Original Article

Isolation and Identification of Some Fungi Causes of Otomycosis and Treatment by Phenolic Extract of *Agaricus Bisporus*

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Abstract

Background: Otomycosis is a superficial fungal infection that often involves the pinna and auditory canal. It is a pathologic condition, *Aspergillus* is the most common fungal species. It is common worldwide, characterized by inflammation, pruritis, scaling, and otalgia. **Objectives:** This study aimed to isolate and identify some fungi cause of otomycosis and treatment by crude phenolic extract of *Agaricus bisporus* as an antifungal. **Materials and Methods:** Ear samples were obtained from 110 patients with otomycosis and verified by reverse transcriptase polymerase chain reaction (PCR). High-performance liquid chromatography (HPLC) results showed the presence of six biologically active compounds in *A. bisporus*. The active substance (phenols) was extracted from the mushroom *A. bisporus* and the concentrations were prepared (6.25, 12.5, and 25 mg/mL) and then tested against ear fungi *Aspergillus niger*, *Aspergillus flavus*, and *Penicillium* using evaluation of the antifungal activity in the extracts and the fungal growth inhibition was calculated individually. **Results:** The results showed that the inhibitory activity of the phenolic extract against *A. niger*, *A. flavus*, and *Penicillium* at a concentration of 6.25 mg/mL showed the lowest inhibition activity, as the diameter of inhibition was (1.63 ± 0.18 cm), while the concentration of 25 mg/mL showed the highest inhibition activity, as the diameter of inhibition was (1.17 ± 0.08) compared to the control (8.67 ± 0.33 cm). **Conclusion:** The crude phenolic extract showed inhibition for fungi that cause otomycosis.

Keywords: *Agaricus bisporus*, *Aspergillus*, otomycosis, PCR, phenolic compounds

Introduction

Otomycosis or fungal otitis externa is a superficial, subacute, or chronic auditory canal infection characterized by inflammation, pruritis, scaling, and otalgia. It is more prevalent in warm and humid climates, particularly in the monsoon season.[1] Nearly about 60 different species of fungi cause otomycosis but the most common is *Aspergillus*. The incidence of otomycosis externa is high.[2]

The otomycosis diagnosis is mainly based on clinical manifestations and mycological examination results. However, differences in the pathogenic fungi species directly affect the positive rate of direct microscopic examination results. It is a common disease, accounting for approximately 10%–20% of ear conduit infections. The *Aspergillus* species, *Aspergillus niger*, and *Aspergillus flavus* have been reported to be the most common pathogens of otomycosis.[3,4]

Aspergillus spp. is the major fungi isolated from otomycosis (*A. niger*, *A. fumigatus*, and *A. flavus*) in 80% of cases, followed by *Penicillium* and *Candida albicans*. Otomycosis may associate with bacterial infection.[5] *Aspergillus niger* is the most prevalent fungi in immunocompetent patients. Other fungi in immunocompromised patients can cause otomycosis like *Penicillium*, *Mucor*, and *Rhizopus*.[6] The main causative

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agent of otomycosis among saprophytes and yeasts. The other causative agents are *Cladosporium* spp., *Alternaria* spp.[7,8]

Agaricus bisporus is a suitable fungus for commercial utilization because of their functional properties, high protein, and unique flavor. However, the caps are one of the most mortal fruits and tend to lose quality directly after harvest with 1 to 3 days shelf life at ambient temperature in marketing conditions and this is largely due to their high respiration rate, high moisture content, and microbial contamination. Due to the lack of suitable means of protection from physical or microbial attack.[9,10]

Phenols are a class of organic compounds that contain a hydroxyl (–OH) group attached directly to an aromatic ring. Due to their unique chemical structure, phenol derivatives exhibit a wide range of biological activities and have attracted attention in the fields of medicine, pharmacology, and normal product chemistry. The diverse bioactivities associated with phenol derivatives make them promising candidates for the development of novel therapeutics and functional materials. Numerous of them possess the capacity to chelate heavy metal ions. Antifungal and antibacterial activity of phytoalexins.[11,12]

High-performance liquid chromatography (HPLC) is a useful technique that can separate a mixture of substances, and it is used in biochemistry and analytical chemistry to identify, quantify, and purify different components of the mixture.[13] HPLC employs various types of stationary phases and the pump that drives the mobile phase(s) and analyte through the column and detector to provide a characteristic retention time for the analyte. The retention period of an analyte varies according to the strength of its interactions with the stationary phase, solvent(s) ratio/composition utilized, and flow rate of the mobile phase. HPLC has a number of advantages, including low organic solvent utilization, minimal sample volume, quick analysis, and high chromatographic resolution.[14]

This study aimed to isolate and diagnose some fungi cause of otomycosis and treatment by the extracted phenolic crud from the *A. bisporus* that contains a bioactive compound to treat the fungi of *A. niger*, *A. flavus*, and *Penicillium* spp.

Materials and Methods

Fungal isolation

The investigation was conducted on 110 samples obtained from patients between November 2022 and March 2023. Patients were selected and included in the study with their permission. a comprehensive clinical history was obtained from each patient. In addition, the following details were recorded: the patient's name, sex, age, affected body part, presence of an inflammatory margin, symptoms, and duration of illness. The samples were collected under aseptic conditions using a sterile cotton swab to diagnose otomycosis, detailed history, clinical examination, otoscopic findings, and laboratory identification of fungus were considered.

Microscopic and macroscopic examination

To diagnose otomycosis, detailed history, clinical examination, otoscopic by the helping of ENT physician, and laboratory identification of fungus was considered.[15] This identification relied on the subsequent:

(1) Colonial characteristics (color, signification pigment) and colony retreats (color, significant pigment).
(2) Microscopical structure (micro and macroconidia: shape, arrangement, and hyphal structures). Multiple preparations from various locations of fungal development were put on a clean slide and stained with lactophenol cotton blue to show spores consisting of large, septate macroconidia and tiny, single-celled microconidia.

All samples were examined directly and planted on the SDA medium and after its growth it was examined microscopically and the use of several tests to confirm the type.

The primers preparation

The primers were lyophilized, they dissolved in the free ddH_2O to give a final concentration of 100 pmol/μL as stock solution and keep a stock at −20 to prepare 10 pmol/μL concentration as work primer suspended, 10 μL of the stock solution in 90 μL of the free ddH_2O water to reach a final volume 100 μL.

The PCR amplification was performed in a total volume of 25 μL containing 1.5 μL DNA, 5 μL Taq PCR PreMix, 1 μL of each primer (10 pmol) then distilled water was added into a tube to a total volume of 25 μL. The reaction components of PCR were as follows: Taq PCR PreMix, 5 μL; Forward primer, 10 picomols/μL (1 μL); Reverse primer, 10 picomols/μL (1 μL); DNA, 1.5 μL; and distilled water,16.5 μL.

Amplification of ITS region

The universal primers (ITS1-ITS2) were used [Table 1] for the amplification of rRNA gene and ITS regions which

Table 1: The sequence of primers that used this study

Primer	Sequence	Primer sequence	Tm (°C)	GC%	Size of Product (bp)
ITS	F	5′- TCCGTAGGTGAACCTGCGG -3′	60.3	50%	550-650
	R	5′ TCCTCCGCTTATTGATATGC-3′	57.8	41%	

is found in all eukaryotes as a preserved regions.[16] The thermal cycling conditions were done as follows: Initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 45 s, 52°C for 1 min, and 72°C for 1 min with extension 2 at 72°C for 5 min using a thermal cycled as in Table 2.

The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by exposure to ultraviolet light after staining by a safe red stain.

Sequencing and sequence alignment of fungi

After confirming the amplification of gene by conventional PCR, 20 µL from PCR reaction with 50 µL of forward primer for these genes were send to Macrogene company to determine the DNA sequencing in these genes. 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.

Collection of mushroom

One species of mushroom, *A. bisporus*, was collected from local markets, and diagnosed based on phenotypic characteristics (the original wild type had a brownish cap and dark brown gills, whereas the new version has a white cap, stalk, and flesh and brown gills), placed in sterile polyethylene bags, in an electric oven, sterile, and dry glass container.

Table 2: The optimum PCR conditions for gene detection

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial denaturation	95°C	5 min	1 cycle
2-	Denaturation -2	95°C	45 s	35 cycle
3-	Annealing	52°C	1 min	
4-	Extension-1	72°C	1 min	
5-	Extension -2	72°C	5 min	1 cycle

Ultrasonic extraction of phenolic compounds

The phenolic compounds were extracted from homogenized plant sample (3 g) using ethanol/water (70/30) solvent. Extraction process was carried out using Ultrasonic Bath (USA) at the room temperature for 1 h. After filtration, 5 mL of liquid extract was used for extraction yield determination. Solvent was removed by rotary evaporator under vacuum (Slovenia), and was dried at 40°C to the constant mass. Dry extracts were stored in the glass bottles at 4°C to prevent oxidative damage until analysis. Quantification of individual phenolic compounds was performed by reversed phase HPLC analysis, using a SYKAMN HPLC chromatographic system equipped with a UV detector), Chemstation, a Zorbax Eclipse Plus-C18-OSD 25 cm, 4.6 mm column. The column temperature was 30°C the gradient elution method, with eluent A (methanol) and eluent B (1% formic acid in water (v/v)) was performed, as follows: initial 0-4 min, 40% B; 4-10 min, 50% B; and flow rate of 0.7 mL/min. The injected volume of samples was 100 µL and standards were 100 µL and it was done automatically using an autosampler. The spectra were acquired in the 280 nm.[17]

Determination of MIC of *A. bisporus* extract

(1) Various amounts of phenol extracts were made, and each volume was separately combined with 100 mL of SDA (Sabouraud Dextrose Agar) to achieve the desired concentrations (6.25, 12.5, and 25 mg/mL). Three replicates were made for each concentration. The mixture of extract and SDA is put onto Petri plates and allowed to harden under sterile conditions.
(2) Mycelial growth from a 7-day fungal culture was placed in the middle of each plate at 28°C, and the vaccinated plates were incubated.

Table 3: Effect of *Agaricus bisporus* mushroom crude phenolic extract at different concentrations on fungal growth or colony on SDA at 28°C ± 2°C

Concentration (mg/mL	Mean ± SE		
	Fungal diameter (cm) of *Aspergillus niger*	Fungal diameter (cm) of *Aspergillus flavus*	Fungal diameter (cm) of Penicillium
Control	8.67 ± 0.33[a]	8.67 ± 0.33[a]	8.67 ± 0.33[a]
6.25 mg/mL	1.63 ± 0.18[b]	1.47 ± 0.03[b]	1.50 ± 0.05[b]
12.50 mg/mL	1.43 ± 0.07[b]	1.47 ± 0.03[b]	1.23 ± 0.03[b]
25.00 mg/mL	1.23 ± 0.03[b]	1.23 ± 0.03[b]	1.17 ± 0.08[b]
LSD	0.634**	0.551**	0.564**
P value	0.0001	0.0001	0.0001

**($P \leq 0.01$)

Mean = a,b; control = a; SE = standard error

(3) After 7 days, fungal growth's diameters were measured and each concentration's antifungal activity was particular by measuring growth inhibition.[18]

Statistical analysis

The statistical analysis system (SAS, 2018) program was used to detect the effect of difference factors in study parameters. Least significant difference –LSD test (analysis of variation-ANOVA) was used to significant compare between means. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study.

Ethical approval

The study was carried out in conformity with the moral standards set forth. Before the sample was taken, it was done with the patients' verbal and analytical consent. A local ethics committee examined and approved the study protocol, subject information, and permission form on January 15, 2023 in accordance with Ref.: CSEC/0123/0053.

Results

Fungal isolation

The results of fungal isolation from patients showed that the types were *A. niger*, *A. flavus* and *Penicillium* spp. The morphological description *A. niger* the vesicle is spherical, and the coccyx is composed of two rows, the first is short and the second is branched, resembling a bottle, the colonies are black.

The morphology of the *A. flavus*, the vesicle is oval, the coccyx is composed of one row of the type of bottle shape, and the colonies are yellowish-green in color. The structure of the colonies of the fungus *Penicillium* spp colonies are yellowish-green. *Penicillium* spp rubens are oval, blue or bluish-green in color, with smooth walls. As shown in Figures 1–3.

The three PCR product samples [Figure 4] were sent for sequence analysis; of some fungi species and 50 µL (10 pmol) from the forward primer for this gene was submitted to the Macrogene business to evaluate the DNA sequencing in these genes. The result of the sequence analysis was analyzed by blast search in the National Centre Biotechnology Information (NCBI) and BioEdit program to detect polymorphism. After sequencing analysis on the blast website, acquired results allowed to determine some fungi ear at the species level. The result indicated the presence of three different species which is *A. niger*, *A. flavus* and *Penicillium* spp. The three species alignments with universal isolate recorded on BLAST program showed 100% identification and 0% gaps [Figures 5–8].

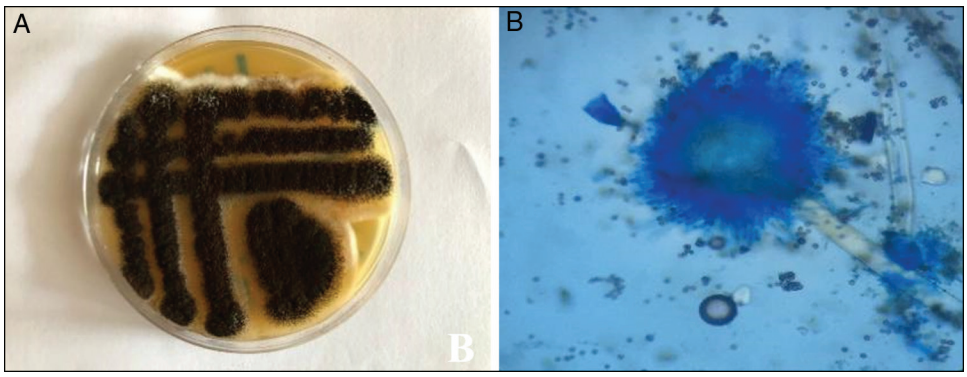

Figure 1: *Aspergillus* species grown on SDA after 7 days of incubation at 28°C ± 2°C: (a) Top view: *Aspergillus niger* (b) microscopic feature of *Aspergillus niger* (40×)

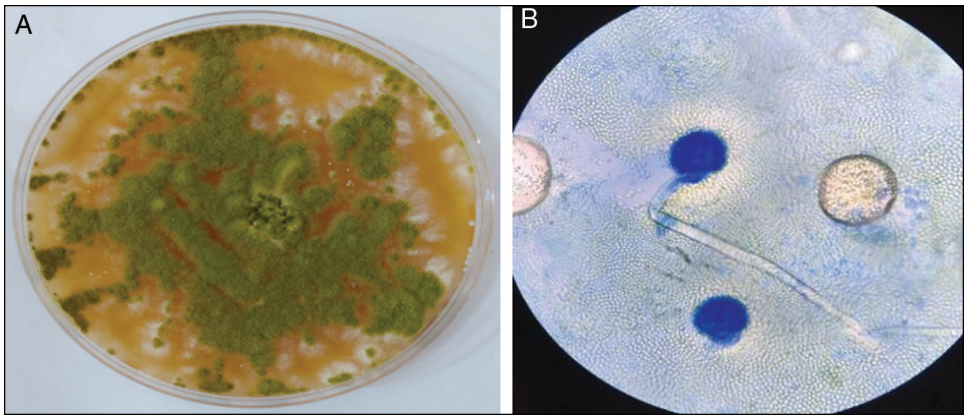

Figure 2: *Aspergillus flavus* grown on SDA after 7 days of incubation at 28°C ± 2°C: (a) Top view (b) microscopic feature of *Aspergillus flavus* (40×)

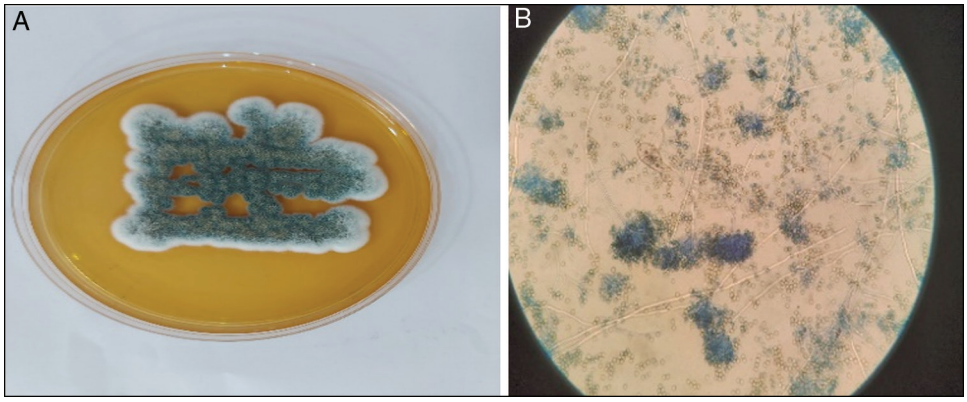

Figure 3: *Penicillium* grown on SDA after 7 days of incubation at 28°C ± 2°C: (a) Top view (b) microscopic feature of *Penicillium* spp (40×)

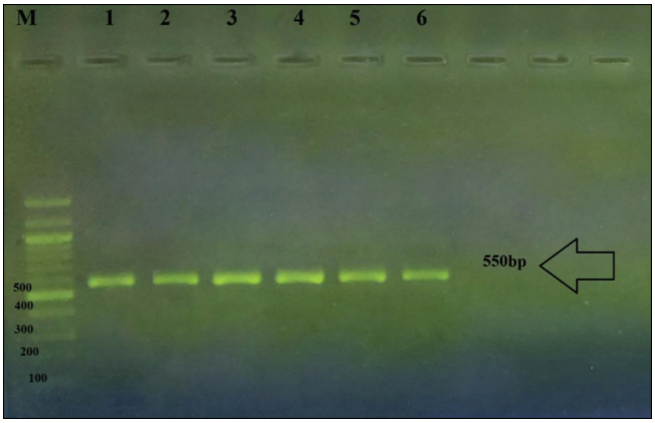

Figure 4: Agarose gel electrophoresis PCR product (550 pb) of ITS. The product was on 1.5% agarose at 5 V/cm². 1× TBE buffer for 1:30 h. M: DNA ladder (100 pb).

The active substance (phenols) was extracted from the *A. bisporus* fungus, and concentrations (6.25, 12.5, and 25 mg/mL) were prepared and then tested against otomycosis using the evaluation of the antifungal activity of the extracts and the inhibition of fungal growth calculated individually. The results showed that the inhibitory activity of the phenolic extract against *A. niger* at a concentration of 6.25 mg/mL showed the lowest inhibitory activity, as the diameter of the colony reached (1.63 ± 0.18 cm), while the concentration of 25 mg/mL showed the highest inhibitory activity, as the diameter of the colony reached (1.23 ± 0.03 cm). The results showed that the inhibitory activity of the phenolic extract against *A. flavus* at a concentration of 6.25 mg/mL showed the lowest inhibitory activity, as the colony diameter reached (1.47 ± 0.03 cm), while the concentration of 25 mg/mL showed the highest inhibitory activity, as the diameter of the colony reached (1.23±0.03 cm) [Table 3]. The results showed that the inhibitory activity of the phenolic extract against *Penicillium* spp at a concentration of 6.25 mg/mL showed the least inhibitory activity, as the colony diameter reached (1.50 ± 0.05 cm), while the concentration was 25 mg/mL. It showed the highest inhibitory activity, with the colony diameter reaching (1.17 ± 0.08 cm) compared to the control (8.67 ± 0.33 cm).

Discussion

Otomycosis is a worldwide disease, in which appropriately 20% of ear infections are caused by fungi and yeasts, Otomycosis is more prevalent because of high humidity, hot weather, and dust in the environments.[19,20] Common symptoms of otomycosis are itching, ear discharge, ear pain, blocking sensation, decreased hearing, and tinnitus.[21,22] In the current study, ear discharge was the more common symptom followed by tinnitus immunocompromised patients having otomycosis were commonly associated with comorbid illnesses like a common cold, cough, diabetes, high blood pressure, and heart problems. The present study, we recorded 60% of *Aspergillus* species, which was correlated with several authors worldwide.[23] *Aspergillus niger* found in this study was 60%, *A. flavus* found was 20% and 20% of *Penicillium* species.

There was a difference in the isolation of fungi in the present study when compared to other studies which may be due to geographical variation.[24] In comparison to females, where more males infected where the percentage was (51.25%) in males and (48.75%) in females.[25] Samples were subjected to direct microscopic examination to identify fungi species the otomycosis isolates were identified at molecular level using PCR.[26] HPLC method is significant to separate and quantify the main drug and any reaction impurities.[27]

The results of HPLC showed the presence of six biological compounds of the fungus used.[28] The active substance (phenols) was extracted from the fungus *A. bisporus*.[29-31] The results indicated that the highest content of total phenols was found in Apigenin. The main phenolic compounds were: Apigenin, Gallic acid, Caffeic acid, Kaempferol, Frulic acid, and Rutin.

The results of the inhibitory activity of the phenolic extract against *A. niger* and *A. flavus* showed that the

Score	Expect	Identities	Gaps	Strand
1068 bits(1184)	0.0	595/597(99%)	0/597(0%)	Plus/Plus

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Sbjct  557  ............................................................  616

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Sbjct  617  ............................................................  676

Query  541  AGAGGATGCTTTGGGTGCGGCCCCCGTCTAAGTGCCCTGGAACGGGCCGTCAGAGAG  597
Sbjct  677  ..........................................................  733
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Figure 5: *Aspergillus niger* strain DUCC5709 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: MT582749.1Length: 1156 Number of Matches: 1 Range 1: 137 to 733 GenBank Graphics Next Match Previous Match

concentration of 25 mg/mL showed the highest inhibitory activity. The results Also showed that the inhibitory activity of the phenolic extract against *Penicillium* spp, at a concentration of 25 mg/mL showed the highest inhibitory activity compared to the control. When the crude phenol extracted from *A. bisporus* concentration increased above 25 mg/mL, the sporulation of the fungi blocked [Figures 9–11].

Conclusion

Otomycosis exists in all ages, especially middle ages, and also in both genders Common symptoms of otomycosis, ear discharge, ear pain, blocking sensation, itching decreased hearing, and tinnitus. The immunocompromised patients have otomycosis. and *Aspergillus* was the most common pathogen in otomycosis. We conclude that the phenol crude extracted from *A. bisporus* exhibited significant antifungal activity by decreasing in diameter of fungi and inhibiting the sporulation process.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

Score	Expect	Identities	Gaps	Strand
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Figure 6: *Aspergillus flavus* isolate DTO 213-I2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence, Sequence ID: MH279408.1 Length: 887 Number of Matches: 1 Range 1: 270 to 862 GenBank Graphics Next Match Previous Match

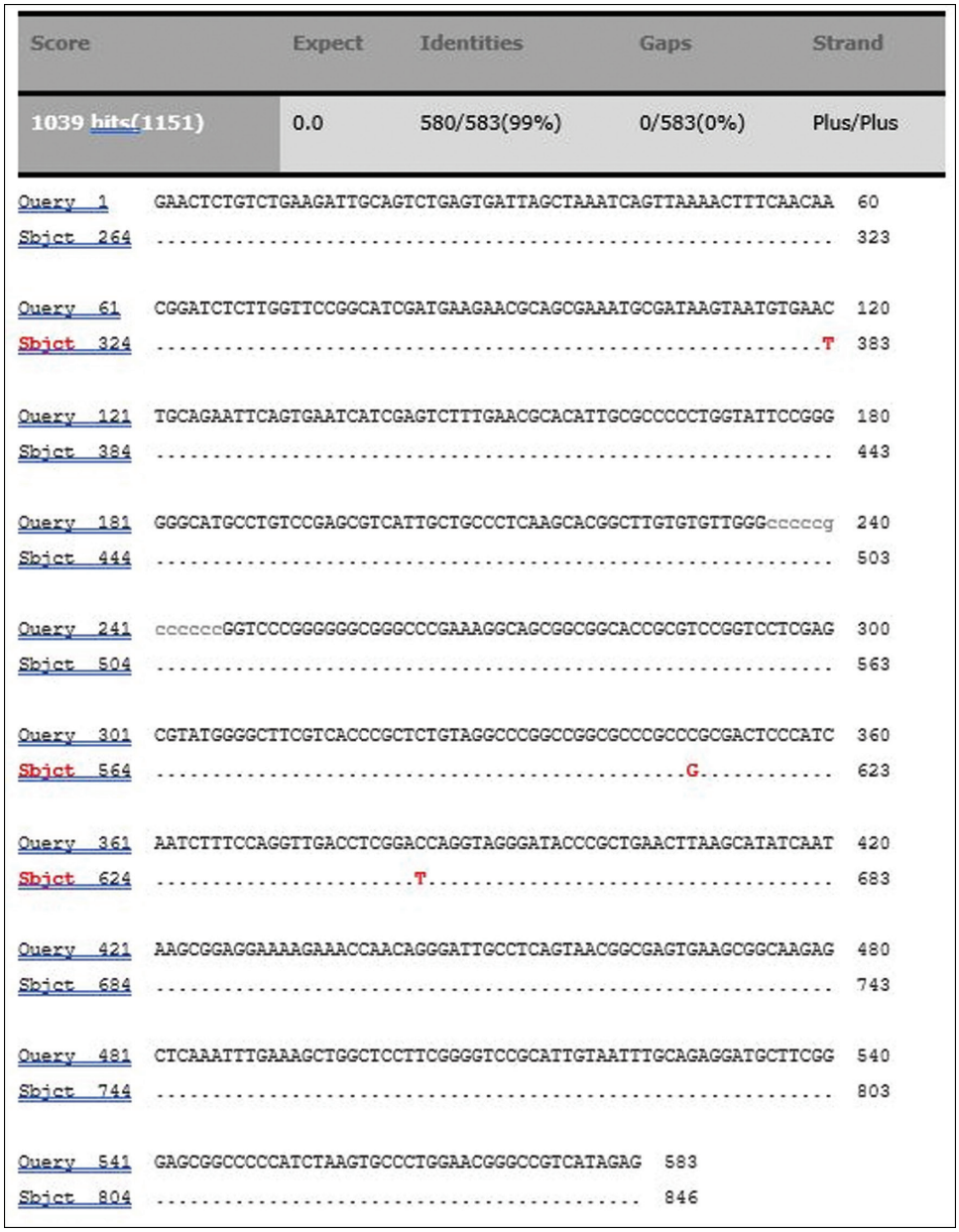

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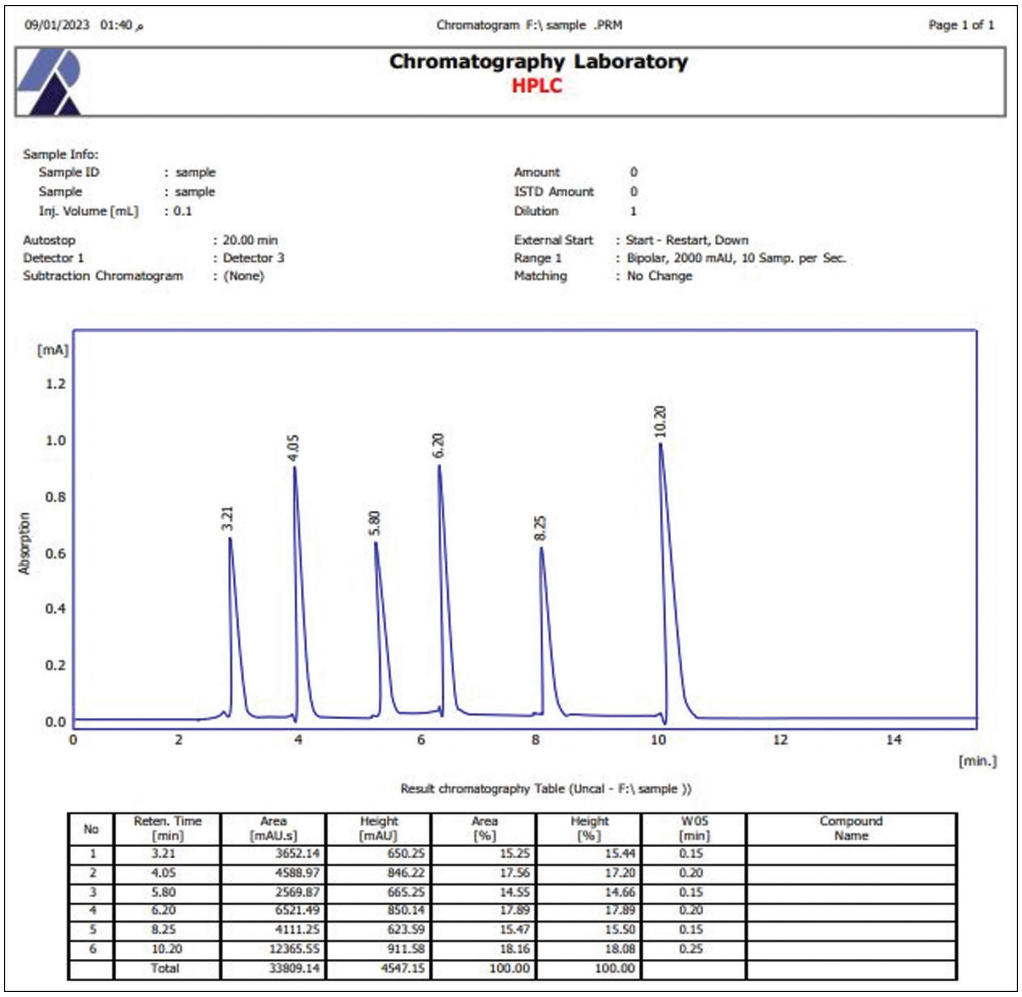
Figure 7: *Penicillium* spp. strain CMV003F5 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence



No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	3.21	3652.14	650.25	15.25	15.44	0.15	
2	4.05	4588.97	846.22	17.56	17.20	0.20	
3	5.80	2569.87	665.25	14.55	14.66	0.15	
4	6.20	6521.49	850.14	17.89	17.89	0.20	
5	8.25	4111.25	623.59	15.47	15.50	0.15	
6	10.20	12365.55	911.58	18.16	18.08	0.25	
	Total	33809.14	4547.15	100.00	100.00		

Figure 8: The detection of the active compounds of *Agaricus bisporus* mushroom extract by the HPLC technology

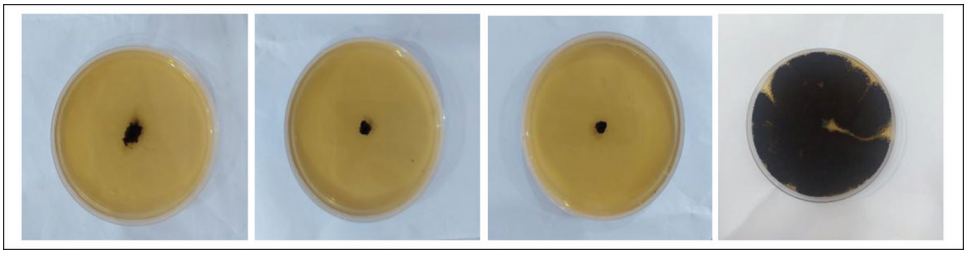


Figure 9: The effect of crude phenol extract for *Agaricus bisporus* mushroom on *Aspergillus niger* growth

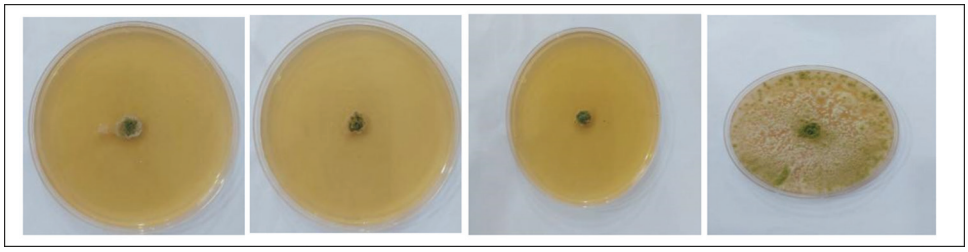


Figure 10: The effect of crude phenol extract for *Agaricus bisporus* mushroom on *Aspergillus Flavus* growth

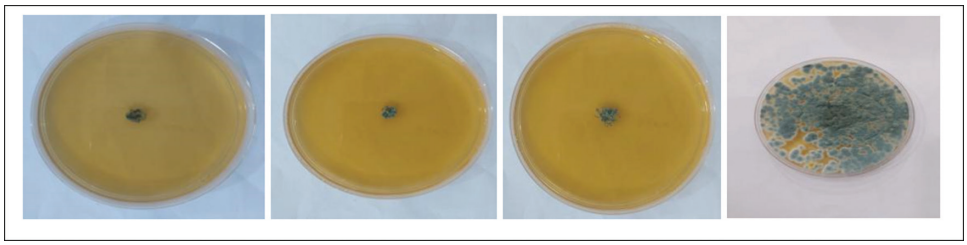


Figure 11: The effect of crude phenol extract for *Agaricus bisporus* mushroom on Penicillium spp, growth

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