Anti-yeast activity of endophytic fungi isolated from medicinal herb *Ocimum sanctum* with emphasis on *Candida albicans*

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Abstract
Endophytic fungi, Lasiodiplodia pseudotheobromae IBRL OS-64 and Muscodor sp. IBRL OS-94 were previously isolated from the leaf of medicinal plant, Ocimum sanctum was screened for their anti-yeast activity against pathogenic yeast. Both fungal isolates were cultivated in submerged fermentation using yeast extract sucrose broth as culture medium enriched with plant water extract and incubated for 20 d at 30°C with an agitation speed of 120 rpm. Preliminary study via agar plug diffusion assay revealed that both fungal isolates possess a fair anti-yeast activity against three out of four test yeast including Candida albicans, C. utilis and Rhodotorula rubra. On the disk diffusion assay, the ethyl acetate extract of L. pseudotheobromae IBRL OS-64 and Muscodor sp. IBRL OS-94 showed significant anti-yeast activity with the size of the inhibition zone ranging in between 9.6±0.5 - 21.3±0.5 mm and 14.4±0.5 - 20.2±0.7 mm respectively. As for ethyl acetate extract from L. pseudotheobromae OS-64, the MIC and MYC values were in the range of 500 – 1000 µg/mL and 2000 – 8000 µg/mL respectively.

On the other hand, the MIC and MYC values of ethyl acetate extract of Muscodor sp. OS-94 ranged between 250 – 500 µg/mL and 1000 – 4000 µg/mL respectively. The time-kill assay demonstrated that the growth of C. albicans cell was dose-dependent in which the extract concentration was lower and higher than the MIC value showing yeastostatic and yeastocidal effects respectively. The scanning and transmission electron microscopes observation revealed that the candidal cells expose to extract experienced severity of morphological deterioration which led to cell death beyond repair and thus suggesting that the fungal extract could be a potential anti-candidal agent.

Keywords: Endophytic fungi, Anti-yeast activity, Agar plug diffusion assay, Disc diffusion assay, MIC and MYC values, Morphological deterioration.

Introduction

A dramatic increase of invasive incident and opportunistic fungal infections by pathogenic yeasts results in a higher rate of morbidity and mortality. According to Kaufman[25], *Candida* spp. especially *Candida albicans* are the main cause of fungal infection in humans approximately 95% and thus lead to a life-threatening disease known as candidiasis if it reaches the human bloodstream.

Usually, infections caused by *Candida* spp. are developed as a result of changes in immune response and the prevalence is supported by their virulence. The virulence of this species is attributed to their morphological plasticity since they are capable to form chlamydospores, hyphae and pseudohyphae depending on growth condition. The ability to form biofilm is one of the most important virulence factors of this species since it can increase resistance to antibiotic[8].

Candida spp. are commonly found on the skin of humans, genitourinary tracts and mucosal surfaces of gastrointestinal and they become opportunistic pathogens in immuno-compromised and immunologically weak patients including AIDS and cancer survivors[24]. Nett[36] reported that *Candida* becomes an invasive pathogen with immunosuppression and thus leads to disseminated disease with mortality approximately 40% and the emergence of drug-resistant strain poses an additional obstacle to treatment. The management of fungal infection has become more complex due to the lack of available antifungal drugs, side effects and drug safety problems[40]. The emergence of resistant fungal and yeast strains and increasing prevalence of their infections in immunocompromised patients led to the need for searching for new effective anti-yeast drugs. As a result, Yenn[61] has reported endophytic fungi for potentially useful pharmaceutical compounds to overcome the drug resistance issues.

Endophytic fungi widely exist inside the healthy tissue of living plants without causing any harm to their host and the symbiotic co-existing over the long period of evolution has established a special relationship among them which influenced the formation of metabolic products in plants and thus affected quantity and quality of drugs derived by medicinal herbs[23].

Endophytes are ubiquitous and widely distributed in host plants and recent reports revealed that every plant harbors at least one species of fungal endophytes and many plants such as woody plants may be colonized by thousands of species[16]. The distribution and population of endophytes can be affected by several factors including the environmental conditions of their hosts, age and genetic background.

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Endophytic fungi play a vital role in an ecosystem by helping plants to a new habitat, increasing their resilience and protecting plants from against abiotic stress such as extreme pH and temperatures and biotic stress including plant pathogens and insects. In return, plants provide nutrients, protection from desiccation, spatial structures and dissemination to the next generation of hosts[31].

Muscodor sp. and *Lasiodiplodia* sp., endophytic fungi have been reported to possess bioactive compounds with antimicrobial activity against a wide range of pathogenic bacteria, yeast and fungi.

Strobel et al[50] reported that *Muscodor albus* E-6, an endophyte fungus isolated from *Guazuma ulmifolia* can produce volatile organic compounds (VOCs) including 2-methyl- butanoic acid, 3-methyl- 2-butenal and 2-methyl-butanoic acid that are able to inhibit several bacteria such as *Escherichia coli* and *Bacillus subtilis*.

On the other hand, an endophytic fungus from *Viscum coloratum*, *Lasiodiplodia* sp. ME4-2 has been reported to formulate indole-3-carboxylic acid and other aromatic metabolites[42]. Meanwhile, Wei et al[57] reported that *Lasiodiplodia pseudotheobromae* F2 can produce lasiodiplines with antibacterial activity against several clinical strains including *Peptostreptococcus* sp. and *Streptococcus* sp. Thus, the present study was designed to screen the anti-yeast activity of *Muscodor* sp. IBRL OS-94 and *Lasiodiplodia pseudotheobromae* IBRL OS-64, the endophytic fungi of *Ocimum sanctum* to combat pathogenic yeast such as *C. albicans*.

Material and Methods

Culture and maintenance of endophytic fungus: The endophytic fungi *Lasiodiplodia pseudotheobromae* IBRL OS-64 and *Muscodor* sp. IBRL OS-94 which were previously isolated from the leaf of *Ocimum sanctum*, were provided by the culture collection division of Industrial Biotechnology Research Laboratory (IBRL), School of Biological Sciences, Universiti Sains Malaysia, Penang. The fungal isolates were cultured on potato dextrose agar (PDA) supplemented with host plant extract (2.0 g/L) and incubated at 30ºC for 7 d. The culture was kept in a fridge at 4ºC until further use and sub-culture was performed monthly to ensure its purity and survivability.

Test microorganisms: A total of four yeast strains (clinical isolates) which were provided by the Industrial Biotechnology Research Laboratory (IBRL), School of Biological Sciences, Universiti Sains Malaysia, Penang were used in the present study. The yeast strains which are *Candida albicans*, *Candida utilis*, *Cryptococcus neoformans* and *Rhodotorula rubra* were maintained on potato dextrose agar (PDA) slants. The cultures were incubated for 48 h at 37ºC and kept in a chiller at 4ºC until further use. Sub-culturing was done once a month to ensure the purity and viability of the cultures.

Culture medium: Yeast extract sucrose (YES) broth consisting of yeast extract, 20 (g/L); sucrose, 40 (g/L) and magnesium sulfate, 0.5 (g/L) nourished with the water extract of *O. sanctum* was used to cultivate endophytic fungal isolates in the shake-flask system. Before that, the water plant extract was prepared by boiling 2 g of the powdered plant materials in 1000 ml distilled water for 30 min. The water plant extract was then filtered using Whatmann no. 1 filter paper and mixed with freshly prepared culture medium. The pH of the culture medium was adjusted to 5.8 and autoclaved for 15 min at 121°C.

Fungal cultivation and Extraction: The cultivation and extraction process were performed according to methods described by Taufiq and Darah[53]. The inoculum was prepared by transferring two mycelial agar plugs into 250 ml Erlenmeyer flasks consisting of 100 mL YES broth. The agar plugs were 1.0 cm in diameter with 4.0 mm thickness and excised from the 7-day old fungal culture. This culture was then incubated at 30°C with an agitation speed of 120 rpm under the dark and static condition for 20 d. The fermentative broth and fungal biomass were separated using Whatmann no. 1 filter paper after 20 d of the incubation period. The fermentative broth was subjected to the extraction process sequentially with hexane, dichloromethane, ethyl acetate and methanol (1:1; v/v) thrice. The organic phase extracts were then collected and evaporated under the reduced pressure.

Yeast suspension: Yeast inoculums were prepared by transferring 3-4 single colonies of 16 to 24 h old yeast culture into 5.0 mL sterile physiological saline and mixing well to obtain cell suspensions approximately 1×10^6 CFU/mL. The size of inoculums was standardized by matching its turbidity with the McFarland 0.5 standard.

Agar plug diffusion assay: Primary screening of antimicrobial activity of the endophytic fungal isolates was studied by employed procedures described by Taufiq and Darah[53]. For agar plugs preparation, the fungal isolate was inoculated onto the PDA agar plate supplemented with host plant extract (2 g/L) and incubated for 20 d at 30°C. The agar plugs were then cut (1 cm in diameter and 4 mm thickness) using a sterile cork borer before placing them on the PDA agar seeded with test yeast and the plates were inversely kept overnight at 4°C to allow diffusion of bioactive compounds and subsequently incubated at 37°C for 24-48 h. The inhibition zones formed around the endophyte agar plugs were measured and recorded.

Disc diffusion assay: The sterile 6.0 mm paper discs were impregnated with 20 µL of fungal extract and placed on potato dextrose agar (PDA). The fungal 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 obtain a final extract concentration of 1 mg/mL. Ketoconazole (30 µg/mL) was used as a positive control whereas 1.0% of DMSO was set

as a negative control. The impregnated disk was placed on the PDA seeded with test yeast and then incubated at 37°C for 24-48 h. After the incubation period, the inhibition zone formed was measured and the results were expressed as mean value ± standard error of the inhibition zone obtained from three separate occasions.

Determination of minimum inhibitory concentration (MIC) and minimum yeastocidal concentration (MYC): Broth microdilution assay was performed to determine the minimum inhibitory concentration (MIC) in a sterile 96-well microtiter plate[34]. The inoculum was prepared as discussed earlier and the fungal extract was subjected to two-fold serial dilution using sterile Sabouraud dextrose broth (SDB) as a diluent to obtain the final extract concentrations ranging between 0.0156 to 8000 µg/mL. The control sets of an experiment consisting of the mixture of sterile SDB medium and microbial inoculum.

After incubation at 37ºC for 24-48 h, an amount of 40 µl of 0.2 mg/mL p-iodonitrotetrazolium violet salt (INT) was then added into each well as a growth indicator. The lowest concentration of extract that inhibits any visible of test microbial growth was considered as MIC whereas the MYC was recorded as the lowest concentration of extract that resulted in a 99.9% growth reduction relative to the control.

Time-kill assay: The experiment was performed following the protocol described by CLSI[11] with some modifications. The *C. albicans* suspension previously prepared was aseptically transferred into the 20.0 ml SDB followed by incubation at 37°C at agitation rate of 150 rpm for 16 to 18 h. The cells' turbidity was measured during the log phase using spectrophotometer at 625 nm wavelength. The extract with different concentrations viz. control (0 mg/mL), 1/2 × MIC (125 mg/mL), MIC (250 mg/mL) and 2 × MIC (500 mg/mL) was mixed with 25 ml of SDB in 50 ml Erlenmeyer flask to obtain the desired concentration and yeast inoculum. The inoculated flasks were then incubated for 48 h in an orbital shaker at 37°C with agitation rate of 150 rpm. A 100 µl aliquot from each treatment flask was pipetted out at predetermined time intervals for viable cell count at every 4 hourly during the time intervals of 0–48 h.

To determine colony-forming unit per milliliter (CFU/mL), the samples were then diluted and spread onto fresh SDA plates before incubation process at 37ºC for 24 h. Upon counting, only the plates with the number of colonies ranging from 30 to 300 were counted. A curve for time–kill study (log CFU/mL vs. time) was plotted and the experiment was carried out in triplicate on separate occasions.

Structural degeneration of microbial cells: Microbial samples were prepared by employing the method described by Taufiq and Darah[54]. The specimen preparations for SEM and TEM were employed method described by Borgers et al[6] and Latha et al[29] respectively. The prepared specimens were then viewed under a scanning electron microscope (Leica Cambridge, S-360, United Kingdom) and transmission electron microscope (Philips CM12, Eindhoven, The Netherlands).

Statistical analysis: All the experiments were carried out in triplicate and the data were expressed as mean ± standard deviation (SD). The t-test (SPSS Version 12.0) was used for comparing the effect of the extract against different yeast strains and control. Statistically significance was assumed at the 0.05 significance level ($p < 0.05$).

Results

Preliminary antimicrobial assay of endophytic fungi: The ability of two endophytic fungal strains *Lasiodiplodia pseudotheobromae* IBRL OS-64 and *Muscodor* sp. IBRL OS-94 to inhibit test yeast was determined through agar plug diffusion assay. Table 1 demonstrated that both endophytic fungi exerted favorable anti-yeast activity against three out of four yeast including *Candida albicans*, *C. utilis* and *Rhodotorula rubra*. For *Lasiodiplodia pseudotheobromae* IBRL OS-64, *C. albicans* was the most susceptible to the fungal isolate with a diameter of inhibition zone in the range of 11 to 20 mm. However, both yeasts strains *C. utilis* and *Rhodotorula rubra* were less susceptible to the fungal isolate with the size of the inhibition zone less than 10 mm.

On the other hand, *C. albicans* and *Rhodotorula rubra* were the most and least susceptible to endophytic fungal strain *Muscodor* sp. IBRL OS-94 with a diameter of inhibition zone bigger than 21 mm and smaller than 10 mm respectively. The present finding also revealed that *Cryptococcus neoformans* was resistant to both endophytic fungal isolates since there are no inhibition zones observed surrounding the impregnated disk.

Table 1
Primary screening of antiyeast activity on agar diffusion assay.

Test yeast	Inhibition zones	
	***L. pseudotheobromae* OS-64**	***Muscodor* sp. OS-94**
Candida albicans	++	+++
Candida utilis	+	++
Cryptococcus neoformans	-	-
Rhodotorula rubra	+	+

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

Evaluation of antimicrobial activity of crude extracts: The anti-yeast activity of ethyl acetate and dichloromethane extracts of *Lasiodiplodia pseudotheobromae* IBRL OS-64 and *Muscodor* sp. IBRL OS-94 was shown in table 2. For *L. pseudotheobromae* IBRL OS-64, the ethyl acetate extract showed anti-yeast activity with the size of the inhibition zone ranging between 9.6±0.5 - 21.3±0.5 mm with *Candida albicans* and *Rhodotorula rubra* as the most and least susceptible to the extract respectively.

Besides, only *C. albicans* was active against the dichloromethane extract of *L. pseudotheobromae* IBRL OS-64 with a diameter of inhibition zone as 11.7±0.5 mm. On the other hand, the ethyl acetate extract of *Muscodor* sp. IBRL OS-94 exhibited anti-yeast activity with the size of the inhibition zone in the range of 14.4±0.5 - 20.2±0.7 mm. *C. utilis* and *C. albicans* were observed to be the least and most susceptible strain against the fungal extract. Meanwhile, the dichloromethane extract of *Muscodor* sp. IBRL OS-94 demonstrated a fair anti-yeast activity against *C. albicans* and *R. rubra* with the diameter of inhibition zone as 12.8±0.5 and 9.1±0.3 mm respectively.

However, two yeast strains such as *C. utilis* and *Cryptococcus neoformans* were inactive against the dichloromethane extract since no inhibition zone was observed. Figure 1 illustrated the diameter of the inhibition zone surrounding the impregnated disks of several fungal extracts against *C. albicans*.

Determination of minimal inhibitory concentration (MIC) and minimal yeastocidal concentration (MYC): Table 3 shows the MIC and MYC values of ethyl acetate extracts of *L. pseudotheobromae* OS-64 and *Muscodor* sp. OS-94 against test yeasts. The findings demonstrated that the MIC and MYC values of ethyl acetate extract from *L. pseudotheobromae* OS-64 were in the range of 500 – 1000 µg/mL and 2000 – 8000 µg/mL respectively. The fungal extract exhibited yeastocidal effect against *Candida albicans* since MYC/MIC ratio was less or equal to 4 and yeastostatic effect against *C. utilis* and *Rhodotorula rubra* was eight.

On the other hand, the MIC and MYC values of ethyl acetate extract of *Muscodor* sp. OS-94 ranged between 250 – 500 µg/mL and 1000 – 4000 µg/mL respectively. Yeastocidal activity of *Muscodor* sp. OS-94 extract was observed against *C. albicans* whereas the fungal extract showed yeastostatic activity against both yeast strains, *C. utilis* and *R. rubra*.

Table 2
Antiyeast activity of different solvent extracts of *L. pseudotheobromae* IBRL OS-64 and *Muscodor* sp. IBRL OS-94 against test microorganism through disk diffusion assay

Test yeasts	Diameter of inhibition zones (mm)								
	L. pseudotheobromae OS-64				*Muscodor* sp. OS-94				
	HE	DC	EA	BU	HE	DC	EA	BU	Control
Candida albicans	-	11.7±0.5	21.3±0.5	-	-	12.8±0.5	20.2±0.7	-	30.3±0.5
Candida utilis	-	-	10.2±0.3	-	-	-	14.4±0.5	-	28.7±0.3
Cryptococcus neoformans	-	-	-	-	-	-	-	-	29.6±0.5
Rhodotorula rubra	-	-	9.6±0.5	-	-	9.1±0.3	15.4±0.3	-	31.4±0.7

Notes: Ketoconazole was used a positive control. HE = hexane, DC = dichloromethane, EA = ethyl acetate, BU = butanol extract.

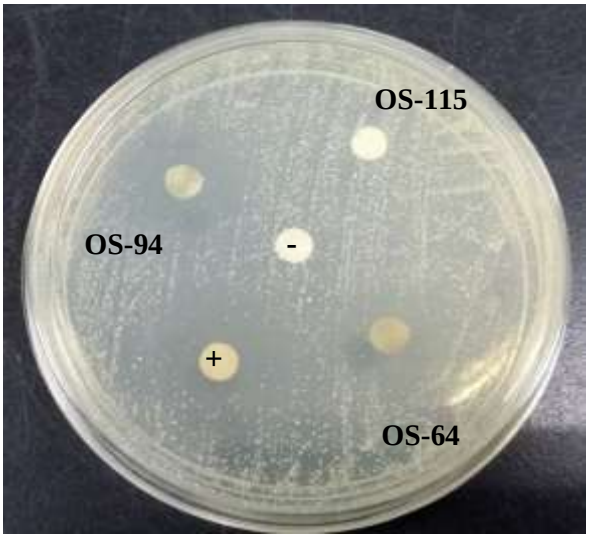

Figure 1: Disk diffusion assay of fungal extracts against *Candida albicans*.

Table 3
Determination of MIC and MYC values of the ethyl acetate extracts of endophytic fungi via broth microdilution assay.

Test yeast	*L. pseudotheobromae* OS-64			*Muscodor* sp. OS-94		
	MIC (µg/mL)	MYC (µg/mL)	Ratio	MIC (µg/mL)	MYC (µg/mL)	Ratio
Candida albicans	500	2000	4	250	1000	4
Candida utilis	500	4000	8	500	2000	4
Cryptococcus neoformans	-	-	-	-	-	-
Rhodotorula rubra	1000	8000	8	500	4000	8

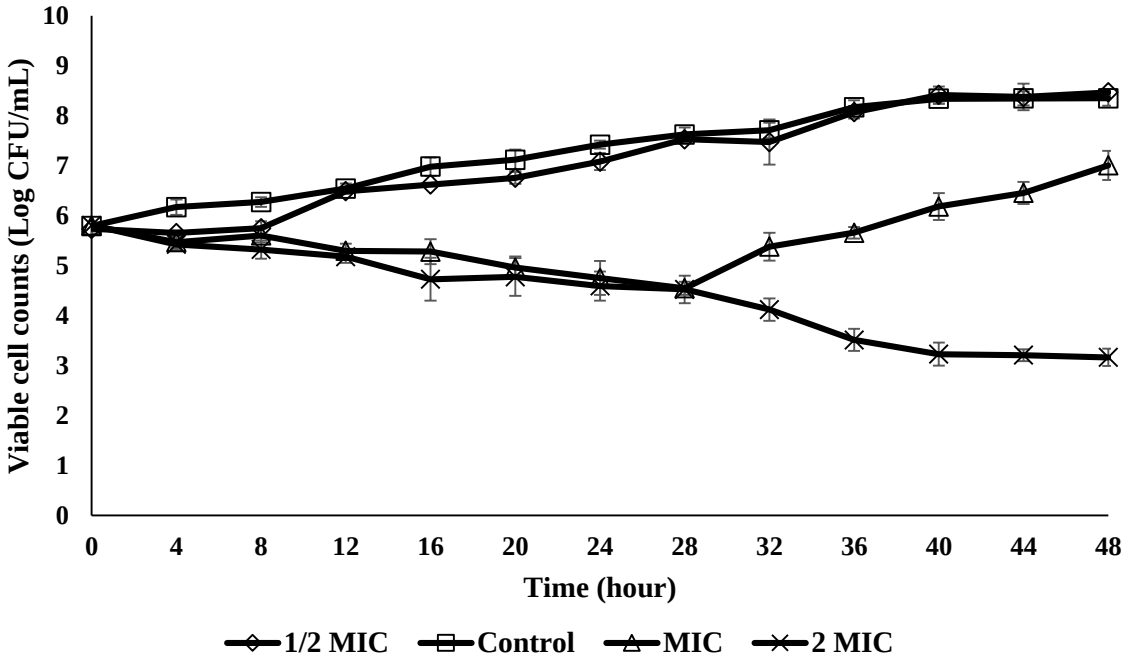

Figure 2: Time-kill curve of the ethyl acetate extract of *Muscodor* sp. IBRL OS-94 against *Candida albicans.*

Time-kill assay: The time-kill curve of the ethyl acetate extract of *Muscodor* sp. OS-94 against *C. albicans* was depicted in figure 2. The growth curve demonstrates four different growth phases of *C. albicans* consisting of lag, exponential, stationary and death phases. However, the growth curve was drastically changed with the addition of the fungal extract. Overall, the findings revealed that the growth of *C. albicans* was reduced as the concentration of fungal extract increased. The post-antibiotic effect was observed at the MIC value of the fungal extract in which the re-growth of candidal cells occurred after 28 h of exposure time. The fungal extract exhibited yeastostatic effect at ½ × MIC (125 µg/mL) and MIC (250 µg/mL) values whereas at 2 × MIC (500 µg/mL), the effect was observed as yeastocidal. The finding revealed that the fungal extract can significantly reduce the candidal cell number from the initial inoculum proving the potency of the extract as anti-candidal agents, especially against *C. albicans*.

Structural degeneration of bacterial cells exposed to the extract: SEM and TEM photomicrographs were studied in order to obtain a detailed observation of what actually occurred to the *C. albicans* cells during exposure to the fungal extract in the time-kill assay. Figure 2 exhibits the SEM photomicrographs of the effect of the ethyl acetate extract derived from *Muscodor* sp. OS-94, an endophytic fungus of *Ocimum sanctum* origin. Figure 2a shows the untreated cells with oval and smooth appearance with some at a budding stage.

On the contrary, some remarkable morphological changes of the cells occurred after 48 h exposed to the fungal extract (Figure 2b). Most of the treated cells were shrunken and formation of cavitation as well as invaginations was observed. The cells were also starting to collapse, in which most of them lost their intact oval structure and some of the cell debris were seen. The findings have shown that the cells have experienced severe structural changes and cytological alterations leading to loss of their metabolic functions. A TEM study was performed for a closer view inside the yeast cell after being treated with the fungal extract (Figure 3).

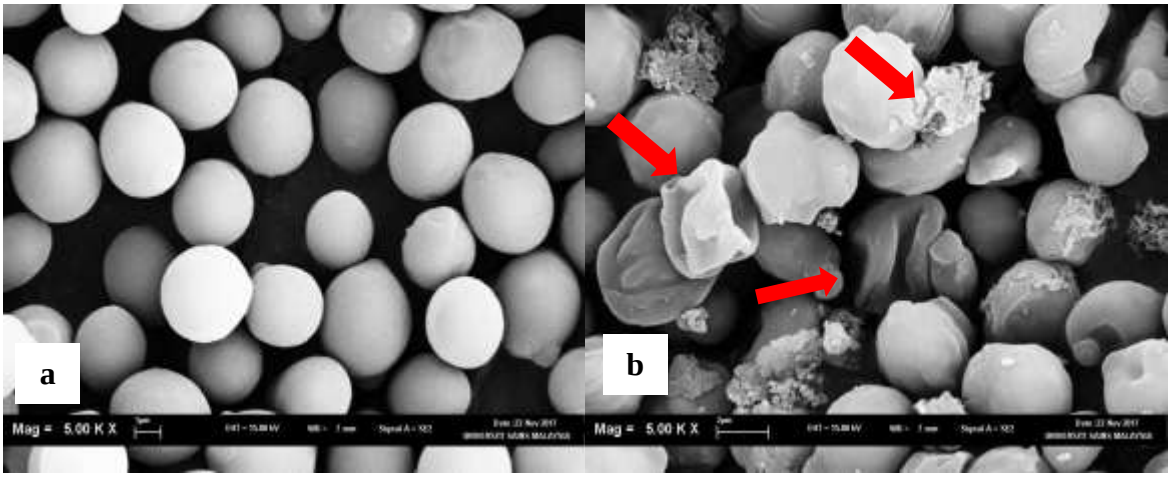

Figure 2: SEM photomicrographs of yeast, *Candida albicans* treated with 250 µg/mL of ethyl acetate crude extract. (a) 0 h [control] (b) 48 h. Scale bars: 100nm.

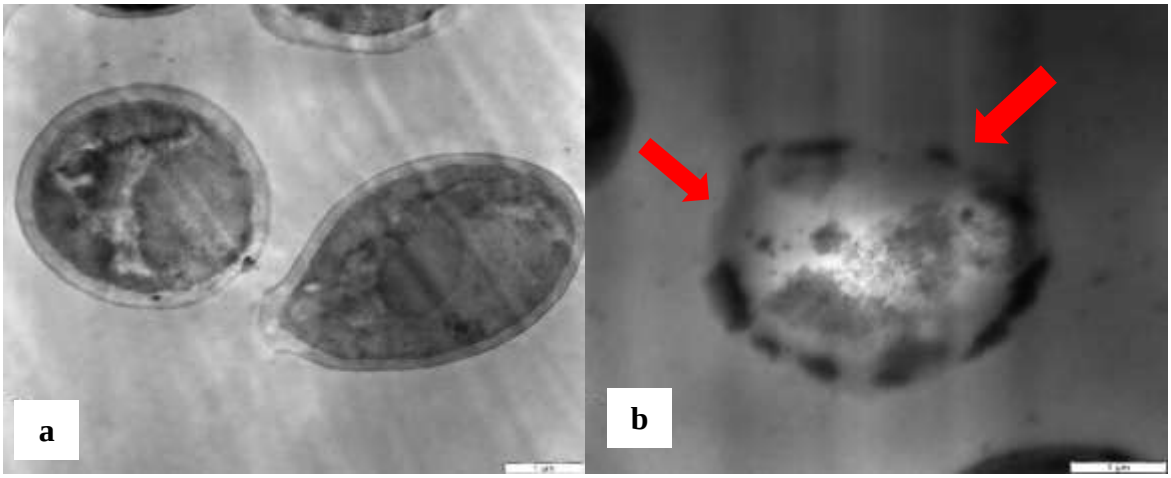

Figure 3: TEM photomicrographs of yeast, *Candida albicans* treated with 250 µg/mL of ethyl acetate crude extract. (a) 0 h [control] (b) 48 h. Scale bars: 100nm.

Figure 3a demonstrates a typical structure of untreated *C. albicans* cells which consist of normal cytoplasm with the cell organelles suspending within it and enveloped by a cell wall and cell membrane.

On the other hand, the disposition and alterations of the cells were seen after 48 h exposed to the fungal extract (Figure 3b). The cytoplasm had leaked out which might be due to the imbalance osmotic pressure caused by alterations of cell membrane permeability. Meanwhile, the cytoplasmic volume decreased and it is clearly observed the cells had undergone severe disorganization inside the cells leading to collapse. Besides, the cell membrane was partially disintegrated and the disorganization of cytoplasm as well as organelles, leading to cell lysis and death beyond repair.

Discussion

Fungal infections are serious health problems, particularly in people with an immune-compromised system and are the main cause of mortality and morbidity worldwide[55]. Candidiasis is an acute or chronic, disseminated, or superficial mycosis caused by a candidal infection in humans and it encompasses infections that range from superficial such as vaginitis and oral thrush to systematic and potentially life-treating diseases. Prevalence candidal infections lead to candidemia usually infected with severely immuno-compromised patients including AIDS, transplant, cancer and non-trauma emergency surgery patients[51].

Candida albicans is the most common opportunistic fungal pathogen of human diseases that dwells on the skin, in the mucosa of gut, oral cavity and urogenital tract as a symbiotic fungus under normal conditions[30]. However, it can be infected when the host becomes immuno-compromised or debilitated and these infections affected mucous or skin membrane and invade the bloodstream and disseminate to internal organs. Long-term stay in an intensive care unit, burns, abdominal surgery and previous administration of immunocompromised agents and broad-spectrum of antibiotics are the risk factors for invasive fungal infections by *C. albicans*[49].

The emergence of drug-resistant strains that limits the available antimicrobial agents has prevented antifungal treatments, particularly infection caused by *Candida albicans*. Odds et al[38] revealed that the increasing number of

yeast strains are developing against antifungal agents, especially *C. albicans* that have been reported to resistant towards available antifungal drugs including fluconazole and amphotericin B. According to Whaley et al[58], fluconazole is commonly prescribed antifungal drug to treat most *C. albicans* infection. As a group of azoles family, fluconazole inhibits 14-α-sterol demethylase encoded by the ERG11 gene which is an enzyme responsible in the biosynthesis of the fungal-specific membrane sterol ergosterol. However, the presence of point mutations in ERG11 has prevented the action of azole and leads to resistance to antifungal drugs. Thus, the discovery of new antifungal agents is a need to combat the resistant fungal strain that limits available antibiotics.

Endophytic fungi are known as one of the sources of anti-yeast and antifungal agents. Several studies reported the use of endophytic fungi from medicinal plant origin as a source of bioactive compounds with pharmaceutical properties. Deshmukh et al[15] reported that endophytic fungi are a microbial community that lives inside all plants with the promise of producing diverse bioactive substances and novel metabolites with medicinal activities having applications in industrial and agricultural setups. A previous study revealed that endophytic fungi isolated from the leaf *Lannea coromandelica* such as *Aspergillus niger, A. flavus, Alternaria alternata* and *Colletotrichum gloeosporioides* possess fair antifungal activity against several fungi including *Candida albicans* and *Malassezia pachydermis*[41].

Besides that, Nath and Joshi[35] revealed that endophytic fungi isolated from *Calotropis gigantean* and identified as *Phomopsis asparagi* and *C. gloeosporioides* exhibited antifungal activity against *C. albicans* with a MIC value of 46.9 and 93.75 µg/mL respectively. The present study revealed that endophytic fungi, *Lasiodiplodia pseudotheobromae* IBRL OS-64 and *Muscodor* sp. IBRL OS-94 possesses anti-yeast activity against several yeast strains particularly *C. albicans*.

The findings were in agreement with Meshram et al[33] who reported the VOCs produced by *Muscodor kashayum*, an endophytic fungus isolated from a medicinal plant, *Aegle marmelos* (Bael tree) which exhibited significant anticandidal activity against *C. albicans*. According to Deshmukh et al[15], *Muscodor* is the most studied genus which produces a synergistic mixture of volatile organic compounds (VOCs) with lethal effects towards a broad spectrum of bacteria, nematodes, human pathogenic fungi and certain insects. However, the reports on the anticandidal activity of *Lasiodiplodia* sp. were very scarce.

The selection of solvent is crucial since it greatly affected the extraction yield and bio-activity of the extracts[1]. The present study has shown that ethyl acetate extracts from both endophytic fungal strains exhibited significant activity against test yeasts. This phenomenon indicated that semi-polar solvent such as ethyl acetate is suitable to extract bioactive compounds from endophytic fungi with antimicrobial activities. Similar observation was reported by Ibrahim et al[22] who revealed ethyl acetate extract of *Aspergillus flavus* IBRL-C8, an endophytic fungus isolated from the leaf of a medicinal plant. *Cassia siamea* exhibited significant anti-candidal activity with the size of inhibition zone as 28.3 ± 2.4 mm. A mid-polar solvent such as ethyl acetate was used as a solvent for the extraction process of fermented broth due to its efficiency for extracting and isolating secondary metabolites of fungal origin[19].

Furthermore, this solvent has the capability to extract broad range compounds such as low molecular weight (phenolic) and high molecular weight (polyphenols) compounds[45]. According to Yadav et al[59], ethyl acetate extraction was the most efficient method in extracting and isolating fungal secondary metabolites. The findings also revealed that different solvent selection affected the antimicrobial efficacy of the fungal extracts. According to Chai et al[9], the variation in the extraction process particularly caused by the extraction process is restricted by the solubility of bioactive substances in the solvent used. Furthermore, ethyl acetate can be used to extract compounds secreted in the fermentative broth without freeze-drying the broth due to the nature of the solvent that is not miscible with aqueous solutions.

Ethyl acetate is suitable to extract various mid-polar compounds including peptides, amino acid derivatives and polyketides[9]. Hexane and butanol were also used to extract bioactive compounds in the present study. However, the findings revealed that there is no anti-yeast activity observed for both solvents. This may be due to the function of hexane to remove unwanted fats, waxes and oils due to their stability, lipid selectivity and convenient boiling point[47] and the capability and efficiency of butanol to extract polar compounds from aqueous medium[26].

According to Yenn et al[60], some of the antimicrobial compounds from endophytic fungi are semi-polar and thus, they can be extracted using mid-polar solvents such as ethyl acetate and dichloromethane. Different extraction solvents exhibited variation in yield and this may due to polarity of the solvent that might influence the quantity and quality of the extracted compounds[18].

The minimum inhibitory concentration (MIC) and minimum yeastocidal concentration (MYC) values of the fungal extracts against several test yeast were determined through broth microdilution assay. In general, broth microdilution assay is more sensitive as compared to disc diffusion assay and it is appropriate procedure for assaying non-polar and polar compounds for determination of MIC and MYC[12,46].

According to Langfield et al[28], this assay involves the preparation of two-fold dilution of antimicrobial agents and is more accurate as compared to disc diffusion assay since the MIC value is generated quantitatively.

In addition, the MYC values of the extract benefit the researchers due to the ability to distinguish between bactericidal and bacteriostatic effects[12]. MIC is the lowest concentration of an antimicrobial agent that inhibits the visible microbial growth after an overnight incubation period[3]. Meanwhile, MYC could be defined as the lowest concentration of antimicrobial drugs needed to kill 99.9% of microbial growth after 24 h of incubation period under standardized conditions[10,63]. The intriguing result from the broth microdilution assay revealed that both endophytic fungi isolates *L. pseudotheobromae* IBRL OS-64 and *Muscodor* sp. IBRL OS-94 were isolated from *Ocimum sanctum* leaf and exerted significant anti-candidal activity.

The ratio of MYC/MIC values of both endophytic fungal extracts was 2 against *Candida albicans* and they were assumed to possess yeastocidal effect. A similar observation was reported. On the other hand, both fungal extracts exhibited yeastostatic effect against *Rhodotorula rubra* since the MYC/MIC ratio was 8.

According to Krishnan et al[27], antimicrobial compounds are considered as bacteriostatic agents when the MBC/MIC ratio is less or equal to 4, whilst bactericidal agents if the MBC/MIC ratio is greater than 4 and thus, in parallel to this, the fungal extract can be considered as yeastocidal and yeastostatic if the MYC/MIC ratio was ≤ 4 and > 4 respectively. In general, MYC of the fungal extracts was significantly higher than MIC values against all test yeast which indicated that the anti-candidal activity of the extracts was dose-dependent. Mu'azzam and Darah[34] reported that the bactericidal drugs are clinically more powerful antibiotic agents compared to bacteriostatic drugs since this type of drug can kill bacteria and is rather effective in severely immune-compromised patients. Therefore, yeastocidal drugs can be recognized as effective antibiotic agents compared to yeastostatic drugs since they can kill the pathogenic yeast rather than inhibiting it.

The time-kill curve has been frequently used to evaluate the efficacy of antimicrobial agents since it can monitor bacterial growth and death over a wide range of concentrations and times[17]. Meanwhile, Pfaller et al[39] stated that the time-kill assay is widely used to determine whether the antimicrobial agents produces time-dependent killing or concentration-dependent killing and this method is useful in determining the tolerance to the lethal activity of antimicrobial agents as well as the synergistic and antagonistic effect between two or more antimicrobial agents. The present finding revealed that the fungal extract exhibited yeastostatic effect on candidal cells at low extract concentration whereas yeastocidal activity was at higher extract concentration.

This observation was in parallel with Ibrahim et al[22] who studied the anti-candidal activity of ethyl acetate extract from *Aspergillus flavus* IBRL-C8, an endophytic fungus of *Cassia siamea* Lamk leaf origin. At higher extract concentration (2 × MIC), the fungal extract of *Muscodor* sp. showed yeastocidal effect against *Candida albicans* in a time- and dose-dependent manner. A similar finding has been reported by Yenn et al[62] who studied the killing kinetic analysis of a novel compound 3-hydroxy-5-methoxyhex-5ene-2,4-dione produced by endophytic fungus identified as *Diaporthe* sp. ED2 against *C. albicans*. The post-antibiotic effect (PAE) has occurred at low extract concentration. The PAE is defined as the suppression of microbial growth persisting after a short exposure to an antibiotic[56].

According to Taufiq and Darah[54], this event occurred due to the remaining resistant microbial cells that would begin to resume their growth. Besides, the re-growth phenomenon was observed in the present study especially in low extract concentration (0.5 × MIC). The finding was in agreement with Skinner et al[48] who reported that fidaxomicin demonstrated a bactericidal effect with re-growth after 24 h exposure in two *Clostridium difficile* strains (NCTC 13366 and ATCC 9689) at 0.5 × MIC concentration.

According to Tam et al[52], this phenomenon was attributed to two distinct sub-populations whereby the preferential killing of susceptible sub-population combined with the selective amplification of resistance sub-population at a specified interaction time. Furthermore, Nielson et al[37] reported that a decline in the killing rate of microbial cells, particularly in a lower extract concentration, is due to the presence of persister cells which is less susceptible to antimicrobial agents.

The SEM and TEM photomicrographs confirm the notable morphological changes in *Candida albicans* cells and some of its organelles are caused by the ethyl acetate extract of *Muscodor* sp. IBRL OS-94 that possesses anti-candidal activity. Morphological changes of growing candidal cells affected by the fungal extract suggested that the extract inhibited the cell wall components. Therefore, it is reasonably presumptive that inhibition by the fungal extract can induce some abnormalities such as crumpled, shrunken cells and disorganization of cytoplasm on treated candidal cells compared to normal yeast. The observation was in agreement with Basma et al[4] who reported that the cell treated with the extract exhibited some notable alterations in the cell wall, the cell membrane and the cytoplasmic volume decreased with structural disorganization within the cell cytoplasm whereas a certain number of cells underwent drastic shape changes and some lysed and collapsed.

According to Darah et al[13], the cell lysis principally may be due to interference in the function of the cell membrane or the disruption in the syntheses of the cell wall and cell membrane. In fact, the majority of anti-yeast and antifungal agents act on sterols and other compounds located in the cell membrane[43].

Yeast and fungi have a similar basic structure of eukaryotic cells but different in chemical components. The proteomics

and genomics studies revealed a shred of concrete evidence that both yeast and fungi are sharing a common origin of their cell wall. Their cell wall is a specific and complex organelle consisting of chitosan, chitin, glucans and glycosylated proteins. These proteins are associated with polysaccharides to form glycoproteins and these components contribute to the cell wall rigidity whereby the synthesis and maintenance of cell wall involves a large number of biosynthetic and signaling pathways[20].

Even though lacking cellulose, the fungi and yeast have other special polysaccharides such as galactosans and mannans which may replace chitin in some fungal cell walls and form a rigid structure of cell wall together with inorganic ions, protein, lipid and polyphosphates[32].

However, the chitin concentration in fungi is higher compared to yeast[5]. The yeast and fungal cell walls are dynamic structures that are crucial in morphogenesis, pathogenesis, cell viability and they also play important role in regulating the response to harsh environmental conditions and imposed stresses such as osmotic pressure whereas at the meantime permitting cells to interact with their surrounding[7,21].

To date, many studies have been carried out to investigate the mode of action by fungal endophytes against yeast and fungal cells particularly on the destruction of the cell wall and cell membrane. The present findings revealed that the endophytic fungal extract greatly impacted the cell wall synthesis and cell membrane as well, congruent with Das et al[14] who reported the bioactive compound from butanol fraction caused cell wall lysis and destruction of cell membrane integrity. Similarly, Nath and Joshi[35] observed the presence of cell abnormalities after treatment with endophytic fungal extracts including disruption of the cell wall, disintegrated cell structures and wrinkled cells indicating the disturbance in the cell wall and cell membrane integrity.

According to Garcia-Rubio et al[20], the fungal cell wall commonly consists of molecules that are not found in the human body and thus, constitute an ideal target for the development of clinical antifungal drugs and the design for immunotherapies. For instance, echinocandins are antifungal drugs that target the synthesis of the β-1,3-glucan of the cell wall in a non-comparative way[2]. On the other hand, fungal cell membranes composed of glycerophospholipids, sphingolipids and sterols and the synthesis of these molecules are one of the main targets of antifungal drugs leading to the destruction of the cell membrane.

For example, validamycin A competitively inhibited the cell wall-bound acid trehalase (Atc1p) of *Candida albicans* and thus, blocked the biosynthesis of phospholipid[44]. Therefore, the exposure of candidal cells to the endophytic fungal extract affected the cell membrane with alteration of some cell organelles since the main metabolic system of cells is depending on the integrity of the cell membrane.

Conclusion

The present finding indicated that extracts of endophytic fungi *Lasiodiplodia pseudotheobromae* IBRL OS-64 and *Muscodor* sp. IBRL OS-94 possessed anti-yeast properties. Therefore, the fungal extracts are suitable to be used to treat yeast infections particularly those caused by *Candida albicans, C. utilis* and *Rhodotorula rubra*.

Exposure of *C. albicans* cells to the *Muscodor* sp. IBRL OS-94 extract leads to alteration and abnormalities of the cell wall and cell membrane indicating the efficacy of the extract as anti-candidal agents. The extracts also could be an important source of bioactive substances useful for developing new anti-yeast drugs.

Acknowledgement

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

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(Received 03rd June 2020, accepted 01st August 2020)