Jurnal
Teknologi

Full Paper

ANTIFUNGAL ACTIVITY OF ENDOPHYTIC FUNGI ASSOCIATED WITH *OCIMUM SANCTUM*

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Article history
Received
4 June 2023
Received in revised form
14 October 2023
Accepted
28 November 2023
Published Online
18 February 2024

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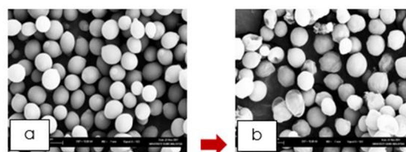
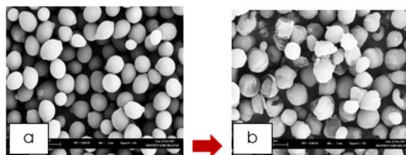
Graphical abstract

Antifungal effect of endophytic fungal extracts



Candida albicans treated with 500 µg/mL of dichloromethane crude extract of *Lasiodiplodia* sp. IBRL OS-64. (a) control, (b) treated



Candida albicans treated with 250 µg/mL of dichloromethane crude extract of *Muscodor* sp. IBRL OS-94. (a) control, (b) treated

Scanning electron microscope (SEM) observation

Abstract

Endophytic fungi of the medicinal herb, *Ocimum sanctum* are believed to possess antifungal activity against pathogenic fungi. Due to the emergence of pathogenic fungi and antibiotic-resistant strains, the search for alternative antimicrobial agents is a need. The present study aimed to evaluate the antifungal activity of endophytic fungi isolated from *O. sanctum*. Plate-to-plate method, disk diffusion assay, and Scanning electron microscopic (SEM) were employed in this study. The finding revealed that fungal isolates *Colletotrichum* sp. IBRL OS-39, *Aspergillus* sp. IBRL OS-65, *Muscodor* sp. IBRL OS-94 and *Muscodor* sp. IBRL OS-98 was able to produce volatile compounds with antifungal activity against pathogenic fungi. On the disk diffusion assay, *Lasiodiplodia* sp. IBRL OS-64, and *Muscodor* sp. IBRL OS-94 displayed good antifungal activity against test fungi with a diameter of inhibition zone between 9.6±0.6 - 14.3±0.6 mm and 11.2±1.2 - 15.7±0.6 mm, respectively. SEM observations revealed remarkable morphological changes in *Candida albicans* treated with the dichloromethane extracts of *Lasiodiplodia* sp. IBRL OS-64 and *Muscodor* sp. IBRL OS-94 with severe cell damage beyond repair and thus leads to cell death.

Keywords: Endophytic fungi, *Ocimum sanctum*, Antifungal activity, Volatile compounds, Dichloromethane extracts

Abstrak

Kulat endofit daripada pokok herba, *Ocimum sanctum* dipercayai mempunyai aktiviti antikulat terhadap kulat pathogen. Disebabkan kemunculan kulat patogen dan strain rintang antibiotik, pencarian untuk alternatif agen antimikrob merupakan satu keperluan. Kajian ini bertujuan untuk menilai aktiviti antikulat oleh kulat endofit yang dipencilkan daripada pokok *O. sanctum*. Kaedah plat ke plat, ujian resapan cakera dan mikroskop pengimbas elektron telah digunakan dalam kajian ini. Dapatan kajian mendedahkan pencilan *Colletotrichum* sp. IBRL OS-39, *Aspergillus* sp. IBRL OS-65, *Muscodor* sp. IBRL OS-94 dan *Muscodor* sp. IBRL OS-98 berupaya menghasilkan sebatian meruap yang mempunyai aktiviti antikulat terhadap kulat patogen. Untuk ujian resapan cakera, *Lasiodiplodia* sp. IBRL OS-64, dan *Muscodor* sp. IBRL OS-94 menunjukkan aktiviti antikulat yang baik terhadap kulat uji dengan diameter zon rencatan masing - masing diantara 9.6±0.6 - 14.3±0.6 mm dan 11.2±1.2 - 15.7±0.6 mm. Pemerhatian SEM mendedahkan perubahan morfologi yang ketara terhadap *Candida albicans* yang telah dirawat dengan ekstrak diklorometana dari pencilan *Lasiodiplodia* sp. IBRL OS-64 dan *Muscodor* sp. IBRL OS-94 dengan kerosakan sel yang teruk dan membawa kepada kematian sel tersebut.

Kata kunci: Kulat endofit, *Ocimum sanctum*, Aktiviti antikulat, Sebatian meruap, Ekstrak diklorometana

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1.0 INTRODUCTION

Endophytic fungi are a class of fungi that coexist symbiotically with their host plants inside their tissues without exhibiting any signs of illness. In their host plants, they are known to biosynthesize a few important secondary metabolites [1]. Endophytes are thought to synthesize plant hormones that help their host plant develop because of their symbiotic relationship. In order to protect their host plant against pathogenic bacteria, many endophytes have the ability to secrete bioactive substances [2]. The bioactive substance released by endophytes, according to Guo [3], has proven effective in the discovery of new medications. In the meanwhile, Stierle [4] made the discovery that endophytes might be involved in the biosynthesis of related plant chemicals. As a result, many researchers have been motivated by this discovery to investigate the potential of endophytic fungi for their unique bioactive substances.

Endophytic fungi with pharmacological potentials such as anticancer, antibiotic, and insecticidal properties are reported to colonize medicinal plants with exceptional ethnobotanical histories [5]. In addition, fungal endophytes from medicinal plants have the capacity to replicate the bioactive substances of their hosts [6]. *Ocimum sanctum*, sometimes referred to as "selasih" locally, is one of many plants that have been documented to be infested by endophytic fungi. Since it has been utilized for thousands of years in the conventional Ayurvedic system, this plant is quite well-known [7]. This traditional medicine plant showed a range of therapeutic properties, including wound healing activity [8], antioxidant activity [9], anti-inflammatory activity [10], and antibacterial activity [11]. *O. sanctum* might be a fantastic source of novel bioactive fungi because it has been shown to have a variety of medicinal potentials.

This study's main goal was to assess the antifungal potential of endophytic fungi that were isolated from the curative plant *Ocimum sanctum*. On a variety of pathogenic fungi, including filamentous fungi and yeasts, the endophytic fungus isolates' antifungal activity has been investigated.

2.0 METHODOLOGY

2.1 Fungal Culture and Maintenance

The endophytic fungal isolates that were previously isolated from *Ocimum sanctum* leaf, *Colletotrichum* sp. IBRL OS-27, *Penicillium* sp. IBRL OS-39, *Lasiodiplodia* sp. IBRL OS-64, *Aspergillus* sp. IBRL OS-65, *Aspergillus* sp. IBRL OS-82, *Muscodor* sp. IBRL OS-94, *Muscodor* sp. IBRL OS-98 were deposited at the Industrial Biotechnology Research Laboratory (IBRL), School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. With the addition of a powdered host plant (2 g/L), the fungal cultures were grown and maintained on potato dextrose agar (PDA) and incubated at 37°C for 7 days. Prior to usage, the fungal cultures were stored at 4°C. Every month, subculturing was carried out to guarantee their viability and purity. On fresh Sabouraud dextrose agar (SDA) (Hi-media) and PDA (Hi-media) slants, test yeasts, and fungi were sub-cultured, respectively. For yeasts, the cultures were incubated for 24 hours at 37°C, and for fungi, for 7 days at 30°C.

2.2 Inoculum Preparation

In order to make the yeast inoculum, 1 or 2 pure yeast colonies were selected and suspended in 10 mL of 0.85% (w/v) sterile physiological saline. The yeast suspension's turbidity was changed to match the 0.5 McFarland standard (about 1×10^6 CFU/mL). Spore density for the test fungi was created by adding 10 mL of 0.85% (w/v) sterile physiological saline to the agar slant containing the culture that had been growing for 7 days. The majority of the fungal spores were suspended in the sterile physiological saline after vigorous shaking. A slide chamber hemocytometer (Neubauer, Germany) was used to measure the density of the spore solution.

2.3 Fungal Fermentation and Extraction

The culture medium, fermentation, and extraction process were prepared and performed according to the methods described by Jalil [12]. As a culture media, yeast extract sucrose (YES) broth was employed. Two 3-day-old mycelial plugs of a fungus were placed into 250 mL Erlenmeyer flasks with 100 mL YES broth and left to incubate for 16 days at 30°C without being disturbed. Using Muslin cloth, the fermenting broth, and fungal biomass were separated, and then the filtrate was produced using filter papers (Whatman, No.1). The filtered broth was then concentrated using a rotary evaporator, dried in a fume hood, and extracted three times with an equivalent volume of dichloromethane (1:1; v/v) to obtain crude ethyl acetate paste.

2.4 Determination of Volatile Antimicrobial Compounds (VOCs)

The plate-to-plate method was performed to test volatile antimicrobial production according to the method described by Stinson [13]. The 14-day-old endophytic isolate cultures were physically attached to the agar plates seeded with test fungi. The two plates were sealed using two layers of Parafilm® and kept at 4°C for 7 days to allow the complete fumigation process of the volatile compounds. Then, the plates were incubated at 30°C for 7 days. The diameter of the test fungal culture was measured to indicate the growth rate, whereas the untreated test microorganisms were used as a control. Data are presented as the percentage of growth in treated

microorganisms compared to untreated microorganisms. Besides, the mycelia of the test fungi that showed positive results were transferred to fresh PDA to maintain their viability.

2.5 Antagonist Activity of Endophytic Fungal Isolates

The antagonistic activity of the endophytic fungal isolates was determined using the dual culture technique against fungi on PDA plates [14]. The 5 mm diameter of the 5-day-old test fungal mycelia was placed on one corner of the PDA medium. The selected endophytic fungi were then inoculated on the other corner of the plates. The plates were incubated for 5 days at a temperature of 37°C. The antagonistic activity was determined according to the following formula,

Antagonistic activity (%) =

$$\frac{(R_c - R_d)}{R_c} \times 100$$

whereby, R_c = Fungal radius in control plates, R_d = Fungal radius in dual culture plates.

2.6 Antifungal Activity of Endophytic Fungal Filtrates

The antifungal activity of the fungal filtrates was determined according to the poison food techniques of Grover and Moore [15]. The fermentative broth was filtered with filter paper (Whatman, No. 1). One milliliter of the filtrate was then introduced into molten potato dextrose agar (PDA). The test fungi were then inoculated into previously prepared PDA and incubated at a temperature of 37°C for 5 days. For control, the test fungi were inoculated in PDA without being supplemented with filtrates. The percentage of growth inhibition was calculated according to the following formula:

Percentage of growth inhibition (%) =

$$\frac{(d_c - d_t)}{d_c} \times 100$$

whereby d_c = diameter of fungal colonies in control, d_t = the diameter of fungal colonies in treatment plates.

2.7 Antifungal Activity of Endophytic Fungal Extracts

This assay was employed to determine the antifungal activity of the endophytic fungal isolate extracts according to the standard method by CLSI [16]. The fungal crude extracts were dissolved in 50% dichloromethane (v/v) for the extract from the fermentative broth. Twenty percent of dichloromethane and 30 µg/mL ketoconazole were used as negative and positive controls, respectively. The plates were subsequently incubated at 37°C for 24 hours.

2.8 Broth Microdilution Assay

This assay was performed to verify MIC and MFC values of the endophytic fungal isolate extracts according to the method described by CLSI [16]. The microbial colonies were suspended in double-strength RPMI 1640 medium (Sigma) containing 0.2% dextrose buffered with 0.165 M 3-(N-morpholino) propanesulfonic acid (MOPS) and Sabouraud Dextrose broth (SDB), respectively, to create the microbial inoculum for the test fungus and yeast. An amount of 100 µl of the extract was added into 100 µl microbial suspension to obtain a final volume of 200 µl in each well and the final concentrations of the fungal extracts ranged from 8000.00 µg/ml to 15.63 µg/ml. The sterility and negative controls were included. To identify microbiological growth, p-Iodonitrotetrazolium Violet (Sigma) was employed at a dosage of 0.2 mg/ml. The colour change from yellow to purple denotes the presence of microbial growth. The MIC value was established as the lowest amount of dichloromethane crude extract that prevents the test bacteria from growing visibly during the incubation period. Reading the MIC values led to the following determination of the MFC for the dichloromethane crude extract. The fungal growth was reduced by 99.9% when MFC was used as the lowest concentration of dichloromethane crude extract compared to the growth control.

2.9 Scanning Electron Microscopic (SEM)

A volume of 0.1 mL of yeast cell suspension was introduced into a 50 mL Erlenmeyer flask containing 18.9 mL of sterile RPMI 1640 medium and 1.0 mL of extract to give a final extract concentration of at 2 × MIC level (2.0 mg/mL) and final yeast cell suspension of 5×10^4 CFU/mL. Control cells were grown in a growth medium without a fungal extract. The mixtures were then incubated at 37°C, 150 rpm for 48 hours. Yeast pallets were subjected to the primary fixation, post-fixation, and dehydration methods as reported by Ibrahim [17]. After that, the specimen was examined with a scanning electron microscope (Leica Cambridge, S-360, UK). All experiments were performed in triplicate.

2.10 Statistical Analysis

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

3.0 RESULTS AND DISCUSSION

A set of chemically varied organic molecules with low molecular weight and vapour pressure in ambient environments are known as volatile organic compounds (VOCs). These volatile substances are typically produced by plants to protect themselves from diseases and pests and to draw pollinators [18]. Recently, it has come to light that endophytic fungi are among the VOC makers with the highest concentrations of alkaloids, cyclohexenes, flavonoids, and terpenes. These substances displayed anti-inflammatory, anticancer, antiproliferative, and antioxidant properties [19]. The results of the plate-to-plate method used to assess the generation of volatile antimicrobials by endophytic fungal isolates are displayed in Table 1. Only four of the seven endophytic fungal isolates were shown to have volatile antifungal activity against test fungi, according to the current investigation. Fungal isolate, *Colletotrichum* sp. IBRL OS-39 showed antifungal activity against *M. fulvum*, *T. rubrum*, and *Rhizopus* sp. with inhibition percentages of 23.2±1.2, 35.2±0.6, and 17.8±1.2, respectively. Besides, three test fungi were inhibited by volatile compounds of *Aspergillus* sp. IBRL OS-65 with inhibition percentage of 34.4±1.2 (*M. fulvum*), 28.2±1.6 (*M. gypseum*), and 41.4±0.6 (*C. albicans*). Endophytic fungal isolate, *Muscodor* sp. IBRL OS-94 inhibited six out of eight test fungi with an inhibition percentage in the range of 33.4±1.6 to 53.6±1.2%. Another *Muscodor* sp. isolate, IBRL OS-98 inhibited *M. gypseum*, *Rhizopus* sp., *C. albicans*, and *C. utilis* with inhibition percentages of 30.2±1.6, 41.6±1.2, 35.2±0.8, and 31.2±1.2%, respectively.

A recent study found that the test fungus *Fusarium sambucinum* and *Phytophthora erythroseptica* were significantly inhibited in their ability to develop mycelial by the volatile compounds produced by *Aspergillus flavus* and *A. niger* [20]. The volatile antifungal activity of *Muscodor* sp. was discovered by several researchers. For instance, Strobel [21] observed that the *Muscodor albus* volatile compounds, which were isolated from the *Cinnamomum zeylanicum* cinnamon tree, exhibited antifungal action against human pathogenic Aspergilli and Candida. Additionally, it has been noted that *Muscodor camphora* produces volatile chemicals that have antifungal activity against a number of test fungi, including *Fusarium solani*, *Rhizoctonia solani*, *Colletotrichum gloeosporioides*, and *C. albicans* [22]. Pena [23] revealed the antifungal activity of volatile compounds synthesized by *Muscodor brasiliensis* LGMF1256 against *Penicillium digitatum* LGMF1507 with 100% growth inhibition.

Table 1 Volatile antifungal activity endophytic fungal isolates against test fungi

Fungal isolates	Growth Inhibition (%)							
	M. fulvum	*T. rubrum*	*M. gypseum*	*F. solani*	*Rhizopus sp.*	*A. niger*	*C. albicans*	*C. utilis*
Colletotrichum sp. IBRL OS-27	-	-	-	-	-	-	-	-
Colletotrichum sp. IBRL OS-39	23.2±1.2	35.2±0.6	-	-	17.8±1.2		-	-
Lasiodiplodia sp. IBRL OS-64		-	-	-	-	-	-	-
Aspergillus sp. IBRL OS-65	34.4±1.2	-	28.2±1.6	-	-	-	41.4±0.6	-
Aspergillus sp. IBRL OS-82	-	-	-	-	-	-	-	-
Muscodor sp. IBRL OS-94	45.7±3.6	37.6 ±2.6	-	-	33.4±1.6	35.2±1.2	53.6±1.2	46.2±0.6
Muscodor sp. IBRL OS-98	-	-	30.2±1.6	-	41.6±1.2	-	35.2±0.8	31.2±1.2

Any organism that can obstruct or suppress the activity or typical growth of plant pathogens, such as bacteria or fungus, is said to be engaged in an antagonistic activity. The current investigation aimed to ascertain the antagonistic activity of several pathogenic fungi against fungal endophytes. As indicated in Table 2, different levels of antagonistic endophytic fungal isolates inhibited test fungi's mycelium growth. The endophyte isolate, IBRL OS-39, showed a growth reduction of more than 50% in comparison to control against *M. fulvum*, *T. rubrum*, *M. gypseum*, and *Rhizopus* sp. Only one endophyte fungal isolate, *Lasiodiplodia* sp. IBRL OS-64, was able to exert an inhibitory impact with an antagonistic index of 42.92.6% on *Fusarium solani*, which was the least inhibited by the endophyte fungal isolates. In addition, *M. fulvum* and *T. rubrum's* mycelial growth was significantly inhibited by four different fungi, *Colletotrichum* sp. IBRL OS-39, *Lasiodiplodia* sp. IBRL OS-64, *Muscodor* sp. IBRL OS-94, and *Muscodor* sp. IBRL OS-98, with growth reductions ranging from 15 to 50%. *Colletotrichum gloeosporioides* has a promising

antagonistic impact against strong fungal infections, according to Rabha [24]. The antagonistic behaviour of *Colletotrichum* sp. toward the test pathogens may be attributed to diffuse metabolites, volatile chemicals, and excessive isolate development that inhibits pathogen growth. It has been previously documented that the fungus *Colletotrichum* sp., *Lasiodiplodia* sp., and *Muscodor* sp. can demonstrate antagonistic activity towards. Against *Fusarium oxysporum*, for instance, *Muscodor kashayum* demonstrated antagonistic action [25]. Besides, *L. theobromae* JF766989, *Colletotrichum* sp. JF766996, *C. gloeosporioides* JF767002, *Colletotrichum* sp. JF767006, *Colletotrichum* sp. JF767004, *Colletotrichum* sp. JF766999, endophytic fungi isolated from *Piper hispidum*, have shown antagonistic activity against phytopathogenic fungi including *Alternaria alternata*, *Colletotrichum* sp., *Phyllosticta citricarpa*, and *Moniliophthora perniciosa* [26].

Table 2 Antagonistic activity endophytic fungal isolates against test fungi

Fungal isolates	Antagonistic index (%)							
	M. fulvum	*T. rubrum*	*M. gypseum*	*F. solani*	*Rhizopus* sp.	*A. niger*	*C. albicans*	*C. utilis*
Colletotrichum sp. IBRL OS-27	-	-	-	-	13.5±1.6	-	-	-
Colletotrichum sp. IBRL OS-39	50.0±0.0	40.0±1.0	62.5±0.4	-	57.2±1.7	-	-	-
Lasiodiplodia sp. IBRL OS-64	41.4±0.4	35.0±0.4	50.4±0.4	42.9±2.6	-	56.9±1.2	46.5±0.6	44.8±0.9
Aspergillus sp. IBRL OS-65	-	-	-	-	-	-	-	-
Aspergillus sp. IBRL OS-82	-	-	-	-	26.5±0.4	-	-	-
Muscodor sp. IBRL OS-94	15.2±0.6	20.6±0.8	-	-	23.9±2.6	65.6±1.6	43.8±1.0	50.3±0.3
Muscodor sp. IBRL OS-98	31.4±0.4	26.1±2.6	-	-	-	-	-	-

The current finding also demonstrated that different endophytic fungal isolates have different antagonistic indices for pathogenic test fungi. This might be brought on by the rate of growth of the isolates or test fungus. The pace and development rate of the colony were significant in defining antagonistic activity, according to Campanile [27]. Most of the fungal isolates were able to suppress the slow-growing test fungi since some of them were fast-growing fungi. Contradictory, rapidly proliferating test fungi also had an impact on the antagonistic index, making it difficult for slow-proliferating fungal isolates to inhibit them.

The primary screening of antifungal activity of fungal endophytes was determined using fungal filtrates. Five culture filtrates of endophytic fungal isolates, *Colletotrichum* sp. IBRL OS-27, *Colletotrichum* sp. IBRL OS-39, *Lasiodiplodia* sp. IBRL OS-64, *Muscodor* sp. IBRL OS-94 and *Muscodor* sp. IBRL OS-98 exhibited antifungal activity against test fungi. As shown in Table 3, fungal isolate, *Colletotrichum* sp. IBRL OS-39 showed a broad spectrum of antifungal activity against seven out of eight test fungi including *M. gypseum* (44.9±4.6%), *M. fulvum* (71.8±4.2%), *T. rubrum* (36.5±2.4%), *Rhizopus* sp. (47.5±1.7%), *A. niger* (82.4±0.7%), *C. albicans* (32.8±0.7%) and *C. utilis* (24.6±1.2%). It is crucial to note the potential of *Colletotrichum* sp. IBRL OS-39 in inhibiting the growth of dermatophytes fungi including *M. gypseum* and *T. rubrum*. Among tested pathogenic fungi, *Rhizopus* sp. was most inhibited by culture filtrates of endophytes with growth inhibition ranging from 35 to 48%. Moreover, it is noteworthy that culture filtrates of isolate *Colletotrichum* sp. IBRL OS-39 and *Muscodor* sp. IBRL OS-94 inhibited the *Aspergillus niger* with high inhibition percentages of 82.4±0.7 and 85.4±1.1, respectively. The ability of *Colletotrichum* sp. to inhibit pathogenic fungi has been previously reported. Packiaraj [28] reported the antifungal activity of *Colletotrichum gloeosporioides* isolated from endemic tree *Cinnamomum malabatrum* against *Candida albicans*. Besides, an endophytic fungus of *Vaccinium dunalianum* var. urophyllum, *Colletotrichum* sp. has been reported to possess antifungal activity against *C. albicans* [29]. Additionally, the test fungus *Rhizopus* sp. is highly susceptible to fungal filtrates. This might be due to the mechanism of antifungal compounds that can disrupt any metabolism in the cell of pathogenic fungi. Shirazi and Kontoyiannis [30] reported the potential of antifungal compounds such as posaconazole and itraconazole to inhibit the mitochondrial respiratory pathway of *Rhizopus oryzae*. On the other hand, the filtrates of *Colletotrichum* sp. IBRL OS-39 showed significant antifungal activity against filamentous fungi. This might be due to the bioactive compound produced by the isolate having a special mode of action that

can restrict filamentous fungi. According to Parks and Casey [31], antifungal compounds such as polyenes, azoles, and allylamine can inhibit the synthesis of ergosterol, which is a prominent component in the cell membrane of fungi.

Table 3 Antifungal activity of culture filtrates of endophytic fungi against pathogenic fungi

Fungal isolates	Growth inhibition (%)							
	M. gypseum	*M. fulvum*	*T. rubrum*	*F. solani*	*Rhizopus* sp.	*A. niger*	*C. albicans*	*C. utilis*
Colletotrichum sp. IBRL OS-27	-	-	-	-	23.4±0.8	-	-	-
Colletotrichum sp. IBRL OS-39	44.9±4.6	71.8±4.2	36.5±2.4	-	47.5±1.7	82.4±0.7	32.8±0.7	24.6±1.2
Lasiodiplodia sp. IBRL OS-64	59.1±2.7	-	-	26.3±1.8	36.9±0.7	40.4±0.6	57.6±1.2	42.4±0.6
Aspergillus sp. IBRL OS-65	-	-	-	-	-	-	-	-
Aspergillus sp. IBRL OS-82	-	-	-	-	-	-	-	-
Muscodor sp. IBRL OS-94	-	-	34.8±0.8	-	34.7±2.2	85.4±1.1	62.9±0.9	58.6±1.2
Muscodor sp. IBRL OS-98	-	-	-	-	30.2±1.2	-	-	-

Table 4 lists the findings of the disc diffusion assay used to determine the endophytic fungal extracts' antifungal activity. All of the test fungi were unaffected by the dichloromethane extract of *Colletotrichum* sp. IBRL OS-27 and *Aspergillus* sp. IBRL OS-82. Furthermore, *Aspergillus* sp. IBRL OS-65 only inhibited *M. fulvum* with a diameter of inhibition zone of 11.30.6 mm, whilst *Colletotrichum* sp. IBRL OS-39 and *Muscodor* sp. IBRL OS-98 were only active against *Rhizopus* sp. with a diameter of inhibition zone of 17.81.2 mm and 10.60.8 mm, respectively. Meanwhile, endophytic fungal isolates, *Lasiodiplodia* sp. IBRL OS-64 and *Muscodor* sp. IBRL OS-94 exhibited antifungal activity against all test fungi except *A. fumigatus* and *M. fulvum* with inhibition zone in the range of 9.6±0.6 - 14.3±0.6 mm and 11.2±1.2 - 15.7±0.6 mm, respectively. In order to extract bioactive compounds from the fermentative broth of fungal isolates, a variety of solvents were employed. This was done because, according to Kandasamy and Arunachalam [32], the extraction solvent is important because it influences the existence and activity of bioactive compounds.

Table 4 Antifungal activity of endophytic fungal isolates against test fungi on disc diffusion assay

Fungal isolates	Diameter of inhibition zone (in mm)						
	Test Fungi						
	T. rubrum	*A. fumigatus*	*M. fulvum*	*A. niger*	*Rhizopus* sp.	*C. albicans*	*C. utilis*
Colletotrichum sp. IBRL OS-27	-	-	-	-	-	-	-
Colletotrichum sp. IBRL OS-39	-	-	-	-	9.2±0.6	-	-
Lasiodiplodia sp. IBRL OS-64	13.0±1.0	-	-	10.5±1.2	9.6±0.6	11.7±0.6	14.3±0.6
Aspergillus sp. IBRL OS-65	-	-	11.3±0.6		-	-	-
Aspergillus sp. IBRL OS-82	-	-	-	-	-	-	-
Muscodor sp. IBRL OS-94	14.0±1.0	-	-	14.5±0.6	11.2±1.2	15.7±0.6	14.7±1.2
Muscodor sp. IBRL OS-98	-	-	-	-	10.6±0.8	-	-
(+) control	**19.7±0.6**	**15.3±0.6**	**18.7±1.0**	**14.5±0.3**	**19.5±0.3**	**20.3±0.6**	**21.3±1.2**

Since dichloromethane is a mid-polar solvent and has the ability to extract molecules with antifungal activity, it was the subject of the current study. The polarity of the organic solvent affects the antimicrobial activity of the fungal extract, and mid-polar solvents are optimum for isolating compounds with antimicrobial activity from fungi, claim Taufiq and Darah [33]. The majority of antibacterial substances in nature, according to Tong [35], exist as mid-polar molecules and can therefore be extracted using either dichloromethane or ethyl acetate. The current research indicated that a variety of test fungi were resistant to the dichloromethane produced by *Lasiodiplodia* sp. and *Muscodor* sp. It is notable that all test fungi except *Rhizopus* sp. were unaffected by the dichloromethane extract of *Colletotrichum* sp. IBRL OS-39. The outcome was in contrast to the earlier research, which showed that *Colletotrichum* sp. IBRL OS-39 fungal filtrates exhibited antifungal efficacy against all test fungi. The various polarity of the bioactive compounds may be the cause of this phenomenon. Solvents such as methanol and hexane, which are either polar or non-polar, can be used to extract some chemical molecules. A variety of chemical compounds with various functions are present in natural goods, and any alterations to those functionalities may have an impact on the polarity of those molecules. The presence of bioactive compounds in natural products and their activity may therefore be impacted by the choice of organic solvent used in the extraction process [33].

To ascertain the in vitro activity of novel antibiotics and their MIC breakpoint, evaluation of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) is widely used. According to Jalil [12], the assessment of the MIC and MBC values of the fungal extract may serve as a helpful signal when choosing the right therapeutic agents and their optimal dosage in pharmaceutical procedures. *Lasiodiplodia* sp. IBRL OS-64 and *Muscodor* sp. IBRL OS-94, two endophytic fungal isolates, were tested against five different fungi, including *Trichophyton rubrum*, *Aspergillus niger*, *Rhizopus* sp., *C. albicans*, and C. utilis. *Lasiodiplodia* sp. IBRL OS-64 extract had MIC and MFC values against test fungi in the ranges of 250–500 and 500–4000 g/ml, respectively, as shown in Table 5. The *Muscodor* sp. IBRL OS-94 extract's MIC and MFC values, meanwhile, ranged from 125 to 500 and 250 to 4000 g/ml, respectively. Since the MFC/MIC ratio was under 4, the fungal extracts from *Lasiodiplodia* sp. IBRL OS-64 and *Muscodor* sp. IBRL OS-94 had a fungicidal impact on *C. albicans* and C. utilis, but fungistatic action on *A. niger*, T. *rubrum*, and *Rhizopus* sp. since the MFC/MIC ratio was above 4. If the MFC/MIC values are less than 4, according to Ganan *et al*. [35], the bioactive compounds have fungicidal activity rather than fungistatic activity.

Clinically, it has been widely accepted that fungicidal/yeastocidal drugs are more potent antibiotics than fungistatic/yeastostatic ones because they can kill fungi and yeast instead of just inhibiting them and because most of them work well in the treatment of critically ill and immunocompromised patients [36].

Table 5 Determination of minimum inhibition concentration (MIC) and minimum fungicidal concentration (MFC) of endophytic fungal isolates extracts

Fungal isolates	*Lasiodiplodia* sp. IBRL OS-64			*Muscodor* sp. IBRL OS-94		
Test fungi	**MIC**	**MFC**	**Ratio**	**MIC**	**MFC**	**Ratio**
T. rubrum	500	4000	8	250	2000	8
A. niger	500	4000	8	250	2000	8
Rhizopus sp.	500	4000	8	500	4000	8
C. albicans	250	500	2	125	250	2
C. utilis	250	1000	4	250	1000	4

The current study also showed that compared to other test fungi, the Candida species is more vulnerable to fungal endophytes. This might be caused by a little variation in the concentration of chitin in candida.

Despite having a similar origin for their cell walls, fungi have a higher chitin content than yeast [37].

The impact of the dichloromethane extract of fungal endophytes on Candidal cells was examined using scanning electron microscopy (SEM). *Candida albicans* was chosen in this study due to its prominent susceptibility to fungal extract. The dichloromethane extracts of *Lasiodiplodia* sp. IBRL OS-64 and *Muscodor* sp. IBRL OS-94 is shown to have different effects on *C. albicans* in Figures 1 and 2, respectively. Untreated candidal cells with oval, smooth surfaces and some buddings are depicted in Figure 1a. Figure 1b, on the other hand, shows *C. albicans* cells that had been exposed to the *Lasiodiplodia* sp. IBRL OS-64 extract and had undergone dramatic morphological changes, including some invaginations, cavitation, and shrinking of the cell surfaces. Most of the *C. albicans* cells in Figure 2a looked to have a smooth surface and an oval shape, and some of them were in the budding stage (control cells). On the other hand, after a 48-hour exposure period, most of the cells treated with the fungal extract (Figure 2b) shrank and lost their intact oval shapes, along with some cell debris.

Overall, the results showed some significant morphological alterations, including invaginations and cavitation that developed on the treated cells' surfaces. In addition, there has also been cell shrinkage and damage, which ultimately result in cell death. According to Shraideh *et al*. [38], who made a similar observation, date extract induced severe cell damage to *C. albicans* cells, resulting in cell collapse and the discharge of cytoplasmic debris, as well as eventual cell death. The candidal cells treated with the fungal extract, however, showed the existence of abnormalities such as disintegrating and wrinkled structures, according to a different earlier study [39].

Numerous earlier research described a potential mechanism or mode of action for how fungal extracts can affect yeast cells. According to Mu'azzam and Darah [37], candidal cells exposed to fungal extract suffered a number of injuries as a result of decreased DNA/protein synthesis, increased membrane permeability and cytoplasmic leakage, and altered cell wall/cell membrane components. According to Selitrennikoff [40], the extract had an impact on the fungal cells by impairing the structure of their cell walls and membranes, leading to cell lysis. According to Bush [41], the presence of the extract changed the organelles in the cell membranes, and any membrane breakdown caused the major metabolic system to fail, resulting in irreversible cell death. Overall, *Lasiodiplodia* sp. IBRL OS-64 and *Muscodor* sp. IBRL OS-64 and IBRL OS-94 fungal extracts induced severe cell damage that was irreparable and most of the cells lost their metabolic function, which resulted in cell death.

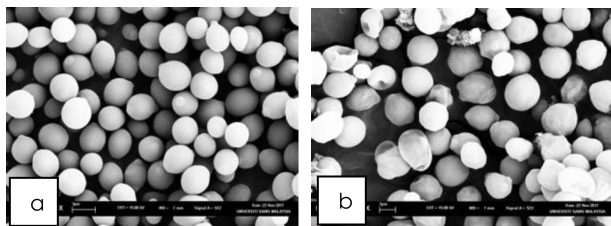

Figure 1 *C. albicans* treated with 500 g/mL of dichloromethane-crude extract of *Lasiodiplodia* sp. IBRL OS-64 is shown in SEM photomicrographs. (a) 0 hour [control] (b) 48 hours. Scale bars: 200nm.

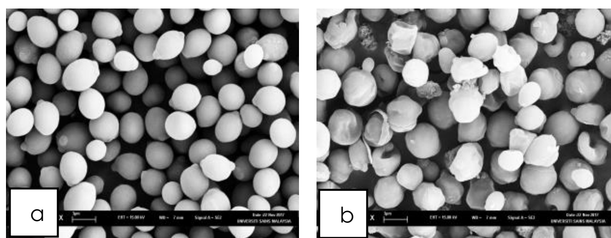

Figure 2 *C. albicans* treated with 250 g/mL of *Muscodor* sp. IBRL OS-94 crude dichloromethane extract in SEM photomicrographs. (a) 0 hour [control] (b) 48 hours. Scale bars: 200nm

4.0 CONCLUSION

In this study, it was discovered that endophytic fungi isolated from the medicinal plant *Ocimum sanctum* have antifungal efficacy against a number of test fungi, including *C. albicans* and *C. utilis*. Two fungal endophytes, *Lasiodiplodia* sp. IBRL OS-64 and *Muscodor* sp. IBRL OS-94, demonstrated notable antifungal activity with a fungicidal effect against *C. albicans* and *C. utilis*. According to SEM analysis, candidal cells exposed to fungal endophyte dichloromethane extracts suffered severe damage that ultimately resulted in cell death.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

Acknowledgement

The authors thanked the School of Biological Sciences, Universiti Sains Malaysia, Penang, and the Ministry of Education, Malaysia for granting the MyBrain15 scholarship to Jalil MTM while completing his Ph.D. study.

References

[1] Owen, N. L. & Hundley, N. 2004. Endophytes - The Chemical Synthesizers inside Plants. *Science Progress*. 87(2): 79-99. https://doi.org/10.3184/003685004783238553.

[2] Sadrati, N., Daoud, H., Zerroug, A., Dahamna, S. & Bouharati, S. 2013. Screening of Antimicrobial and Antioxidant Secondary Metabolites from Endophytic Fungi Isolated from Wheat (*Tricum durum*). *Journal of Plant Protection Research*. 53(2): 128-136. https://doi.org/10.2478/jppr-2013-0019.

[3] Guo, B., Wang, Y., Sun, X. & Tang, K. 2008. Bioactive Natural Products from Endophytes: A Review. *Applied Biochemistry and Microbiology*. 44(2): 136-142. https://doi.org/10.1134/S0003683808020026.

[4] Stierle, A., Strobel, G. & Stierle, D. 1993. Taxol And Taxane Production by *Taxomyces andreanae*, an Endophytic Fungus of Pacific Yew. *Science*. 260: 214-216. https://doi.org/10.1126/science.8097061.

[5] Ibrahim, M., Kaushik, N., Sowemimo, A., Chhipa, H., Koekemoer, T., Venter, M. & Odukoya, O. A. 2017. Antifungal and Antiproliferative Activities of Endophytic Fungi Isolated from the Leaves of *Markhamia tomentosa*. *Pharmaceutical Biology*. 55(1): 590-595. https://doi.org/10.1080/13880209.2016.1263671.

[6] Venieraki, A., Dimou, M. & Katinakis, P. 2017. Endophytic Fungi Residing in Medicinal Plants have the Ability to Produce the Same or Similar Pharmacologically Active Secondary Metabolites as their Hosts. *Hellenic Plant Protection Journal*. 10: 51-66. https://doi.org/10.1515/hppj-2017-0006.

[7] Pattanayak, P., Behera, P., Das, D. & Panda, S. K. 2010. *Ocimum sanctum* Linn. A Reservoir Plant for Therapeutic Applications: An Overview. *Pharmacognosy Reviews*. 4(7): 95-105. https://doi.org/10.4103/0973-7847.65323.

[8] Shetty, S., Udupa, S., Udupa, L. & Somayaji, N. 2006. Wound Healing Activity of *Ocimum sanctum* Linn with Supportive Role of Antioxidant Enzymes. *Indian Journal of Physiology and Pharmacology*. 50: 163-168. PMID: 17051736.

[9] Trevisan, M. T., Vasconcelos Silva, M. G, Pfundstein, B., Spiegelhalder, B. & Owen, R. W. 2006. Characterization of the Volatile Pattern and Antioxidant Capacity of Essential Oils from Different Species of the Genus Ocimum. *Journal of Agricultural and Food Chemistry*. 54: 4378-4382. https://doi.org/10.1021/jf060181.

[10] Kelm, M. A., Nair, M.G., Stasburg, G.M. & DeWitt, D. L. 2000. Antioxidant and Cyclooxygenase Inhibitory Phenolic Compounds from *Ocimum sanctum* Linn. *Phytomed*. 7: 7-13. https://doi.org/10.1016/S0944-7113(00)80015-X.

[11] Singh, S., Malhotra, M. & Majumdar, D. K. 2005. Antibacterial Activity of *Ocimum sanctum* L. Fixed Oil. *Indian Journal of Experimental Biology*. 43: 835-837.

[12] Jalil, M. T. M., Ibrahim, D. & Suhaimi, N. S. M. 2020. Time-kill Study and Morphological Changes of *Proteus mirabilis* Cells Exposed to Ethyl Acetate Crude Extract of *Lasiodiplodia pseudotheobromae* IBRL OS-64. *Malaysian Journal of Microbiology*. 16(3): 219-228. https://doi.org/10.21161/mjm.190605.

[13] Stinson, M., Ezra, D., Hess, W. M., Sears, J. & Strobel, G. 2003. An endophytic *Gliocladium* sp. of *Eucryphia cordifolia* Producing Selective Volatile Antimicrobial Compounds. *Plant Science*. 165: 913-922. https://doi.org/10.1016/S0168-9452(03)00299-1.

[14] Landum, M. C., Felix, Md-R., Alho, J., Garcia, R., Cabrita, M. J., Rei, F. & Varanda, C. M. R. 2016. Antagonistic activity of fungi of *Olea europaea* L. against *Colletotrichum acutatum*. *Microbiological Research*. 183: 100-108. https://doi.org/10.1016/j.micres.2015.12.001.

[15] Grover, R. K. & Moore, J. D. 1962. Toxicometric Studies of Fungicides against Brown Rot Organisms *Sclerotinia fructicola* and *S. laxa*. *Phytopathology*. 52: 876-880.

[16] CLSI. 2017. Performance Standards for Antifungal Susceptibility Testing of Yeasts. 1st ed. CLSI supplement M60. Clinical Laboratory Standards Institute. Wayne, PA.

[17] Ibrahim, D., Lee, C. C., Yenn, T. W., Zakaria, L. & Sheh-Hong, L. 2015. Effect of the Extract of Endophytic Fungus, *Nigrospora sphaerica* CL-OP 30, Against the Growth of Methicillin-Resistant *Staphylococcus aureus* (MRSA) and *Klebsiella pneumonia* cells. *Tropical Journal of Pharmaceutical Research*. 14(11): 2091-2097. https://doi.org/10.4314/tjpr.v14i11.20,

[18] Rowan, D. D. 2011. Volatile metabolites. *Metabolites*. 1(1): 41-63. https://doi.org/10.3390/metabo1010041.

[19] Naik, B. 2018. Volatile Hydrocarbons from Endophytic Fungi and Their Efficacy in Fuel Production and Disease Control. *Egyptian Journal of Biological Pest Control*. 28: 69. https://doi.org/10.1186/s41938-018-0072-x.

[20] Aydi Ben Abdallah, R., Jabnoun-Khiareddine, H., Mejdoub-Trabelsi, B. & Daami-Remadi, M. 2015. Soil-borne and Compost-borne *Aspergillus* Species for Biologically Controlling Post-harvest Diseases of Potatoes Incited by *Fusarium sambucinum* and *Phytophthora erythroseptica*. *Journal of Plant Pathology & Microbiology*. 6: 313. https://doi.org/10.4172/2157-7471.1000313.

[21] Strobel, G. A., Dirkse, E., Sears, J. & Markworth, C. 2001. Volatile Antimicrobials from *Muscodor albus*, a Novel Endophytic Fungus. *Microbiology*. 147: 2943-2950. https://doi.org/10.1099/00221287-147-11-2943.

[22] Meshram, V., Kapoor, N., Chopra, G. & Saxena, S. 2017. *Muscodor camphora*, a New Endophytic Species from *Cinnamomum camphora*. *Mycosphere*. 8(4): 568-582.

[23] Pena, L. C., Jungklaus, G. H., Savi, D. C., Ferreira-Maba, L., Servienski, A., Maia, B. H. L. N. S., Annies, V., Galli-Terasawa, L. V., Glienke, C. & Kava, V. 2019. *Muscodor brasiliensis* sp. nov. Produces Volatile Organic Compounds with Activity against *Penicillium digitatum*. *Microbiological Research*. 221: 28-35. https://doi.org/10.1016/j.micres.2019.01.002.

[24] Rabha, A. J., Naglot, A., Sharma, G. D., Gogoi, H. K. & Veer, V. 2014. In Vitro Evaluation of Antagonism of Endophytic *Colletotrichum gloeosporioides* Against Potent Fungal Pathogens of *Camellia sinensis*. *Indian Journal of Microbiology*. 54(3): 302-309. https://doi.org/10.1007/s12088-014-0458-8.

[25] Meshram, V., Kapoor, N. & Saxena, S. 2013. *Muscodor kashayum* sp. nov. – A New Volatile Anti-microbial Producing Endophytic Fungus. *Mycology*. 4(4): 196-204. https://doi.org/10.1080/21501203.2013.877990.

[26] Orlandelli, R. C., Almeida, T. T., Alberto, R. N., Polonio, J. C., Azevedo, J. L. & Pamphile, J. A. 2015. Antifungal and Proteolytic Activities of Endophytic Fungi Isolated from *Piper hispidum* Sw. *Brazilian Journal of Microbiology*. 46(2): 359-366. https://doi.org/10.1590/S1517-838246220131042.

[27] Campanile, G., Ruscelli, A. & Luisi, N. 2007. Antagonistic Activity of Endophytic Fungi Towards *Diplodia corticola* Assessed by In Vitro and in Planta Tests. *European Journal of Plant Pathology*. 117: 237-246. https://doi.org/10.1007/s10658-006-9089-1.

[28] Packaraj, R., Jeyakumar, S., Ayyappan, N., Adhirajan, N., Premkumar, G., Rajarathinam, K. & Muthuramkumar, S. 2016. Antimicrobial and Cytotoxic Activities of Endophytic Fungus *Colletotrichum gloeosporioides* Isolated from Endemic Tree *Cinnamomum malabatrum*. *Studies in Fungi*. 1(1): 104-113. https://doi.org/10.5943/sif/1/1/10.

[29] Tong, X., Shen, X. & Hou, C. 2018. Antimicrobial Activity of Fungal Endophytes from *Vaccinium dunalianum* var. urophyllum. *Sains Malaysiana*. 47(8): 1685-1692. https://doi.org/10.17576/JSM-2018-4708-07.

[30] Shirazi, F. & Kontoyiannis, D. P. 2013. Mitochondrial Respiratory Pathways Inhibition in *Rhizopus oryzae* Potentiates Activity of Posaconazole and Itraconazole via Apoptosis. *PloS One*. 8(5): 1-6. https://doi.org/10.1371/journal.pone.0063393.

[31] Parks, L. W. & Casey, W. M. 1996. Fungal sterols. In: Prasad, R. & Ghannoum, M. A. (eds). Lipids of Pathogenic Fungi. CRC Press, Florida. 63-82.

[32] Kandasamy, M. & Arunachalam, K. D. 2008. Evaluation of In Vivo Antibacterial Property of Seaweeds of South-east Cost of India. *African Journal of Biotechnology*. 7: 1958-1961. https://doi.org/10.5897/AJB08.120.

[33] Taufiq, M. M. J. & Darah, I. 2020. Biological Activity of *Lasiodiplodia pseudotheobromae* IBRL OS-64 Extracts, an Endophytic Fungus Isolated from Medicinal Herb, *Ocimum sanctum* Against Foodborne Diarrhea-caused Bacteria. *Pharmacognosy Journal*. 12(4): 897-904. https://doi.org/10.5530/pj.2020.12.127.

[34] Tong, W. Y., Nurul, J. Z., Nurhaida., Tan, W. N., Melati, K., Latiffah, Z. & Ibrahim D. 2014. Antimicrobial activity of *Phomopsis* sp. ED2 residing in Medicinal Plant *Orthosiphon stamineus* Benth. *Annual Research & Review in Biology*. 4(9): 1490-1501. https://doi.org/10.9734/ARRB/2014/8060.

[35] Ganan, M., Lorentzen, S. B., Agger, J. W., Heyward, C. A., Bakke, O., Knutsen, SH, Aam, B. B., Eijsink, V. G. H., Gaustad, P. & Sorlie, M. 2019. Antifungal Activity of Well-defined Chitooligosaccharide Preparations against Medically Relevant Yeasts. *PLoS ONE*. 14(1): e0210208. https://doi.org/10.1371/journal.pone.0210208.

[36] Fauci, A. S., Kasper, D. K., Longo, D. L., Braunwald, E., Hanser, S. L., Jameson, J. L. & Loscalzo, J. 2008. *Harrison's Principles of Internal Medicine*. 17th edition, McGraw-Hill Professional, New York.

[37] Mu'azzam, K. A. A. R. & Darah, I. 2019. *Ceratobasidium ramicola* IBRLCM127, an Endophytic Fungus Isolated from *Curcuma mangga* Valeton & Zijp with Strong Anti-candidal Activity. *Journal of Applied Pharmaceutical Science*. 9(11): 86-92.

https://doi.org/10.7324/JAPS.2019.91111.

[38] Shraideh, Z. A., Abu-Elteen, K. H. & Sallal, A. J. 1998. Ultrastructural Effects of Date Extract on *Candida albicans*. *Mycopathologia*. 142: 119-123. https://doi.org/10.1023/a:1006901019786.

[39] Nath, A. & Joshi, S. 2017. Anti-candidal Effect of Endophytic Fungi Isolated from *Calotropis gigantean*. *Revista De Biologia Tropical*. 65(4): 1437-1447.

[40] Selitrennikoff, C. P. 2001. Antifungal Protein. *Applied and Environmental Microbiology*. 67(7): 2883-2894. https://doi.org/10.1128/AEM.67.7.2883-2894.2001.

[41] Bush, K. 2012. Antimicrobial Agents Targeting Bacterial Cell Walls and Cell Membranes. *Revue Scientifique et Technique*. 31(1): 43-56. https://doi.org/10.20506/rst.31.1.2096.