Isolation and diagnosis of *Alternaria alternata* fungi which is associated with some local and imported food products the possibility of controlling of growth fungus production of toxicity using some biological and chemical processes

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Abstract:

Alternaria alternata is a fungus that Infect plants, Contamination of food products ,some disease infection to humans,. This fungus produces many toxins, including Altratoxin II , for high frequency fungus in contaminated food . A number of nutrients have been collected to determine the frequency of fungi *A. alternata*, a fungus that has a leading role in the infection of raw materials in the food industry,the variant *Alternaria alternata* was used using microscopic and micro methods, including PCR technique and detection of fungal toxins. Several methods were used, including HPLC, to prove the fungus production of venom and to measure the toxic concentration of isolates isolates from food samples. 9 isolates of Altratoxin II were found. The highest concentration was 7.5499 mg / ml of isolation for non canned of chipse, biological and chemical agents were used to discourage as much as possible the fungal growth of laboratory fungi. *Pleurotus ostreayus* was used as a biological resistance agent, a non-toxic fungus for humans. It also works to break down the toxins in the culture media , which grows the fungus by Inhibition Growth of fungi in the food circles where the most significant effect was in the concentration of 30% and the rate of inhibition was 62.19% on the sold media, and 64.85% of the dry weight on the liquid media, and this encourages the use of . *P. ostreayus* with food products as a substance protected from pollution and the growth of fungi . It also contains a high percentage of carbohydrates, proteins, fats, salts, vitamins and other nutrients . Of the chemicals used for this purpose calcium carbonate CaCo3 in inhibiting the growth of fungus *A. alternata*, significantly reduced the ability of fungi to produce toxins. The most inhibitory concentrations were 30 % with 43.90% inhibition of the sold media, and 46.00% from the dry weight on the liquid media,. Interaction between the two treatments was the most effective at 30% concentration and 73.17% in the Sold media , 70.60 dry weight on the liquid media .

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Introduction:

Many toxins are infiltrated into our bodies hidden through the food without knowing the passage or the presence of not only after causing the Damage of organs, the entry of toxins into the human body either directly, for example, enter through the mouth through consumption with food or inhalation of fungi produced toxins either through the respiratory system or through direct contact with fungus, where the nutrient promotes the growth of the fungus either during the different stages of production or during transport or storage period, or indirectly through the consumption of products originating from animals that have already fed on fodder contaminated with fungal toxins , and so on toxins are especially dangerous for children . Contamination of nutrients with fungi produced by fungal toxins is one of the most important problems that threaten human life in many developing countries, especially those that lack good food storage conditions. This is a major risk, requiring countries to provide food for food security (Makun, 2010).

For fungal toxins, many properties dissolve most of it well in organic solvents, resist digestion, resist degradation during digestive processes in the human digestive system, resist high temperatures and therefore have host effectiveness in destroying them. Some toxins can be destroyed only at 250 ° C, although this degree is not accessible in heat treatment of pasteurization in the preparation of certain foods such as milk. Also, toxins cannot be eliminated at cooking temperatures for most foods (Jumaili, 2014). The International Agency for Research on Cancer (IARC) has classified a number of these toxins as human carcinogens, affecting the vital functions of organisms in several forms, and ultimately destroying them if they are taken in quantities beyond the internationally agreed limits permitted in human food or feedstock Human food (Creppy, 2002). .

General characteristics of pathogenic fungi *Alternaria* spp. An important fungus producing toxins is pathogenic fungi *Alternaria* sp. And special species *A. alternata* (Fakhr Al-din, 2017), that the *Alternaria sp* fungi is due to the missing fungi Fungi imperfecti (Berbee *et al*., 2003, Agrios,2005)and its composition as single-cell cones in a single form or in the form of chains, the conidial spores are released and the air currents are easily released resulting in their wide spread . The conidates of this fungus are grated by the melanin pigment obtained by a brownish brown color. The importance of this function is to give fermentation to the innate cones that are represent a protective shield against harsh environments and inappropriate conditions (Dipak et al,2013) . .

Laboratory tests were carried out for fungi to detect fungal toxins, and it was found that the production of methyl ether alternariol , alternariol , AltratoxinII , AltratoxinI ,targeting Stefanie et al. (2012), in a study the toxic effect of Alternaiol, Altertoxin II and Alternaiol monomethyl ether on DNA of the Chinese rat, pointed out the ability to induce strong genetic mutations, which caused a defect in the sequence of the nitrogen bases on the DNA, which in turn caused genetic abnormalities in the case of feeding the animals On the feed containing these toxins.

Various factors were used to control the fungus, including the use of bilogic and chemical methods, including the use of Trichoderma spp. (Al-Ziadi 2011). In this study, the fungus fungus Pleurotus ostreayus was tested as a biological agent for resistance to pathogenic fungi Pleurotus ostreayus: The fungus used for this purpose is an agricultural basidia that has medicinal benefits because it contains chemical compounds (Mateus et al., 2014), where the fungus is highly competitive against a large number of fungi contaminated with food . The innate yarn contains immunosuppressant's, antifungal agents and toxins (Daba, 2008). .

, The chemical agent was also tested for calcium carbonate (Caco3) in this stud , where previously used many chemicals to resist pathogens, and are very effective methods in the control of pathogenic fungi and the removal of fungal toxins from contaminated materials, Chemical agents have been used to resist pathogens and are very effective in combating pathogenic fungi and removing fungal toxins from contaminated substances without causing any satisfactory public health effects and eliminating toxins without causing harmful changes to the agricultural crop or product if applied (Shekhar *et al*., 2009). Where they directly affect the cell of pathogenic fungi through a change in the permeability of cellular membranes and its competitive effect with the cell on the nutrients, as well as the oxidation and inhibition of pathogenic fungus enzymes (Jalili *et al*., 1985). Of the chemicals used for this purpose CaCO3 calcium carbonate in inhibiting the growth of pathogenic fungi, including *A. Alternaria*, significantly reduce the ability of fungi production of toxins (Depasquale *et al*, 1990).

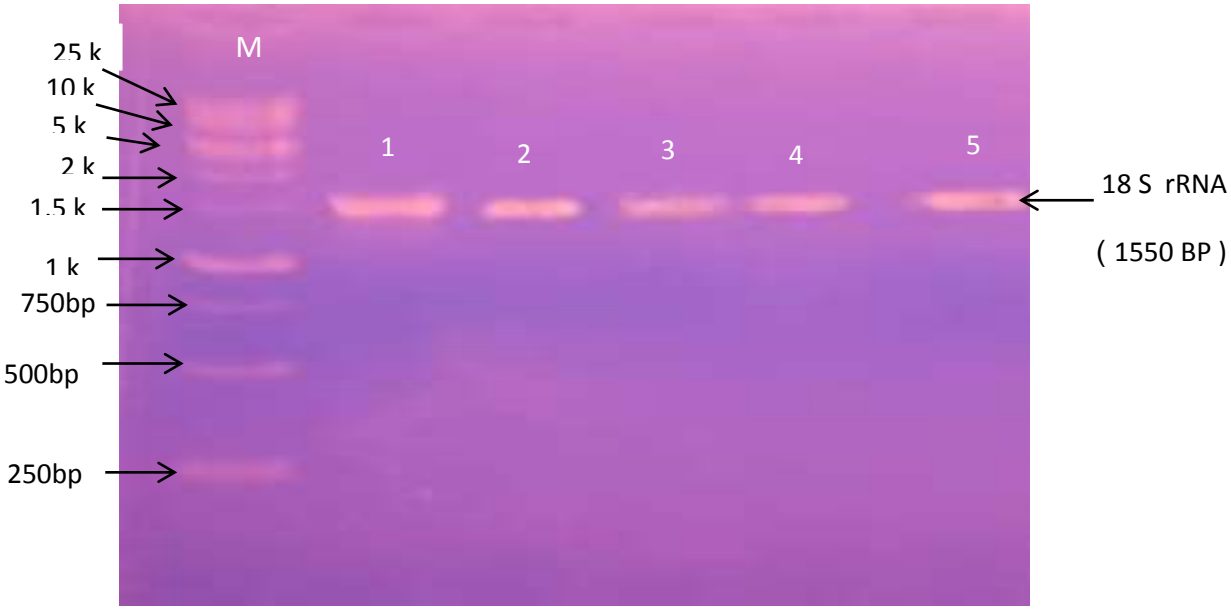
Working methods :

Food Sampling: Samples were collected from various local and imported food sources from the markets of different areas of Diwaniyah governorate , randomlyand at a suitable quantity for the laboratory examination of each sample. The food collected from different types of canned and uncooked food was collected manually, (Chips,Almonds, pistachios, walnuts), fruits, and vegetables for the period (October - December 2017), which numbered (184) food samples.

Isulation and diagnosis of isolated fung : Each type of sample was divided into two groups . The first consisted of samples washed with distilled water only. The second group sterilized surface sterilization with sodium hypochlorite solution at a concentration of 2% for (2 minutes) , then washed with distilled water and three times to remove the traces of sterile material. And was then implanted in pre-prepared plastic dishes for this purpose containing a PDA medium ((Reza, 2015) , isolated fungus diagnosis from food to sex and species were identified based on the morphology features such as color, colony form and colony base, as well as microscope features of the size, shape and composition of conical and spores (Moustafa, 1982), and after its diagnosis, the percentage of its frequency was calculated by the equation below :

$$\text{Percentage of fungal frequency (species)} = \frac{\text{Number of fungal colonies}}{\text{Total number of colonies of fungal species in dishes}} \times 100$$

In addition to using PCR technique to confirm the diagnosis of the fungus, Arif et al. (2012) indicated that molecular diagnostics can be done using a reliable PCR method as shown below



Relay outputs on the agarose gel(%1) and voltages (70) for an hour of DNA multiplier A. alternata with the ITS-1 initiatorusing PCR technique where : (1-5)

A.alternaria M: DNA Ladder marker (25 k - 250 bp

Detection of fungal toxins in Alternata alternaria using HPLC technology

The analysis was carried out in the laboratories of the Department of Environment and Water of the Ministry of Science and Technology. The toxins detected by *A.alternata* were detected by Altertoxin II (ATX II) using the Skyam HPLC technique and using a mobile phase (DW: 5% Formic acid : methanol (20 : 5: 75) and the C18-ODS separation column (25 cm and 4.6mm) using the Fluorescence detection detector. The flow rate (1ml / min) . , Using the following equation described by Akiema et al. (1999), the concentration of toxins in the fungus samples was calculated:

$$\text{Model concentration} = \frac{\text{Sample area}}{\text{Standard}} \times \text{Degradation factor} \times \text{Concentration of the standard solution}$$

Effect of Biologycal Resistant *(Pleurotus ostreayus*) in the Growth of the A. alternata on the Solid Food Medium P.D.A. (Contradictory Phenomenon):

Double Culture technique was used in Petri dishes containing the pre-prepared food medium P.D.A. Potato Dextrose Agar. After the laboratory experiment, the degree of antibody for each fungus was determined by the five-step standard of measurement (Bell et al., 1982).

Effect of *P.ostreayus* fungus extract on the growth of *A.alternata* on the solid medium:

To determine the efficacy of *P.ostreayus* in the growth of the fungus *A. Alternata* followed the method of Dixit et al. (1976), the Poisoned Food Technique, where three strains of the *P.ostreayus* fungi were prepared (10,20,30 %) Potato s Dextrose Agar (PDA) with the food medium . Transfer spaces (5 mm) from the end of the radial growth of the fungus *A. alternata* and the age of 7 days translate to the ptridish, and three replicates for each concentration and placed in the incubator for (7 days) in the degree of 25˚c , while the comparison treatment included the Petri dishes without adding. After the incubation period is completed, the growth rate of the fungus is measured in the treatments for the different concentrations and then the percentage of inhibition is calculated ((Shaaban and Al-Malah, 1993). According to the following equation: .

$$\text{Percentage of inhibition} = \frac{\text{fungus diameter in the treatment petridishes}}{\text{The rate of fungus diameter in comparison petridishes}} \times 100$$

Effect of chemical treatment concentrations Caco3 on the growth of the fungus *A.alternata*:To determine the effectiveness of calcium carbonate tested in the radial growth of the fungus *A. alternata* followed the way Dixit et al., (1976), a food technology poisoned (Poisoned Food Technique) returned the steps mentioned in above where three concentrations of calcium carbonate preparation of a (10,20 , 30)mg/ml and then the percentage of inhibition is calculated.

Effect of Caco3 Interaction and *P.ostreayus* Growth in A. Alternata:
To determine the effect and effectiveness of the tested chemicals and the biomass of the biomass in the growth of the fungus *A. alternata*, follow the steps mentioned in previously Three concentrations of CaCo3 and three different strains of the extracte *P.ostreayus*.

Effect of biological resistance *P.ostreayu*s on the dry weight of pathogenic fungi in the liquid media:

To test effectiveness of the fungus bio-resistance tested in the dry weight of the fungus *A. alternata* prepare three concentrations of *P.ostreayu*s extract which (10,20,30) , Using a cylindrical piercing two 5 mm tablets are transferred from the 7 day at the age of the *A.alternata* to each vial, and three replicates for each

concentration and placed in the incubator for 7 days in the degree of 25 ± 2˚c , with control of transactions without any add, where we are working on a liquid media nomination using the Filter paper, the movement of the stock information weight in advance to the electric oven at 60 ˚c until completely dry, then weighed nomination papers and presents them the weight of the filter paper before use : (The weight of the filter paper after drying - the weight of the filter paper before use - Weight of the patch) by using the balance of the sensitive . We obtain the dry weight of the fungal growth in the liquid media calculated the percentage of inhibition. The percentage of inhibition is calculated as in the following equation: .

$$\text{Percentage of inhibition} = \frac{\text{The weight of } A.alternata \text{ in comparison bottle} - \text{the weight of the } A.alternata \text{ in the treatment bottles}}{\text{Fungus weight in comparison bottles}} \times 100$$

Effect of chemical treatments CaCo3 on the dry weight of pathogenic fungi in the liquid media: Following the same steps mentioned in previously, only three calcium carbonate concentrations CaCo3 (10,20,30) mg/ml are prepared instead of the *P.ostreayu*s .

Interaction between chemical and biomass resistance in the dry weight of A.alternata in the liquid media : To test the efficacy of the oyster and chemical treatment Caco3) tested in the dry weight of A. Alternata follows the same steps mentioned in previously .

Results and discussion : .

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Isolation and diagnosis of fungi : The results of isolation and diagnosis of fungi from the food samples under study revealed the isolation of a number of contaminated fungi of the food samples , of these fungus isolates were isolated from *A. alternate*, as in Table (1) and had a frequency (11.05) in Non-sterile samples superficially. While in superficial sterile samples, the frequency of fungi (16.54) Of isolated fungi. A strong indicator of the ability of this fungus to grow successfully in different environments, it also has a high competitiveness against a number of other fungi contaminated with food (Hassan, 2015).

The results of HPLC use in the detection of toxins produced in pathogenic fungus *A. alternata* : The results of the HPLC analysis shown in Table (2) and the associated charts show that *A.alternata* has the ability to secrete four types of toxins **:** Alternaiol (AOH) , Alterteroxin ATX (II) altratoxin I(ATX I) ,alternuen(ALT), the highest concentration of toxins was Altertoxin II, the highest concentration of ATX II was in the Chips (7.5499 µg / ml) , while the lowest concentration of the toxin (0. 5105 µg / ml)) , fungal toxins affect human health and life at it consumes any nutrient on which the fungi have grown and produced toxins, which is a real problem for humans, as it affects animals when fed on fungus. They affect a few concentrations (Stefanie et al., 2012).

Effect of Bio-Resistant *P.ostreayu*s in the growth of *A. Alternata* on the Solid Food Media. (Contradictory Phenomenon):

The results of the experiments on the study of the resistance of *P.ostreatus* to fungi isolated from the food samples are the ability of the fungus to inhibit the growth of *A. alternate* in its entirety. As noted in the PDA media, the degree of antimicrobial was measured according to the five-standard standard (Bell *et al*., 1982). These results are consistent with the results obtained by (Jubouri, 2011), which showed the nature of the contrast between the fungus and the pathogenic fungi. This is due to the high resistance of the *P.ostreayu*s to fungal through the direct intrusion on the fungal spinning and the diffraction around it, in addition to its ability to analyze the cell walls through secretion Said (2017) suggests that the fungus secrete substances that kill or discourage other fungi, and note in (2) how the fungus control the *P.ostreayu*s on the fungus *A. Alternata* with the formation and formation of fungal cones.

Table (1) Percentage of fungicide frequency in foodstuffs :

fungus Name of	Fungi isolated from local and imported food	
	Non-sterile surface	sterile surface
Aspergillus niger	***344 (19.81)**	***109 (15.41)**
Penicillium italicum	**213 (12.26)**	**93 (13.15)**
Penicillium notatum	**193 (11.11)**	**79 (11.17)**
Aternaria alternata	**192 (11.05)**	**117 (16.54)**
Aspergillus fumigatus	**97 (5.58)**	**51 (7.21)**
***Candida* sp .**	**97 (5.58)**	**35 (4.95)**
Fusarium oxysprum	**89 (51.2)**	**61 (8.62)**
Rhizopus stolonifer	**82 (4.72)**	**11 (1.55)**
***Cladosporium* sp .**	**70 (4.03)**	**26 (3.67)**
Apergillus flavus	**67 (3.85)**	**23 (3.25)**
***Aspergillus* sp .**	**54 (3.11)**	**13 (1.83)**
Alternaria brascola	**53 (3.05)**	**24 (3.39)**
***Trichoderma* sp .**	**46 (2.64)**	**19 (2.68)**
***Penicillium* sp .**	**41 (2.36)**	**15 (2.12)**
***Geotrichum* sp.**	**30 (1.55)**	**11 (1.55)**
Aternaria sp .	**27 (1.55)**	**9 (1.27)**
Mucor sp .	**7 (0.40)**	**3 (0.42)**
Stemphylum sp .	**6 (0.34)**	**2 (0.28)**
Drechslera sp .	**2 (0.11)**	**0 (0)**
47.57 = X ²		
0 = P. value		

Significant difference at $P < 0.05$

*The results shown in the table represent the rate of three replicates. Note that the number of fungal colonies of non-sterilized foodstuffs amounted to (1736), and the macrophages were superficial (707)

Table (2) Determination of Alterxox II (ATX II) toxins produced by *A. alternata* fungi:

No.	Name of the sample	The poison concentration of the sample ppm =(µg /ml)
1	Not canned of Chipse	7.5499 (µg/ml)
2	Chipse 1 TOP	3.8367 (µg/ml)
3	Fashar (corn)	3.8367 (µg/ml)
4	Dry grape	2.5961 (µg/ml)
5	Apple	2.0907 (µg/ml)
6	Chipse Mito	1.7064 (µg/ml)
7	Cocorioh	1.6349 (µg/ml)
8	Wheat	0.7480 (µg/ml)
9	Almond importer	0.5105 (µg/ml)

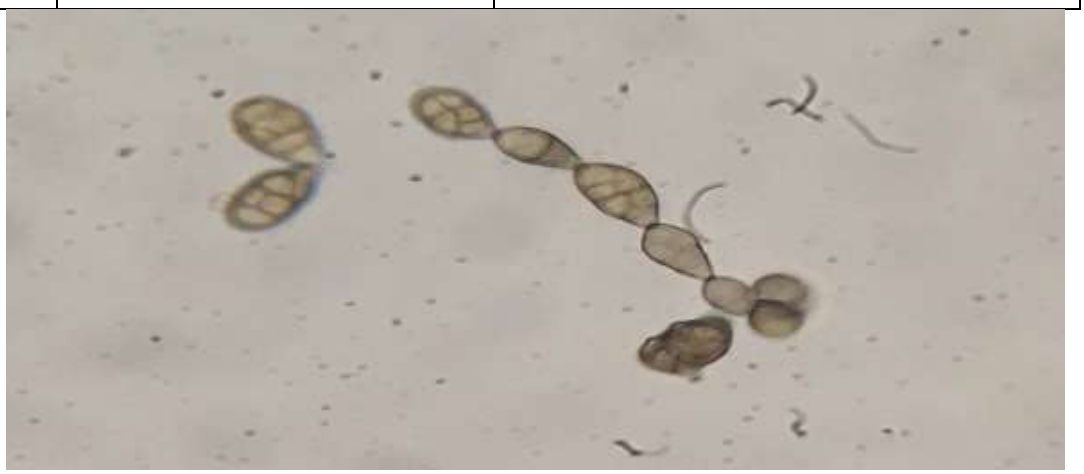

Figure (1) Microscopic appearance of conidia of *Alternaria alternata* isolated from food sample. Conidia develop on the apices of conidiophores, taper towards their distal ends :

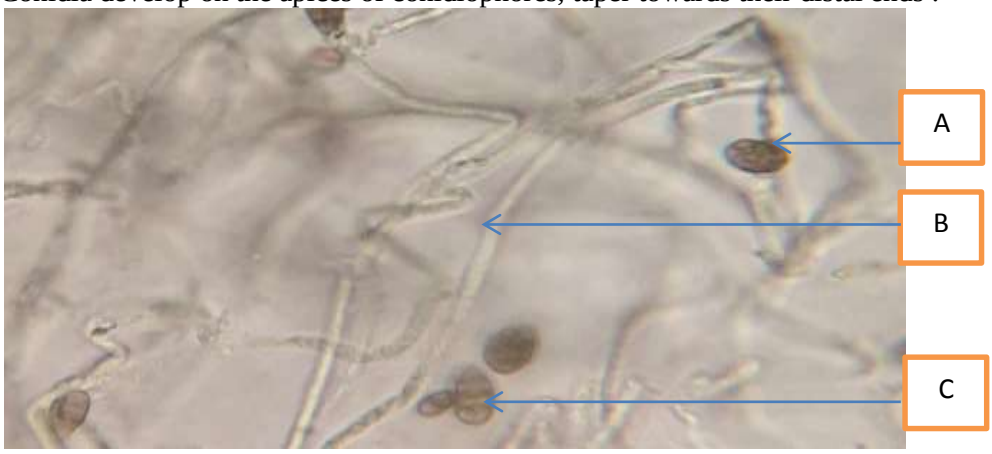

Figure 2 : shows the control of the fungus and the effect on pathogen *A.* *alternata*

A) - Disturbance in the shape of the cone of the fungus *A. alternata*, B)- The growth of the fungus fungus of the fungus, C)-The normal form of the fungus *A.alternata*

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Effect of *P.ostreayu*s on radial growth of pathogen *A. alternata* on the solid media:

The results showed that significant differences were found in table (3). The highest percentage of inhibition of A.alternata was found in the center containing 30% dilution of the *P.ostreayu*s, where the mean diameter of the colonies was 3.1 ± 0.25 cm, ie, a 62.19% , Concentration rate 10% The mean diameter of the colony diameters was 0.15 ± 4.5 cm with 45.12% inhibiting the growth of the treated fungi compared to the colony diameter in the control treatment (0.20 ± 8.2 cm) while the growth in the 20%) With 56.09% inhibition. Here, it is worth noting that the growth of the fungus was weak, whereas in the control treatment, the growth was heavy. The results of the study agree with Chowdhwy (2015) that the species belonging to the fungus are characterized by the presence of many antimicrobial agents, antioxidants, bio-active substances, high tolerance to colonization and rapid growth of fungal spinning on the agricultural media (Vamanu 2012).

Effect of chemical treatments calcium carbonate (CaCO3) on the growth of pathogenic fungi of *A. alternata* on the solid media :

The results indicated in Table (3) that the highest percentage of inhibition of *A.alternata* was found in the center containing 30 mg / ml of CaCo3. The mean diameter of the colonies was (4.63± 0.12) cm , (43.90%) inhibition, while the lowest concentration was 10 mg The growth rate of the colonies was (5.2 ± 0.26) cm, with a decrease of (36.58%) in the growth of the tested fungi compared to the colony diameter in the control treatment of (8.2 ± 0.20) cm, while the growth in the concentration was 20 mg / ml. The growth rate of the colonies was (4.8 ± 0.17) cm with an inhibition of (41.46%) . With Qasim (2017), who pointed out that the addition of CaCo3 to the center of the plant inhibits the growth of certain fungi, including *Aspergillus flavu*s The calcium carbonate changes the mean to the basal state when hydrophilic hydrolysis is dissolved by hydrolysis. As fungi are known to favor growth in intermediate or low acid states . Any change in the PH value of the mean to the basal conditions affects the nutrients of the growth of the fungus in the center and its readiness, which in turn affects the rate of growth or the effect of carbonate on the water effort in the media is more negative, Water is ready for growth of fungus, or affects absorbency Necessary nutrients from the vegetative medium (Koller et al., 1982).

Effect of Interaction of Chemical Processes and *P.ostreayu*s Fungus in the Growth of *A.alternata :*

The results of the test of the effect of chemical Processes and the ***P.ostreayu*s** of the fungus *A.alternata* isolated from the food in Table (3) indicated that the ***P.ostreayu*s** and Caco3 had a significant effect on the growth of *A. alternata,* .The diameter of the colonies of *A. alternata* colonies was 30% for the *P.ostreayu*s Fungus and Caco3 (2.23 ± 0.18) cm and for the inhibition percentage (73.17%). In the 10% concentrations, the diameter of the colonies of fungal colonies (3.1 ± 0.20) cm with an inhibition rate of 62.19%). . while the growth rate was (2.43 ± 0.14) cm, with an inhibition rate of 70.73% .

Table (3): **Effect of chemical Processes and bio-resistance on the growth of the fungus *A.alternata.* :**

Concentration mg/ml for Caco3 and precentation of dilutions for extract	Calcium carbonate equation		Treatment of extract P.ostreayus		Treatment of intraction Caco3 and extract P.ostreayus	
	Diameter (cm)	Inhibition (%)	Diameter (cm)	Inhibition (%)	Diameter (cm)	Inhibition (%)
10	5.2±0.26	36.58	4.5±0.15	45.12	3.1 ±0.20	62.19
20	4.83±0.17	41.46	3.6 ±0.17	56.09	2.43 ±0.14	70.73
30	4.63±0.12	43.90	3.1±0.25	62.19	2.23±0.18	73.17
Control	8.2±0.20	-	8.2±0.20	-	8.2±0.20	-
L.S.D.	0.732		1.459		0.469	

Effect of *P.ostreayus* on the dry weight of pathogenic fungi in the liquid media :

The results of the effect of the fungus on the dry weight of the fungus Alternaria alternata showed the results of the effect of the fungus on the radial growth of the fungus. The larval fungus again showed its high inhibitory capacity in the growth of *A.alternate* in Table (4) and the mean of the weight dry the fungus in the center of P.D.B. In the 30% dilution it was (1.10 ± 0.08) g) with an inhibition ratio of (64.85%) . While in the 10% dilution the weight of fungal growth was (1.54 ± 0.51) g with 50.79% inhibition and 20% in the concentration the mean weight was (1.31 ± 0.16) g with an inhibitory rate of (58.14 %) compared to the control treatment of (3.13 ± 0.05) g. Cloud This test shows the effect of the *P.ostreayus* on the growth of pathogenic fungi .

Effect of CaCo3 chemical treatments on the dry weight of pathogenic fungi in the liquid media :

Table (4) shows that the highest percentage of inhibition of *A.alternata* was found in the center containing 30 mg / ml of CaCo3. The fungal colony weight was (1.69 ± 0.13) g with an inhibition of (46.00%) , while the lowest inhibition was 10 mg / The mean weight of the colonies was (1.90 ± 0.21) g with (39.29%) inhibition of the growth of the tested fungi compared to the average of the weights of the colonies in the control treatment of (3.13 ± 0.05) g , while the growth in the concentration was 20 mg / ml. The mean weight of colonies was (1.81 ± 0.20) g with 42.17% inhibition .

Effect of Interaction between Chemical and Bio-Resistant *P.ostreayus* in dry Weight of *A. alternata* in Liquid Media :

The laboratory results showed in Table (4) the effect of interference on the dry weight of the fungus. The highest inhibitory rate was 70.60% in the 30% concentration of the *P.ostreayus* and calcium carbonate. The dry weight was (0.92 ± 0.04) g. The percentage of inhibition in concentration 10% was (63.57%) where weight was 1.14 ± 0.12 g , while the inhibition rate was (67.09%) with weights (1.03 ± 0.02). We observed a decrease in the weight of the fungus in the liquid media compared to the control treatment Where the weight was (3.13 ± 0.05) g .

Table (4) : Effect of chemical treatment and bio-resistance on the growth of the fungus *A.alternata in the liquid media :*

Concentration mg/ml for Caco3 and precentation of dilutions for extract	Calcium carbonate equation		Treatment of extract P.ostreayus		Treatment of intraction Caco3 and extract P.ostreayus	
	The weight (g)	Inhibition (%)	The weight (g)	Inhibition (%)	The weight (g)	Inhibition (%)
10	1.90±0.21	39.29	1.54±0.15	50.79	1.14±0.12	63.57
20	1.81±0.20	42.17	1.31±0.16	58.14	1.03±0.02	67.09
30	1.69±0.13	46.00	1.10±0.08	64.85	0.92±0.04	70.60
Control	3.13±0.05	-	3.13±0.05	-	3.13±0.05	-
L.S.D.	0.278		0.572		0.305	

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