells had undergone severe morphological changes and cytological alterations and these damaged cells lead to loss of their metabolic functions.

Discussion

Muscodor sp. is an endophytic ascomycete genus which constitutes a valuable contribution of fungal diversity and is introduced by the discovery of *Muscodor albus* isolated from *Cinnamon zeylanicum* from Honduras.[15] To date, 21 species of *Muscodor* have been described and identified based on the biological, physiological, cultural characteristics, chemical profiles, morphological, and genetic features.[16] These fungi are classified in the family *Xylariaceae* and possessed a special molecular identity compared to other genera in the family.[17,18] *Muscodor* species are volatile producing endophytes that can be found in Central/Southeast Asia, Australia, and also Central/South America.[19] They have been reported to produce a variety of volatile compounds with prominent biological effects against bacteria, fungi, insects, and potentially used as a natural biocontrol.[19,20] For instance, Pena[21] reported *Muscodor brasiliensis* produces volatile compounds with antifungal activity against *Penicillium digitatum* with a percentage of inhibition in between 70 to 100%. Besides, the volatile organic compounds (VOCs) of *Muscodor* sp. W-S-41, an endophytic fungus isolated from gramineous plants has been reported to possess strong antifungal activity against *Pythium ultimum*, *Penicillium digitatum*, and *Botrytis cinerea* with complete inhibition and killing of mycelial growth.[9] Besides, a fungal endophyte of *Cinnamomum camphora*, *Muscodor tigerii* has been observed to reduce the growth of *Candida albicans* in the presence of VOCs with 50 – 60% of growth reduction.[22] Meanwhile, Ezra[17] reported that, a fungal endophyte of *Cinnamomum zeylanicum* origin (*Muscodor albus*) can produce a variety of volatile substances with promising antibacterial activity against several test bacteria including *Bacillus subtilis* and *Escherichia coli*.

The intriguing result from the present study is the culture filtrate and fungal biomass of endophytic fungal isolate, *Muscodor* sp. IBRL OS-94 that previously isolated from the leaf medicinal plant, *Ocimum sanctum* exerted significant antimicrobial activity. Primary screening through agar plug diffusion assay revealed that the fungal endophytes isolate, *Muscodor* sp. IBRL OS-94 exerted a broad-range antimicrobial activity since it can inhibit two fungi, two yeasts, five Gram-negative bacteria and five Gram-positive bacteria. The finding was in agreement with the results reported by Sahani[23] who revealed the endophytic fungi isolated from medicinal plants, *Aloe vera* and *Ocimum sanctum* exhibited broad-range antimicrobial activity towards Gram-negative and Gram-positive bacteria. It is noteworthy that, the susceptibility of some test microorganisms towards fungal isolate/extract was different in agar plug diffusion and disk diffusion assay. For instance, the fungal isolate was inactive against *Trichophyton rubrum* in agar plug diffusion assay but, the fungal extract exhibited antifungal activity against *T. rubrum* in disk diffusion assay. This phenomenon might be due to the concentration of bioactive compounds, depth of agar medium, and diffusion rate. According to Bonev,[24] the formation of inhibition zone was influenced by diffusion behavior (dissipative diffusion), diffusion rate, and depth of agar, loss of substrate during diffusion, antibiotic degradation, and antibiotic removal by the microbial film. Besides, the different strains of test microorganisms with different resistant mechanisms affected their susceptibility towards the fungal extract. For instance, previous studies reported the resistance and low susceptibility of *Shigella* sp. strains towards the antimicrobial drug, fluoroquinolone due to gene coding (a QRDR in the gyrA gene) for a region associating with NA occurred in all NA-resistant strains that lead to the mutation of the gene.[25] The mutation in codon contributes to the high resistance of *Shigella* sp. strains towards antibiotic agents.[26] Besides that, *Streptococcus agalactiae* was observed to be more resistant towards antibiotics

and this may be due to its ability to produce slime which is a desirable virulence factor during cell colonization especially in aggregation and biofilm formation.[27,28] Also, the resistance of *Streptococcus* sp. towards antibiotic agents such as macrolide may be attributed to methylation of the ribosome that preventing erythromycin from binding with its ribosomal methylase that encoded by erm genes.[29] On the other hand, the mode of action of fungal extract plays a vital role in contributing to the higher susceptibility of microbial strains towards antibiotics. Some of the antibiotic agents can interfere in gene transcription and inhibit the certain physiological function of bacteria.[30] For instance, antimicrobial agents can block the biosynthesis of mycolic acid in *Mycobacterium tuberculosis*, and thus, change in expression of the corresponding enzymes.[31] Therefore, increase the susceptibility of microbial strains against antimicrobial agents.

Disk diffusion assay is the best choice of method to screen the antimicrobial activity from fungal biomass and their culture filtrates and is recommended by many researchers.[32] This assay is usually used in the preliminary screening for antimicrobial activity due to its ability to rapidly identify the activity of bioactive metabolites presents within the fungal endophytes extract.[33] The results of secondary screening revealed that Gram-positive bacteria were susceptible compared to Gram-negative bacteria. The finding corroborated the previous reports that event endophytic fungal extracts are more active against Gram-positive bacteria. The differential sensitivity of both bacterial strains may be due to the composition of the cell wall and the efficacy of the antimicrobial agents. According to Norajit,[34] the cell wall of Gram-positive bacteria is more sensitive to many antimicrobial compounds. The phenomenon may be due to the inherent tolerance of Gram-negative bacteria such as the presence of lipopolysaccharides layer and periplasmic space and the nature and composition of antimicrobial agents.[35] Generally, the cell wall of Gram-negative bacteria consists of complex structures such as the outer membrane layer, thin peptidoglycan layer, and periplasm compared to Gram-positive bacteria composed of a thick layer of peptidoglycan.[36] The outer membrane layer is a special structure that differentiates between both bacteria comprised of lipopolysaccharides, proteins, and phospholipids separating the external environment from the periplasm and it has restricted flow and serves as selective barrier that prevents antibiotic compounds but, allows the transportation of valuable nutrients to the cell. The membrane is also anchored by membrane proteins known as porins which act as a selective channels that allow the transportation of specific size hydrophilic substances into the periplasm.[37,38] Besides that, the concentration of extract also plays a vital role in inhibiting bacterial growth and the low concentration of antibiotic agents leads to insufficient in executing the inhibitory action, and thus, the bacteria become resistant to the antibiotics.[39] On the other hand, yeast, and fungi were least susceptible to the fungal extract compared to bacteria. The resistance of fungal species could be due to their morphological structure since fungi are composed of the thicker cell wall and contain a higher percentage of chitin.[40] Chitin, a long-chain polymer of N-acetylglucosamine, is a primary component of cell walls in fungi and laid down in microfibrillar bundles to form a thick and tough wall structure, which acts as an efficient permeability barrier.[41] Thus, the component restricts the permeable of antimicrobial compounds into the cell walls of fungi and yeast.

Broth microdilution assay was carried out to determine the MIC and MLC values of the fungal extract towards several test bacteria and fungi. This assay involves the preparation of two-fold dilution of antimicrobial agents and more accurate as compared to disc diffusion assay since the MIC value is generated quantitatively.[42,43] Besides, the MLC value from the broth dilution assay might benefit the researcher due to the ability to distinguish between bactericidal/fungicidal and bacteriostatic/fungistatic effects.[43,44] In general, broth microdilution assay is more sensitive as compared to disc diffusion assay and it is appropriate procedures for assaying non-polar and polar compounds for determination of MIC and MLC.[44,45] MIC is the lowest concentration of an antimicrobial agent that inhibits the visible microbial growth whereas, MLC can be defined as the lowest concentration of antimicrobial drug needed to kill 99.9% of microbial growth after 24 h of incubation period under standardized conditions.[46,47] The current results demonstrated that extracellular fungal extract exerted a bactericidal effect against almost all test Gram-positive bacteria and some Gram-negative bacteria. It is noteworthy that, the fungal extract has significant antibacterial activity against *Staphylococcus aureus* and *Streptococcus mutans* since MLC/MIC ratio was 1. The same value of MLC and MIC indicated the high sensitivity of test bacteria towards the antibacterial compounds in the extract. This finding in parallel with Yenn[48] that reported MRSA has high sensitivity towards the ethyl acetate of an endophytic fungus, *Phomopsis* sp. ED2 due to similar MLC and MIC values. According to Ocampo,[49] antimicrobial agents can be divided into two groups which are bacteriostatic or bactericidal drugs, in which bacteriostatic drugs can only inhibit bacterial growth without killing the cells whereas, the bactericidal drugs resulted in cell killing. Silva[50] defined bactericidal/fungicidal activity as a reduction of 99.9% microbial growth which approximately > 3 log10 CFU/mL in the original inoculum whilst, bacteriostatic/fungistatic activity was defined as a reduction of microbial growth less than 99.9% (less than 3 log10 CFU/mL) of the original inoculum concentration of microbial cells. Furthermore, antimicrobial agents can be considered to possess a bacteriostatic/fungistatic effect if the MLC/MIC ratio less than or equal to 4-folds whilst, bactericidal/fungicidal activity if the MLC/MIC ratio more than 4-folds.[51] Besides, MLC of the fungal extracts was significantly higher than MIC values against almost all test microorganisms indicated that the antimicrobial activity of the extracts was dose-dependent. A previous study reported

that bactericidal/fungicidal drugs are clinically more powerful antibiotic agents compared to bacteriostatic/fungistatic drugs since this type of drug can kill bacteria and rather effective in severely immunocompromised patients.[3]

The present findings also revealed that the endophytic fungus, *Muscodor* sp. secrete more bioactive compounds extracellularly into the fermentative broth compared to intracellular. The result was in agreement with Ibrahim[52] who reported an endophytic fungus of *Cassia siamea* origin produced anti-candidal compounds extracellularly. Normally, fungal endophytes secreted their secondary metabolites extracellularly and seldom keeping them intracellularly. According to Yenn,[53] this phenomenon may be due to the role of secondary metabolite substances as protector agents from pathogen and thus, they are more suitable to be produced extracellularly. In contrast, another study reported that the extract from fungal biomass (intracellularly) of *Phomopsis* ED2, an endophyte of *Stamineus orthosiphon* origin exhibited better antimicrobial activity and they claimed that more antimicrobial substances were associated with the fungal biomass compared to fermentation culture.[54] The current finding speculated the secretion and formulation of secondary metabolites by endophytic fungi is depends on their species and role in the host plant.

SEM analysis was performed to investigate the effect of the fungal extract on the cells of yeast, Gram- negative, and Gram- positive bacteria. The current findings revealed the extract affected the cell of Gram-positive bacteria with some abnormalities including the formation of cavities and cell debris, congruent with Ibrahim[55] that reported the morphological changes of Gram-positive bacteria exposed to ethyl acetate extract of an endophytic fungus, *Nigrospora sphaerica* CL-OP30 with the formation of cell debris and cavities on the bacterial cells. They also revealed that some of the treated bacterial cells were lysed, shrunk, and completely collapsed. Similarly, Borthakur and Joshi,[56] reported the treated pathogen, *Staphylococcus aureus* showed some cellular deformities such as sticky membrane and leakage of the internal compositions. They postulated that the cell of Gram-positive bacteria exposed to macrofungal extract exhibited perforation in the cell wall membrane followed by the leakage of inner compositions. On the other hand, Gram-negative bacteria treated with the fungal extract showed crumpled, flaccid, shrunk, and cell death beyond repair. Similarly, Sofy[57] revealed that the Gram-positive bacteria treated with *Thymus vulgaris* aqueous extract appeared to be shrunk, stuck together, melted and even some were empty. They also confirmed that the treated cells were lost their shape and integrity followed by the ultimate cell death. Besides, Chatterjee[58] reported some deformations and prominent degradation of cell wall structures were observed on *Escherichia coli*, and *Salmonella* Typhimurium exposed to the extract of *Alternaria alternata* AE1, a fungal endophyte from *Azadirachta indica*. They also observed end-to-end joining of bacterial cells upon treatment indicating an effect of antimicrobial agents on normal cell division function and presumed that the massive distortion of morphological structures of bacteria cells may be due to the mode of action of antimicrobial agents on the bacterial cell wall. As for yeast, the *Candida albicans* cells exposed to the fungal extract were shrink with the formation of dents observed. Sukmawati[59] reported that the candidal cell treated with the extract of *Auricularia auricula* becomes shrunken, dents, and formation of a bulge on one side of the cell. They also reported the formation of protrusions on candidal cells indicating the inability of peptidoglycan cells damaged by antimicrobial substances along with intracellular pressure and thus, cytoplasm exits and the bulge appears in regions attenuated with antimicrobial agents. Generally, photomicrographs SEM observations confirmed the physical deformation and considerable morphological alterations to test Gram-negative, Gram-positive bacteria, and yeast exposed to the ethyl acetate extract of *Muscodor* sp. IBRL OS-94.

Many previous studies were done in an attempt to observe the effects of fungal extracts and their mode of actions on different bacteria and yeast to support the antimicrobial activities after exposing them to the extracts. For bacteria, a previous study revealed that endophytic fungal extract, *Trichoderma asperelloides* affected bacterial cells by inducing cellular damages including cell wall and cytoplasmic membrane disturbance.[60] Besides, Yenn[61] suggested that the mode of action of endophytic fungal extract, *Penicillium purpurogenum* ED76 causes significant damage to bacterial cells including invaginations of the cell wall. As for yeast, the majority of the previous studies observed the fungal extracts affected the fungal cell wall and membrane structures. For instance, Supaphon[62] revealed that the mycelial extract of *Trichoderma* sp. PSU-ES38 significantly inhibited the *Candida albicans* NCPF 3153 with considerable structural alterations such as shrinkage, deformation, broken, and collapsed cells. They also postulated that the active extracts affect the fungal cell wall as well as the cell membrane. Besides, Ibrahim[52] studied the SEM and TEM photomicrographs and observed the morphological abnormalities experienced by *C. albicans* cells exposed to the ethyl acetate extract of *Aspergillus flavus*, an endophytic fungus isolated from the leaf of *Cassia siamea* Lamk. They suggested the fungal extract inhibits the cell wall components and thus, induce the abnormal cell wall formation. Thus, the present findings suggested that the fungal extract of *Muscodor* sp. IBRL OS-94 efficiently inhibits the yeast and bacterial growth and the bioactive compounds in the extract affected the cell wall and cell membrane which lead to cell death.

Conclusion

The present study corroborates previous studies that showed the existence of antimicrobial activities in the endophytic fungal extract of *Muscodor* sp. The fungal extract has promising and significant antimicrobial activity

against Gram-positive, Gram-negative bacteria, and yeast. It also has a significant effect on inhibiting yeast and bacterial growth by disturbing the cell wall and membrane. However, that ethyl acetate crude extract needs to be further characterized and purified in order to obtain pure/single bioactive compound(s) and so that, the individual antimicrobial activity analysis can be suggested based on the present study.

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Author Contributions

JMTM and HNHM: Did all experiments. ID: The principle investigator and supervisor. JMTM, HNHM and ID: Prepared and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

Authors have declared that no competing interests exist.

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