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ISOLATION AND IDENTIFICATION OF FUNGI IN FRESH AND DRIED FRUITS AND THE POSSIBILITY OF CONTROLLING TOXIN-PRODUCING FUNGI USING *PLEUROTUS OSTREATUS* FILTRATE AND CALCIUM CARBONATE

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ABSTRACT

The current study aims to identify fungi associated with fresh and dried fruits available in local markets in Al-Diwaniyah city, in addition to detecting the aflatoxin B1-producing fungus *Aspergillus flavus* using high-performance liquid chromatography (HPLC) technology. Samples were collected from shops and local markets in Diwaniyah city and some of its districts during the period (February - May 2025) and several fungal species were isolated: *Aspergillus niger, A. flavus, Penicillium expansum, A. Parasiticus, Rhizopus stolonifer, P. italicum, A. carbonarius, A. ochraceus, A. fumigatus, A. terreus, Alternaria Alternate*. Significant differences were observed in fungal isolate frequencies between unsterilized samples compared to surface-sterilized fruit samples. Specific fungal species were characterized using both morphological (microscopic and macroscopic) and molecular methods to assess the aflatoxin-producing capacity of *A. flavus.* Phenotypically identification was performed using standard classification keys, while molecular confirmation was performed using polymerase chain reaction (PCR). HPLC results indicated that the highest concentration of aflatoxin B1 was due to the filter isolate *A. flavus* from the unsterilized dried grape sample, which was 215.9 ppb. The filtrate of *P. osteatus* and calcium carbonate demonstrated a significant ability to reduce the radial growth and dry weight of the isolated fungus. The combined treatment showed the strongest effect, achieving the highest inhibition of radial growth (74.8%) and dry weight (70.8%) at a concentration of 30%, surpassing the effectiveness of the filtrate alone (68.3% and 66.0%) and calcium carbonate alone (49.1% and 48.7%). These results highlight a clear synergistic interaction that enhances the antifungal efficacy.

KEYWORDS: Aflatoxin B1, HPLC, Fruit contamination, Fungal toxins, *A. flavus*, *P. ostreatus*, Calcium carbonate.

INTRODUCTION

Food contamination by fungi can cause a noticeable decline in the quality and even destruction of food. Deterioration of food and mycotoxin formation relies on several factors, such as the type of food and its constitution and handling and storage conditions.[1] The formation of mycotoxin by some species of fungi is compelling and more subtle to control their growth.[2] Mycotoxin is a toxic secondary metabolite produced naturally by some mold.[3] However, chief mycotoxin-producer fungi belonging to *Aspergillus spp* (produces aflatoxin).

Fresh and dried fruits are susceptible to microbial contamination, At any point throughout their development and preparation. Certain organisms may begin to grow before the fruits reach the processing facility under favorable environmental conditions, and they may continue to grow until the products are dried, at which point personnel and equipment may contaminate the finished goods. Mold can contaminate dry fruits and spread swiftly, even if only a small portion of fruit peel is contaminated.[4][5]

Food deterioration can be caused by microorganisms, with fungus being the most prevalent organisms that cause food rotting globally.[6] Not only can the presence of some fungus in food cause food to deteriorate, but eating this food can also cause fungal illnesses and other health issues.[7] Some molds naturally produce a toxic secondary metabolite called mycotoxin[8][9], which can have immediate or long-term negative effects on humans and animals, including oestrogenic, carcinogenic, mutagenic, teratogenic, and atherogenic effects.[10,11][12] *Aspergillus*, *Fusarium*, *Penicillium*, and *Alternaria* are among the few molds that produce mycotoxins; however, the main mycotoxin-producing fungi are *Aspergillus* species (which produce aflatoxin), Penicillium species (which produce ochratoxin), and Fusarium species (which produce T-2, HT-2 toxin, deoxynivalenol, nivalenol, zearalenone, and fumonisins).[13] Because they generate aflatoxin, a poisonous mycotoxin that has a negative impact on human health, *A. flavus* and *A. parasiticus* are especially significant for community health.[14][15] Human development is hampered by aflatoxin, which also weakens the immune system, raises the risk of cancer, and can be fatal in situations of severe acute exposure.[16][17] In ideal environmental conditions (favorable temperature and moisture), some of the toxigenic fungal species may have contaminated the dried fruit from the fields or spoiled it during drying and storage. These species produced mycotoxins like aflatoxin and ochratoxin, which when consumed by humans cause mycotoxicosis.[7],[18]

Mycotoxins are a class of low-molecular-weight metabolic toxins produced by certain molds, which have dangerous properties and are referred to as mycotoxins.[19] The contamination of certain fruits, such as figs, apricots, and fresh and dried grapes, with fungi and their toxins is a problem that concerns most developed countries, especially those without good storage conditions.[20],[21] Mycotoxins in food products pose a significant risk to human health.[22] According to the Food and Agriculture Organization of the United Nations (FAO), mycotoxin contamination could affect 25% of the world's food supply.[23] Mycotoxins have a low molecular weight, so the immune system is unable to distinguish and recognize them. These toxins are among the most potent known, and even low levels, which cause them to accumulate in the tissues of certain organs such as the liver and kidneys, can cause significant disturbances. These toxins are produced by the fungus *Aspergillus spp*., known for its ability to produce aflatoxins.[24] They were detected using HPLC analysis.

This study aims to examine the ability of *A. flavus* fungus to produce aflatoxin B1 from fresh and dried fruit in the markets of Diwaniyah city, and to study the possibility of controlling its growth using *P. ostreatus* fungus as a biological control agent, and calcium carbonate as a chemical resistance agent.

MATERIALS AND METHODS

Isolation and Identification of Fungi

From February to May 2025, samples of fresh and dried fruit were collected from local markets and stores in the province of Al-Diwaniyah and its districts. Each sample was divided into two groups. The first group was thoroughly cleaned with sterile distilled water, while the second group was surface-sterilized with sodium hypochlorite solution before being left to air dry at room temperature. The fungi were identified based on their morphological characteristics and classified at the genus and species levels. Macroscopic features, such as colony color, texture, and morphology, as well as the appearance of the culture plates' reverse sides, were initially used to make the identification. Additionally, structural characteristics such the size, shape, and arrangement of spores and sporangia were examined under a microscope. Additionally, the taxonomic categorization of the isolated fungi was confirmed by molecular identification utilizing Polymerase Chain Reaction (PCR) techniques.

Preparation of the Filtrate

A nutrient medium of potato dextrose broth (PDB), from HIMEDIA, India, was used to prepare filters of both *A. flavus* and *P. ostreatus* in glass beakers. Each beaker contained 100 mL of the medium, which was then autoclaved at 121°C and 15 pounds /inch2 for 15 minutes. After cooling, the antibiotic chloramphenicol was added to the medium at a concentration of 250 mg/L. Each beaker contained two 5 mm diameter fungal discs, seven days old. The beakers were incubated at 25°C for three weeks, with regular shaking every two days. The filtrates were filtered through 0.22 µm filters and stored at 4°C until use.

Detection of mycotoxins using HPLC technique

Detection of the toxic fungal filtrate

The test was conducted in the laboratories of the Department of Environment and Water, Ministry of Science and Technology, according to the method proposed by.[25] High-performance liquid chromatography (HPLC) was used to detect mycotoxins. A Skyamn model, manufactured in Germany, was used for the HPLC analysis. The transport phase consisted of a mobile phase of acetonitrile: distilled water (30:70). Vertical separation was performed using a C18-ODS column (25 cm × 4.6 mm) to separate the mycotoxins. A fluorescence detector (excitation wavelength 365 nm, emission wavelength 445 nm) was used to detect the mycotoxins. The flow rate of the transport phase was 0.7 mL/min. Large molecules and membranes from biological samples were broken down using ultrasound in 50 mL of methanol at a volumetric ratio of 70 mL to 40 mL in 30 mL of water, followed by centrifugation for 5 minutes. An immunochromatography column was used at a flow rate not exceeding 3 mL/min (the column was pre-treated with 20 mL of distilled water). The column was cleaned with 20 mL of distilled water to remove contaminants, and any remaining water was then air-dried. 1.4 mL of methanol was added to the column, and it was air-dried to obtain the extract. The extract was diluted with 2 mL of water, then passed through a 0.55 µm filter, and the filter was subsequently inserted into a high-performance liquid chromatograph (HPLC). The mycotoxin concentration was calculated according to the following equation C = m/V, where C is the concentration, m is the mass of the solute dissolved, and V is the total volume of the solution as described by.[25]

Polymerase Chain Reaction (PCR) Technique for Molecular Diagnosis of *A. flavus*

The PCR reaction was performed for molecular determination purposes, using GoTaq® G2 Green Master Mix from Promega, USA. A 25 µL final reaction volume including 12.5 µL of PCR Master Mix 2X, 1 µL of forward primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′), 1 µL of reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), and 4 µL of template DNA, and 6.5 µL of nuclease-free water. Initial denaturation at 95°C for five minutes, thirty cycles of denaturation at 95°C for thirty seconds, annealing at 58°C for thirty seconds, and extension at 72°C for one minute comprised the PCR conditions. A last extension step was carried out for five minutes at 72°C.

Antagonism evaluation of *P. ostreatus* against the fungus *A. flavus* in vitro

(The Dual culture technique)

The dual culture technique was used to cultivate the fungi in 9 cm diameter Petri dishes containing solid nutrient medium (PDA). The dish was divided into two equal halves. Using a cork punch, a hole was made in the first half, which was then inoculated with a 5 mm diameter disc of 7-day-old *P. ostreatus*. A hole was made in the middle of the second half of the dish, and a 5 mm diameter disc of *A. flavus* was then inoculated with a 7-day-old culture. The experiment was repeated three times. Control dishes contained the fungi of each species separately. The dishes were then incubated at 25°C for 7 days. The antagonism score for each fungus was then calculated according to a five-point scale described in reference.[26]

The effect of *P.ostreatus* filtrate, calcium carbonate and their interactions treatments on the radial growth of the fungus *A.flavus*

P. ostreatus filtrate was used as a biological resistance agent in different (10,20,30 mg/ml) concentrations, while calcium carbonate was used as a chemical control agent, using the Poisoned Food technique (PFT).[27] The biological and chemical factors were added in different concentrations and overlapped to the PDA culture medium, which was then poured into Petri dishes (9 mm) with three replicates for each concentration. The control dishes were left without any in addition, after the solidification of the medium, a hole was made in the middle and a disk of contaminated mushrooms (5 mm) was placed in it. The dishes were incubated at a temperature of 25 °C. After 7 days, the growth of the fungal colonies was observed and their growth rate was calculated by taking the growth rate of two perpendicular diameters of the developing colonies, and then the percentage was calculated to inhibit.

The effect of calcium carbonate, *P.ostreatus*, and their interactions on the dry weight of the fungus *A.flavus*

To evaluate the impact of the two treatments (chemical and biological and their interaction), The filtrate and calcium carbonate solution[28],were mixed with the liquid medium to determine the same concentrations in redial growth. The medium was distributed by 50 ml in each flask. 250 ml flasks were used for the experiment. Two tablets measuring 5mm were taken from the end of radial growth of the fungus *A. flavus* at the age of 7 days using a cork piercing put in each flask. And with three replicates for concentration were placed in the incubator for 7 days at a temperature of 25 °C. The control treatments were not supplemented in any addition. The Every two days and after the completion of the incubation period was through, flasks were stirred. During that time, various measurements were made of the mushrooms' dry weight the sensitive scale concentrations. Weighing the filter sheets and measuring before using them, the weight of the filter paper was subtracted from them before use (Weight of the filter paper after drying - the weight of the filter paper before use - the weight of the inoculum) The precise dry weight of the fungal growth in the liquid culture medium is obtained and the percentage of inhibition is then calculated.[29]

3. RESULTS

3.1 Fungal Isolation and Identification

The results showed the presence of several fungal species in fresh and dried fruit samples collected from markets in Diwaniyah Governorate and its districts. The identified fungi included: *A. niger, A. flavus, Penicillium expansum. A. parasiticus, Rhizopus stolonifer, P. italicum, A. carbonarius, A. ochraceus, A. fumigatus, A. terreus, Alternaria alternata*. The prevalence of fungi varied between sterile and non-sterile samples (Table 1). A. niger recorded the highest prevalence in both cases, reaching 17.72% in non-sterile samples and 20.29% in sterile samples.

Other fungal species showed lower frequencies, including *A. flavus* (15.18% and 17.99%), *P. expansum* (12.88% and 15.27%), *A. parasiticus* (10.70% and 12.13%), and *Rhizopus stolonifer* (9.23% and 2.51%) in non-sterile and sterile samples, respectively.

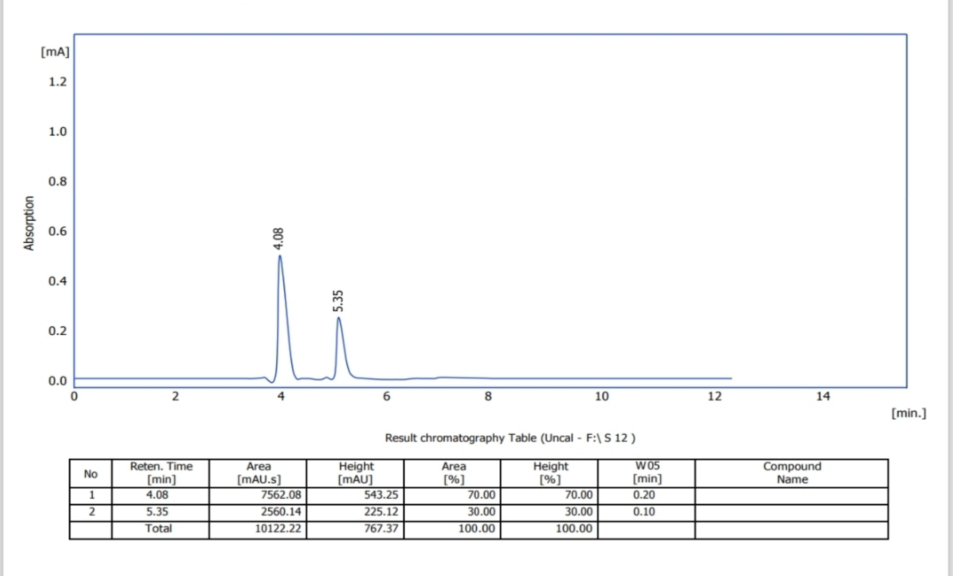
The fungi (*P. italicum, A. terreus, A. fumigatus, A. ochraceus, Alternaria alternata, and A. carbonarius*) were recorded at frequency rates of (8.74% and 7.11%, 3.91% and 3.34%, 5.52% and 4.81%, 5.98% and 6.06%, 2.64% and 2.51%, and 7.36% and 7.94%) respectively.

Table 1: Fungi isolated from fresh and dried fruits and frequency percentage.

Fungal Species	Non-sterilized	Surface-sterilized	Reduction %
A. niger	154 (17.72%)	97 (20.29%)	37.0%
A. flavus	132 (15.18%)	86 (17.99%)	34.8%
P. expansum	112 (12.88%)	73 (15.27%)	34.8%
A. parasiticus	93 (10.70%)	58 (12.13%)	37.6%
Rhizopus stolonifer	81 (9.32%)	12 (2.51%)	85.2%
P. italicum	76 (8.74%)	34 (7.11%)	55.3%
A. carbonarius	64 (7.36%)	38 (7.94%)	40.6%
A. ochraceus	52 (5.98%)	29 (6.06%)	44.2%
A. fumigatus	48 (5.52%)	23 (4.81%)	52.1%
A. terreus	34 (3.91%)	16 (3.34%)	52.9%
Alternaria alternata	23 (2.64%)	12 (2.51%)	47.8%
Total isolates	869	478	
Grand total	**1347**		
X2total	**109.83**		
P value	**< 0.0027**		

Aflatoxin B1 Detection Using High-Performance Liquid Chromatography (HPLC) technique

HPLC analysis confirmed that some fungal isolates are capable of producing aflatoxin B1 (AFB1). The highest concentration (215.9 ppb) was recorded in *A. flavus* isolated from unsterilized dried grape (blackcurrant) samples, while the lowest concentration (51.4 ppb) was observed in *A. flavus* isolated from sterilized fresh fig samples, as shown in Figure 1. The retention time for AFB1 was recorded at 4.08 minutes, which is consistent with the standard pattern for aflatoxin B1.



Result chromatography Table (Uncal - F:\ S 12)

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	4.08	7562.08	543.25	70.00	70.00	0.20	
2	5.35	2560.14	225.12	30.00	30.00	0.10	
	Total	10122.22	767.37	100.00	100.00		

Figure 1: Standard results for detection of Aflatoxin B1 toxins by HPLC technology.

Estimation of aflatoxin B1 produced by the *A. flavus*

The fungus *A. flavus* can produce aflatoxin B1. HPLC analysis (Table 2) showed that some contaminated fungal isolates from fresh and dried fruit are capable of producing aflatoxin B1. The highest concentration of aflatoxin B1 was recorded in an *A. flavus* isolate from a sample of unsterilized dried grapes (blackcurrant), reaching 215.9 ppb, while the lowest concentration was recorded in a sterilized fresh fig sample from the same fungus, at 51.4 ppb for the toxin AFLB1. To confirm that this value corresponds to the standard area of the toxin shown in Figure 1, the results showed a retention time of 4.08 minutes. A simple test using standard solution and solvent was performed only once, and no value was detected at this stage. The appearance of a second value in the retention time (5.35 minutes) indicates that this value is related to the standard substance, aflatoxin B1. Mycotoxins are a serious problem because consuming any food contaminated with or grown on mycotoxins can have a harmful effect on human health and life.

Table 2: Estimation of aflatoxin B1 (AFB1) toxins produced by the *A. flavus*.

No	Name	Con (ppb)
1.	Unsterilized dried grapes	215.9
2.	Unsterilized fresh grapes	174.9
3.	Sterilized dried grapes	88.7
4.	Sterilized fresh grapes	65.7
5.	Unsterilized dried apricots	200.1
6.	Unsterilized fresh apricots	166.0
7.	Sterilized Dried Apricots	79.8
8.	Sterilized fresh apricots	60.5
9.	Dried figs (unsterilized)	186.9
10.	fresh unsterilized figs	150.8
11.	Sterilized Dried Figs	70.6
12.	Sterilized Fresh Figs	51.4

Polymerase chain reaction (PCR) technology is used for the molecular analysis of an *A. flavus* isolate

To identify *A. flavus* using polymerase chain reaction (PCR): Fungal DNA amplification by PCR showed a band at 595 base pairs using ITS primers, confirming the identity of the *A. flavus* isolates (Figure 2).

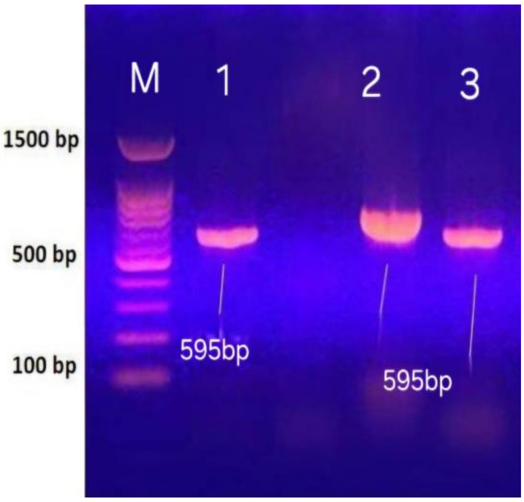


Figure 2: Agarose gel electrophoresis image that showed the PCR product analysis of ribosomal RNA (ITS1) gene in *A. flavus* isolates. Where M: marker (100-1500bp) and the lane (1-3) was showed positive *A. flavus* isolates at (595bp) PCR product.

The growth of *P. ostreatus* and *A. flavus* in dual culture technique

The dual culture technique showed that *P. ostreatus* inhibited the growth of *A. flavus* on PDA medium. The inhibition was classified as first-order inhibition on a five-point scale.

According to the five-step method, the results of this technique, as shown in Figure (3), demonstrate the ability of *P.ostreatus* to inhibit the growth of *A. flavus* on PDA medium. According to the scale[30], a five-point scale for assessing fungal antagonism in dual culture tests, calculations revealed that the fungus was antagonistic of the first order. The nature of the antagonistic relationship between *P. ostreatus* and *A. flavus* was clarified, showing that the longer the incubation period, the faster the cells grew in the culture medium due to the secretion of various lytic enzymes and their strong antagonistic ability to compete for nutrients and space.

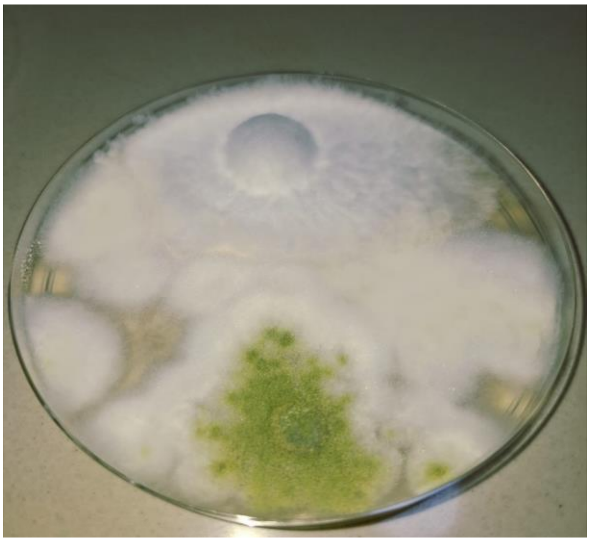


Fig. 3: Growth of *P.ostreatus* against *A.flavus* in dual culture.

Effect of *P.ostreatus* filtrate's on the radial growth of *A.flavus* on PDA medium

The *P. ostreatus* filter showed significant inhibition of fungal growth. The highest inhibition rate (68.3%) was observed at a concentration of 30%. Lower inhibition rates were observed at 20% (57.7%) and 10% (48.4%) compared to the control sample (8.2 cm).

Effect of calcium carbonate on the radial growth of *A. flavus* on PDA medium

Calcium carbonate inhibited fungal growth in a concentration-dependent manner. The highest inhibition rate (49.1%) was recorded at a concentration of 30 mg/ml, while the lowest (37.4%) was recorded at a concentration of 10 mg/ml.

Effect of calcium carbonate filtrate and *P. ostreatus* on the radial growth of *A. flavus* on PDA medium

The combined treatment with *P. ostreatus* filtrate and calcium carbonate demonstrated enhanced antifungal activity. The highest inhibition rate (74.8%) was recorded at a concentration of 30%.

Table 3: The Effect of *P.ostreatus* filtrate and calcium carbonate and their interaction on the radial growth of *A.flavus*.

Treatment	**Concentration**	**Mean Radial Growth (cm) ± SD**	**Growth Inhibition (%) vs. Control**	**Statistical Significance ($p < 0.05$)**
P. ostreatus filtrate (1)	10 %	4.23 ± 0.55	48.4%	p < 0.0019
	20 %	3.47 ± 0.15	57.7%	p < 0.0063
	30 %	2.60 ± 0.45	68.3%	p < 0.0041
Calcium Carbonate filtrate (2)	10 %	5.13 ± 0.21	37.4%	p < 0.0078
	20 %	4.67 ± 0.21	43.0%	p < 0.0058
	30 %	4.17 ± 0.25	49.1%	p < 0.0041
Combined Treatment (1+2)	10 %	2.93 ± 0.32	64.2%	p < 0.0047
	20 %	2.30 ± 0.20	72.0%	p < 0.0028
	30 %	2.07 ± 0.25	74.8%	p < 0.0036
Control (Untreated)	-	8.20 ± 0.00	0%	H

Effect of *P. ostreatus* filter on the dry weight of *A. flavus* in PDB medium

P.ostreatus filter showed significant inhibition of fungal growth. The highest inhibition (66.0%) was recorded at a concentration of 30%. Lower inhibition rates were recorded at 20% (58.2%) and at 10% (52.8%).

Effect of calcium carbonate on the dry weight of *A. flavus* in PDB medium

Calcium carbonate inhibited fungal growth in a concentration-dependent manner. The highest inhibition rate (48.7%) was recorded at a concentration of 30 mg/ml, while the lowest inhibition rate (41.2%) was recorded at a concentration of 10 mg/ml.

Effect of Calcium Carbonate Filter and *P. ostreatus* on the Dry Weight of *A. flavus* in PDB Medium

Regarding the effect of the interaction between calcium carbonate and *P. ostreatus* on the dry weight of *A. flavus* in liquid medium, compared to the effects of other treatments, this treatment achieved the highest inhibition rate of *A. flavus* growth, reaching 70.8% at a concentration of 30%. The inhibition rate was 67.9% at a concentration of 20%, and 65.4% at a concentration of 10%, as shown in Table (4).

Table 4: The effect of *P.ostreatus* filtrate and calcium carbonate and their interaction on the radial growth of *A. flavus* in PDB.

Treatment	**Concentration**	**Mean Mycelial Weight (g) ± SD**	**Growth Inhibition (%) vs. Control**	**Statistical Significance ($p < 0.05$)**
P.ostreatus Filtrate	10 %	1.50 ± 0.30	52.8%	p < 0.0033
	20 %	1.33 ± 0.15	58.2%	p < 0.0035
	30 %	1.08 ± 0.13	66.0%	p < 0.0048
Calcium Carbonate ($CaCO_3$)	10 %	1.87 ± 0.15	41.2%	p < 0.0051
	20 %	1.70 ± 0.10	46.5%	p < 0.0038
	30 %	1.63 ± 0.15	48.7%	p < 0.0011
Combined Treatment	10 %	1.10 ± 0.10	65.4%	p < 0.0031
	20 %	1.02 ± 0.08	67.9%	p < 0.0019
	30 %	0.93 ± 0.02	70.8%	p < 0.0028
Control (Untreated)	-	3.18 ± 0.00	0%	q

DISCUSSION

The growth of P. ostreatus and A. flavus in dual culture technique

P. ostreatus's ability to stop *A. flavus* from growing on PDA culture media is shown in Figure (3) of the five-step procedure[30], which also makes reference to the standardizing scale. The fungus was first-degree hostile, according to calculations. These findings corroborate the antagonistic connection between *P. ostreatus* and other contaminated or pathogenic fungus that was shown in.[31] Because different lytic enzymes are secreted for food and location, the longer the incubation period, the faster the cells grow in the medium.[32]

The Effect of *P. ostreatus* extract, Calcium Carbonate, and Their Combination on the Radial Growth of *A. flavus* in PDA Medium

According to the current findings, *P. ostreatus* extract clearly inhibits *A. flavus* radial growth on PDA medium. Radial growth is significantly reduced as a result of this impact, and the inhibition percentage rises from 48.4% at a 10% concentration to 68.3% at a 30% concentration. This concentration-dependent response shows that the filtrate's inhibitory efficacy is increased by the buildup of antifungal components at greater levels. These findings provide compelling evidence that the build-up of bioactive compounds in the mushroom filtrate is associated with the antifungal effectiveness of *P. ostreatus.*[33]

The production of extracellular enzymes like chitinase, beta-glucanase, and laccase, along with phenolic metabolites and antimicrobial peptides, is commonly thought to be responsible for *P. ostreatus* antifungal action. These substances impede spore germination in fungi that produce toxins, damage membrane permeability, and aid in the disintegration of fungal cell walls.[34][35] Recent research has shown that oyster mushroom species have similar inhibitory effects on aflatoxin-producing fungi, suggesting that they could be useful biological control agents in food preservation systems.[36] These chemicals interfere with fungal hyphae elongation and colony expansion, which explains the study's steady decrease in radial growth. The results are consistent with recent studies that demonstrate bioactive compounds extracted from the fungus *A. flavus* and other fungi that produce aflatoxin can be severely inhibited by *P. ostreatus* through oxidative stress and membrane instability. Differences in inhibition ratios between studies can be explained by differences in fungal strain pathogenicity, metabolite composition, extraction method, and culture conditions.[37]

Although it had a lesser impact than the fungal filtrate, calcium carbonate also showed decreased radial development. At the maximum dose of 30%, the inhibition reached 49.1%, suggesting moderate antifungal effectiveness. Fungal growth can be hindered by calcium carbonate's capacity to raise environmental pH, decrease water availability, and change ionic balance.[38] Since *A. flavus* prefers slightly acidic to neutral environments, any change in pH may inhibit mycelial growth by reducing enzyme effectiveness and food uptake. Treatments based on minerals are becoming more widely acknowledged as feasible and safe substitutes for managing fungal contamination after harvest.[39]

The greatest radial growth inhibition was achieved by the combination therapy, which reached 74.8% at a concentration of 30%. This improved performance points to a cooperative relationship between the physicochemical stress brought on by calcium carbonate and the biochemical action of the *P. ostreatus* filtrate. Fungal structures are directly damage by the filtrate, and calcium carbonate helps by altering the surrounding environment, which causes several inhibitory forces to act at once. Because they target infections through complementary mechanisms and lessen the chance of adaptation, these combination techniques are becoming more and more important in contemporary fungal control systems.[40]

Therefore, the differences in inhibition rates can be explained by the mechanism of action of each agent. Calcium carbonate has indirect environmental effects, biological filtrates give direct antifungal metabolites, and their combination integrates both processes to produce better suppression than each treatment alone.

The Effect of *P. ostreatus* extract, Calcium Carbonate, and Their Combination on the Dry Weight of *A. flavus* in PDA Medium

Reductions in fungal dry weight indicated inhibition of internal metabolic activity and overall mycelial development, and the dry-weight assay validated the antifungal potential of the studied therapies in liquid culture. *P. ostreatus* filtrate decreased dry biomass by up to 66.0% at 30% concentration, indicating that its inhibitory effect went beyond colony diameter to impact fungal physiology at the cellular level.[41]

This decrease in dry weight suggests disruption of respiration, nutritional uptake, and metabolic pathways necessary for the formation of biomass. According to recent research, antifungal metabolites derived from biological sources can lower mycelial mass in *A. flavus* by causing oxidative stress, interfering with mitochondrial activity, and impairing energy metabolism.[37] The biomass loss seen in the current study was probably caused by these factors.[42]

Dry weight was consistently but moderately reduced by calcium carbonate, with a maximal inhibition of 48.7%. Its impact is more environmental than biochemical, in contrast to the filtrate. $CaCO_3$ may change osmotic conditions and decrease nutrient solubility in liquid media, limiting the effectiveness of fungal growth.[38]

Furthermore, calcium carbonate produces less-than-ideal circumstances for fungal metabolism by altering pH and decreasing moisture-related availability. Its inhibitory percentages were lower than those of the physiologically active filtrate, which can be explained by these indirect effects.[43]

Once more, the combined treatment had the greatest impact, with dry-weight inhibition reaching 70.8%. This demonstrates that the two medicines' interaction affects deeper physiological mechanisms in addition to surface growth suppression. A wider metabolic collapse results from the filtrate's disruption of intracellular activity and calcium carbonate's weakening of external environmental support. Because fungal cells must react to chemical toxicity and environmental stress at the same time, such multifactorial suppression is especially effective.[42]

The different biological endpoints that were examined can account for variations in the inhibition percentages between dry weight and radial growth. While dry weight quantifies the overall accumulation of biomass, radial growth represents the expansion of surface colonies. While certain therapies may disrupt intracellular metabolism, others may have a greater impact on hyphal extension. As a result, a small variance in these parameters is anticipated and shows that each treatment affects fungal development in a variety of ways.[4])[37]

The conclusion that *P. ostreatus* filtrate is the primary antifungal component and calcium carbonate functions as an amplifying agent is generally supported by the dry-weight results. The best method for inhibiting *A. flavus* growth and biomass accumulation is their combination.[40],[35]

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