# Study the inhibitory effect of *Grifola frondosa* mycelium extract in some types of organisms growth that cause bread spoilage

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## Abstract:

This study aims to study the effect of *Grifola frondosa* fungus hyphae extract at different concentrations. Hyphae (10, 20, 30, and 40 g) were transferred to 100 ml of sterile distilled water once and to 100 ml of 70% ethyl alcohol again. The culture media used in the experiment were prepared according to their purpose (PDA, NA, NB) and according to the instructions of the supplier company. The alcoholic (ethanol 70%) and aqueous extract of *Grifola frondosa* fungus hyphae were also prepared by transferring (10, 20, 30, 40) g/100 ml of alcohol or water, each separately. The effect of the two extracts on bacteria *(Eshericha coli, Bacillus cereus* and *Staphylococcus aureus*) and fungi (*Aspergillus niger, Fusarium oxysporum, Rhizopus stolonifer*) that cause bread spoilage was studied. A qualitative chemical detection of the most important active compounds in the two extracts was carried out by detecting tannins, glycosides, flavonoids, saponins, alkaloids, steroids-terpenoids, anthraquinones, carbohydrates and proteins. A completely randomized design (CRD) with three replicates was used and means were compared according to the least significant difference (LSD) test at $P \leq 0.05$. The results showed that flavonoids and steroid-terpenoids were more prominent in the 70% ethyl alcohol extract, while tannins, glycosides, alkaloids, steroid-terpenoids, carbohydrates and proteins were more prominent in the aqueous extract. Saponins and anthraquinones were not detected chemically in either extract. The results also showed that the concentrations (10, 20 and 40 g/100 ml) slightly inhibited the bacteria and fungi under study. The concentration of 30 g/100 ml for both extracts showed a clear effect in increasing the diameter of the halo of the inhibition zone. This study demonstrated the potential of using alcoholic and aqueous extracts at a concentration of 30g/100ml as a natural preservative and inhibitor of the growth of some bacteria and fungi that cause bread spoilage. Therefore, they can be considered a promising application in the bakery industry and a potential replacement for the synthetic preservatives used in baked goods.

**Keywords**: Grifola frondosa fungal hyphae, alcoholic and aqueous extract, microbial inhibition, bread, mycelium extract

## Introduction:

One of the oldest food industries that accompanied the development of human civilizations is the bread industry. It is one of the most widely consumed food products and a staple food for most countries around the world, especially developing countries, due to its nutritional value, ease of preparation in bakeries and homes, and the variety of its types (17). However, it is a highly perishable food due to its exposure to the growth of microorganisms such as fungi and bacteria, which causes a loss of its quality and nutritional value, negatively affecting its suitability for human consumption (9). Bread spoilage is one of the most important economic and food problems due to its significant financial losses and waste of food resources (10). This, in turn, has motivated researchers to find effective, sustainable, and safe solutions to extend the shelf life of bread without affecting its nutritional value or the health of the individual consuming it (19). Among the proposed modern solutions, the use of natural extracts, including extracts of

susceptible fungi, stands out. For food, as natural solutions to preservatives instead of chemical ones, due to the antimicrobial and antioxidant properties of these extracts. (3) Edible fungi, such as *Agaricus bisporus*, *Pleurotus ostreatus*, and *Grifola frondosa*, are known to contain a wide spectrum of active bioactive compounds such as polyphenols, beta-glucans, terpenoids, and flavonoids, all of which contribute to the fungal activity. The effectiveness of these compounds increases depending on the extraction method, as the nature of the solvent used (water or alcohol) affects the quality and quantity of the extracted compounds, which is reflected in the strength of the inhibitory activity against microorganisms (5). Recent studies have shown that aquatic fungal extracts contain hydrophilic compounds such as polysaccharides, while hydrophobic compounds such as phenols and some terpenoids are concentrated in alcoholic extracts. Therefore, the effectiveness of each extract may vary depending on its chemical content, which calls for a comprehensive comparative study to determine the best in terms of inhibiting microorganisms that cause bread spoilage (4). Microorganisms that cause bread spoilage, particularly mold species (*Aspergillus*, *Penicillium*, *Fusarium*, and *Rhizopus*), are a major problem, as they grow rapidly in humid, warm conditions. Bacterial spoilage, however, is less common and is often caused by the growth of *Bacillus* species. In order to contribute to providing safe, natural solutions to extend the shelf life of bread and reduce reliance on artificial preservatives, in line with public health trends and the growing global demand for natural and healthy food products, the importance of the current research lies in its aim to study the effect of mycelium extract of the fungus (*Grifola frondosa*) in inhibiting the growth of some types of organisms that cause bread spoilage. This research aims to study the effectiveness of *Grifola frondosa* extracts, both aqueous and alcoholic, in inhibiting the growth of these microorganisms, using bioactivity testing techniques.

**Materials and Methods:**

• Preparation of culture media: These were prepared according to the instructions of the supplier (HIMDIA):

- Potato dextrose agar (PDA): Prepared by dissolving 39 g/L of distilled water. This medium was used to activate and produce mycelium of the fungus Grifola frondosa and to estimate the diameter of the inhibition zone of alcoholic and aqueous extracts of Grifola frondosa hyphae on isolates of mold species (Aspergillus niger, Fusarium oxysporum, and Rhizopus stolonifer). Plates were incubated at 25°C. Hyphal growth was monitored for three days. - Nutrient agar (NA) medium: prepared by dissolving 28 g/L of distilled water. It was used to estimate the diameter of the inhibition zone of Grifola frondosa fungal hyphae extract in Eshericha coli, Bacillus cereus, and Staphylococcus aureus bacteria (14).

- Nutrient broth (NB) medium: prepared by dissolving 18 g/L of distilled water to activate Eshericha coli, Bacillus cereus, and Staphylococcus aureus isolates. The media were sterilized at 121°C for 15 minutes and a pressure of 15 psi (pounds per inch) (MacFaddin, 2000). The media were cooled to approximately 55°C and distributed into disposable Petri dishes with a diameter of 8.5 cm, with 25 ml per dish. The dishes were inoculated with bacteria and incubated at 37°C. For 18 hours (22)

- Method for preparing Grifola frondosa hyphae extract:

Grifola frondosa hyphae plates grown on PDA were scraped under the optimal conditions specified in this study. (10, 20, 30, and 40 g) of hyphae were transferred to 100 ml of sterile distilled water once and to 100 ml of ethyl alcohol (70%) again in separate test tubes to prepare the aqueous and alcoholic extract concentrations, respectively. They were mixed until homogeneous using an electric mixer. The cells were ultrasonicated at 20 Hz for 8 minutes, then centrifuged at 1370xg. The resulting mixture was filtered through 0.4-mesh filter paper, then through a membrane filter with 0.22-micrometer openings to sterilize it from microbial contaminants (6).

6- Effect of aqueous and alcoholic extracts on bacteria and fungi that cause bread spoilage:

N.A. medium for bacteria and PDA. medium for fungi were prepared, poured into plates, and left to solidify completely. Activated bacteria and fungi that cause bread spoilage were densely streaked on the N.A. and PDA plates, each separately, on the surface of the medium. Several holes were made in each plate using a cork punch, and 5 microliters of each concentration of alcoholic and aqueous extracts of *Grifola frondosa* hyphae were transferred to each hole. The plates were then incubated at 37°C for bacteria and at 25°C for fungi for 48 hr. The average diameter of the halo formed around the holes in the plate was measured (12). The results were compared with holes filled with 5 microliters of distilled water. The results were recorded by measuring the diameter of the inhibition zones in millimeters using a regular ruler.

7- Qualitative chemical detection of the most important active compounds in the hyphae extract of the fungus *Grifola frondos*.

The methods developed by (11) were followed to detect tannins, glycosides, flavonoids, saponins, alkaloids, steroid-terpenoids, anthraquinones, carbohydrates, and proteins, which were carried out in the current research:

1- Tannin detection:

2 grams of hyphae were boiled in 10 ml of distilled water, the solution was filtered, and then allowed to cool. The solution was divided into two parts. To the first part, a 1% lead acetate solution was added to indicate the presence of tannins by the formation of a gelatinous precipitate. To the second part, a 1% ferric chloride solution was added. A bluish-green color was observed, indicating the presence of tannins. 2- Detection of glycosides:

Mix equal parts of Fehling's reagent A and B with the extract and heat in a boiling water bath for 10 minutes. The presence of glycosides was determined by the presence of sugars, as seen in a red precipitate.

3- Detection of alkaloids:

Boil 2 g of hyphae with 10 ml of 4% hydrochloric acid. Filter the solution after cooling, and test 0.5 ml of the filtrate in a watch glass with each of the following reagents:

Mayer's reagent: A white precipitate indicates the presence of alkaloids.

Dragendroff's reagent: An orange precipitate indicates the presence of alkaloids.

4- Detection of flavonoids:

Solution (A) was prepared by dissolving 2 grams of hyphae in 1 ml of 95% ethyl alcohol, then filtering the solution. Solution (B) was prepared by adding 2 ml of 50% ethyl alcohol to 10 ml of 50% potassium hydroxide solution. When equal amounts of each solution were mixed, the appearance of a yellow ring indicated the presence of flavonoids.

5- Detection of saponins:

Add 2 ml of cold distilled water to 2 ml of the aqueous extract, shaking continuously for one minute. The presence of saponins was indicated by the appearance of bubbles.

6- Detection of steroids-terpenoids: Lieberman-Borchard test:

Place 2 ml of fungus hyphae extract in a test tube, then add 1 ml of chloroform. Slowly add 1 ml of concentrated sulfuric acid to the wall of the tube. Observe for the appearance of a purple or green color at the border between the two layers. The presence of steroids-terpenoids is indicated by the appearance of a purple or green color.

7- Detection of anthraquinones – Ammonia test:

Add 1 ml of ammonia ($NH_3$) solution to 2 ml of a methanol extract of fungus hyphae. Their presence is indicated by the appearance of a pink layer in the basal layer.

8- Detection of Carbohydrates – Molisch Test:

Add 2 drops of a 5% alpha-naphthol solution in methanol to 1 ml of aqueous hyphae extract, then slowly add 1 ml of concentrated sulfuric acid to the wall of the tube to form two layers. The appearance of a purple ring between the two layers indicates the presence of carbohydrates in the extract.

9- Detection of proteins (amino acids) Biuret test:

Add 1 ml of sodium hydroxide (NaOH) solution to 1 ml of aqueous hyphae extract, then add 2 drops of 1% copper sulfate ($CuSO_4$) solution to the mixture. We observed the solution turn purple, indicating the presence of proteins (amino acids).

Statistical analysis:

A completely randomized design (CRD) was used with three replicates. The means were compared using the least significant difference (LSD) test at a probability level of 0.05 (2).

## Results and discussion:

1- Results of qualitative chemical detection of the active ingredients in the *Grifola frondosa* hyphae extract:

Table (1) shows the results of qualitative chemical tests to detect the chemical nature and active ingredients present in the aqueous and alcoholic extracts of *Grifola frondosa* hyphae, compared to distilled water (treatment). The control group) showed that glycosides, tannins, carbohydrates, and proteins were distinctly present in the aqueous extract as well as in the alcoholic extract. The presence of alkaloids, flavonoids, and steroid-terpenoids was also clearly observed in the alcoholic extract as well as in the aqueous extract. Saponins and anthraquinones showed negative detection in both extracts. The nature of the solvent used to prepare the aqueous extract (water) was characterized by being a strong polar solvent with the ability to dissolve polar compounds such as carbohydrates, proteins, tannins, and glycosides. Therefore, these compounds appeared in the aqueous extract in a distinct manner. As for the alcoholic extract (ethanol 70%), it is less polar than water and has the ability to dissolve compounds with medium to low polarity such as alkaloids, flavonoids, and steroid-terpenoids. This may have provided an opportunity to detect them in the alcoholic extract more quickly and clearly. The cell wall structure of the *Grifola* hyphae may have *Grifola* frondosa compounds such as chitin and beta-glucan, which are substances that may hinder the release of some compounds unless a suitable solvent such as 70% ethanol is used. This in turn suggests that alcohol may penetrate the cell wall better to extract semi-polar compounds such as alkaloids and flavonoids. The absence of saponins and anthraquinones in both extracts may be due to their absence in the hyphae of the fungus *Grifola frondosa*, or perhaps their presence in very small quantities that cannot be detected by qualitative methods, or the type of saponins and anthraquinones present is insoluble in both solvents. These results are in agreement with (27) that highly polar compounds have the ability to dissolve in water more than other solvents. (25) also confirmed the presence of glycosides in the hyphae and fruiting bodies of some edible fungi such as Sinigrin, Glucoberin, Glucocleomin, Glucocapparin and Glucobrassicin, which play a role Essentially increases the speed and density of growth of hyphae of these fungi.

The presence of active chemical compounds in the aqueous and alcoholic extracts of *Grifola frondosa* hyphae is often attributed to their antimicrobial (both bacterial and fungal) properties. Tannins may play an important role by binding to proteins on the surface of bacterial and fungal cells that cause bread spoilage, disrupting the function of cell membranes and enzymes. They also create an unfavorable environment for microbial growth by precipitating proteins and inactivating essential enzymes within the damaged cell. Tannins have been shown to have antibacterial activity against bacteria such as E. coli and S. aureus, and even some fungi such as Candida albicans. Glucosides, on the other hand, produce toxic compounds upon decomposition (such as cyanogenic glycosides) and act as natural antibiotics that disrupt DNA synthesis and enzymes within the microbial cell. 24)

confirmed that alkaloids possess powerful antibacterial and antifungal properties by intertwining with DNA and RNA, which hinders DNA translation and replication. They also affect membrane permeability, increasing membrane permeability. This, in turn, stimulates the leakage of vital cell components out of the cell. Flavonoids (1) have been shown to inhibit the synthesis of nucleic acids and proteins and have the ability to interact with cell membranes, which contributes to the release of cellular contents. Regarding steroids and terpenoids, (23) confirmed that limonene (a terpenoid found in the essential oils of some plants) has an antifungal effect against Candida albicans by stimulating apoptosis in infected fungal cells. Limonene interferes with the structure of ergosterol, a key component of fungal cell membranes, disrupting cell membrane function and destroying the cell. Carbohydrates do not directly act as antimicrobials, but they may act as carriers for other active compounds. (13) reviewed the antibacterial effectiveness of chitosan in their study, confirming its antibacterial activity against both Gram-positive and Gram-negative bacteria. They also predicted that chitosan could be a promising alternative to traditional antibiotics in combating bacterial infections. (7) indicates that plant-source proteins have antimicrobial properties by disrupting the activity of enzymes or destroying cell membranes of *E. coli* and *Candida*.

**Table (1) Results of qualitative chemical detection of the active components in the hyphae extract of the fungus Grifola frondosa:**

| compound | Reagents used | Detection index | Test results for Grifola frondosa hyphae extracts | | |
|---|---|---|---|---|---|
| | | | Alcoholic extract | Aqueous extract | Distilled water |
| **Tannins** | Lead acetate 1% | Formation of a white, gelatinous precipitate | + | +++ | --- |
| | Fehlenck's reagent A | Appearance of a blue-green color | + | + | --- |
| **Glucosides** | Fehlenck's reagent B | Red precipitate | ++ | +++ | --- |
| **Alkaloides** | Mayer's reagent | White precipitate | ++ | ++ | --- |
| | Dragendroff's reagent | Orange precipitate | ++ | ++ | --- |
| **Flavonoides** | 95% ethyl alcohol + KOH | Appearance of a yellow ring | +++ | + | --- |
| Saponins | Cold distilled water | Appearance of air bubbles | – | – | – |
| Steroids-Terpenoids | Liebermann-Borchard test: | Appearance of a purple or green color | +++ | ++ | - |
| Anthraquinones | Add $NH_3$ to a methanolic extract → a pink layer appears in the basal layer | Color change indicates anthraquinones | - | - | - |
| Carbohydrates | Molich's test | Appearance of a purple ring | ++ | +++ | |
| Proteins/Amino Acids | Biuret's test | Appearance of a purple-blue color | ++ | +++ | |

2- Results of the effect of alcoholic and aqueous extract of mycelium of the fungus (*Grifola frondosa*) in inhibiting the growth of some types of bacteria that cause bread spoilage:

| Extract Type | Extract Concentration | Inhibition diameter/mm | | |
|---|---|---|---|---|
| | | *Eshericha coli* | *Bacillus cereus* | *Staphylococcus aureus* |
| Ethyl alcohol | 10 | 5 | 7 | 5 |
| | 20 | 7 | 13 | 9 |
| | 30 | 14 | 17 | 19 |
| | 40 | 8 | 11 | 11 |
| Aqueous extract | 10 | 2 | 5 | 4 |
| | 20 | 5 | 11 | 6 |
| | 30 | 7 | 13 | 15 |
| | 40 | 4 | 9 | 7 |
| Distilled water | 0 | 0 | 0 | 0 |
| L.S.D(0.05) | | 0.87 | 5.7 | 5.9 |

The results shown in Table (2) showed the effect of the alcoholic and aqueous extract of *Grifola frondosa* fungus at concentrations of (10, 20, 30, 40)% in inhibiting the growth of *Eshericha coli*, *Bacillus cereus*, and *Staphylococcus* aureus, which cause bread spoilage, and at a significant level ($p \leq 0.05$), it inhibited the growth of the three types of bacteria, albeit to varying degrees. The diameter of the inhibition zone for *Eshericha coli* in the ethyl alcohol extract reached (5, 7, 14, 8 mm), *Bacillus cereus* (7, 13, 17, 11 mm), and *Staphylococcus aureus* (5, 9, 19, 11 mm). As for the aqueous extract, they were (2, 5, 7, 4), (5, 11, 13, 9 mm), and (4, 6, 15, 7 mm), respectively. It seems that *E. coli* (Gram-negative) has a thin wall of peptidoglycan and an outer membrane rich in lipids and lipopolysaccharides, which means more resistance to polar substances (such as the aqueous extract), but *B. cereus* (Gram-positive) has a thick wall of peptidoglycan and does not contain an outer membrane, which indicates less resistance to the aqueous extract. *S. aureus* (Gram-positive) has a thick peptidoglycan wall containing teichoic acid, thus achieving moderate and sometimes high resistance (26). (15) confirmed that the aqueous extract of some edible fungal hyphae often contains polar compounds such as flavonoids and tannins, which are more effective against Gram-positive bacteria because the thick membrane does not impede the entry of these compounds. Therefore, *B. cereus* and *S. aureus* are more sensitive, while *E. coli* is more resistant due to the lipid-rich outer membrane. Therefore, it can be said that the 30% aqueous extract achieved the best results.

The alcoholic extract of the fungus *(Grifola frondosa)* hyphae presented another pattern of bacterial inhibition. The alcoholic extract (70% ethanol) achieved the extraction of more diverse compounds described as polar and non-polar, such as phenols, terpenes, and aldehydes. It can also dissolve components of the outer membrane of Gram-negative bacteria, which created a site Effective against all three species, the effect was on *S. aureus*, which was highly sensitive, *B. cereus*, which was moderately sensitive, and *E. coli* showed a greater effect compared to water, due to the ability of alcohol to dissolve lipids in the membrane. (6) confirmed that the effect of the alcoholic extract on Gram-positive bacteria was greater than on Gram-negative bacteria, represented in this experiment by *E. coli.* This indicates that some of the active components in the extract are transferred to the bacterial cells, including tannins, which have an inhibitory nature for microorganisms and are known to absorb water, making it unavailable

to bacteria growing on the medium in which they are found (19), thus deforming them and losing the basic function of the cell (20). Kazanji (2017) explained that the difference in the sensitivity of bacteria to aqueous or alcoholic extracts is due to the composition of the cell wall and the organization of the outer membrane of Gram-negative and Gram-positive bacteria. This is what some studies have shown that the plant extract has a greater effect on Gram-positive bacteria. Its effect on Gram-negative bacteria is due to differences in the outer layers of the cell wall of Gram-positive and Gram-negative bacteria. Gram-negative bacteria have exceptional outer membranes that are not found in Gram-positive bacteria. However, Gram-positive bacteria do not have such a structure for cell walls and outer membranes. Antibacterial substances easily damage the cell wall and cytoplasmic membrane, leading to cytoplasmic expulsion and coagulation, resulting in cell death.

2- Results of the effect of alcoholic and aqueous extracts of mycelium of the fungus (*Grifola frondosa*) on inhibiting the growth of some fungi that cause bread spoilage:

It is noted from Table (2) that the alcoholic extract (70% ethanol) had a strong effect on the growth of the fungus *Aspergillus niger*, as the diameter of the inhibition zone increased from 7 mm at a concentration of 10% to 15 mm at a concentration of 30%, then decreased to 9 mm at a concentration of 40%. Its effect on the fungus *Fusarium oxysporum* was The inhibition zone diameter increased from 5 mm at 10% concentration to 13 mm at 30% concentration, then decreased to 8 mm at 40% concentration. The fungus *Rhizopus stolonifer* had the greatest effect in increasing the inhibition zone diameter from 13 mm at 10% concentration to 20 mm at 30% concentration, then decreased to 11 mm at 40% concentration. The aqueous extract of *Grifola frondos* hyphae showed a weak effect on the growth of *Aspergillus niger*, as the inhibition zone diameter increased from 2 mm at 10% concentration to 7 mm at 30% concentration, then decreased to 6 mm at 40% concentration. For *Fusarium oxysporum*, the diameter of the inhibition zone increased from 2 mm at 10% concentration to 8 mm at 30% concentration, then decreased to 7 mm at 40% concentration. *Rhizopus stolonifer* demonstrated significant resistance to the aqueous extract, as the diameter of the inhibition zone increased from 4 mm at 10% concentration to 11 mm at 30% concentration, then decreased to 3 mm at 40% concentration. Distilled water (the control treatment) showed no effect on fungal growth, as all inhibition zones were zero. The results indicate that the alcoholic extract of *Grifola frondos* hyphae demonstrated a greater inhibitory effect on fungal growth than the aqueous extract. This is consistent with previous studies that reported that active compounds such as phenols and flavonoids are present at higher concentrations in alcoholic extracts, enhancing their antifungal activity. (21) indicated that alcoholic extracts of *Agaricus bisporus* were more effective against *Aspergillus flavus* than aqueous extracts. It is clear that increasing the concentration of the alcoholic extract up to 30% resulted in an increase in the diameter of the inhibition zone, indicating that the extract concentration plays an important role in enhancing antifungal activity. However, the decrease in activity at a concentration of 40% may be due to a toxic effect on the fungi or a change in the chemical composition of the extract at higher concentrations. *Rhizopus stolonifer* was more sensitive to alcoholic extracts, recording the highest diameter of the inhibition zone at a concentration of 30%. Meanwhile, *Aspergillus niger* and *Fusarium oxysporum* showed a lesser response, which may indicate differences in cellular composition or resistance mechanisms between these fungi (16). Natural extracts of *Grifola frondos* hyphae, especially alcoholic ones, may be a promising alternative to chemical fungicides, given their high efficacy and safety for human consumption. Despite all these results, further studies are necessary to determine the optimal concentration and application methods to ensure the safety of food consumed.

Table (2) Results of the effect of alcoholic and aqueous extracts of mycelium (*Grifola frondosa*) on inhibiting the growth of some fungi that cause bread spoilage.

| Extract Type | Extract Concentration | Inhibition diameter/mm | | |
|---|---|---|---|---|
| | | ***Aspergillus niger*** | ***Fusarium oxysporum*** | ***Rhizopus stolonifer*** |
| Ethyl alcohol | **10** | **7** | **5** | **13** |
| | **20** | **9** | **6** | **17** |
| | **30** | **15** | **13** | **20** |
| | **40** | **9** | **8** | **11** |
| Aqueous extract | **10** | **2** | **2** | **4** |
| | **20** | **5** | **3** | **8** |
| | **30** | **7** | **8** | **11** |
| | **40** | **6** | **7** | **3** |
| Distilled water | **0** | **0** | **0** | **0** |
| **L.S.D(0.05)** | | **3.5** | **9.7** | **5.3** |

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