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Isolation and Evaluation of Antifungal Metabolites from Endophytic Fungi Against Some Pathogenic Fungi

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Abstract

Background and aim: The aim of this study was to find endophytic fungi with potential antifungal activities against some pathogenic fungi. Materials and methods: Antifungal activities of 24 endophytic fungal strains, including *Fusarium, Aspergillus, Cylindrocladium, Penicillium, Chaetomium, Alternaria, Cladosporium, Nigrospora, Aureobasidium, Bipolaris*, and sterile mycelia, were tested against *Candida albicans* using a visual agar plate assay method. Endophytic fungus F-12-BSU, with the highest antifungal activities, was identified using ITS1/4 sequencing and cultured on potato dextrose broth medium for 21 days at 26°C. The culture filtrate was extracted using methanol, ethanol, and ethyl acetate, water and concentrated by a rotary evaporator. Antifungal susceptibility testing against *C. albicans, C. tropicalis, C. glabrata, Cryptococcus neoformans, Aspergillus fumigatus, A. flavus, Trichophyton rubrum,* and *T. mentagrophytes* was performed by CLSI microbroth dilution method (M-27-A3, M38-A2).Results: The results of ITS sequencing led to the identification of *A. aculeatus* as an endophytic fungus (F-12-BSU). Regarding the minimum inhibitory concentration (MIC), methanolic extract showed the MIC50 and MIC90 values of 0.32 and 0.64 µg/ml against *C. albicans,* respectively. Meanwhile, fluconazole had the MIC50 value of 2 µg/ml against this *Candida* species. Furthermore, *C. tropicalis, C. glabrata,* and *C. krusei* had the MIC50 values of 0.01, 0.04, and 0.032 µg/ml, respectively, compared to fluconazole with the MIC50 values of 4, 16, and 16 µg/ml. Filamentous fungi of *A. fumigatus* and *A. flavus* had the MIC50 values of 0.32 and 0.08 µg/ml, respectively, while fluconazole had the MIC50 values of 128 and 64 µg/ml, respectively, against these species. Additionally, *Cryptococcus neoformans, T. rubrum,* and *T. mentagrophytes* with the MIC50 value of more than 5 µg/ml had the highest MICs against the crude extract of endophytic fungus, compared to fluconazole with the MIC50 values of 8, 4, and 8 µg/ml, respectively. Conclusion: Based on the findings of the present study, the methanolic extract of *A. aculeatus* as an endophytic fungus can be a suitable substitute for antifungal drugs, which can be used against the pathogenic fungi after standardization and purification.

Keywords: Entophytic Fungi, Pathogenic Fungi, Secondary Metabolites, Antifungal Activity.

Introduction

In today's advanced societies, the prevalence of fungal infections, mainly those caused by *Candida albicans*, is on a growing trend. This increased prevalence can be due to the use of chemotherapy, advancement of transplantation, administration of immunosuppressive and cytotoxic medications, spread of blood diseases, long-term use of antibiotics, growing incidence of diabetes mellitus, and use of adrenal cortical hormones drugs (Turner et al., 2014) Little research has been conducted on antifungal agents in comparison to the number of studies addressing antibacterial drugs. Considering the increasing trend of fungal diseases, there is an urgent need to perform further studies in this field. A number of researchers believe that the production of antifungal drugs is a challenging issue since fungal cells are eukaryotes and similar to human cells (Li et al., 2015; Mazu et al., 2016).

Candida albicans are among the most important opportunistic and pathogenic fungi in humans, causing numerous diseases from superficial mucosal infections (e.g., vaginitis and oral candidiasis) to systemic fungal infections. These *Candida* species are frequently

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isolated from human fungal infections and are the fourth cause of blood infection in nosocomial infections, specifically in patients with HIV/AIDS (Tortorano et al., 2006; Melo et al., 2011; Zida et al., 2017). The ability of *C. albicans* to generate single-celled yeast, chlamydoconidia, true hyphae, and budding cell forms can be effective in its invasive penetration into the tissues (Torey et al., 2016). The formation of biofilms by *C. albicans* increases the resistance of these organisms to the majority of antifungal drugs (Silva et al. 2017).

Fluconazole is one of the most important drugs in azole group that is widely used in the treatment of fungal infection. There is a growing trend of fluconazole resistance among *C. albicans*. On the other hand, amphotericin B widely used in the treatment of systemic candidiasis reportedly is associated with several adverse effects, such as nephrotoxicity, on human cells (Hoehamer et al., 2010). Effort has been made to develop antifungal drugs with the ability to kill *C. albicans* and deal with antifungal drug-resistant strains while having few side effects (Mazu et al., 2016). The word 'endophyte' is composed of two Greek words, namely endo and phyto, meaning inside and plant, respectively. Endophytes are fungi and bacteria; however, actinomycetes and mycoplasmas have been also categorized under this group. These organisms are abundantly found in plants and spend all or part of their lives either intracellularly or intercellulary without any apparent infections in the plant. Endophytes play a key role not only in plant growth and food absorption, but also in protecting the plant against pests. Almost all known plants can be the habitat of these endophytes. The interaction between fungal endophytes and their host plant is very diverse and can vary from mutualistic to antagonistic and rarely parasitic (Ribeiro et al., 2018; Gouda et al., 2016). Scientists have estimated that approximately 1.5 million fungal species exist in the world, while only 100,000 cases have been discovered. Meanwhile, there may be at least one million species of endophytic fungi (Sharma et al., 2016).

Today, researchers have paid special attention to the considerable properties of secondary metabolites of endophytic fungi. These organisms have been used for their anticancer, immunomodulatory, antioxidant, antiparasitic, antiviral, antifungal, antituberculosis, and antimalarial properties, as well as for their role in the production of the metabolites that are pest repellents. These secondary metabolites owe their properties to various biosynthetic pathways belonging to a variety of structural groups, including terpenoids, alkaloids, steroids, and other metabolites (Gouda et al., 2016).

Only one endophyte species can produce several types of secondary metabolites that have biological effects. Studies show that endophytes produce secondary metabolites that mimic those produced by their host plants. They can utilize the same path used by the plant for the production of secondary metabolites. Endophytes are a potential source of secondary metabolites that can be colonized in the roots, stems, leaves, petioles, seeds, and fruits of the plants (Murali et al., 2017; Premjanu et al., 2016).

In addition, endophytes are important sources for the production of such enzymes as pectinase, cellulase, lipase, amylase, and laccase, which are widely used in the industry (Patil et al., 2015). According to the literature, about 51% of biologically active substances are derived from the endophytic fungi (Supaphon et al., 2013). Based on the recent studies, the production of secondary metabolites by endophytic fungi can be extremely influenced by the conditions of the culture medium and its constituent compounds, such as the ratio of carbon to nitrogen. In addition, the production of these metabolites can be affected by temperature, aeration conditions, co-culturing of two endophytic fungi, and coexistence of endophytic fungi with endophytic bacteria (Supaphon et al., 2013; Ranjbariyan et al., 2014; Deshmukh et al., 2018; Ranjbariyan et al., 2011).

Increased prevalence of invasive fungal infections, limited access to common drugs, and resistance of pathogenic fungi to these drugs underscore the need for using natural products as important substitutes for the isolation of newly emerging molecules for the treatment of fungal diseases. In this regard, endophytic fungi can be considered as the main source of these metabolites (Ribeiro et al., 2018; Jouda et al., 2016; Deshmukh & Verekar, 2013). In the last two decades, the use of the secondary metabolites of endophytic fungi as a source of important biological properties has grown considerably. Taxol, which is obtained from Taxomyces andreanae, has been the most effective and successful anticancer drug derived from an endophytic fungus (Gouda et al., 2016; Zilla et al., 2013; González-Menéndez et al., 2018). Furthermore, the results of several studies confirm the ability of the endophytic fungi to produce a range of secondary metabolites that have anticancer properties (e.g., Taxol, vinblastine, camptothecin, and podophyllotoxin) and antifungal metabolites (e.g., cryptocandin, enfumafungin, CR377, ambuic acid, jesterone, moriniafungin, parnafungins, and phaeofungin). Endophytic fungi are the sources of new natural metabolites; however, low production and the lack of in vitro expression of cryptic gene clusters limit the extraction of these metabolites from fungal endophytes. Gene mutation and transformation, mixed culture, and use of additives (e.g., epigenetic modifiers or absorbent polymer resins) can stimulate endophytic fungi to have more products (González-Menéndez et al., 2018).

With this background in mind, the present study aimed to detect and extract the antifungal metabolites obtained from the endophytic fungi collection of Bu-Ali Sina Botanical Research Center in Hamedan, Iran. In addition, this study involved the evaluation of the inhibitory effect of fungal endophytes against yeasts (e.g., *C. albicans*, *C. krusei*, *C. glabrata*, and *C. tropicalis*), filamentous fungi (e.g., *Aspergillus flavus* and *A. fumigatus*), and dermatophytes (e.g., *Trichophyton rubrum* and *T. mentagrophytes*).

Materials and Methods

Fungal endophytes

In this research, endophytic fungi were obtained from the Botanical Research Center of Bu-Ali Sina University of Hamedan, Iran. A total of 24 endophytic fungal strains of different species, including *Fusarium, Aspergillus, Cylindrocladium, Penicillium, Chaetomium, Alternaria, Cladosporium, Nigrospora, Aureobasidium,* and *Bipolaris*, as well as some other fungal species (sterile mycelia), were selected for this study. The fungi were initially cultured on a potato dextrose agar (PDA; HI Media, India) for purification. Chloramphenicol was added to the culture medium to prevent bacterial growth. After 2-5 days, fungal colonies were formed on the medium at 27°C and transferred to another new PDA from hyphal tip. This process was repeatedly performed until having a pure culture of fungal endophytes (Bhardwaj et al., 2014).

Fungal strains

The evaluation of the antifungal properties of the metabolite produced by endophytic fungi was performed using the clinical fungal isolates obtained from the culture collection of fungi from Department of Mycology, Pasteur Institute of Iran (http://fa.pasteur.ac.ir/Pages.aspx?id=1152). These organisms included *C. albicans* (ATCC 10231), *C. krusei* (PFCC 1179), *C. glabrata* (PFCC 772), *Cryptococcus neoformans* (PFCC 589), and *C. tropicalis* (PFCC 1456), as well as the filamentous fungi of *A. flavus* (PFCC 1004), *A. fumigatus* (PFCC 155), *Trichophyton rubrum* (PFCC 867), and *T. mentagrophytes* (PFCC 592).

Preliminary screening for antifungal activity

For the purpose of evaluating antifungal activity, a suspension was prepared from the colonies of *C. albicans* (ATCC 10231), and then cultured on PDA for 48 h at a concentration of 0.5 McFarland (Deshmukh et al., 2018). Subsequently, it was cultured on PDA medium by sterile swabs using streak method. In the next stage, the fungal endophyte was cultured at the center of the medium.

After 48-72 h, the surrounding of the endophytic fungal colony was assessed for the zone of inhibition. Therefore, the anti-*Candida* activity of the fungal endophytes was subjected to preliminary screening. Accordingly, all endophytic fungi were evaluated in terms of anti-*Candida* activity. In addition, endophytic fungus *Aspergillus* (F-12 BSU), which had an acceptable inhibitory effect against *C. albicans* in the preliminary screening method, was used as the endophyte of choice in the following studies (Santiago et al., 2012).

Molecular identification of endophytic fungus (F-12 BSU)

Fungal DNA was extracted following the kit instructions of Sinaclon Company, Iran. Then, they were subjected to polymerase chain reaction (PCR) using two primers, namely ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTGATATGC). The PCR conditions included initial denaturation at 95°C for 10 min, 35 cycles at 95°C for 1 min, at 55°C for 1 min, at 72°C for 2 min, and a final extension at 72°C for 8 min. The qualitative and quantitative assessments of the extracted DNA were accomplished using electrophoresis gel and NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) at the wavelengths of 260 and 280 nm, respectively. The sequencing of the extracted DNA was conducted by Vista Gene Company, and then edited by Finch TV and Bio Edit software. After using the NCBI BLAST service to perform a similarity search on NCBI Genbank, the fungal accession number was obtained (Sharma et al., 2016).

Extraction of fungal metabolites

Endophytic fungus *Aspergillus* (F-12 BSU) was used to prepare the crude extract. To this end, 10^6 colony forming unit/ml of the grown endophytic fungal suspension was added to a liquid medium in four Erlenmeyer flasks (1 L), each containing 200 cc potato dextrose broth, along with dextrin (1.5%), poly peptone (2%), KH_2PO_4 (0.2%), copper sulfate (0.1%), iron sulfate (0.1%), and $FeSo_4\ 7H_2O$ (0.1%) at a pH of 6.5.In this medium, fungal colonies were maintained under a constant condition at 26°C using a shaking mode of 140 rpm for 21 days. In the next stage, the contents of the medium were filtered after centrifugation at 3,000 rpm using Whatman filter grade 1 (Whatman, Maid stone, UK). Subsequently, water, ethanol, methanol, and ethyl acetate were added to the filtered culture. After 24 h, the compound was stirred for 10 min. The culture filtrate was separated after keeping it in a passive state for 5 min using Buchner funnel. The preliminary extract was placed on a rotary evaporator (R110, BUCHI, Switzerland), and the crude extract was obtained at 40°C with a decrease in the pressure. This process was carried out three times to achieve the proper amount of the crude extract. A part of the obtained crude extract was dissolved with dimethyl sulfoxide and maintained at 4°C for next step (Patil et al., 2015; Medina et al., 2005; Madki et al., 2010).

Evaluation of antifungal activity using agar well diffusion method

In order to investigate antifungal activity, 20 ml Sabouraud glucose agar (SGA, Hi Media, Mumbai, India) was added to a Petri dish. After the solidification of the culture medium, 0.5-2.5×10^3 CFU/mL of C. albicans (ATCC 10231) suspension with 0.5 McFarland turbidity was cultured on the medium using a sterile swab. Afterwards, a hole, 6 mm in diameter, was created at the medium by a sterile cork borer, and 100 µl of the crude extract was added to the medium.

The culture medium was then maintained at 25°C for 48-72 h. Subsequently, the zone of inhibition was evaluated using fluconazole as a control. In addition to showing proper anti-Candida activities in the preliminary screening, endophytic fungus Aspergillus (F-12 BSU) demonstrated suitable inhibitory effects against C. albicans in extracting by methanol. Therefore, it was entered into the next stage of the research for determining the minimum inhibitory concentration (MIC) (Premjanu et al., 2016; Magaldi et al., 2004; Kumar et al., 2014).

Evaluation of minimum inhibitory concentration and antifungal activity of methanolic extract using broth microdilution

In order to determine the MIC, microdilution technique was employed using the standard method of the Clinical and Laboratory Standards Institute (CLSI 2008; M38-A2 and M27-A3). At first, 100 µl of the medium (RPMI-640) containing L-glutamine and no bicarbonates supplemented with both 0.3 g of L-glutamine per liter and 0.165 M MOPS buffer (34.54 g/l) was added to the wells of each horizontal row of the special 96-well U-shaped microdilution plate. Afterwards, 100 µl of the crude extract, prepared in dimethyl sulfoxide, was added to the first well in series, followed by adding 100 µl of the extract to the second well, until obtaining a concentration range of 0.05-5 µg/mL of the extract derived from the endophytic fungus. In order to prepare the fungal suspension, the pure culture of *C. albicans* (ATCC 10231), which was grown on PDA medium for 48 h and homogeneous cell suspensions spectrophotometrically measured at the 530 nm and a percent transmission within the range of 75-77% using McFarland 0.5 turbidity standard as a reference.

Therefore, the final densities of the stock inoculum suspensions of the tested isolates had a range of 2.5-5×10^3 CFU/ml. Ultimately, 100 µl of *C. albicans* suspension was added to the wells. Only 200 µl of the medium was added to the well number 11, which was selected as a negative control. In addition, 100 µl of culture and 100 µl of the *C. albicans* suspension were added to the well number 12 as a positive control. After incubation at 35°C in an aseptic condition for 24-48 h, the turbidity created at the bottom of the wells was evaluated using a visual method.

The first well in which growth inhibition was 50% relative to the positive control well was considered as MIC50. On the other hand, the well in which 90% growth inhibition was observed, compared to the positive control well, was regarded as MIC90. Fluconazole, obtained from Sigma-Aldrich Corporation (USA), was used as a drug control in a range of 0.5-256 µg/ml. To determine the susceptibility to filamentous fungi, a fungal suspension cultured on PDA for 7 days was used, and its turbidity was adjusted using sterile normal saline with 0.5 McFarland turbidity. In addition, M38-A2 protocol was applied for filamentous fungi at a concentration of 2×10^4 spore/ml. The test was carried out in duplicate (Leite et al., 2014; Miron et al., 2014).

Results

Preliminary screening for the assessment of anti-Candida property

A total of 24 strains of endophytic fungi obtained from the Botanical Research Center of Bu-Ali Sina University of Hamadan, Iran, were analyzed in this study. Out of the 24 strains, only one strain of fungal endophyte (F-12 BSU) showed inhibitory effects against *C. albicans* and was selected to be used in future studies (Figure 1).

Agar well diffusion

For the evaluation of the culture filterate, we used endophytic fungi (F-12 BSU), along with water, methanol, ethanol, and ethyl acetate. The crude methanolic extract of endophytic fungus *Aspergillus* (F-12 BSU), which had the highest inhibitory activity against *C. albicans*, compared to the other applied solvents, was also utilized in this study (Fig 2).

Molecular identification of endophytic fungus

Molecular evaluation of the PCR results using ITS1 and IT4 sequences showed that the endophytic fungus *Aspergillus* (F-12 BSU) was *A. aculeatus* (Accession No. KY204005.1) (Figs 3, 4).

Antifungal susceptibility determination using broth microdilution method

Table 1 presents the results related to the antifungal susceptibility of *C. albicans* (ATCC 10231), *C. krusei* (PFCC 1179), *C. glabrata* (PFCC 772), *Cryptococcus neoformans* (PFCC 589), and *C. tropicalis* (PFCC 1456), *A. flavus* (PFCC 1004), *A. fumigatus* (PFCC 155), *Trichophyton rubrum* (PFCC 867), and *T. mentagrophytes* (PFCC 592). The evaluation of antifungal susceptibility of *C. albicans* by broth microdilution showed the MIC50 and MIC90 of 0.32 and 0.64 µg/mL, respectively, which were indicative of a favorable inhibitory effect, compared to fluconazole as a standard drug with an MIC50 value of 2 µg/ml.

Furthermore, *C. tropicalis, C. glabrata*, and *C. krusei* had the MIC50 values of 0.01, 0.04, and 0.032 µg/ml, respectively, compared to fluconazole with the MIC50 values of 4, 16, and 16 µg/ml. *A. fumigatus* and *A. flavus* had the MIC50 values of 0.32 and 0.08 µg/ml, respectively, while fluconazole had the MIC50 values of 128 and 64 µg/ml, respectively, against these species. Additionally, *Cryptococcus neoformans, T. rubrum,* and *T. mentagrophytes* with the MIC50 value of more than 5 µg/ml had the highest MICs against the crude extract of endophytic fungus, compared to fluconazole with the MIC50 values of 8, 4, and 8 µg/ml, respectively.

Discussion

The polymorphic fungus *C. albicans* may exist in the digestive and urogenital systems of a healthy human body as a commensal organism. Any changes in the immunological balance of the body or any impairments in the immune system will facilitate the onset of the disease by this fungus. *Candida albicans* is the most important fungal organism in nosocomial infections and accounts for 90% of fungal infections in humans. Moreover, this species is a pathogenic agent accounting for 70-90% of vulvovaginitis cases, which can cause a range of infections from superficial to systemic ones in humans (Zida et al., 2017; Kumar et al., 2014; Kumar et al., 2012).

In a review study investigating the natural resources against *C. albicans* within a period of 46 years (i.e., from 1969 to 2015), 142 anti-*Candida albicans* compounds were indicated in 111 research papers. In this regard, 63 (44.37%) reports were from Asia, whereas 40 (28.17%), 29 (20.42%), 9 (6.34%), and 1 (0.7%) cases were reported in the United States, Africa, Europe, and Oceania continents, respectively. The results of the mentioned study are indicative of the existence of potential anti-*Candida* sources in Asia (Zida et al., 2017). The increased incidence of *C. albicans* infection and the unexpected enhancement of resistance to antifungal drugs underscore the need for performing research on the sources for producing antifungal drugs (Mishra et al., 2017; Khan etal., 2013). Introduction of a clinical drug requires the implementation of costly and complicated methods, sometimes taking a long time of about 12 years, thereby significantly delaying the opportunity to introduce a new drug. Endophytic fungi are among the most important natural resources serving this end. The first and perhaps the most important step in introducing a drug is the investigation of its lack of cellular cytotoxicity (Torey et al., 2016; Kumar et al., 2014).

New antifungal drugs should have a wide range of antifungal properties, as well as high specificity and low toxicity without any antagonistic effects against other commercial drugs. Although the achievement of this goal seems difficult, it should be regarded as a general principle in designing and constructing antifungal drugs (Bondaryk et al., 2017). In the present study, a crude extract was derived from the culture filtrate of endophytic fungus *A. aculeatus* (F-12 BSU) extracted by methanol solvent, which had a higher anti-*Candida* activity, compared to other solvents. The results of antifungal susceptibility testing by broth microdilution showed that the compound had the MIC50 and MIC90 values of 0.32 and 0.64 µg/mL, respectively, against *C. albicans* (ATCC 10231). Accordingly, this compound showed a suitable inhibitory effect, compared to fluconazole as a standard drug (MIC50: 2 µg/ml). Furthermore, it showed proper inhibitory activities against *C. tropicalis, C. krusei*, and *C. glabrata* with the MIC50 values of 0.01, 0.04, and 0.32 µg/ml, respectively (Table 1). However, *Cryptococcus neoformans, T. rubrum,* and *T. mentagrophytes* dermatophytes had the highest MICs against the endophytic fungus extract.

In a study investigating the production of metabolites from endophytic fungi, the fungi from Ascomycota family had a more acceptable superiority in the production of anti-*Candida* metabolites, compared to those of basidiomycetes family (Weber et al., 2007). According to the antimicrobial peptide database, 756 antifungal peptides have been extracted from various organisms, including humans, animals, birds, reptiles, amphibians, insects, and microbes. These antifungal peptides cause fungal cell destruction in various ways, such as pore formation in lipid membranes and prevention of cell wall synthesis (Zhao et val., 2013).

In a study, cerulenin was obtained from endophytic fungus *Phomompis*, which is an inhibitory metabolite against polyketide and fatty acids and inhibits the growth of *C. albicans*. On the other hand, ascosterosides A and B were obtained from the endophytic fungus *Ascotricha amphitricha*, which has an inhibitory effect against 1,3-beta-glucan in *C. albicans*. Isofusidienol was produced by *Chalara* species, which is an endophytic fungus isolated from *Artemisia vulgaris* exhibiting antifungal activities against *C. albicans*.

Helovic acid, Leucinostatin A, and Phomol obtained from endophytic fungi *Aspergillus*, *Acremonium*, and *Phomopsis* species, respectively, have anti-fungal properties. The arundifungin metabolite, obtained from the endophytic fungus *Arthrinium arundis*, has

inhibitory activities against 1,3-beta-glucan in *C. albicans* (Bondaryk et al., 2017). In a study, more than 30% of the metabolites were isolated from *Aspergillus* and *Penicillium* species, and the most commonly used liquid medium for the extraction of secondary metabolite was PDB. Furthermore, in the mentioned study, the commonly used organic solvents were ethyl acetate and methanol (38). Another example of antimicrobial agent production from fungal endophytes is the isolation of anti-*Helicobacter pylori* substances from the endophytic fungus. *Aspergillus* species metabolite can inhibit *Helicobacter pylori*, which is an etiological agent of chronic active gastritis and a significant determinant in peptic and duodenal ulcer diseases gastritis (Zhao et val., 2013). Cryptocandin derived from endophytic fungi *Cryptosporiopsis quercina* has antifungal properties against *C. albicans* and *Histoplasma capsulatum* (i.e., a causative agent of histoplasmosis), and also inhibitory effects against *T. rubrum* and *T. mentagrophytes*. This metabolite inhibits 1,3-beta-glucan synthase enzyme and is considered as one of the most important anti-*Candida* drugs(Kaul et al., 2012).

Sordaricin is an antifungal metabolite isolated from endophytic fungus *Xylaria* species. This metabolite has an inhibitory activity against *C. albicans* (18). Phenolic compounds are obtained from endophytic fungus *Pestalotiopsis mangiferae*, which has a strong inhibitory power against *C. albicans* with a MIC value of 0.039. In addition, calvasterol A and ganodermaside D compounds are derived from *Phomopsis*, which has an inhibitory effect against *C. albicans*.

The entophytic fungus *Guignardia* has guignardone N and guignardic acid compounds, showing inhibitory activities against *C. albicans* and synergistic properties with fluconazole. In addition, phialomustin and altenusin are extracted from endophytic fungi *Phialophora mustea* and *Alternaria alternata*, respectively, and have inhibitory effects against *C. albicans* (Gouda et al., 2016; Deshmukh et al., 2018). In the final phase of fungal growth, fungi produce various products that are not essential for their growth. These products are called secondary metabolites that have different functions and activities (39). *Aspergillus* has 339 species, including pathogenic species for humans and animals (e.g., *A. fumigatus*, *A. terreus*) and species (e.g., *A. flavus* and *A. ochraceous*) that play a role in the production of toxics, such as aflatoxin.

Secondary metabolites are including polyketides, nonribosomal peptides, ribosomal peptides, terpenes, and alkaloids that are synthesized by their specific enzymes, and primary metabolites include enzymes, organic acids, and proteins. Bioinformatics analysis has predicted the presence of two highly conserved genes, namely polyketide synthases (PKS) and nonribosomal peptide synthases (NRPS), in the genome of endophytic fungi, which are involved in the biosynthesis of various secondary metabolites. Polyketides are a group of structurally diverse compounds used in the production of human and veterinary medicines, including antibiotics (e.g., erythromycin and tetracycline), antiparasitic compounds (e.g., avermicin), and antitumor compounds (e.g., daunorubicin) (Sharma et al., 2016). Polyketides are among the most abundant secondary metabolites secreted by PKS. These organic materials have complex chemical compounds used in pharmaceutical industries. Drugs, such as doxycycline, erythromycin, and clarithromycin, are categorized in this group of medications. In this respect, non-ribosomal peptides are also among important genetic groups that secrete important metabolites, including penicillin, cephalosporin, and vancomycin.

Aspergillus species are among the most significant fungal species that can produce cyclic peptides, most of which are created by non-ribosomal peptides. Cyclic peptides are the most important secondary metabolites that are synthesized by plants, bacteria, and fungi. However, fungi are the most well-known organisms secreting these cyclic peptides with amazing biological properties that can synthesize a wide range of peptide chain from dipeptide to decapeptide. Among the cyclic peptides, one can refer to echinocandins and pneumocandin that have anti-*Candida* properties, and cyclosporine, which has immunosuppressive properties. Mulundocandin and deoxymulundocandin are derived from *A. sydowii* and *A. mulundensis*, respectively, and have inhibitory activities against yeasts and filamentous fungi.

Furthermore, aculeacin A is obtained from *A. acleatus*, which has strong anti-*Candida* activities with 1,3-beta-glucan synthase inhibitor. Pneumocandin is isolated from *Zalerion arboricola*, which has an anti-*Candida* effect with 1,3-beta-glucan synthase inhibitor. In addition, sclerotides A and B are derived from *A. sclerotiorum*, which has inhibitory power against *C. albicans*. Meanwhile, sclerotide B has cytotoxic and antimicrobial properties, in addition to its anti-*Candida* activities. Caspofungin acetate is a semi-synthetic derivative and a cyclic lipopetide from pneumocandin B extracted from *Glarea lozoyensis*. Micafungin and echinocandin inhibit fungal growth through affecting the fungal cell wall. It is noteworthy that many of these metabolites have been isolated from endophytic fungi. Cyclic peptides are produced with a weight of 2-3 kDa and have antifungal properties. Aculeacin, derived from *A. aculeatus*, and echinocandine are among these metabolites, showing antifungal activities against *C. albicans* by inhibiting 1,3-beta-glucan (Vicente et al., 2003).

Based on several scientific studies investigating the function of metabolites with antifungal activities, when an extract has a MIC value of less than 100 mg/mL, it is considered an effective or significant metabolite. In addition, if the MIC value of the crude extract is 100-625 mg/mL, it is categorized in the moderate group. In this review study, it was shown that out of 142 antifungal metabolites obtained from natural sources, 40 (28.20%) and 24 (16.90%) cases had effective and moderate activities against *C. albicans*, respectively. Therefore, the metabolite obtained in the present research was significantly effective against *C. albicans* (Zida etal., 2017).

Conclusion

According to the results of the present study, the secondary metabolites produced by *A. aculeatus* (F-12 BSU) had a suitable inhibitory effect against *C. albicans* and other non-*albicans* species, compared to fluconazole, which is a standard antifungal drug. Consequently, this compound can be a good candidate for drug preparation against *C. albicans* and other pathogenic fungi after performing further research regarding its purification and standardization.

Conflicts of Interests

The authors report no conflicts of interests.

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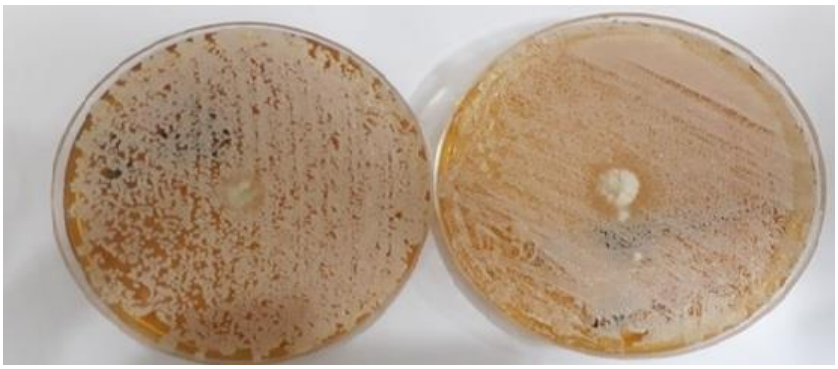

Fig 1. Preliminary screening for antifungal producing endophytic fungi.

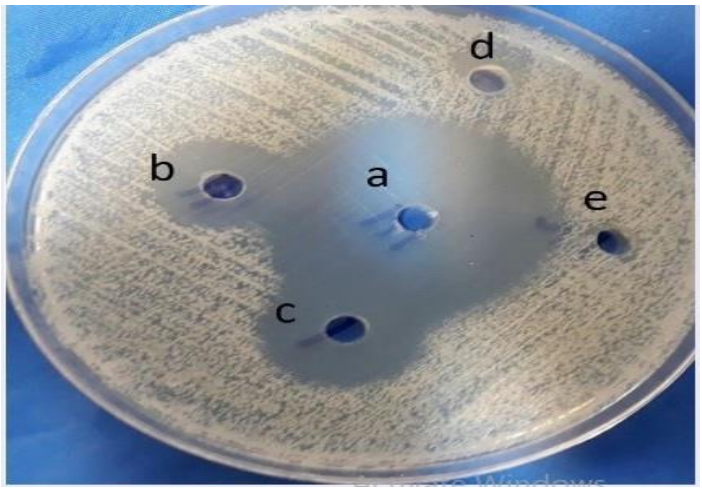

Fig 2. Comparison of the culture solvent extract of Aspergillus aculeatus against Candida albicans ATCC 10231. (a) methanolic extract; (b) ethanolic extract; (c) ethyl acetate extract; (d) water extract; (e) MeOH control.

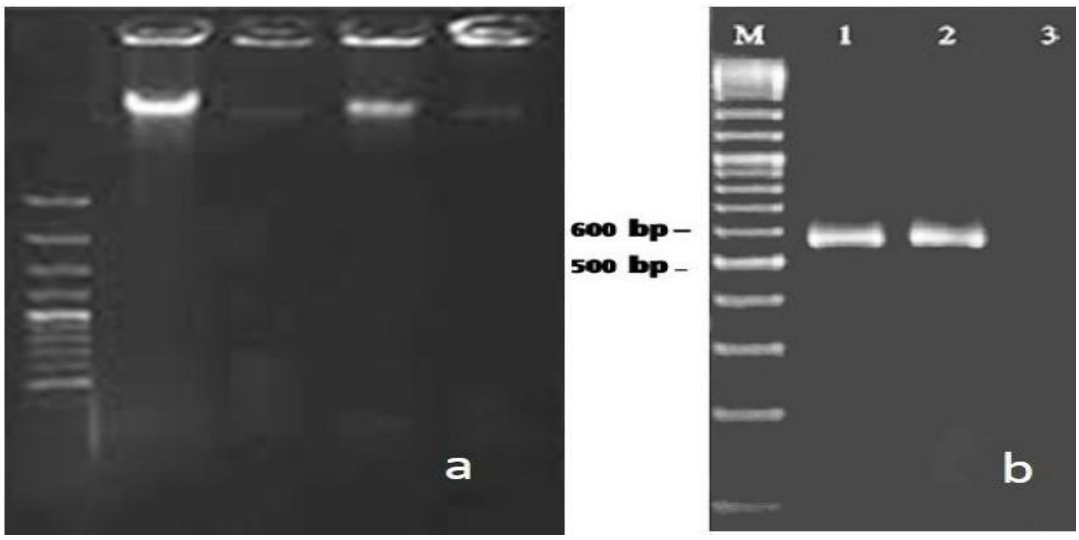

Fig 3. (a) DNA extraction electrophoresis on 1% agarose, (b) ITS- PCR product (500-600bp).

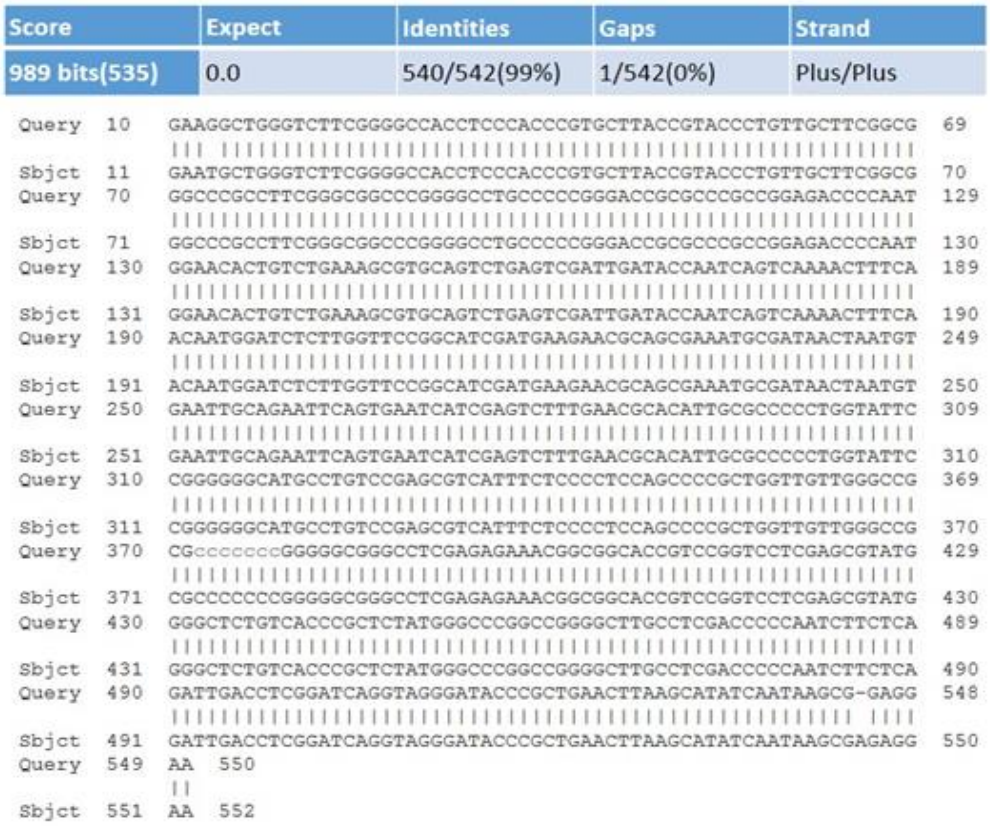

Fig 4. Aspergillus aculeatus isolate (F-12 BSU) ITS sequence.

Table 1. Antifungal susceptibility of the methanolic extract of *Aspergillus aculeatus* against some pathogenic fungi

Fungal species	Methanolic extract MIC (µg/ml)			Fluconazole MIC (µg/ml)		
	Range	50	90	Range	50	90
Candida albicans	0.005-5	0.32	0.64	0.5-256	2	4
C. tropicalis	0.005-5	0.01	0.04	0.5-256	4	8
C. krusei	0.005-5	0.04	0.16	0.5-256	16	32
C. glabrata	0.005-5	0.32	0.64	0.5-256	16	32
Cryptococcus neoformans	0.005-5	>5	>5	0.5-256	8	16
Aspergillus fumigatus	0.005-5	0.32	1.28	0.5-256	128	256
A. flavus	0.005-5	0.08	0.32	0.5-256	64	128
Trichphyton rubrum	0.005-5	>5	>5	0.5-256	4	64
T. mentagrophytes	0.005-5	>5	>5	0.5-256	8	64