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Isolation and Identification of *Aspergillus niger* Causes Otomycosis and Treatment by Phenolic Extract of *Agaricus bisporus*

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Abstract

This study aimed to isolate and characterize fungus *Aspergillus niger* and treated by crude phenolic extract from *Agaricus bisporus* mushroom that contains a bioactive compound to treat fungi. Thirty samples were taken of suffered patients with otomycosis. Phenol extract was performed by HPLC technology, Molecular diagnosis of fungal isolation by PCR technique. Results show that the microscopic and culture examination on sabouraud dextrose agar medium showed that the fungal isolates *A. niger* had a percentage of (60%). The molecular diagnosis of fungal isolates using the polymerase chain reaction technique. HPLC showed being present of six biologically active substances in *A. bisporus*. Active substance (phenols) was extracted from the fungus *A. bisporus*, and the concentrations were prepared (6.25, 12.5, 25 mg/ml) and then tested against *A. niger* fungus using the assessment of antifungal activity, the antifungal activity of the extracts and the inhibition of fungal growth were calculated individually. The outcomes demonstrated that the extract's inhibitory activity Phenolic against *A. niger* a concentration of 6.25 mg/ml showed lowest inhibition activity, the diameter of the colony was (1.63 ± 0.18cm), while the concentration of 25 mg/ml was the maximum of inhibitory activity, the diameter of the colony, was (1.23±0.03 cm) compared to the control (8.67 ± 0.33 cm).

Keywords: *Aspergillus niger*, Otomycosis, *Agaricus bisporus*, Phenolic, HPLC technique

1. Introduction

Otomycosis is a fungal infection of the external auditory canal that can be superficial, subacute, or chronic. It occasionally has consequences that affect the middle and inner ear. Prickling is the most common symptom of fungal-induced otomycosis (1). The most widespread variety of *Aspergillus* is *Aspergillus niger*. *Aspergillus niger* derives its name from the Latin aspergillum, meaning "holy water sprinkler," due to its resemblance to a sprinkler under a microscope. Black mold can frequently be caused by it. Additionally, it has milder opportunistic traits than other species, giving it the nickname "black mold," and it is more likely to cause pneumonia in impaired people. Some strains of *Aspergillus niger* produce mycotoxins (2). A capable microbial cell factory for the synthesis of organic acids is *Aspergillus niger* (3). The *Aspergillus* genus contains more than 340 distinct species (4).

Most instances of aspergillosis occur in immunocompromised patients (5). Extrapulmonary aspergillosis includes keratitis and sinusitis (6). These infections, however, are less serious and frequently treatable with antifungal medications. Here, we describe a rare instance of *Aspergillus niger* otomycosis-related left central tympanic membrane perforation. Citric acid has been manufactured industrially using the filamentous fungus *Aspergillus niger*; current output exceeds 2 million tonnes annually (7). *Aspergillus niger* is popular due to its simplicity in cultivation and tolerance of common industrial fermentation conditions (8). An ideal organism for use in industries. In addition to its current applications, *Aspergillus niger* also has the ability to create succinic and other important compounds (9) and contains significant quantities of oxalic and citric acids (10).

Plants produce phenolic chemicals primarily to aid in their development, growth, and defense. During the interactions between the plant's biotic and abiotic stresses, these aromatic benzene ring molecules play a crucial role. They are a crucial component of the secondary metabolites found in plants and are crucial for a number of physiological and mechanical processes. They might serve as pathogen and insect repellents as well as attractants for helpful organisms (11).

One of the most popular edible mushrooms worldwide, *Agaricus bisporus* has a long history of being beneficial for people's health. People commonly consume the mushroom as a food item. It is also known as a champignon, button mushroom, or white mushroom. *Agaricus bisporus* has a lot of metabolites and other biologically active substances, such as indole, phenolic compounds, fatty acids, sterols, statins, vitamins, trace elements, minerals, and simple sugars or carbohydrates (12).

2. Materials and Methods

2.1. Sample Collection

Thirty samples of *Aspergillus niger* from different ages and genders were collected for the period between December 2022 and March 2023. The samples were cultured on SDA media for the purpose of isolation and the initial diagnosis of pathogenic fungi.

2.2. Identification of *Aspergillus niger*

In this investigation, *Aspergillus niger* was identified based on its morphology (13). This identification relied on the subsequent: Colonial characteristics (color, significant pigment) and colony retreats (color, significant pigment). Microscopic structure (microconidia and macroconidia: their shape, arrangement, and hyphal structures). Multiple Lactophenol cotton blue was used to show spores made up of big, septate macroconidia and microscopic, single-celled microconidia on samples from distinct fungal development sites.

2.3. Preparation of Phenolic compounds

Phenolic compounds were extracted using ultrasound from a homogenized plant sample (3 g) using an ethanol/water (70/30) solvent. The extraction process was placed for an hour at room temperature utilizing an Ultrasonic Bath (USA). 5 mL of the liquid extract was used to determine the extraction yield after filtering. In Slovenia, the solvent was extracted using a rotary evaporator while under vacuum, and it was then dried at 40°C to a consistent mass. In order to prevent oxidative damage until analysis, dry extracts were kept in the glass bottles at 4°C. By using a Zorbax Eclipse Plus-C18-OSD, Chemstation, and a SYKAMN HPLC chromatographic system equipped with a UV detector, reversed-phase HPLC analysis was used to quantify specific phenolic chemicals in a 4.6mm column, 25 cm. The gradient elution method used a column with an ambient temperature of 30 ^{o}C (14).

2.4. Determination of total phenolic compounds.

Using a typical Folin-Ciocalteu reagent, the total amount of phenolic components in the ethanolic extract was calculated. 100 l of the extract, 500 l of the Folin-Ciocalteu reagent (Merck, Germany), and 1.5 ml of 20% sodium carbonate made up the reaction mixture. After being blended using a vortex mixer, the sample was finally diluted with distilled water to a final amount of 10 ml. After a two-hour process, the 765 nm absorbance was measured, and the phenolic content was estimated using a calibration curve created with gallic acid (Sigma-Aldrich, Germany). Gallic acid equivalent (GAE) was used to express the total amount of phenolic compounds per gram of dry weight (15).

2.5. Preparation of different concentrations of plant extracts

Phenols extracts were prepared by dissolving a certain weight of each sample extract according to the concentrations (6.25, 12.5, 25) mg/ml of sample extracts that were prepared using the dilution equation following (16):

(C1V1 = C2V2)

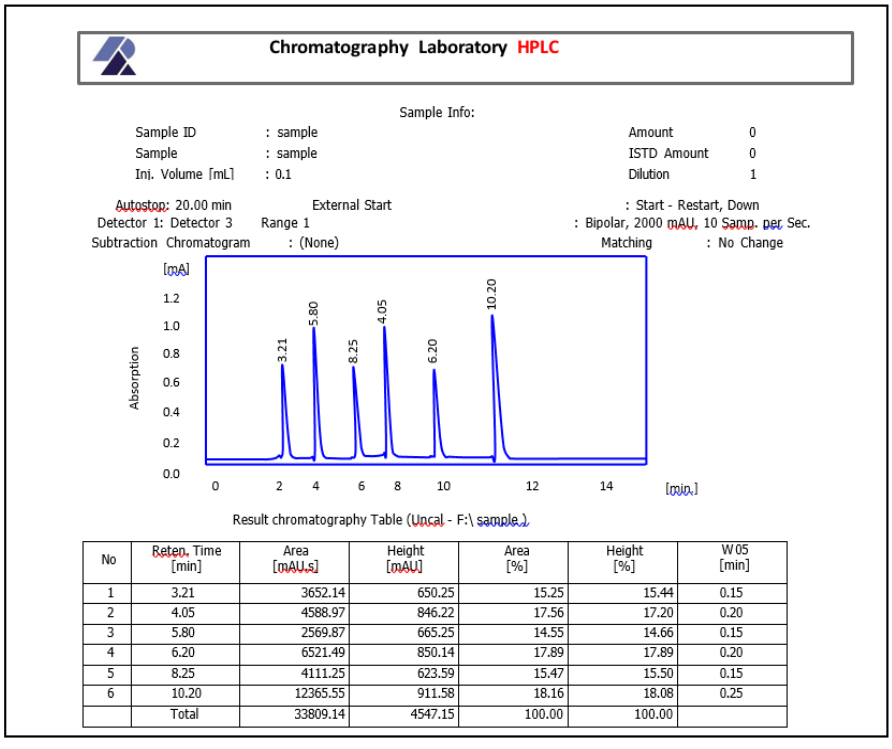
2.6. Determination of Minimal Inhibitory Concentration of *Agaricus bisporus* extract on isolated Otomycosis:

The appropriate concentrations (6.25, 12.5, 25 mg/ml) of phenol extract were obtained by making different volumes, which were then separately combined with 100 ml of SDA (Sabouraud Dextrose Agar). For each concentration, three replicates were created. The extract and SDA combination is added to Petri plates and allowed to solidify while being kept sterile. millimeters of mycelial growth from a 7-day fungal culture were placed in the middle of each plate at 28°C, and the inoculated plates were incubated. fungal growths After seven days, measured diameters were taken and, each concentration's antifungal activity was determined by measuring the growth inhibition (17).

3. Results

3.1. Chromatography Laboratory HPLC

The result of extracting the compounds included *Agaricus bisporus* mushroom phenolic compounds by HPLC technology as shown in **Figure (1).**



No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W 05 [min]
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 1. The detection of the active compounds of *Agaricus bisporus* extract by the HPLC technology.

HPLC results showed the presence of six (6) biologically active compounds in *Agaricus bisporus*. Present phenolic compounds were estimated by HPLC analysis, and the antifungal characteristics of phenolic acids Several studies against fungi have also reported their presence, as well as human pathogenic fungi (18,19). The findings showed that Apigenin has the greatest concentration of total phenols. Major phenolic compounds were: Apigenin (62.15 ppm), Gallic acid (60.25 ppm), Caffeic acid (23.55ppm), Kaempferol (24.12 ppm), Ferulic acid (40.25 ppm) and Rutin (22.58 ppm), where total phenolic content (mg Gallic / 100 gm) (112.05) (**Table 1**).

Table 1. Total phenolic content,

Name	Con (ppm)	Name	Con (ppm)
Apigenin	62.15	Gallic acid	60.25
Caffeic acid	23.55	Kaempferol	24.12
Ferulic acid	40.25	Rutin	22.58
Total phenolic content (mg Gallic / 100 gm)			112.05

Otomycosis was diagnosed, namely *Aspergillus niger*, with a percentage of (60%) as in **Table (2)**.

Table 2. Frequency and percentage of Otomycosis isolated from patients.

Otomycosis	No	Percentage (%)
Aspergillus niger	30	60.00

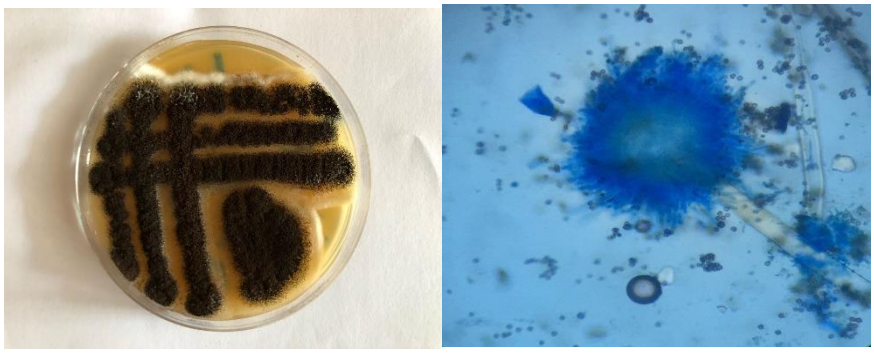
Aspergillus niger was the most prevalent fungus species isolate that causes Otomycosis globally (20, 21, 22). The most common appearance was *Aspergillus niger* (60%) (**Figure 2**). The current study showed that the gender factor affected the extent of infection (**Table 3**). The rate of infection for the age of ≤ 10 years is in **Table (4).**

Table 3. Distribution of patients with Otomycosis according to gender**.**

Otomycosis species	No	Male No. (%)	Female No. (%)	P-value
Aspergillus niger	30	16 (53.33%)	14 (46.67%)	0.782 NS

Table 4. Distribution of patients with Otomycosis according to age.

Clinical type	Age (year)				
	10-20	21-30	31-40	41-50	Total
Aspergillus niger	3	10	10	7	30



A **B**

Figure 2. *Aspergillus* species grew on SDA after seven days of incubation at 28±2°C. (A): Top view: *Aspergillus niger*. (B): microscopic feature of *Aspergillus niger* (40 X).

3.2. .Identification of the fungi by PCR

PCR (polymerase chain reaction) has been effective in detecting a wide range of fungi and could be an effective diagnostic tool. This outcome is comparable to those of earlier research on the identification of fungus using sequencing analysis of the ITS region, which provides faster, more accurate, and more reliable diagnosis at the species and subspecies levels compared to traditional laboratory methods (23) (**Table 5** and **Figure 3**).

Table 5. Ribosomal RNA gene(ITS).

Gene : 5.8S ribosomal RNA gene(ITS)						
No. of sample	**Type of substitution**	**Location**	**Nucleotide**	**Source**	**Sequence ID with compare**	**Identities**
1	Transversion	310	G\T	*Aspergillus niger*	ID: MT582749.1	99%
	Transition	385	C\T			

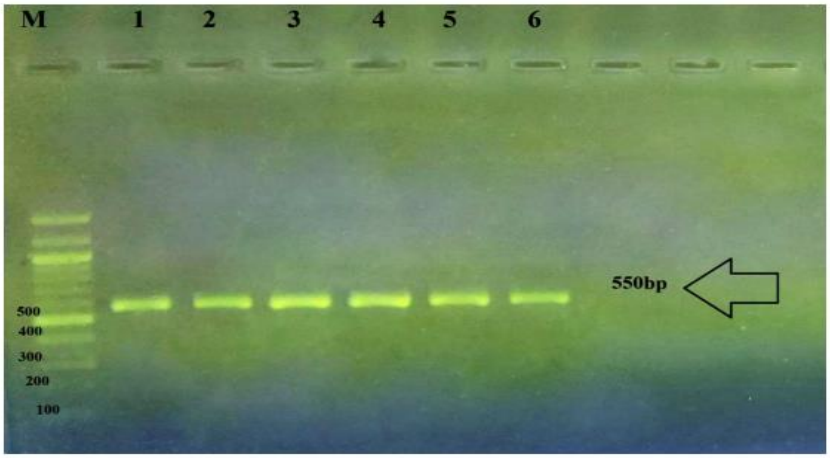


Figure 3. PCR product the band size .The product was electrophoresis on 1.5% agarose at 5 volt/cm2. 1x TBE buffer for 1:30 hours. M: DNA ladder (100).

The active substance (phenols) was extracted from the fungus *Agaricus bisporus*, and the concentrations were prepared (6.25, 12.5, 25 mg/ml) and then tested against the *Aspergillus niger* fungus using the evaluation of the extracts' antifungal activity and the calculation of the amount of fungal growth inhibition. The results demonstrated that the extract phenolic inhibitory activity against *Aspergillus niger* was lowest at a concentration of 6.25 mg/ml, with a diameter of inhibition of (1.63±0.18 cm), and highest at a concentration of 12.5 mg/ml, with a diameter of inhibition of (1.43 ± 0.07 cm), and highest at a concentration of 25 mg/ml, with a diameter of inhibition of (1.23 ± 0.03 cm), compared to the control (8.67 ±0.33 cm) (**Table 6** and **Figures 4 and 5**).

Table 6. Effect of *Agaricus bisporus* mushroom crude phenolic extract at different concentrations on fungal growth or colony on SDA at 28±2 °C.

Concentration (mg/ml)	**Mean ± SE**
	Fungal diameter(cm) of *Aspergillus niger*
Control	8.67 ±0.33 a
6.25 mg/ml	1.63 ±0.18 b
12.5 mg/ml	1.43 ±0.07 b
25.00 mg/ml	1.23 ±0.03 b
LSD	0.634 **
P-value	0.0001

** (P≤0.01).

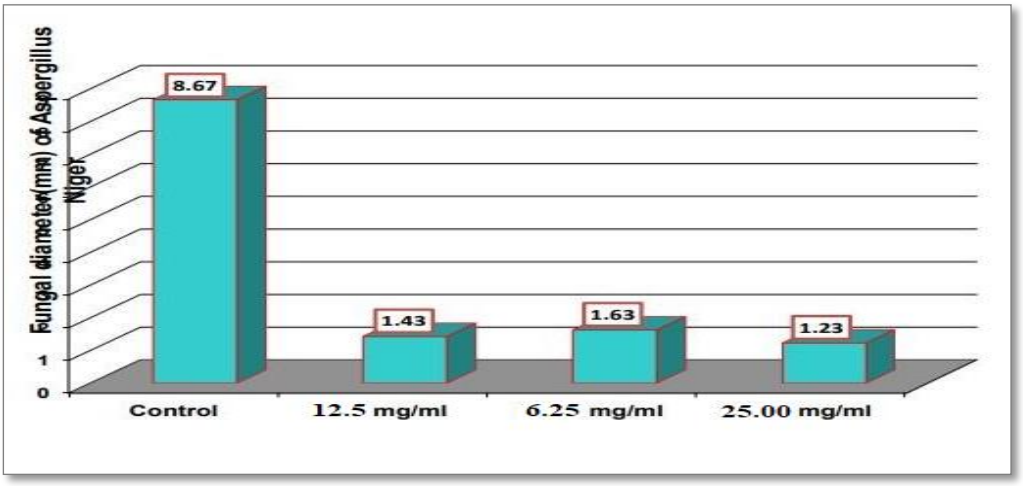


Figure 4. The effect of crude phenol extract for *Agaricus bisporus* mushroom on *Aspergillus niger* growth**.**

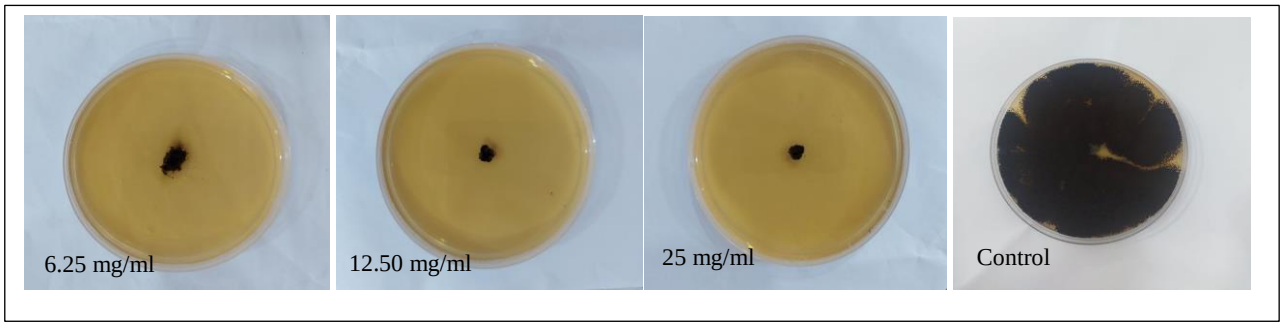


Figure 5. Effect of *Agaricus bisporus* mushroom phenolic extract at different concentrations on fungal growth or colony on SDA at 28±2 °C//*Aspergillus niger*.

4. Discussion

Aspergillus niger is one of the most widespread species; they may flourish even in nutrient-poor conditions. To prevent the spread of spores, *Aspergillus niger* and other filamentous fungus must be handled carefully (24). Ear fungi can develop as a result of spore dust contamination of the ear canals. The quantity of proteins and carbohydrates, humidity, and temperature in the human auditory canal create an excellent habitat for the growth of these fungi (25). Immune-compromised individuals (26). The ear canal is thought to be primarily colonized by *Aspergillus* (27). According to our study, males are more likely than females to contract a fungus (53% of males and 46% of females, respectively). Risk factors include being exposed to tainted water, plucking at one's ears frequently, and persistent otitis media. It results in symptoms like tinnitus, hearing loss, otalgia, otorrhea, and fullness in the ears (28, 29). The edible fungus known as *Agaricus bisporus*, sometimes known as the white mushroom, is widely used throughout the world. Results showed the inhibitory activity of the phenol extract against *Aspergillus niger*, and a concentration of 25 mg/ml was most effective, as the diameter of the inhibition was (1.23 ± 0.03 cm).

5. Conclusion

In this study, *Aspergillus niger* was identified as the most common agent of otomycosis. and accurate identification of *Aspergillus niger* by morphological characteristics. The results showed the inhibitory activity of the phenolic extract against *Aspergillus niger* and that a concentration of 25 mg/ml was most efficient.

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Conflict of Interest
The authors declare that they have no conflicts of interest.

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