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ORIGINAL STUDY

Evaluation of Antifungal Properties of *Shiitake* and the Effects of Its Extracts on Some Pathogenic Fungi

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ABSTRACT

The present study aims to investigate the inhibitory effect of shiitake mushrooms on some common fungi found in the soil of Mosul City. The fungal extract from Shiitake was prepared using ethanol or water. Different concentrations of the extract were prepared to analyze the antifungal properties of the mushroom by first making a stock solution and then adding specific amounts to the plates containing (Potato Dextrose Agar) PDA medium to achieve the desired concentrations. The active components found in Shiitake were pinpointed through the use of HPLC. The findings revealed how the aqueous extract of Shiitake affected Trichoderma sp. Penicillium sp. with the impact intensifying as the extract concentration levels rose. In the case of Rhizopus sp. and Aspergillus sp. the influence was noticeable, at a concentration of 0.5 units. Increased with concentrations. Furthermore the ethanolic extract exhibited efficacy against Trichoderma sp., Penicillium sp. and Rhizopus sp. At concentration levels leading to a decrease in growth diameter as the concentration increased. As for Aspergillus sp. its impact was more pronounced at concentrations. Analysis using HPLC revealed that phenolic compounds such as Chlorogenic Acid, Quercetin, Caffeic Acid, Lutine and Vanillic Acid were identified, along with their retention times and concentrations in mg/g for both types of extracts. Notably Vanillic acid and Caffeic acid demonstrated effectiveness compared to compounds present in Shiitake mushrooms. In conclusion, it is evident that shiitake mushrooms contain levels of compounds particularly vanillic acid and caffeic acid, which play a vital role, in combating fungal infections.

Keywords: Fungi, Mushroom, Phenolic, Shiitake, Vanillic acid

1. Introduction

In the realm of exploration, there is a quest to discover substances that can impede the proliferation of pathogenic fungi serving as ecofriendly alternatives, to synthetic antifungal agents, in farming and industrial settings (da Cruz Cabral, Pinto, and Patriarca, 2013). Various fungi such as *Trichoderma sp.*, *Penicillium sp.*, *Rhizopus sp.*, and *Asperigllus sp.* are among the microorganisms that cause health damage to humans, animals, and plants (Imran et al., 2021). For instance, *Trichoderma sp.* causes many difficult-to-treat diseases and is typically found in the soil, leading to respiratory, skin, gastrointestinal, eye infections, and, in rare cases, central nervous system infections such as meningitis (Oliveira et al., 2023). *Rhizopus sp.* causes several diseases, particularly in immunocompromised individuals, including mucormycosis, which can affect the nasal and sinus regions and spread to the brain, more common in individuals with

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uncontrolled diabetes, pulmonary mucormycosis affecting the lungs in immunocompromised patients such as cancer patients undergoing chemotherapy, and cutaneous mucormycosis occurring through skin wounds or burns (Monika and Chandraprabha, 2022). *Penicillium sp.* can cause several diseases, particularly in immunocompromised individuals or specific environments, including fungal pneumonia, sinusitis from spore inhalation leading to congestion, pain, and headache, skin infections, otitis media, and allergic reactions causing symptoms like sneezing, itching, and rash (Azzam, Cayme, and Martinez, 2020).

While known for producing antibiotics like penicillin, some species can be pathogenic under certain conditions. *Aspergillus sp.* causes various diseases, especially in immunocompromised individuals, including pulmonary aspergillosis affecting the lungs with symptoms like cough, fever, and shortness of breath, and sinusitis leading to congestion and pain (Russo et al., 2020). Mushrooms are generally regarded as useful products with multiple applications in the food and health industry. Shiitake mushrooms, scientifically known as Lentinula edodes. Given the advancements, in research and concerns like resistance (Ahmad et al., 2023). The mushrooms are valued for their rich nutritional content boasting a variety of essential minerals such as potassium, magnesium, manganese, iron and phosphorus along with vitamins. They serve as a source of nutrients due to their abundance of both macro and micronutrients coupled with beneficial compounds, like polysaccharides, antioxidants, dietary fiber and ergosterol (Usman, Murtaza, and Ditta, 2021).

Bioactive compounds can be helpful for maintaining the good health of the users and preventing them from diseases. Shiitake mushroom contains phenolics, polysaccharides, sterols, that play role in bodily functions resulting in improved health of the individuals (Ahmad et al., 2023). Shiitake mushrooms (*Lentinula edodes*) are generally ranking as the second largest edible mushroom, with increased production and usage over recent years. Shiitake mushrooms not only provide nutrition but also offer various health benefits, with numerous reports highlighting their nutritional and health advantages ranging from health improvement to disease prevention (Amin et al., 2022). The discovery of antibiotics stands as one of the significant breakthroughs of the century, aiding medical sciences in treating fatal diseases, now treatable with antibiotics and antifungals. However, the emergence of resistance poses a serious challenge to medical sciences.

The present study aims to investigate the inhibitory effect of shiitake mushrooms on some common fungi found in the soil of Mosul City.

2. Materials and methods

2.1. Isolation of fungi

To isolate types of fungi (*Trichoderma sp.*, *Penicillium sp.*, *Rhizopus sp.*, *Aspergillus sp.*) from the soil, the following steps were taken:

Collection of Soil Samples: Soil samples were collected from two different areas in Mosul City.

1. **Preparation of Fungal Suspension:**

The serial dilution method was used to prepare fungal suspensions from soil samples. Concentrations from 10^{-1} to 10^{-10} were prepared. 1 ml of each concentration, particularly from dilutions 10^{-3} and 10^{-10}, was taken.

2. **Culturing of Fungi:**

1 ml of each dilution was cultured on PDA (Potato Dextrose Agar) medium. Streptomycin was added to the medium at a concentration of 1 mg/l to prevent bacterial growth.

3. **Distribution of Suspension:**

After adding the microbial suspension to the medium, the plate was swirled to the right and left to evenly distribute the available components.

4. **Incubation:**

Plates were incubated at a temperature of 26.5°C for 7 days. Plates were monitored every 3 to 7 days to observe fungal growth.

5. **Separation of Fungi:**

After the incubation period, the fungi growing on the medium were separated. Fungi were identified based on morphological and microscopic characteristics.

2.2. Preparation of fungal extract from Shiitake for biological evaluation

Edible *Shiitake* mushrooms were used to prepare a fungal extract to evaluate their biological properties. The following steps were taken to achieve this:

1. **Sample Preparation:**

25 grams of dried mushroom powder were carefully weighed.

2. **Extraction Using Petroleum Ether:**

The dried mushroom powder was mixed with 200 ml of petroleum ether solvent at room temperature. The mixture was placed on a magnetic stirrer for 72 hours to ensure the extraction of petroleum

ether-soluble compounds. After the extraction period, the mixture was filtered using filter paper to separate the liquid from the solid material. The liquid extract was transferred to a rotary vacuum evaporator to remove the petroleum ether under reduced pressure, yielding the crude extract. The raw extract was dried in an oven at a temperature, below 40˚C to keep the compounds intact.

2.3. Extraction using ethanol

200 ml of 70% ethanol was mixed with the leftover material from the petroleum extraction process. The stirring and filtration steps were repeated, similar to the petroleum phase. The ethanol extract was moved to a vacuum evaporator to eliminate the ethanol under reduced pressure followed by drying of the extract in an oven at a temperature not exceeding 40˚C.

2.4. Extraction using water

Following ethanol extraction, 200 ml of water was introduced to the remaining material. The mixture underwent stirring on a stirrer within a temperature range of 40 and 60˚C, for extracting water compounds. After filtration, the aqueous extract was concentrated using a vacuum evaporator. Then dried in an oven at a temperature not surpassing 40˚C.

2.5. Sterilization of Shiitake extracts

Steps Taken to Ensure the Safety of *Shiitake* Fungal Extracts. To guarantee the safety of *Shiitake* Fungal extracts and prevent contamination the following measures were implemented for sterilizing both alcoholic extracts;

1. High Concentration Sterilization:

The aqueous and alcoholic extracts underwent sterilization using a water bath set at 50˚C for a duration of 15 minutes.

2. Microfiltration Sterilization:

For this method, a 0.22 micron membrane filter (Millipore) was employed to sterilize the alcoholic extracts through the disc and well technique as outlined by Al Saadi in 2015.

2.6. Antifungal properties assessment of Shiitake extracts

In order to prepare the concentrations of extracts for assessing their antifungal efficacy the following procedures were carried out;

1. Preparation of Stock Solution;

A gram of the extract was dissolved in 5 ml of a solvent to achieve a concentration level of 200 mg/ml.

2. Preparation of Desired Concentrations;

By adding volumes (0.5 ml, 1 ml, 1.5 ml 2 ml, 2.5 ml), from the stock solution, to Petri dishes containing PDA medium various required concentrations were prepared efficiently. The dishes liquid volume was adjusted to reach extract concentrations of 19.5 mg/ml, 19 mg/ml 18.5 mg/ml 18 mg/ml and 17.5 mg/ml. This method allows for assessing the properties of extracts, at different concentrations to determine their effectiveness and find the ideal concentration for antifungal activity.

2.7. Using high performance liquid chromatography (HPLC) active compounds in Shiitake were identified as follows

1. Preparation of Samples;

Dried extracts were processed to create solutions with concentrations using solvents like methanol or distilled water.

2. HPLC Analysis of Samples;

The prepared samples were injected into an HPLC system with a detector (UV VIS). Chromatographic columns filled with phases were utilized for analyzing fungal compounds.

3. Operational Settings;

Operating parameters such as flow rate, column temperature and mobile phase composition were adjusted for compound separation. The mobile phase typically comprised a blend of solvents and water, in varying ratios.

2.8. Detection and analysis

Chromatographic data (chromatograms) for each sample were collected, showing peaks representing different compounds in the extract. Retention times and other characteristics of the peaks were compared with known standards to identify the active compounds. This method identified the biologically active compounds in the *Shiitake*extracts using HPLC, contributing to the understanding of the chemical composition of the mushroom and evaluating its potential biological properties.

2.9. Statistical analysis

SPSS software was used and ANOVA was employed to compare the means between different groups (various extract concentrations on different types of fungi). Values represent the mean ± standard deviation. This provides an idea about the data distribution around the mean and the degree of dispersion. The data were then subjected to LSD test to find the least significant difference.

3. Results

3.1. Observations from (Table 1)

- Trichoderma sp.: The effect increases with concentration from 0 to 2.5, where the diameter of fungal growth ranges from 5.00 ± 0.00 to 0.00 ± 0.00.
- Penicillium sp.: The diameter of fungal growth decreases with increasing concentration, ranging from 7.00 ± 0.00 to 0.00 ± 0.00.
- Rhizopus sp.: The effect is noticeable at higher concentrations, starting at 1.5 with a diameter of 1.57 ± 0.15 and reaching 0.00 ± 0.00 at 2.0 and 2.5.
- Asperigllus sp.: The effect starts at a concentration of 0.5 and continues to increase with higher concentrations, reaching 0.00 ± 0.00 at 2.5.

Table 1. Effect of aquatic extract on the tested fungi species.

Fungi Type	Concentration (mg/ml)	Fungi Type Effect	Extract Effect
	0	0.5	1.0
Trichoderma sp.	5.00 ± 0.00 ih	4.60 ± 0.26 hg	4.63 ± 0.15 hg
Penicillium sp.	7.00 ± 0.00 k	3.30 ± 0.10 f	2.70 ± 0.20 e
Rhizopus sp.	9.00 ± 0.00 n	9.00 ± 0.00 n	2.73 ± 0.15 e
Asperigllus sp.	7.67 ± 0.58 l	5.57 ± 0.15 j	4.60 ± 0.10 hg

3.2. Observations from (Table 2)

- Trichoderma sp.: There is a noticeable effect at lower concentrations, with the diameter of fungal growth ranging from 6.67 ± 0.58 to 0.00 ± 0.00.
- Penicillium sp.: The effect is similar to the aquatic extract, with the diameter of fungal growth decreasing with increasing concentration until it reaches 0.00 ± 0.00.
- Rhizopus sp.: A noticeable effect is seen at concentrations of 1.5 and 2.0, with the diameter of fungal growth decreasing with increasing concentration until it reaches 0.00 ± 0.00.
- Asperigllus sp.: The effect is clear at higher concentrations, with the diameter of fungal growth decreasing with increasing concentration until it reaches 0.00 ± 0.00 at 2.5.

Table 2. Effect of ethanol extract on the tested fungi species.

Fungi Type	Concentration (mg/ml)	Fungi Type Effect	Extract Effect
	0	0.5	1.0
Trichoderma sp.	6.67 ± 0.58 k	4.57 ± 0.06 g	3.30 ± 0.10 f
Penicillium sp.	8.33 ± 0.58 m	3.30 ± 0.10 f	2.70 ± 0.20 e
Rhizopus sp.	9.00 ± 0.00 n	9.00 ± 0.00 n	9.00 ± 0.00 n
Asperigllus sp.	8.33 ± 0.58 m	5.10 ± 0.17 i	4.83 ± 0.21 ihg

3.3. Observations from (Table 3)

- The most significant effect is observed at higher concentrations (2.0 and 2.5), where the diameter of fungal growth significantly decreases to 0.00 ± 0.00.
- The effect increases with concentration, indicating the effectiveness of the extract at higher concentrations.

Table 3. Concentration effect.

Concentration (mg/ml)	Effect on All Fungi
0	7.63 ± 1.35 F
0.5	5.55 ± 2.17 E
1.0	4.31 ± 2.02 D
1.5	2.14 ± 1.21 C
2.0	0.88 ± 0.78 B
2.5	0.00 ± 0.00 A

3.4. Observations from (Table 4)

- The analysis shows the concentration of various phenolic compounds in both ethanolic and aqueous extracts of Shiitake mushrooms.
- The phenolic compounds identified include Chlorogenic Acid, Quercetin, Caffeic Acid, Lutine, and Vanillic Acid, with their respective standard detention times and concentrations in mg/g for both extract types.

This data offers an insight into how extracts, from Shiitake mushrooms exhibit properties and the levels of active phenolic compounds present in these extracts.

4. Discussion

Clear from the findings that both water based and alcohol based Shiitake mushroom extracts effectively hindered the growth of fungi in the experiments. The impact of these extracts became more notable as their concentrations increased, particularly demonstrating effects at the concentrations (2.0 and

Table 4. Phenolic compounds in acid hydrolysis of Shiitake mushrooms.

Sequence	Phenolic Compounds	Standard Detention Time (min)	Concentration in Ethanolic Extract (mg/g)	Concentration in Aqueous Extract (mg/g)
1	Chlorogenic	4.087	3.03	3.03
2	Quercetin	5.257	2.76	2.76
3	Caffeic Acid	6.520	7.55	7.55
4	Lutine	7.323	2.91	2.91
5	Vanillic Acid	11.977	14.39	14.39

2.5 mg/mL) where fungal growth was completely stopped. *Trichoderma sp.* and *Penicillium sp.* Displayed sensitivity to the Shiitake extracts showing effects at elevated concentrations. *Rhizopus sp*. Exhibited sensitivity compared to species but its response to the extract was evident at higher concentrations. Similarly *Aspergillus sp.* Exhibited reduction in growth at concentrations. These findings suggest that Shiitake mushroom extracts contain components that could serve as antifungal agents. These components have potential for use in developing treatments for infections, especially those that are resistant to conventional medications (Kim et al., 2022). The Shiitake extract demonstrates an effect, on the growth of various fungi tested particularly at higher concentrations. Notably the water based extract shows a inhibitory effect compared to the ethanolic extract especially at lower concentrations. This implies that the Shiitake extract might contain ingredients of effectively stopping the growth of fungi. Higher doses (2.0 and 2.5) display an impact, on all tested fungal strains indicating the extracts ability to reduce fungal growth as concentration increases. The inhibitory effect escalates with concentrations suggesting that using increased amounts of the extract can lead to outcomes. To sum up the data in the table reveals that both aqueous and ethanolic Shiitake extracts exhibit an effect on the fungi tested, particularly at higher concentrations. These findings could be valuable for applications in controlling growth (Erdoğan Eliuz, 2022). It is worth noting that all five phenolic compounds listed in Table 4 exhibit concentrations in both aqueous extracts. This implies that extracting phenolics using either ethanol or water can be equally effective when isolating these compounds from Shiitake mushrooms (Sharpe et al., 2021).

There are variations in concentrations, among phenolic compounds. For instance, Vanillic Acid demonstrates the concentration (14.39 mg/g) whereas Quercetin shows the concentration (2.76 mg/g). This suggests that Shiitake mushrooms contain differing levels of phenolics reflecting their biochemical characteristics (Chaipoot et al., 2023). Phenolic substances such, as Vanillic Acid and Caffeic Acid have the ability to combat pathogenic fungi through several mechanisms (Rashad, Aseel, and Hammad, 2020; Alnuaimi and Alabdaly, 2023). They interfere with components of the cell wall of pathogenic fungi then weaken its structure and make it more prone to collapse (Oliveira Silva et al., 2022). These substances can also interact with proteins and fats in the cell membrane of pathogen causing changes in membrane permeability and leakage of contents ultimately resulting in pathogen cell death (Wang, Xu, and Liu, 2022). Additionally phenolic compounds can impede the activity of fungal enzymes for growth and reproduction disrupting key biological processes of the fungus (Rashad, Aseel, and Hammad, 2020). They also possess antioxidant properties that help mitigate damage caused by radicals, in cells ultimately leading to cellular damage and death. Some phenolic compounds are capable of inhibiting the respiratory chain, which halts energy production and leads to cell demise (Rahman et al., 2021). These combined mechanisms make phenolic compounds effective factors in combating fungi and inhibiting their growth and spread (Al Aboody and Mickymaray, 2020).

Using High-Performance Liquid Chromatography (HPLC) is considered an accurate and effective method for measuring concentrations of chemical compounds (Kanu, 2021). Precision in retention times and concentrations enhances the reliability of these results. The effects of aqueous and ethanolic Shiitake extracts on the growth of four fungal species (Trichoderma sp., Penicillium sp., Rhizopus sp., Aspergillus sp.) at different concentrations were measured. Effects are quantified by average fungal growth (in millimeters). In Tables 1 to 3, we analyzed the concentration of phenolic compounds in aqueous and ethanolic Shiitake extracts. These compounds are known for their antioxidant and antimicrobial properties, which help interpret the results in Table 4. The effect of phenolic compounds such as Vanillic Acid - distinguished by the highest concentration (14.39 mg/g) in both aqueous and ethanolic extracts - contributes to the high efficacy of these extracts in inhibiting fungal growth. This effect is clearly shown in Table 4, where we note that both aqueous and ethanolic extracts at high concentrations (2.0 and 2.5) completely eliminate fungal growth. Caffeic Acid

- at a concentration of 7.55 mg/g - also contributes to the inhibitory effect on fungal growth, especially at moderate concentrations (1.5 mg/g). This explains the significant reduction in fungal growth when using these concentrations in Table 4. Chlorogenic, Quercetin, and Lutein, with relatively lower concentrations, still contribute to the overall effectiveness of the extracts. We note that the effect of these compounds becomes more apparent at lower concentrations (0.5 and 1.0 mg/g).

Aqueous extracts show strong effects on fungal growth with a noticeable decrease in growth with increasing concentration, leading to complete elimination of growth at concentrations of 2.0 and 2.5 mg/gThe research shows that aqueous extracts are effective, in stopping the growth of fungi because they contain levels of compounds like Vanillic Acid and Caffeic Acid. Although ethanol extracts also have an effect they seem slightly less potent than aqueous extracts at certain concentrations. This difference could be due to how phenolic compounds are extracted or how ethanol affects the fungi themselves (Rahman et al., 2021; Alabsy and Alabdaly, 2022). The findings confirm that phenolic compounds, Vanillic Acid and Caffeic Acid play a role in preventing fungal growth. These compounds are recognized for their properties, which help protect against infections (Zulhendri et al., 2021). The effectiveness of the extracts is dependent on their concentration. As the concentration increases, the impact on fungi becomes more pronounced leading to growth reduction and complete elimination in some instances. The strong inhibitory properties of Shiitake extract can be harnessed for industrial purposes to combat fungi. Future research can delve into identifying compounds in the extract and assessing their effects on types of fungi.

5. Conclusion

This study highlights that Shiitake mushrooms contain levels of compounds that can be efficiently extracted using ethanol or water. These compounds, Vanillic Acid and Caffeic Acid play a role, in safeguarding against fungal infections making Shiitake extracts promising for various biological and medical uses. These discoveries could help improve the utilization of Shiitake mushrooms, in health and nutrition related contexts because of their antioxidant and antifungal characteristics.

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Conflicted interest

None.

Ethical approval

All ethical approvals necessary to complete this study were obtained from College of Education for Girls University of Mosul, Mosul, Iraq.

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