Isolation And Identification of Fungi Contaminated with Cardamom and Black Pepper and Detection of Mycotoxins Produced from Them in Al-Najaf Governorate

Zahraa Haider Reda Al-Musawi[1], Hussein Sami Naji Kermasha[2]
[1, 2] College of Education for Girls - University of Kufa /Iraq

Abstract
the current study included the isolation and identification of fungi in some types of spices contaminated with fungi in the province of Najaf, Samples of spices were collected in August of the year 2022 from two different places in Al-Najaf Governorate, which included the city center and a spice store belonging to the Saffron Company, where 2 samples were collected from the two places at a rate of 100 grams for each sample, and these samples were placed in tightly closed bags to ensure that the sample was not contaminated. From the air, the name of the sample, the date of taking the sample, the place and its number were written on it, then it was transferred to the Graduate Studies Laboratory / College of Education for Girls / Department of Life Sciences / University of Kufa and kept at the temperature of the laboratory until investigation of fungal isolates within a period not exceeding 3 days. where potato dextrose medium PDA was used to grow the fungus, and the results of sequence showed that the fungus found in cardamom is *Aspergillus flavus* and in Black pepper is *Aspergillus niger*, and the results appeared in *Aspergillus flavus* isolated from cardamom, the percentage of mycotoxins were as follows: aflatoxin 0,0 ochratoxin1,3, and T2 33,2, As for the results of the toxins isolated from the black peper the fungus *Asparigllus niger*, they were as follows: Afha 1.1, Ochratoxin 2,4, and T2 9,3. while growing the fungus in the above-described method, but on day 28, it showed that the percentage of *aspergillus flavus* in afla 0,0, ochratoxin 1, and T2 19,6 While the results of *Aspergillus niger* for aflatoxin8.5 and ochratoxin 6,9 and T2 54,2
Keyword: Aspergillus flavus , PDA , cardamom , Black pepper , Aspergillus Niger

1. Introduction

A spice is either the dried seed, fruit, root, bark, flower, or flower of a plant or herb used in small or large amounts for flavor, color, or as a preservative as used in traditional medicines. Modern and ancient science has made these spices readily available, increasing their popularity ("Mol. Targets Ther. Uses Spices - Mod. Uses Anc. Med.," 2010).
Where spices are part of our daily routine, such as black and white pepper, which are considered Indian spices. Which is polluted during the harvest period, and after the harvest and storage period. Which has a role in increasing the contamination of mycotoxins, and this pollution results in heavy losses for the producing countries (Shiva Rani, 2021).
Isolating fungi and identifying them from different sources was and still is necessary in order to reach an accurate diagnosis of species, including them in the scientific classification, evaluating their effects and giving strains, for environmental treatment and biological control (Blackwell,2011).
Mycotoxins are a group of toxic by-products of metabolism produced by fungi belonging mainly to the genera Aspergillus, Penicillium, Fusarium, and Alternaria. Being endowed with a large structural diversity of these toxic compounds, they have a wide reputation for adverse effects, which include carcinogenic, hepatotoxic, and toxic effects. Nephrotoxic, teratogenic, haematological, immunotoxic, hormonal or reproductive mycotoxins pose a difficult food safety challenge because they are unavoidable in nature and unpredictable in cultivated crops (Selamat & Iqbal, 2016).
Contamination of foods with fungi poses a threat to human and animal health, especially those that produce mycotoxins. Mycotoxins do not enter into any biological interaction of actual importance in the growth of fungi, but they are considered secondary metabolic compounds with low molecular weight, which play a role in increasing virulence and developing pathogenicity and produce In the early stages of development (Age, 2001). Where there are four types of aflatoxins produced by Aspergillus flavus and A.Parasiticus whose specific chemical properties have been identified, namely B1, B2, G1, G2 (Al-Abdalall, 2009)
Ochratoxins are metabolic by-products of Aspergillus and Penicillium, which pose a health hazard through food contamination. Ochratoxin A (OTA) remains the single most potent component of this group of mycotoxins. OTA has a long half-life in humans and is therefore easily detectable in serum. Food-related studies have confirmed a link between nephrotoxicity present in humans and daily household intake of OTA. OTA has been reported to contribute to endemic nephrotoxicity and carcinogenicity in humans and animals. OTA produces kidney tumors and substances. DNA and chromosomal aberrations in the kidney. OTA may be fetotoxic, teratogenic, and immunotoxic only at doses higher than those that cause nephrotoxicity. The

doi.org/10.31838/hiv23.03.303 Received: 28.11.22, Revised: 19.12.22, Accepted: 12.01.23.

occurrence of predominantly endemic nephrotoxicity has been reported in northeastern Europe since the early 1950s. However, recent studies have cautioned that OTA and other toxins, such as aristolochic acid, Renal diseases appear very similar. Thus there is a need for simultaneous comprehensive studies on the occurrence of toxins(Reddy & Bhoola, 2010).

The safety and purity of foods from fungi and their toxins are binding matters that must be highlighted, and to achieve this, the diagnosis must be accurate for fungi that produce toxins than others. For the diagnostic aspect, mycologists used the traditional method of diagnosis armed with purely phenotypic characteristics to identify fungal species (Al-Zamel, 2009).

Aim of the study

1. Isolation and purification of fungi isolated from spices and testing their ability to produce mycotoxins
2. Phenotypic and molecular diagnosis by PCR technique of fungal isolates isolated from spices
3. Estimating the moisture content of some spice samples and comparing them with different Arab and international standards

Sample collection

Samples of spices were collected in August of the year 2022 from two different places in Al-Najaf Governorate, which included the city center and a spice store belonging to the Saffron Company, where 2 samples were collected from the two places at a rate of 100 grams for each sample, and these samples were placed in tightly closed carton bags to ensure that the sample was not contaminated. From the air, the name of the sample, the date of taking the sample, the place and its number were written on it, then it was transferred to the Graduate Studies Laboratory / College of Education for Girls / Department of Life Sciences / University of Kufa and kept at the temperature of the laboratory until investigation of fungal isolates within a period not exceeding 3 days.

2. Method of Isolation and Diagnosis

Potato Dextrose Agar (PDA)

The PDA culture medium was prepared according to the manufacturer's instructions by dissolving 39 g of the medium powder in one liter of distilled water, then it was distributed in a glass flask with a capacity of 1000 ml, and its nozzles were closed with a tight cotton stopper. lb/in2 and at a temperature of 121°C for 15 minutes, then the medium was left to cool at 45°C, then added to the Glomox antibiotic medium at a rate of 250 mg/L. The medium was used to grow fungal isolates. After that, the dishes were poured and the ground spices were spread lightly and evenly on the dish. The isolates of the fungi belonging to the spices were grown on the PDA culture medium, then the dishes were incubated at a temperature of 25 ± 2 ° C for a period of 7 days. After 7 days of incubation, the plate was extracted from the incubator, and purification was made for each type of fungus that grew in the plate, where 4 plates were made for each isolate, then the isolation was taken to make PCR.

PCR diagnosis

DNA extraction from fungal isolates

As indicated in the use of PCR (Carvalho-Pereira et al., 2020) The fungal isolates of the spices were grown on the PDA culture medium, then the plates were incubated at a temperature of 25 ± 2 ° C for 7 days, and the DNA was extracted and purified.

1. The fungus was activated on the PDA culture media by taking a single spore after 7 days of incubation.
2. Crush 100-500 mg of mycelium.
3. 20 microliters of Proteinase K enzyme and 180 microliters of Universal Digestion Buffer were added to the sample and mixed with a Vortex device, and the tubes were transferred to a water bath at a temperature of 56 °C for 30-60 minutes.
4. 100 microliters of Universal Buffer PF was added to the tubes and mixed using a Vortex device, then the tubes were incubated in the freezer at a temperature of -20°C for 30 minutes.
5. The mixture was transferred to the Centerfuge and centrifuged at 12000 rpm/min for 5 minutes at room temperature. The upper supernatant was removed from the mixture and added to a new 1.5 ml Eppendrof tube.
6. Add to the mixture 200 microliters of Universal Buffer BD and mix with the Vortex device in order to isolate each of the genetic material, protein and other molecules, as well as help in the adhesion of silica to the DNA. Now the tubes contain silica.
7. Add 200 microliters of Ethanol with a concentration of 96 - 100% and mix with a Vortex device. To get rid of the filtrate, the mixture is transferred to an EZ-10 Tube and centrifuged at a speed of 12000 rpm for two minutes.
8. 500 microliters of Universal PW Sohition solution was added to the tube, and placed in the Centerfuge apparatus at a speed of 12000 rpm for one minute, then the filter tube was transferred to a new collection tube.
9. 500 microliters of Universal Wash Sohition solution was added to the tube, then centrifuged for one minute at a speed of 12,000 rpm and transferred to a new collection tube, and centrifuged again at the same speed for two minutes to dry the EZ-10 Tube membrane and transfer the latter to a new tube with a volume of 1.5 ml. .
10- 100 microliters of TE Buffer solution was added directly to the center of the EZ-10 Tube and left for one minute at room temperature, then centrifuged at 12000 r/min for one minute for the purpose of DNA concentration, then transferred to an Eppendrof tube.

Amplification of the extracted DNA

According to(In, 2020) microtubes containing 5 µL of Master Mix were prepared, to which 5 µL of extracted DNA, 2 µL of ITS4 primer (1 µL Reverse and 1 µL Forword) and the nitrogenous base sequence were added. From (3 - 5) (TCC TCC GCT TAT TGA

TAT GC), then 25 microliters of sterile ionic water was added, the materials were mixed using a Vortex device, then the tubes were transferred to a thermocycler for the PCR reaction for the purpose of DNA amplification, represented by separating the DNA strand (Denaturation) and the association of primers with the separated strand (Annealing) and the lengthening of the DNA chain (Extension)

Gel electrophoresis

The agarose gel was prepared by dissolving 1 gm of agarose in (90) ml of sterile distilled water and (10) ml of (10X TBE buffer) solution, then placing the mixture in a thermometer until it reached the boiling point, then cooled to 65 °C after that. Add 5 microliters of (Ethidium bromide), then shake the mixture lightly for the purpose of mixing, then pour it into the designated place in the electrophoresis device and put a UV sterilized comb in the mixture for the purpose of making holes in the gel, then leave it at room temperature for 30-45 minutes until it hardens. Then the comb was removed and 5 microliters of Ladder was added to the first hole (which contains pieces of standard DNA), then the same amount of extracted DNA was added to the rest of the holes, then the cover of its device was placed and the electrophoresis was carried out at 70 volts for 60 minutes for the purpose of Detection of the PCR products represented by the extracted and amplified DNA bundles and compared with the standard ladder, then the sample was sent to the Korean company Macrogen to obtain the sequence of the nitrogenous bases.

products of secondary metabolism

Contaminated medium prepared by adding 24 g of PDB medium to 1000 ml of distilled water, adding 250 mg of Glomox antibiotic to the medium and sterilized by autoclave

Then,3 discs were taken from the pure fungal isolates by means of a cork puncture. The diameter of the disc was 1 cm from the end of the pure isolate at the age of 7 day , with four replicates for each isolate, with continuous stirring of the flask to ensure a homogeneous distribution of fungal spores. A vibrating incubator was used for this purpose, after which the flask were incubated at a temperature of 25 ± 2 C for three weeks and then for 28 days also to stimulate the fungus to produce poison. (Ishii et al., 1974; Lyose et al., 2006). After that, a piece of cotton was placed on the mouth of the flask to prevent contamination. A paper was placed on which was written the inoculation day, the sample number and type, and the day the sample will purified. It was placed in the shaking incubator for growth. The mixture was filtered at the age of 21 and at 28 days using filter papers and milipores, and the solution was placed in a test tube and do the Toxicology test was measured using the ELISA device and the result as the table 1 , 2

3. Results and Discussion

The results in Table 1 show that the toxicity between *Aspergillus Flavus* isolated from Cardamom and *Aspergillus Niger* isolated from showed Black pepper that the percentage of aflatoxin toxin in both of them was The result of an approach ranging from *A.flavus* in afla 0,0 and in A.niger was 1,1 , while the percentage of ochratoxin was estimated to be higher in *Aspergillus Niger* about 2.4 than it was in *Aspergillus Flavus* is 1.3, while the percentage of T2 was higher in *Aspergillus Flavus* about33.2 than in *Aspergillus Niger* is9,3.

Table 1: Comparison of the toxicity of fungi in a period of 21 days to *Aspergillus flavus and Aspergillus niger*

Fungi name	Sample type	Toxin ratio of Aflatoxin	Toxin ratio of Ochretoxin	Toxin ratio of T2 Toxin
Aspergillus flavus	Cardamom	0.0	1,3	33,2
Aspergillus niger	Black pepper	1,1	2,4	9,3

The results shown in Table (2) The results of the second table showed at the age of 28 that the fungus *Aspergillus flavus* about 0.0 and *Aspergillus niger* about 8,5 in aflatoxin, while the ochratoxin in the fungus *Aspergillus niger* 6,9 was higher than that of *Aspergillus flavus* 1 , and the toxicity of T2 *Aspergillus niger* about 54,2 was higher than the toxicity of *Aspergillus flavus* about 33,2. The permissible levels of aflatoxins is <20 While the percentage of ochratoxin is <5 And the T2 ratio is <150

Table 2: Comparison of the toxicity of fungi in a period of 28 days to Aspergillus flavus and Aspergillus niger

Fungi name	Sample type	Toxin ratio of Aflatoxin	Toxin ratio of Ochretoxin	Toxin ratio of T2 Toxin
Aspergillus flavus	cardamom	0,0	1	33,2
Aspergillus niger	Black pepper	8,5	6,9	54,2

The results showed that A. niger (uniseriate) or low toxin-producers, although this was not confirmed quantitatively. Sultan & Magan (2010).

Children are particularly affected by exposure to mycotoxins, which lead to stunted growth, delayed development liver damage, and liver cancer. As for adults, there is a disparity in their exposure to mycotoxin damage, but they are also at risk. No animals are immune to mycotoxins. Mycotoxins are among the most known carcinogens After entering the body, it is metabolized by the liver into an epoxide or reactive hydroxyl to form the less harmful aflatoxin M1(Wannop, 1961). It is known that the growth of fungi and the production of mycotoxins in food are affected by many factors, such as substrate composition, temperature, pH, and microbial interactions. The physical and chemical properties

undergo changes during maturation to which the fungus is exposed, which affects the production of mycotoxins. Aspergillus flavus grows and produces mycotoxins in a wide range of temperatures and water activity (Casquete et al., 2017).

A.f strains cannot produce AFs due to the absence of a polyketide synthase gene or gene mutation. AF strains competitively eliminate AF strains in the field, giving an additional advantage to stored grain. Several microbiological, molecular and field methods were used to select the appropriate biological control agent, which explains why Aspergillus flavus did not produce mycotoxins of the aflatoxin type on days 21 and 28 (Khan et al., 2021).

Recent studies found that the production of aflatoxin by Aspergillus flavus decreases when it is cultivated with some fungi. The researchers noted that the co-cultivation of A. flavus with A. niger makes the fungus work on a significant decrease in the formation of aflatoxin beyond the simple displacement of one fungus from the other and support this work. The early concept was that competition with A. flavus isolates incapable of producing aflatoxin could address endemic aflatoxin contamination in the world. This type of biological control is currently the most widely used biological control method worldwide to reduce aflatoxin contamination of crops, as aflatoxin contamination represents a difficult problem for human and animal health (Ehrlich, 2014).

Despite its importance, pepper is vulnerable to mycotoxin contamination along the production chains by Aspergillus species which exhibit immense ecological and metabolic differences . These fungi are capable of growing on a great variety of food commodities and animal feed materials when the conditions of temperature, relative humidity, and product moisture are favorable for development. Pepper crop has been reported as aflatoxin-contaminated a major group of mycotoxins, produced by species of Aspergillus, primarily *A. flavus* Contamination of pepper with aflatoxins may start at field (pre-harvest) conditions or during drying, storage or processing stages (post-harvest). Due to its health and economic impacts, different (Tsehaynesh et al., 2021).

Reference

Age, B. (2001). *Major Groups of. January*, 1–3.

Al-Abdalall, A. H. A. (2009). Production of aflatoxins by Aspergillus flavus and Aspergillus niger strains isolated from seeds of pulses. *Journal of Food, Agriculture and Environment*, *7*(2), 33–39.

Al-Zamel, F. A. (2009). Detection and diagnosis of mycobac terium tuberculosis. *Expert Review of Anti-Infective Therapy*, *7*(9), 1099–1108. https://doi.org/10.1586/ERI.09.92

Carvalho-Pereira, J., Fernandes, F., Araújo, R., Springer, J., Loeffler, J., Buitrago, M. J., Pais, C., & Sampaio, P. (2020). Multiplex PCR based strategy for detection of fungal pathogen dna in patients with suspected invasive fungal infections. *Journal of Fungi*, *6*(4), 1–16. https://doi.org/10.3390/jof6040308

Casquete, R., Benito, M. J., Córdoba, M. de G., Ruiz-Moyano, S., & Martín, A. (2017). The growth and aflatoxin production of Aspergillus flavus strains on a cheese model system are influenced by physicochemical factors. *Journal of Dairy Science*, *100*(9), 6987–6996. https://doi.org/10.3168/jds.2017-12865

Ehrlich, K. C. (2014). Non-aflatoxigenic Aspergillus flavus to prevent aflatoxin contamination in crops: Advantages and limitations. *Frontiers in Microbiology*, *5*(FEB). https://doi.org/10.3389/fmicb.2014.00050

In, S. (2020). *What are the 4 steps of PCR? | AAT Bioquest*. 3–4. https://www.aatbio.com/resources/faq-frequently-asked-questions/What-are-the-4-steps-of-PCR

Khan, R., Ghazali, F. M., Mahyudin, N. A., & Samsudin, N. I. P. (2021). Biocontrol of aflatoxins using non-aflatoxigenic aspergillus flavus: A literature review. *Journal of Fungi*, *7*(5), 7050381. https://doi.org/10.3390/jof7050381

Molecular Targets and Therapeutic Uses of Spices - Modern Uses for Ancient Medicine. (2010). *Molecular Targets and Therapeutic Uses of Spices - Modern Uses for Ancient Medicine*. https://doi.org/10.1142/9789812837912

Reddy, L., & Bhoola, K. (2010). Ochratoxins-food contaminants: Impact on human health. *Toxins*, *2*(4), 771–779. https://doi.org/10.3390/toxins2040771

Selamat, J., & Iqbal, S. Z. (2016). Food safety: Basic concepts, recent issues, and future challenges. *Food Safety: Basic Concepts, Recent Issues, and Future Challenges*, *11*, 1–160. https://doi.org/10.1007/978-3-319-39253-0

Shiva Rani, S. (2021). Fungal Contamination of some Common Spices. *Comparative Study of M Oleifera and M Ovalifolia Survival Rates in Central Namibia*, *5*(1), 1–4. https://doi.org/10.24966/pscr-3743/100014

Tsehaynesh, T., Abdi, M., Hassen, S., & Taye, W. (2021). Aspergillus SPECIES AND AFLATOXIN CONTAMINATION IN PEPPER (Capsicum annuum l.) IN WEST GOJJAM, ETHIOPIA. *African Journal of Food, Agriculture, Nutrition and Development*, *21*(1), 17178–17194. https://doi.org/10.18697/ajfand.96.18815

Wannop, C. C. (1961). The Histopathology of Turkey "X" Disease in Great Britain. *Avian Diseases*, *5*(4), 371. https://doi.org/10.2307/1587768