Journal of Kerbala for Agricultural Sciences Issue (4), Volume (11), (2024)

Evaluating biological agents and plant extracts against *Aspergillus niger* in pomegranate fruit rot and *Ochratoxin A* prevention

Fatima Haider Abd-zaid Al-fatlawi, Yasir Naser Alhamiri

Plant Protection department, Agriculture College, University of Kerbala, Kerbala, Iraq.
***Corresponding author e-mail: faima.h@s.uokerbala.edu.iq**
https://doi.org/10.59658/jkas.v11i4.2803

Received:

Aug. 05, 2024

Accepted:

Sept. 15, 2024

Published:

Dec. 15, 2024

Abstract

The aim of the research was to conduct an ecological and biological survey of the extent of pomegranate fruit rot disease and to identify the fungi responsible for these cases in Iraq. The study involved isolating and identifying pathogenic *Aspergillus niger* isolates capable of producing the *mycotoxin Ochratoxin* A and evaluating the effects of certain plant extracts, agricultural fungicides, and the yeast *S. cerevisiae* in protecting pomegranate fruits from *A. niger* attack during storage. The isolation and identification results revealed that all samples were contaminated with various fungal isolates, with a contamination rate of 100% .The storage experiment results showed that percentage of disease severity, coriander extract significantly reduced the severity to 8.53%, compared to 23.23% for the pathogen-only treatment, with a fungal inhibition rate of 63.28%. This was followed by the treatment with sage extract, which reduced the severity to 10.13% with an inhibition rate of 56.39%. Lastly, the plant-based pesticide *Palizin* and the biological agent *(S. cerevisiae*) achieved reductions in disease severity of 11.86% and 10.73%, respectively, with inhibition rates of 45.94% and 53.80% compared to the control treatment. The results showed that treating stored pomegranate fruits with alcoholic coriander extract reduction in the levels of *Ochratoxin A*, production, with a reduction percentage of 6.92%. The *A. niger* fungal isolate was able to resist the effect of the plant extract and produce the mycotoxin *Ochratoxin A*, at a rate of 57.8 microgram/kg, compared to the pathogen-only treatment, which resulted in toxin production of 62.1 microgram/kg.

Keywords: *Punica granatum* L., Fruit rot, *A.niger*, *Ochratoxin A* mycotoxin.

Introduction

The pomegranate is one of the oldest fruits known to humans, and its nutritional and medicinal values were recognized early on, leading to its cultivation for these benefits. It is a comprehensive pharmacy for treating and preventing diseases [1]. Pomegranate trees are attacked by various fungal pathogens in the field and during storage, causing numerous diseases, the most significant of which is pomegranate fruit rot. A major cause of this disease is the fungi *Aspergillus niger* [2]. This fungus is considered significant and is found worldwide in various ecosystems. It is a causa-

tive agent of black heart disease in pomegranates, affecting the fruits and causing their decay. It also affects the flowers, making it difficult for farmers to identify the disease during the growing season. remains intact, but the internal content is hollow and damaged [3].

Studies indicate that *Aspergillus spp*. Particularly those producing mycotoxins like *aflatoxins* and ochratoxins, not only constitute a large group of fungi associated with pomegranate fruit rot and are responsible for fruit deterioration but also pose a health risk to consumers of pomegranate fruits or derived products [4]. Biological and novel control methods have been adopted to reduce the significant economic losses caused by these pathogens. Although chemical pesticides are effective, this approach does not align with modern strategies that aim to reduce pesticide use due to their negative impacts on the environment, non-target organisms, human health,human health, and the emergence of resistant strains. Therefore, most studies have focused on identifying alternative methods that are both effective and environmentally friendly, such as using antagonistic organisms to combat pathogens and developing their efficiency as an alternative strategy. Many studies have focused on the role of the yeast *Saccharomyces cerevisiae* in combating these diseases, demonstrating high efficacy against soil-borne pathogens under laboratory conditions and reducing the impact of pathogens under greenhouse and field conditions [5].

One of the most important control methods currently used to combat post-harvest disease pathogens is plant extracts. These extracts are promising alternatives to chemical resistance methods, as many have proven effective against fungal, bacterial, and other pathogens. They are cost-effective, safe to use, and do not leave toxic residues on plants and fruits. Moreover, they are easily accessible due to their natural abundance [6]. The study aimed to evaluate the efficiency of certain biological agents, such as *S. cerevisiae* and plant extracts from coriander, sage, and others, in combating *Aspergillus niger* isolates, which cause pomegranate fruit rot and prevent them from producing the mycotoxin *Ochratoxin A*.

Materials and Methods

Collection of pomegranate fruit samples infected with fruit rot disease

Pomegranate fruit samples were collected from eight different agricultural sites. Local pomegranates were randomly gathered from several orchards, while imported fruits were collected from specialized local fruit markets. The sampling covered various agricultural areas, including sites in the Kerbala and Babylon governorates, to ensure that the survey represented all cases of fruit rot in pomegranates. The survey included five sites in Kerbala: Al-Huseiniyah district, Center district, Al-Hur, Al-Hindiyah and Ain Al-Tamr. There are three sites in Babylon: Al-Musayb, Saddat Al-Hindiyah, and Al-Mashru. For each site, three sub-samples were collected, each weighing 2-3 kg, and placed in bags labeled with the sample code, the site of collection, and the collection date. The samples were then transported to the Mycology and Mycotoxin Laboratory in the Department of Plant Protection, College of Agriculture, University of Kerbala.

Isolation and identification of fungi associated with pomegranate fruit rot disease

Fungi associated with pomegranate fruit rot disease were isolated: The infected fruits were thoroughly washed and surface-sterilized using a 2% sodium hypochlorite solution for one minute. They were then rinsed with sterile distilled water to remove any residual sterilizing solution, and excess water was removed using sterile filter paper. Subsequently, The sterilized sections were transferred with sterile tweezers to Petri dishes containing a suitable growth medium. Five fruits were placed in each petri dish (P.D.A.), with 100 pomegranate fruits per sample. The Petri dishes were incubated at 25±2°C. After four days, the fungal colonies growing on the plates were examined under a microscope for identification [7]. Identification was performed according to the classification key [8]. and the morphological characteristics described by [9,10,11].

Pathogenicity testing of *Aspergillus niger* isolates from pomegranate fruits

Pathogenicity testing was conducted on eight fungal isolates selected from those isolated in this study from pomegranate fruits (one isolate per sample). This selection aimed to reduce the number of isolates and identify the most virulent ones for use in subsequent tests and field trials. The pathogenicity experiments were conducted in a refrigerated storage room at the College of Agriculture, University of Kerbala. Healthy pomegranate fruits were placed in metal trays lined, with each serving as an experimental unit containing five healthy fruits. The fruits were sterilized for 3 min, in a 1% NaOCl solution and then rinsed with sterile distilled water (S.D.W.).

The fungal inoculum (fungal suspension) was prepared by growing the fungi on .D.A.P.D.A. medium for 7 days at 25 + 2 C. Then the fungal spores were harvested by adding 10 ml of sterile distilled water to each of the plates on which the fungus was grown. Then a sterile glass rod was passed over the surface of the colonies to facilitate the process of separating the spores from the conidiophores. After that, the spore suspension was collected, and the numbers were calculated using a hemocytometer slide. The spore concentration of the fungal species was adjusted to (0.6 spore/ml).

The artificial inoculation of pomegranate fruits with fungal isolates was performed using two distinct methods. The first method involved injecting a suspension of each fungal isolate at a concentration of 5 x 104 conidia/ml (0.5 ml per fruit) into the fruit through the calyx area, with three replicates per isolate using a 15 ml syringe. The second method involved scratching the fruits and spraying them with the fungal suspension (making superficial wounds on the fruit skin without reaching the seeds), with three replicates and five fruits per replication, where 1 ml of the suspension was sprayed on each fruit. Three replications were treated with sterile distilled water (S.D.W.) as controls. After 21 days at a temperature of 22°C, the results of both methods were assessed.

Detection of fungal isolate capability to produce the mycotoxin *Ochratoxin A* in pomegranate fruits

Extraction of *Ochratoxin A*

Pomegranate seeds, dried and derived from the cultivation of pathogenic fungal isolates, were ground using an electric mill to obtain fine and homogeneous particles. Ten grams of the powder from each sample were added to an extraction solution (methanol CH_4O: potassium chloride KCl 4%) in a 1:9 ratio, with a volume of 60 ml. The resulting mixture was homogenized using a shaker (45 min, 200 rpm, at room temperature). After 24 hours, the mixture was filtered through Whatman no.2 filter paper (primary filtrate). 30 ml of this filtrate was mixed well with 30 ml of 30% ammonium sulfate $(NH_4)_2SO_4$ solution and then filtered again using the same method [12].

The initial toxin extract was purified using a chromatographic column (50 cm × 12 mm) with silica gel activated by heating in an electric oven (130°C for 45 min). Then, 0.5 g of anhydrous sodium sulfate (Na_2SO_4) was added to create a uniform base for the silica gel. Chlorofluorocarbons ($CHCl_3$) were added until the column was approximately half-filled. Subsequently, 2.0 g of silica gel was added slowly, followed by another 0.5 g of anhydrous sodium sulfate (Na_2SO_4). The prepared extract was added slowly to the column, and the eluent (25 mL) was collected. Separation was achieved by adding 50 mL of chlorofluorocarbons ($CHCl_3$) to the mixture, which was then placed in a separating funnel and shaken well. The upper layer was discarded, and the process was repeated twice. The remaining filtrate was passed over 10 g of anhydrous sodium sulfate (Na_2SO_4). The solvent was evaporated, and the remaining solution was stored in a dark vial (2.0 mL at -20°C) until qualitative and quantitative analysis was performed [12].

Qualitative detection and quantitative estimation of the mycotoxin *Ochratoxin A* using HPLC technology

Chromatographic analysis was conducted at the Ministry of Science and Technology / Environmental and Water Research and Technology Directorate using a high-performance liquid chromatography (HPLC) system. The analysis employed a C18 reversed-phase column (150 mm × 4.6 mm, 3.5 µm) and a fluorescence detector (F.L.D., exc = 333 nm, em = 460 nm; gain = 100). The sample was filtered with a 1 ml/min flow rate and an injection volume of 10 microliters. The mobile phase consisted of a mixture of acetonitrile/water/acetic acid (99:99:2, v/v/v), and the column temperature was maintained at 30°C [13].

Molecular diagnosis of the *Aspergillus niger* isolate producing the mycotoxin *Ochratoxin A*

The fungal isolate (KTAN) of *Aspergillus niger* was targeted due to its high virulence in causing pomegranate fruit rot and its capacity to produce the mycotoxin *Ochratoxin A.* T.N.A.he isolate was characterized through D.N.A. D.N.A. sequencing to study its genetic and genomic composition and compare it with global isolates. PCR products were sent to Macrogen Inc. in South Korea for nucleotide sequencing of the Internal Transcribed Spacer (ITS) region. Upon receiving the nucleotide se-

quences of the fungal isolates, the sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) to compare them with available data in the National Center for Biotechnology Information (NCBI) gene bank for similar globally characterized fungal isolates [14].

The fungal isolate was recorded as not matching any nucleotide sequences 100% in NCBI. Additionally, nucleotide analysis was performed using MEGA X version 11 software to analyze the isolates and construct a phylogenetic tree showing the evolutionary relationships among these isolates and similar isolates registered at the NCBI. The phylogenetic tree, constructed using the Neighbor-Joining method, was based on the ITS region's nucleotide sequence for each isolates [15].

Preparation of alcoholic plant extracts from selected plants

Alcoholic plant extracts were prepared from several plants, including sage (*Salvia officinalis*), cinnamon (*Cinnamomum cassia*), coriander (*Coriandrum sativum*), thyme (*Thymus vulgaris*), clove (*Syzygium aromaticum*) and sesame (*Sesamum indicum*). For each plant, 20 grams of powdered material was placed in a 500 ml glass flask, and 200 ml of 70% ethanol was added. The mixture was then placed in a shaker for 24 hours at 35°C. Afterward, the mixture was filtered using Whatman No.1 filter paper. The filtrate was transferred to 10 cm diameter glass beakers, and the ethanol was evaporated in an oven at 40°C until completely removed. The resulting plant powder was stored in dark, airtight tubes and kept in a freezer at -18°C until use [16,17].

Evaluation of the efficacy of various plant extracts, chemical pesticides, and the biological agent against the growth of *Aspergillus niger* in the Laboratory

Activation of *Saccharomyces cerevisiae* yeast and testing its effectiveness in inhibiting the growth of *Aspergillus niger* isolate

The yeast *Saccharomyces cerevisiae* was grown in Nutrient Yeast Dextrose Broth (NYDB). The method involved adding 1 ml of a 3-day-old yeast suspension (dilutions 6th, 8th, and 10th) to a Petri dish containing Potato Sucrose Agar (P.S.A.) medium, and the dish was gently swirled to distribute the yeast suspension. A 0.5 cm diameter disc from a 7-day-old fungal culture was then placed at the center of each dish. Three plates were used for each treatment, and three additional plates were kept without yeast as controls. The plates were incubated at 25±2°C for 7 days, after which the average fungal growth and the percentage inhibition were calculated to determine the most effective concentration for inhibiting fungal growth [18].

Testing the Effect of Plant Extracts on the Growth of *Aspergillus niger* Isolate in the Laboratory

The effect of six different plant extracts (sage, coriander, cinnamon, thyme, sesame and Clove) were tested on the growth of pathogenic fungal isolates from decayed pomegranate fruits. The prepared plant extracts (dissolved in 10 ml of ethanol) were mixed with a petri dish (P.D.A.) that had been cooled to 45°C at a concentration of 2 ml per 100 ml of medium. The mixture was then poured into Petri dishes, with three replications for each extract. After the medium had solidified, a 0.5 mm disk of the fungal isolates used in the study was placed using a cork piercer in the medium

mixed with the plant extract, with replicates of the control treatment being made (by adding 2 ml of ethyl alcohol to 100 ml of the culture medium and pouring it into plates, and inoculated with fungi). The plates were incubated at 25±2°C for a week (when the control growth was complete). The average diameter of the growing colony was measured, the results were recorded, and the percentage of inhibition was calculated, on the basis of which the most effective and inhibiting plant extracts were selected.

Testing the efficacy of certain pesticides against the growth of *Aspergillus niger* isolate in the Laboratory

The effectiveness of two agricultural pesticides, the botanical-origin chemical pesticide Palizin 65% .L.S.L. and the red pepper extract pesticide Tondexir 80% E.C., was tested against the growth of pathogenic fungal isolates. Prepared the culture medium (P.D.A.) in glass flasks with a capacity of 250 ml and sterilized using an autoclave at a temperature of 121°C and a pressure of 15 Ib/in, 20 min. After sterilization, the flasks were allowed to cool to 45°C, and the pesticides were added to the medium at concentrations close to those recommended by the manufacturers: 1, 2, and 3 g/L for each pesticide. The medium was mixed thoroughly and poured into Petri dishes. Once the medium had solidified, the plates were inoculated by placing a 0.5 cm diameter disc of the fungal isolates in the center of each plate using a sterile punch. A control plate with medium without any pesticide was also prepared. The plates were incubated at 25°C for one week. The percentage of inhibition was calculated, and the most effective and inhibitory concentration and pesticide were selected based on these results [19].

Testing the effect of alcoholic plant extracts of sage and coriander, the pesticide (Palizin), and *Saccharomyces cerevisiae* yeast in protecting pomegranate fruits from *Aspergillus niger* infection in storage

After conducting previous laboratory tests, the most effective pesticide, Palizin, was selected, along with the two most efficient plant extracts, sage extract and coriander extract, as well as the sixth effective dilution of the yeast *S. cerevisiae*. The study examined the effects of the plant extracts and *S. cerevisiae* yeast in combination with the pesticide Palizin against fungal isolates' growth and protecting pomegranate fruits from their attack. The fungal isolate suspension, *S. cerevisiae* yeast suspension, plant extracts, and pesticide concentration were prepared as outlined in previous laboratory experiments. Simultaneously, the refrigerated storage was sterilized and equipped with experimental units of healthy pomegranates, with three replications for each treatment and five fruits per replication. The pomegranate fruits were scratched and treated with all control factors individually 24 hours before inoculating the fruits with the pathogenic fungal isolates to allow stabilization on all fruit surfaces. The following day, the pomegranates were artificially inoculated with the fungal isolates (1 ml of fungal suspension per fruit). Three replicates were left as a control treatment without any additions in each group. The experiment included six treatments: a control treatment (no addition), treatment with the pathogen only (*A. niger*), treatment with the pathogen + sage extract (ex. S.o + *A. alternata*), treatment

with the pathogen + coriander extract (ex. C.s + *A. niger*), treatment with the pathogen + Palizin pesticide (Palizin + *A. niger*) and treatment with the pathogen + yeast (*S. cervi*. + *A. niger*). After 21 days of the experiment, the results were calculated by determining the percentage of infection and the percentage of infection severity for each of the factors used and assessing the production of fungal toxins. These results were compared with the control and pathogen-only treatments to identify the best treatments used.

Results and Discussion

Isolation and diagnosis of fungi associated with pomegranate fruit rot disease

The isolation and identification results showed that all samples taken from infected pomegranate fruits, collected from five different areas in Karbala Governorate and three areas in Babil Governorate, were contaminated with various fungal isolates at a contamination rate of 100% (Table 1). The isolation and identification revealed 196 fungal isolates belonging to several fungal genera. *Aspergillus niger* was the most prevalent, followed by *Alternaria alternata* and then the genus *Rhizopus spp.*

Table (1): Fungi isolated from pomegranate fruits infected with fruit rot disease

Isolated Fungi	Kerbala Governorate					Babylon Governorate			Total
	Al- Husseiniyah district	Al- Hindiyah district	Ain Al-Tamr	Center district	Al-Hur district	Saddat Al-Hindiyah	Al-Musayb	Al-Mashru	
	KH	KT	KA	KC	KR	BH	BM	BS	
A. niger	7	3	6	5	8	3	11	8	52
A. flavus	2	4	3	0	5	9	2	1	26
A.ochraceus	2	0	3	1	3	3	0	0	12
Aspergillus terreus	2	0	0	2	0	1	0	1	6
Alternaria.alternata	3	5	8	8	7	9	2	7	48
P.expansum	1	0	3	0	0	2	0	0	6
Botrytis sp	0	0	2	2	0	6	0	2	12
Rhizopus	7	5	6	1	5	1	2	7	34
	21	17	34	19	28	34	17	26	196

The results showed that the percentage of occurrence for the isolated fungi (Table 2) recorded the highest occurrence rate of 100% for *A. niger*, *A. alternata*, and *Rhizopus spp.* These were followed by *A. flavus* and *A. ochraceus*, with occurrence rates of 87.5% and 62.5%, respectively. Meanwhile, the results for the percentage frequency of fungal species showed that *Aspergillus niger* had the highest frequency at 26.53%, followed by *Alternaria alternata* at 24.49%, *A. flavus* at 13.27% and *A. ochraceus* at 6.12%.

Table (2): Percentage of occurrence and frequency of fungi isolated from pomegranate fruits

Isolated fungi	Total isolates (%)	Percentage of appearance (%)	Frequency percentage (%)
A. niger	52	100	26.53
Alternaria.alternata	48	100	24.49
A. flavus	26	87.5	13.27
A.ochraceus	12	62.5	6.12
Aspergillus terreus	6	50	3.06
P.expansum	6	37.5	3.06
Botrytis spp	12	50	6.12
Rhizopus spp	34	100	17.35

Pathogenicity of the studied fungal isolates

The results showed a significant variation in the pathogenicity of the fungal isolates in causing pomegranate fruit rot disease. Out of eight fungal isolates of *A. niger*, three exhibited high pathogenicity. There were notable differences between the tested fungal isolates and the methods used in artificial inoculation (injection or scratching) regarding their impact on the infection rate of pomegranate fruit rot (Table 3). Three fungal isolates, specifically *A. niger-2* (KRAN), *A. niger-5* (KTAN), and *A. niger-8* (BMAN), showed the highest virulence with a 100% infection rate in the scratching method. In contrast, the injection method resulted in a 100% infection rate for all fungal isolates. Therefore, the scratching method was adopted for artificial inoculation with fungi in subsequent pomegranate storage experiments.

Table (3): Percentage of infection in the pathogenicity test of the selected fungal isolates

Fungal isolate	Sample isolated from it	Isolate code	Injury in injection treatment (%)	Injury in scratch treatment (%)
A. niger 1	KH	*KHAN*	100	80
A. niger 2	KR	*KRAN*	100	100
A. niger 3	KC	*KCAN*	100	93.33
A. niger 4	KA	*KAAN*	100	86.66
A. niger 5	KT	*KTAN*	100	100
A. niger 6	BH	*BHAN*	100	73.33
A. niger 7	BS	*BSAN*	100	80
A. niger 8	BM	*BMAN*	100	100
L.S.D $_{0.05}$			0.8858	2.8589

The results also showed significant differences among the tested fungal isolates in terms of their impact on the percentage severity of pomegranate fruit rot disease (Table 4). The three highly virulent fungal isolates demonstrated infection severity rates of 52%, 42% and 30% for the injection method. In contrast, the scratching method resulted in severity rates of 43%, 37%, and 24% for the isolates *A. niger-5* (KTAN), *A. niger-2* (KRAN), and *A. niger-8* (BMAN), respectively.

Table (4): Percentage of infection severity in the pathogenicity test for selected fungal isolates

Fungal isolate	Sample isolated from it	Isolate code	Injury in injection treatment (%)	Injury in scratch treatment (%)
A. niger 1	KH	*KHAN*	32	11
A. niger 2	KR	*KRAN*	42	37
A. niger 3	KC	*KCAN*	18	14
A. niger 4	KA	*KAAN*	20	18
A. niger 5	KT	*KTAN*	52	43
A. niger 6	BH	*BHAN*	22	18
A. niger 7	BS	*BSAN*	19	17
A. niger 8	BM	*BMAN*	30	24
L.S.D 0.05			1.4542	1.9969

Detection of *A. niger* Isolates' Ability to Produce *Ochratoxin A* in Pomegranate Fruits

The chromatographic analysis using HPLC technology indicated that all three *A. niger* isolates—*A. niger-5* (KTAN), *A. niger-2* (KRAN), and *A. niger-8* (BMAN)—were capable of producing the mycotoxin *Ochratoxin A*. Among these, the isolate *A. niger-5* (KTAN) demonstrated a high ability to produce *Ochratoxin A* in pomegranate fruits, with levels reaching 72.5 microgram/kg, compared to the control (fruits not treated with the pathogen), which recorded 0.00%. The other two isolates, *A. niger-2* (KRAN) and *A. niger-8* (BMAN), showed lower production levels of 23.56 and 21.66 microgram/kg, respectively. Therefore, *A. niger-5* (KTAN) was selected as the most virulent and highest *Ochratoxin A*, producing isolate for subsequent research experiments.

Nucleotide Sequence Analysis of *A. niger-5* (KTAN) Isolate

The nucleotide sequence analysis results for the *A. niger* isolate from pomegranate fruits, which exhibited high virulence and a strong ability to produce the mycotoxin *Ochratoxin A*, confirmed its identity as an *Ochratoxin A*-producing fungus. This fungal isolate has been registered with the National Center for Biotechnology Information (NCBI) under the accession number PQ034726.1. The molecular nucleotide sequences showed a high degree of similarity, ranging from 99.82% to 99.46%, with the ITS genetic region compared to equivalent nucleotide sequences retrieved from the NCBI GenBank using the BLAST program. Additionally, nucleotide analyses were performed using the MEGA program to analyze the isolates and construct a phylogenetic tree, illustrating the relationships between this isolate and similar isolates recorded at the NCBI based on the ITS region's molecular nucleotide sequence.

By comparing the nucleotide sequences of the *A. niger* Y.n.191.Fatima .N.A.D.N.A. band isolated from pomegranate fruits with data available from the National Center for Biotechnology Information (NCBI), a genetic similarity of 99.64% to 99.29% was observed with all *A. niger* isolates (Table 5). The phylogenetic tree (Fig. 1) showed that this isolate has a significant genetic affinity (same genetic line-

age) with the Chinese isolate recorded under accession number MF067456.1. It also displayed secondary similarity with the Iranian (MZ474847.1) and Nigerian (OQ085118.1) isolates, indicating a secondary genetic lineage. In contrast, it appeared in separate clades from other fungal isolates, particularly the Indian (MK633877.1) and Malaysian (GU362012.1) isolates, due to significant genetic divergence.

Table (5): Comparison of the nucleotide sequence similarity percentages of the ITS gene region of the fungal isolate *A. niger* . Y.n.191.Fatima and other fungal isolates of the same fungus registered worldwide in NCBI

Fungal name	Isolate code	Place of isolation origin	Accession number	Sequence similarity	Registration date
A.niger	*Y.n.191.Fatima*	Iraq	PQ034726.1	100	2024
A.niger	breed P.D.A.	china	MF067456.1	99.65	2017
A.niger	isolate DNik4	IRAN	MZ474847.1	99.64	2021
A.niger	isolate D9	Nigeria	OQ085118.1	99.29	2022
A.niger	isolate N72	India	MK633877.1	99.46	2019
A.niger	isolate .P.M.U.P.M. A7	Malaysia	GU362012.1	99.46	2011

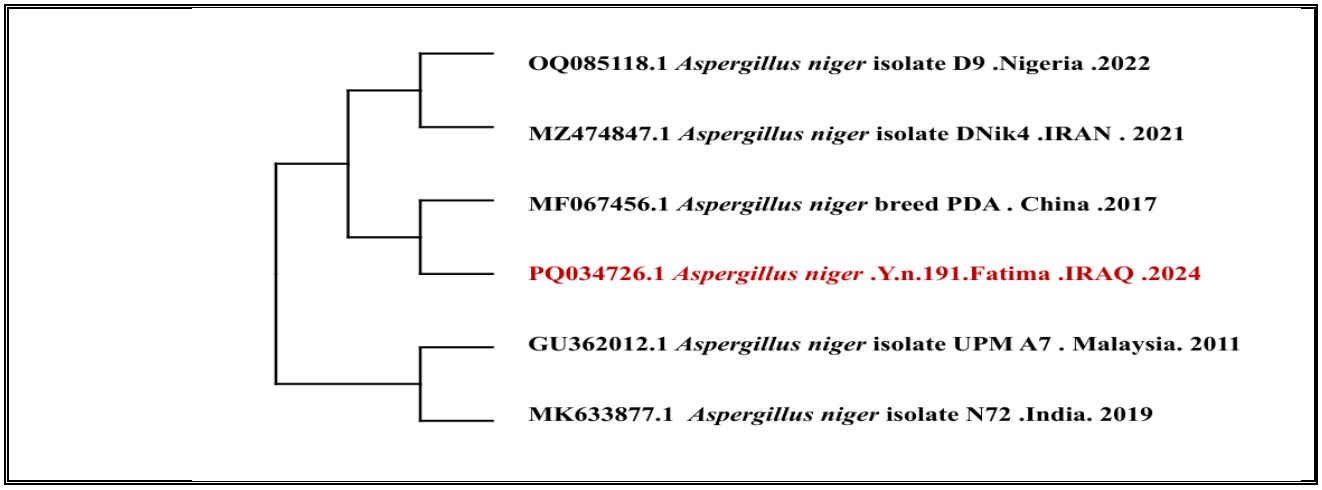


Figure (1): Genetic tree of the fungal isolate A.niger. Y.n.191.Fatima

Effect of plant extracts on the growth of pathogenic fungal isolates in the Laboratory

The results indicated that all plant extracts inhibited the growth of fungal isolates to varying degrees. Some extracts exhibited high inhibitory effectiveness, with inhibition percentages reaching 100%. Specifically, the sage and coriander extracts completely prevented fungal growth, achieving the highest inhibition percentage of 100% compared to the pathogen control, which showed 0.00%. Other extracts, including thyme, Clove, sesame, and cinnamon, also demonstrated significant inhibitory effects with percentages of 79%, 73%, 73%, and 72%, respectively, compared to the control

at 0.00%. Based on these results, sage and coriander extracts were selected as the most effective plant extracts for subsequent experiments.

These findings are consistent with the study by [20], which reported that a 20 µL/mL ethanol extract of coriander (*Coriandrum sativum*) was highly effective in inhibiting the growth of pathogenic fungi. Similarly, [21] highlighted the inhibitory efficacy of ethanol extracts from sage (*Salvia officinalis*). In their study, five concentrations of each extract (5%, 10%, 15%, 20%, and 25%) were tested for their effect on fungal growth inhibition of soil-isolated *A. niger*. The results demonstrated that ethanol extracts significantly inhibited fungal growth in the culture medium.

Evaluation of the Efficacy of the Pesticides Palizin and Tondexir and the Yeast *S. cerevisiae* Against Fungal Isolates in the Laboratory

The results indicated that the chemical pesticide of plant origin, Palizin, exhibited the highest inhibition rate against the pathogenic fungal isolates, with an inhibition percentage of 87.03% (Table 6). The pesticide Tondexir inhibited at 77.77% compared to the control, which showed 0.00% inhibition. Notably, a 0.3% concentration of Palizin completely prevented fungal growth, achieving 100% inhibition, compared to the pathogen control at 0.00%. Similarly, Tondexir at the same concentration achieved a high inhibition rate of 88.88%. Previous studies have confirmed that using plant-derived chemical fungicides is a safe and alternative method for controlling fungi affecting crops post-harvest [22].

Regarding the yeast *S. cerevisiae*, the sixth dilution of the yeast suspension completely inhibited the growth of the *A. niger* isolate, with a 100% inhibition rate. In contrast, the eighth and tenth dilutions showed varying inhibition percentages of 88.88% and 83.33%, respectively. Therefore, the sixth dilution was selected for use in subsequent experiments.

Table (6): Efficiency of the fungicides Palizin and Tondexir and the yeast *S.cervisiae* against the growth of fungal isolates in the Laboratory

Factors	Concentration (%)	Percentage of Inhibition (%)	Effect average (%)
Palizin	00	0.0	87.03
	0.1	77.77	
	0.2	83.33	
	0.3	100	
Tondexir	00	0.0	77.77
	0.1	66.66	
	0.2	77.77	
	0.3	88.88	
S.cervisiae	0.00	0.0	90.73
	$1*10^{-6}$	100	
	$1*10^{-8}$	88.88	
	$1*10^{-10}$	83.33	
L.S.D 0.05		**2.1012 (Only for higher concentration of factors)**	

Evaluation of the Effectiveness of Plant Extracts (Coriander and Sage), Pesticide (Palizin), and Yeast (*S. cerevisiae*) in Protecting Pomegranate Fruits from Pathogenic Fungal Isolates in Storage

The results indicated that, after 21 days of the experiment, the coriander extract demonstrated the highest effectiveness in reducing disease incidence, with an incidence rate of 73.33% compared to the pathogen-only treatment, which reached 100%. The percentage of fungal inhibition caused by coriander extract was 26.67% (Table 7, Fig. 2). This was followed by the biological agent (*S. cerevisiae*), which reduced disease incidence by 80%, with an inhibition rate of 20%.In comparison, the sage extract and the plant-derived pesticide (Palizin) showed a similar reduction in disease incidence, with a decrease of 86.66% for both treatments and an inhibition rate of 13.34% compared to the control.

Regarding disease severity, coriander extract significantly reduced the severity to 8.53% compared to the pathogen-only treatment, which was 23.23%, with a fungal inhibition rate of 63.28%. This was followed by sage extract, which reduced disease severity to 10.13% with an inhibition rate of 56.39%. Both the pesticide (Palizin) and the biological agent (*S. cerevisiae*) showed a final reduction in disease severity, with rates of 11.86% and 10.73%, respectively, and inhibition rates of 45.94% and 53.80% compared to the control.

Table (7): Effect of alcoholic plant extracts of sage, coriander, pesticide (Palizin) and yeast *S.cervisiae* on the percentage of infection with pathogenic fungal isolates in the storage

Treatment	Percentage of infection (%)	Inhibition percentage (%)	Severity of infection (%)	Severity inhibition (%)
Control	0.0	---	0.0	---
(*A.niger*)only	100	0.0	23.23	0.0
(Path)+(*S.cervi.*)	80	20	10.73	53.80
(Path)+(*Palizin*)	86.66	13.34	11.86	45.94
(Path)+(ex.*S.o*)	86.66	13.34	10.13	56.39
(Path)+(ex.*C.s*)	73.33	26.67	8.53	63.28
L.S.D 0.05	**Injury occurrence**		**Injury severity**	
	2.2331		**0.8788**	

(A.niger)+ (ex.S.o)

(A.niger)+ (ex.C.s)

Control

(*A.niger*) Only

(*A.niger*)+ (*S.cervi*.)

(*A.niger*) +(*Palizin*)

Figure (2): Models of the effect of plant extracts, pesticide (Palizin), and S.cervisiae in protecting pomegranate fruits.

Detection of the effect of alcoholic coriander extract in controlling *A.niger* fungus from producing mycotoxin *Ochratoxin A* in pomegranate fruits in storage

The results of HPLC chromatographic analysis showed that the fungal isolate A.niger with the code (KCAA) isolated from infected pomegranate fruits and collected from the center district of Kerbala Governorate showed a high ability to produce the mycotoxin Ochratoxin A in pomegranate fruits stored in the pathogen treatment only (21 days) and at high levels of 62.1 microgram/kg compared to the control treatment (fruits not treated with the pathogen) amounted to 0.00% (Fig. 3 and 4). At the same time, the results showed that treating the stored pomegranate fruits with alcoholic coriander extract led to inhibiting the pathogen's ability to attack pomegranate fruits and reducing the rate and severity of infection. This caused a partial reduction in the levels of *Ochratoxin A* production, as the percentage of toxin reduction reached 6.92%. The fungal isolate *A.niger* was able to resist the effect of the plant extract and produce the mycotoxin *Ochratoxin A* at a rate of 57.8 µg/kg.

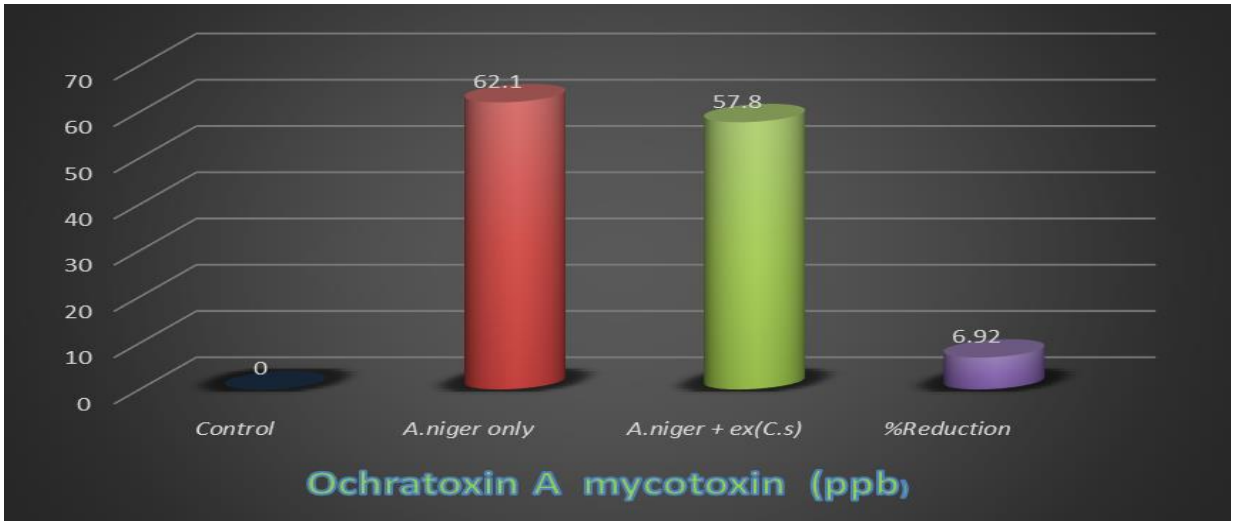
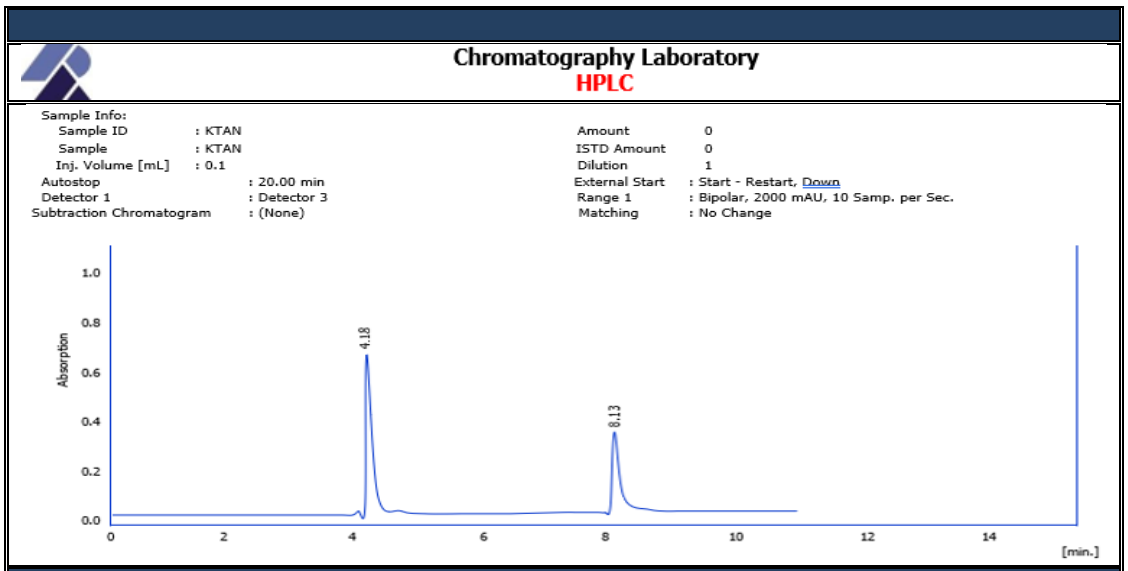


Figure (3): Effect of coriander extract on *A.niger* fungus in producing the mycotoxin *Ochratoxin A*.

These results are consistent with previous studies [23], which indicate that *Ochratoxin A* is one of the main and most widespread toxins in pomegranate fruits, with significant and potentially negative effects on humans when consuming contaminated fruit or food. Its main producer is *A. niger*.



Quantitative and qualitative estimation of *Ochratoxin A* produced by *A.niger* isolate in pomegranate fruits.

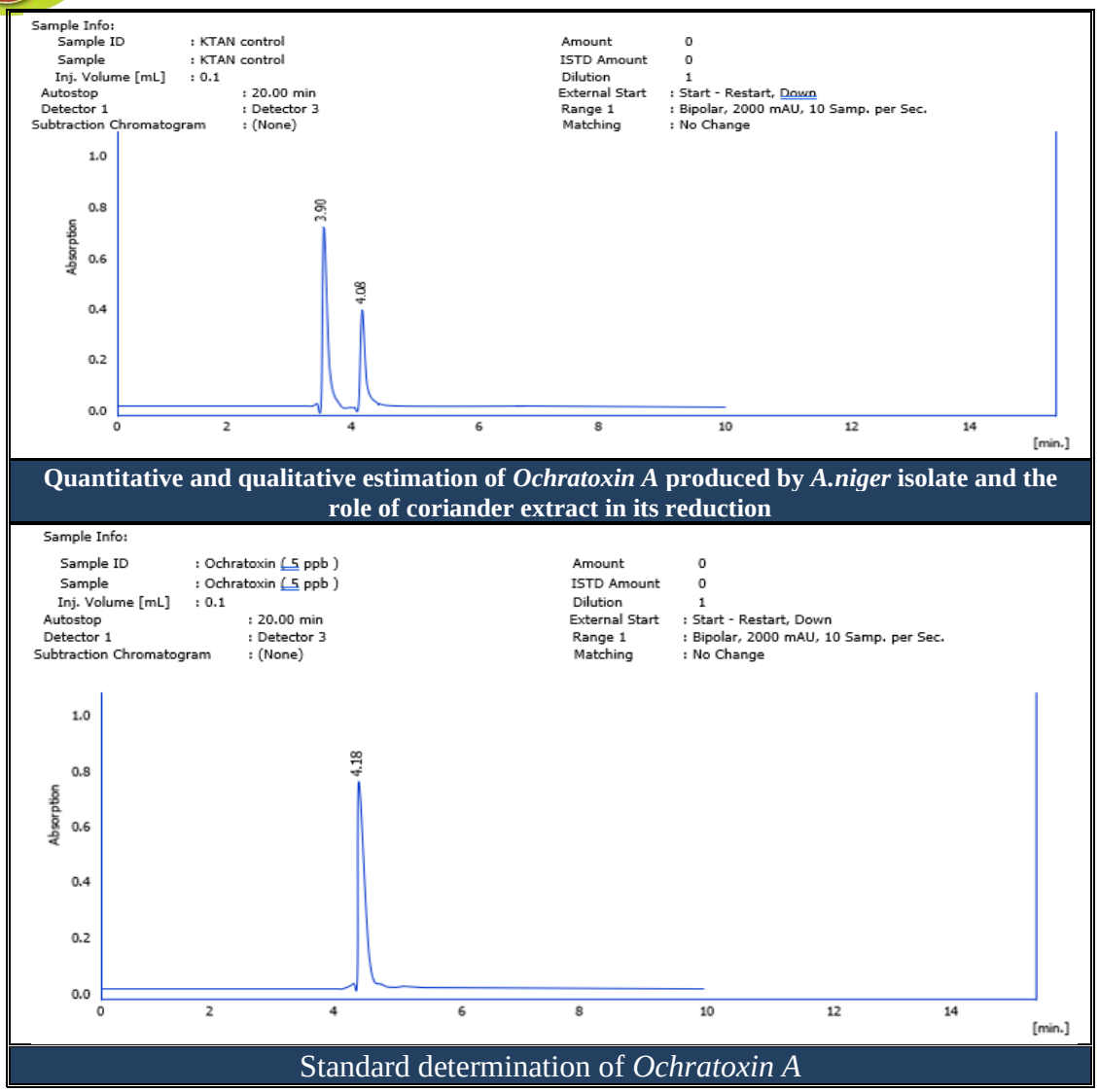


Figure (4): Detection of the ability of *A.niger* to produce the mycotoxin *Ochratoxin A*

Based on the information provided, it can be concluded that the most common fungi isolated from infected pomegranate fruits belong to the genus Alternaria sp. These fungi are known for their toxicity, as they can produce a wide range of mycotoxins. The results of molecular diagnosis confirmed their toxic properties by comparing them with known toxic fungi. Moreover, specific inhibitory substances, such as coriander and sage extracts, proved effective in restricting the growth and reproduction of these fungi.

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