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Isolation and identification of fungi contaminated with some local and imported chips and the possibility of controlling one of the toxin-producing fungi by using pleurotus ostreatusFiltrate and Sodiumbicarbonate treatments

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***Abstract*---**Samples of chips were collected from the markets and shops of the center and districts of Al-Diwaniyah Governorate for the period from (October - November 2021) and several types of fungi were isolated, Aspergillus niger , A.flavus, A.parasiticus, Aspergillus sp1,Aspergillus sp2,Aspergillus terreus,Mucor, Penicillium natatum and Rhizopus stolanifer. The results of the HPLC technique showed that the highest concentration of Aflatoxin B1 toxin was due to the filterate of Aspergillus terreus isolate of the chips sample, which amounted to 193.9 ppb, and this isolate was phenotypically diagnosed using taxonomic keys as well as molecularly diagnosed by polymerase chain reaction (PCR) technique in addition to base sequence analysis phylogenetic tree analysis for the purpose of comparing the isolate of the contaminated fungus A.terreus with the isolates previously registered in the National Center for Biotechnology Information (NCBI). Pleurotus.ostreatus filtrate and sodium bicarbonate proved their inhibitory ability for both radial growth and dry weight of the contaminated fungus isolate, where the highest inhibition rate reached64.44% for radial growth at 30% concentration to treat the interaction between P.ostreatus filtrate and sodium bicarbonate, while the highest inhibition rate was 57.03% for dry weight at 30% for same treat.

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Introduction

Chips being the trades largely by a very important segment of society children,beside consumed by adults,but less so , however, they provide a suitable medium for the growth of mold and toxin-producing fungi and the presence of mycotoxins in food products poses a serious threat to human health. Therefore, investigation of its presence in diets isof paramount importance [13]. The Food 1and Agriculture Organization of the United Nations (FAO) estimates that approximately 25% of the world's food and feedstuffs are at risk of mycotoxin contamination [18]. The term mycotoxin describes a chemically diverse group of low molecular weight metabolic by products produced by molds that have toxic effects to both humans and animals [7,8] The immune system is unable to distinguish and detect these toxins due to their molecular weight Low level, which leads to its accumulation in the tissues of certain organs such as the liver and spleen, and these toxins are among the most powerful known toxins, and small concentrations of them reach less than (10 ppm) cause serious diseases. They are resistent to treatments used in manufacturing and cooking processes [3]. The current study dealt with the issue of mycotoxins called aflatoxin in order to provide more information about these toxins, especially the first type, Aflatoxin B1. These toxins are produced by the fungus Aspergillus sp., These fungi are characterized by the ability to produce Aflatoxin. And it was found through HPLC examination. The differant concentrations of AFLB1 toxin is due to the fungi *A.terreus*, where represents the focus of this research, and *P.ostreatus* were used as a biological control agent and sodium bicarbonate, which represents the chemical resistance factor.

Materials and Methods

Isolation of fungi from chips

Samples of chips (chips of all kinds) from the markets and shops of the center and districts of Al-Diwaniyah Governorate, to isolated several types of fungi and diagnosed byMorphologic and Microscopic featuers and molecularly diagnosed by PCR.

Diagnostics of the isolated fungi
Phenotypic and microscopic diagnosis

It was diagnosed with fungi isolated from the chips to the level of genus and the species, depending on the outer appearance of the colony Morphological features such as color and shape, and the dish base has also been relying on some of the characteristics microscopic such as the size of The size of the sporozoites and spores, and their shape and arranged according taxonomic bases approved the use of taxonomic keys contained in [9,20].

Filtrates preparation

Fungal filtrates (*A.terreus* and *P. ostreatus*) were prepared using PDB food medium in glass beakers with an amount of 100 ml of medium for each beaker and sterilized by autoclave at a temperature of 121°C. and a pressure of 15 pounds / ing2 for 15 minutes and after cooling the antibiotic Chloramphenicol 250 Mg/L was added to the medium and the beakers were inoculated. Two tablets (5 mm) in diameter of 7 days old mushrooms were placed in each flask and the flasks were incubated at 25 °C for 3 weeks with continuous shaking every two days. Filters with a diameter of 0.22 microns and keep the filter until use at a temperature of 4 ℃. Detection of mycotoxins using HPLC technique: This technique was conducted to detectAFLB1 toxins using an HPLC Skyam model device and under HPLC working conditions:

- Vector phase consisting of Mobile phase = acetonitrile: D.W: (60: 40).
- Column Separation
- Column = C18-ODS (25 cm * 4.6mm).
- Using Detector = florescent Ex = 365 nm, Em = 445 nm.
- Flow rate = 0.7 ml / min.

Biological samples are subjected to ultrasonic vibration to break up cells, macromolecules and membranes in 50 ml in a volume ratio of 70 ml methanol: 30 ml water for 40 minutes and centrifuge for 5 minutes. Immunoaffinity column at less than 3 mL/min (the column was pre-treated with 20 mL of distilled water). The column was washed with 20 ml distilled water to remove impurities and air dried to remove residual water. The extract is taken by pouring 1.4 mL methanol into the column and leaving it in air. Then the extract is diluted with 2 ml of water and then passed at 0.55 μm through a filter and the filter is injected into the HPLC. Using the equation described by [1]the mycotoxin concentration of the isolated fungi filtrate was calculated.

Molecular diagnosis of *A.terreus* using polymerase chain reaction (PCR) technology

For the purpose of diagnosing the isolate of the fungus isolated in this study, the polymerase chain reaction (PCR) was tested, using the (Maxime PCR PreMix (i-Taq), kit supplied by the Korean iNtRoN company. The PCR was prepared.) with a total volume of 20 microliters, which contains one microliter of each of the anterior initiator (GGAAGGRTGATTTATTAG:ITS1) and posterior (TCCTCTAAATGACAAGTTTG) [21]as well as one microliter of DNA extracted from fungus. All of the above ingredients were put into the tube supplied by the manufacturer and the volume was completed to 20 microliters with (Nuclease-free water). The process of doubling the DNA of *A. Terreus* isolate was carried out by following the following steps and conditions of the PCR reaction: Initial denaturation of the DNA for 5 minutes at a temperature of 95° C, followed by 30 cycles consisting of a denaturation process. Final denaturation for a period of 45 seconds at a temperature of 72° C. The process of primer annealing lasts for 30 seconds at a temperature of 58° C. Then initial elongation of the PCR-amplified product for a period of 1 Minute at 95 C, and at the end of the reaction, the final elongation step is completed and at 72° C [22].

The Dual culture technique of *P.ostreatus* and *A.terreus* growth

The dual culture technique was used to culture the fungi in 9 cm diameter Petri dishes containing the solid medium (PDA). The plate was divided into two equal halves, and by using a cork piercing, a hole was made in the first half and inoculated with a 5 mm diameter disc of *P.ostreatus* at the age of seven days, and a hole was made in the center of the second half of the plate and inoculated with a 5 mm disc of the fungus *A.Terreus* at the age of 7 days and by three replications. As for the control dishes, they contain mushrooms individually and for both types. After that, the dishes were incubated in the incubator at a temperature of 25 °C for a period of 7 days. After that, the degree of antagonism for each mushroom was calculated according to the five-step scale mentioned by [6], consists of five grades.

The effect of *P.ostreatus*, Sodium bicarbonate and their interaction treatments on the radial growth of the fungus *A. terreus*

Dixit *et al*, (1976) followed the Poisoned Food Technique, where *P. ostreatus* filtrate was used as a biological resistance agent with different concentrations (%30, 20%, 10%), and sodium bicarbonate as a chemical control agent ($NaHCO3$). With the same concentrations, an interaction was made for the biological and chemical factors and for the same concentrations to control the fungus contaminated with chips *A.terreus*, where different concentrations of both factors were added and overlapped to the PDA culture medium and the medium was poured into Petri dishes (9 mm) with three replicates for each concentration, while 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 ^{0}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 *P.ostreatus*, Sodium bicarbonate and their interaction treatments on the dry weight of the fungus *A. terreus*

For the purpose of testing the effect of the two treatments (biological and chemical and their interaction) on the dry weight of the fungus *A.terreus,* 250 ml flasks were used, and the filtrate and sodium bicarbonate solution were mixed with the liquid medium to obtain the three concentrations of 30,20,10%, the medium was distributed by 50 ml in each beaker. By means of a cork piercing, two tablets measuring 5 mm were taken from the end of the radial growth of the fungus *A. Terreus* at the age of 7 days and placed in each beaker, and with three replicates for each concentration and placed in the incubator for 7 days at a temperature of 25 ˚ C. As for the control treatments, they were left without any addition. The flasks were agitated every two days and after the completion of the incubation period, the dry weight of the mushrooms was measured in different concentrations using the sensitive scale. The filter papers were weighed and 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), we get the exact dry weight of the fungal growth in the liquid culture medium and then the percentage of inhibition is calculated [19].

Results and Discussion

Isolation and identification of fungi

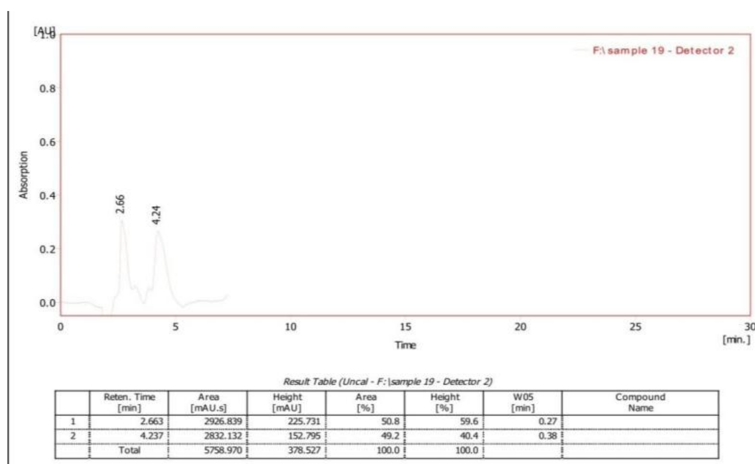
The current study included the isolation of many Species of fungi associated with chips These samples were collected from the markets and shops of the center and districts of Al-Diwaniyah Governorate, and many of them were diagnosed, namely: Aspergillus *niger* , A. *terreus* , A. *flavus,* A. *parasiticusMucor,* Aspergillusp1, Aspergillus sp2*, Penicillium natatum* and *Rhizopus stolanifer.* The results inTable (1) showed that there were significant differences in the frequency of fungi diagnosed in superficially sterilized and non-sterilized dried fruits. Sterile as its effect is limited to the surface fungi that contaminate chips. The results also showed that the replication of the fungus A. niger is the highest in the treatment of superficially non-sterilized chips, where the recurrence rate was 62.22% when compared with other isolated fungi, while its recurrence rate in sterilized samples was 8.66%. As for *A.terreus*, its frequency in non-sterile fruits was 24(9.09)% and 10(5.52)% in sterilized chips. This is consistent with [10,14] that this type of fungi possesses a high ability to produce secondary metabolic products within The polyketide family and these products are well characterized because of their biological or toxicological activities.The frequency of the contaminated fungus *Rhizopus stolonifer* in agricultural soils was7 (2.65)% and4 (2.2)% in non-sterile and sterile treatments, respectively.

Table 1
Fungi isolated from dried chips and their frequency percentage

Name of the isolated fungus	The percentage of fungal recurrence in dried fruits			
	Superficial non-sterili	Superficial sterili	x2	P value
Aspergillus *niger*	160 (60.6)	120(66.29)	1.49	0.222
Aspergillus *flavus*	21(7.95)	9(4.97)	1.51	0.218
Asper gillus *parasiticus*	22(8.33)	18(9.94)	0.341	0.559
ASpergillus sp1	3.(1.13)	2(1.1)	0.001	0.957
ASpergillus sp2	4(1.51)	2(1.1)	0.136	0.712
ASpergillus *terreus*	24(9.09)	10(5.52)	1.93	0.164
Mucor	5(1.89)	4(2.2)	0.054	0.816
Penicillium	18(6.81)	12(6.62)	0.006	0.938
Rhizopus stolanifer	7(2.65)	4(2.2)	0.058	0.810
Total isolates	264(100)	181(100)	30.96	0
X2	758.6	640.4		
p value	0	0		

HPLC technique for the detection of Aflatoxin B1 mycotoxin produced by isolated fungi

The results of HPLC technique Table (2) showed that some fungal isolates contaminated with dried fruits have the ability to produce Aflatoxin B1 toxin, and that the highest concentration of AFLB1 toxin was recorded in the isolate of *A. Terreus* of currant sample, which amounted to 193.9 ppb, while the lowest concentration was recorded in a sample for the fungus A. niger, where it reached 53.6 ppb forAFLB1 toxin, and these results are similar to what was indicated [16,17]. The results showed the value of the retention time (4.24) minutes, which represents the standard area of the poison shown in Appendix (1), and to ensure that this value belongs to the standard substance, a simple test was made where the solvent was injected only once, and no value appeared at this time and the Standard solution was injected once A second value appeared in the time of detention (2.66) minutes, and this indicates that this value belongs to the standard substance, which is the AflatoxinB1 poison.



Result Table (Uncal - F:\sample 19 - Detector 2)

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	2.663	2926.839	225.731	50.8	59.6	0.27	
2	4.237	2832.132	152.795	49.2	40.4	0.38	
	Total	5758.970	378.527	100.0	100.0		

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

Molecular diagnosis of *A. terreus* isolate using polymerase chain reaction (PCR) technology

The results showed the possibility of doubling PCR-amplified products with a size of 550 base pairs, bp by polymerase chain reaction (PCR) and in the presence of ITS1 and ITS4 (Fig.2).

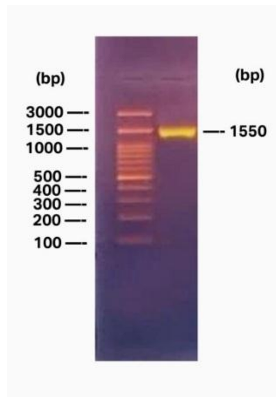


Figure 2. PCR product multiplier using PCR form of isolated fungus isolation in this study from raisins and using its ITS 1 and ITS4 pairs of Primer. M=(Molecular-weight size marker), NC: Negative comparison

The results of the nucleotide sequence analysis of the PCR product from the fungal isolate and using the BLAST program to compare with the data available at the US National Center for Biotechnology Information (NCBI), where found by comparing the sequence of the nitrogenous bases of the fungus *A.Terreus* isolated in this study, there is a 100% similarity with many *A.Terreus* isolates previously registered in the National Center for Biotechnology Information (NCBI), including those diagnosed in China (OM250078.1) and India (HQ393867). Figure 3 and Figure 4).

Figure 3. Similarity in sequence alignments of DNA products (PCR-amplified products) multiplied by the isolation of isolated *A.Terreus* fungi in this study and other isolations of the same mushrooms previously registered at the National Center for Biotechnology Information (NCBI)

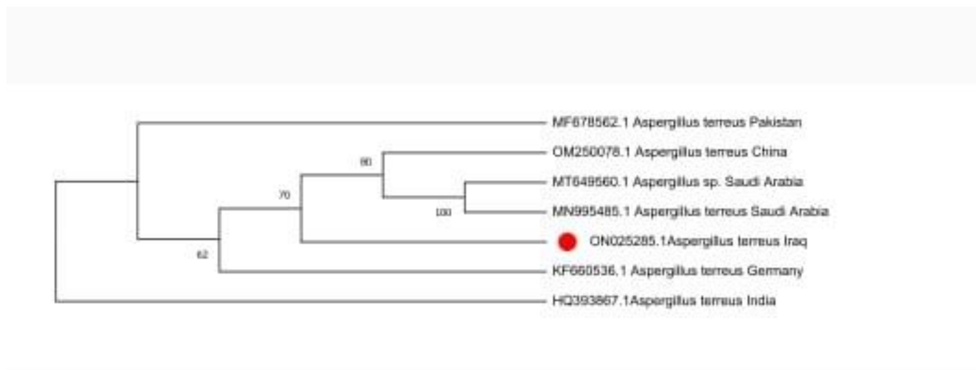


Figure 4. Neighbor-Joining tree) showing the genetic relationship *A.Terreus* isolate in this study and other isolates previously registered in the National Center for Biotechnology Information (NCBI)

The dual culture technique of *P.ostreaus* and *A.terreus* growth

The results of this technique showed in Figure (5) the ability of *P.ostreaus* to inhibit the growth of *A.Terreus* on PDA culture media, according to the five-step scale of standardization mentioned by [6]. The degree of antagonism to the fungus was calculated and it was of the second degree. These results are in agreement with what showed [2], which showed the nature of the antagonism between *P.ostreaus* and other contaminated or pathogenic fungi. The cells by secreting different lytic enzymes in addition to the high antagonistic ability to compete for food and place and the speed of its growth in the medium the longer the incubation period.

Fig 5. Growth of *P.ostreaus* against *A.Terreus* in dual culture

Effect of *P.ostreaus* filtrate on the radial growth of *A.terreus* on PDA

The results of Table (3) showed that the treatment of *P.ostreaus* infiltrate with its different concentrations gave significant differences, as the highest inhibition rate of *A.Terreus* was 57.03% in PDA medium containing 30% of *P.ostreaus* infiltrate, and the growth rate of colonies diameters was 3.86 cm, while The average colony diameter was 7.78 cm and 35.73% inhibition in the growth of the tested fungi at 10% concentration. The average colony diameters were 6.18 cm and the inhibition rate was 31.25% at 20% concentration. In comparison with the average diameters of colonies in the control treatment, which amounted to 9.0 cm, the effect is observed. The clear concentrations of *P.ostreaus* filtrate on the radial growth of contaminated fungi of chips where the relationship is direct between the concentration and the rate of inhibition, this study agrees with what indicated [12] that this fungus is considered one of the medicinal fungi that possesses effective defense materials and is used as an anti-inflammatory and toxin.

Effect of sodium bicarbonate on the radial growth of *A.terreus* on PDA

The results of this test mentioned in Table (3) showed the effectiveness of sodium bicarbonate with its different concentrations and its significant effect on the radial growth of *A.terreus* in comparison with the control treatment, and its effect on the growth of pathogenic fungi increased with increasing concentration, as the highest percentage of inhibition of the fungus was in the culture medium containing PDA concentration. 30% of sodium bicarbonate, where the average colonies diameter was 3.80 cm and the percentage of inhibition was 57.77, while

we noticed the lowest percentage of inhibition at the concentration 10%, and the average diameter of the colonies was 5.83 cm, with a percentage of inhibition of 35.18%, while at the concentration of 20%, the growth rate of the colonies diameter was 5.70 cm, with a percentage of Inhibition reached 36.66% compared to the average diameters of colonies in the control treatment of 9.0 cm. The reason for the effectiveness of sodium bicarbonate is that it is a basic salt and affects the pH value of the medium, which has a negative effect on the growth of fungi [4].

Effect of interaction of *P.ostreaus* and sodium bicarbonate filtrate on the radial growth of *A.terreus* on PDA medium

The results of testing the effect of the interaction of *P.ostreaus* filtrate and sodium bicarbonate on the radial growth of *A.Terreus* in Table (3) showed that there were significant differences between the different concentrations of *P.ostreaus* filtrate and sodium bicarbonate, which significantly affected the growth of fungus. The results show that the rates of fungi colony diameters are proportionately Reversible with the concentration of each of the filtrate and bicarbonate, and note the superiority of the results of the antifungal filtrate and the chemical together in inhibiting the radial growth of the fungus *A.terreus* when comparing the use of each of them separately, as the average diameters of contaminated fungi colonies at 30% concentration for this treatment were 3.30 cm and 64.44% inhibition, and at 20% concentration, the average colonies diameters were 3.73 cm and 58.51% inhibition, and these two concentrations represent more effective than 10% concentration. The average diameter of the colonies was 2.86 cm and the inhibition rate was 68.14% compared to the average diameter of the colonies in the control treatment of 9.0 cm.

Table 3
The effect of *P. ostreaus* filtrate and sodium bicarbonate and their interaction on the radial growth of *A.terreus*

Con.	P.ostreatus Filtrate treatment(2)		sodium bicarbonate treatment(1)		Interfere between1+2		C. Inhibition average ± S.D
	Diameter (cm)	Inhibition %	Di.(cm)	In. %	Di.(cm)	In. %	
10%	5.78±0.70	35.75±7.78	5.83±1.89	35.18±21.03	2.86±1.09	68.14±12.18	41.34±12.6
20%	6.18±0.07	31.20±0.79	5.70±1.08	36.66±12.02	3.73±0.11	58.51±1.28	43.2±+16.3
%30	3.86±0.32	57.03±3.57	3.80±0.26	57.77±2.94	3.20±0.60	64.44±6.75	63.7±8.18
Control	9±0		9±0		9±0		
Inhibition average of treatm	.	9.71		9.71			16.82

ent ± S.D							
L.S.D	Con.	0.74	Treat.	0.64	Inter.	1.28	

Effect of *P.ostreaus* filtrate on dry weight of fungus *A.terreus* in PDB

The results of Table (4) showed the inhibitory ability of *P. ostreaus* filtrate in the growth of *A.terreus* on PDB liquid medium, and a significant decrease in dry weight rates was observed in comparison with the control treatment, where the average dry weight of polluted *A.Terreus* ranged in a concentration of 43.87% of PDB medium. The prepared preparation was about 1.74 g with an inhibition rate of 42.58%, while at 20% concentration, the weight of the fungal growth was 1.78 g and an inhibition rate of about 41.61%. The average dry weight in it was 1.81 g, which means the higher the concentration of the fungal filtrate in the liquid medium, the higher the inhibition rate. These results are consistent with what was reached by [5] on the effect of the mushroom filtrate on the growth of the contaminated fungus *A.alternata*.

Effect of sodium bicarbonate on the dry weight of the fungus *A.terreus* in PDB

Table (4) shows that there are some significant differences in the treatment of sodium bicarbonate, and the highest percentage of inhibition of the fungus *A. Terreus* was 45.48% at the concentration of 30% of the bicarbonate, as the average weight of the fungal colonies was 1.69 g, and at the 20% concentration, the average weight of the colonies was 1.70 g and the percentage of inhibition 45.16%, while we notice the lowest inhibition rate of 36.45% at the 10% concentration, where the average colony weights were 1.97 g, compared to the average colony weight in the control treatment3.10 g. This indicates a direct relationship between the concentration of bicarbonate and its inhibitory ability. These results are consistent with [4], in which he showed that sodium bicarbonate had significant effects on the dry weights of the tested fungi and that there were significant differences between the concentrations used where the inhibitory effect increased by increasing the concentration.

The effect of the interaction between sodium bicarbonate and *P. ostreaus* filtrate on the dry weight of *A.terreus* mushroom in liquid medium

This treatment was more effective in the effect compared to the effect of using both bicarbonate and *P. ostreaus* filtrate individually, and the highest inhibition rate for *A.terreus* was 65.80% at 30% concentration, where the average dry weight of poisonous fungus was 1.06 g, while at 20% concentration, it was The average dry weight was 1.33 g and an inhibition rate of 57.09%, and the average dry weight of the colonies of pathogenic fungi was 1.54 g and an inhibition rate of 50.32% at a concentration of 10% compared to the average weight of colonies in the control treatment, which amounted to 3.10 g as shown in Figure (4).

Table 4
Effect of sodium bicarbonate and *P.ostreaus* filtrate and their interaction on the dry weight of *A.Terreus* in PDB

Con.	P.ostreatus Filtrate treatment(2)		sodium bicarbonate treatment(1)		Interfere between1+2		C. Inhibition average ± S.D
	Weight (gm)	Inhibition %	Weight (gm)	In. %	Weight (gm)	In. %	
10%	1.81±0.01	41.61	1.97±0.49	36.45	1.54±0.08	50.32	42.68±1.13
20%	1.78±0.01	42.58	1.70±0.007	45.16	1.33±.01	57.09	42.36±5.12
%30	1.74±0.01	43.87	1.69±0.05	45.48	1.06±0.14	65.80	57.73±7.76
Control	3.10±0		3.10±0		3.10±0		
Inhibition average of treatment ± S.D		42.68±1.13		42.36±5.12		57.73±7.67	
L.S.D	Con.	5.41	Treat.	8.28	Inter	10.81	

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