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Antifungal Activity of *Trichoderma orientale* FMR 12486 Crude Extract against Some Human Pathogenic Fungi

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Abstract

This work aimed to investigate the prevalence of pathogenic fungi and evaluate the antifungal activity of *Trichoderma orientale* FMR12486 crude extract against pathogenic fungi isolated from patients attending the National Center for Thoracic and Respiratory Diseases (having a history of tuberculosis) and consultant of Dermatology of Baghdad hospital, Iraq. A total of 80 clinical specimens were collected: 20 skin scrapings specimens and 60 sputum specimens. The results of direct examination by KOH 10% and culture showed that 11 (55%) cases from 20 skin specimens were positive for fungal infections, while in the sputum specimens, 28 (47%) cases from 60 were positive. *Candida albicans* represented the most common fungal infection isolated from sputum specimens which represented 18 cases at a rate of 64.2%, followed by *Candida tropicalis* 5 (17.9%), *Aspergillus fumigates* 2 (7.1%), *Aspergillus flavus* 1 (3.6%), *Candida glabrata* 1 (3.6%) and *Candida krusei* 1 (3.6%). While in the skin scrapes, *Candida parapsilosis* was the common which represented 3 cases at a rate of 27.3%, followed by *Trichophyton mentagrophyte* 2 (18.2%), *Microsporium canis* 2 (18.2%), *C. albicans* 2 (18.2%), *Trichophyton rubrum* 1 (9.1%), *C. tropicalis* 1 (9.1%). *T. orientale* isolates were collected from the gardens soil of the University of Baghdad and were then identified depending on the morphological feature of the colony and microscopic characteristics. To confirm identification, PCR technique was used in which DNA of *T. orientale* was extracted at a concentration of 54 - 294 $\mu\text{g}/2\text{ g}$ wet mycelium with a purity of 1.6 - 1.8. DNA samples were amplified with primers *ITS-1* and *ITS-4*, And the results confirmed that the isolate was *T. orientale* strain FMR12486. This isolate was processed with ethyl acetate using a standard extraction method and the final amount of crude extract being 6 grams. Different concentrations of crude extract 0.5, 1, 2 and 4 mg/ml were prepared and used against fungal pathogens isolated using the agar-well diffusion method. The results showed high antifungal activity against pathogenic isolates, significantly recording maximum inhibition zones of 20 and 22 mm against *C. parapsilosis* at concentrations of 0.5 and 1 mg/ml, respectively. It was followed by *T. mentagrophyte* and *C. glabrata*. 16, 20, and 16, 20 mm that were recorded at concentrations of 2 and 4mg/ml, a significant maximum inhibition zone was recorded 26 mm against *C. glabrata*, followed by *C. parapsilosis*, *M. canis* and *T. mentagrophyte* (24, 26; 16, 24 and 20, 24mm respectively). Just *Trichophyton rubrum* fungus in this study showed resistance to all concentrations of the crude extract where the inhibition zone was zero. The results of the current study showed that *T. orientale* strain FMR12486 crude extract can be used to make an effective drug to treat human fungal infections.

Keywords: *Dermatophytes*, *Aspergillus* spp., *Candida* spp., *Trichoderma orientale*, PCR - *ITS* region.

الفعالية الحيوية لمستخلص الفطر *Trichoderma orientale* FMR 12486 الخام ضد بعض انواع الفطريات الممرضة للإنسان

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الخلاصة:

يهدف هذا العمل إلى التحري عن انتشار الفطريات المسببة للأمراض وتقييم الفعالية المضادة للفطريات للمستخلص الخام لفطر *Trichoderma orientale* FMR12486 ضد الفطريات المسببة للأمراض والمعزولة من المرضى الذين ارتادوا المركز الوطني للأمراض الجلدية في مستشفى بغداد، العراق. تم جمع 80 عينة سريرية؛ 20 عينة من الجلد و 60 عينة من البلغم. أظهرت نتائج الفحص المباشر (بواسطة 10% KOH) ونتائج الزرع بأن 11 (55%) حالة من أصل 20 عينة جلدية كانت إيجابية للعدوى الفطرية ، بينما في عينات البلغم كانت 28 (47%) حالة من أصل 60 حالة إيجابية. حيث كانت *Candida albicans* المعزولة من عينات البلغم أكثر أنواع العدوى الفطرية شيوعاً إذ بلغت 18 حالة بنسبة (64.2%) تليها 5 *Candida tropicalis* (17.9%) ، 2 *Aspergillus fumigates* (7.1%) ، 1 *Aspergillus flavus* (3.6%) ، 1 *Candida glabrata* (3.6%) و 1 *Candida krusei* (3.6%)، بينما في عينات الجلد كانت *Candida parapsilosis* هي الأكثر شيوعاً حيث بلغت 3 حالات بنسبة (27.3%) تليها 2 *Trichophyton mentagrophyte* (18.2%) ، 2 *Microsporium canis* (9.1%) ، 1 *Trichophyton rubrum* (9.1%) و 1 *C. tropicalis* (9.1%). تم جمع عزلة *T. orientale* من تربة حدائق جامعة بغداد وشخصت اعتماداً على السمات المظهرية للمستعمرة والخصائص المجهريّة. ولتأكيد التشخيص، تم استخدام تقنية PCR فقد تم استخلاص الحمض النووي بتركيز نهائي (54-294) ميكروغرام/2غم من الغزل الفطري الرطب وبنقاوة (1.6-1.8). تم تضخيم عينة الحمض النووي مع كل من البوادئ (*ITS-4&ITS-1*). أكدت نتائج PCR أن العزلة كانت *T. orientale* strain FMR12486. تمت معاملة العزلة بأسيتات الإيثيل وباستخدام طريقة الاستخلاص القياسية وكانت الكمية النهائية للمستخلص الخام 6 غم. تم تحضير تراكيز مختلفة (0.5 ، 1 ، 2 و 4 ملغم/مل) من هذا المستخلص الخام واستخدمت ضد الممرضات الفطرية المعزولة وباستخدام طريقة الانتشار بالحفر. أظهرت النتائج فعالية تثبيطية قصوى ضد العزلات الممرضة، حيث سجلت معنوياً منطقة التثبيط القسوى (20 ، 22 ملم) ضد *C. parapsilosis* عند التراكيز (0.5 ، 1 ملغم/مل) على التوالي يليها *T. mentagrophyte* (16 ، 20) و *C. glabrata* (16 ، 20) ملم بينما عند التراكيز (2 و 4 ملغم/مل) سجلت منطقة تثبيط قصوى (26 ملم) ضد الفطر *C. glabrata* يليها *C. parapsilosis* و *T. mentagrophyte* (24 ، 24; 24 ، 20; 24 ، 16) ملم على التوالي. التثبيط صفر. أظهرت نتائج هذه الدراسة أن المستخلص الخام لفطر *Trichophyton rubrum* هو الفطر الوحيد الذي أظهر مقاومة لجميع تراكيز المستخلص الخام حيث كانت منطقة التثبيط صفر. أظهرت نتائج هذه الدراسة أن المستخلص الخام لفطر *T. orientale* FMR12486 يمكن استخدامه لصنع دواء فعال لعلاج الفطريات المسببة للأمراض البشرية.

INTRODUCTION

The incidence of severe fungal infections caused by opportunistic fungi has raised in recent years, especially in immunocompromised patients and endemic mycoses remain a major public health problem in several countries [1]. Dermatophytes as keratinophilic fungi can infect keratinous tissues of skin (the stratum corneum layer), hair and nail in humans via their keratinase enzymes [2]. Tinea is a mycosis caused by dermatophytes. The common manifestations of tinea are Tinea capitis, Tinea pedis, and *Tinea unguium* or onychomycosis [3]. Dermatophyte fungi belong to three genera (*Trichophyton*, *Microsporium*, and *Epidermophyton*) and the global burden

of their infection is estimated to be 20–25% [4]. Candidiasis is a fungal infection caused by any type of genus *Candida* which is the most prominent cause of fungal infections and a very ubiquitous infectious disease [5]. The vital mechanism responsible for candidiasis is the host's organic weakness or lower immunity status [6]. *Candida* is almost found as normal flora on the skin and the mucous membrane lining the gastrointestinal, respiratory, and vaginal tract [7]. *Aspergillus fumigatus* is one of the most dangerous fungi that causes infection in individuals with lower immunity causing aspergillosis which affects the respiratory tract. Hence, the primary site of entry is the host's lungs [8]. Mainly, infection results from the inhalation of airborne spores which are small enough to reach the alveoli of the respiratory system. Most patients suffering from aspergillosis have an impaired immune system that is often evoked by leukemia, neutropenia, tuberculosis or after prolonged treatment with steroids, such as SOT patients. The mortality rate of aspergillosis among these patients lies between 30 to 90% [9]. Fungi constantly develop resistance to synthetic antifungals and most of these antifungals are relatively expensive. While others have negative side effects such as hepatotoxicity. As well as some antifungals may interact with other medicines causing a high risk. For these reasons, researchers are looking for new antifungal agents and the recognition of resistance [10], [11]. *Trichoderma* is well known to be a biocontrol agent. Different mechanisms have been reported as being responsible for their biocontrol activity such as the secretion of chitinolytic enzymes, competition for nutrients and space, mycoparasitism, and production of inhibitory compounds (antibiotics and chemicals)[12] [13]. A member of a large group of secondary metabolites isolated from different *Trichoderma* species has been reported to have antimicrobial activities [14]. Therefore, this study was aimed at determining the prevalence of pathogenic fungi and testing the antifungal activity of a crude extract of the *T. orientale* strain (FMR12486) against fungal pathogens isolated from patients attending the tuberculosis clinic and dermatology consultant at Baghdad Hospital, Iraq.

Materials and Methods

Isolation and Identification of Pathogens

A total of 20 samples were collected from patients infected with dermatitis clinically diagnosed by a dermatologist after attending a dermatological consultant at Baghdad hospital. At the same time, a total of 60 sputum specimens from different patients having a history of tuberculosis, were obtained from the National Center for Thoracic and Respiratory Diseases. Skin scrapings were collected by a scalpel blade from the active margin of the lesion and carried on folded paper. All specimens were diagnosed directly under the microscope. Each specimen was placed on a slide that had been dripped with 10% KOH, heated (30°C) for 5 minutes and was then allowed to cool down. Each sample was covered with a coverslip in order to be examined under a microscope. Specimens were inoculated into Petri dishes containing Sabouraud dextrose agar (OXOID, England) containing 500 mg of cycloheximide (SantaCruz, USA) and incubated at 28±2°C. Cultures were first examined after 7 days, and then every two weeks for approximately 4 weeks. Filamentous fungi were diagnosed depending on the appearance of the colonies on the medium and by examining morphological characteristics under a microscope according to [15]. *Trichophyton mentagrophytes* and *Microsporum canis* have already been diagnosed [16]. The *Candida* API (Biomérieux, France) is used to identify yeasts in 18-24hr where it allows 12 biochemical tests to be performed. The reaction results were read visually depending on the color changes according to the reading table and the identification was obtained by the list of numerical profiles.

Isolation and Identification of *Trichoderma orientale*

Thirty soil samples were collected using sterile metal spoons at a depth of approximately 5 cm from various locations in the University of Baghdad gardens, placed in polyethylene bags, and were transported directly to the laboratory for processing.

Ten grams of each soil sample was placed in a conical flask (volume of 250 ml) containing 100ml of sterilized distilled water, and then shaken on an electrical shaker to have a homogeneous suspension. Serial dilutions were then prepared (10^{-1} , 10^{-2} and 10^{-3}). One ml was added to Petri dishes containing potato dextrose agar (OXOID, UK) from the third dilution and incubated at $26\pm 1^{\circ}\text{C}$ for 5 days. After the growth of fungi on PDA, colonies were sub-cultured on another PDA medium in order to obtain a pure culture. *Trichoderma spp.* was diagnosed morphologically (colony morphology and microscopic examination) according to [17].

Identification of *Trichoderma orientale* using the Molecular Method

In order to identify fungal species, PCR and gene sequencing were used to confirm the diagnosis. DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep™ according to the manufacturer's protocol. DNA samples were amplified using primers ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) (Integrated DNA Technology Company, USA). PCR was performed in a total volume of 25 μl consisting of 1.5 μl DNA, 5 μl Taq PCR PreMix, 1 μl of each primer and distilled water to a complete volume of 25 μl . A thermal cycler (Applied Biosystems GeneAmpPCR System 9700) was used to amplify DNA. The conditions of the thermal cycles were as the following:

1. Initial denaturation (One cycle) for 3 minutes at 94°C .
2. Final denaturation (Thirty-five cycles) for 40 sec at 94°C , annealing for 35 sec at 56°C , and initial extension for 35sec at 72°C .
3. Final extensions (One cycle) for 10 min at 72°C .

All PCR products were separated by agarose gel 1.5% electrophoresis, added with red stain (Intron Korea), and visualized by exposure to UV light 320 nm. The *ITS* gene was sequenced by AB13730XL, Applied Biosystem, Macrogen Company, USA. Homology search was conducted using BLAST program which was available at (NCBI) [18].

Fungal Crude Extract Preparation

Modified Czapek's medium was prepared under sterilized conditions as following: 0.1g/l $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1g/l CaCl_2 , 0.2g/l FeCl_3 , 0.5g/l $(\text{NH}_4)_2\text{SO}_4$, 1.5g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2g/l Na_2HPO_4 , 7g/l KH_2PO_4 , 30g/l Glucose, and 150g/l Sugarcane bagasse. Two-block of 10mm diameter from *Trichoderma orientale* FMR12486 (cultured on PDA for 6 days) was added to the prepared Czapek's broth medium of 500ml in the flask (1L) and incubated in the incubator shaker for 14 days at $27\pm 2^{\circ}\text{C}$ in the dark. Next 500 ml of ethyl acetate (EtOAc) (HIMEDIA, India) was added to the medium and shaken at 121 rpm for 24 hours overnight to extract the antifungal compound. After completion of the extraction process, we concentrated the crude extract at $37\pm 2^{\circ}\text{C}$ using a rotary evaporator (Galenhamp, UK) [19].

Preparation of Inoculums

To prepare inoculums of filamentous fungi, serial dilutions of pathogenic fungi suspensions were prepared. The fresh fungi colonies (grown on SDA) were flooded with 1ml of sterilized (0.9% saline), and a transfer pipette tip was moved gently along the colonies. The suspension (conidia and hyphae) was withdrawn and transferred to a sterile tube by a sterilized pipette. Heavy particles were allowed to settle for 5min and then the upper homogeneous suspension was transferred to another sterile tube, covered with a cap tightly, and vortexed for 15sec. To prepare 10^{-1} dilution, only 1ml of this suspension was added to 9ml of sterilized 0.9% saline (Pioneer,

Iraq). NCCLS M27-A2 method was used to prepare and standardize the inoculum density for yeasts. To prepare inoculums, yeasts were sub-cultured from sterile vials onto SDA then passaged (to ensure their purity and viability) and incubated at $37\pm 2^{\circ}\text{C}$. Five colonies of approximately 1mm diameter, were selected from 24 hr cultures of *Candida* spp. and then suspended in 5ml of sterile 0.145-mol/L saline (8.5 g/L NaCl; 0.85% saline). This suspension was vortexed for 15sec. Cell density was adjusted by spectrophotometer (adding enough sterile saline to increase transmission) to that produced by a 0.5 McFarland standard at 530 nm W.L. A stock suspension produced by this method contains approximately 1×10^6 to 5×10^6 cells/ml. McFarland standard was prepared according to Alexander *et al.* [20].

Agar Well Diffusion Assay

Trichoderma orientale crude extract was diluted with 100% dimethyl sulfoxide (Sigma-Aldrich, Germany) to obtain different concentrations of 0.5, 1, 2 and 4 mg/ml. One ml suspensions of 10^{-1} and 10^{-3} dilutions of pathogenic filamentous fungi were mixed homogeneously with SDA medium using the pour plate method on a Petri dish (6cm). Other fungal yeast suspensions were spread over the SDA surface (in 14 cm Petri dishes) by using a sterile cotton swab. Then a well of 5mm was made in the medium by using a sterile Cork-borer. 100 μl of each concentration of the *T. orientale* crude extract was transferred into separate wells. Dimethyl sulfoxide was used as a negative control. Before reading the results, plates were incubated at $37\pm 2^{\circ}\text{C}$ for 1 day in *Aspergillus* spp. and *Candida* spp. *Trichophyton* spp. And *Microsporium canis* were incubated at $28\pm 2^{\circ}\text{C}$ for 4 and 6 days respectively. The diameters of the inhibition zone were recorded and then the average diameter was calculated. This assay was achieved with three replicates.

Statistical Analysis

The Statistical Analysis System-SAS (2012) program was used to study the effects of different concentrations on study parameters. The least significant difference - LSD test was used to significantly compare means in this study.

Results and Discussion

Prevalence of Pathogenic Fungi

A total of 80 clinical samples were cultured, of which thirty-nine (39) samples were positive for fungal growth giving a prevalence rate of (48.8%). The number of collected samples consisted of 60 (75%) sputum and 20 (25%) skin scrape samples (Table 1). Most patients suffering from fungal infection had an impaired immune system that is often evoked by some diseases such as tuberculosis. Among sixty patients, twenty-eight (28) sputum samples showed positive results for fungal infections after culturing with a prevalence rate of (47%) (Table 1). The results of this study were close to the results obtained from the study of Yadu *et al.* [21] where he found the prevalence rate of 49% in same study in India. Also, the prevalence reported by Mwaura *et al.* [22] was 44.18% who studied fungal infections from sputum samples among pulmonary tuberculosis patients in Kenya. The reasons for increased prevalence meant the lowering of the immune system due to tuberculosis, and the prolonged use of anti-tuberculosis drugs which promote the growth and reproduction of the fungus flora, and in turn, aggravate the course of the underlying process in the lung tissues [23]. On the other hand, eleven skin scrapes samples (among twenty cases) showed positive results for fungal infections by both direct KOH examination and culture with a prevalence rate of 55% (Table 1). This was in relative agreement with Najem *et al.* [24] who found that direct KOH examination and cultures were positive in 52.2%. Also, it was lower than the prevalence of 78.7% as reported by Al-Hmadani *et al.*[25] in Al-Najaf government, Iraq.

Table 1: Prevalence of human pathogenic fungi among clinical samples

Type of Sample	Number of Samples	Number Negative	Number Positive	Prevalence Rate (%)
Sputum	60	32	28	47%
Skin	20	9	11	55%
Total	80	41	39	48.8%

Among the 28 positive sputum cultures, there were *Aspergillus fumigatus* (2 isolates), *Aspergillus flavus* (1 isolate), (*Candida albicans* (18 isolates), *Candida tropicalis* (5 isolates), *Candida glabrata* (1 isolate) and *Candida krusei* (1 isolate). All *Candida* spp. were diagnosed according to API Candida kit) (Table-2) (Figures 1, 2, 4). Other 11 positive cultures of skin infections were *Trichophyton mentagrophyte* (2 isolates), *Trichophyton rubrum* (1 isolate), *Microsporium canis* (2 isolates), *C. albicans* (2 isolates), *C. tropicalis* (1 isolate) and *Candida parapsilosis* (3 isolates) (Table 3) (Figures 3, 4). *A. fumigatus* was isolated in 2 (7.1%) cases which is lower than 16.5% and 11.5% as reported by Ali and Almothaffar [26] in India and Al-Charrakh *et al.* [27] in Iraq among pulmonary complaints patients. The prevalence of *A. flavus* was 3.6% which was close to 1.2% reported by Mwaura *et al.* [22] in Kenya. While Hmood [28] reported that in Iraq the prevalence of *A. flavus* isolation from pulmonary patients was 33.3%. The association of *Candida* and *Mycobacterium tuberculosis* patients has increased the concerns for studying various *Candida* species and their significance in pulmonary tuberculosis patients during recent years. Also *C. albicans* was the common fungi isolated from sputum samples (64.2%) which correlated with the study of Babita and Prabhat [29] where they isolated *C. albicans* in 44.4% cases among pulmonary tuberculosis patients. And three types of dermatophytes (*T. rubrum*, *T. mentagrophyte* and *M. canis*) were obtained from skin scrape samples. *M. canis* and *T. mentagrophyte* (zoophilic fungi that cause ringworm infections) were isolated with a percentage of 18.2% from patients who had tinea corporis and a history of direct or indirect contact with animals. This result was consistent with Greene [30] and Al-Hmadani *et al.* [25] who reported that *M. canis* is a major infectious agent in dermatophytosis. The results were also consistent with Ali [31] and Sowmya *et al.* [32] who showed that *T. mentagrophytes* was the most frequent fungus from isolated clinical samples. On the other hand, the present study disagreed with Samia *et al.* [33] and Saleh [34] who showed that *T. rubrum* was the most frequent isolate. *T. rubrum* (Anthropophilic fungus) was isolated in the present study with a percentage of 9.1%. The infection may have spread due to overpopulation which led to contact between infected and healthy persons who facilitated the transmission and spread of the infection, as well as via sharing clothes, towels and combs in large families and through the use of unsterilized shaving tools. At another hand, *C. parapsilosis* was the most common fungus isolated from skin scrapes. This result agreed with Bonassoli *et al.* [35] who mentioned that *C. parapsilosis* was the most frequently isolated species. Patients who used topical antifungal treatment before taking the sample showed negative results for cultures.

Table 2: Distribution of fungal species among sputum specimens

Fungal Isolates	Sputum Isolates (N)	Total (N%)
<i>Aspergillus fumigatus</i>	2	(7.1%)
<i>Aspergillus flavus</i>	1	(3.6%)
<i>Candida albicans</i>	18	(64.2%)
<i>Candida tropicalis</i>	5	(17.9%)
<i>Candida glabrata</i>	1	(3.6%)
<i>Candida krusei</i>	1	(3.6%)
Total	28	(100%)

N: Number of isolates; (%): Percentage

Table 3: Distribution of fungal species among skin scrapes specimens.

Fungal Isolates	Skin Scrapes (N)	Total (N%)
<i>Trichophyton mentagrophyte</i>	2	(18.2%)
<i>Trichophyton rubrum</i>	1	(9.1%)
<i>Microsporium canis</i>	2	(18.2)
<i>Candida albicans</i>	2	(18.2)
<i>Candida tropicalis</i>	1	(9.1)
<i>Candida parapsilosis</i>	3	(27.3)
Total	11	(100%)

N: Number of isolates; (%): Percentage

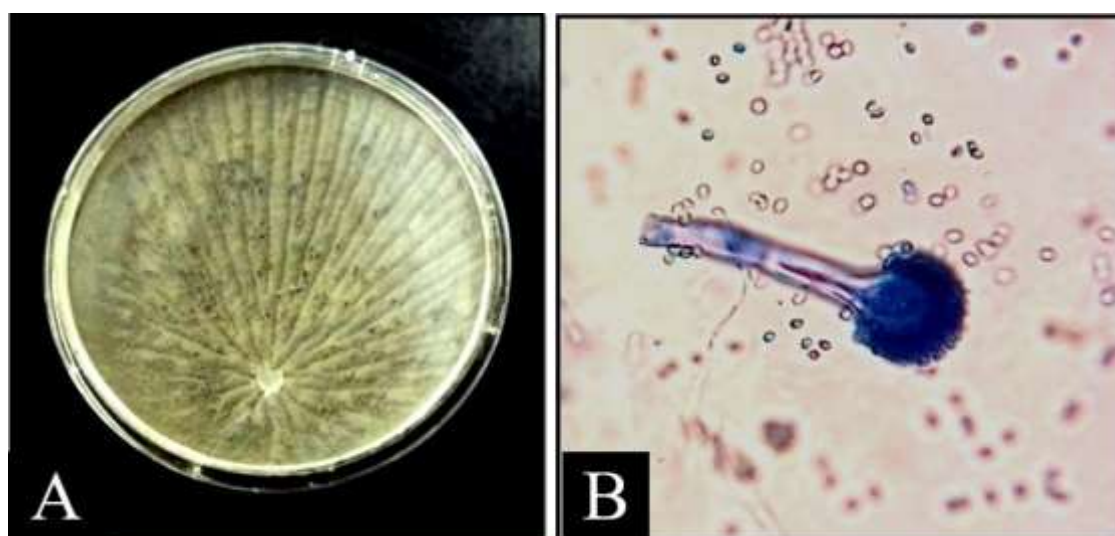


Figure 1: (A): *Aspergillus fumigatus* grown on SDA medium at $35\pm 2^{\circ}\text{C}$ for 5 days of incubation, (B): Microscopic features of *Aspergillus fumigatus* at 40X.

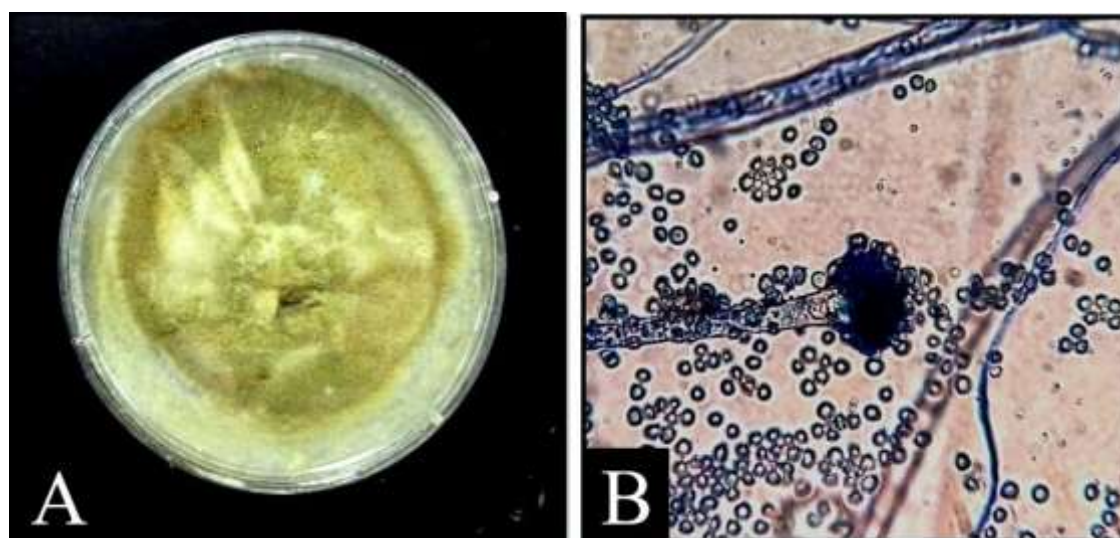


Figure 2: (A): *Aspergillus flavus* grown on SDA medium at $35\pm 2^{\circ}\text{C}$ for 5 days of incubation, (B): Microscopic features of *Aspergillus flavus* at 40X.

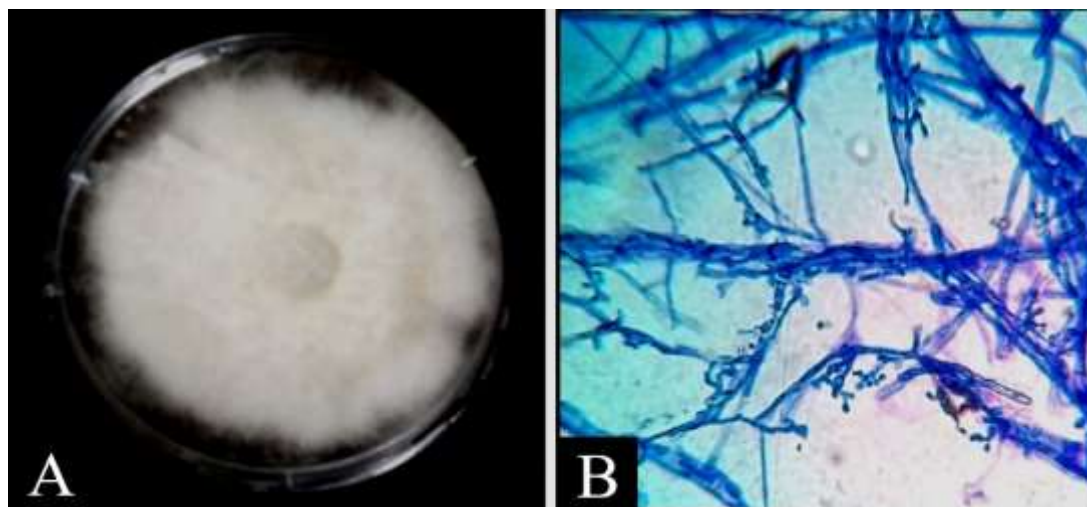
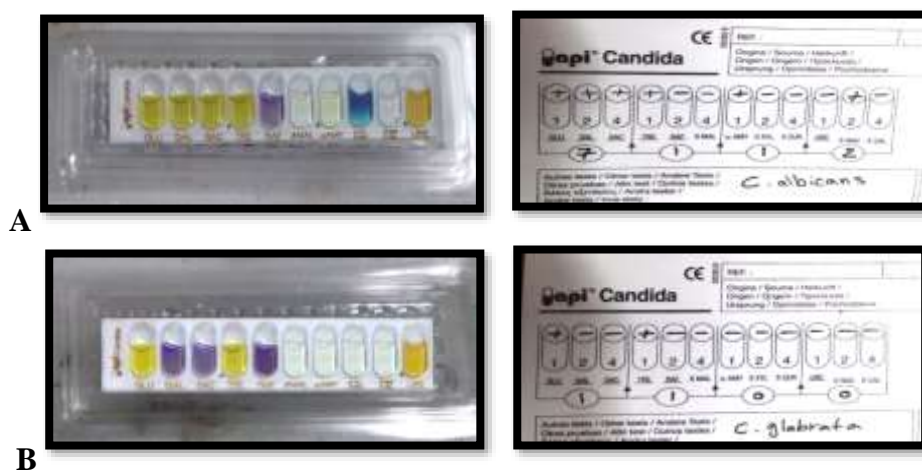


Figure 3: (A): *Trichophyton rubrum* grown on SDA medium at $28\pm 2^{\circ}\text{C}$ for 10 days of incubation, (B): Microscopic features of *Trichophyton rubrum* at 40X.

Identification of *Trichoderma orientale*

In this study, five samples from thirty soil samples showed a positive growth for *Trichoderma* species. One of them was detected as positive for *Trichoderma orientale* (Figure 5). This fungus was identified depending on the morphological feature of the colony and the microscopic characteristics. It was necessary to confirm its species through molecular methods. DNA of *T. orientale* was extracted at a concentration of 54 - 294 $\mu\text{g}/2\text{g}$ of wet mycelium with 1.6-1.8 purity that was measured by nanodrop (the purity of DNA was confirmed by agarose gel electrophoresis). The DNA sample was amplified with primers (ITS-1 & ITS-4) and the isolates yielded a unique product size of approximately ~565bp. PCR results confirmed that the isolate was *T. orientale* strain FMR12486 which matched with a universal isolate recorded on the BLAST program and showed 100% identification with the presence of one genetic polymorphism (transversion) at site 417 (G>N) (Figure 7).



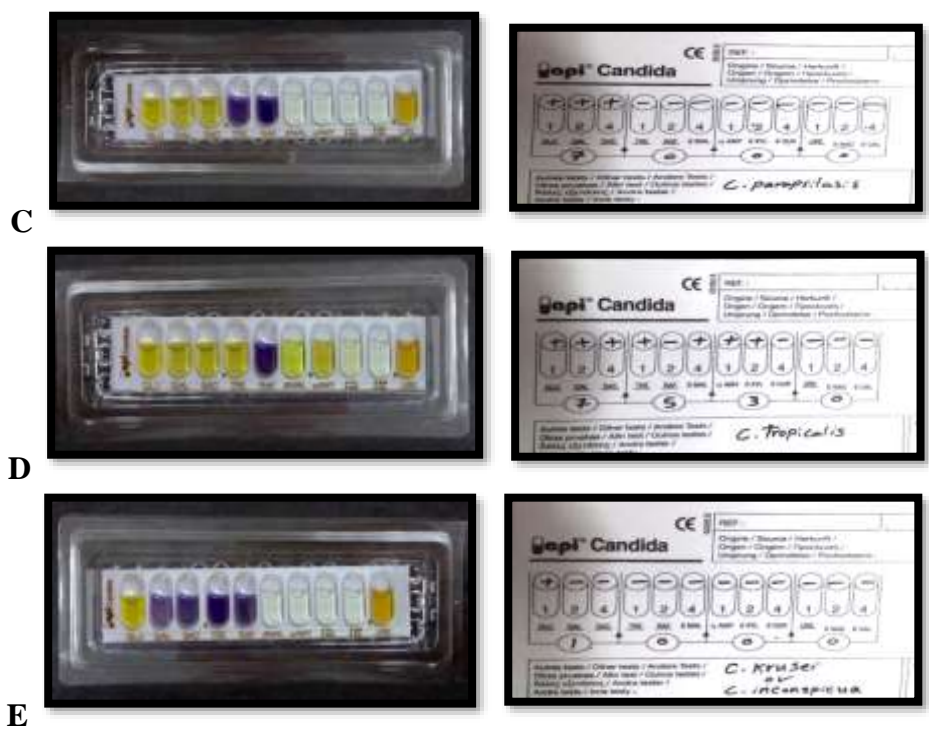


Figure 4: Identification of *Candida* species by Api Candida Kit (A) *Candida albicans* (B) *Candida glabrata* (C) *Candida parapsilosis* (D) *Candida tropicalis* (E) *Candida krusei*, incubation period 24 hr at 36°C ± 2°C.

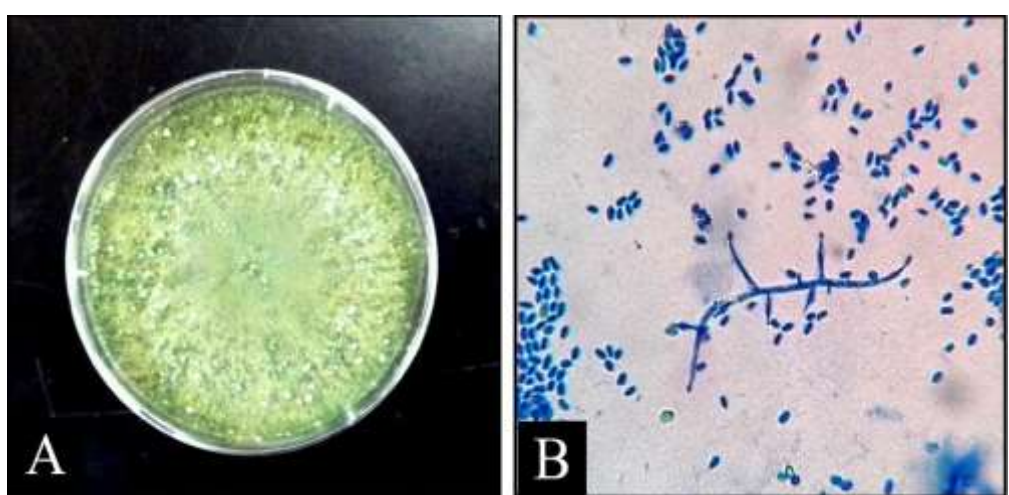


Figure 5: (A): *Trichoderma orientale* grown on PDA medium at 25±2°C after 7 days of incubation, (B): Microscopic features of *Trichoderma orientale* at 40X.

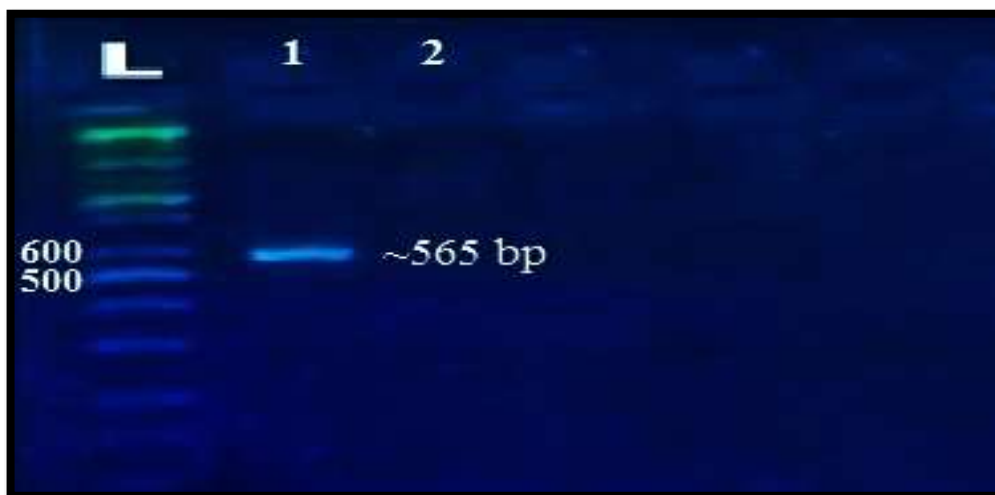


Figure 6: Agarose gel electrophoresis of PCR products showing band size ~565 bp. Products were electrophoresed at 2% L: DNA ladder (100), lane (1) *Trichoderma* isolate, lane (2) negative control sample.

Score	Expect	Identities	Gaps	Strand
957 bits (518)	0.0	519/520(99%)	0/520(0%)	Plus/Plus
Query 1	AACCCCAATGTGAACGTTACCAATCTGTTGCCTCGGCGGGATTCTCTTGCCCCGGGCGCG	60		
Sbjct 22	AACCCCAATGTGAACGTTACCAATCTGTTGCCTCGGCGGGATTCTCTTGCCCCGGGCGCG	81		
Query 61	TCGCAGCCCCGGATCCCATGGCGCCCGCGGAGGACCAACTCCAAACTCTTTTCTCTCTC	120		
Sbjct 82	TCGCAGCCCCGGATCCCATGGCGCCCGCGGAGGACCAACTCCAAACTCTTTTCTCTCTC	141		
Query 121	CGTCGCGGCTCCCGTCGCGGCTCTGTTTTATTTTGGCTCTGAGCCTTCTCGGCGACCT	180		
Sbjct 142	CGTCGCGGCTCCCGTCGCGGCTCTGTTTTATTTTGGCTCTGAGCCTTCTCGGCGACCT	201		
Query 181	AGCGGGCGTCTCGAAAATGAATCAAACCTTCAACAACGGATCTCTTGGTTCTGGCATCG	240		
Sbjct 202	AGCGGGCGTCTCGAAAATGAATCAAACCTTCAACAACGGATCTCTTGGTTCTGGCATCG	261		
Query 241	ATGAAGAACGCAGCGAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGA	300		
Sbjct 262	ATGAAGAACGCAGCGAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGA	321		
Query 301	ATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCAT	360		
Sbjct 322	ATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCAT	381		
Query 361	TTCAACCCTCGAACCCCTCCGGGGGTCGGCGTTGGGGGATCGGCCCTCACCGGGCCGC	420		
Sbjct 382	TTCAACCCTCGAACCCCTCCGGGGGTCGGCGTTGGGGGATCGGCCCTCACCGGGCCGC	441		
Query 421	CCCCGAAATACAGTGGCGGTCTCGCCGACGCTCTCATGCGCAGTAGTTTGACACTCGC	480		
Sbjct 442	CCCCGAAATACAGTGGCGGTCTCGCCGACGCTCTCATGCGCAGTAGTTTGACACTCGC	501		
Query 481	ACCGGAGCGCGGCGCGGCCACAGCCGTAACACCCCAA	520		
Sbjct 502	ACCGGAGCGCGGCGCGGCCACAGCCGTAACACCCCAA	541		

Figure 7: Sequencing of *Trichoderma orientale* strain FMR 12486 isolate.

Antifungal Activity of *Trichoderma orientale* Crude Extract against Pathogenic Fungi

The *Trichoderma orientale* crude extract was concentrated by the rotary evaporator to the final quantity of 6 gm. Results showed that all isolates of *Aspergillus fumigates*, *Aspergillus flavus*, *Trichophyton mentagrophyte*, *Microsporium canis*, *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis* and *Candida krusei* were susceptible to *Trichoderma orientale* crude extract

at a concentration of 0.5 mg/ml. While *Candida albicans* was susceptible at 1mg/ml concentration of crude extract. And at the concentrations 0.5 and 1mg/ml, *C. parapsilosis* inhibition zone was significantly higher than other pathogenic fungi, followed by *T. mentagrophyte* and *C. glabrata* (20, 22; 16, 20 and 16, 20 mm) respectively (Table 4) (Figure 8, 10). *C. glabrata* was the most sensitive fungus to the concentration of 2mg/ml where inhibition zone was 26mm, followed by *C. parapsilosis* and *T. mentagrophyte* (24 and 20 mm) respectively (Table 4) (Figure 8, 10). A concentration of 4 mg/ml showed *C. glabrata* inhibition zone was the highest and followed by *C. parapsilosis*, *M. canis*, and *T. mentagrophyte* 26, 24, 24 and 24mm respectively (Table 4) (Figure 8, 9, 10). Also, it can be observed that *T. rubrum* was resistant to *T. orientale* crude extract concentrations when the inhibition zone was zero. The inhibition zone of pathogenic fungi increased with the increase in crude extract concentration; *T. mentagrophyte* inhibition zone was 16mm at the concentration of 0.5mg/ml. This inhibition zone increased to 20 and 24mm when the concentrations was increased to 1, 2 and 4mg/ml respectively (Table 4). These results were similar to Aasi *et al.* [36] who showed that the lower concentration of 0.5mg/ml of *Trichoderma harzianum* crude extract achieved a high antifungal activity against *Trichophyton mentagrophyte* and *Microsporum canis*.

Table 4: Growth inhibition zone of human pathogenic fungi by *Trichoderma orientale* strain FMR 12486 crude extracts at different concentration at pH 5.5 on SDA medium.

Fungi	Inhibition Zone (mm)				LSD value
	0.5 mg/ml	1 mg/ml	2 mg/ml	4 mg/ml	
<i>Candida albicans</i> **	0.0± 0.0	4.0± 0.6	8.0± 0.6	10.0± 0.6	2.81*
<i>Candida tropicalis</i> **	6.0± 0.6	10.0± 0.6	10.0± 0.6	10.0± 0.6	3.65*
<i>Candida parapsilosis</i> **	20.0± 0.6	22.0± 0.6	24.0± 0.6	24.0± 0.6	3.52*
<i>Candida glabrata</i> **	16.0± 0.6	20.0± 0.6	26.0± 0.6	26.0± 0.6	4.17*
<i>Candida krusei</i> **	4.0± 0.57	10.0± 0.6	12.0± 0.6	12.0± 0.6	3.58*
<i>Aspergillus fumigatus</i> ***	8.0± 0.6	8.0± 0.6	10.0± 0.6	12.0± 0.6	2.75*
<i>Aspergillus flavus</i> ***	8.0± 0.6	10.0± 0.6	12.0± 0.6	14.0± 0.6	3.63*
<i>Trichophyton mentagrophyte</i> ****	16.0± 0.6	20.0± 0.6	20.0± 0.6	24.0± 0.6	3.09*
<i>Trichophyton rubrum</i> ****	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0 NS
<i>Microsporum canis</i> *****	10.0± 0.6	14.0± 0.6	16.0± 0.6	24.0± 0.6	3.52*
LSD value	1.44 *	1.53 *	1.53 *	1.53 *	---

* (P<0.05), NS: Non-Significant.

±Standard error for the average of three replicates.

** incubation period for 2 day at 35±2C°.

*** incubation period for 2 days at 28±2 C°

**** incubation period for 4 days at 28±2 C°.

***** incubation period for 6 days at 28±2 C°.

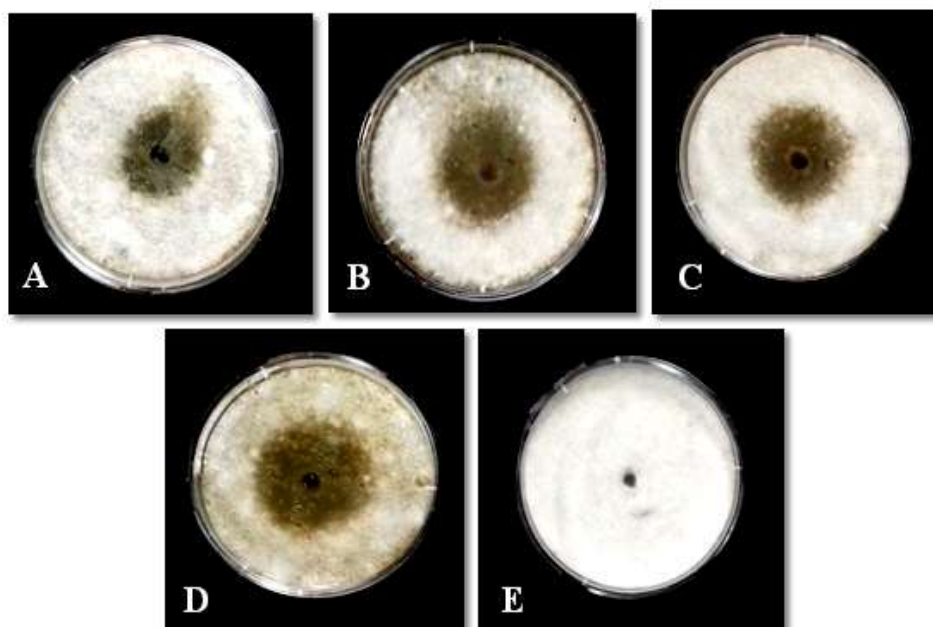


Figure 8: Antifungal activity of *Trichoderma orientale* crude extract against *Trichophyton mentagrophyte* on SDA at $28\pm 2C^{\circ}$ after 4 days incubation using agar well diffusion method. (A) 0.5mg/ml, (B) 1mg/ml, (C) 2mg/ml, (D) 4mg/ml, (E) Control.

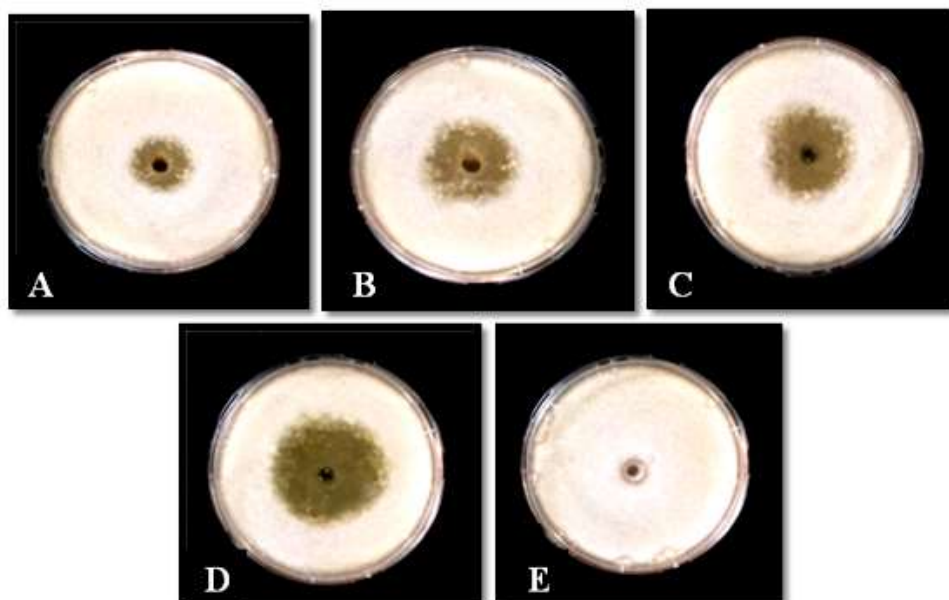


Figure 9: Antifungal activity of *Trichoderma orientale* crude extract against *Microsporium canis* on SDA at $28\pm 2C^{\circ}$ for 6 days incubation using agar well diffusion method. Concentrations: (A) 0.5mg/ml, (B) 1mg/ml, (C) 2mg/ml, (D) 4mg/ml, (E) Control.

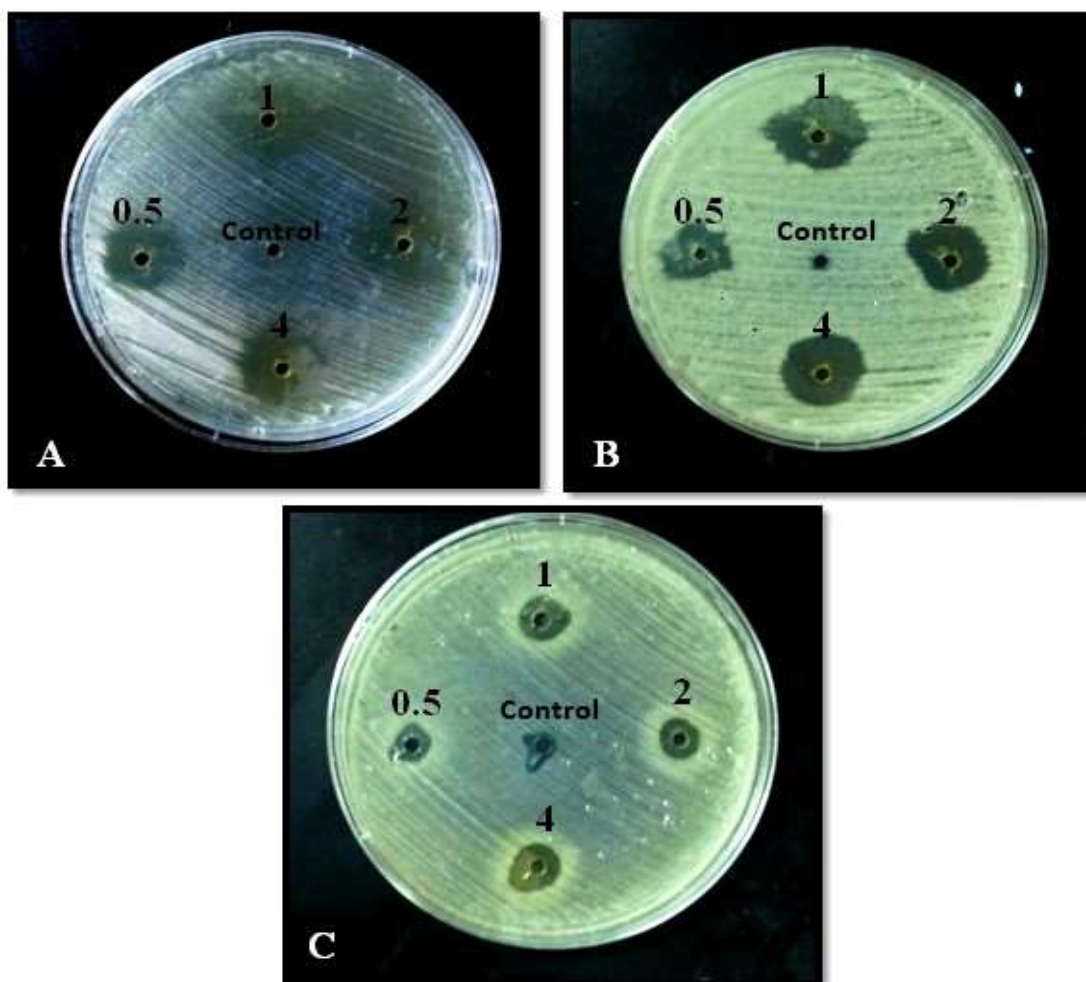


Figure 10: Antifungal activity of *Trichoderma orientale* crude extract against *Candida spp.* on SDA at $35\pm 2C^{\circ}$ after 1 day incubation using agar well diffusion method (A) *Candida parapsilosis*, (B) *Candida glabrata*, (C) *Candida tropicalis*

Conclusions

1. *Candida albicans* was the most common yeast isolated from sputum specimens, while in the skin scrapes *Candida parapsilosis* was the common.
2. In this study, all pathogenic fungi were inhibited by *T. orientale* crude which showed a high antifungal activity, except *T. rubrum* which showed resistance to *T. orientale* crude extract.
3. *T. orientale* FMR 12486 can be used to extract an effective treatment to eliminate human pathogenic fungi rather than the use of chemical antibiotics.

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References

- [1] Bongomin, F., Gago, S., Oladele, R. O., and Denning, D. W., "Global and Multi-national Prevalence of Fungal Diseases Estimate Precision.," *Journal of fungi*, vol. 3, no. 4, pp. 1-29, 2017.

- [2] Martinez-Rossi, N. M., Bitencourt, T. A., Peres, N., Lang, E., Gomes, E. V., Quaresimin, N. R., Martins, M. P., Lopes, L., and Rossi, A., "Dermatophyte Resistance to Antifungal Drugs: Mechanisms and Prospectus.," *Frontiers in microbiology*, vol. 9, p. 1108, 2018.
- [3] L. Ilag, "Ringworm Disease-Causes, Diagnosis, and Treatment: AMYCOT®, a Novel Natural Treatment for Ringworm and other Tinea Infections," *Journal of Dermatology and Clinical Research*, vol. 6, no. 1, p. 1114, 2018.
- [4] Kakande, T., Batunge, Y., Emmanuel, E., Ambrose, S., Abimana, J., Akinola, S., Muhwezi, R., Adam, A., Onkoba, S., Aliero, A., Atuheire, C. and Kato, C., Ntulume, I., "Prevalence of Dermatophytosis and Antifungal Activity of Ethanolic Crude Leaf Extract of *Tetradenia riparia* against Dermatophytes Isolated from Patients Attending Kampala International University Teaching Hospital, Uganda.," *Dermatology Research and Practice*, vol. 2019, pp. 1-13, 2019.
- [5] D. J. V., "Candida albicans Hyphae: From Growth Initiation to Invasion," *Journal of fungi*, vol. 4, no. 1, pp. 1-10, 2018.
- [6] De Oliveira Santos, G. C., Vasconcelos, C. C., Lopes, A., de Sousa Cartágenes, M., Filho, A., do Nascimento, F., Ramos, R. M., Pires, E., de Andrade, M. S., Rocha, F., and de Andrade Monteiro, C., "Candida Infections and Therapeutic Strategies: Mechanisms of Action for Traditional and Alternative Agents," *Frontiers in microbiology*, vol. 9, p. 1351, 2018.
- [7] Carroll, K. C., Hobden, J. A., Miller, S., Morse, S. A., Mietzner, T. A., Detrick, B., Mitchell, T. G., Mckerrow, J. H., and Sakanari, J. A., Jawetz, Melnick & Adelberg's medical microbiology. 27th edition, New York: Mcgraw-Hill Education, 2016.
- [8] Li, Z., Lu, G., and Meng, G., "Pathogenic Fungal Infection in the Lung," *Frontiers in immunology.*, vol. 10, p. 1524, 2019.
- [9] Taccone, F.S., Van den Abeele, A.M., Bulpa, P., Misset, B., Meersseman, W., Cardoso, T., Paiva, J.A., Blasco-Navalpotro, M., De Laere, E., Dimopoulos, G., Rello, J., Vogelaers, D., Blot, S.I. and AspICU Study Investigators., "Epidemiology of invasive aspergillosis in critically ill patients: clinical presentation, underlying conditions, and outcomes," *Crit Care*, vol. 19, no. 1, pp. 1-7, 2015.
- [10] Krishnasamy, L., Krishnakumar, S., Kumaramanickavel, G. and Saikumar, C., "Molecular Mechanisms of Antifungal Drug Resistance in Candida Species," *Journal of Clinical and Diagnostic Research.*, vol. 12, no. 9, pp. 1-6, 2018.
- [11] Bondaryk, M., Staniszevska, M., Zielińska, P. and Urbańczyk-Lipkowska, Z., "Natural antimicrobial peptides as inspiration for design of a new generation antifungal compounds," *Journal of Fungi*, vol. 3, no. 3, pp. 20-46, 2017.
- [12] Lahuf, A., Kareem, A., Al-Sweedi, T. and Alfarttoosi, H., "Evaluation the potential of indigenous biocontrol agent *Trichoderma harzianum* and its interactive effect with nanosized ZnO particles against the sunflower damping-off pathogen, *Rhizoctonia solani*," in *IOP Conference Series: Earth and Environmental Science*, Istanbul-Turkey, 2019.
- [13] Leylaie, S. and Zafari, D., "Antiproliferative and Antimicrobial Activities of Secondary Metabolites and Phylogenetic Study of Endophytic *Trichoderma* Species from Vinca Plants," *Frontiers in microbiology*, vol. 9, p. 1484, 2018.
- [14] N. Rajendran, "Antimicrobial activity of crude extracts from Mangrove-derived *Trichoderma* species against human and fish pathogens," *Biocatalysis and Agricultural Biotechnology*, vol. 6, pp. 189-194, 2016.
- [15] Tille, P. M., and Forbes, B. A., *Bailey and Scott's diagnostic microbiology*. Thirteenth edition, St. Louis, Missouri: Elsevier, 2014.
- [16] C. V. Sciortino, *Atlas of Clinically Important Fungi*, Hoboken, New Jersey: Wiley-Blackwell, 2017.
- [17] Samuels, G.J. and Hebbbar, P. K., *Trichoderma: Identification and Agricultural Applications*, Minnesota: The American Phytopathological Society, 2015.
- [18] NCBI, "National Center for Biotechnology Information," National Library of Medicine, [Online]. Available: https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome.

- [19] Liu, S., Lo, C., Chen, C., Liu, M., Chen, J. and Peng, K., "Efficient isolation of anthraquinone-derivatives from *Trichoderma harzianum* ETS 323," *Journal of biochemical and biophysical methods*, vol. 70, no. 3, pp. 391-395, 2007.
- [20] Alexander, B. D., Procop, G. W., Dufresne, P., Fuller, J., Ghannoum, M. A., Hanson, K. E., Holliday, D., Holliday, N. M., Kovanda, L., Lockhart, S.R., Ostrosky-Zeichner, L., Schuetz, A. N., Wiederhold, A. P. and Zelazny, A. M., M27 Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts - 4th Edition, Wayne, PA 19087 USA: Clinical and Laboratory Standards Institute, 2017.
- [21] Yadu, R., Rao Nawange, S., Mohan Singh, S., Sethi Gutch, R., Gumasta, R. and Nawange, M., "Prevalence of opportunistic fungal pathogens in Pulmonary Tuberculosis patients from Madhya Pradesh, Central India.," *Journal of Microbiology and Biomedical Research*, vol. 1, pp. 1-12, 2015.
- [22] Mwaura, E.N., Matiru, V. and Bii, C., "Mycological Findings of Sputum Samples from Pulmonary Tuberculosis Patients Attending TB Clinic in Nairobi, Kenya," *Virology and Mycology*, vol. 2, no. 3, pp. 1-6, 2013.
- [23] Kwizera, R., Parkes-Ratanshi, R., Page, I. D., Sekaggya-Wiltshire, C., Musaaazi, J., Fehr, J., Castelnuovo, B., Kambugu, A., and Denning, D., "Elevated *Aspergillus*-specific antibody levels among HIV infected Ugandans with pulmonary tuberculosis.," *BMC pulmonary medicine*, vol. 17, no. 1, p. 149, 2017.
- [24] Najem, M. H; Al-Salhi, M. H. and Hamim, S. S., "Study of Dermatophytosis prevalence in Al-Nassiriyah city- Iraq.," *World Journal of Pharmaceutical Sciences*, vol. 4, no. 4, pp. 166-172, 2016.
- [25] Al-Hmadani, A. H., Al-Dhalimi, M. A., and Alrufae, M. M. A., "Epidemiologic study of dermatophytosis in Al-Najaf government," *Al-Kufa Journal for Biology*, vol. 6, no. 1, pp. 53-67, 2014.
- [26] Ali, R., AL-Wattar M.A. and Almothaffar, W., "Detection of galactomannan Antigen in the Serum of Iraqi Patients with Suspected Invasive Aspergillosis," *Journal of Faculty of Medicine Baghdad*, vol. 58, no. 1, pp. 71-78, 2016.
- [27] Al-Charrakh, A., Al-Malaky, K. and Imran, Z., "Diagnostic Aspergillosis in the sputum of patients with pulmonary complains and antifungals susceptibility of triazoles and caspofungin using e-test.," *International Journal of Medical Science and Clinical invention*, vol. 2, pp. 1448-1455, 2015.
- [28] B. Hmood, "Detection of Gliotoxin in Patients with Pulmonary Aspergillosis," *Journal of University of Babylon*, vol. 25, no. 2, pp. 639-650, 2017.
- [29] Babita, S. and Prabhat, K., "Prevalence of Mycotic Flora with Pulmonary Tuberculosis Patient in," *International Journal of Contemporary Medical Research*, vol. 3, no. 9, pp. 2563-2564, 2016.
- [30] C. E. Greene, *Infectious diseases of the dog and cat*. 3rd, Missouri: Saunders Elsevier, St. Louis, Mo., 2006.
- [31] W. Ali, Epidemiological study of tinea capitis and therapeutic evaluation of some antifungal and herbal drug of Al-Qadisiya governorate., Baghdad: M. Sc. Thesis. College of Science, Univ. of Baghdad., 2009.
- [32] Sowmya, N.; Appalaraju, B.; Surendran, P. and Srinivas, C. R., "Isolation, Identification and comparative analysis of SDA and DTM for dermatophytes from clinical samples in a tertiary care hospital.," *Journal of Dental and Medical Sciences*, vol. 13, no. 11, pp. 68-73, 2014.
- [33] Samia, A.G., Nehal E.Z, Hala, B., omnia, A.S. and Hadia, A.S., "Genotypic identification and antifungal susceptibility pattern of Dermatophytes isolated from clinical specimens of Dermatophytosis in Egyptian patients," *Egyptian dermatology online journal*, vol. 2, no. 1, pp. 4-9, 2006.
- [34] T. Saleh, "Occurrence of Ring worm infections among primary Schools Children in Misan," *Misan journal of academic studies*, vol. 9, no. 17, pp. 130-142, 2010.
- [35] Bonassoli, L. A., Bertoli, M., and Svidzinski, T. I., "High frequency of *Candida parapsilosis* on the hands of healthy hosts," *The Journal of hospital infection*, vol. 59, no. 2, pp. 59-62, 2005.

- [36] Aasi, S. R. and Al-araji, A. M., "The inhibitory effect of *Trichoderma harzianum* CA-07 crude extract against *Trichophyton mentagrophyte* and *Microsporium canis.*," *Iraqi Journal of Science*, vol. 59, no. 3B, pp. 1387-1395, 2018.