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Effect of thyme oil, temperature, and storage period on the reduction of Ochratoxin A produced by *Aspergillus niger* in almond fruits

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Abstract

An experiment was conducted in one of the laboratories of the College of Agriculture - University of Kufa, to evaluate the effect of thyme oil and temperatures (10°C, 20°C, and 30°C) on the concentration of Ochratoxin A (OTA) produced by *Aspergillus niger* in Almonds stored for one and two months. The results showed significant differences in toxin concentrations according to the type of treatment, the treatment with fungus only, recorded the highest concentration (74.68 µg kg^{-1}) compared to the control treatment, which recorded a concentration of (5.39 µg kg^{-1}), confirming the strong toxigenic potential of the fungus to produce the toxin. In contrast, the treatment combining of thyme oil with the fungus significantly reduced the toxin concentration (32.40 µg kg^{-1}), indicating the effectiveness of the oil as an antifungal and toxin reducer. It was also found that increasing the storage temperature significantly increased OTA concentrations, with values of (12.9, 29.4, and 35.1 µg kg^{-1}) at 10, 20, and 30°C, respectively. Extending the storage period from one to two months also resulted in a significant increase in the toxin level (from 20.3 to 31.4 µg kg^{-1}). The highest value was recorded in the treatment with fungus only at 30°C for two months (124.8 µg kg^{-1}). Therefore, these results confirm that thyme oil can serve as an effective natural strategy for reducing the risk on contamination of Almonds with Ochratoxin A, especially under suboptimal storage conditions.

Keywords: *Prunus dulcis* L., Ochratoxin A, Thyme oil, Temperature, Storage period.

Introduction

Almond (*Prunus dulcis* L.) is a type of nut that belongs to the family Rosaceae and the order Rosales. Almonds have been known for a long time to be a source of vital nutrients [1]. Nuts in the field or in storage can be susceptible to a number of fungi. Some fungi can cause damage to the fruit but also produce mycotoxins that have a direct or indirect impact on human health. The most common mycotoxin producing fungi in storage that can infect almond fruits are *Rhizopus* spp, *Penicillium* spp, and *Aspergillus* spp [2].

In order to preserve these fruits from fungal infections, many methods have been used to limit their spread, especially during storage. The most prominent of these methods is the use of chemicals such as propionic acid [3], and fumigation using methyl bromide [4,5]. The frequent use of chemicals and pesticides has led to numerous negative consequences, with environmental pollution and negative effects on human and animal health resulting from their accumulation in the food chain appearing to be the most prominent [6]. Given the economic and health impacts of fungal contamination, there is an urgent need for safe, eco-friendly, and effective alternatives to chemical control [7]. In this context, it is recommended to store nuts at temperatures below 15°C and relative humidity below 70% to reduce the risk of fungal or mycotoxin contamination [8].

Aspergillus is considered one of the genera rich in species, as it belongs to the Ascomycota section, which comprises six subgenera, 27 sections, and over 400 species [9]. These species produce various secondary metabolites, including mycotoxins, which contribute to their pathogenicity [10]. The main reasons that explain the predominance of *A. niger* species are their ability to utilize a wide range of organic substances and adapt to many different environments, in addition to their ability to produce large numbers of conidia that are highly resistant to environmental stress and are easily dispersed by air due to their small size [11]. Many fungi of this genus infect crops in the field and accompany seeds during storage, thus causing seed contamination and a decrease in germination rate, in addition to the secretion of mycotoxins such as ochratoxins and aflatoxins, which makes grains contaminated with these types of fungi unfit for consumption and inefficient in germination [12].

Ochratoxin A is considered one of the most prominent mycotoxins. It belongs to the group of ochratoxins and is commonly produced by *Aspergillus spp.* and *Penicillium spp.* [13]. Ochratoxin A (OTA)the most common and toxic form, is the primary concern among ochratoxins [14,15]. It is considered one of the most biologically active fungal compounds with a highly toxic effect [16], making it relatively resistant to conventional cooking processes [17]. OTA is highly stable, resistant to common food processing conditions, which complicates its removal from contaminated products [18,19]. OTA is considered one of the five most prevalent and contaminating toxins in food and feed [20].

Ochrotoxin contamination is widespread and occurs during the growth, storage, and transportation of various foods and feeds, including cereals, legumes, coffee beans, grapes, cocoa beans, nuts, spices, and animal-derived products [21]. The European Food Safety Authority (EFSA) has set the tolerable weekly intake (TWI) for ochrotoxin A in nuts at 10 µg/kg in 2023. This is in line with the limits set by the National Organization for Standardization and Quality Control in Iraq (C.O.S.Q.C.) in specification number 5144 for mycotoxins [22].

Thyme oil, which is the common oil of wild thyme (*Thymus vulgaris*), is considered one of the most important plant oils used to reduce fungal growth, including toxin-producing fungi such as *A. niger*, thereby limiting the production of these toxins. The main reason for the effectiveness of thyme oil in inhibiting fungi is due to its content of many compounds, including thymol and carvacrol [23]. The antifungal nature of thymol is attributed to its ability to change the morphology of fungal hyphae, causing clumping of the hyphae which results in reducing their diameters and degradation of their walls. In addition, thymol is lipophilic, enabling it to interact with the fungal cell membrane, altering the permeability of the cell membrane by allowing the loss of large molecules from the cell, leading to cell death and consequently fungal death [24]. Carvacrol is a non-toxic compound with broad antimicrobial activity, acting mainly by disrupting fungal cell membranes [25,26]. The purpose of this study was to evaluate the effect of thyme oil, different temperatures, and storage period on reducing or eliminating ochratoxin A produced by *Aspergillus niger* in almond fruits, in order to identify the optimal conditions and methods for minimizing the accumulation of this mycotoxin.

Materials and Methods

Sample Collection

Samples were collected on 1-2/9/2024 from five different regions of Al-Najaf Al-Ashraf Governorate / Iraq, namely: the Old City, Al-Amir neighborhood (southern Najaf), Al-Askari neighborhood (northern Najaf), Al-Abbasiya subdistrict, and Al-Radhawiya subdistrict, with 250 g for each sample. The samples were then packed in paper bags to protect them from moisture, labeled with indicators of the region and the replicate, and brought to the laboratory for purification from impurities, if any, and for use in direct and indirect isolation.

Isolation of *Aspergillus niger* Associated with Almond Fruits

The direct plate method was used as a preferred technique for detecting and isolating fungi from almonds.These materials must be sterilized before planting. Sterilization removes surface contamination from dust and other sources and allows the fungi present within the material to grow. This process provides an effective measure of fungal presence, particularly toxin-producing ones. If all fungi present in these materials are to be isolated, sterilization is not necessary, in which case direct plating is more practical [27].

Molecular Identification of *Aspergillus* Isolate Using Polymerase Chain Reaction (PCR) Technique

The fungal DNA was extracted using the ZR BashingBead™ kit and the extracted DNA was stored in TEB solution at -20°C. Then, Electrophoresis was performed to identify the resulting band at 100 bp. The primers ITS1 forward and ITS4 reverse, with a size of 550–600 bp, were used to perform the polymerase chain reaction. Samples were prepared using the Maxime PCR Pre Mix Kit (i-Taq) with a volume of 5 microliters, to which 1 microliter of each of the mentioned primers and 1.5 microliters of DNA were added, then the final volume was completed to 25 microliters in the tube after adding 16.5 microliters of sterile distilled water. The device was run according to the program shown in Table 1.

After PCR, the purity and quantity of DNA were checked using Nanodrop 2000 and 2% agarose gel electrophoresis [28]. The samples were then exposed to high contrast light (302 nm) after adding Redsafe red dye to determine the band length to 100 bp. The samples were then sent to Macrogen Korea, and the sequences were analyzed using Basic Local Alignment Search Tool (BLAST) for comparison with the data available at the National Center for Biotechnology Information (NCBI).

Table (1): Polymerase chain reaction (PCR) conditions program.

No.	Step	Temperatur	Time	Cycle
1	Initial Denaturation	94°C	5 min.	1
2	Denaturation	94°C	45 sec.	35
3	Annealing	52°C	45 sec.	
4	Extension-1	72°C	45 sec.	
5	Extension -2	72°C	7 min.	1

Extraction and Quantification of Ochratoxin A Using HPLC Technique

Ochratoxin A was extracted from almond samples following the method of [29], where the almond sample is first prepared by grinding it to a fine powder. A suitable solvent (usually methanol and water mixture, or sometimes another solvent) is then added to the ground sample, such as the sample tissue to remove micotoxine such as Ocratoxin A. To allow the mixture to dissolve toxins in the solvent for some time after the mixture is fully mixed, the solution is filtered to remove fixed, and the detection of OTA was performed using High-Performance Liquid Chromatography (HPLC), known for its high sensitivity and accuracy in mycotoxin detection [30]. An HPLC device, model SYKAM (Germany), belonging to the College of Science and Technology.

Reduction of Ochratoxin A in Almond Fruit Samples

After extracting and measuring the concentration of Ochratoxin A using HPLC technique, the percentage of its reduction by thyme oil was estimated according to Abbott's formula as cited by [31], as follows:

$$\text{Reduction percentage} = \frac{\text{Control Concentration} - \text{Treatment Concentration}}{\text{Control Concentration}} \times 100$$

Storage Experiment

A storage experiment was conducted to test the effect of thyme oil at a concentration of 10% on the growth of *A. niger* on almond fruits. This experiment was carried out using a Completely Randomized Design (CRD) with three factors and three replications. The first factor included five different treatments: Control treatment (without the addition of any substance), Sterile distilled water (20 mL added to 180 g of almonds. Distilled water was used as a single treatment as it is a component of the spore suspension, in order to evaluate its potential effect),thyme oil (20 mL of thyme oil added to 180 g of almonds), *A. niger* suspension (180 g of almonds inoculated with 20 mL of fungal filtrate), and Fungal suspension + thyme oil (20 mL of fungal filtrate + 20 mL of thyme oil). The second factor involved two storage period (one and two months), while the third factor included three temperature levels (10, 20, and 30°C).

Statistical analysis

After collecting and tabulating the study data, they were statistically analyzed using a randomized complete block design (CRD) and the least significant difference test (L.S.D $_{0.05}$) was used to compare and separate the means [32]. This was done using the Genstat12.1 statistical analysis program.

Results and discussion

Polymerase chain reaction (PCR) products

Nucleotide sequence analysis of *Aspergillus* sp.

After examining the DNA of the fungus used in this research, the sequence of nitrogenous bases in the ITS region at 537 bp (Fig1) was compared with data recorded on the NCBI. The results showed a 99% similarity to other *Aspergillus niger* isolates. The isolate was registered on GenBank and named AJ7 under accession number PV700217. Nucleotide sequence analysis using Mega software was also used to analyze the isolates and draw a phylogenetic tree. The analysis showed a 99–100% similarity to the other isolates (Fig 2).

Figure (1): Electrophoretic separation of DNA on 1.5% agarose gel showing a distinct band at 537 bp after 1.5 hours of electrophoresis at 5 V/cm².

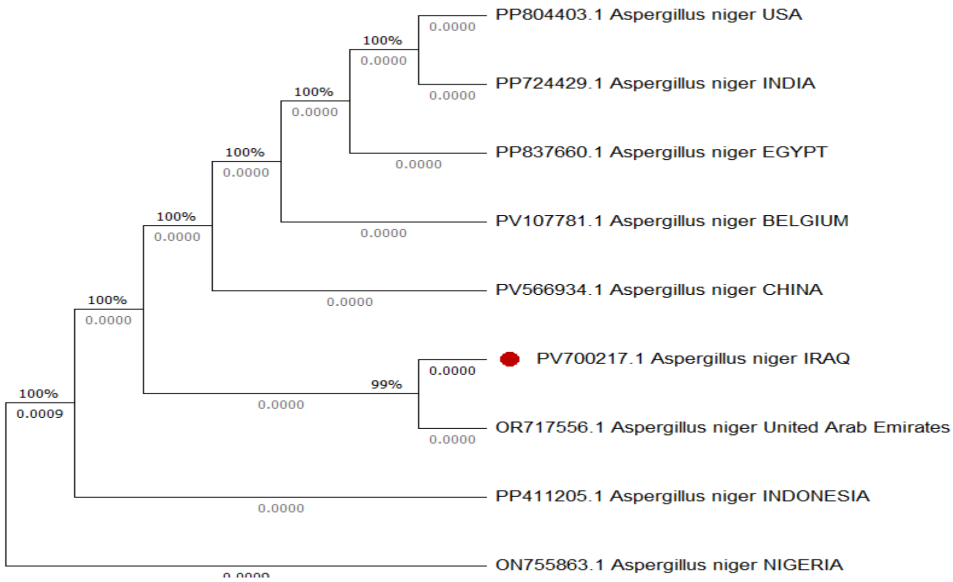

Figure (2): Phylogenetic tree of the fungal isolate *Aspergillus niger* AJ7, showing 99–100% similarity with other isolates of the same species from different countries.

Effect of thyme oil and temperature on the amount of ochratoxin A produced by *A. niger* in almond fruits after one and two months of storage

The results (Table 2) showed that there was a significant effect when adding the fungus *A. niger* to almond fruits on the concentration average of the toxin (OTA), which recorded (74.68 µg kg^{-1}), compared to the control, which recorded (5.39 µg kg^{-1}). While thyme in the treatment (thyme oil + fungus) significantly reduced the toxin concentration, recorded (32.40 µg kg^{-1}) compared to the treatment (fungus only). The results showed that storing almond fruits at a temperature of (10, 20 and 30°C) significantly increased the toxin concentration an average of (12.9, 29.4, and 35.1 µg kg^{-1}), respectively. There were significant differences in the storage period for (one and two months) in the toxin concentration, recorded (20.3, 31.4 µg kg^{-1}), respectively. While the interaction treatment (fungus only at a temperature of 30°C for two months), gave the highest average of toxin concentration of (124.8 µg kg^{-1}), compared to the other treatments.

The reason for the increase in fungal growth with the rise in temperature, reaching the highest production at 30°C, may be due to the *A. niger* fungus entering the optimum temperature range, which lies between 30 and 35°C, and according to what [33], stated, that the *A. niger* fungus increases its activity at these temperature levels. While the lowest growth of the fungus was at a temperature of 10 °C because the fungus remains in a state of inactivity until conditions improve [34]. Therefore, the low production of toxin for the fungus *A. niger* after treatment with thyme oil may be due to the effect of the active compounds in the oil, particularly thymol and carvacrol on the vital processes of the fungus, as these compounds are lipophilic and interact with the fungal cell membrane and affect its permeability, causing loss of ionic balance and leakage of part of the cell components, which ultimately leads to disruption of vital processes and consequently cell death. These findings are consistent with those reported by [35] in their study on *Aspergillus flavus*.

Another study on *Fusarium graminearum* demonstrated that thymol inhibits spore production and hyphal growth of the fungus, and reduces the content of ergosterol, a major component of the fungal cell membrane. This affects the integrity of the membrane and hinders fungal growth and reproduction [36]. The results obtained by [37] indicated that the active compounds in thyme oil, including thymol and carvacrol, retained their inhibitory activity on fungal growth and spore production even after 45 days, with a significant decrease in spore growth and ergosterol content.

Table (2): Effect of thyme oil, temperature, storage period and their interaction on the concentration of ochratoxin A (µg kg^{-1}) produced by *A. niger* in almond fruits.

Treatments	Month1			Months2			Means
	10°C	20°C	30°C	10°C	20°C	30°C	
Control	3.68	4.78	6.66	4.14	5.06	7.99	5.39
Distilled Water	2.76	3.22	3.32	2.89	3.23	3.52	3.16
Thyme Oil	2.15	2.08	3.16	2.04	2.31	3.05	2.47
Fungus only	11.6	82.6	99.4	18.8	110.9	124.8	74.68
Fungus + Thyme Oil	6.5	30.1	41.8	8.8	49.8	57.4	32.40
Mean across months	20.3			31.4			
Mean across temperatures	10°C = 12.9			20°C = 29.4		30°C = 35.1	
$L.S.D_{0.05}$	Treatments		Temperatures		Months		Interaction
	7.01		5.43		4.44		17.18

Effect of thyme oil and temperature on the reduction of ochratoxin A produced by *A. niger* after (one and two months) of storage in almond fruits

The results (Table 3) showed that the treatment (thyme oil + fungus) achieved significant reduction rates with an average of (58.95%), while adding thyme oil alone to the almonds achieved a significant reduction rate for the toxin (OTA), recording an average of (52.92%). This is logical due to the addition of thyme oil to sterilized almonds and storage under conditions that prevent contamination. On the other hand, the treatment (sterile distilled water) showed a moderate effect in reducing the toxin, recording an average of (38.42%), which is less than the thyme oil treatment. As for the fungus only treatment and the control treatment, it was noted that they did not record any reduction rate, which is expected in the absence of any inhibitory or antifungal agent.

It was also noted that storing almonds at temperatures (10, 20 and 30°C) resulted in a significant increase in the reduction percentage with increasing temperature, reaching (27.08, 29.85, and 33.24%), respectively. The results also showed that there were significant differences in the storage period (one and two months) in the reduction percentage, as it recorded (28.24, 31.88%), respectively. The results also demonstrated the presence of significant differences between treatments, temperatures, and storage period, which supports the reliability of these results in evaluating the effectiveness of thyme oil as a natural inhibitor of *A. niger* fungus toxicity in almonds.

There are many studies that have shown the effectiveness of thyme oil in inhibiting the growth of certain mycotoxigenic fungi and the extent of its effect in reducing the toxin OTA depending on storage conditions and incubation period. Among them is what [23], reached regarding the role of phenolic compounds of thyme oil and thymol in inhibiting the growth of fungi (*A. niger*, *A. carbonarius* , and *A. ochraceus*) and reducing toxin OTA at a 60% percentage.

Table (3): Effect of thyme oil, temperature, storage period and their interaction on reduction ochratoxin A (%) produced by *A. niger* in almond fruits.

Treatments	**Month1**			**Months2**			**Means**
	10°C	20°C	30°C	10°C	20°C	30°C	
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Distilled Water	25.00	32.64	50.15	30.43	36.37	55.94	38.42
Thyme Oil	41.58	56.49	52.55	50.72	54.35	61.83	52.92
Fungus only	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fungus + Thyme Oil	43.67	63.56	57.95	79.40	55.09	54.01	58.95
Mean across months	28.24			31.88			
Mean across temperatures	10°C = 27.08			20°C = 29.85		30°C = 33.24	
L.S.D $_{0.05}$	Treatments		Temperatures		Months		Interaction
	0.0435		2.149		1.755		6.797

The study highlights the effective role of thyme oil as a natural antifungal agent capable of reducing the production of Ochratoxin A of the fungus *Aspergillus niger* in the almond fruits. The results also confirm that the increase in storage temperature and an increase in period is an important contribution to toxic concentrations, which indicates that the state of storage is an important factor in increasing the risk of toxic pollution. Therefore, the use of thyme oil is a promising and durable strategy to reduce toxic pollution in stored agricultural products, especially in high temperatures and extended storage periods, which leads to an increase in food security and quality.

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