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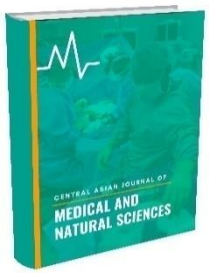

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Isolation and Identification of Fungi Contaminated with Some Local and Imported Raisins and the Possibility of Controlling One of the Toxin-Producing Fungi by Using *Ganoderma Lucidium* Filtrate and Calcium Carbonate Treatments

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Abstract: Samples were collected from the shops, and local markets in the city of Al- Diwaniyah for the period from (October – December 2022) and multiple species of fungi were isolated: *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus tubingensis*, *Aspergillus teereus*, *Penicillium natatum*, *Rhizopu stolanifer*, *Alternaria Alternata*, *Fusarium sp*, Some significant differences were observed in the fungi isolated from raisins and currant the frequency percentage highest in the treatment of non-sterilized from sterilized. *A.tubingensis* diagnosed phenotypically using classification keys and molecularly diagnosed by PCR technique (polymerase chain reaction). The results of the HPLC technique observed that the highest concentration of Aflatoxin B1 was due to the filterate of *Aspergillus tubingensis* isolate of the currant sample, which equalize to 114.9 ppb. *Ganoderma lucidium* filtrate and calcium carbonate shown their potential to significantly reduce the radial activity and dry weight of the this fungal isolate, where the maximum inhibition rate was76.85% for dry weight for radial activity at 30% concentration to treated the interplay between *G.lucidium* filtrate and calcium carbonate, while the highest inhibition rate was76.66 % for dry weight at 30% for same treat.

Key words: Contamination food, Raisins, Fungal toxins, *Aspergillus tubingensis*, *Ganoderma lucidium*.

Introduction

The contamination of some types raisins and currant and general foods with fungi and their toxins are problems that intimidation to most advanced nations , especially those which don't have good storage conditions[14,17]. This has received much attention recently for these nations to provide free access to safe, wholesome food sources. Humans are protected from these pollutants by achieving food security and without harming animals. Mycotoxins found in food products constitute a major hazard to human

health[15]. According to the Food and Agricultural Organization of the United Nations (FAO), mycotoxin contamination could affect 25% of the world's food supplies are susceptible to contamination with mycotoxins [18]. A category of lower molecular weight metabolic produced by molds that have hazardous properties are referred to as mycotoxin[10]. Include human and animal [19]. Because of lower molecular weight, the immune system has no ability to discriminate and recognize these toxins. These toxins are some of the most potent ones now known, and low levels of them, which cause their buildup in the tissues of certain organs like the liver and kidney, can cause significant disorders. In order to learn more about these mycotoxins, particularly the first type, Aflatoxin B1, the current study focused on the problem of mycotoxins known as Aflatoxin B1. These toxins are produce by the fungus Aspergillus spp. which is known for its ability to produce Aflatoxin [21]. And it was discovered using an HPLC analysis. Different AFLB1 toxin concentrations are caused by the fungi *A.tubingesis*, which is the subject of this study, *G. lucidium*, which was used as a biological control agent, and calcium carbonate, which is the chemical resistance factor.

Material and Methods

Isolation and diagnosis

Samples of raisins and currant were collected from Al-Diwaniyah province shops and local markets for the period (October-December 2022) .The contaminated fungi used in this isolated where each type of samples was separate in two groups, first groups were washed well with sterile water and second group washed with sodium hypochlorite[12] left to dry at room temperature. Was diagnosed with fungus isolated from the raisins and currant at the species and genus levels, based on the colony's outward appearance. Color and shape are morphological characteristics, and the dish foundation has also been dependent on some of the tiny features, such as the size of the sporozoites and spores, as well as their shape and microscopic featuers[14] and molecularly diagnosed by PCR [22].

Filtrates preparation

PDB food medium was used to create the fungal filtrates (*A. tubingensis* and *G.lucidium*) in glass beakers, 100 ml of medium and disinfected in an autoclave at 121°C and 15 pounds /inch2 of pressure for 15 minutes. Following which the medium was allowed to cool, the antibiotic chloramphenicol 250 Mg /L was added to the medium , and the beakers were infused. Each flask contained two 5 mm-diameter tablets of mushrooms that had been grown for seven days. The flasks were incubated at 25 °C for three weeks with frequent shaking every two days. Filters having a 0.22 micron diameter should be stored at 4 °C until use HPLC technique for mycotoxin detection this technique model Skyamn:

working using vector phase consisting of mobile phase =acetonitrile: D.W: (30 :70),column separation ,column = C18-ODS (25cm * 4.6mm), using detector=florescent EX=365nm, Em=445nm and flow rate=0.7 ml /min.

Macromolecules and membranes from biological samples are broken up calls by ultrasonic vibration in 50 ml of methanol at a volume ratio of 70 ml: 40 minutes in 30 ml of water followed by 5 minutes of centrifugation. a column of immunoaffinity at not more than 3 mL/min (the column was pre-treated with 20 mL of distilled water)The column was cleaned with 20 cc of distilled water to get rid of contaminants, and any remaining water was then dried by air. By adding 1.4 mL of methanol to the column and drying it in the open air, the extract is obtained. The extract is then diluted with 2 ml of water, passed through a filter at 0.55u m, and the filter is then introduced into the HPLC. Implementing the formula given in the mycotoxin level of the measured filtrate of the isolated fungi [1].

Polymerase chain reaction (PCR) technology for the molecular diagnosis of *A. tubingensis*

The isolation of the fungus isolated in this investigation needed to be diagnosed, and the The Korean favorgen company's (Maxime PCR PreMix (go- Taq) kit was used to test the polymerase chain reaction (PCR). The PCR was set up. 20 microliters in total, one microliter of the anterior initiator (TGCGGAAGGATCATTACCGAG: ITS1), and posterior initiator (AATGGTTGGAAAACGTCGGC) [20], and one microliter of fungus derived DNA. The manufacturer's tube was filled to the recommended capacity with 20 microliters (Nuclease-free water) after the aforementioned substances were added. The following procedures and conditions were used to double the DNA of the *A. tubingensis* isolate was completed by following the PCR reaction parameters and methods listed below: DNA is initially denaturated for 5 minutes at 95 °C, then for five thirty cycles that involve a denaturation procedure. Final denaturation was performed for 5minutes at 72 °C. At a temperature of 58 °C the primer annealing process lasts for 30 seconds. The PCR-amplified product is first extended for 1minute at 72 °C, and after the conclusion of the process, the final elongation phase is done at 72 °C.

The Dual culture technique of *G.lucidium* and *A.tubingensis* 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 *G.lucidium* at the age of 7days, 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.tubingensis* at the age of 7 days and by 3 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 5 step scale mentioned by [7], consists of five grades.

The effect of *G.lucidium*, calcium carbonate and their interactions treatments on the radial growth of the fungus *A.tubingensis*

G. lucidium 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[11]. 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, *G.lucidium* , and their interactions on the dry weight of the fungus *A.tubingensis*

To evaluate the impact of the two treatments (chemical and biological and their interaction), The filtrate and calcium carbonate solution[18] 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. tubingensis* 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 befor use (Weight of the filter paper after drying - the weight of the filter paper befor 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 [8].

Results and Discussion

Isolation and identification fungi

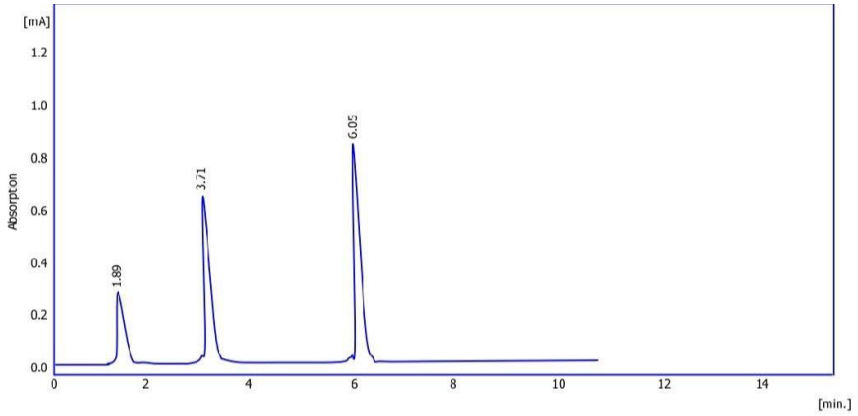
The present investigation involved the identification of many fungal species linked to These samples came from the marketplace and stores in the heart and surrounding areas of Al-Diwaniyah Governorate and many of them had the following diagnoses: *Aspergillus niger*, *A.tubingensis*, *A.flavus*, *A.terreus*, *Fusarium sp*, *Alternaria alternata*, *Penicillium natatum*, and *Rhizopus stolanifer* were among the diagnoses made. The findings in Table (1) demonstrated that there were significant variations in the frequency of fungi identified in dried fruits[18] that had undergone superficial sterilization and those that had not. Because it only affects the surface fungi carried on fungi-contaminated dried fruit. These results are consistent with what. That confirmed the effect of sodium hypochlorite on externally carried fungi. The results also indicate that the *Aspergillus niger* percentage is the highest frequency in the treatment surficial of non-sterilized of this fungus about 52.87%, frequency in the sterilized treatment was 67.56% for same fungus , the reason is due to the ability of this fungus to form large numbers of reproductive units even in inappropriate environmental conditions, as well as its small diameters less than 15 nanometers and thus a great ability to spread and contaminate dried fruit. As for *A.flavus* fungus, its frequency in treating superficially non-sterilized and sterilized was 12.58% and 9.72% respectively, *A.tubingensis* was 9.35% and 2.16% respectively. These results are consistent with what he mentioned. The frequency percentage of fungi *A.terreus* and *P. natatum* was 2.15% ,5.75% in the treatment of non-sterilized sample and 1.62%, 7.56% in the sterilized respectively. These results are consistent with mentioned that the fungi accompanying grains are *P. natatum*, *A. alternate*, etc. The *A. alternata* fungi are among the most important contaminated fungi for local and imported food products [4] which has a frequency of 2.15% in non- sterilized and 1.62% in the treatment of sterile sample. The contaminated fungus found in agricultural soils *Rhizopus stolonifer* its frequency percentage reached 13.30% and 8.64%, respectively, in non-sterilized and sterile treatments, *Fusarium sp*. The frequency in treatment of non-sterilized grains was 1.79% and 1.08%, respectively.

Table (1) Fungi isolated from dried fruits and 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	147 (52.8)	125(67.56)	0.782	0.182
Aspergillus flavus	35(12.5)	18 (9.72)	0.693	0.218
Rhizopus stolanifer	37(13.3)	16 (8.64)	0.539	0.238
Aspergillus tubingensis	26(9.35)	4 (2.16)	0.836	0.130
Penicillium natatum	16(5.75)	14 (7.56)	0.774	0.172
Aspergillus terreus	6(2.15)	3 (1.62)	0.168	0.273
Alternaria alternate	6(2.15)	3 (1.62)	0.143	0.295
Fusarium sp	5(1.79)	2 (1.08)	0.463	0.253
Total isolates	278(100)	185 (100)	36.67	0
X2	273	243		
P value	0	0		

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

The HPLC results table (2) showed up that some contaminated fungal isolates from dried fruits where took three isolates can created the toxin Aflatoxin B1 and the highest concentricity was recorded for AFLB1 toxin high in the *A.tubigensis* isolate from the black currant sample, equal to 114.9 ppb, while the little concentricity was recorded in the sample black raisins for the fungs *A.niger*, where it reached 74.1 ppb for the AFLB1 toxin and In order to confirm that the value corresponds to the standard area of the poison indicated in Appendix (1), the results showed the value of the retention time (3.71) minutes. Using a standard solution and the solvent only once each, a straightforward test was conducted. At this point, no value was detected. The fact that a second number emerged in the detention time (6.05) minutes shows that this value is associated with the standard material[1], which is the toxin Aflatoxin B1.



Result chromatography Table (Uncal - F:\ sample 5))

No	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	1.89	1632.08	312.49	15.00	15.00	0.10	
2	3.71	7014.55	786.55	35.00	35.00	0.25	
3	6.05	9365.00	961.49	50.00	50.00	0.35	
	Total	18011.63	2060.53	100.00	100.00		

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

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

The outcomes demonstrated the capacity to double PCR-amplified products with a size. Polymerase chain reaction (PCR) of 518 base pairs, with the presence of ITS1 and ITS3 (Fig.2)

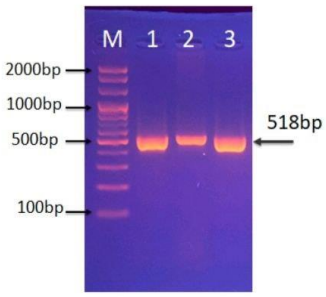


Fig 2: Agarose gel electrophoresis image that showed the PCR product analysis of ribosomal RNA (ITS1) gene in *Aspergillus tubingensis* isolates. Where M: marker (2000-100bp) and the lane (1-3) was showed positive *Aspergillus tubingensis* isolates at (518bp) PCR product.

The growth of *G. lucidium* and *A. tubingensis* in dual culture technique

According to the five-step method, the results of this technique demonstrated in Figure (3) G. lucidium capacity to prevent the growth of *A. tubingensis* on PDA culture media.[5] mentions the standardizing scale. Calculations revealed that the fungus was antagonistic to a second- degree. The nature of the antagonistic relationship between *G.lucidium* and other contaminated or pathogenic fungus was demonstrated in [13], and these data support that finding. The longer the incubation period, the faster the cells grow in the medium due to the secretion of various lytic enzymes and the strong antagonistic capacity to compete for food and position.

Fig 3: Growth of *G.lucidium* against *A.tubingensis* in dual culture

Effect of *G.lucidium* filtrate's on radial growth of *A.tubingensis* on PDA

Table (3) findings revealed that the treatment of *G. lucidium* infiltration at various doses resulted in substantial variations, with the highest inhibition rate of *A.tubingensis* was 76.66% whereas the average colony diameter was 2.10 cm in PDA medium concentration 30 % of *G.lucidium* filtrate, and 40.36 % inhibition rate in the growth of the tested fungi at 10% concentration and the average colony diameter was 5.36 cm. The average colony diameter was 2.58 cm, and at a 20% concentration, the inhibition rate was 71.29 %. The result is shown in comparison to the average colony diameters in the control treatment, which were 9.0 cm. The obvious *G. lucidium* filtrate concentrations on a radial growth of this study came up with what noted [3] that this fungus is classified one of the medicinal fungi that has effective defense substances and has been employed as an anti-inflammatory and toxin. The relationship between concentration and the rate of inhibition is straightforward in the tainted fungi of dried fruits.

Calcium carbonate's effect on *A. tubingensis* radial growth on PDA

The outcomes of this experiment, which are listed in Table 3, demonstrated the efficiency of calcium carbonate at various concentrations and its considerable impact on the radial The highest rate of fungus inhibition was in the culture medium comprising PDA concentration, and its effect on the growth of pathogenic fungi grew with increasing concentration. growth of *A. tubingesis* in comparison to the control treatment. 30 mg/ml of calcium carbonate, average colony diameter of 3.68 cm, and inhibition percentage of 59.07% . An average diameter of a colonies was 6.58 cm with an inhibition level of 26.84 % at the concentration of 10mg/ml , whereas at the concentration of 20mg/ml ,the growth rate of a colonies diameter was 4.45 cm, and the percentage of inhibition was 50.55 %, as opposed to the control treatment's average colony diameter of 9.0 cm. Calcium carbonate is a basic salt and influences the pH of the medium, which has a detrimental effect on the development of fungus, it is effective [16].

Effect of calcium carbonate filtrate and *G.lucidium* on the radial growth of *A.tubingensis* on PDA medium

The findings of evaluating the interaction between salt and *G. lucidium* filtrate The effects of calcium carbonate and *G. lucidium* filtrate at various concentrations on the radial growth of *A. tubingensis* were demonstrated in Table (3) and had a significant impact on fungal growth. The findings demonstrate that the rates of fungi colony diameters are commensurately reversible with the concentration of each filtrate and carbonate, and they highlight the superiority of the findings acquired when the antifungal filtrate and chemical were used in combination to stop the fungus *A. tubingensis* from growing in a radial fashion after contrasting their individual applications, as the average contaminated fungal colony diameters for this treatment at 30% concentration were 2.08 cm and 76.85 % inhibition, and the average colony diameters of 2.25 cm and 74.99% inhibition at 20% concentration. These two concentrations are more efficient than 10% concentration, as evidenced by the average colony diameters of 2.76 cm and 69.25 % inhibition . In comparison to the control treatment's average colony diameter of 9.0 cm[6].

Table (3) The effect of *G.lucidium* filtrate and calcium carbonate and their interaction on the radial growth of *A.tubingensis*

Concentration C.carbonate mg/ml percentages G.lucidium	G.lucidium filtrate treatment (2)		Calcium carbonate Treatment(1)		Interfere between 1+2		Concentration Inhibition average ± S.D
	Diameter (cm)	Inhibition%	Di.(cm)	In.%	Di.(cm)	In.%	
10	2.51±5.36	40.36	0.14±6.58	26.84	1.32± 2.76	69.25	2.204.90
20	0.62± 2.58	71.29	0.72± 4.45	50.55	0.25± 2.25	74.99	1.13± 3.09
30	0.26± 2.10	76.66	1.78± 3.68	59.07	0.14± 2.08	76.85	1.20± 2.62
Control	0± 9		0± 9		0± 9		
Inhibition average of treatment± S.D	2.00± 3.35		1.61± 4.90		0.53± 4.90		1.90± 4.22
L.S.D	Con.	0.17	Treat.	0.18	Inter.	0.21	

Effect of *G.lucidium* filtrate on *A.tubingensis* dry weight in PDB

Table (4) findings demonstrated *G. lucidium* filtrate's ability to inhibit the growth of *A. tubingensis* 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 contaminated *A. tubingensis* ranged in a 30% concentration highest inhibition rate 82.54 % of PDB medium, weight rates were around 0.52 g of the produced compound had an inhibition rate of 78.29 %, whereas 0.64 g of the fungal growth at a 20% concentration. The average dry weight in 10% concentration it was 0.30 g and the inhibition rate was 64.76%, which means the higher the fungal filtrate concentration in the liquid medium the higher the inhibition rate. These results are consistent with what was reached by [7] on the effect of the mushroom filtrate on the growth of the contaminated fungus *A.tubingensis*.

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

Table (4) demonstrates some substantial variations in the treatment of calcium carbonate, with the largest percentage of inhibition of the fungus *A. tubingensis* at the concentration of 30 mg/ml of the carbonate was 80.19% , as the average weight of its colony growth was 0.62 g, and at the 20mg/ml concentration, with the average weight of the colonies being 1.31 g and also the percentage of

inhibition being 67 %, whereas we appreciate the lowest inhibition rate of 54.44 % at the 10mg/ml concentration, where its average colony weights had been 1.35 g. This demonstrates a clear connection between the carbonate content and its capacity to inhibit .These outcomes are in line with [4], where he demonstrated that calcium carbonate significantly affected the dry weights of the tested fungi. There were substantial variations in the doses employed, and as the concentration rose, the inhibitory effect increased by increasing the concentration.

Effect of the interaction between calcium carbonate and *G. Lucidium* filtrate on the dry weight of *A. tubingensis* mushroom in liquid medium

When compared to the effects of employing other treatments[8], this one was The highest inhibition rate for *A. tubingensis* was 83.99 % at 30% concentration, where its average dry weight of a toxic fungus was 0.47 g, while at 20% concentration, the inhibition rate was 81.98 %, the average dry weight was 0.53 g, and also the average dry weight compared to an average weight of colonies in the treated control. while was 0.31 g and an inhibition rate of 64.08 % at a concentration of 10%, as shown in table (4) [9].

Table (4) The effect of *G.lucidium* filtrate and calcium carbonate and their interaction on the radial growth of *A.tubingensis* in PDB

Concentration mg/ml percentages of G.lucidium filterate	G.lucidium filtrate treatment (2)		Calcium carbonate Treatment(1)		Interfere between 1+2		Concentration Inhibition average ± S.D
	Weight (gm)	Inhibition %	Weight (gm)	In.%	Weight (gm)	In.%	
10	0.05± 0.30	64.76	1.35± 1.27	54.44	0.31± 1.07	64.08	0.31± 1.07
20	0.48± 0.64	78.29	0.50± 1.31	67	0.46± 0.53	81.98	1.13± 3.09
30	0.45± 0.52	82.54	0.59± 0.62	80.19	0.46± 0.47	83.99	1.18± 2.61
Control	0± 2.98		0± 2.98		0± 2.98		
Inhibition average of treatment± S.D	0.43± 0.73		0.84± 1.08		0.46± 0.69		
L.S.D	Con.	0.18	Treat.	0.16	Inter.	0.13	

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