Isolation and identification fungi contaminated onion bulbs, detection *Aspergillus* spp. toxins, and inhibiting its growth by *Moringa oleifera* extract

Sadeq Mohammed Ali, Rafid M. AbdulAali, Usamah A. Alkarim A. Almunam Alshimaysawe, Ahmed Mohammed Ali

Department of Plant Protection, Faculty of Agriculture, University of Kufa, Najaf, Iraq.

Corresponding author sadeq.almusailmawi@uokufa.edu.iq

DOI: https://doi.org/10.36077/kjas/2024/v16i2.16108

Received date: 8/3/2024

Accepted date: 8/5/2024

Abstract

The study aimed to isolate and diagnose fungi contaminating onion fruits and secreting mycotoxins, and to study the effect of *Moringa oleifera* extract on the growth of the fungus *Aspergillus niger*. The results of isolating fungi from onions showed the presence of the following fungi: *Aspergillus niger*, *Fusarium* sp., and *Rhizopus stolonifer*. It became clear that there was a predominance of the fungus *A. niger*, then the fungus *Fusarium* sp., followed by the fungus *Rhizopus stolonifur*, as their frequency rates reached 53.33, 36.66, and 10.00%, respectively, while the occurrence rate reached 83.33, 50.00, and 33.33%, respectively. In this study, molecular diagnosis of the fungus *A. niger* was carried out using internal transcribed spacer (ITS) genetic markers, and the results of chemical analysis using thin layer chromatography (TLC) showed that 9 of the 16 isolates were *A. niger*, isolated from onions, produced ochratoxin A with a production rate of 56.25%. The moringa extract inhibited the growth of the fungus *A. niger*, as the percentage of inhibition increased with increasing concentration, and the highest percentage of inhibition was at a concentration of 15%, which reached 47.32%. The percentage of inhibition was at concentrations of 5 and 10 (12.82 and 26.15%).

Keywords: onions, mycotoxins, moringa extract, *Aspergillus niger*

Introduction

The original homeland of onion (*Allium cepa* L.) is northern Iran or the region extending from Palestine to India on the continent of Asia. Onions are classified as a vegetable crop of the Amaryllidaceae family (18).

The United States of America, the United Kingdom, Australia, Bulgaria, Japan and Spain are among the leading countries in its production, as their production rate per unit area reached 47,461 tons/hectare, while the average production rate per unit area of the countries neighboring Iraq reached 24.23 tons / hectare. The global production rate per unit area reached 19.89 tons/ha (14), while the best local production rate in Iraq per unit area reached 10.67 tons/ha in 1997 (9). The problem of food contamination with toxin-producing fungi is one of the important problems that threatens the quality and safety of food and the health of the consumer. The fungus *A. niger* is one of the most well-known species of the genus *Aspergillus* that causes black rot on fruits and vegetables (15). The mycotoxin aflatoxin is the most widespread mycotoxin, and it is a secondary metabolic compound produced by fungal species belonging to the genus *Aspergillus* spp. (24).

Mycotoxins are an important problem in all parts of the world because they affect public health, and they often cause chronic effects, in addition to causing acute effects in the event of exposure to them in high doses, especially the liver, kidneys, and immune system, causing kidney failure, liver cancers, and a weakened immune system (19). Among the fungi that most produce mycotoxins are some types of *Aspergilli*, *Penicillium*, *Alternaria*, and *Fusarium*. These growth on several food products and produce mycotoxins. Some of the first three types contribute mainly to damage

to fruits and crops and to the production of mycotoxins in their fruits (8).

Onions are infected with many pathogens, including fungi, so the study aimed to

isolating the fungi accompanying onion samples, diagnosing them, and determining the extent of the presence of fungi producing mycotoxins, testing the ability of these isolates to produce mycotoxins and detecting them using thin layer chromatography (TLC) technology and testing the effect extract of Moringa leaves in the inhibition of growth of *Aspergillus niger*.

Materials and Methods

Collect onion samples

Six samples of onion bulbs (white, yellow, and red) were obtained from several locations (retail markets), amounting to 1 kg. To obtain toxin-producing fungal isolates, they were transferred to the Mycology Laboratory at the College of Agriculture - University of Kufa to isolate and diagnose the fungi accompanying those samples.

Preparing the culture media

A- Potato Dextrose Agar (P.D.A)

The medium was prepared by taking 200 grams of peeled potato tubers, cut into small pieces, and boiling them with distilled water in a volume of 500 cm3 for 20-30 minutes in a glass beaker. After the end of the boiling period, the mixture was filtered through a piece of gauze. To obtain the extract, dissolve 20 grams of dextrose sugar and 17 grams of addition the agar in another 500 ml, then add the potato filtrate to it and bring the volume to 1 liter. Distribute it in glass jars as needed, close tightly with cotton plugs, and sterilize them using an autoclave at a temperature of 121°C for 20 minutes. After the sterilization period ends, the jars are left to cool. The antibiotic chloramphenicol was added to it at a rate of 250 mg/L before the medium solidified and then poured into petri dishes (10).

Preparation of plant extract

Preparation of moringa extract

Some moringa leaves were taken from general garden in AL-Najaf Governate. Put 10 grams of moringa leaf powder in 100 ml of sterile distilled water and leave it for 24 hours. After that, the plant extract were removed by filtering with Whattman NO1 filter papers. Then, centrifugation was carried out at 3000 rpm for ten minutes, and this extract was considered the basis at a concentration of 10%. Then the filtrate was taken, in sterile bottles, and placed in the refrigerator until use. Concentrations (5-10-15)% were prepared by taking proportions of the filtrate and placing it in sterile distilled water according to the required concentration (11).

Isolation and identification of fungi associated with onions

The onion-mentioned above samples were brought and isolated from them by direct cultivation on P.D.A medium. The fruits were cut into small pieces (1 cm) and were surface sterilized with a 2% sodium hypochlorite solution for two minutes. After that, the pieces were washed with sterile distilled water and then placed on filter papers for disposal. of free water, then the fruit pieces were planted in plastic dishes (9 cm diameter) containing P.D.A medium, by placing four pieces 3 cm away from the edge of the dish and a fifth piece in the middle. The process was repeated three times (replicates), after which all the dishes were incubated at 25 °C, three days (16), and after the end of the incubation period, the isolates of the developing fungi were purified and then identified based on the taxonomic characteristics mentioned by Domsch et al, (12) and Hoching and Pitt, (14).

The occurrence and frequency rates of the fungi were calculated according to the following two equations:

$$Occurence\ percentage\ (\%) = \frac{\text{No.of sample that Genus or species appeared}}{\text{Sample total number}} \times 100$$

$$Frequency\ percentage\ (\%) = \frac{\text{Indivdual sample isolation}}{\text{Total isolations}} \times 100$$

Detection of ochratoxin A using thin plate chromatography (TLC) technique

The method of Balzer et al. (7) was followed in detecting ochratoxin A, which is as follows:

1- The fungal isolate *A.niger* was grown on PDB medium by placing three week-old discs of fungus with a diameter of 5 mm in 250 ml flasks for each fungal isolate, which were then incubated at a temperature of 30°C ± 2°C for 21 days.

2- Filter the extract through Whatman No. Filter paper. 4.

3- Place the filtrate in a 250 ml separatory funnel, then add 25 ml of hexane to it to get rid of fats (defatting). Then shake the funnel gently for 30 seconds, expelling the accumulated gases whenever necessary (at least twice), and leave it. On the rack for one minute until the two layers separate. The upper layer was removed took the lower layer, and repeated the process three times to ensure fat removal.

4- 25 mL of distilled water, 8 ml of saturated sodium bicarbonate solution (NaHCO3), and 25 ml of chloroform were added to the lower layer. After three minutes, the upper and lower layers were separated, and the lower layer was kept. I repeated the process twice.

5- Transfer the extract to a 100 ml separating funnel and add 15 ml of 1 M hydrochloric acid prepared simultaneously and 20 ml of chloroform, shake well and leave the funnel on the stand for one minute. The lower layer was taken, and 20 ml of chloroform was added to the upper layer and extracted again. The two lower layers were collected and passed through a filter paper containing a layer of anhydrous sodium sulfate (Na2SO4) to get rid of the remaining water.

6 - The filtrate was taken and placed in an electric oven at a temperature of 40°C until dry, then it was dissolved in 1 ml of chloroform. The presence of ochratoxin was detected using thin-plate chromatography (TLC) technology with dimensions of 20 cm, where the plates were activated in an electric oven at 105 °C for an hour before use (6). Using a separation system for ochratoxin A consisting of toluene: chloroform: ethyl acetate: formic acid, in volume ratios of 35: 25: 25: 15, prepared simultaneously. A light straight line was drawn on the TLC plate at a distance of 1.5 cm from the base of the plate. 15 microliters of the standard toxin ochratoxin were taken using a capillary tube and placed on the line at 2 cm from the left edge of the plate and at 2 cm from the spot for the standard toxin. Extract samples of the fungal isolates were placed. *A.niger* at the same distance and in an amount equal to the amount of standard poison. The spots were left to dry and then placed in the separation basin containing the system referred to above. They were monitored until the separation system reached approximately 2 cm from the upper end of the plate. The plates were taken out and dried under Laboratory conditions for 5 minutes, then treated with a basic solution (sodium hydroxide), and then examined under ultraviolet radiation at 360 nanometers. The presence of ochratoxin was detected by matching the migration coefficient Rf and fluorescence color of the standard toxin with the color and migration coefficient of samples

of extract of the fungal isolates from ochratoxin A.

Molecular diagnosis of *Aspergillus niger* isolates Using PCR technique

The PCR test was applied for the most toxic isolates all using pairs of universal primers ITS1 (5`-TCCGTAGGTGAACCTGCGG-3`) and ITS4 (5`- TCCTCCGCTTATTGATATG ATGC-3`) for targeting the ITS region of fungi (23). The PCR test was performed using the Ready-To-Go PCR Beads kit (GE Healthcare, UK) and following the manufacturer`s instructions. Two microliters of total DNA isolated from the pathogenic fungus were added to each the reaction.

The PCR amplification program started with initial denaturation for 5 min at 95°C, then 40 cycles consisting of three steps: denaturation at 94°C for 60 seconds, primer annealing at 59°C for 60 seconds and extension at 72°C for 1 min. A final extension step for 5 min at 72°C was included. PCR products were electrophoresis analyzed and visualized on 1.5 % agarose gels stained with ethidium bromide using a UV transilluminator (Edvotek Inc, USA) (23).

The effectiveness of moringa extract on the growth of the fungus *A.niger* on P.D.A medium

Moringa extract was added in proportions of 5, 10, and 15 ml/100 ml to the P.D.A. medium and poured into the dishes. After solidifying the plates, they were inoculated with discs with a diameter of 5 mm taken from the edge of the colonies of each fungus grown on P.D.A. medium. The process was repeated three times (replicates). Three plates containing P.D.A. medium (control treatment) were inoculated with discs of P.D.A. on which the fungus *A.niger* was grown. The plates were then incubated at a temperature was 25 ± 2°C for a week. After that, the diameters of the fungal colonies were calculated, and according to the implemented parameters, the percentage of inhibition was extracted according to the Abbott equation mentioned by Shaaban and Al-Mallah (20), where:

$$\textbf{Inhibition}\% = \frac{\textbf{R1} - \textbf{R2}}{\textbf{R1}} \times \textbf{100}$$

R1 = Maximum radial growth of the fungus colony (control treatment).

R2 = Maximum radial growth of the studied fungus colony in the treatment dishes

Results and discussion

1- Isolation and diagnosis of fungi contaminating onion bulbs

The results of isolating fungi from onions showed the presence of the following fungi: *Aspergillus niger*, *Fusarium* sp, and *Rhizopus stolonifur*

It became clear that there was a predominance of the fungus *A. niger*, then the fungus *Fusarium* sp., followed by the fungus *Rhizopus stolonifur*, as their frequency rates reached 53.33, 36.66, and 10.00%, respectively, while the occurrence rate reached 83.33, 50.00, and 33.33%, respectively. (Table 1). The reason for the dominance of the *Aspergillus* genus may be due to its widespread in the environment, which comes from its ability to form huge numbers of reproductive units that are resistant to unfavorable environmental conditions and which form plankton in the air because their diameter is less than 15 millimetres, thus reaching many places, as they can enter warehouses through windows and openings. In addition to its growth in wide ranges of temperature and humidity, as some species of the genus *Aspergillus* are characterized by growing in ranges of temperatures ranging

from 5 to 45 degrees Celsius or higher (17) and (14).

Table (1) shows the frequency and occurrence of fungi contaminating onions

Associated fungi	Frequency ratio %	Occurrence rate %
A. niger	53.33	83.33
Fusarium sp.	36.66	50.00
Rhizopus stolonifer	10.00	33.33

The ability of some isolates of the fungus *A. niger* to produce ochratoxin A by thin-plate chromatography (TLC).

The results of chemical analysis using thin layer chromatography (TLC) showed that 9 of the 16 isolates were *A. niger*, isolated from onions, produced ochratoxin A with a production rate of 56.25% (Table 2). The fungus isolates varied in their toxin production, and the isolate An7 was the most toxin-producing isolate based on the intensity of fluorescence under ultraviolet rays. The variation of isolates in the production of ochratoxin A is due to the genetic ability of the isolate.

These results are consistent with what Ali mentioned (1). Which indicated that 20% of *A. niger* isolates isolated from dates are capable of producing ochratoxin A. These results are in line with what was reported by Al-Ghazali and Ali (4), who indicated that 24% of the isolates of the fungus *A. niger* isolated from apple and pear fruits produce the toxin (ochratoxin A). And it is close to what Abd ElKhalek (1) mentioned about the ability of 9 isolates from a total of 16 isolates of *A. tubingensis* which was a producer of ochratoxin A with a production rate of 65.25%. Abboud & Ali (2) found that four isolates (of six) of *A. flavus* produced aflatoxins.

Table (2) the ability of a number of isolates of the fungus *A. niger* to produce ochratoxin A isolated from onion fruits using the thin plate chromatography (TLC) method.

Fungal isolation	Ability to produce ochratoxin A
An1	+
An2	-
An3	++
An4	-
An5	+
An6	-
An7	+++
An8	+
An9	-
An10	++
An11	-
An12	+
An13	-
An14	++
An15	+

An16	-

(+) an ochratoxin A-producing isolate, (-) a non-ochratoxin A-producing isolate.

Molecular diagnosis of *Aspergillus niger* isolates Using PCR technique

The results of D.N.A extraction from *Penecillium* spp. isolates showed. After exposing it to the polymerase chain reaction (PCR) showed that the D.N.A product can be (PCR-amplified product) to the expected size about 524 N-bp using the forward and reverse primers (ITS1 and ITS4) (Figure 1). The results of D.N.A amplification of the fungal isolate under study of *Aspergillus niger* is approachable, confirming other former studies (22) for ITS1 and ITS4 forward and reverse primers, which gave amplification results that ranged between (450 - 870) base pairs, which is considered among the sizes for these fungal species.

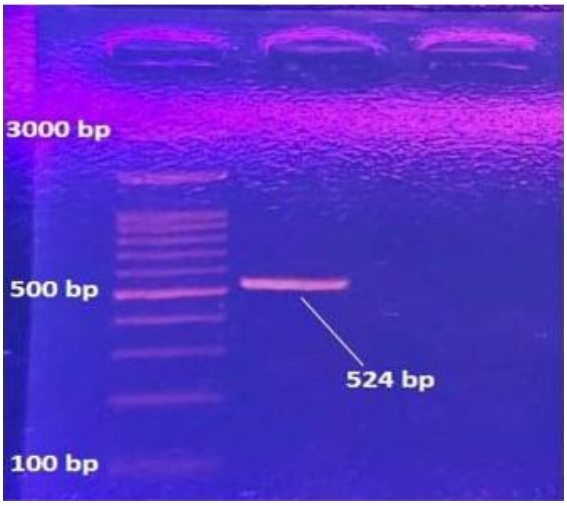

Figure 1. The PCR products of *Aspergillus niger* isolated in this study D.N.A amplified using the primer pair (F.R.) ITS1 and ITS4. M = 1 Kbp D.N.A ladder marker

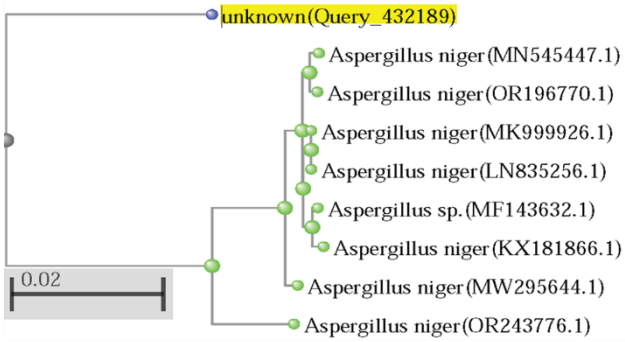

Figure 2. The genetic tree of the pathogenic fungus *Aspergillus niger*, performed based on the sequences of its nitrogenous bases for the ITS-rDNA region, in addition to the sequences of global strains of the same pathogenic fungus obtained from the GenBank data repository. Genetic distances were calculated using the neighbor-joining method.

The results also showed that the studied isolate belongs to the pathogenic fungus *A. niger* and that this isolate is a new isolate registered in the U.S. National Center for Biotechnology Information (NCBI) under the accession number: PP732188. Based on the sequences of the nitrogenous bases of the ITS-rDNA region, and the sequences of global strains of the same pathogenic fungi obtained from the GenBank data repository. The genetic distances were calculated using the neighbor-joining method, as shown in Figure (2).

Evaluation the effectiveness of different concentrations of moringa extract on inhibiting the growth of *A. niger*

Moringa extract inhibited the growth of the fungus *A. niger*, as the percentage of inhibition increased with increasing concentration, and the highest percentage of inhibition was at a concentration of 15%, which reached 47.32%. The percentage of inhibition was at a concentration of 5 and 10 (12.82 and 26.15%), respectively. Table (3) Figure (3). The hot extract of the Moringa plant at a concentration of 5% with an inhibition rate of 26.80%, and the reason for the inhibitory action of the

aqueous extract of these plants on fungal growth may be due to the aqueous extracts of plants containing chemical compounds that act as antifungals (25) . Many previous studies have indicated the effectiveness of medicinal plants and their volatile oils in inhibiting toxin-producing fungi (3) and (20).

Table (3) Effect of different concentrations of moringa extract on growth of the fungus *A. niger* on P.D.A medium.

Treatments	Average diameter of colonies (cm)	Inhibition rate %
Control	8.03	0.00
%5	7.00	12.82
%10	5.93	26.15
%15	4.23	47.32
L.S.D. 0.05	0.540	1.331

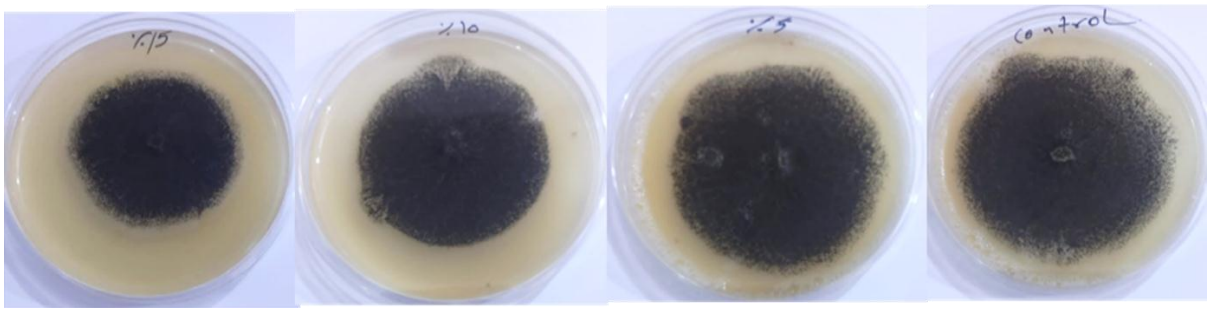

Figure 3. shows an evaluation of the effectiveness of different concentrations of moringa (5%, 10%, and 15%) in inhibiting the diagonal growth of the fungus *A. niger*

Conclusion

This study showed contamination of onions with the fungi *A. niger*, *Fusarium* sp., and *R. stolonifer*. The ability of the fungus *Aspergillus niger* to secrete ochratoxin A isolated from onions. Moringa leaves extract provided the highest suppression against *A. niger* by using 15% concentration.

Conflict of Interest

The authors have no conflict of interest.

References

1. **Abd ElKhalek, A. Q. 2023.** The isolation and diagnosis of polluted fungi of walnut fruits, and the detection of its mycotoxins under different storage conditions and the potential of protecting it by using mustard oil. Master thesis, Faculty of Agriculture, University of Kufa. Iraq.
2. **Abboud, A. K. A. K. and S. M. Ali .2024.** Evaluation of Mustard oil and temperature in the toxicity reduction of *Aspergillus flavus* in Walnut fruits. Kufa Journal for Agricultural Sciences, 16(1), 98-112. https://doi.org/10.36077/kjas/2024/v16i1.10507.
3. **Aldred, D; V. Cairns-Fuller and Magan, N. 2008.** Environmental factors affect efficacy of some

essential oils and resveratrol to control growth and ochratoxin A production by *Penicillium verrucosum* and *Aspergillus westerdijkiae* on wheat grain. Journal of Stored Products Research, 44(4), 341-346.

4. **Al-Ghazali, M. T. B. and S. A. Ali. 2014.** Study of the toxic effects of ochratoxin A produced from a local isolate of the fungus *Aspergillus niger* on some biochemical and histological parameters of male and female white rats. Kufa University Journal of Life Sciences.
5. **Ali, S. M. 2015.** Isolation and diagnosis of the most important fungi contaminating the fruits of the Zahdi and Khastawi date varieties, detection of aflatoxin (B1) and ochratoxin (A) and their control using extracts of sweet bean and sesame seeds. Atrokha Ph.D thesis, University of Kufa, College of Agriculture.
6. **Al-Jumaili, S. A. 1996.** Integrated resistance against infection with the fungus *Aspergillus flavus* and contamination with aflatoxin B1 in field pistachio crops. Doctoral thesis. College of Agriculture, University of Baghdad.
7. **Balzer, I.; C. Boddanic and Pepljujak, S. 1978.** Rapid thin layer Chrmatographic method for determining Aflatoxin B1, Ochratoxin A, and Zearalenon in Corn. Journal of the Association of Official Analytical Chemists, 61, 584 – 585.
8. **Barkai-Golan, R. 2008**. *Aspergillus* mycotoxins. In: *Mycotoxins in Fruits and Vegetables* (R. Barkai-Golan, N. Paster, ed.), Elsevier, CA, U.S.A., pp 118–138.
9. **Central Bureau of Statistics 1998.** Department of Agricultural Statistics, Ministry of Planning, Iraq.
10. **Collee, J.G.; A.G. Fraser and Marmion, B.P. 1996.** Medical Microbiology. 14th edition.Churchill Livingston. USA., 937.
11. **Dakhil, A. H. and U. A. Alshimaysawe.2023.** Morphological and molecular identification of the root and stem base rot pathogen on broad bean crop and control it using some Trichoderma biological agents**.** Kufa Journal for Agricultural Sciences .15(2):43-56**.** https://doi.org/10.36077/kjas/2023/v15i2.10323
12. **Domsch, K. H.; W. Goms and Anderson, TH. 1980.**Compendium of soil fungi. Academic press., P.P: 941.
13. **FAO. 2003.** Production yearbook. Food and Agriculture Organization of the United Nation. Rome, Italy.
14. **Hocking, A.D. and J. I. Pitt .1997**. Fungi and food spoilage (Second ed.) Shampan and Hill. U.K., pp 989.
15. **Kamaz, B. A. N. and M. M. Diwan.2017.** Effect of some *Aspergillus niger* isolates on onion rotting of onion bulbs and resistance to the hot aqueous extract of ginger. Kufa Journal of Agricultural Sciences. 9 (4) 181 - 199.

16. **Mikhail, S. and B. Turki.1982.** Seed diseases. Dar Al-Kutub for Printing and Publishing - University of Mosul.
17. **Moubasher, A. H.; S. I. I. Abdel-Hafez; H. M. Abdel-Fattah and Mohrram, A. 1982.** Fungi of wheat and broad –bean staw composts. Mycopathology, 78, 161-168.
18. **Randle, W.M. 1997.** Onion flavor chemistry and factors influencing flavor intensity. American Chemical Society ,5, 42-52.
19. **Richard J.L. 2007**. Some major mycotoxins and their mycotoxicoses-An overview. International Journal of Food Microbiology, 119, 3–10.
20. **Saratha, V. and S.P. Subramanian. 2010.** Evaluation of antifungal activity of Calotropis gigantea latex extract:An in vitro study. International Journal of Pharmaceutical Sciences and Research, 1(9), 88-96.
21. **Shaaban, A. and N. M. Al-Mallah. 1993.** Pesticides. Directorate of Dar Al-Kutub for Printing and Publishing / University of Mosul.
22. **Susca, A.; G. Stea; G. Mulé and Perrone, G. 2007.** Polymerase chain reaction (PCR) identification of *Aspergillus niger* and *Aspergillus tubingensis* based on the calmodulin gene. Food Additives and Contaminants, 24(10), 1154-1160.
23. **White, T.J.; T. Bruns; S. Lee and Taylor, J. 1990.**Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: M. A. Gelfand, D. H. Sninsky and T. J. White (eds.), PCR Protocols: a guide to methods and applications. Academic Press, San Diego, USA. pp. 315–322.
24. **Yurkakul, O., K.; F. S. Kocasari; N. Yalcin and Keyvan, E. 2019.** Survey of ochratoxin a in coffee, dried grapes and grape pekmes samples in Burdur Turkey. Veterinary Medicine, 38, 46-54.
25. **Zait, A. Q. and S. M. Abu Al-Feith. 2021.** Antagonistic efficacy of some plant extracts against *Aspergillus niger* isolated from *Allium cepa* L. Al-Bayan Scientific Journal