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Detection of mycotoxin ochratoxin in some coffee beans and powders available in the local markets

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Abstract

Introduction: Contamination of coffee beans with mycotoxins is common in various countries, especially in imported ones. Transportation and storage play a major role in the contamination of pre- and post-harvest samples with mycotoxins.

Objectives: The detection of ochratoxin is considered as one of the types of mycotoxins that can contaminate various foods including cereals, beans, dried fruits and coffee in different countries around the world.

Materials and Methods: This cross-sectional study has been conducted on fifteen samples of coffee beans and powders (three replicates for each sample) including Turkish, Brazilian, Syrian, Jordanian and Italian varieties, were randomly collected from local markets in Baghdad city from March to June 2022. Isolation and identification of fungi were conducted out by cultivating coffee samples on potato dextrose agar medium. Polymerase chain reaction was utilized for the detection of *Aspergillus* spp. in coffee samples that produced mycotoxins. High-performance liquid chromatography (HPLC) technology was employed to detect the mycotoxin ochratoxin in all coffee samples.

Results: Results showed the presence of the causative agents *Aspergillus* spp. in eight samples of coffee in this study. The main cause of secretion of the mycotoxin ochratoxin in coffee beans and powder was *Aspergillus* spp. Eight isolates of *Aspergillus* spp. were diagnosed by polymerase chain reaction technique. All eight isolates belonged to *Aspergillus flavus*, as indicated by the appearance of a band with a molecular weight of 600 bp. HPLC technique showed that most of coffee samples were contaminated especially Turkish Syrian, and Jordanian samples (0.91, 0.80, and 0.73 µg kg^{-1} respectively), while low concentrations of ochratoxin were recorded in Italian and Brazilian coffee samples (0.44, 0.39 µg kg^{-1} respectively). Therefore, there are no significant differences between the brands (P>0.05).

Conclusion: The results of this study showed a high level of mycotoxin concentration (ochratoxin) in the Syrian, Jordanian and Turkish samples. However, its concentration was lower in both the Brazilian and Italian coffee samples when using one of the modern practices of HPLC. Therefore, it is necessary to understand and study the importance and impact of these toxins on both human health and the country's economy.

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Introduction

Coffee is a popular beverage made from roasted coffee seeds that are grown in over 70 countries, the original home of coffee is Yemen, coffee is spread through the Red Sea to different parts of the world (1,2). Coffee has health benefits for humans because of its refreshing taste and aroma, as well as its ability to stimulate all human senses. Drinking coffee in specific quantities can have a positive effect on the health and activity of the body (3). According to the food and agriculture organization, the annual global consumption of coffee is estimated at seven million tons, coffee is enjoyed in various ways, both in different regions and within individual countries (4). There are two well-known types of coffee; Arabica coffee, and Robusta coffee. Arabica coffee contains around 12%-18% fat, along with chemical compounds such as caffeine, chlorogenic acid, diterpenes, and trigonelline. Caffeine, an antioxidant, helps fight free radicals that produce toxic compounds like hydroperoxides and peroxides. In addition to providing the body with energy for daily tasks, coffee consumption may also reduce the risk of cancer (5). Chlorogenic acid is a phytochemical found in coffee beans. It has been described as having the ability to lower blood sugar levels to normal levels and is likely to have an anti-diabetic effect. In addition to its use for lowering blood

Key point

The presence of mycotoxins ochratoxin in foods, especially in coffee beans and powders, affects the safety and health of the consumer. Therefore, rapid detection and observation are essential in preserving the type of food.

pressure, recent studies have indicated that chlorogenic acid can improve the mood of individuals and help treat and fight bacterial infections (6). Mycotoxins are natural, low-molecular weight compounds produced during the secondary metabolism of certain fungi belonging to the genera *Penicillium* spp., *Rhizopus* spp. and *Fusarium* spp. (7). Mycotoxins are cyclic hydrocarbon compounds that rarely have open chains. Due to their low-molecular weight, they are considered resistant to harsh environmental conditions. Additionally, they are non-antigenic, meaning they do not have the ability to stimulate the human immune system to produce antibodies (8). Ochratoxin A is considered one of the types of mycotoxins that can contaminate various foods, including cereals, beans, dried fruits and coffee. It is produced by different types of fungi such as *Penicillium*, and *Aspergillus*. Ochratoxin A was first isolated from coffee in 1974 (9). Several types of fungi such as *Aspergillus,* and Penicillium can attack coffee beans and produce the mycotoxin ochratoxin A when exposed to specific environmental conditions.

Objectives

This study aimed to detect ochratoxin A in coffee samples from local markets. The most accurate and effective method for measuring contamination in grains is through the use of high-performance liquid chromatography (HPLC).

Material and Methods

This cross-sectional study has been conducted on fifteen samples of coffee beans and powders (three replicates for each sample) including Turkish, Brazilian, Syrian, Jordanian and Italian varieties, were randomly collected from local markets in Baghdad city from March to June 2022. This study was directed to detect ochratoxin A in coffee samples by analytical methods and knowing the type of cause through genetic detection.

Collection of samples

The aim of current was to collect samples of coffee beans and powders from local markets in Baghdad for the period from March 2022 to June of the same year. A total of 15 samples were collected, three replicates for each sample, weighing 1 kg per sample. The samples were stored in polyethylene bags and originated from Turkey, Jordan, Brazil, Syria, and Italy was shown in (Table 1). They were transferred to the laboratory and kept in a cool place until testing was carried out.

Isolation and identification of fungi in coffee samples

The method described by Greeff-Laubscher et al (10) was followed in investigating the types of *Aspergillus* spp. Present in the coffee beans and powder used in the study. Potato dextrose agar was conducted as a selective medium to isolate *Aspergillus* spp. The dishes were then incubated at 28 °C for 5-7 days and monitored throughout the incubation period. After the completion of the incubation process, the fungi present in the dishes were diagnosed according to the study by da Mota et al (11).

Genetic diagnosis of Aspergillus flavus using PCR technology

The polymerase chain reaction (PCR) technique was conducted to confirm the identity of the fungal isolates described in the process involved the following steps;
first, previously purified isolates suspected to be *Aspergillus* spp., eight isolates were grown on a medium of yeast extract and liquid sucrose in small plastic tubes (slants) with a capacity of 10 ml. Then a 5 mm disc was taken from the edge of the mushroom colony that had been previously grown on potato dextrose agar medium and left in the incubator for five days. The growing mycelium was then harvested, washed thoroughly with distilled water multiple times to remove any residual yeast medium, and placed in an Eppendorf tube with a volume of 2 mL. The tube was then stored in a freezer at -20 °C for later use described by Susca et al (12).

Extraction and purification of DNA from Aspergillus spp

The DNA was extracted using the standard kit for extracting DNA from mycelium, which was prepared by the Korean company Biobasic. The steps in the protocol attached with the standard kit were followed, and the electrophoresis technique was conducted with an agarose gel at a concentration of 1% to detect the DNA fragments.

Primers for Aspergillus flavus

The standard AccuPower® PCR PreMix kit from Bioneer Company was employed for the examination. The reaction mixture was prepared in 2ml tubes containing the reaction components, along with specific primers for *Aspergillus flavus*, Forward :5-CGGAGACACCACGAACTCTG-3 and reverse 5-CCCTACCTGATCCGAGGTCA-3. The resulting DNA fragment from the reaction was 600 bp,

Table 1. Coffee samples collected from local market

N	Sample	Origin	Date of production	Date of expiry	Volume /gram
1	Lavazza	Italia	2022/01/13	2023/1/10	500
2	Bin- Alameed	Jordan	2022/2	2023/1	500
3	Brazilian classic	Brasilia	2022/1/2	2023/12/31	500
4	Bin- Hamwi	Syria	2021/11/29	2022/11/28	500
5	Turk Kahvesi	Turkey	2021/9/20	2022/9/19	500

demonstrated by Kumar et al (13). After the preparing of the reaction mixture, the tubes were briefly spun in the Exispin device for five seconds. Then the tubes were transferred to the PCR thermal cycler to start the DNA amplification process. The cycler was programmed with optimal thermal cycling conditions as described by Abd El-Aziz et al (14). Initial denaturation at 94 °C for 10 minutes, followed by 35 cycles of denaturation at 94 °C for 50 minutes, extension at 72 °C for one-minute, final extension at 72 °C for seven minutes, and cooling at 4 °C. Electrophoresis was then performed using 1.5% agarose gel with DNA ladder solution (1000 bp).

Prepare the agarose gel

First, we weight 1g of agarose and dissolve it in 100 mL of tris-borate-EDTA in a glass beaker. We placed the beaker on a heat source until the agarose is completely dissolved. Add 0.1 µL of ethidium bromide dye and allow the mixture to cool. Then we poured the agarose into a mold, sealing the two ends with adhesive tape to prevent leakage. We allowed the agarose solidify before fixing the comb in place. Then we placed the mold in the electrophoresis device cavity and immerse it in TBE transfer buffer. Meanwhile, we removed the comb from the template. After, we mixed 5 µL of DNA with 3 µL of dye loading DNA dye. Additionally, we injected the mixture into the gel using a micropipette. To continue the process, we turned on the electrophoresis device at 70 V for 50 minutes. After a minute, we allowed the dye to flow through the gel template. Once migration is complete, then we positioned the gel was on ultraviolet device to visualize the DNA bands, and photographed the results.

Extraction and quantification of ochratoxin from coffee

First the chemicals and equipment used in this study were analytical grade, methanol, acetic acid, acetonitrile, benzene, and chloroform (Italy). The ochratoxin A mycotoxin was extracted from imported coffee following the method described by Peng et al (15). This involved weighing 50 grams of coffee from each sample, grinding it with a special mill, and then extracting it with 100 ml of a solution consisting of methanol and water (80:20 v/v) using a high-speed mixer for five minutes. The extract was then filtered through Whatman filter paper (No. 1), while 10 ml of the filtrate was collected. The volume was then brought up to 50 mL with distilled water.

Ochratoxin analysis by using HPLC technique

The ochratoxin A (OTA) mycotoxin was estimated by injecting 50-100 µL of coffee extract and fungal isolates, respectively, into a HPLC device made by the Japanese company Shimadzu. In our study, all analysis was carried out using this system and the following steps: the first step of cleaning the samples was carried out according to the method described by Zapaśnik et al (16), using ochratoxin test immune-affinity columns. These columns have the property to detect up to 100 mL of toxin in the coffee sample. To clean the extracts containing mycotoxin, 10 mL of the final filtrate was taken and passed through immune-affinity columns at a rate of one drop per second. The column was then washed three times with 10 mL of distilled water. Mycotoxin of the OTA attached to the column was removed with 4 mL of methanol per second, then the product was dried with a nitrogen stream at 45 °C using an evaporator. The residues were dissolved in 200 µL of acetonitrile contain water and acetic acid, mixed well with a Vortex, and kept at 4 °C.

Standard solutions and cleaning columns

The ochratoxin standard stock solution provided by Sigma Corporation, was one ml at the concentration of 50 µg kg^{-1}. It was then dissolved in benzene; acetic acid (99:1 v/v) and stored at (-20 °C). A calibration curve was created with the expected levels of ochratoxin in samples at six points (0, 0.5, 1.0, 2.0, 3.0, 6.0 µg kg^{-1}). Then 20 µL of each dilution was injected into the HPLC system three times, and all concentrations were stored at 4°C was described by Chen et al (17).

The calibration curve for ochratoxin was established by injecting concentrations of 0, 0.5, 1.0, 2.0, 3.0, and 6.0 µg kg^{-1}, with three replicates for each concentration. The averages were calculating and shown in Figure 1.

Statistical analysis

The Statistical Analysis System (SAS) program was conducted to detect the effect of difference factors in study parameters. Fisher's exact test was also employed to significant percentages (0.05 probabilities) according to the method demonstrated by Lukow and Weatherall (18).

Results

Isolation and identification of fungi in coffee beans

The results of the isolation and identification of the coffee samples under study revealed the presence of 8 isolates belonging to the fungus *Aspergillus spp.* This predominance of this saprophytic fungus is likely due to its wide spread distribution in various soils, as well as its ability to thrive in harsh environmental conditions such as

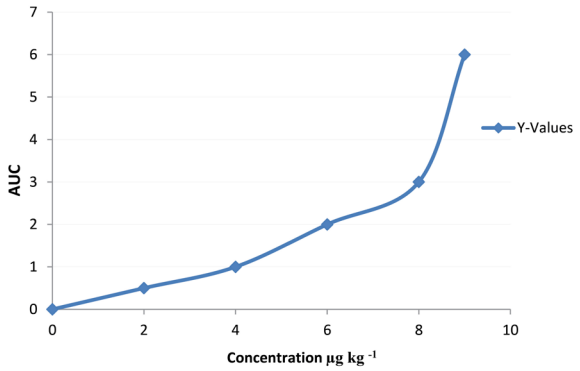

Figure 1. The standard curve of ochratoxin from (0-6 µg kg^{-1}). AUC: Area under the ROC curve.

drought and high temperatures. Additionally, *Aspergillus* spp. exhibits enzymatic diversity through the production of many enzymes. Furthermore, it has the capacity to produce a significant number of conidia for reproduction to survive hostile conditions.

Genetic diagnosis of Aspergillus spp. using PCR technology

The eight isolated strains of *Aspergillus* spp. were identified through in vitro diagnosis *Aspergillus flavus* fungus. This was specifically determined using the PCR technique, indicated by the presence of a band with a molecular weight of 600 bp in all test isolates. Additionally, the presence of a DNA Marker with a molecular weight of 1000 was observed on an agarose gel (Figure 2).

Ochratoxin analysis using HPLC technique

Table 2 displays the levels of ochratoxin found in the coffee samples analyzed. Out of the 15 samples of coffee grains and powders, most were found to be contaminated with ochratoxin above the permissible limit (standard level of mycotoxin). Approximately 54% of the samples had levels greater than or equal to (≥2 µg kg^{-1}). The high concentration of ochratoxin was found in Turkish, Syrian, and Jordanian coffee samples (0.91, 0.80 and 0.73 µg kg^{-1} respectively), while Italian and Brazilian coffees showed lower concentrations of mycotoxin (0.44 µg kg^{-1}, 0.39 µg kg^{-1} respectively) indicated in Table 2. Therefore, there are no significant differences between the brands ($P > 0.05$).

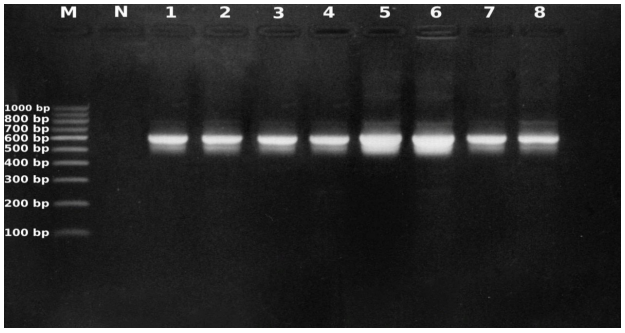

Figure 2. The electrophoresis of the PCR reaction product (600 bp) on an Agarose gel of *Aspergillus* isolates from coffee samples. N (control), Lanes: (M) DNA Ladder (1000 bp), L; 1 - 8 (*Aspergillus flavus*).

Table 2. The levels of ochratoxin in the analyzed coffee bean samples

Trade market	<2 µg kg^{-1}	≥2 µg kg^{-1}	*P* value
Italia	0.30	0.44	0.479 NS
Jordan	0.51	0.73	0.479 NS
Brasilia	0.24	0.39	0.479 NS
Syria	0.67	0.80	0.479 NS
Turkey	0.75	0.91	0.479 NS
P value	0.328 NS	0.371 NS	---

P>0.05; Non-significant. Fisher's exact test was conducted to significant percentages (0.05 probabilities).

Discussion

The results of isolation and identification in our study were in agreement with the finding by Abe et al (19), that found all types of coffee contaminated with *Aspergillus* spp., *Aspergillus* isolates were able to secrete toxins in coffee samples, However, our study disagreed with finding by Chen et al (17) which found fungi that produce mycotoxins in 5 out of 11 Brazilian coffee samples, with *Penicillium* being the type of fungus identified. This fungus requires a cold environment to produce mycotoxins, which is why it was found in Europe and Western countries, rather than hot places. Our study was agreement with the findings of studies by Hwang et al (20) that examined commercial coffee powder in Malaysia, Culliao and Barcelo (21) revealed that *Aspergillus, and Penicillium* are a natural pollutant in coffee, moving from the field to storage. The appearance of *Aspergillus* spp. colonies in coffee powder indicated air pollution. The contamination of these fungi in coffee products may be due to inadequate heat treatment. During roasting, packaging, storage or transportation, or as a result of poor environmental conditions.

The polymerase chain reaction used in this study was in agreement with findings from the study by Santos-Ciscon et al (22), isolated 19 *Aspergillus flavus* strains out of 87 from coffee samples, with a molecular weight of 600 bp. *Aspergillus flavus* is known to pose the greatest risk of mycotoxins in Brazilian coffee beans. However, this study was disagreement with finding by Sartori et al (23), which reported that most coffee samples were contaminated with *Aspergillus ochraceus.* This discrepancy could be attributed to environmental factors, the method of packing coffee samples, and the lack of airtightness in the packaging.

The results of the ochratoxin examination using HPLC and p value (high concentration of mycotoxin were found in Turkish, Syrian, and Jordanian coffee samples (0.91 µg kg^{-1}, 0.80 µg kg^{-1} and 0.73 µg kg^{-1} respectively), while low-concentration (0.44, 0.39 µg kg^{-1} respectively) are found in Italian and Brazilian coffees, in this report were agreement with a finding by Zapaśnik et al (16), it was discovered that 20 out of 40 samples of coffee were contaminated with mycotoxins at high levels. The main causes of contamination were identified as poor storage practices both before and after harvesting, as well as inadequate packaging. This study is agreement with the finding by Alshannaq and Yu (24), who found high levels of food and grain contamination with mycotoxins. This contamination was caused by poor storage and transportation practices, as well as moisture and the failure to utilize modern storage methods, the ochratoxin is characterized as one of the most common toxins, and although the kidney is the target organ, it has many negative effects on the health of society and humans. Its significance lies in its impact on the digestive system, as it increases intestinal permeability by reducing the expression of tight junctions and stimulating oxidative stress. This leads to apoptosis of intestinal cells, compromising the height of the villi and altering the

immune system reducing cytokines expression), making individuals more susceptible to secondary infections. Consequently, ochratoxin damages intestinal integrity and reduces nutrient absorption. The study was agreement with the finding by Peng et al (15), which revealed several rapid tests and analyses to detect contamination with mycotoxins. It was accurate and flexible in determining the concentrations of mycotoxins using methods such as HPLC, which demonstrated the ability to produce excellent results in measuring mycotoxins concentrations. On the contrary, our study contradicted the findings by Gonzalez et al (25), as they found a high level of contamination in coffee and roasted coffee beans with ochratoxin. They confirmed that roasting can impact mycotoxins, but the chemical composition of ochratoxin enables it to withstand heat.

We recommend future research on detecting the mycotoxin ochratoxin using gas chromatography-mass spectrometry. This method plays a crucial role in identifying mycotoxin components and has a significant impact on societal benefit.

Conclusion

The results of this study showed a high level of mycotoxin concentration (ochratoxin) in the Syrian, Jordanian and Turkish samples. However, its concentration lowered in both the Brazilian and Italian coffee samples when using modern HPLC practices.

Limitations of the study

The primary concern is detecting mycotoxins that contaminate coffee, a staple in many consumers' diets.

Acknowledgments

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Conflicts of interest

The author declares that he has no competing interests.

Ethical issues

The research conducted in this study adhered to the principles outlined in the Declaration of Helsinki. The Market Research and Consumer Protection Center/University of Baghdad, Iraq, and ethics committee approved the study (#MRCPC-29). Moreover, ethical issues (including, double publication, data fabrication, plagiarism) have been completely observed by the author.

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