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The inhibitory effect of *Trichoderma harzianum* CA-07 crude extract against *Trichophyton mentagrophyte* and *Microsporium canis*

Surra Rasan Aasi*, Alaa Mohsen Al-Aaraji
Department of Biology, University of Baghdad, College of Science, Baghdad, Iraq

Abstract

The study aimed to identify *Trichoderma harzianum* isolates morphologically and by using PCR teqnique and evaluate the antifungal activity of *T. harzianum* CA-07 crude extract against *Trichophyton mentagrophytes* and *Microsporium canis* from patients with dermatophytosis. *T.harzianum* isolates were collected from the soil of Baghdad University gardens and they were identified depending of morphological features on plate and microscopic examination. The genomic DNA of *T.harzianum* isolate was extracted at a final concentration of (400-600) µg / 2-3 g of wet mycelium and at a purity of 1.6-1.8and DNA sample was amplified with each of universal primers (ITS-1& ITS-4) to be used for detection of *Trichoderma* species. The crude extract was extracted from *T.harzianum* strain CA-07 by ethyl acetate with finalyield of 4.8gm., then different crude extract concentrations (0.5, 1, 2 and 4 mg/ml) were used against the clinical pathogenic fungi (*T.mentagrophyte* and *M.canis*) using agar well diffusion method. The results of identification of *Trichoderma harzianum* isolates by using PCR confirmed that the isolate was *T.harzianum* strain CA-07,and their crude extract exhibited significantly high antifungal activity against *T. mentagrophyte* and *M. canis* with high growth inhibition zones(14, 12mm) respectively at the lower concentration (0.5mg/ml)of crude extract.

Keywords: *Trichoderma harzianum*, PCR - *ITS* region, *Trichophyton mentagrophytes*, *Microsporium canis*.

التأثير المثبط للمستخلص الخام لفطر *Trichoderma harzianum* CA-07 ضد الفطرين *Trichophyton mentagrophyte* و *Microsporium canis* المسببة للأمراض الجلدية البشرية

سرى رسن عاصي*، علاء محسن الاعرجي
قسم علوم الحياة ، كلية العلوم ، جامعة بغداد، بغداد، العراق

الخلاصة

هدفت هذه الدراسة إلى تشخيص عزلات الفطر *Trichoderma harzianum* مظهريا وباستخدام تقنية تفاعل البلمرة المتسلسل وتقدير الفعالية التثبيطية للمستخلص الخام لفطر *T. harzianum* CA-07 ضد الفطرين *Trichophyton mentagrophytes* و*Microsporium canis* المعزولين من مرضى يعانون من فطريات جلدية والذين تم تشخيصهم سريريًا من قبل اطباء الأمراض الجلدية. تم جمع عزلات الفطر *T. harzianum*من تربة حدائق جامعة بغداد وشخصت اعتمادا على الصفات المظهرية على الطبق وتحت المجهر. تم استخلاص الحمض النووي الوراثي (DNA) لعزلة *T. harzianum* بتركيز نهائي يتراوح من

*Email: alaa_mohsen7@yahoo.com

400 – 600 ميكروغرام / 2–3 غرام من الغزل الفطري الرطب وبنقاوة من 1.6–1.8و تم تضخيم عينة الـ DNA مع كل من البوادئ العالمي(ITS–1 وITS–4) لتستخدم للكشف عن نوع فطر *Trichoderma* . تم استخلاص المستخلص الخام من سلالة الفطر *T. harzianum* CA–07 بواسطة الاثيل اسيتيت وبأنتاج نهائي يعادل 4.8غم. ثم استخدمت تراكيز مختلفة من هذا المستخلص الخام (0.5 ، 1 ، 2 ، 4 ملغم / مل) ضد الفطرين *T. mentagrophyte* و *M. canis* باستخدام طريقة الانتشار في الحفر. واظهرت نتائج التشخيص بواسطة الـ PCR ان العزلة كانت من نوع *T. harzianum* CA–07 وان المستخلص الخام لهذه السلالة يمتلك بشكل ملحوظ فعالية تثبيطية عالية ضد الفطرين *T. mentagrophyte* و *M. canis* مع مناطق تثبيط النمو عالية بلغت (14 ، 12 ملم) على التوالي عند اقل تركيز (0.5 ملغم / مل) من المستخلص الخام.

Introduction

Dermatophytes are fungi that require keratin for growth and they can cause superficial infections of the skin, hair and nails [1]. These fungi include three species(*Trichophyton Epidermophyton and Microsporum*) that affect approximately 25% of the general population worldwide causing tinea or ring worm (skin worm) [2]. Among the dermatophytes, *Trichophyton mentagrophytes* is one of the major causative agents of dermatophytosis in humans and animals, it is a worldwide cause of athlete's foot, and also it can cause tinea capitis, tinea corporis, tinea barbae and tinea cruris. In many instances these infections are sporadic [3]. *Microsporum canis* is also a common agent of dermatophytosis, it is a frequent cause of tinea capitis and tineacorporis in humans, especially children [4]. Cats and dogs are the main sources of *M. canis* infection as zoonosis [5]. The fungi *Trichoderma* SPP are proven to be a potential source of bioactive molecules, *Trichoderma harzianum* release antibiotics and other chemicals that are kill pathogens or inhibit their growth [6, 7]. However, the colony morphology serves to identify fungi of this genus; it is insufficient to distinguish the species, which makes it necessary to confirm the species through molecular methods, such as sequencing assay [8]. According to [9] there are several strains of *Trichoderma* genus that have the ability to produce many enzymes such as protease, cellulase and hemicellulase, it is therefore commercially used in sugar food factories for the production of cellulase enzyme to digest sugars. Based on this study, the researchers discovered that *Trichoderma* crude extract showed a high inhibition activity against *Trichophyton* spp. that can cause human fungal infections [10]. Treatment of fungal disease such as dermatophytosis can be managed by topical antifungal agents, but the severe cases require a systemic therapy with griseofulvin, itraconazole or terbinafine [11, 12]. These therapies may also cause dangerous side effects especially when they are combine with many common medicines, this led researchers looking for new antimycotic agents which can inhibit fungal growth without harming the host [13, 14]. The aim of this study was the identification of *T. harzianum* isolate using byPCR and evaluating the antifungal activity of *T. harzianum* crude extract against *T. mentagrophytes* and *M. canis*.

Materials and Methods

Isolation and Identification of dermatophytes

A total of 20 specimens were collected from different patients with dermatophytosis which were clinically diagnosed by Dermatologist, were recruited in the current study after attending to the Consultant of Dermatology in Baghdad hospital of Medical City. Skin Scrapes were collected by disposable scalpel blades of the solid type held vertically to the skin. When the lesion has a definite edge, the material was taken from the active margin also transport swabs were used as a good back-up tool. The scrapings were collected and transported in folded paper and then be carefully folded and secured by a paper clip. All clinical specimens were subjected to direct microscopic examination by using potassium hydroxide (KOH 10%), The specimen was placed on a surface of clean slide flooded with drops of 10% KOH heated gently (30C^{o}) for about 5-10 minutes, then, the slide was left to be cooled and the cover slip was placed and examined under the low (10X) and high (40X) microscope lens. Each skin scrape specimens were inoculated by sterile scalpel in to petri dish containing autoclaved Sabouraud's dextrose agar with 500mg cyclohexamid to identify the pathogenic fungi and incubated at 28±2 °C. Cultures were firstly examined after 7 days, and then twice weekly for at least 3-4weeks.*Trichophyton mentagrophytes* and *Microsporium canis* were diagnosed based on the morphology of the colony and microscopic examination (morphological characteristics) according to [15].

Isolation and identification of *Trichoderma harzianum*

Thirty soil samples were collected from different locations of Baghdad University gardens at a depth within (4-5 cm) using a metal spatula that was sterilized every time with 70% alcohol. The samples were kept in new polythene bags, sealed and transported to the laboratory immediately for the mycological examination. Ten gm of soil samples were put in a 250ml conical flask containing 100ml sterile distilled water. The flask was shaken on an electric shaker to get a homogenous suspension and serial dilutions of the soil sample such as (10^{-1}, 10^{-2} and 10^{-3}) were prepared. One ml of (10^{-3}) dilution for each sample was added to petri dish containing potato dextrose agar medium (OXOID, England), then it was incubated at 25±2°C for five days. After growing of different colonies on PDA plates, the fungal cultures were then transferred and subcultured to have a pure culture. *Trichoderma* spp. were diagnosed depending on the morphology of the colony and microscopic examination (morphological characteristics) according to [16].

Identification of *Trichoderma harzianum* by PCR and gene sequence:

The morphological features of the fungus serve to identify fungi of this genus, it is insufficient to distinguish the species, which makes it necessary to confirm the diagnosis through molecular methods. So, *Trichoderma harzianum* diagnosis by PCR and gene sequence.

DNA extraction

DNA was extracted using ZR Fungal/Bacterial DNA MiniPrep™ according to manfactures protocol.

PCR Amplification of *ITS* gene:

The primer pair *ITS1* (TCC GTA GGT GAA CCT GCG G) and *ITS4* (TCC TCC GCT TAT TGA TAT GC) (Integrated DNA Technologies company, USA) using for amplification of *ITS*gene.PCR amplification was performed in a total volume of 25µl containing 1.5µl DNA, 5 µl *Maxime*PCRPreMix master mix / i-StarTaq (Intron/Korea), 1µl of each primer (10 pmol) then distilled water was added into tube to a total volume of 25µl.The thermal cycling conditions were done as follows: Denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45s, 58°C for 1 min and 72 °C for 1min with final incubation at 72 °C for 7 min using a thermal Cycler (Gene Amp, PCR system 9700; Applied Biosystem). The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by exposure to ultraviolet light (320nm) after red stain staining (Intron Korea).

Sequencing and Sequence Alignment

Sequencing of *ITS* gene of 4 fungi isolates was performed by AB13730XL, Applied Biosystem, Macrogen Company, USA. Homology search was conducted using Basic Local Alignment Search Tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm.nih.gov) and BioEdit program. The results were compared with data obtained from Gene Bank published ExPASY program which is available at the NCBI online.

Extraction of antifungal compounds

In this study, modified Czapek's medium was used to test the production of inhibitory substances of crude extract. *Trichoderma* isolates were grown on PDA for 6 days, then, two block of (10mm) in diameter from *Trichoderma* agar culture were added to the autoclaved prepared modified Czapek's medium (2g/l Na_2HPO_4, 1.5g/l $MgSO_4.7H_2O$, 7g/l KH_2PO4, 0.2g/l $FeCl_3$, 0.1g/l $ZnSO_4.7H_2O$, 0.1g/l $CaCl_2$, 0.5g/l $NH_4)_2SO_4$, 30g/l Glucose, 150g/l Sugarcane bagasse)in flasks and incubated in the dark for 14 days at 27±2C^{o}with shaking. For extraction of antifungal compounds from modified Czapek's medium 800ml of ethyl acetate (EtOAc) (HIMEDIA, India) was added and placed on a shaker at 121 rpm (overnight). The extraction was completed with a 24 hour period. Extraction of antifungal extracts was employed by using rotary evaporator (Gallenhamp, England) at 37 C^o taking into consideration the boiling point of the solvents (EtOAc, 88°C) [17].

Preparation of the inoculum for *T. mentagrophyte* and *M. canis*

In *vitro* antifungal activity of the ethyl acetate crude extracts of *Trichoderma harzianum* was studied against *T. mentagrophyte* and *M. canis.* For the susceptibility testing of filamentous fungi, the inoculum prepared by serial dilution method of *T. mentagrophyte* and *Microsporium canis* suspensions.

Inoculums suspension was prepared as follow:

1. Filamentous fungi were maintained on SDA using (6cm) petri dishes until their growth was complete.
2. The fresh colonies were covered with approximately 1ml of sterilized 0.9% saline, and the suspension was prepared by gently probing the colonies with the tip of a transfer pipette.
3. The resulting mixture of conidia and hyphal fragments was withdrawn and transferred to a sterile tube. After heavy particles are allowed to settle for 3 to 5 minutes, the upper homogeneous suspension is transferred to a sterile tube, the cap tightened and mixed with a vortex mixer for 15 seconds.
4. One ml of this suspension was added to 9ml of sterilized 0.9% saline to prepare 10^{-1} dilution.

Agar Well Diffusion Method

The *Trichoderma harzianum* crude extract was diluted in 100% dimethyl sulfoxide (Sigma-Aldrich, Germany) to give different concentrations of (0.5, 1, 2, 4mg/ml). The suspension (1ml) of 10^{-1}dilution of each *T. mentagrophyte* and *M. canis* were homogeneously mixed with SDA medium using the pour plate method in 6 cm Petri dishes. Then a well of (5mm) was made in the medium by using sterile cork borer. 100µl of each concentration of the *Trichoderma harzianum* crude extract was transferred into separate wells. Dimethyl sulfoxide was used as a negative control. Plates are incubated at 28±2°C for 4 days before determining results. The diameter of the inhibition zone was recorded for each replicate and the average diameter was calculated.

Statistical analysis

The Statistical Analysis System- SAS (2012) program was used to effect of difference concentration in study parameters. Least significant difference -LSD test was used to significant compare between means in this study.

Results and discussion

In these results out of 20 cases of skin scrapes samples were tested, eleven (11) of them were positive for fungal infections by both direct microscopic examination and cultured on media were giving a prevalence rate of 55%. The negative results of culture may be due to that most cases have topical antifungal medications before sampling, or may be the sampling was not from the active border of the lesion which invaded by fungi. Among the 11 positive skin scrape cultures there were two isolate *Trichophyton mentagrophyte* and two isolate *Microsporium canis*(Figure-1). They are the most frequently isolated species mainly from patients with tinea corporis. The higher frequency with *Microsporiumcamis* and *Trichophyton mentagrophytes* may be explained to the direct or indirect contact with domestic animals such as cattle because they are zoophilic fungi and cause many ringworm infections. The results agreed with [18] who mentioned that *Microsporium canis* was the main causative agent of dermatophytes infection. Also, present result agreed with [19] who mentioned that *Trichophyton mentagrophytes* was the most common isolate from the clinical samples.

From a total of 30 soil samples cultures, 5 samples were tested positive for *Trichoderma* species were obtained after morphological identification. From five *Trichoderma* isolates, there were (1 isolate)*Trichoderma harzianum* (Figure-3).*Trichoderma harzianum* isolate showed the formation of concentric ring that are typical of *Trichoderma* species which is consistent with the characteristics previously described for this fungus. After finishing the DNA extraction should measurement the concentration and purity of DNA by Nanodrop, the result showed a concentration between (54 - 294) and purity (1.6-1.8).Then the purity of DNA was confirmed by agarose gel electrophoresis. The molecular characterization was based on the *ITS* region where the nuclear rDNA region spanning the ITS1, ITS2 and 5.8S rRNA gene was amplified for the *Trichoderma* isolate. Theinter genic spacer region was successfully amplified and a distinct product size was consistently obtained and the isolate yielded a unique product size of approximately ~550-600bp. The amplicon obtained of this region was in the size range of (~565 bp), (Figure-4).PCR and gene sequence results indicate the presence of species *Trichoderma harzianum* strain CA-07. This species alignments with universal isolate recorded on BLAST program showed 100% identification and 0% gaps (Figure-5).

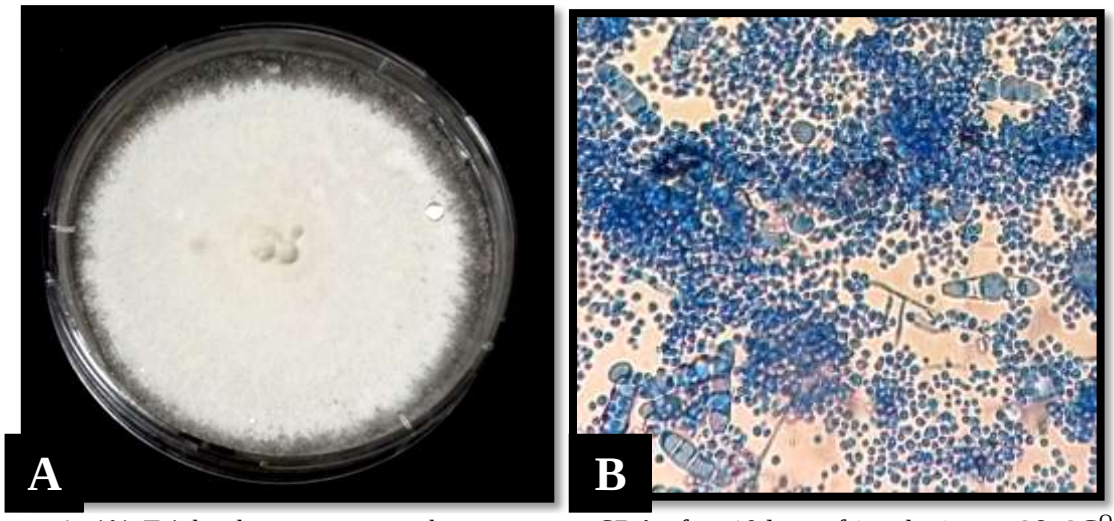

Figure 1- (A):*Trichophytonmentagrophyte*grown on SDA after 10days of incubation at 28±2C^{O}, (B): microscopic features of*Trichophyton mentagrophyte* stained with lactophenol cotton blue (40X).

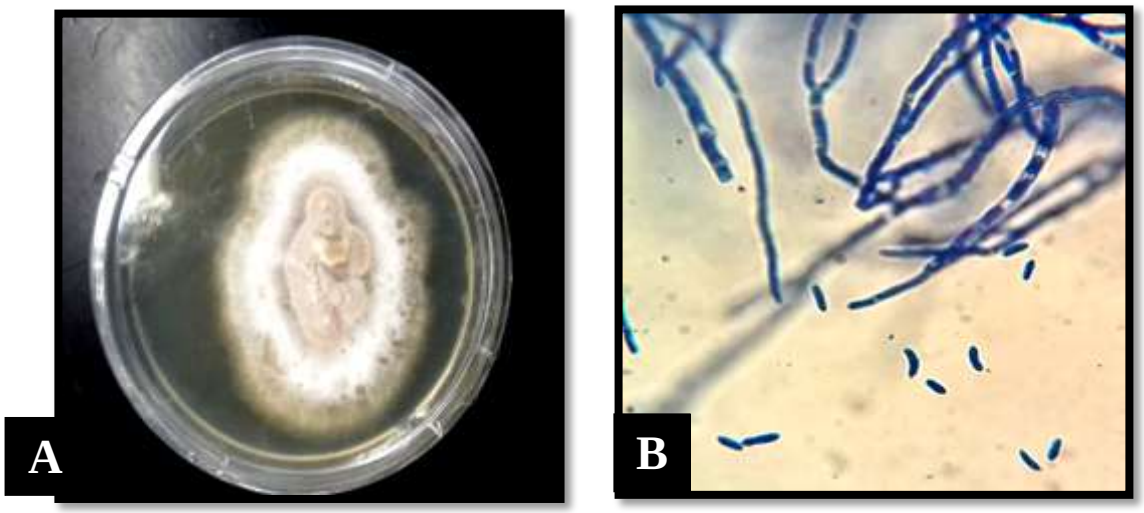

Figure 2- (A): *Microsporiumcanis* was grown on SDA after10days of incubation at28±2C^{O}, (B): Microscopic features of *Microsporium canis* was stained with lactophenol cotton blue (40X).

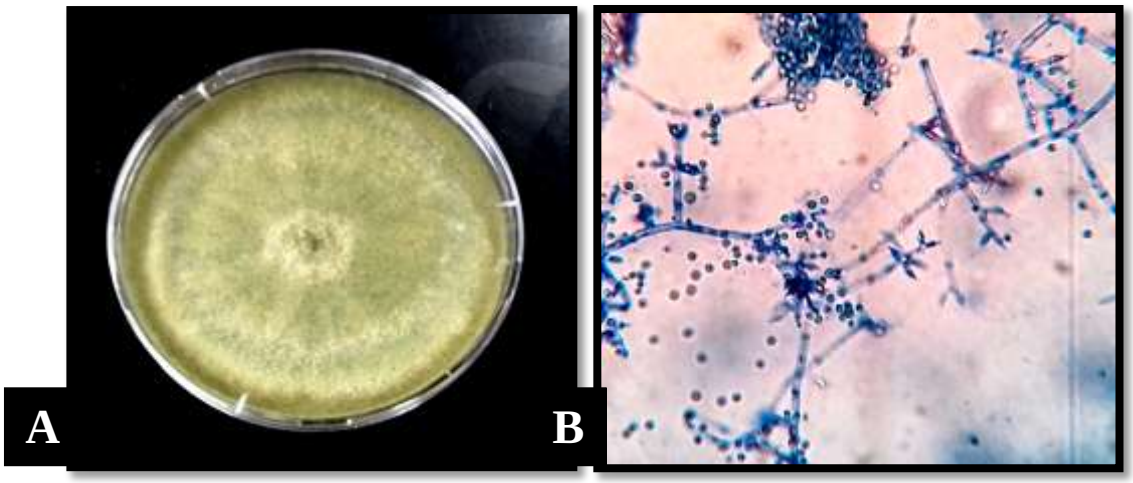

Figure 3-(A): *Trichoderma harzianum* grown on PDA at 25±2°C after 7 days of incubation,
(B): Microscopic features of *Trichoderma harzianum* stained with lacto phenol cotton blue (40X).

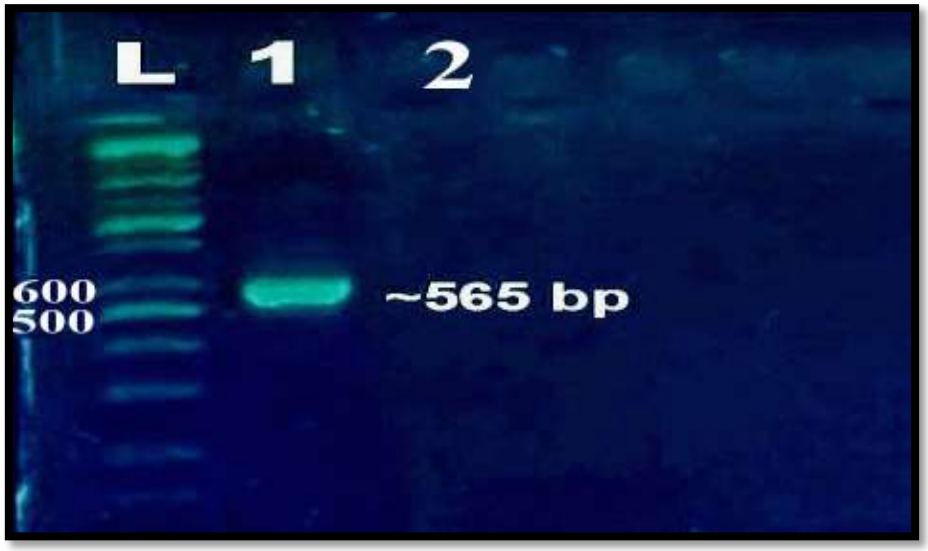

Figure 4- Agarose gel electrophoresis of PCR product the band size ~565 bp. The product was electrophoresis on 2% L: DNA ladder (100), lane (1) *Trichoderma* isolate, lane (2) negative control sample (no DNA template).

Score	Expect	Identities	Gaps	Strand
970 bits(525)	0.0	525/525(100%)	0/525(0%)	Plus/Plus

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Query  1    TCGGCGGGATCTCTGCCCCGGGTGCGTCGCAGCCCCGGACCAAGGCGCCCGCCGGAGGAC  60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  54   TCGGCGGGATCTCTGCCCCGGGTGCGTCGCAGCCCCGGACCAAGGCGCCCGCCGGAGGAC  113

Query  61   CAACCAAAACTCTTTTTGTATACCCCCTCGCGGGTTTTTTATAATCTGAGCCTTCTCGGC  120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  114  CAACCAAAACTCTTTTTGTATACCCCCTCGCGGGTTTTTTATAATCTGAGCCTTCTCGGC  173

Query  121  GCCTCTCGTAGGCGTTTCGAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTG  180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  174  GCCTCTCGTAGGCGTTTCGAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTG  233

Query  181  GCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAAT  240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  234  GCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAAT  293

Query  241  CATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAG  300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  294  CATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAG  353

Query  301  CGTCATTTCAACCCTCGAACCCCTCCGGGGGGGTCGGCGTTGGGGATCGGCCCTCCCTTAG  360
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  361  CGGGTGGCCGTCTCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTT  420
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  414  CGGGTGGCCGTCTCCGAAATACAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGTAGTT  473

Query  421  TGCACACTCGCATCGGGAGCGCGGCGCGTCCACAGCCGTTAAACACCCAACTTCTGAAAT  480
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  474  TGCACACTCGCATCGGGAGCGCGGCGCGTCCACAGCCGTTAAACACCCAACTTCTGAAAT  533

Query  481  GTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATAT  525
           |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  534  GTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATAT  578
```

Figure 5- Sequencing of *Trichoderma harzianum* strain CA-07 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large

subunit ribosomal RNA gene, partial sequence. Sequence ID: KX579941.1Length: 578Number of Matches: 1.

Estimation of antifungal activity of *Trichoderma harzianum* crude extract against *Trichophyton mentagrophyte and Microsporium canis* using agar well diffusion method

After crude extract concentrated using rotary evaporator, the final quantity of ethyl acetate crude extract was 4.8gm of *Trichoderma harzianum*. Results showed that the lower concentration 0.5mg/ml of crude extract achieved a high antifungal activity against *Trichophyton mentagrophyte* and *Microsporium canis*. The growth inhibition zone of human pathogenic fungi increased with increasing of crude extract concentrations (Table-1). At the concentrations (0.5 and 1mg/ml) *Trichophyton mentagrophyte* growth inhibition zone was (14 and14mm) respectively, this growth inhibition zone increased to (18 and 20mm) respectively, when the concentrations increased to **(**2 and 4mg/ml) respectively (Figure-6). At the concentration0.5mg/ml *Microsporium canis* growth inhibition zone was**(**12mm), this growth zone inhibition increased to (16, 20 and 22mm) when concentrations increased to (1, 2 and 4mg/ml) respectively (Figure-7).

Table 1- Growth inhibition zone of human pathogenic fungi by *Trichoderma harzianum* CA-07 crude extract at pH 5.5

Fungi	Inhibition Zone (mm)				LSD value
	0.5 mg/ml	1 mg/ml	2 mg/ml	4 mg/ml	
[**]***Trichophyton mentagrophyte***	14.00 ± 0.57	14.00± 0.57	18.00± 0.57	20.00± 0.57	3.06 *
[***]***Microsporium canis***	12.00 ± 0.57	16.00± 0.57	20.00± 0.57	22.00± 0.57	3.82 *

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

[**] Incubation period was 4 days at 28±2C^{o}.
[***] Incubation periodwas 6 days at 28±2C^{o}.

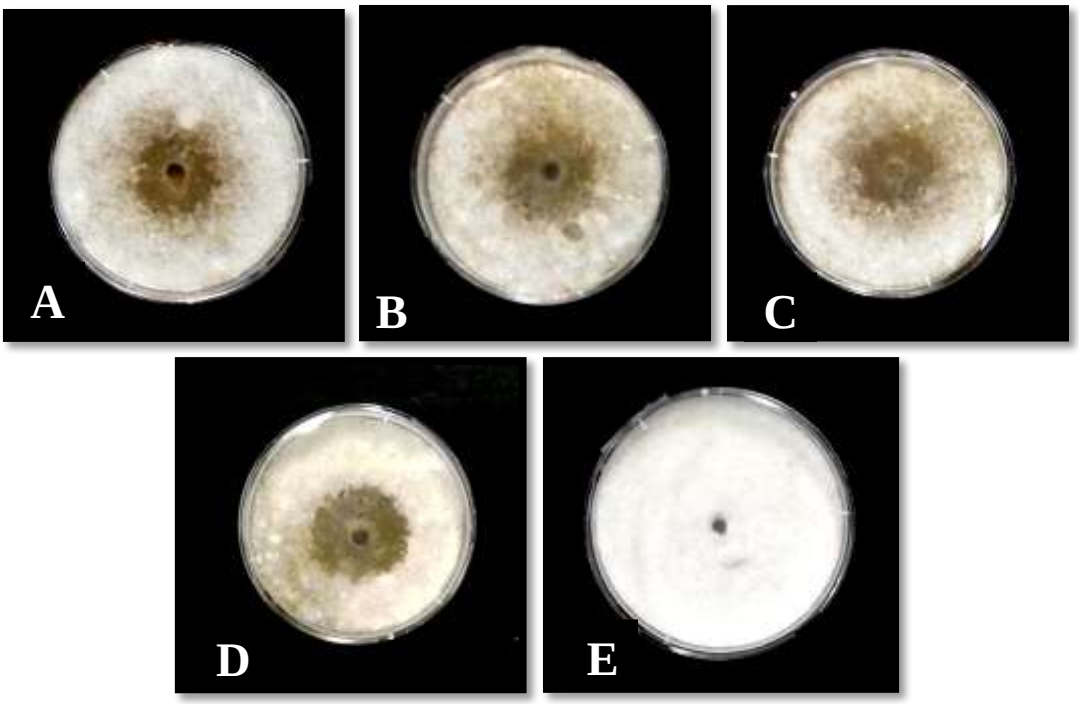

Figure 6- Antifungal activity of ethyl acetate crude extractsof *Trichoderma harzianum* CA-07 isolate against *Trichophyton mentagrophyte* on SDA, after 4 days at28±2C^{o}using agar well diffusion method (diameter of the well 5 mm). (A) 0.5mg/ml, (B) 1mg/ml,(C) 2mg/ml, (D) 4mg/ml, (E) control.

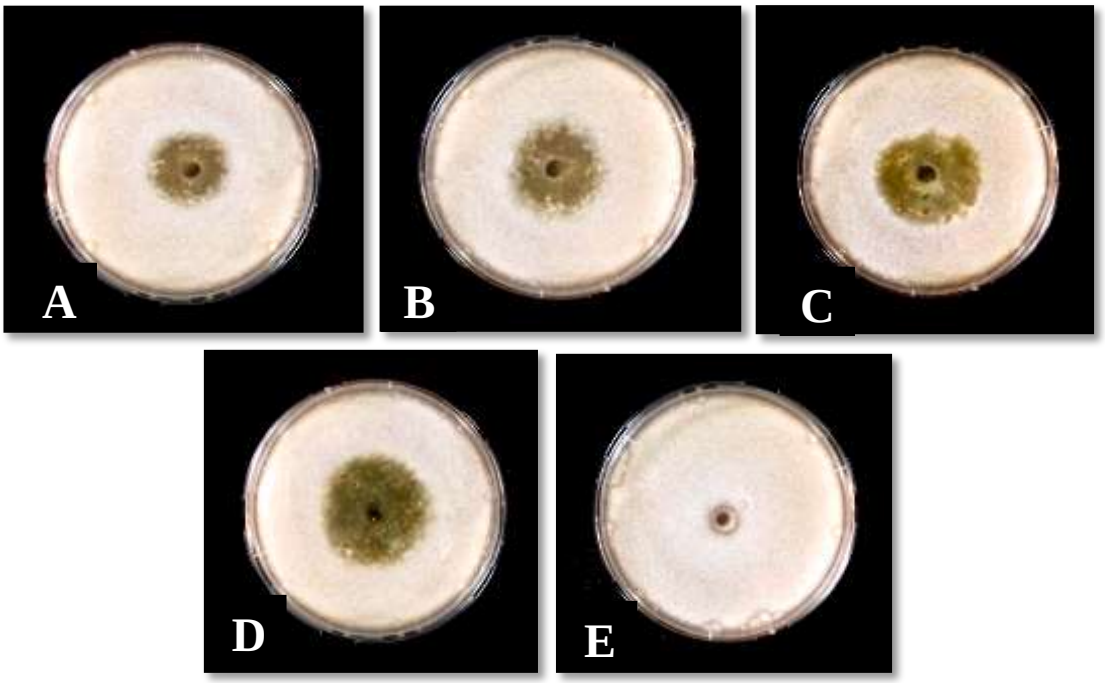

Figure 7- Antifungal activity of ethyl acetate crude extracts of *Trichoderma harzianum* CA-07 isolate against *Microsporium cani*s on SDA, after 6 days at28±2C^{o}using agar well diffusion method (diameter of the well 5 mm). (A) 0.5mg/ml, (B) 1mg/ml,(C) 2mg/ml, (D) 4mg/ml, (E) control.

In this study the results in accordance with [10] in that the *Trichoderma harzianum* showed greatest inhibiting activities toward *Trichophyton mentagrophytes*. The extracts from *Trichoderma* isolates had different active pharmacological compound which could be responsible for their different antimicrobial activities.

Conclusion

The crude extract concentrations of *T. harzianum* CA-07showed a high antifungal activity against *T. mentagrophyte* and *M. canis* was tested in this study. The extract from *T. harzianumCA*-07 can be used as an effective treatment to eliminate human pathogenic fungi rather than the use of chemical antibiotics.

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Refferences

1. Kim, S-H., Jo, I.H., Kang, J., Joo, S.Y. and Choi J-H. **2016**. Dermatophyte abscesses caused by *Trichophyton rubrum* in a patient without pre-existing superficial dermatophytosis: a case report. *BMC Infectious Diseases*. **16**: 298.
2. Kamal, R., Aneja , Joshi, R., Sharma, C. ; Surain, M. , Aneja and Ashish. **2012**. Biodiversity of dermatophytes: an overview. *Rev. Plant Pathol.* **5**: 299-314.
3. Marais A. and Osuch E. **2017**. Common cutaneous dermatophyte infections of the skin and nails. *South African Family Practice* .**59**(3): 33-40.
4. Pasquetti, M., Min, A.R.M., Scacchetti, S., Dogliero, A. and Peano, A. **2017**. Infection by *Microsporum canis* in Paediatric Patients: A Veterinary Perspective. Butaye P, ed. *Veterinary Sciences.* **4**(3): 46.
5. Murmu, S., Debnath, C., Pramanik, A.K., *et al*. **2015**. Detection and characterization of zoonotic dermatophytes from dogs and cats in and around Kolkata. *Veterinary World*. **8**(9): 1078-1082.
6. Erna Prawita Setyowati, SUT Pratiwi, Triana Hertiani and Oka Samara. **2017**. Bioactivity of Fungi *Trichoderma reesei* Associated with Sponges Stylissa flabelliformis Collected from National Park West Bali, Indonesia. *Journal of Biological Sciences*, **17**: 362-368.
7. Leelavathi, M.S., Vani, L. and Reena, P. **2014**. Antimicrobial activity of *Trichoderma harzianum* against bacteria and fungi. *Int. J. Curr. Microbiol. App. Sci.* **3**: 96-103

8. Mendoza, J.L.H; Pérez, M.I.S.; Prieto, J.M.G.; Velásquez, J.D.Q.; Olivares, JGG and Langarica, H.R.G. **2015**. Antibiosis of *Trichoderma spp* strains native to northeastern Mexico against the pathogenic fungus *Macrophomina phaseolina*. *Brazilian Journal of Microbiology*. **46**(4): 1093-1101.

9. Verma, M., Brar, S. K., Tyagi, R. D., Surampalli, R. Y., Valero, J. R. **2007**. Antagonistic fungi, *Trichoderma* spp. Panoply of biological control . *Biochem. Engineer. J*. **37**: 1-20.

10. Al-Rijabo, M. A. and Al-Obaidy, O. M. **2011**. *Trichoderma harzianum* and *T. viride* Crude extracts effect on species of *Trichophyton* which caused skin human disease. Department of Biology College of Science Mosul University. Rafidainjournal of science. **22**(2A): 16-27.

11. Sahoo, A.K. and Mahajan, R. **2016**.Management of tinea corporis, tinea cruris, and tinea pedis: A comprehensive review. *Indian Dermatology Online Journal*. **7**(2): 77-86.

12. Kaul S, Yadav S, Dogra S. **2017**.Treatment of Dermatophytosis in Elderly, Children, and Pregnant Women. *Indian Dermatology Online Journal*.**8**(5): 310-318.

13. Bondaryk, M.; Staniszewska, M.; Zielińska, P. and Urbańczyk-Lipkowska, Z. **2017**. Natural antimicrobial peptides as inspiration for design of a new generation antifungal compounds. *Journal of Fungi*. **3**(3): 46.

14. Mazu, T.K., Bricker, BA., Flores-Rozas, H. and Ablordeppey, S.Y. **2016**. The Mechanistic Targets of Antifungal Agents: An Overview. *Mini reviews in medicinal chemistry*.**16**(7): 555-578.

15. Tille, P. M., and Forbes, B. A. **2014**. Bailey & Scott's diagnostic microbiology. Thirteenth edition. St. Louis, Missouri: *Elsevier*.

16. Samuels, G.J. (2006). *Trichoderma*: Systematics, the sexual state, and ecology. *Phytopathology* .**96**: 195-206.

17. Liu, S., Lo, C. ; Chen, C. ; Liu, M.; Chen, J. and Peng, K. **2007**. Efficient isolation of anthraquinone-derivatives from *Trichoderma harzianum* ETS 323. *Journal of biochemical and biophysical methods*. **70**: 391-5.

18. Al-Hmadani, A. H., Al-Dhalimi, M. A., and Alrufae, M. M. A. **2014**. Epidemiologic study of dermatophytosis in Al-Najaf government. Al-Kufa Journal for Biology. **6**(1).

19. Sowmya, N.; Appalaraju, B.; Surendran, P. and Srinivas, C. R. **2014**. Isolation, Identification and comparatative analysis of SDA and DTM for dermatophytes from clinical samples in a tertiary care hospital. *IosR Journal of Dental and Medical Sciences*. **13**(11): 68-73.