Isolation And Identification of Fungi Contaminated with Some Types of Spices and Detection of Mycotoxins Produced from Them in Al-Najaf Governorat

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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 nutmeg is Aspergillus flavus and in turmeric is Aspergillus niger ., and the results appeared. Aspergillus flavus isolated from nutmeg, the percentage of mycotoxins were as follows: aflatoxin 0,0 ochratoxin1,1, and T2 31,1, As for the results of the toxins isolated from the turmeric the fungus Aspariglis niger, they were as follows: Afha 0,0, Ochratoxin 2,9 , and 2 27,3. while growing the fungus in the above-described method, but on day 28, it showed that the percentage of aspergillus flavus in afla 0,8, ochratoxin 0,6, and T2 22,8 While the results of Aspergillus niger for aflatoxin 0,5 and ochratoxin 1,9 and T 2 67,1
Keyword: Aspergillus flavus , PDA ,Nutmeg , turmeric , Aspergillus Niger

1. Introduction

Spices have been known since ancient times, and this is due to the characteristics of their color, taste and sometimes pungent smell. However, it can serve several additional functions as a source of nutrients, antioxidants, preservation, and pesticides, in addition to medicinal plants. It is also important in the economy of some countries in trade (Fasoyiro, 2015) .

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 metabolic byproducts secreted mainly by fungi of the following genera Aspergillus, Fusarium, Penicillium, and Alternaria. Mycotoxins have been reported in many different types of foods such as black pepper, white pepper, cumin, Nutmeg , turmeric and coriander. Many mixed spices, Aflatoxins (AFs), Ochratoxion A (OTA) are primary metabolites in Aspergillus spp. and Penicillium spp. In spices, including hot pepper, which is considered more susceptible to the invasion of fungi causing mycotoxins, especially during long and inappropriate storage periods. Which leads to the production of mycotoxins. Conditions, processing methods, and spice handling practices may influence fungal growth and subsequent mycotoxin production. And the impact on humans must be a regulatory limit approved by many countries around the world (Syamilah et al.,2022).

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)

Ochratoxin is a group of mycotoxins produced by Aspergillus or Penicillium. They are weak organic acids. The Ochratoxin family consists of three members, A, B, and C, which differ slightly from each other in terms of their chemical structures. A is the most abundant and most toxic of the three because it mainly affects the kidneys, as is the case with other

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mycotoxins. This toxin can be found in a variety of foods that have been contaminated by the fungus during growth, harvesting, storage, and shipping (Government of Hong Kong, 2006).

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).

The use of molecular diagnostics has a remarkable accuracy, specificity, and speed to differentiate between species and subspecies of fungi, in contrast to the traditional tests used in diagnosis (Liu et al., 2000).

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.

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

2. Results and Discussion

The results in Table 1 show that the toxicity between Aspergillus Flavus isolated from nutmeg and Aspergillus Niger isolated from turmeric showed that the percentage of aflatoxin toxin in both of them was non-existent, while the percentage of ochratoxin was estimated to be higher in Aspergillus Niger about 2.9 than it was in Aspergillus Flavus is 1.1, while the percentage of T2 was higher in Aspergillus Flavus about31.1 than in Aspergillus Niger is27.3.

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	Nutmeg	0.0	1,1	31,1
Aspergillus niger	Turmeric	0,0	2,9	27,3

The results shown in Table (2) The results of the second table showed at the age of 28 that the fungus Aspergillus flavus about 0.8 contained a higher toxicity than Aspergillus niger about 0.5 in aflatoxin, while the ochratoxin in the fungus Aspergillus niger1.9 was higher than that of Aspergillus flavus 0.6 , and the toxicity of T2 Aspergillus niger about 67.1 was higher than the toxicity of Aspergillus flavus about 22.8. 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	Nutmeg	0.8	0.6	22.8
Aspergillus niger	Turmeric	0,5	1,9	67.1

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). Among the fungal contamination of various spices, the presence of mycotoxins was reported in the nutmeg plant, which are toxins that are carcinogenic to humans and pets. This study was conducted on 52 types of different spices, including nutmeg. , B. These isolates belong to the aflatoxins *Aspergillus niger* . nutmeg It is the most popular spice all over the world. In addition to their culinary uses, a number of functional properties of aromatic herbs and spices are also well described in the scientific literature. However, spices and herbs grown mainly in tropical and subtropical regions can be contaminated with toxic fungi and thus mycotoxins (Thanushree et al., 2019)

Fungi and their production of mycotoxins are affected by factors such as ozone dose, temperature, pH, and moisture content of the sample as critical parameters that determine the efficiency of ozone fumigation and mycotoxin decontamination (Daou et al., 2021).

Ochratoxin a (OTA) exhibits carcinogenic, nephrotoxic, teratogenic, immunotoxic, and neurotoxic properties. It has also been associated with nephropathy in humans. OTA is a small molecule soluble in water and it is chemically constituted by a combination of an amino acid (phenylalanine) and a polyketide to carbon 10, contains one chlorine atom necessary for its biological activity, and it is stable when exposed to heat (Ota, 2014).

T-2 is the most common cause of poisoning from consumption of contaminated grain-based foods and feeds reported in humans and animals. The food and feed most contaminated with T-2 toxin are made from wheat, barley and corn. After presentation or ingestion, T-2 is immediately absorbed from the gastrointestinal tract or through the respiratory mucous membranes and transported to the liver as the primary organ responsible for toxin metabolism. Depending on age, method of exposure and dose, poisoning is manifested by vomiting, refusal to feed, gastric necrosis and skin irritation, which is rarely observed in mycotoxin poisoning. In order to eliminate T-2 toxin, various decontamination techniques have been found to mitigate the concentration of T-2 toxin in agricultural commodities.(Janik et al., 2021).

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