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# Purification , Characterizations and Detoxification of Ochratoxin A (OTA) in wheat and maize and its Possibilities of it Control by Medicinal Plant Extract

A thesis

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By

**Eman Salman Rahaif Hilo AL-Bdeery**

M.Sc. University of Al-Qadisiyah -2017

Supervised by

**Prof Dr. Ibtihal Muiz Abdul Mahdi Al-Hussaini**

University of Babylon / College of Science

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# Dedication

*To my mother, father who continue to learn, grow and develop and who has been the source of encouragement and inspiration to me throughout my life, Words never enough to thanks.*

*To my brothers and my sisters, who have never left my side and are very special.*

*To every one supported me.*

*I dedicate the fruit of my humble work*

***Eman***

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة بابل / كلية العلوم
قسم علوم الحياة

# تنقية وخصائص وازالة سمية الاوكراتوكسين من الحنطة والذرة وامكانية السيطرة عليها بواسطة بعض مستخلصات النباتات الطبية

اطروحة مقدمة الى
مجلس كلية العلوم - جامعة بابل كجزء من متطلبات نيل درجة الدكتوراه فلسفة في العلوم - علوم الحياة

من قبل
**ايمان سلمان رهيف حلو البديري**

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بأشراف

**أ. د. ابتهال معز عبد المهدي الحسيني**

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

**يُؤْتِي الْحِكْمَةَ مَن يَشَاءُ ۚ وَمَن يُؤْتَ الْحِكْمَةَ فَقَدْ أُوتِيَ خَيْرًا كَثِيرًا ۗ**
**وَمَا يَذَّكَّرُ إِلَّا أُولُو الْأَلْبَابِ**

صدق الله العلي العظيم

سورة البقرة اية (٢٦٩)

# الخلاصـــــــــة :

اجريت الدراسة الحالية في كلية العلوم - قسم علوم الحياة - مختبر عزل الفطريات والسموم الفطرية وذلك للفترة بين شهر شباط ٢٠٢٠ لغاية شهر ايلول ٢٠٢٠. هدفت الدراسة الى عزل وتشخيص الفطريات المرافقة لحبوب نباتي الحنطة والذرة بعد الحصاد، وكذلك الكشف عن انتاج الاوكراتوكسين A المستخلص من *Aspergillus niger* و*Aspergillus ochraceus* المعزول من حبوب الحنطة *Triticum aestivum* والذرة *Zea may* في محافظتي بابل والديوانية . كما تضمنت الدراسة استخدام تقنية TLC و HPLC للكشف عن وجود السم اضافة الى استخدام تقنية RT-PCR للكشف والتحري عن الجين المسؤول عن انتاج ( Ochratoxin A (OTA في *A.niger* و *A.ochraceus* ، بالاضافة الى الكشف عن المركبات الفعالة في المستخلص الكحولي والمائي لاوراق نبات الزيتون *Olea europaea L.* و ازهار الشفلح *Capparis spinosa* ودراسة تأثيرها التثبيطي على الفطريات اعلاه.

اظهرت النتائج عزل عدة انواع من الفطريات المرافقة لحبوب الحنطة والذرة المأخوذة من سايلوات واسواق محافظة بابل ، حيث شخصت الانواع التالية *A.fumigatus A.parasiticus A,flavus A.terreus A.niger, A.ochraceus, Al.alternata , Rhizopus sp., Fusarium sp. Mucor sp., Negrosporum sp, Penicillium spp.*وتم تشخيص الفطريات قيد الدراسة بأستخدام عدة مفاتيح تصنيفية.

بينت النتائج ان الفطر *A.niger* هو الاكثر تكرار في سايلو بابل والديوانية وفي اسواق بابل اذ بلغت النسب 16.12% و 25% و 49.04% على التوالي وايضا كان اعلى ظهور في نفس الاماكن وبنسب 12% و 100% و 87.5% على التوالي ، بينما سجل الفطر *Fusarium sp.* اقل نسب الظهور والتردد اذ بلغت نسبة التكرار 1.67% والظهور20% في سايلو بابل وبتكرار 1.01% وظهور 37.5% في اسواق محافظة بابل في حين لم يسجل له اي ظهور او تكرار في سايلو الديوانية ، كما لم يتم عزل فطر *A.ochraceus* ، وكل ذلك الفطريات المعزولة من نبات الحنطة. اما نبات الذرة فقد اظهرت النتائج ان *A.flavus* ذو ظهور عالي بلغ 70% و 71% و 55.35% تلاه الفطر *A.niger* بظهور بلغ 20% و 27.21% وكذلك 66% في المناطق قيد الدراسة ، سايلو بابل وسايلو الديوانية واسواق بابل على التوالي. كما ان بعض الفطريات المعزولة قد ظهرت في بعض الاماكن في حين لم تظهر في اماكن اخرى .

تم تشخيص الفطر *A.ochraceus* جزيئيا اذ سجلت ١١ عزلة من مجموع ١١ عزلة تعود للفطر *A.ochraceus* من خلال استخدام بادئ ITS2 عند الوزن الجزيئي 420bp .

تم الكشف عن وجود Ochratoxin A في الفطريات المعزولة من حبوب الحنطة والذرة بأستخدام TLC و HPLC ، بينت نتائج TLC ان 5 من اصل 6 عزلات للفطر *A.niger* كانت منتجة للسم وجميع عزلات *A.ochraceus*المستخدمة في . بينت نتائج HPLC ان OTA المستخلص من *A.ochraceus* والمعزول من حبوب الذرة بلغ اعلى تركيز (98.3, 124.9) ppb ، على التوالي، بينما السم المستخلص من *A.niger* المعزول من حبوب الحنطة بلغ اعلى تركيز (8.14, 3.50) ppb، على التوالي عن ذلك المستخلص من حبوب الذرة.

شملت الدراسة الجزيئية (الترحيل الكهربائي لنواتج PCR و RTpcr )، بينت نتائج الترحيل الكهربائي بأستخدام هلام الاكاروز لتحليل نواتج PCR للـ 18s rRNA Its gene من DNA المستخلص من فطر *A.ochraceus* كما كشفت نتائج Real-time PCR لكل من *A.niger* و *A.ochraceus* عن احتوائها على الجين المسؤول عن انتاج سم OTA .

اظهرت نتائج الدراسة احتواء كل من اوراق الزيتون وازهار الشفلح على القلويدات، الفينولات، التربينات، الفلافونيدات، الكلايكوسيدات، والتانينات. كما كشفت الفعالية التضادية لمستخلصات اوراق الزيتون وازهار الشفلح الكحولية والمائية ان كل من الجنسين *A.niger* و *A.ochraceus* قد تأثر بالمستخلصين وبدرجات متفاوتة، بلغ التركيز المثبط الادنى MIC 5% عند استخدام المستخلص المائي لاوراق الزيتون 29.91% بينما التركيز 26.44% للمستخلص الكحولي لنفس النبات وقد سجل المستخلصان تركيز التثبيط الادنى 92.45% لتركيز 20% مستخلص مائي و 92.66% لمستخلص كحولي لنفس التركيز ولنفس النبات . اما بالنسبة لازهار الشفلح فقد بلغ التركيز المثبط الادنى (MIC) عند التركيز 20% 92.16% و 92.89% للمستخلص المائي والكحولي على التوالي، ومن خلال النتائج تبين ان لنباتي الزيتون والشفلح تأثير كبير على نمو الفطرين *A.niger* و *A.ochraceus* ولكلا المستخلصين قيد الدراسة .

# Summary

The current study was conducted in the department of Biological Faculty of Science- Laboratory of isolating fungi and mycotoxin, from February 2020 to September 2020. The study aimed to isolate and diagnosis the fungi in wheat (*Triticum aestivum*) and maize (*Zea may*s) grains during post-harvest and detect the ochratoxin A (OTA) extracted from *Aspergillus niger* and *Aspergillus ochraceus* isolated from wheat and maize grains in the provinces of Babylon and Al-Qadisiyah. The study included the use of thin layer chromatography(TLC) and high performance liquid chromatography(HPLC) techniques to detect the presence of ochratoxin A, in addition to , use Real-time PCR (RT-PCR) to detect the gene responsible for the production of OTA in A.*niger* and *A.ochraceus*. The phytochemical components of *Olea europaea L.* leaves and *Capparis spinosa L.* flowers were also detected, and their inhibitory effect on the *A.niger* and *A.ochraceus* where tested.

The results showed the isolation of several types of fungi associated with wheat and maize grains obtained from Silos and Markets, where the following types were diagnosed: *A.fumigatus A.parasiticus A,flavus A.terreus A.niger, A.ochraceus, Al.alternata* , *Rhizopus sp., Fusarium sp. Mucor sp., Negrosporum sp, Penicillium spp.*

The results showed that *A.niger* was the most frequent in the silo of Babylon and Al-Diwaniyah and in the markets of Babylon, as the percentages reached 16.12%, 25% and 49.04%, respectively, and also the highest appearance was in the same places at rates of 12%, 100% and 87.5%, respectively, while *Fusarium sp*. record the lowest rates of appearance and frequency, as the frequency reached 1.67%, and the appearance was 20% in the Babylon silo, and the frequency was 1.01%, and the appearance of 37.5% in the markets of the province of Babylon,

while no appearance or frequency was recorded in the Diwaniyah silo, and *A.ochraceus* was not isolated, and all that fungi Isolated from the wheat plant. As for maize plant, the results showed that *A.flavus* had a high appearance of 70%, 71% and 55.35%, followed by *A.niger* with an appearance of 20% and 27.21%, as well as 66% in the areas under study, Babylon silo, Al-Diwaniyah silo and Babylon markets, respectively. Also, some isolated fungi appeared in some places, while they did not appear in other places.

*A.ochraceus* was molecularly identified as 11 isolates were recorded out of the total 11 isolates belonging to *A.ochraceus* by using the ITS2 starter at a molecular weight of 420bp.

The presence of OTA in grains was determined by TLC and HPLC. The results of TLC indicated that 5 of 6 isolates of *A.niger* were produced of OTA and 4of 4 isolated of *A.ochraceus* produced of OTA. The results of HPLC indicated that the concentration of OTA extract from *A.ochraceus* that isolated from maize grains recorded the highest concentration (98.3 , 124.9) ppb respectively , while the concentration of OTA extract from *A.niger* that isolated from wheat grains recorded the highest concentration (8.14 , 3.50) ppb respectively, than that isolate from maize grains.

Molecular study which includes (electrophoresis of PCR product and Quantitative Reverse Transcription Real-Time PCR in *A.niger* and *A.ochraceus* ) . The results of RT- PCR showed presence the gene that responsible for production of OTA in *A.niger* and *A.ochraceus*

The secondary metabolites analysis of *Olea europaea L.* leaves and *Capparis spinosa L.* flowers extracts revealed the presence of phenols, alkaloids, terpenes, flavonoids, glycosides and tannins. Antagonistic

activity of the alcoholic and aqueous extracts of olive leaves and *C.spinosa* flowers revealed that both *A.niger* and *A.ochraceus* were affected by the two extracts to varying degrees, the minimum inhibitory concentration (MIC) reached 5% when using the aqueous extract of olive leaves was 29.91%, while the concentration was 26.44% for the alcoholic extract of the same plant. The two extracts recorded a minimum inhibition concentration of 92.45% for a concentration of 20% aqueous extract and 92.66% for an alcoholic extract of the same concentration and for the same plant. As for the flowers of *C.spinosa*, the minimum inhibitory concentration (MIC) at the concentration reached 20% 92.16% and 92.89% for the aqueous and alcoholic extracts, respectively. Through the results, it was found that the two plants *O.europaea L.* and *C.spinosa* had a significant effect on the growth of *A.niger* and *A.ochraceus* and for both extracts under study.

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## *List of Abbreviation*

| Abbreviation | Complete Terms |
|---|---|
| **ANOVA** | A one way analysis of variance |
| **BHI** | Brain heart infusion |
| **HPLC** | High performance liquid chromatography |
| **ITS** | The internal transcribed spacer |
| **MIC** | Minimal inhibitory concentration |
| **OTA** | Ochratoxin A |
| **PCR** | Polymer chain reaction |
| **PDA** | Potato dextrose agar |
| **PK** | Protein kinase |
| **RT** | Real-time |
| **SDA** | Sabouraud dextrose agar |
| **TLC** | Thin layer chromatography |
| **UV** | Ultra violet |

## *Conclusion*

1. *Samples* of wheat and maize grains collected from Babylon and Al-Diwanyiah Silos and markets were all contaminated with fungi, and *A.niger* was the most frequency and appearance in wheat samples , *A.flavus* was the most frequency and appearance in maize grains.
2. The HPLC results show that the concentration of ochratoxin A in *A.ochraceus* is higher than the concentration in *A.niger*.
3. Gene expression results show that all the isolated of the *A.niger* and *A.ochraceus* that secret the ochratoxin A have the gene that responsible for the production of the toxin.
4. Results revealed that plant extract *O.europaea L.* leaves and *C. spinosa* flowers have a good activities against *A.niger* and *A.ochraceus*. Growth and increase inhibitory effect with increasing of their concentration.

## ***Recommendation***

1. Periodic and continuous examination of stored grains (wheat and maize) to determine the amount of contamination with fungi.
2. Establishing laws specifying the permissible quantities of mycotoxins, especially ochratoxin A, in food and feed.
3. Conducting studies on the anther genes responsible for the production of anther mycotoxins.
4. Use laboratory animals to study the effects of OTA .
5. Study the effect of plant extracts on laboratory animals.
6. Test the plant extracts as antibacterial and antioxidant.
7. Further investigation on the effect of plant extract against growth parameter of *Aspergillus* sp.

# Introduction:

Mycotoxins are secondary metabolites produced by a wide variety of filamentous fungi, including species from the genera *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria* and *Claviceps* that grow under different climatic conditions on agricultural commodities (Majeed *et al.*, 2018). Mycotoxins are ubiquitous and contaminate various feedstuffs and agricultural crops and induce a range of harmful effects (Jolly *et al.*, 2011). These metabolites are produced and found in many feeds and foodstuffs especially in plants during their pre-and post-harvest, transportation, processing and storage and are detected in cereal crops (Juan *et al.*, 2014) and in peanuts (Afolabi *et al.*, 2015). Aflatoxin, ochratoxin, fumonisin, deoxynivalenol and zearalenone are all considered the major mycotoxins produced in food and feedstuffs (Wagacha and Muthomi, 2008). Among the dangerous mycotoxins; aflatoxin, ochratoxin A and fumonisins (FB1 and FB2) represent the greatest health risk in tropical Africa (Manjula *et al.*, 2009). Asia (Li *et al.*, 2014) and the rest of the world (Alborch *et al.*, 2012). Mycotoxins are capable of causing disease and death in both humans and livestock (Bennett and Klich, 2003).

Ochratoxin (OT) is produced mainly by *Penicillium* and *Aspergillus* species such as *Penicillium verrucosum*, *penicillium nordicum*, *Aspergillus ochraceus*, and *Aspergillus carbonariusm*, while new producers are continuously being discovered (Davolos and Pietrangel, 2014). OTA occurs ubiquitously in plant products such as cereals, beans, groundnuts, raisins, coffee, beer and wine, as well as in certain animal products (Jorgesen,2005) **.** Cereals (wheat, barley, and oats) are the main source of human exposure to OTA (Duarte *et al*., 2010).

The *Aspergillus* is one of the widespread fungi, which includes many species that cause human and animals diseases, food spoilage, production of mycotoxins as secondary metabolites, and on the other hand, it is beneficial through fermentation of food. The lifestyles of *Aspergillus* species associated with plants range from saprophytes and symptomless endophytes to weak and opportunistic phytopathogens. The shift between these lifestyles is the result of global transcriptome changes, primarily affecting secondary metabolite (SM) production (Reverberi *et al.,* 2013). The principal and well-known mycotoxins produced by the *Aspergilli* are ochratoxin A (OTA) and aflatoxins (AFs), as well as less-prominent toxins like patulin (Keller *et al.*, 2005). These toxins are found in different agricultural commodities (Varga *et al.*, 2004), and are tightly regulated with different threshold limits depending on the matrix (Cano *et al*., 2016).

The effectiveness of plant extracts is due to the fact that they contain active substances that affect the growth of pathogenic microbs, fungi, bacteria, and these effective compounds resulting from the photosynthesis process, included tannins, glycosides, terpenes, and other active substances .The olive (*Olea europaea L.*) leaf is known to be resistant in nature to microorganisms and insect attack, and much research has focused on the antimicrobial activity of compounds contained in olives and olive oil. Phenolic compounds including oleuropein, tyrosol, hydroxytyrosol, caffeic acid, gallic acid, syringic acid, p-coumaric acid and luteolin, isolated from olive fruit an leaves, have been shown to inhibit or delay the rate of growth of a range of microorganisms (Korukluoglu *et al*., 2004); thus, they might be useful as an alternative food additive (Tassou and Nychas 1994). Markin *et al*,

(2003) tested water extract of olive leaf against some microorganisms and found that *Candida albicans* was killed within 24h.

*Capparis spinosa* is one of the most important economical and medicinal plants which has been used in folk medicine and considered as a very good source of glucosinolates (glucocapparin, glucoiberin, sinigrin, and glucobrassicin), flavonoids, phenolic acids, and alkaloids, all of which are known to provide health-improving benefits due to their various biological activities (Tlili *et al*.,2017). A wide range of biological activities such as antioxidant, anti-inflammatory, anticancer, antimicrobial, antimutagenic, and antidiabetic effects have been reported for this plant (Zhang and Ma,2018). Furthermore, leaves of *C. spinosa* are one of the most affluent sources of quercetin which is considered as the main bio-antioxidant and hepatoprotective ( Kalantari *et al*.,2018).

## Aim of the study

Detection and Characterizations Ochratoxin A in some economic grains that contaminated with fungi and the possibilities of its control by medicinal plant extracts.

## Objectives of the study

1- Isolation and identify of the fungi species that may contaminated with ochratoxins A from wheat grain and maize In stores .

2- Detection and purification of the Ochratoxin A from the fungi by using TLC and HPLC techniques .

3- Evaluation the genes- expression that responsible for the production of Ochratoxin A from fungi species .

4- Determining the phytochemicals medicine plants and controlling on the growth mold with ochratoxin A by some plants extract.

# 2. Literature Review

## 2.1: Mycotoxins

Mycotoxins are toxic chemical products formed as secondary metabolites by a few fungi species that colonize crops and contaminate them with toxins in the field or after harvest (Moss,1996). They are produced during growth and multiplication of fungus when micro ecological conditions are favourable (Sumalan *et al*., 2011). It was found that plant-derived feedstuffs may be contaminated with mycotoxins, and mycotoxins have been found in animal products and in egg, milk and dairy products from animals fed with rations containing such feedstuffs (Meucci *et al*., 2010). One mold species may produce many different mycotoxins, and several species may produce the same mycotoxin. The spectrum of toxins produced in a commodity largely depends on one or more fungal species/strains contaminating the commodity, type and composition of commodity, environmental conditions, climatic factors, and also handling practices such as pre-harvest agricultural practices, harvesting, drying, storage, and processing (Chilaka *et al.*, 2016; Ogara *et al.*, 2017). The toxic effect of mycotoxins reveals boundary less distribution and harm of health and economic attributes.

Mycotoxins are invisible, tasteless, chemically stable and resistant to temperature and storage. They are resistant the normal feed manufacturing processes. Mycotoxin producing fungi can be divided into two groups (Weidenbörner, 2007; Krska 2016) .Field fungi (such as *Fusarium sp*.) typically produce mycotoxins in the field (“pre-harvest”) and Storage fungi (such as *Aspergillus* and *Penicillium sp*.) typically occur after harvest (“post-harvest”) However, in special cases like under unusually hot or dry conditions *Aspergillus* and *Penicillium* species can also affect crops during the growing season. On the other hand, field fungi can continue growing and produce mycotoxins during transport and storage (Dawlal *et al.*,2012) .

### 2.1.1: Occurrence and distribution of mycotoxins

Mycotoxins are ubiquitous. They can occur in cereals, cereal products and foods, feeds, animal products and soil. Animal feeds commonly harbor mycotoxins are wheat bran, pea hulls and maize grain. Concentrated animal feedstuffs harbor the growth of mycotoxins. Mycotoxins can be transferred from feed to food of animal origin, as this food represents a significant route of exposure for humans. Apart from their toxicological effect in animals, they carry-over through animal derived products, such as meat, milk and eggs and transfer them into the human food chains (Demissie, 2018). Also, they may be distributed in pre-harvest period (time of plant growing), post-harvest during processing, packaging, distribution and storage of food products. Mycotoxin contamination intensity in crop varies geographically (Pereira *et al.,* 2014; Marta and Bedaso, 2016). Conclusively, all crops and cereals which are stored improperly under favorable temperature and prompting humidity for a long time facilitate mold growth and can be subject to mycotoxin contamination (Ahmad and Jae-Hyuk, 2017); no boundary can limit fungal growth and mycotoxin production unless appropriate measures are taken.

### 2.1.2: Major types of mycotoxins

Mycotoxins contaminate food and feed and affect food security throughout the world, and their effect is higher, especially in low and middle-income countries (Antonio *et al.*, 2018). Researchers have isolated and characterized more than 400 mycotoxin types. The most important and highly toxic mycotoxins include; aflatoxin, ochratoxin A, trichothecenes, zearalenone, fumonisins B1 and B2 (FUMB1, FUMB2), tremorgenic toxins, and ergot alkaloids (Margherita *et al.*, 2012). The major fungi causing frequent and problematic contamination of foods and feeds with mycotoxins are members of the fungal genera *Aspergillus*, *Fusarium* and *Penicillium* (Ahmad and Jae-Hyuk, 2017)

### 2.1.2.1: Aflatoxins

Aflatoxins are poisonous carcinogens which interfere with the immune system and are produced by certain molds (*Aspergillus flavus* and *Aspergillus parasiticus*) (Jef *et al*., 2015) which grow in soil, decaying vegetation, hay, and grains of primarily found in hot, humid climates, colonizing mostly the aerial parts of plants (Marin *et al*., 2013). Mostly aflatoxins have related structure (Eaton and Groopman, 1994) .Aflatoxins have earned significant attention because of their deleterious effects on human and livestock health as well as on the international trade of foodstuffs. There are 20 known types of aflatoxins which are mainly classified into aflatoxin B1 (AFB1), B2, G1, G2, M1 and M2 based on structure, chromatographic and fluorescent characteristics (Ephrem, 2015).

AFB1 has higher toxicity and mainly metabolized by liver in to AFB1-8, 9-exo-epoxide and 8, 9-endo-epoxide which bind to DNA to form 8, 9-dihydro-8-(N7-guanyl)-9-hydroxy - (AFB1-N7-Gua) and AFB1-N7-Gua could be converted to two secondary lesion which is an apurinic site where more stable ring is opened. This implies that aflatoxins have an effect on amino acid metabolism. The major human cytochrome P450 (CYP) enzymes involved in aflatoxin metabolism are CYP3A4, 3A5, 3A7, and 1A2 (Marin *et al.*, 2013).

Drought and stress increase aflatoxin spread in the field and can be produced due to insufficient drying of contaminated crops before storage or stored under humid conditions (Jef *et al.*, 2015). Due to their stability to severe processes of roasting, extrusion, baking, and cooking, aflatoxins also induce a great problem in processed foods, such as roasted nuts and bakery products and it can be found alone or simultaneously, as well as co-occurring with other mycotoxins such as OTA (Marin *et al.*, 2013). Mokif *et al.*,2014 study the aflatoxin B in some food and possibility of reducing the toxic effect by olive oil and green tea**.**

### 2.1.2.2: Ochratoxins

Ochratoxin A (OTA) was first identified and characterized from fungal cultures in South Africa (Van der Merwe *et al.*, 1965). It is a phenylalanyl derivative of a substituted iso-coumarin (R)-N-[5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-y1)-carbonyl]-L phenylalanine .Ochratoxin A is produced by two main genera of fungi, *Aspergillus* and *Penicillium* with main producing species of *Aspergillus* Section *Circumdati*, *Aspergillus* Section *Nigri*, *Penicillium verrucosum,* and *Penicillium nordicum* (EFSA, 2006a).

Ochratoxin A is the most toxic member of the ochratoxin which is structurally similar to the amino acid phenylalanine. Thus, it has an inhibitory effect on a number of enzymes that use phenylalanine as a substrate, particularly Phe-tRNA synthetase, resulted in the inhibition of protein synthesis. Ochratoxin A is a mitochondrial poison, which causes cellular damage, oxidative burst, lipid peroxidation, and oxidative phosphorylation. Furthermore, it increases cell apoptosis and it is a stable and heat resistant which is not damaged by common food preparation temperature (above 250oC for several minutes reduce its concentration (Marin *et al.*, 2013).

### 2.1.2.3: Fumonisins

Fumonisins are fusarium toxins first discovered in 1988 (Gelderblom *et al*. 1988) cited in Marasas (2001) and constitute the large family of compounds (Antonio *et al.,* 2018) which are produced by a number of fungi most dominantly *Fusarium verticillioides* and *Fusarium proliferatum.* Other fungal species, including *F. dlamini, F. nygamai* and *F. napiforme* also produce fumonisins (EFSA, 2005a). Fumonisins have strong structural similarity to sphinganine which are the precursor of sphingolipids.

There are about 12 types of known fumonisin types and the most important ones are FB1, FB2, and FB3 of which FB1 is most toxic. They are the mostly found in maize grown in warmer areas. Since *F. verticillioides* and *F. proliferatum* grow in a wide range of temperatures only at relatively high water activities (aw > 0.9), FBs are formed prior to harvest or during the early stage of storage and its concentration does not increase during storage except under extreme conditions. They are fairly heat-stable, and toxicity can be minimized only during processes where temperature is beyond 150°C (Marin *et al.,* 2013). The chemical name of this mycotoxin is 1,2,3-propanetricarboxylicacid,1,10-[1-(12-amino-4,9,11-trihydroxy-2 methyl tridecyl)-2-(1-methylpentyl)-1,2-ethanediyl]ester.

### 2.1.3: Importance of mycotoxins

Mycotoxicoses in human like other toxicological syndromes can be categorized as acute or chronic. Acute toxicity has a rapid onset and an obvious toxic response, while chronic toxicity is characterized by low dose exposure over a long time period leading to cancer and other generally reversible effects (James, 2005).

Ochratoxin A and citrinin cause nephropathy in animals and they have also been implicated as the cause of Balkan endemic nephropathy in humans. Both OTA and citrinin are well known nephrotoxins. OTA is also carcinogenic to rodents (Creppy *et al*., 1985) and possesses teratogenic (Arora and Feldegg, 1983), immunotoxic (Stormer and Lea, 1995), neurotoxic (Harbison, and Sanchezramos, 2006), mutagenic (Stetina and Votava, 1986), and genotoxic (Meisner *et al*.,1983) properties. Compared to OTA, ochratoxin B is rarely found and very less toxic. Ingestion of clover hay containing slaframine causes salivary episodes that last from several hours to over 3 days in ruminants and horses (Gupta, 2007).

Aflatoxin contributes factor for the disease like Kwashiorkor and Reye’s syndrome when children suffering it (Blunden *et al*., 1991); immunosuppression in children (Turner *et al*., 2003). Despite this, ruminants are less affected than non ruminant animals. However, production (milk, beef or wool), reproduction and growth can be altered when ruminants consume mycotoxin contaminated feed for extended periods of time (Hussein and Brasel, 2001).

Health effects occur in companion animals, livestock, poultry and humans because aflatoxins are potent hepatotoxins, immunosuppressant, and mutagens and carcinogens (Eaton and Gallagher, 1994). Zearalenone has major effects on reproduction that can lead to hyperestrogenism. Prepubertalswine are the most sensitive species. Typical clinical signs of hyperestrogenism are swelling of the vulva, increase in uterine size and secretions, mammary gland hyperplasia and secretion, prolonged oestrus, anestrus, increased incidence of pseudo-pregnancy, infertility, decreased libido, and secondary complications of rectal and vaginal prolapses, stillbirths and small litters (Gupta, 2007).

Fumonisins (B1 and B2) toxic metabolites that are usually found in corn have been implicated in field cases of porcine pulmonary oedema (PPE) (Colvin *et al*., 1993), and equine leukoencephalomalacia (ELEM) (Wilson *et al*., 1990). Experimentally, fumonisin has been shown to cause liver damage in multiple species including pigs, horses, cattle, rabbits, and primates (Gumprecht *et al*., 1995) as well as species-specific target organ toxicity, such as lung in pigs (Haschek *et al*., 1992), brain in horses (Ross *et al*., 1993), kidney in rats, rabbits, and sheep (Gumprecht *et al*., 1995; Voss *et al*., 1989), and oesophagus in rats and pigs (Casteel *et al*., 1994; Lim *et al*., 1996).

Epidemiologic data has linked ingestion of corn contaminated with *F. verticillioides* to human oesophageal cancer (Rheeder *et al*., 1992), and fumonisins have been shown to be hepatocarcinogenic in rats and mice (Gelderblom *et al*., 1988; Howard *et al*., 2001).

### 2.1.4: Factors favoring fungal proliferation and mycotoxin production

The favoring conditions for mycotoxin production relate mainly to poor hygienic practices during transportation, improper storage, processing, high temperature and moisture content and heavy rains (Bhat and Vasanthi, 2003). The demand for the storage of food substances has been increased due to the increasing population.

Researchers have found a variety of factors which favor the production of mycotoxins. Those are grouped as physical, chemical, and biological factors. Physical factors include environmental conditions temperature, relative humidity, and insect infestation while chemical factors include the use of fungicides or fertilizers as well as biological factors depend on the interactions between the colonizing toxigenic fungi and the substrate, in fact some plant species are more susceptible to colonization while environmental conditions may increase the vulnerability of others are more resistant (Margherita *et al.*, 2012). In other ways thus factors can be either intrinsic, extrinsic, processing or implicit each of which including moisture content, water activity, substrate type, plant type and nutrient composition; climate, temperature, oxygen level; drying, blending, addition of preservatives, handling of grains; insect interactions, fungal strain and microbiological ecosystem respectively, figure (2-1) (Gabriel and Puleng, 2013).

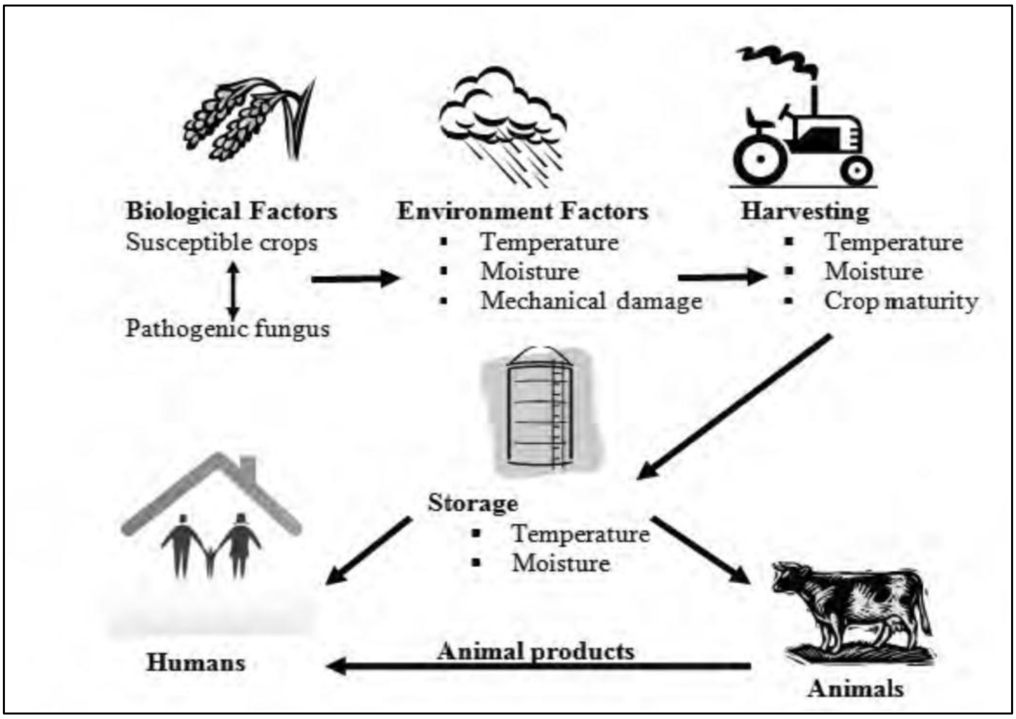


**Figure (2-1): Factors affecting the mycotoxin production in the food chain(Gabriel and Puleng, 2013).**

### 2.1.5 Some Mycotoxin analysis techniques

Determination of mycotoxin level in food sample is usually accomplished by certain steps: sampling, preparation, extraction followed by a cleanup and detection which is performed by many instrumental and non-instrumental techniques (Ahmad and Jae-Hyuk, 2017) .

#### 2.1.5.1: Chromatographic techniques

This technique is the most commonly used method for mycotoxin analysis. Thin layer chromatography (TLC) is one of earliest quantitative method for mycotoxin screening based on visual assessment or instrumental densitometry. However, recent advances in mycotoxin analysis have introduced fast and convenient chromatographic technologies for both detection and quantification such as high performance liquid chromatography (HPLC) coupled with ultraviolet, diode array, fluorescence or mass spectrometry detectors and ultra HPLC with reduced column packing material. Highly advanced coupling liquid chromatography techniques, mass-spectrometry and HPLC coupled mass spectrometric or fluorescence detectors are frequently used in mycotoxins analysis while other chromatographic techniques are rarely used because of limited sensitivity and specificity. HPLC-FLD (HPLC coupled with fluorescence) is used for single mycotoxin analysis and HPLC-MS/MS (HPLC coupled with mass spectrometry) is the best choice for simultaneous determination of multiple mycotoxins (Ahmad and Jae-Hyuk, 2017).

These are the ultimate methods used for the identification/confirmation of the identity of mycotoxins, including those which are masked and do not fluoresce or do not absorb visible UV light. Such methods allow the identification and sometimes the quantization of many mycotoxins in a single sample. As mycotoxins are real problems for health, there will always attract attention and, certainly, methods for their analysis will continue to improve. Because of the potential co-occurrence of such contaminants, the challenge is to develop screening methods for their rapid simultaneous detection of multiple families of mycotoxins from the same sample. But the differences in their chemical and physical properties and of concentration range of interest have made simultaneous detection very difficult. In this regard HPLC technique coupled with mass spectrometry or multiple detectors have good prospects(Ahmad and Jae-Hyuk, 2017).

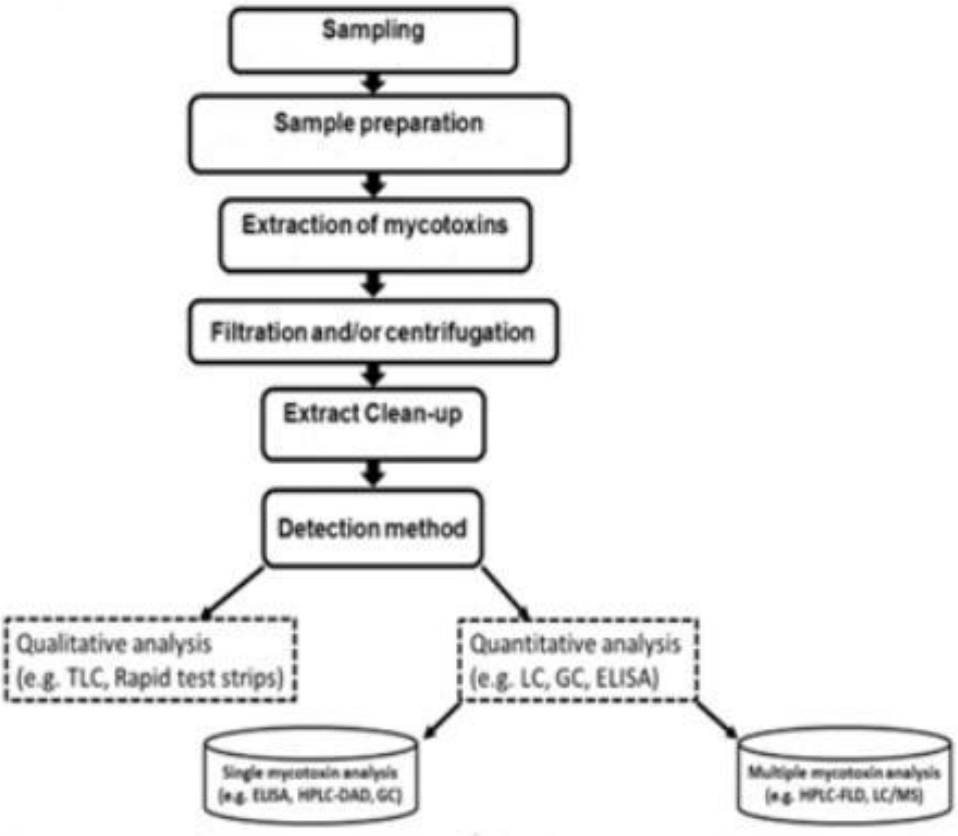


**Figure (2-2): Common steps in mycotoxin analysis (Source: Ahmad and Jae-Hyuk, 2017).**

### 2.1.5.2: Biosensor technology

Biosensors enable the detection of the analyte in a sample because of the interaction between the analyte and biological sensitive elements such as enzymes, tissues, nucleic acids or antibodies. The interaction results in a signal which can be detected by a transducer (optical or physicochemical detection) and is transformed in an utilizable measured variable (Kristine and Florian, 2018).

### 2.1.5.3: Molecular techniques

#### 2.1.5.3.1: DNA and aptamer based biosensors

It is reported that DNA biosensor based method is used to analyze AFM1 in milk samples. In this technique, thiol-modified single stranded DNA probe is immobilized on a monolayer of cysteamine and gold nanoparticles. The DNA biosensor particularly bound the AFM1 and detection of the process is carried out with electrochemical impedance spectroscopy and cyclic voltammetric techniques (Dinckaya *et al.,* 2011). Another form to use DNA in biosensors is aptamer based technique. Aptamers are peptide molecules (DNA or RNA duplex structures) which can bind with specific analyte. Chen *et al.*, 2012) reported a DNA duplex structure with an anti-OTA-aptamer such as fluorophore and quencher and binding ochratoxin A to this structure leads to an increase of the fluorescence (Kristine and Florian, 2018).

#### 2.5.3.2 Molecular imprinting polymers

It is a synthetic technique designed to imitate natural recognition entities viz antibodies and biological receptors. This highly selective molecular technique uses cross-linked polymers which are electrochemically prepared by the reaction of monomer and cross linker in the presence of mycotoxins (Ahmad and Jae-Hyuk, 2017).

### 2.1.6: Control of mycotoxins problems

Control of Mycotoxins is for the purpose of public health importance and economic improvement in the country. Hence, a number of strategies for reduction and control of mycotoxins have been considered in different areas of world including African countries. The control of mycotoxins involves: 1, Prevention of mould or fungus growth in crops and other feedstuffs; 2, Decontamination of mycotoxin contaminated feeds/foods as a secondary strategy; 3, Continuous surveillance of mycotoxins in agricultural crops, animal feedstuffs and human food (Dawlal *et al*.,2012).

### 2.2: Ochratoxins A in *A.niger* and *A.ochraceus*

Ochratoxin A (OTA) is the main mycotoxin in the group of ochratoxins (Ringot *et al*. 2005) and is produced by several fungal species of the genera *Aspergillus* and *Penicillium* (Maleř *et al*. 2003; Valero *et al.* 2006; Cavin *et al.* 2007). The major OTA-producing species are *Penicillium* (*P.*) *verrucosum, Aspergillus* (*A.*) *ochraceus*, and *A*. section *Nigri* (Magan and Aldred 2005). *P. verrucosum* is the major OTA-producing fungus in northern Europe, while *A. ochraceus* is more important in warmer climatic zones (Cairns-Fuller *et al.* 2005).

More *Aspergillus* species have been found to produce OTA, for example, *A. melleus, A. sulphureus, A. alliaceus*, *A. sclerotiorum* (Maleř *et al*. 2003; Bayman and Baker 2006; Palumbo *et al*. 2007), *A. albertensis*, *A. lanosus* (Bayman and Baker 2006; Palumbo *et al*. 2007), *A. glaucus*, *A. ostianus*, and *A. petrakii* (Maleř *et al*. 2003; Bayman and Baker 2006). In *Penicillium*, OTA has also been detected, for example, in *P. nordicum* (Larsen *et al.* 2001; Bayman and Baker 2006), *P. chrysogenum* (Maleř *et al*. 2003; Bayman and Baker 2006). OTA has been detected, for example, in cereal grains, grapes, wine, grape juice, dried vine fruits, coffee, legumes, beer, nuts, cocoa, spices (Bayman and Baker 2006; Clark and Snedeker 2006).

The occurrence of OTA in meat products is due to its transmission into tissues of animals fed with contaminated feed (Guillamont *et al.* 2005). The highest reported occurrences of OTA contamination have been found in cereal grains, and to a lesser extent in grapes, wine, grape juice, and dried vine fruits (Clark and Snedeker 2006). The highest OTA content, among grapes and their derivatives, has been found in dried vine fruits (OTA is a thermostable molecule) (Boudra *et al.* 1995; Czerwiecki *et al*. 2002) that is not completely eliminated during the food processing operations and, therefore, it appears in the final products (Bullerman and Bianchini 2007).

*P. verrucosum* and *A. ochraceus* are considered the main producers of OTA in cereals. Battilani *et al.* (2003) found that the most ochratoxigenic strains isolated from grapes belonged to *A. carbonarius*. Also, this species is the most probable source of OTA in dried vine fruits (Abarca *et al.* 2003). In coffee, *A. carbonarius* and *A. ochraceus* are the most potent OTA producers (Bucheli and Taniwaki 2002). The fungi imperfecti are a major group of fungi in soil, the isolation of fungi such as *Penicillium* and *Aspergillus* is common (Nesci *et al.* 2006).

According to Visconti *et al.* (2008) the main source of OTA in the wine food chain is the infection of grapes by *Aspergillus* spp. belonging to *A.* section *Nigri* in the field. Cereals can also be infected with ochratoxigenic fungi in the field (Miller 1995). Post harvest contamination of food by ochratoxigenic fungi and sequential production of OTA will occur, if environmental conditions are favourable. For example, harvesting of cereals with a high content of water or their inefficient drying or storage under humid conditions may result in higher levels of OTA in them (Jrgensen and Jacobsen 2002). Out of cereals, for example rice is an aquatic plant and is usually harvested at high moisture levels (Zinedine *et al*. 2007). If rice crop is infected by ochratoxigenic fungi and if the environmental conditions during storage are favourable, OTA will be produced (Pena *et al.* 2005).

*Penicillium* spp. and *Aspergillus* spp. are typical storage fungi that are able to develop at low water activity (*a*w) (Ramakrishna *et al.* 1996; Waldemarson *et al.* 2005), for example, the minimal water activity for the growth of *P. verrucosum* is about 0.80 (Cairns- Fuller *et al.* 2005). Sweeney and Dobson (1998) reported that significant levels of OTA production by *P.*

*verrucosum* can occur at the *a*w as low as 0.86 and at 4°C. According to Cairns-Fuller *et al*. (2005), the highest production of OTA by this fungus was at 0.93–0.98 *a*w at 10–25°C. *A. ochraceus* produces OTA at minimal water activity of 0.80 (Adebajo *et al.* 1994; Ribeiro *et al.* 2006).

In the investigation by Ramos *et al.* (1998), optimal water activity for OTA production by this fungus was 0.98. Pardo *et al.* (2004) found optimal water activity as 0.99. OTA is produced by *A. ochraceus* at 12°C to 37°C with an optimum at 31°C (Sweeney & Dobson 1998). According to Pardo *et al.* (2004), optimal temperature is 30°C. Varga and Kozakiewic (2006) reported that the climatic and geographic differences influence fungal contamination and OTA contamination of grapes. For example, in Europe an increase in OTA levels occurred in wines coming from southern regions of Europe. *Aspergillus* spp. are opportunistic pathogens and are responsible for several disorders in various plants (Varga *et al.* 2004). In grapes, *Aspergillus* spp. belonging to *A.* section *Nigri* develop particularly in damaged grapes during ripening although they may occur and form OTA on grapes from veraison to harvest (Visconti *et al*. 2008).

OTA concentrations tend to increase with the grape maturity (Rousseau 2004). Berry damage is the primary factor affecting the disease development and OTA accumulation in berries. The damage may be due to birds, insects, infection by other fungi, or rain (Leong *et al.* 2006; Visconti *et al.* 2008). Some grape varieties may display greater susceptibility than others to *Aspergillus* bunch rots. Data obtained in the investigations suggested that varieties had no direct effect on the incidence of black *Aspergillus* spp. on undamaged bunches, however, some varieties were more susceptible to berry splitting and hence would be at a greater risk of *Aspergillus* rots (Leong *et al.* 2006).

The type of agricultural practices involved in the crop production also influences the amount of OTA. In the study of Juan *et al.* (2008), the organic cereal samples showed a highest incidence of contamination. Czerwiecki *et al.* (2002) analysed over 200 samples of Polish cereal grain from the 1997 harvest obtained from conventional and ecological farms. OTA contamination of rye from ecological farms was over six times more frequent than that from conventional cultivation.

The OTA content in wheat and barley samples from ecological farms was also higher. Jørgensen and Jacobsen (2002) also found that a multiyear mean concentration of OTA was higher in organically grown rye than in conventionally grown rice. The concentration of OTA was higher in rye than in wheat with both conventionally and organically grown rye and wheat. In a Danish survey, Elmholt and Rasmussen (2005) analysed organically cultivated grains and found that most of the harvested samples contained *P. verrucosum* prior to drying other factor affecting the mycotoxin production by toxigenic fungus is the presence of other microorganism nonproducing the mycotoxin (Maleř *et al.* 2003).

Human exposure to OTA is worldwide (Guillamont *et al.* 2005; Clark and Snedeker 2006). OTA has been detected in human serum and milk in different countries (Clark and Snedeker 2006). The contaminated foods have been recognised as a possible threat to human health (Jodlbauer *et al.* 2002).

Toxic effects of OTA are various, the most relevant being nephrotoxicity and nephrocarcinogenicity in rodents (Cavin *et al.* 2007). In 1993, the International Agency for Research on Cancer classified OTA as a possible human carcinogen (Valero *et al.* 2006). It has been speculated that OTA may be associated with the human disease Balkan endemic nephropathy (BEN) and the onset of urinary tract tumours. However, in the workshop organised by the European branch of the International Life Sciences

Institutes, it was concluded that there is no convincing evidence from human epidemiology to confirm the association between OTA exposure and the prevalence of BEN or Urinary Tract Tumours. In 2006, the Scientific Panel on Contaminants in the Food Chain of the European Food Safety Authority stated that the epidemiological data on human carcinogenicity are incomplete and did not justify OTA classificationas a human renal carcinogen (Cavin *et al.* 2007).

OTA has also been reported to be immunosuppressive and teratogenic in animals (Bayman and Baker 2006; Bejaoui *et al.* 2006). Because of the detrimental effects of mycotoxins, some strategies have been developed to prevent the growth of mycotoxigenic fungi and also to decontaminate and/or detoxify foods and feeds (Kabak *et al.* 2006). These strategies include: – the prevention of mycotoxin contamination, – the detoxification of mycotoxins present in foods and feeds, – inhibition of mycotoxin absorption in the gastrointestinal tract .

### 2.2.1 : Chemistry of Ochratoxins

Ochratoxins (Ochratoxin A: OTA, Ochratoxin B: OTB and Ochratoxin C: OTC) are toxic metabolites of different fungi; their structure consists of a dihydro-isocoumarin moiety linked with a phenylalanine through an amide bond (Figure 2-3). Furthermore, OTA and OTC contain a parachlorophenol part as well. OTA (C20H18ClNO6; IUPAC name: N-{[(3R)-5-chloro-8-hydroxy-3- methyl-1-oxo-3,4-dihydro-1H-isochromen-7-yl]carbonyl}-L-phenylalanine; molecular weight: 403.8) is a white, odorless, heat stable, crystalline solid agent (melting point: 168–173 ″C) with poor aqueous solubility (Pohland *et al*.,1992; Pohland *et al*.,1982).

OTA does not completely disappear during baking (Vidal *etal*.,2015) furthermore, OTA resists against three hours of high pressure steam sterilization at 121 ″C (Trivedi *et al*., 1992), and even at 250 ″C it is only partially degraded (Boudra *et al*.,1995). Even during coffee roasting OTA is only partially decomposed (Blanc *et al*.,1998; Van der Stegen *et al*.,2001) one of the identified products is 14-(R)-ochratoxin A (about 25%) that shows slightly lower cytotoxicity in vitro (Cramer *et al*., 2008). Due to the structure of OTA, the mycotoxin exhibits strong fluorescence property (Poor et *al*.,2012; Poor *et al*., 2013). Depending on the microenvironment, OTA exists in non-ionic, monoanionic (OTA´), and dianionic (OTA2´) form

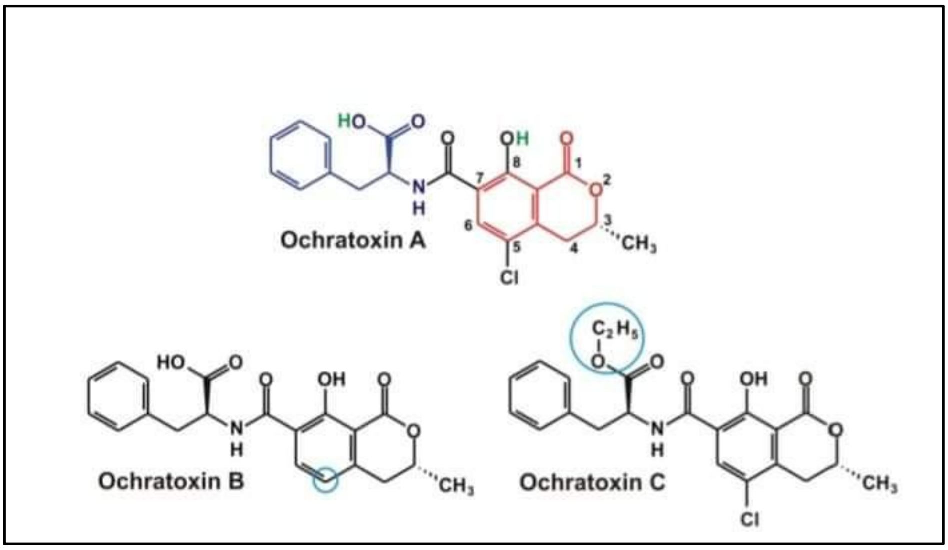


**Figure (2-3) : Chemical structures of Ochratoxin A (Pohland *et al*.,1992)**

### 2.2.2.:Classification of Ochratoxin A Gene Cluster

The adaptation of an organism to environmental conditions requires the cooperation of several genes that contribute to its survival. In a metabolic chain, the product of an enzymatic reaction must be taken very quickly by the following enzyme to ensure a correct speed of formation of the final product and avoid being destroyed by the possible reactions. So an enzymatic assembly in the form of complex or enzymatic cluster (Sidhu 2002; Yu and Keller, 2005) is therefore a necessity. In fungi, several clusters coding for polyketides have been studied. These clusters include clusters for the biosynthesis of mycotoxins (aflatoxins, fumonisins, ergot alkaloid, paxillin), antibiotics (cephalosporin and penicillin), melanins and some pharmaceutical products (lovastatin and compactin).

### 2.2.2.1: *Aspergillus* species

Given that mycotoxin biosynthetic genes are often coordinately regulated (Sidhu 2002; Yu and Keller, 2005) and arranged in clusters (Brown *et al* 1996; Yu *et al*., 2004), and having established that OTA production in permissive and restrictive medium appears to be dependent on *pks* gene transcript levels expression, O'Callaghan *et al*. (2006) attempted to identify other putative OTA biosynthetic genes in *A. ochraceus* by comparing their expression profiles with that of the OTA *pks* gene in permissive and restrictive medium. In their study, they focused on two putative cytochrome P450 monooxygenase genes. Both genes displayed a high degree of similarity to other monooxygenase genes encoding enzymes involved in the biosynthesis of polyketide secondary metabolites. Specifically, the p450-H11 gene is very similar to the averantin oxidoreductase gene involved in aflatoxin biosynthesis (Yu *et al* ., 1997), while the p450-B03 gene is not only similar to the trichodiene oxygenase gene from the trichothecene biosynthetic pathway in *Gibberella zeae* (Ward *et al* ., 2006) but also displays significant similarity to the *cyp* A gene in the aflatoxin biosynthetic pathway from *A. flavus* (Ehrlich *et al*., 2004).

A strong correlation between the amount of *pks* and cytochrome P450 transcripts present and the amount of OTA accumulation was observed by the authors indicating a possible role for these genes in OTA biosynthesis. Additionally, O'Callaghan *et* al., 2006 reported on the cloning of the full length pks gene and on the cloning part of a novel nonribosomal peptide synthetase (*nrps*) gene 900 bp upstream of the pks gene. Both the *pks* and *nrps*genes appear to be transcribed in the same direction but they are not in the same reading frame. Thus they appear to encode two separate proteins rather than a single hybrid type PKS-NRPS.

Alignment study revealed that the *pks* gene characterized by O'Callaghan *et al.* shares more than 98% identity with a PKS gene *awlc35-12* (or *aolc35-12*), identified in *A. westerdijkiae* (Atoui *et al*.,2006; Dao *et al*.,2005). This finding shows that *A. ochraceus* and *A. westerdijkiae* contain a similar gene. The *pks* gene also displayed similarities of 60% to a hypothetical PKS gene corresponding to the OTA cluster in *A. niger* (Pel *et al*.,2007) and only 38% to a *awks1* gene required for the biosynthesis OTA in *A. westerdijkiae* (Bacha *et al*.,2009).

Based on the high degree of similarities between the *awlc35-12* and *pks* genes, Bacha *et al.* (2009) assumed that *awlc35-12* could be involved in OTA biosynthesis in *A. westerdijkiae*. Given that *awks1* is different from *pks*, they concluded that two PKS may be involved in the biosynthesis of OTA in *A. westerdijkiae*. To date, only four cases have been reported that involve two different fungal PKSs essential for a single polyketide: a set of two unusual type I multifunctional PKSs for the biosyntheses of lovastatin and compactin in *A. terreus* and *P. citrinum*, respectively, ( Hendrickson *et al*.,1999; Kennedy *et al*.,1999; Abe *et al*.,2002) and two PKS have been reported to be involved in the biosynthesis of zearaleone in *G. zea* (Kim *et al*.,2005) and T toxin in *C. heterosphorus* (Baker *et al*.,2006).

In *A. carbonarius*, a cDNA-AFLP differential technique was performed by Botton *et al.* (2008) in two strains of *A. carbonarius*, antagonists to the ability of producing OTA, allowing the identification of 119 differentially expressed sequences putatively involved in the regulation of OTA biosynthesis. The differential conditions were achieved by growth on different minimal media, either supporting or inhibiting OTA production. A connection was pointed out between the biosynthesis of the toxin, vegetative growth and sexual/asexual developmental progression. A putative model for OTA biosynthesis regulation has been elaborated by the authors (Botton *et al*.,2008).

### 2.2.2.2: *Penicillium* species

Recently, most of the gene cluster responsible for the biosynthesis of OTA in *Penicillium* has been characterized. A 10 kb genomic DNA fragment of *P. nordicum* has been cloned (Geisen *et al*.,2004 . Karolewiez and Geisen,2005, Farber and Geisen, 2004, Karolewiez and Geisen,2005], which carries three long open reading frames. One open reading frame (*otapksPN*) is a polyketide synthase, which is different from PKS genes found in *Aspergillus* species. The second open reading frame (*npsPN*)has homology to non-ribosomal peptide synthetases, and the third open reading frame (*aspPN*) has homology to fungal alkaline serine proteinases. In a systematic analysis, the influence of the most important growth parameters including temperature, water activity and pH were analyzed by Real Time PCR and microarrays. Interestingly ,all analyzed external parameters resulted in a similar expression profile of the OTA biosynthesis genes. Interestingly the *otapksPN* gene is present only in *P. nordicum* but not in *P. verrucosum*, indicating genetic differences exist also between both OTA producing *Penicillium* species (Geisen *et al*.,2004; Farber and Geisen, 2004, Karolewiez and Geisen,2005).

### 2.2.3: Detection of Ochratoxigenic Species by PCR

Early and rapid detection of potential OTA producing fungi is important to reduce the negative impacts of OTA. Usual identification and quantification methods of food-borne fungi require multiple steps. Morphological and physiological tests were time-consuming and often, mycological expertise was necessary (Farber and Geisen, 2004; Farber *et al*.,1997).

The PCR (Saiki *et al*.,1988) has replaced laborious and time consuming microbiological analysis by amplification of specific genomic markers rather than growing the living organism under study. Within the last 10 years PCR systems have been developed for the detection and differentiation of major species and groups of mycotoxigenic fungi. One of the most important factors in the set up of such method is the reliability of the primer set designed and the targeted DNA sequence of interest organism (Saiki *et al*.,1988). Recently, various pairs of PCR primers were developed to set up novel diagnostic approaches for OTA producers in the *Aspergillus* and *Penicillium* genera.

#### 2.2.3.1: Gene-based approaches for the diagnosis of ochratoxigenic fungi

Several genes such as rRNA, β-tubulin, elongation factor 1 α and the calmodulin genes provide highly conserved as well as variable sequence regions. Based on the alignment of a partial sequence of calmodulin genes from representative strains of *A. niger* group, *A. carbonarius*, *A. japonicus* and *A. aculeatus*, Perrone *et al*. (2004) detected a high degree of homology between strains assigned to *A. carbonarius* (99.98%) and also between strains of *A. japonicus* and *A. aculeatus* (99.40%).

Based on variable regions, the authors found three different regions suitable for designing species-specific PCR primers. The assay was useful in screening vast numbers of isolates of black aspergilli from grapes in order to evaluate the toxigenic potential connected with this commodity. Recently, Susca *et al.* (2007) developed species-specific primers based on partial calmodulin gene sequences to identify *A. carbonarius* and *A. niger* by PCR. Moreover, a primer pair targeting the conserved regions of the *A. carbonarius* calmodulin gene has also been developed by Mulè *et al.* (2006), allowing only the amplification of *A. carbonarius* strains.

Morello *et al.* (2007) found genetic variation between the β-tubulin gene sequences from *A. ochraceus* and *A. westerdijkiae* allowed to design a primer-pair specific to *A. westerdijkiae*. Using these primers, no PCR product from DNA of *A. ochraceus* was obtained while all isolates of *A. westerdijkiae* gave positive results.

The coding portions of many fungal 18S, 5.8S and 28S rDNA genes are highly conserved and primers to these regions have been generated (White *et al*.,1990). The internal transcribed spacer sequences (ITS-1 and ITS-2) and IGS (Martínez-Culebras and Ramón,2007) contains hypervariable regions leading to most of the intraspecies sequence diversity ( Kappe *et al*.,1996; Zhou *et al*.,2000; Vanittanakom *et al*.,2002; Hinukson *et al*.,2005).

The ITS region is amplified from the target fungus and sequenced to identify regions of DNA unique to the fungus of interest. Polymorphism within the ITS region is generally (but not always) at the level of species, rather than between isolates of the same species, making it an ideal target for the development of species-specific PCR assays. For example the species-specific primers have been designed on the basis of ITS sequence to discriminate the main species included in section *Nigri:* A. *japonicus*, *A. heteromorphus*, *A. ellipticus* and the two morphologically indistinguishable species of the *A. niger* aggregate: *A. niger* and *A. tubingensis* (Gonzalez-

Salgado *et al.*,2005). Other species-specific PCR-based assays to ochratoxigenic species were developed for *A. carbonarius* and *A. ochraceus* based on ITS (Patino *et al.*,2005).

Restriction digestion analysis of the ITS products was tested to assess its effectiveness as a rapid method to identify different isolates of black *Aspergillus* species from grapes (Martínez-Culebras and Ramón,2007).

Among the PCR based approaches, Accensi *et al.* (1999) used a PCR-RFLP technique to distinguish *A. niger* and *A. tubingensis* isolates. The authors used the restriction enzyme *RsaI* to digest the amplified ITS region of the isolates, and observed that isolates of the *A. niger* species complex exhibit two different RFLP patterns, N and T corresponding to *A. niger* and *A. tubingensis* isolates, respectively. Other restriction enzymes has also been used for species identification recently using PCR-RFLP analysis of the ITS region (Martínez-Culebras and Ramón,2007). The authors used *HhaI*, *NlaIII* and *RsaI* to distinguish between *A. niger*, *A. tubingensis*, *A. carbonarius* and *A. aculeatus* isolates came from grapes. Zanzotto *et al.* (2006) used PCR-RFLP analysis of the ITS, IGS and β-tubulin genes to distinguish between OTA-producing and non-producing isolates of the *A. niger* aggregate

#### 2.2.3.2: Using mycotoxin biosynthetic gene for identifying of ochratoxigenic fungi

In some instances, species specific detection is not always of primary objective. It may be more relevant to determine whether the producers of particular mycotoxins are present rather than to identify exactly what the species are. This is particularly important where a mycotoxin can be produced by a number of species. Many mycotoxin biosynthetic genes are present within gene clusters, and some of these appear to have undergone horizontal transfer from one species to another and are now present in several species (Sidhu,2002; Niessen,2007).

Regions of homology within mycotoxin biosynthetic gene from the different species can be used to develop primers to detect the presence of the relevant mycotoxigenic species. This strategy was successfully applied for aflatoxin producers (Shapira *et al*.,1996; Geisen, 1996), trichothecene-producing fungi (Niessen andVogel, 1997;Paterson,2006; Bluhm *et al*.,2002), fumonisin-producing *Fusarium* species (Paterson ,2006; Bluhm, 2002; Gonzalez-Jaen *et al*.,2004) and also for producers of patulin (Paterson,2006; Paterson,2006).

In *A. ochraceus*, the sequence of AoLC35-12 was found to join a sequence encoding for acyl transferase domain of a polyketide synthase (Atoui et al.,2006; Dao *et al*.,2005), which is involved in the OTA biosynthesis pathway ( Claydon *et al*.,1979). Dao *et al*. (2005) designed two sets of specific primers, AoLC35-12L/AoLC35-12R and AoOTAL/AoOTAR, from AoLC35-12. The set primers AoOTAL/AoOTAR specifically detected *A. ochraceus* by PCR method. Because *A. ochraceus* and *A. westerdijkiae* were only recently dismembered into two species, the primer-pairs designed until now are not specific either to *A. ochraceus* or to *A. westerdijkiae*, *i.e.*, they recognize both species. The second primer pair AoLC35-12L/AoLC35-12R was found to detect OTA producing fungi *A. carbonarius, A. melleus, A. ochraceus, A. sulfureus, P. verrucosum* and citrinin producing *P. citrinum* and *Monascus ruber*. In *A. carbonarius*, five different KS domain sequences (AcKS9, AcKS10, Ac12RL3, AcLC35-4 and AcLC35-6) were identified by Atoui *et al*. (2006).

Two different PCR species specific detection systems were developed based on primers designed from AcKS10 (Selma *et al*.,2007) and Ac12RL3

(Atoui *et al*.,2007) proved to be highly specific, yielding amplification only in *A. carbonarius* strains, the main OTA producer in grapes. In the genus *Penicillium*, a PCR method for differentiation and detection of two ochratoxingenic *Penicillium* species, *P. nordicum* and *P. verrucosum*, has been developed. It is based upon two genes of the OTA biosynthetic pathway, namely the OTA polyketide synthase gene (*otapksPN*) and a non-ribosomal peptide syntethase gene (*otanpsPN*) from *P. nordicum* (Geisen *et al*.,2004; Farber and Geisen, 2004).

*P. verrucosum* gives consistently only a positive reaction with the primers for the otanpsPN gene, whereas *P. nordicum* is positive for both genes. The PCR reaction is negative with all of other food related fungal species tested. This system has been used to analyze 62 *Penicillium* strains isolated from cured meat products or ripening rooms.

## 2.4: Secondary Metabolities

Plants are autotrophic organisms. In addition to the primary metabolism present in all living beings, they have secondary metabolism that allows them to produce and accumulate compounds of a very diverse chemical nature. The compounds derived from secondary metabolism in plants are called secondary metabolites (SM) (Tiwari and Rana,2015) .

The SM of the plants constitute a large and varied group of organic compounds that are synthesized in small quantities; they have no direct function in essential processes such as photosynthesis, respiration, solute transport, protein synthesis, nutrient assimilation, and the differentiation or formation of carbohydrates, proteins, and lipids. They appear in plants as a result of chemical conversions and even when many of their functions are unknown, it is believed that SM are related to the defense of the plant against predators and pathogens, they also act as allelopathic agents that influence growth, survival, and reproduction of other plants, attract seed pollinators and serve to face adaptation to sudden changes in temperature, humidity, light intensity and drought(Berini *et al*.,2015).

The SM of the plants have a differential distribution between taxonomic groups in the Kingdom of the plants, and therefore they are useful for Systematic Botany(Anuj *et al*.,2019) . The study of biological functions and the structure of SM are of great importance because from this knowledge, it has been possible to use them in different industries. Many SM are used as aromas, resins, gums, flavor enhancers, as insecticides and herbicides(Zillich *et al*., 2015). On the other hand, the majority of SM have found utility in the pharmaceutical industry, given a large number of pharmacological activities that are known about them (Raskin *et al*.,2002).

The main classes of SM in plants, some techniques for their extraction from natural sources and phytochemical screening, as well as the main pharmacological activities described for fundamental classes of SM. Classes of SM in plants Several criteria have been considered for the classification of SM: chemical structure (presence of rings or sugars), composition (containing nitrogen or not), their solubility in organic solvents or water, and the biosynthetic pathway. Of them, the most common criterion used for grouping the SM in plants has been the biosynthetic pathway. According to this, the SM in plants can be divided into three large groups: terpenes, phenolic compounds, and alkaloids(Ávalos García and Pérez-Urria Carril,2009) .

**2.4.1: Terpenes:** they constitute the largest group of SM in plants to which more than 40,000 different molecules are allocated (Ávalos García and Pérez-Urria Carril,2009). From the chemical point of view, they are nonsaponifiable lipids since fatty acids do not intervene in their formation.

They are also known as isoprenoids, since the basic structural unit that forms them is the isoprene molecule (Vranová *et al*.,2012).

They are classified according to the number of isoprene units they contain. The most straightforward class of all is hemiterpenes with a single isoprene unit and five carbons in its structure. The best-known hemiterpene is isoprene, a volatile product that emerges from photosynthetically active tissues. With two groups, the terpenes are classified in monoterpenes, with three units in sesquiterpenes, with four in diterpenes, with six in triterpenes, with eight in tetraterpenes, and with more than 10 in polyterpenes(Taiz and Zeiger,2010).

Many plants contain terpenes in their flowers and fruits as mixtures of volatile compounds with specific odors; among them, we can mention lemon, mint, eucalyptus, ginger, and great basil (Tiago *et al*.,2017). Terpenes have several biological functions and participate in both the primary metabolism and the secondary metabolism of plants. In the central metabolism they are photosynthetic pigments (carotenes), electron carriers (ubiquinone and plastiquinone) regulators of plant growth and development (giberilins, strigolactones, brassinosteroids), are part of cell membranes (phytosterols) and participate in protein glycosylation (Loreto *et al*.,2014).

In secondary metabolism they participate as defense molecules, toxic compounds and food deterrents for insects. In some plants they are the responsible molecules for attracting pollinators, or they function as dispersers (Veitch *et al*.,2008)they are synthesized from primary metabolites by two pathways: that of mevalonic acid, active in the cytosol, in which three molecules of acetyl-CoA condense to form mevalonic acid that reacts to form isopentenyl diphosphate (IPP) or the pathway of methyleryritol phosphate

(MEP) that functions in chloroplasts and also generates IPP (Tiago *et al*.,2017).

**2.4.2: Phenolic compounds:** they are chemical compounds containing a hydroxyl group directly attached to an aromatic hydrocarbon. Chemically, phenolic compounds are a very diverse group of SM. The simplest representative of this class is phenol (Velderrain-Rodríguez *et al*.,2014). The most important criterion for classifying in the molecule. According to this criterion, the phenolic compounds are classified into simple phenols, acidic phenols, acetophenones, and phenylacetic acids, hydroxycinnamic acids, coumarins, flavonoids, biflavonyls, benzophenones, xhantones, stilbenes, quinones and betacyanins .Lignans, neolignans, tannins, and phlobaphenes also belong to this group. The latter are polymers and have more complex structures ( Dai and Russell,2010).

Phenolic compounds are synthesized in plant cells by the shikimic acid pathway or the malonate/acetate pathway (or both, for example, flavonoids) (Beart *et al*.,1985) . The shikimic acid pathway provides the synthesis of phenylalanine and cinnamic acids and their derivatives (simple phenols, phenolic acids, coumarins, lignans, and phenyl propane derivative (Vattem *et al*.,2005) . The polyacetate pathway provides quinones and xanthones. The mixed pathways combine precursors of both the shikimic acid pathway and the polyacetate pathway. This is the case of flavonoid (Cheynier *et al*.,2013) .

Phenolic compounds fulfill various functions in plants: they oxidize quickly and act as antioxidants (Surco-Laos *et al*.,2016) , they act as plant growth inhibitors, seeds accumulate significant amounts of phenols that act as filter so that oxygen does not reach the embryo and inhibit its germination ( Weidner *et al*.,2014) . Phenols also accumulate on surfaces of leaves, capturing up to 90% of UV radiation46. Phenols confer aromas and colors to the fruits making them appetizing for herbivores, which favors the dispersion of seeds through feces( Valenta *et al*.,2013) (. Plants compete with each other to preserve their territories, and in this process (allelopathy) the phenols participate (John and Sarada, 2012) . Plants also defend themselves against the attack of pathogens by synthesizing phytoalexins that are toxic to microorganisms and their presence prevents infections ( Ahuja *et al*.,2012). Phenols also protect plants by generating bitter flavors or textures that are unpleasant for herbivores (Marsh *et al*.,2019)

**2.4.3: Alkaloids:** alkaloids constitute another large and diverse group of SM that includes molecules isolated primarily from vascular plants. Plants generally produce a complex mixture of alkaloids, in which a significant constituent dominates. In a given plant the biosynthetic origin of the alkaloids present is common, even if their structures are slightly different (Roberts and Wink, 1983). Another interesting observation is that the concentration of alkaloids varies considerably from one part to another of the same plant, and even in some parts it may not contain those at all (Ng and Or,2015)

Alkaloids are also found in fungi, bacteria, and animals (Bérdy ,2005). They include an atom of nitrogen in their structure, are toxic compounds and respond to common precipitation reactions (Matsuura and Fett-Neto ,2017). Even when there is no uniform classification of alkaloids, several criteria have been used in order to classify them: biosynthetic origin, presence of basic heterocyclic nucleus in the structure, pharmacological properties, and distribution in plant families (Kumar, 2014). Among these criteria, the biosynthetic origin of the alkaloids has been used quite frequently. According to this criterion the alkaloids are classified as true alkaloids, protoalkaloids, and pseudoalkaloids( Aniszewski, 2007).

Pure alkaloids strictly comply with the fundamental characteristics of the alkaloids. The majority of the alkaloids found in plants belong to this group. They contain an intracyclic nitrogen, have basic character and are compounds of high reactivity, even in small quantities. In plants, they can be found free, although they predominate as salts. The precursor compounds of the true alkaloids are amino acids (L-ornithine, L-lysine, L-tyrosine, L-tryptophan, L-histidine, and L-arginine). Some pure alkaloids have been derived from anthranilic and nicotinic acids (Pelletier, 1983). The protoalcaloides constitute a smaller class in number. In this group, the nitrogen atom is not part of the heterocycle, and they derive from L-thyroid, L-tryptophan, and L-ornithine. They can also be considered aromatic amines (Babbar, 2015).

The pseudoalkaloids contain heterocyclic rings with nitrogen but are not derived from amino acids. They are formed by subsequent incorporation of nitrogen into compounds originally free of this element. To this group belong terpenic alkaloids(Pelletier, 1983). Although the presence of alkaloids is not vital for the plant, there is evidence that indicates the roles that these substances play in vegetables. As for the functions they fulfill, at first, they were considered waste products of nitrogen metabolism, nitrogen reservoirs in the plant, and were even mentioned as growth regulators. Today it is accepted that the role they play is to defend the plant against insects and herbivores due to its toxicity and deterrent capacity. While some serve to protect the plant from predators or microorganisms (toxic or repellent substances), others do so to compete with other plant species in a given habitat (allelopathic substances) (Azimova and Marat, 2013).

Alkaloids have remarkable physiological properties and toxicological that are exerted primarily on the nervous system central, with predominance in some of its levels . For these reasons, they can be used as drugs. Prolonged use of any of these compounds produced in man accustoming, which constitute true drug addictions, with physical and psychic dependence and an increase in the tolerance ( Aniszewski, 2007). To date, around 15,000 alkaloids have been isolated from plants. If it is considered to have been examined less than 25% of the upper plant species of the planet, it is clear that there is still a wide field for his research. Because of its pharmacological and medicinal importance there is an excellent motivation to continue with the chemical-biological study of the alkaloids. This is one of the most important secondary metabolites of plants with therapeutic interest (Azimova and Marat, 2013).

### 2.5: *Olea europaea L*

The olive tree (*Olea europaea L.*) is cultivated in many parts of the world, but the Mediterranean region is the main area of agricultural production, it represents approximately 98% of the growing around the world. Apart from the Mediterranean region, the olive tree is cultivated on a large scale in the Arabian Peninsula, India and Asia .Most of the plant parts of *Olea europaea* are used in traditional system of medicine in world. Oil is taken with lemon juice to treat gallstones (Sheth,2005). Leaves are taken orally for stomach and intestinal diseases and used as mouth cleanser (Bellakhdar *et al*.,1991).

Decoctions of the dried fruit and of dried leaf are taken orally for diarrhoea and to treat respiratory and urinary tract infections (Razzack, 1980). Hot water extract of the fresh leaves is taken orally to treat hypertension and to induce diuresis (De la Ribeiro, 1986). Seed oil is taken orally as a cholagogue, to remove gall stones, in nephritis associated with the lead intoxication. To prevent hair loss, oil is applied every night on the scalp then shampooed the next morning (Zargari, 1992).

Seed oil is taken orally as a laxative and applied externally as an emollient and pectoral (Al-Khalil, 1995). Decoction of dried leaves is taken orally for diabetes (Alarcorn-Aguilara *et al.*,1998). Tincture of leaves is taken orally as a febrifuge (Gastaldo, 1974). Fruit is applied externally to fractured limb (Ghazanfar and Al-Sabahi, 1993). Fruit is used externally as a skin cleanser (Fujita *et al.*,1995). Hot water extract of dried plant is taken orally for bronchial asthma (Vardanian, 1978). Infusion of the fresh leaf is taken orally for as an anti-inflammatory (Pieroni *et al*.,1996).

Epidemiological data obtained from clinical studies have consistently demonstrated that the Mediterranean diet, rich in olive oil, fruits, vegetables, and grains is correlated with a lower than average risk of coronary heart disease (Kushi *et al*.,1995). The natural antioxidants, including oleuropein, from the olive tree may play a role in the prevention of cardiovascular diseases through a decreased formation of atherosclerotic plaques by inhibiting LDL oxidation (Visoli *et al*.,1994). An olive leaf extract was reported in a laboratory study to have vasodilating effects, seemingly independent of vascular endothelial integrity (Zarzuelo *et al*.,1993).

Traditional uses support olive leaf and olive oil in cardiovascular disease prevention (Giugliano, 2000). Animal experiments in rabbit and rat preparations found a hypotensive effect of oleuropein, possibly via direct action on smooth muscle. Oleuropeoside also may exert vasodilator activity. Additionally, olive leaf extracts may possess antispasmodic, vasodilator, and anti-arrhythmic properties (Khayyal *et al*.,2002). Anti-viral activities Olive leaf extract has reported antiviral activity, reportedly caused by the constituent calcium elenolate, a derivative of elenolic acid (Heinze *et al*.,1975).

The isolated calcium salt of elenolic acid was tested as a broad-spectrum antiviral agent active against all viruses tested (40). Some viruses inhibited by calcium elenolate in vitro include rhinovirus, myxoviruses, Herpes simplex type I, Herpes simplex type II, Herpes zoster, Encephalomyocarditis, Polio 1, 2, and 3, two strains of leukemia virus, many strains of influenza and para-influenza viruses (Renis, 1975).

### 2.6: *Capparis spinosa L.*

Due to the global trend of expanding the use of plant raw materials in the manufacture of pharmaceutical preparations instead of chemicals with harmful side effects, the use of those raw materials with appropriate active substances and the appropriate dose has become more beneficial and safer in the treatment of many diseases (Ghamdi *et al.*,2017) The *Capparis Spinosa* is a perennial shrub whose height ranges between (30-80) cm, and most of it lies on the ground, unless there is something attached to it, it can grow high, the evergreen plant has a bluish green color, the branches are creeping or its outgrowth, easy to break, the leaves are thick with ears Thorny, large flowers bloom in the morning in white color with a tendency to pink and wither before noon, giving a beautiful red color (Al-Asady *et al*.,2012)

The fruit is fleshy in shape resembling a pear and is carried on a long neck, and when the fruit ripens it turns from yellowish green to crimson bright and its taste is sweet from the inside and bitter from Outside (Aliyazicioglu *et el.*,2013) *C. spinosa* plants are one of the richest plants with active substances. The plant contains flavonoids, terpenoids and alkaloids. (Alsabri *et al*.,2012) It also contains resins, glucosinolates, coumarins, saponins, tannins, sterols, in addition to organic acids and fatty acids (Al-Smail *et al*.,2020) Relieve stomach pain if properly prepared with vinegar .( Al-Snafi, *et al*,2015) .

The roots are used as a diuretic, which is astringent, appetizing, menstrual, tonic, and repellent to intestinal worms. It is also used for treating infections, and foreskin is used to treat rheumatism in the joints. (Ashkan, *et al*.,2020) As for the flower buds, they are used as an organ regulator, diuretic and tonic, and for the treatment of scurvy and specialists in alternative medicine believe that *C. spinosa* is at the forefront of medicinal plants that should be taken care of by those who are keen to recover from rheumatic diseases, high blood sugar, and people with bloating and liver disorders (Benzidane *et al*.,2010).

As the study aimed to detect the chemical content (Phytochemical Screening) of *C. Spinosa* for overland growth in Iraq and to diagnose some classes of chemical compounds present in it for the purpose of evaluating it from the chemical point of view, extracting phenols for quantitative evaluation of flowers and fruits and studying the inhibitory effect of them against some pathological bacteria, And its effect on some blood parameters such as blood clotting outside and inside the organism.

# 3. Materials and Methods

## 3.1: Study Supplies

### 3.1.1: Equipments:

The following equipments and tools which were used in this study listed in Table **(3-1):**

**Table (3-1): Equipments and tools used in the laboratory experiments.**

| No. | Equipment and Tools | Company | Country |
|---|---|---|---|
| 1 | Autoclave | Gallenkamp | England |
| 2 | Camera | Nikon | Japan |
| 3 | Capillary tube | Superestar | India |
| 4 | Cell Disrupter Genie vortex | Scientific Industries | USA |
| 5 | Compound light microscope | Olympus | Japan |
| 6 | Distiller | Fisons | Germany |
| 7 | Electric oven | Memmert | England |
| 8 | Electrophoresis apparatus | Shandon | Canada |
| 9 | Eppendorf tubes | Biobasic | England |
| 10 | Filter paper | Whatman 4 | England |
| 11 | Gel Electrophresis | Shandon,scientific | Germany |
| 12 | High speed centerfuge | Hettich | Germany |
| 13 | High speed cold centerfuge | Eppendorf | England |
| 14 | Hot plate | Gallenkamp | Japan |
| 15 | High Performance Liquid Chromatography | National | Germany |
| 16 | Incubator | Memmert | China |
| 17 | Micropipette 0.5-10µL, 10-100 µL, 100-1000 µL | CYAN | USA |
| 18 | MiniOpticon Real Time PCR | BioRad | USA |
| 19 | PCR thermocycler T100 | BioRad | China |
| 20 | Petri dishes | Sailbran | Holand |
| 21 | PH- meter | Philips | USA |
| 22 | PowerPac HC Electrophoresis Power Supply | BioRad | Germany |
| 23 | Rotary evaporator | Memmert | England |
| 24 | Sensitive electronic balance | Gallenkamp | India |
| 25 | Slides and cover slides | Superestar | India |
| 26 | Test tube | Superstar | England |
| 27 | Thin Layer Chromatography | Unisonics | USA |

| | | | |
|---|---|---|---|
| 28 | Tube Rack Double Panel Microcentrifuge PCR Centrifuge Tube Holder | P-ABC | Korea |
| 29 | Ultra violet | Bioneer | USA |
| 30 | Vortex mixer | Tallboys | Japan |
| 31 | Warring blender | National | Germany |
| 32 | Water bath | Tafesa | Germany |
| 33 | Water distillatory | Gallenkamp | England |

### 3.1.2: Biological and chemicals Materials

The biological and chemicals which were used in this study listed in Table (3-2).

**Table (3-2):. Biological and Chemicals Materials**

| No. | Chemicals | Company | Country |
|---|---|---|---|
| 1 | Absolute ethanol | Fluka | Switzerlan |
| 2 | Acetic acid | BDH | England |
| 3 | Agarose Gel | Biobasic | Canada |
| 4 | Chloroform | BDH | England |
| 5 | Copper sulphate | Scharlau | Spain |
| 6 | DEPC water | Bioneer | Korea |
| 7 | Dimethyl silphoxide (DMSO) | BDH | England |
| 8 | DNA Marker ladder 1500-100bp | iNtRON | Korea |
| 9 | DNA Marker ladder 2000-100bp | Bioneer | Korea |
| 10 | Ethanol alcohol | BDH | England |
| 11 | Ethidium bromide | Biobasic | Canada |
| 12 | Ferric chloride | Scharlau | Spain |
| 13 | Formalin | BDH | England |
| 14 | Free nuclease water | BioLabs | UK |
| 15 | HCL | BDH | England |
| 16 | Isopropanol | BDHCHEN-LAB | Belgium |
| 17 | KCL | Scharlau | Spain |
| 18 | Ladder | Bioneer | Korea |
| 19 | Mercuric chloride | BDH | England |
| 20 | Methanol alcohol | BDH | England |
| 21 | NaCL | BDH | England |
| 22 | NaOH | BDH | England |
| 23 | Picric acid | Himedia | India |

| | | | |
|---|---|---|---|
| 24 | Potassium hydroxide | BDH | England |
| 25 | Potassium tartarate | Scharlau | Spain |
| 26 | Protinase K enzyme | Bioneer | Korea |
| 27 | Sodium acetate | CDH | India |
| 28 | Stander ochratoxin A | Promega | America |
| 29 | sulfuric acid | Scharlau | Spain |
| 30 | TBE buffer | Biobasic | Canada |

### 3.1.3: Agar Culture Media used in the Study

**Table (3-3): Culture media used in the Laboratory Experiments.**

| No | Media | Company | Uses |
|---|---|---|---|
| 1 | Brain Heart Infusion Broth (BHI Broth) | Hi Media laboratories Limited (India) | used for the purpose of molecular diagnosing of *A.niger* and *A.ochraceus.* |
| 2 | Czapek Dox Agar | | used for the purpose of isolating and diagnosing *Aspergillus sp.* |
| 3 | Potato's Dextrose Agar (PDA) | | used for the purpose of isolating and diagnosing fungi. |
| 4 | Sabouraud Dextrose Agar medium (SDA) | | used for the purpose of isolating and diagnosing fungi. |
| 5 | Sabouraud Dextrose Broth | | used for the purpose of molecular diagnosing (RT-pcr) of *A.niger* and *A.ochraceus* |

### 3.1.4: The Plants used in the Study

**Table (3-4): The Plants used in the Laboratory Experiments.**

| No | Local name | Scientific name | Family | Used part |
|---|---|---|---|---|
| 1 | Wheat | *Triticum aestivum* | Poaceae | Grains |
| 2 | Maize | *Zea mays* | Poaceae | Grains |
| 3 | Olive | *Olea europaea L* | Oleaceae | Leaves |
| 4 | Caper | *Capparis spinosa* | Capparidaceae | Flowers |

## 3-2: Methods

### 3.2.1: Samples Collection

The Grains (Wheat and Maize) were collected from Silo of Babylon and AL Diwaniyah Governorate and Babylon Markets, at 3kg per sample. The samples were placed in sterile paper bags and labeled and brought to the laboratory.

### 3.2.2:Agars culture Media used in the Study:-

#### 3.2.2.1: Potato dextrose agar Medium (PDA)

Medium was prepared according to the manufacturer's instructions, by dissolving 39 grams of the agar in 1 liter of distilled water , sterilized by autoclave a temperature of 121°c for 20 minutes and then cooled to 45°c , after that adding chloramphenicol in the concentration of 250 mg /l used the agar for the purpose of isolating and diagnosis of fungi.

#### 3.2.2.2: Sabouraud Dextrose Agar Medium (SDA)

Medium was prepared according to the manufacturer's instructions, by dissolving 65gm from Sabourauds Dextrose Agar in 1 liter of distilled water , sterilized by autoclave a temperature of 121 °c for 20 minutes and then cooled to 45°c ,after that adding chloramphenicol in the concentration of 250 mg /l used the agar for the purpose of isolating and diagnosis of fungi.

### 3.2.2.3: Czapek Dox Agar

Medium was prepared according to the manufacturer's instructions, by suspend 49gram in 1 liter of distilled water. Heat to boiling to dissolve the medium completely. sterilized by autoclave a temperature of 121 °c for 20 minutes and then cooled to 45°c ,after that adding chloramphenicol in the concentration of 250 mg /l used the agar for the purpose of isolating and diagnosing *Aspergillus* sp.

### 3.2.2.4: Sabouraud Dextrose Broth (Sabouraud Liquid Medium)

Medium was prepared according to the manufacturer's instructions, By dissolving 30gm from Sabourauds Dextrose Broth in 1 liter of distilled water , sterilized by autoclave a temperature of 121 °c for 20 minutes and then cooled to 45°c ,after that adding chloramphenicol in the the concentration of 250 mg /l used the agar for the purpose of isolating and molecular diagnosis of fungi

### 3.2.2.5: Brain Heart Infusion Broth (BHI Broth)

Medium was prepared according to the manufacturer's instructions, by Suspend 37 grams of the medium in one liter of distilled water. Mix well and dissolve by heating with frequent agitation. Boil for one minute until complete dissolution. sterilized by autoclave a temperature of 121 °c for 20 minutes and then, cooled to 45°c ,after that adding chloramphenicol in the concentration of 250 mg /l used the agar for the purpose of isolating and molecular diagnosis of fungi

### 3.2.3: Fungi Isolation from Grains

The Fungi associated with Wheat and Maize grains used in this research were isolated by surface sterilization of the grains using a solution of sodium hypochlorate at a concentration of 1 % for a 2-5 minutes and then washed with sterile distilled water three times. The seeds was culture in Petri dishes contained PDA medium with 10 grains in each dish and in three Repeated and dishes were placed in the incubator at 25˚temperture for a period of 7 days. Then the plates were examined for developing fungi and the percentage of fungi frequency and appearance was calculated as follows:

$$\textbf{1- Percentage of frequency\%} = \frac{\text{Number of isolated fungus}}{\text{Total number of isolated fungi}} \times 100$$

$$\textbf{2-Percentage of appearance \%} = \frac{\text{Number of samples per fungus}}{\text{Total number of samples}} \times 100$$

### 3.2.4: Diagnosis of Fungi

#### 3.2.4.1: Diagnosis by Using Classification Keys

The Fungi isolated from Wheat and Maize were diagnosed to the species types depending on the Morphological features of colony, such as the shape, color and diameter of the colony, as well as on the Microscopic characteristics such as the shape , size, color and composition of the stands, spores and other structures according to the classification principles mentioned in the sources (McClenny, 2005).

### 3.2.4.2: Diagnosis by Using PCR

#### 3.2.4.2.1: Primers:

PCR and Real Time PCR primers were designed in this study using NCBI-Genbank database and primer 3 plus online. These primers were provided by Macrogen company from Korea as following table (3-5):

**Table (3-5): PCR and Real Time PCR primers**

| Genes name | Sequences | bp |
|---|---|---|
| ***Aspergillus ochraceus* rRNA gene** | **TCCCACCCGTGTATACCGTA** | **420bp** |
| | **CCTACAAGAGCGGGTGACAA** | |
| **ochratoxin A biosynthetic gene** | **AACTCGCCACGCAGAAAAAG** | **139bp** |
| | **TCGGACGCCTGTTTGATTTC** | |
| **Actin housekeeping gene** | **TTGCAAACGCGTGTCACTAC** | **144bp** |
| | **AACACGAGGTCGAACACTTG** | |

*A. ochraceus* genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene, isolate *A. niger* and *A.ochraceus* culture CBS:513.88 ochratoxin A biosynthetic gene cluster, complete sequence *A. niger* CBS 513.88 actin, mRNA

### 3.2.4.2.2: Molecular study Kits

### 3.2.4.2.3: PCR detection Kits

**Table (3-6):** The PCR detection Kits used in this study with their company and country of origin:

| No. | Kit | Company and Country |
|---|---|---|
| **1** | **G-spin Total DNA extraction kit** | **iNtRON (Korea)** |
| | CL buffer lysis buffer | |
| | BL buffer binding buffer | |
| | Proteinase K | |
| | WA buffer | |
| | WB buffer | |
| | Elution buffer | |
| | GD column | |
| | Collection tube 2ml | |
| **2** | **Maxime PCR PreMix kit** | |
| | Taq DNA polymerase | |
| | dNTPs (dATP, dCTP, dGTP, dTTP) | |
| | Tris-HCl pH 9.0, KCl, & $MgCl_2$ | |
| | Stabilizer and loading dye | |

## 3.2.4.2.4: Methods

### 3.2.4.2.4.1: Fungal DNA extraction

Fungal genomic DNA was extracted from *A. ochraceus* isolates by using (**G-Spin DNA extraction kit with modification**) as and done according to company instructions as following steps:

**1-Sample Preparation :**

a- 1 ml cultured fungal cells was transferred to 1.5 ml microcentrifuge tube then centrifuged at 10000 rpm for 1 minute then the supernatant was discarded.

**2-Cell Lysis steps :**

a- 180µl GT buffer and 250mg glass bead were added to the tube and the cell pellet suspended by vortex, then 20 µl of Proteinase K was added and the mixtures were incubated at 60ºC for 30 minutes. During incubation periods the mixtures tubes were inverted every 3 minutes.

b- 200µl GB buffer were added to each tube and mixed by vortex for 10 seconds. Then the tubes incubated at 60ºC for 10 minutes with inverted the tubes every 3 minutes through incubation periods.

**3-DNA binding steps :**

a- 200µl absolute ethanol were added and immediately mixed by vortex, then precipitates if happen was broken by pipetting.

b- A GD column was placed in a 2 ml collection tube and all mixtures were transferred (including any precipitate) to the GD column. Then centrifuged at 10000 rpm for 1 minute. The 2 ml collection tubes that contains the flow-through were discarded and placed the GD column in a new 2 ml collection tube.

**4-Washing steps :**

a- 400µl W1 buffer were added to the GD column, then centrifuged at 10000rpm for 1 minute. The flow-through was discarded and placed the GD column back in the 2 ml collection tube.

b-600µl Wash Buffer were added to the GD column. Then centrifuged at 10000 rpm for 1 minute. The flow-through was discarded and placed the GD column back in the 2 ml collection tube. and the tubes were centrifuged again for 2 minutes at 12000 rpm to dry the column matrix.

**5-Elution steps :**

a- The dried GD column was transferred to a clean 1.5ml microcentrifuge tube and 100µl of pre-heated elution buffer were added to the center of the column matrix.

b- The tubes were let stand for at least 3 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 1 minutes to eluted the purified DNA.

### 3.2.4.2.2: Estimation of extracted total DNA:

The extracted total DNA was checked by using Nanodrop (**Thermo Scientific NanoDrop Lite UV Visible Spectrophotometer**. **USA**) that measured DNA concentration (ng/µL) and checked the RNA purity at absorbance (260 /280 nm) as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).

2. A dry wipe was taken and cleaned the measurement pedestals several times. Then carefully pipetted 2µl of free nuclease water and placed onto the surface of the lower measurement pedestals for blank the system.

3. The Nanodrop sampling arm was lowered and 1µl DNA sample measured.

### 3.2.4.2.3: PCR Master mix Preparation

PCR master mix reactions for all genes was prepared by using (Maxime PCR PreMix kit) and this master mix done according to company instructions as following in table (3-7):

**Table (3-7):** Standard PCR master mix protocol:

| PCR Master mix | Volume |
|---|---|
| DNA template 5-50ng | 5μL |
| Forward primer (10pmol) | 2μL |
| Reveres primer (10pmol) | 2μL |
| PCR water | 13μL |
| Total volume | 20 μL |

### 3.2.4.2.4: PCR Thermocycler Conditions

PCR thermocycler conditions protocol for each gene was calculated by using **Optimase ProtocolWriter™** online application and done by using convention PCR thermocycler following table (3-8):

**Table (3-8): PCR thermocycler conditions protocol:**

| No. | Gene name | Initial denaturation Temp./time | Denaturation Temp./time | Annealing Temp./time | Extension Temp./time | Cycle | Final extension Temp./time | Hold Temp./time |
|---|---|---|---|---|---|---|---|---|
| | 18SrRNA gene ITS2 | 95°C/ 4 min | 95°C/ 30 sec | 59°C/ 30 sec. | 72°C/ 60sec. | 32 | 72°C/ 5min | 4°C/forever |

### 3.2.4.2.5: PCR product analysis

The PCR products were analyzed by agarose gel electrophoresis method as in the following steps:

1- 1.5% Agarose gel was prepared in using 0.5X TBE and dissolving in microwave for 5 minutes, and left to cool for 50°C.

2- Then 3µl ethidium bromide stains were added into agarose gel solution.

3- Agarose gel solution was poured in tray after fixed the comb in proper position and left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray.

4- The gel tray was fixed in electrophoresis chamber and filled by 0.5X TBE buffer.

5- 10µl PCR products were loaded in to each well with added 5µl (DNA marker Ladder) in first well. Then electric current was performed at 100 volt and 80 AM for 1hour.

5- PCR products were visualized by using UV Transilluminator.

### 3.2.5: Test The Ability of *A.niger* and *A. ochraceus* Isolated to Produce Ochratoxin A

#### 3.2.5.1: Culture of *A.niger* and *A. ochraceus* Isolated

The fungi isolated were purified on a sterile PDA media, the discs from the isolate were cultured with a diameter of 5mm and with 3 replicates per isolate, and these discs were placed in the center of the dishes, after which they were incubated at 30˚c for 7 days.

#### 3.2.5.2: Ochratoxin A Extraction

The Ochratoxin A was extracted from *A.nige*r and *A. ochraceus* according to Macdonald *et al*.,(1999), where three dishes were taken from the growth fungi for a week at a temperature of 30˚c and cut the grown medium of fungi in each plate and put these pieces in an electric blender and add 100 ml of a mixture consisting of water and chloroform in a ratio of 1:1, after which the mixture was filtered by a 4 layers of muslin cloth and then transfer the filtrate to a separating funnel and shake well. This extract was evaporated to dryness at 45˚C and the dried extract was stored at 4 ˚c .

#### 3.2.5.3: Dertermination of Ochratoxin A in *A.niger* and *A. ochraceus*

##### 3.2.5.3.1:Thin Layer Chromatography (TLC)

The TLC technique was used to detect the ability of *A.niger* and *A.ochraceus* isolated from wheat and maize grain to produce their ochratoxin A. ( 9:1) methanol : distilled water separation system used as mobile phase. A light straight line was made on the TLC plate 1.5 cm away from the base of the lower plate was applied by a capillary tube on the line, the spots were left to dry, then the TLC plates were placed in separation basin containing a mixture of methanol and distilled water 9:1 respectively, and monitored until the mobile phase reached a distance of 1.5 cm from the upper end of the plate. The plate was left to dry and then examined under ultraviolet ray with a wavelength of 365nm. The revealed the presence of the toxin by matching the location and color of the fluorescent of the extract . (Sobolev and Dorner, 2002). Rf was calculated by:

$$\mathbf{R_f = \frac{Distance\ of\ Sample\ (cm)}{Distance\ of\ Solvent\ \ (cm)} \times 100}$$

### 3.2.5.3.2: High Performance Liquid Chromatography (HPLC)

Ochratoxin A was extracted from samples (5ml or 5gm) by homogenization with 20 mL acetonitrile : $H_2O$ (6:4, v=v) for 2 minutes. The extract was filtered and 4 mL of filtrate was diluted with 25mL phosphate buffer saline pH 7.4 (PBS). The samples were degassed in a sonic bath for 30 minutes, than the pH was adjusted to 7.2 using 2 M sodium hydroxide 5 mL of acetonitrile is added to the sample and then stored until the analysis is performed. The mobile phase was an isocratic acetonitrile : D.W: formic acid (50: 47: 3) at a flow rate 1.2 ml/min, column was C18-ODS( 25cm *4.6 mn) and the detector Florescent (Ex = 365 nm, Em= 445 nm) (Skarkova *et al*.,2013).

### 3.2.6: Quantitative Reverse Transcription Real-Time PCR in *A.niger* and *A.ochraceus.*

The quantitative Real-Time PCR technique was performed for quantification detection and relative gene expression analysis of ochratoxin A genes and normalized by housekeeping (actin) gene in *A. niger* and *A.ochraceus* isolates. The method was carried out according to( Dao *et al*.,2005) and included the following steps:

### 3.2.6.1: Real Time PCR detection Kits

**Table (3-9): The Real Time PCR expression Kits used in this study with their companies and countries of origin:**

| No. | Kit | Company | Country |
|---|---|---|---|
| **1** | **easy-BLUE™ Total RNA Extraction Kit** | **iNtRON** | **Korea** |
| | Trizol reagent 100ml | | |
| **2** | **WizBio RT Prmix kit Hexammer** | **Wizbio** | **Korea** |
| | RocketScript Reverse Transcriptase (200U) | | |
| | 5X reaction buffer | | |
| | dNTP 250µM | | |
| | DTT 0.25mM | | |
| | RNase Inhibitor (1U) | | |
| **3** | **Go Taq®Qpcr Master miz** | **Promega** | **USA** |
| | 2X qPCR mix (1ml) | | |

### 3.2.6.2: Total RNA extraction

Total RNA were extracted from *A. niger and A.ochraceus* isolates by using (easy-BLUE™ Total RNA Extraction Kit) and done according to company instructions as following steps:

1- Fungal isolates were inoculated on Sabouraud dextrose broth and incubated at 37ºC into reach fungal cells (OD600:0.8-1.0), the fungal cells were harvested by centrifuge at13000rpm for1min then, supernatant removed.

2- The fungal pellets were suspended by added 1ml easy-blue (Trizol reagent) and vigorously vortex in room temperature for 10 sec.

3- 200µl chloroform was added to each tube and shaken vigorously for 1 min.

4- The mixture was incubated on ice for 5 minutes. Then centrifuged at 13000 rpm, 4C°, for 15 minutes.

5- Supernatant was transferred into a new 1.5ml microcentrifuge tube, and 500µl isopropanol was added. Then, mixture mixed by inverting the tube 4-5 times and incubated at 4C° for 10 minutes. Then, centrifuged at 13000 rpm , 4C° for 10 minutes.

6- Supernatant was discarded, and 1ml 80% Ethanol was added and mixed by vortex again. Then, centrifuge at 13000 rpm, 4C° for 5 minutes.

7- The supernatant was discarded and the RNA pellet was left to air to dry.

8- 100µl Free nuclease water was added to each sample to dissolve the RNA pellet, Then, the extracted RNA sample was kept at -80.

### 3.2.6.3: Estimation of extracted total RNA:

The extracted total RNA was checked by using Nanodrop (**Thermo Scientific NanoDrop Lite UV Visible Spectrophotometer**. **USA**) that measured RNA concentration (ng/µL) and checked the RNA purity at absorbance (260 -280 nm) as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, RNA).
2. A dry wipe was taken and cleaned the measurement pedestals several times. Then carefully pipetted 2µl of free nuclease water and placed onto the surface of the lower measurement pedestals for blank the system.
3. The Nanodrop sampling arm was lowered and 1µl RNA sample measured.

### 3.2.6.4: DNase I treatment

The extracted RNA were treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using samples (DNase I enzyme kit) and done according to method described by Promega company, USA instructions as follow:

**Table (3-10): DNase I enzyme kit**

| Mix | Volume |
|---|---|
| Total RNA 1µg | 10µL |
| DNase I enzyme | 1µL |
| 10X buffer | 4µL |
| DEPC water | 5µL |
| Total | 10µL |

After that, The mixture was incubated at 37C° for 30 minutes. Then, 1µl stop reaction was added and incubated at 65C° for 10 minutes for inactivation of DNase enzyme action.

### 3.2.6.5: cDNA synthesis

The DNase treated total extracted RNA samples were used in cDNA synthesis step from mRNA transcripts by using **(**AccuPower® RocketScript$^{TM}$ RT PreMix) and this kit was done according to company instructions as following in the table (3-11):

**Table (3-11): cDNA synthesis kit**

| RT mix | Volume |
|---|---|
| Total RNA 100µg | 10µL |
| Random Hexamer primer (50pmol) | 1µL |
| DEPC water | 9µL |
| Total | 10ul |

After that, these RT mix components that mentioned in table above placed in AccuPower® RocketScript$^{TM}$ RT PreMix kit strip tubes that containing all other components which needed to cDNA synthesis such as (Reverse Transcriptase, 5 x Reaction Buffer, DTT, dNTP, and RNase Inhibitor). Then, all the strip tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes, and then incubated in Thermocycler (BioRad-USA) as following thermocycler conditions protocol:.

**Table (3-12): thermocycler conditions protocol**

| Step | Temperature | Time |
|---|---|---|
| cDNA synthesis (RT step) | 42 °C | 1 hour |
| Heat inactivation | 95 °C | 5 minutes |

### 3.2.6.6: Real-Time PCR (qPCR) master mix preparation

qPCR master mix was prepared by using **(RealMODTM Green SF 2X qPCR mix Kit)** based on SYBER green dye amplification in Real-Time PCR system and the qPCR master mix was prepared as following:

**qPCR master mix for target genes and housekeeping gene:**

` qPCR master mix for target genes and housekeeping gene was prepared as following in the table (3-13):

**Table (3-13): qPCR standard master mix protocol**

| qPCR master mix | volume |
|---|---|
| cDNA template (10ng) | 5µL |
| Forward primer(10pmol) | 1 µL |
| Reverse primer (10pmol) | 1 µL |
| qPCR Master Mix | 10 µL |
| Nuclease free water | 3 µL |
| Total | 20 µL |

After that, these qPCR master mix component that mentioned above placed in qPCR white plate strip tubes and mixed by Exispin vortex and centrifuge for 5 minutes, then placed in MiniOpticon Real-Time PCR system.

### 3.2.6.7: qPCR Thermocycler conditions

qPCR Thermocycler conditions was done according to qPCR kit instruction and used by using **Optimase ProtocolWriter™** online for primers annealing calculation as following in the table (3-14):

**Table (3-14): qPCR Thermocycler conditions:**

| qPCR step | Temperature | Time | Repeat cycle |
|---|---|---|---|
| Initial Denaturation | 95 °C | 10min | 1 |
| Denaturation | 95 °C | 20 sec | 40 |
| Annealing\Extension Detection(scan) | 58 °C | 30 sec | |
| Melting | 65-95°C | | 1 |

### 3.2.6.8: Data analysis of qPCR

The data results of qPCR for target and housekeeping gene were collected and the expression analysis (fold change) used analyzed by using (Livak method ) that described by (Livak and Schmittgen, 2001) as following equations:

$\Delta C_T$ (Test) = CT (target gene, test) – CT (HKG gene, test)

$\Delta C_T$ (Control) = CT (target gene, control) – CT (HKG gene, control)

$\Delta\Delta C_T = \Delta C_T$ (Test)– $\Delta C_T$ (Control)

Fold change (target / HKG) = $2^{-C_T} \Delta\Delta C_T$

### 3.2.7: preparation the plant material

*O.europaea L*. leaves and *C.spinosa* flowers were collected from Babylon city, after cleaning leaves and flowers, and then washed two times with running tap water and once with distilled water and dried under shade for ten days at room temperature. Each dried part was grinded and stored in airtight container to avoid the humidity effect and then stored at room temperature until further use.

### 3.2.8: Qualitative Secondary Metabolites Tests :

### 3.2.8.1: Preparation of Extracts:-

#### 3.2.8.1.1: Aqueous Extract:-

Ten g from each plant (*O. europaea L.* leaves and *C.spinosa* flowers) (air-dried powder) was taken and put in a conical flask (500 ml), and 250 ml of distilled water was added , and boiled on slow heat for 2h, then filtered through 8 layers of muslin cloth and centrifuged at 5000 rpm for 10 min. The supernatant was collected (Parekh and Chanda, 2008). The extract was concentrated in an oven at 45$^{o}$c until dryness. Dried extracts were stored at 4$^{o}$c for further use (Jain and Singh, 2013).

#### 3.2.8.1.2: Alcoholic Extract:-

Methanol solvent was used (Harborne, 1984). 10 gm of the powdered plant (*O. europaea L.* leaves and *C.spinosa* flowers) were soaked in the conical flask (250 ml) containing 200 ml of solvent, plugged with cotton, and then put in a horizontal shaker at 140-220 rpm for 24 h. Then it was filtered through 8 layers of muslin cloth and centrifuged at 5000 rpm for 10 min, the supernatant was collected (Parekh and Chanda, 2008). The extracts was concentrated in an oven at 45$^{o}$c until dryness. Dried extracts was stored at 4$^{o}$c for further use (Jain and Singh, 2013).

#### 3.2.8.1.3: Terpenes extract Preparation:

10 g of powder dry matter of the plant was extracted with a solvent, (200 ml of chloroform) in the oven for 24 hours at 40$^{o}$c , the sample concentrated in a rotary evaporator, then terpenes dried in the oven at 40-45$^{o}$c and stored in the refrigerator until use (Harborne, 1984).

### 3.2.8.2: Test for Alkaloids:

#### A- Mayer's Reagent

This reagent prepared by dissolving 13.5 gm from Mercuric chloride and 5 gm from KI in 1000 ml distilled water , add 1-2 ml from reagent to 5 ml from extract. A white or creamy precipitate was indicated the test as positive (Harborne , 1984).

#### B- Dragendorff's Reagent

Twenty g from Bismuth Nitrate in 40 ml distilled water and 16 g from sodium Iodide in 40 ml distilled water, mixed together and added 1-2 ml from this reagent to 5 ml from the extract, a prominent orange color was indicated the test as positive (Qaisar *et al.*,2009).

#### C- Tannic acid Reagent

Two ml from 10% Tannic acid solution was added to 5 ml from extract, Alkaloids give orange color precipitate (Neelima *et al.*, 2011).

#### D- Hager's Test:

Hager‘s reagent is saturated solution of Picric acid ($C_6H_3N_3O_7$), after added a few drops from this this reagent appear yellow color precipitate, that's positive indicator to the presence of alkaloids (Neelima *et al.*, 2011).

### 3.2.8.3: Test for Phenols:

Lead acetate test .The extract (50 mg) was dissolved in 5 mL of distilled water. To this, 3ml of 10% lead acetate were added. A bulky white precipitate indicated the presence of phenol compounds (Tamilselvi *et al.*, 2012).

### 3.2.8.4: Test for Saponins:

Mixed one ml of extract with one ml of distilled water, then shaked vigorously, a stable foam indicate the presence of saponin (Anyasor *et al*., 2010).

### 3.2.8.5: Test for Tannins:

**A- Ferric chloride**

A portion of extract was dissolved in water, then filtrate it, Ferric chloride ($FeCl_3$) (1%) was added. The appearance of bluish green color indicates the presence of tannins (Anyasor *et al*., 2010).

**B- Gelatin test**:

To a 1 % gelatin solution, add little 10 % sodium chloride. If a 1 % solution of tannin is added to the gelatin solution, tannins cause precipitation of gelatin from solution ( Shah and Yadav, 2015).

### 3.2.8.6: Test for Flavonoids:

Melted 10 gm of plant powder in 50 ml of ethanol (95%) and then nominated by A. 10 ml of ethanol (50%), added to 10 ml of potassium hydroxide (50%) and nominated by B. Mix equal amount of solution A and B, the appearance color yellow as evidence of a flavonoids (Jaffer *et al*., 1983).

### 3.2.8.7: Test for Glycosides

Using Fehling's test to determinate the presence of glycosides by mixed equal volume of Fehling's A (Copper sulphate in distilled water) and Fehling's B (Potassium tartarate and sodium hydroxide in distilled water) , few drops of this reagent was added to 5 ml of the extract and boiled, red precipitate was formed, if sugars are present (Neelima *et al*., 2011).

### 3.2.8.8: Test for Terpenes

Dissolve 1 ml of plant extract in 2 ml chloroform, added to the solution a drop of anhydrous acetic acid and a drop of concentrated sulfuric acid, the appearance of brown color recorded as a positive indicator to the presence of terpenes (Bargah, 2015).

### 3.2.9: Minimum Inhibitory Concentration (MIC)

Method that used to determine the minimum inhibitory concentration is agar dilution plate method with modified (Wiegand *et al*.,2008). Blending 2 ml from each concentration (5, 10, 15, 20) % with 18 ml of potato dextrose agar (PDA) which solvent and cooled to 50 ˚c, in addition to control plate that not contain extract, added 0.2 ml , left plates 30 min then incubated at 30˚c for 7 days.

### 3.2.10: Statistical Analysis

Data were analyzed statistically using SPSS program through an ANOVA. Means were compared by LSD test under level of probability (0.05) to determine the significant difference between the different treatment assayed.

# 4: Results and Discussion:

## 4.1: Isolation and Diagnosis fungi in wheat and maize grains :

In total, 490 fungal isolates were obtained from wheat and maize grains samples in Babylon and Al-Qadisiayh provence Silo and Babylon Markets, based on morphological cultural characteristics as colony color, margins, texture, and colony reverse colors, several different species were identified, belonged to four different genera, namely: *Rhizopus* sp. *A.fumigatus*, *A.parasiticus A,flavus A.terreus A.niger*, *Alernaria alternate*, *Fusarium sp.*, *Mucor sp.*, *Penicillium spp.* ,*A.ochraceus* , *Negrosporum sp*. Wheat and maize grains samples and the names of the isolates obtained were displayed in Table (14-5) Figure (4-4).

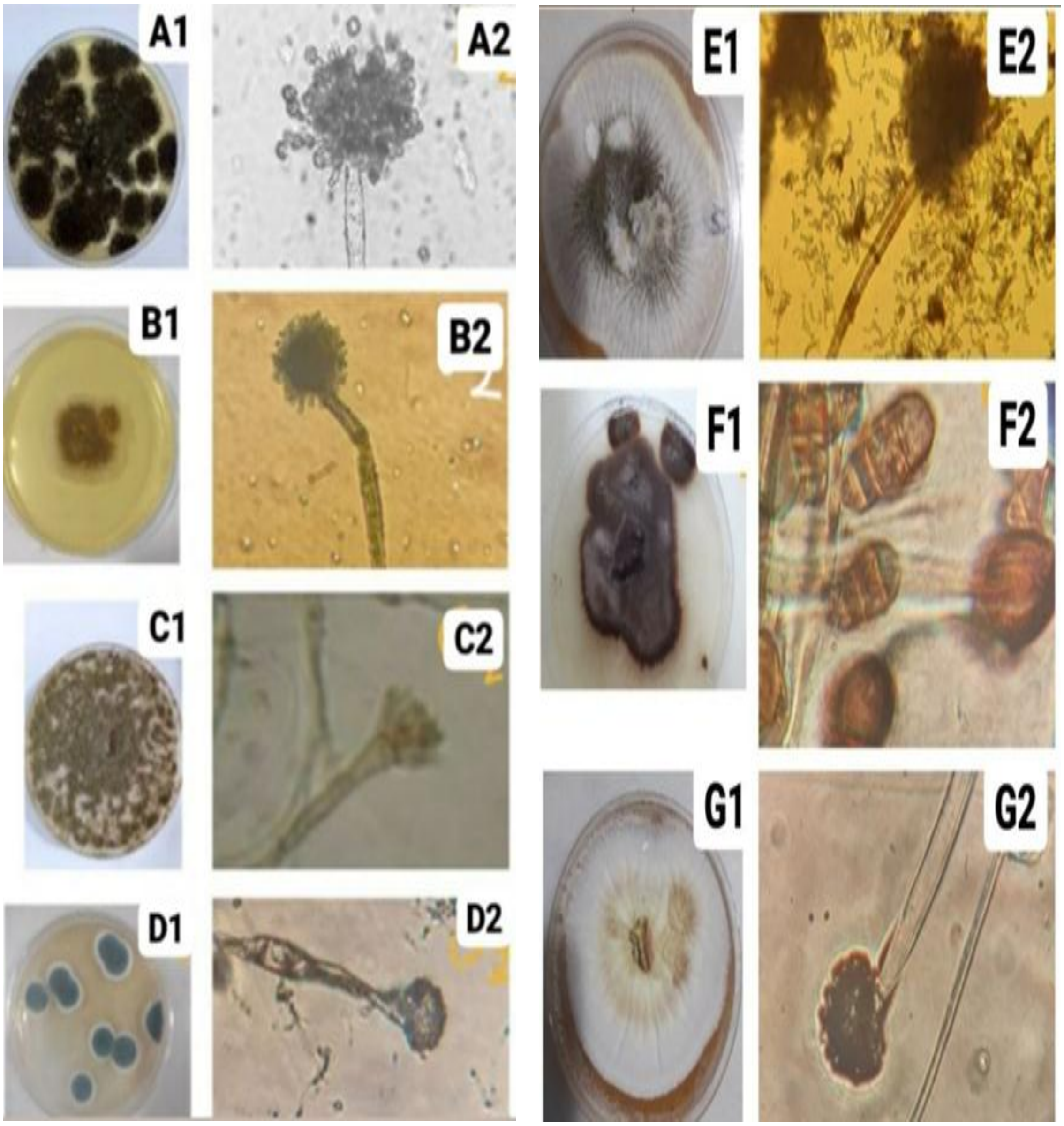


**Figure 4-4: Photographs of the colonies of *A. niger* (A), *A. ochraceus* (B), *A. flavus*(C), *A. fumigatus* (D), *Aspergillus spp*. (E), *Alternaria alternate* (F), *A. terreus* (G), *Aspergillus spp*.**

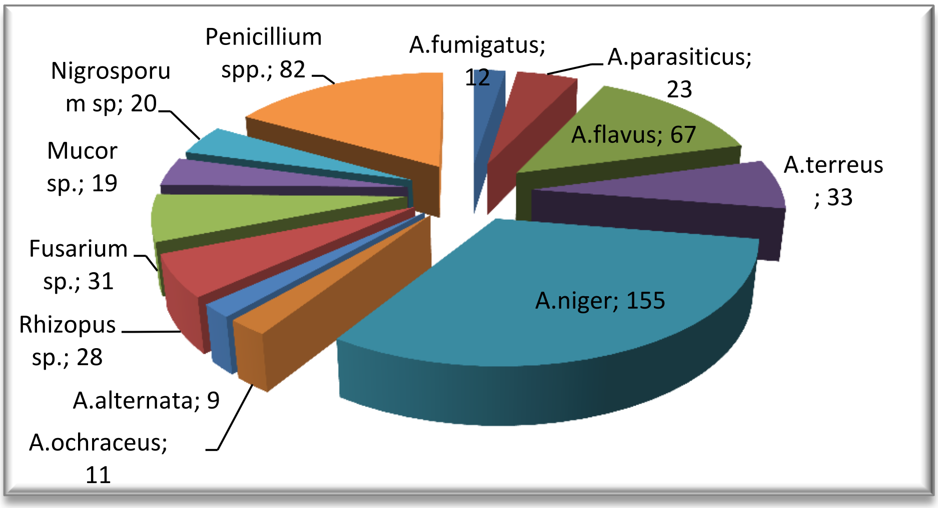


**Figure 4-5: Number of fungi species isolated from wheat and maize grains**

**Table (4-15): Number of fungi species isolated from wheat and maize grains**

| **Fungi species** | **Number of isolates** |
|---|---|
| ***A.flavus*** | **67** |
| ***A.fumigatus*** | **12** |
| ***A.nigr*** | **155** |
| ***A.ochraceus*** | **11** |
| ***A.parasiticus*** | **23** |
| ***A.terreus*** | **33** |
| ***A.alternata*** | **9** |
| ***Rhizopus sp.*** | **28** |
| ***Fusarium sp.*** | **31** |
| ***Mucor sp.*** | **19** |
| ***Nigrosporum sp*** | **20** |
| ***Penicillium spp.*** | **82** |
| **Total** | **490** |

## Table 4-16: frequency and appearance ratios of fungi in Wheat grains

| Fungus | Babylon silo | | Al-Qadisiayh silo | | Babylon Markets | |
|---|---|---|---|---|---|---|
| | Frequency % | Appearance % | frequency % | appearance % | Frequency % | Appearance % |
| ***A.flavus*** | 19.35 | 10 | 0.22 | 50 | 11.35 | 62.5 |
| ***A.fumigatus*** | _ | _ | 1.22 | 38.93 | 0.32 | 12.5 |
| ***A.niger*** | 16.12 | 120 | 25 | 100 | 49.04 | 87.5 |
| ***A.parasiticus*** | 12.90 | 40 | 4.76 | 22.22 | 3.22 | 37.5 |
| ***A.terreus*** | 2.22 | 201 | _ | _ | 5.99 | 75 |
| ***Al.alternata*** | 12.90 | 10 | 0.70 | 33 | _ | _ |
| ***Rhizopus sp.*** | 22.58 | 40 | 0.53 | 33 | _ | _ |
| ***Fusarium sp.*** | 1.67 | 20 | _ | _ | 1.01 | 37.5 |
| ***Mucor sp.*** | _ | _ | 0.24 | 26.23 | 0.13 | 25 |
| ***Penicillium sp.*** | 3.22 | 70 | 13.99 | 20.21 | 11.11 | 50 |

## Table 4-17: frequency and appearance ratios of fungi in maize grains

| Fungus | Babylon silo | | Al-Qadisiayh silo | | Babylon Markets | |
|---|---|---|---|---|---|---|
| | % Frequency | Appearance % | Frequency % | % Appearance | % Frequency | % Appearance |
| ***A.flavus*** | **16.12** | **70** | **30.30** | **71** | **19.01** | **55.32** |
| ***A.terreus*** | **12.90** | **20** | **22.09** | **42.85** | **42.02** | **33** |
| ***A.niger*** | **22.58** | **20** | **20.34** | **27.21** | **11.56** | **66** |
| ***A.parasiticus*** | **19.35** | **10** | _ | _ | **0.33** | **21** |
| ***A.ochraceus*** | _ | _ | **4.22** | **9.09** | **2.38** | **11** |
| ***Rhizopus sp.*** | _ | _ | **7.88** | **9.09** | **1.76** | **11** |
| ***Fusarium sp.*** | **9.67** | **10** | _ | _ | _ | _ |
| ***Negrosporum sp.*** | _ | _ | **0.21** | **3.11** | **20.30** | **22** |
| ***Mucor sp.*** | **12.90** | **20** | **2.77** | **3.11** | **18.33** | **33** |
| ***Penicillium sp.*** | **3.22** | **50** | **11.11** | **18.18** | **32.05** | **45** |

Table 4-15 show the highest number of species founded in *A.niger* about 155 isolates while *A.flavus* record about 67 isolates, *Penicillium spp*. Record about 82 isolates and *A.ochraceus* record only 11 isolates from maize grains only. The results in Tables 4-16, 4-17 indicate that the *A.niger* and *A.flavus* recorded the highest frequency and appearance in wheat and maize grains, compared with some other fungi. Where the *A.niger* recorded the highest frequency in wheat grains and A.*flavus* it had the highest frequency in maize grains. Table 4-18, 4-19 shows *A.niger* frequency in Babylon silo was16.78±0.73 and Al-Qadisiayh silo 28.24±8.14 and Bayblon markets0.28±0.15 in wheat grains, while in maize grains record 25.27±3.04, 21.05±1.02, 11.29±9.42, respectively . Several studies have shown that mycotoxin producing fungi, especially *Aspergillus sp*. Are major pollutants in wheat and maize grains, and the present study was agree with many studies, in on study indicated that *A.niger* found in wheat, barley and maize (Jedidi et al.,2017). Riba *et al*.,(2008), Riba *et al*.,(2010) find the presence of different Aspergillus species in freshly harvested cereals grains. In Eygpt El Shanshoury *et al*.,(2014) found that *A.niger, A.flavus, Penicillium spp. And Fusarium spp*.in samples of cereal grains(wheat and maize). Also Abe *et al*., (2015) recorded *Fusarium* and *Aspergillus* as the highest diversity in maize grains in Brazil. There are other studies, in Tunisian one study found *Alternaria* sp. Was dominance in wheat grains (Belkacem-Hanfi *et al*.,2013). Castella and Cabanes,( 2014) found that *F.equistei* has been commonly detected in barley and wheat cultivated in Mediterranean countries. And *F.verticillioides* and *F.nygamaiare* being the first mostly present in maize (Waalwijke *et al*.,2008). Maryam and Abubakr, (2017) isolated species of *Penicillium sp*. from the grains. And *Penicillium sp*. Were the most frequent fungi isolated from wheat (Jedidi *et al*., 2018).

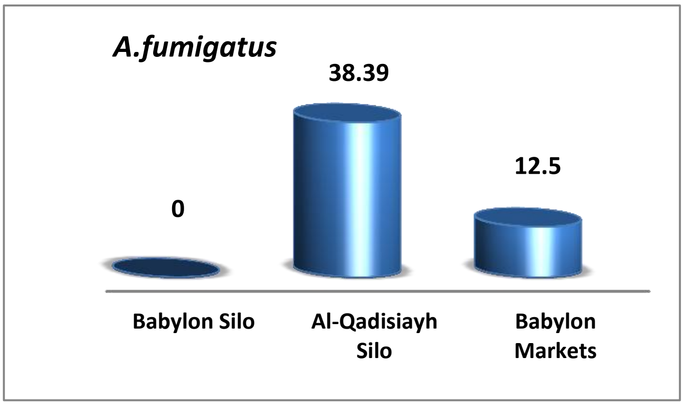


**Figure (4-6): Percentage of appearance of *A.fumigatus* isolated from wheat grains**

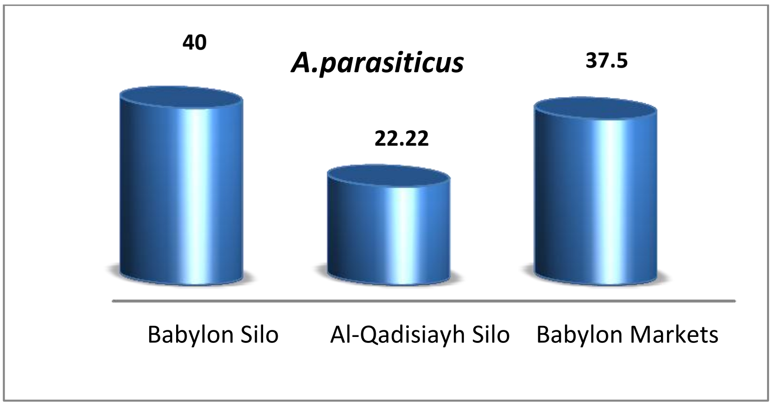


**Figure (4-7): Percentage of appearance of *A.parasiticus* isolated from wheat grains**

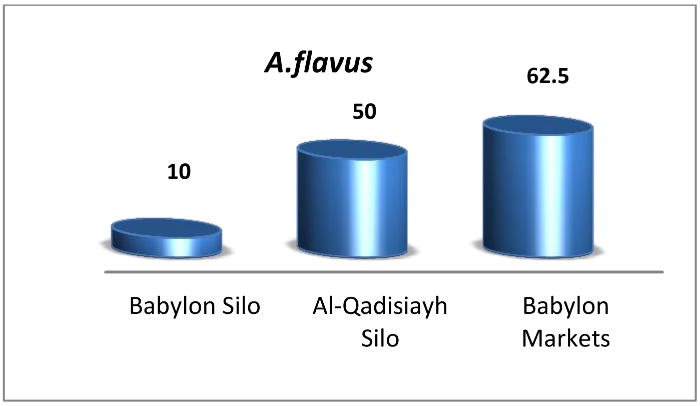


**Figure (4-8): Percentage of appearance of *A.flavus* isolated from wheat grains**

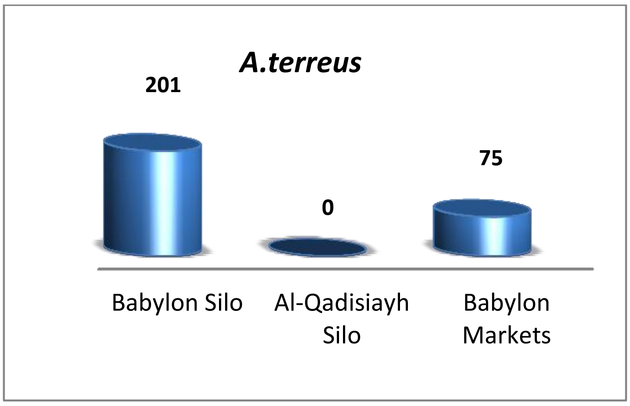


**Figure (4-9): Percentage of appearance of *A.terreus* isolated from wheat grains**

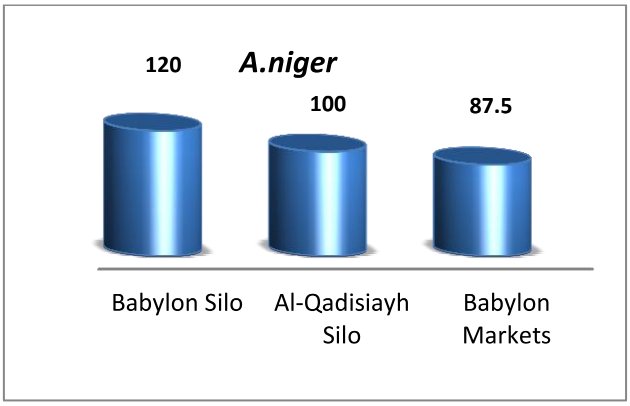


**Figure (4-10): Percentage of appearance of *A.niger* isolated from wheat grains**

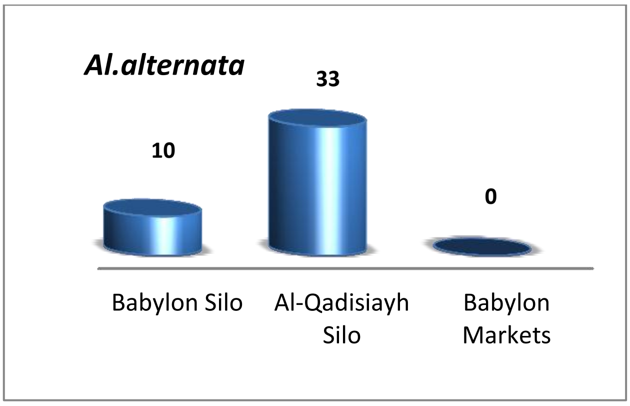


**Figure (4-11): Percentage of appearance of *Al.alternata* isolated from wheat grains**

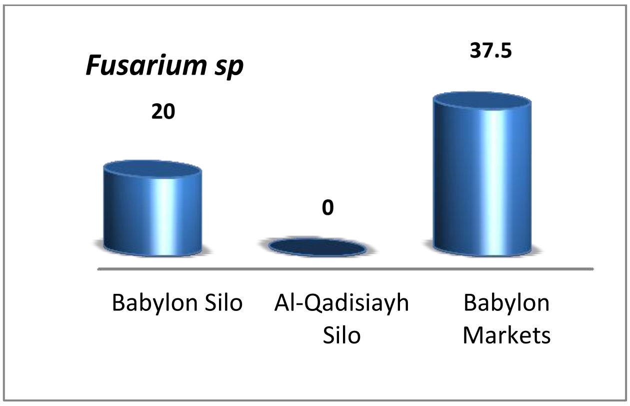


**Figure (4-12): Percentage of appearance of *Fusarium sp.* isolated from wheat grains**

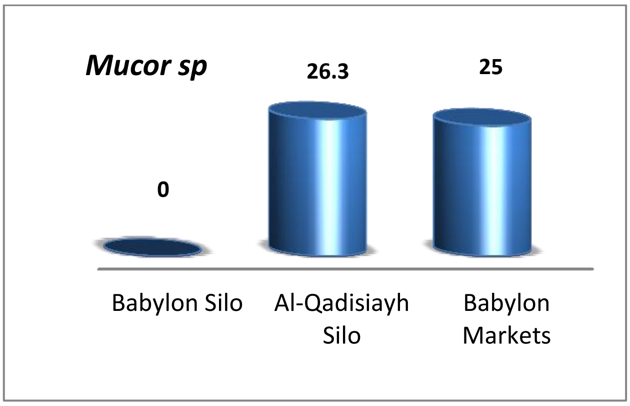


**Figure (4-13): Percentage of appearance of *Mucor sp.* isolated from wheat grains**

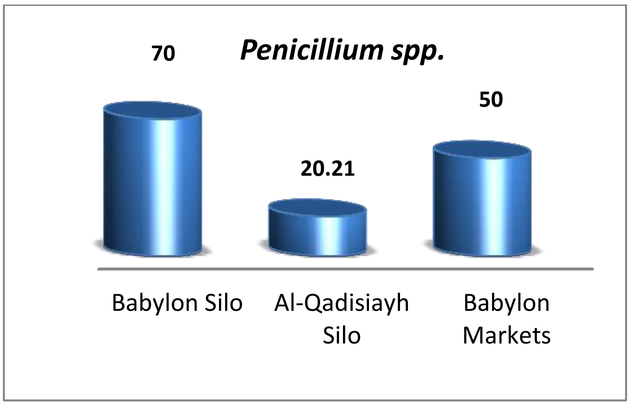


**Figure (4-14): Percentage of appearance of *Penicillium spp.* isolated from wheat grains**

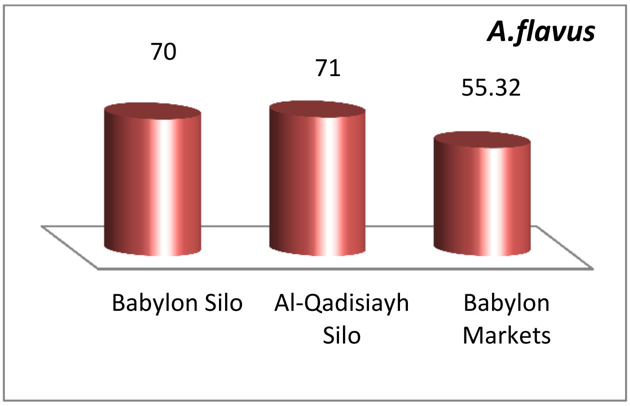


**Figure (4-15): Percentage of appearance of *A.flavus*. isolated from maize grains**

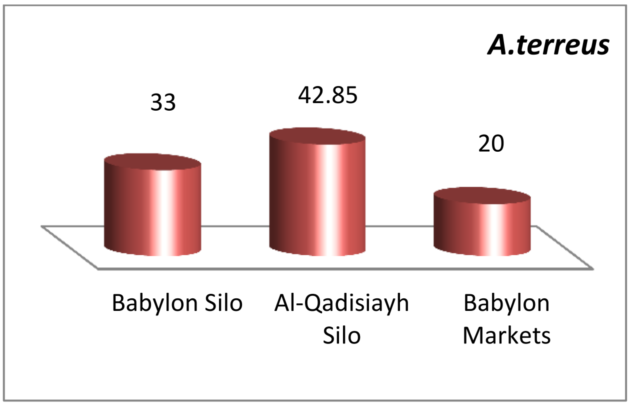


**Figure (4-16): Percentage of appearance of *A.terreus* isolated from maize grains**

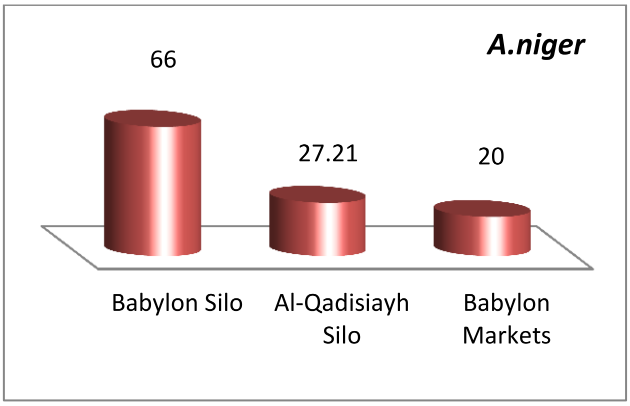


**Figure (4-17): Percentage of appearance of *A.niger* isolated from maize grains**

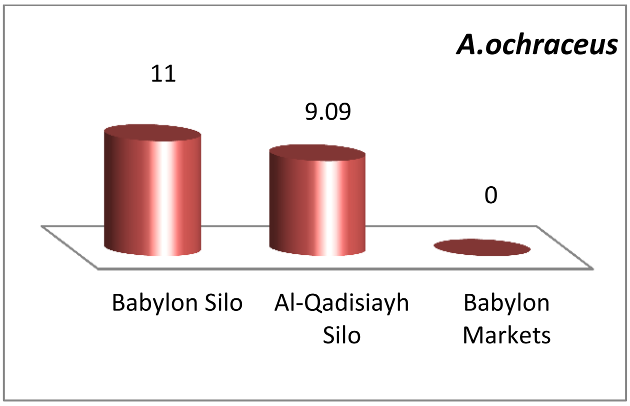


**Figure (4-18): Percentage of appearance of *A.ochraceus* isolated from maize grains**

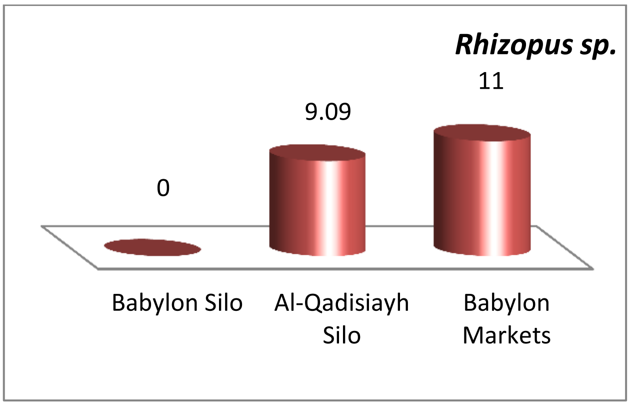


**Figure (4-19): Percentage of appearance of *Rhizopus sp.* isolated from maize grains**

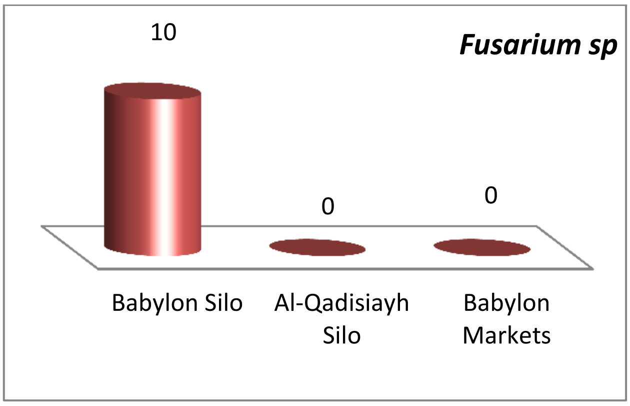


**Figure (4-20): Percentage of appearance of *Fusarium sp.* isolated from maize grains**

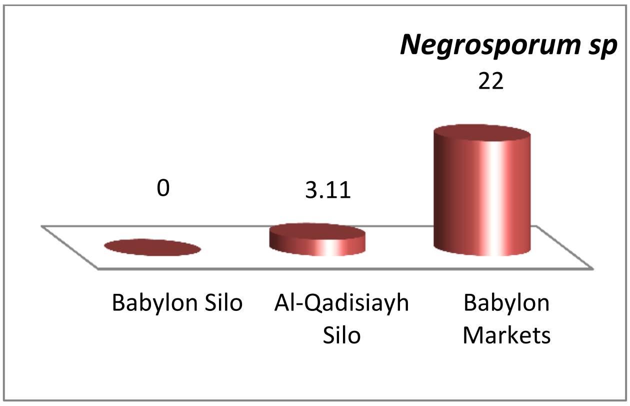


**Figure (4-21): Percentage of appearance of *Negrosporum sp.* isolated from maize grains**

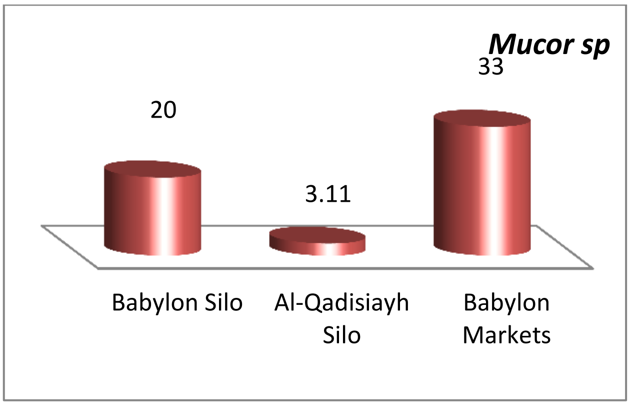


**Figure (4-22): Percentage of appearance of *Mucor sp.* isolated from maize grains**

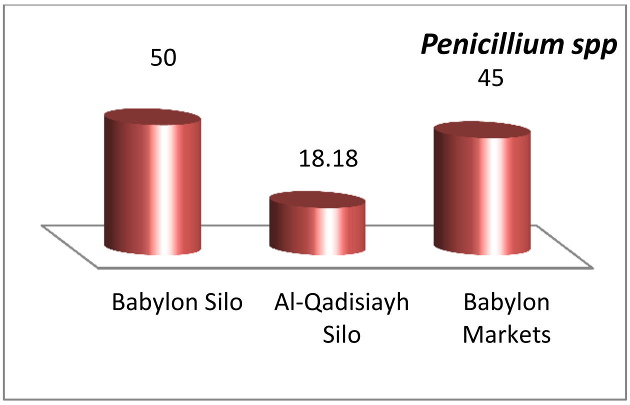


**Figure (4-23): Percentage of appearance of *Penicillium spp.* isolated from maize grains**

**Table 4-18: frequency of fungi in wheat grains**

| **Type of Fungi** | **Place (Mean ±SD)** | | | |
|---|---|---|---|---|
| | **Babylon silo** | **Al-Qadisiayh silo** | **Babylon Markets** | **Mean ± SD** |
| ***A.flavus*** | 16.92±6.08 | 0.62±0.35 | 10.59±1.35 | 9.38±7.77 |
| ***AFumigatus*** | 0±0 | 1.27±0.45 | 0.28±0.15 | 0.52±0.62 |
| ***A.Niger*** | 16.78±0.73 | 28.24±8.14 | 56.82±6.75 | 33.95±18.6 |
| ***A.parasiticus*** | 8.82±6.22 | 4.52±3.007 | 6.09±3.18 | 6.47±4.24 |
| ***A.terreus*** | 1.08±1.06 | 0±0 | 6.84±0.74 | 2.64±3.25 |
| ***Al. alternata*** | 10.8±1.82 | 0.64±0.39 | 0±0 | 3.81±5.33 |
| ***Fusarium spp.*** | 1.65±0.74 | 0±0 | 1.07±0.21 | 0.91±0.82 |
| ***Mucor spp.*** | 0±0 | 0.16±0.07 | 0.21±0.08 | 0.12±0.1 |
| ***Rhizopus sp.*** | 23.71±4.39 | 0.85±0.29 | 0±0 | 8.19±11.85 |
| ***Penicillium spp.*** | 5.94±3.23 | 18.09±3.55 | 6.94±4.82 | 10.33±6.76 |
| **$LSD_{0.05}$** | 1.887 | | | 2.887 |
| **$LSD_{0.05}$ interaction** | 5 | | | |

**Table 4-19: frequency of fungi in maize grains**

| **Type of Fungi** | **Place (Mean ±SD)** | | | |
|---|---|---|---|---|
| | **Babylon silo** | **Al-Qadisiayh silo** | **Babylon Markets** | **Mean ± SD** |
| ***A.flavus*** | 13.44±4.24 | 32.49±3.99 | 23.58±8.73 | 23.34±9.59 |
| ***A.niger*** | 25.27±3.04 | 21.05±1.02 | 11.29±9.42 | 19.2±7.95 |
| ***A.ochraceus*** | 0±0 | 5.02±0.96 | 4.86±3.01 | 3.29±2.93 |
| ***A.parasiticus*** | 17.29±5.44 | 0±0 | 0.25±0.11 | 5.85±9 |
| ***A.terreus*** | 11.67±1.35 | 22.15±2.68 | 34.1±12.08 | 22.64±11.54 |
| ***Fusarium spp.*** | 7.66±1.86 | 0±0 | 0±0 | 2.55±3.94 |
| ***Negrosporum spp.*** | 0±0 | 0.29±0.22 | 21.4±2.54 | 7.23±10.7 |
| ***Mucor spp.*** | 11.26±1.69 | 9.05±5.44 | 18.16±0.14 | 12.82±5.007 |
| ***Rhizopus sp.*** | 0±0 | 5.9±2.71 | 2.69±2.31 | 2.86±3.12 |
| ***Penicillium spp.*** | 6.22±3.16 | 12±0.78 | 21.86±9.82 | 13.36±8.58 |
| **$LSD_{0.05}$** | 2.245 | | | 4.098 |
| **$LSD_{0.05}$ interaction** | 7.098 | | | |

## 4.2: Molecular Identification of *A.ochraceus* by using PCR

Polymerase Chain Reaction (PCR) was used to support the diagnosis of *A.ochraceus* and it was produced OTA. The results of extracted DNA proved in figure (4-24) which contain 11 isolate of *A.ochraceus*, the extracted genomic DNA of these isolates were used as a template for amplification with primers of internal transcribed spacer (ITS), the results revealed that 11/11 isolates were classified as *A.ochraceus* after band electrophoresis and UV-trans illuminated of the product . The internal transcribed spacer (ITS) regions of the fungal ribosomal DNA (r RNA) has been used as one of the techniques for species identification because it is faster, specific, accurate species determination and less feasible to be affected by exterior effects such as temperature and change chemotherapy (Girgis *et al.,*2006; Kong *et al.*, 2008).

This results agree with many studies , in one study, Patiño *et al.*, (2005) developed specific PCR assay for detection of *A. ochraceus* species based on ITS sequences. The primer-pair denoted OCRA1/OCRA2 was designed on the basis of ITS sequence comparison of several strains of *Aspergillus* species. Ochratoxin A contamination in coffee beans was found to be associated with *Aspergillus carbonarius*, *A. niger*, and *A. ochraceus* . A multiplex PCR method that detected PCR amplicons of 809, 372, and 260bps for detection of *A. carbonarius*, *A. niger*, and *A. ochraceus* species, respectively, in coffee beans was developed (Sartori *et al.* 2006) . A highly specific multiplex PCR method was developed using species-specific and mycotoxin metabolic pathway gene primers for detection of *Fusarium* and *Aspergillus* species (Sadhasivam *et al.*, 2017). Stored wheat grain samples (34) were analyzed for the presence of mycotoxin-producing fungi by PCR and mycotoxin productionnby LC/MS/MS. The analyses had a strong correlation, and contamination of six samples with at least one mycotoxin, above EU regulatory limits was confirmed by both the methods. In a Korean study, multiplex PCR and high-performance liquid chromatography (HPLC) analyses were used to assess the ability of mycotoxin production in 32 A. niger isolates (Kim *et al*. 2014). Though multiplex PCR and HPLC analyses of *A. niger* isolates showed that OTA-producing strains exhibited positive PCR patterns for ochratoxin biosynthetic genes, it did not explain the presence of positive PCR products in the non-mycotoxin-producing strains.

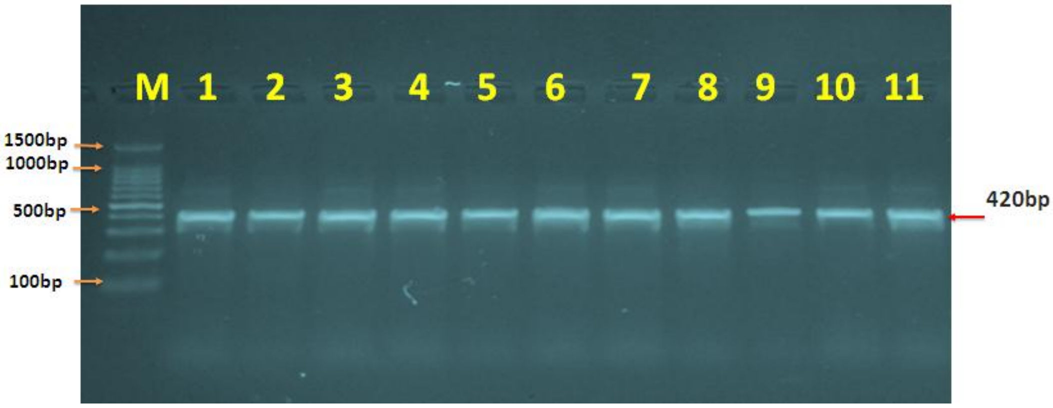
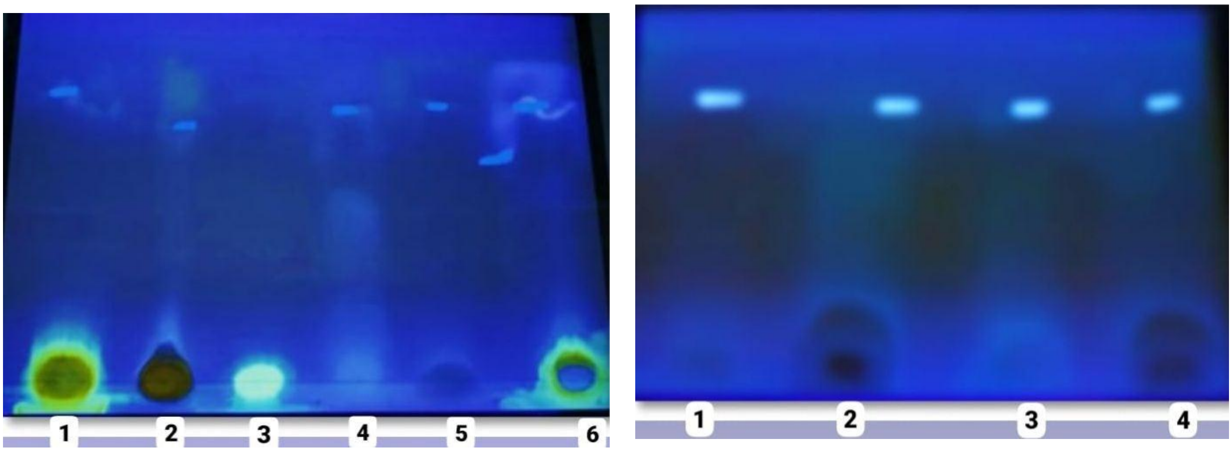


Figure (4-24): Agarose gel electrophoresis image that showed the PCR product analysis of 18S rRNA gene ITS2 gene from extracted DNA of *Aspergillus ochraceus* isolates. Where M: marker (100-1500bp) and the positive *Aspergillus ochraceus* (1-11) isolates were showed at (420bp) PCR product.

## 4.3: Detection The Ability of *A.niger* and *A.ochraceus* isolated on production of OTA by Chromatographic Methods

### 4.3.1: Detection of OTA by TLC

The results explain the detection of the presence of OTA in wheat and maize grains was done by using TLC on class plates silica gel 20x20 cm when examined by visible and UV light. Table (4-20) explain the number, properties and $R_f$ value of each band in the extract by using mobile phase system methanol : water. 6 strains of *A.niger* , strains 2 and 4 have 2 spots, while strains 1, ,5 have only one spot and strain 3 have no spot. 4 stains of *A.ochraceus* strain 3 and 4 have 2 spots , and 1,2 have one spot. The fungi strains also varied in their mycotoxin production based on spot size and brightness intensity under ultraviolet ray. This study is agree with Braicu *et al*.,(2007), found that 12.5% of wheat and 9% of maize samples were contaminated with OTA. Boudra *et al*., (1995) extracted OTA from *Aspergillus ochraceus* contaminated wheat using acidified chloroform and analyzed it by bidirectional TLC. Samples were loaded on silica gel aluminum sheet activated for 1h at 100°C and developed first with mobile phase of anhydrous diethyl ether and later with toluene/ethyl acetate/formic acid (6:3:1) and exposed to UV for OTA detection. Pittet and Royer (2002) reported a rapid, low-cost method for detection of OTA in green coffee using bidirectional TLC with the detection limit of 10 • µg/kg. Two varieties of green coffee obtained from Thailand, India, Kenya, Uganda, Salvador, and Guatemala were extracted using dichloromethane containing 0.1 M phosphoric acid and qualitatively screened using bidirectional TLC method that gave 98% OTA recovery. In Ghana, maize kernels and groundnut seeds were studied for aflatoxin contamination, and the distribution of aflatoxin-producing potential of *Aspergillus* species were associated with both crops (Agbetiameh *et al*. 2018). Out of 326 maize and 183 groundnut samples, aflatoxin level exceeded the threshold limits of 15 and 20ppb, respectively, set by the Ghana Standards Authority for 15% of maize and 11% of groundnut samples.



**A** **B**

**Figure 4-25: Thin layer chromatography (TLC) of ochratoxin in (A): *A.***

**. *niger and* (B): *A.ochracoues***

**Table (4-20): RF value of OTA detected by TLC for *A.niger* and *A.ochraceus* from wheat and maize grains**

| Fungi | Isolate No. | Spot No. | RF % |
|---|---|---|---|
| ***A.niger*** | 1 | 1 | 31.21 |
| | 2 | 2 | 37.21 |
| | 3 | – | – |
| | 4 | 1 | 30.39 |
| | 5 | 1 | 32.1 |
| | 6 | 1 | 35.07 |
| | | 2 | 34.92 |
| ***A.ochraceus*** | 1 | 1 | 36.22 |
| | 2 | 1 | 33.21 |
| | 3 | 1 | 35.07 |
| | 4 | 1 | 32.45 |

### 4.3.2: Detection of OTA by HPLC

The chromatography conditions were adjusted starting from injection of the standard solution.. Table (4-21) shows the linearity from OTA analysis's by proposed method. Figure (4-26) presents the chromatograms obtained from OTA standard solution (0.25 ppb). The retention time 8.517 min and the area 302.176.

**Table (4-21): HPLC Standard Ochratoxin A ( 0.25 ppb )**

| No | Reten. Time [min] | Area [mAU.s] | Height [mAU] | Area [%] | Height [%] | W 05 [min] |
|---|---|---|---|---|---|---|
| **1** | **8.517** | **302.176** | **56.166** | **100.0** | **100.0** | **0.09** |
| | **Total** | **302.176** | **56.166** | **100.0** | **100.0** | |

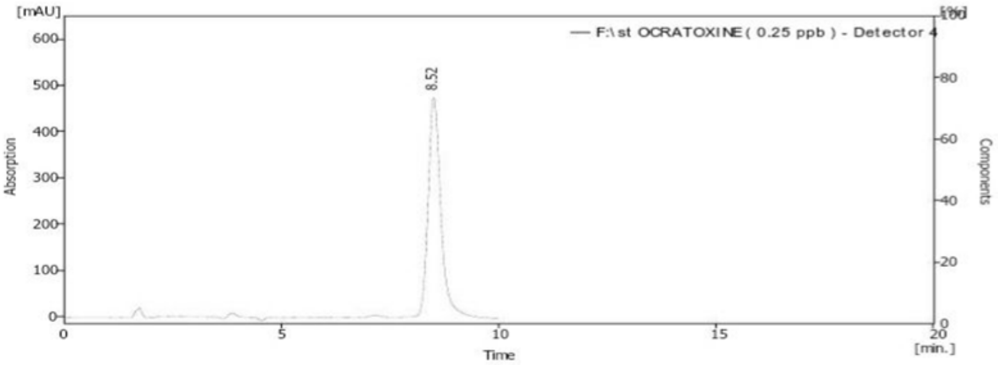


**Figure (4-25): HPLC chromatogram standard Ochratoxin A ( 0.25 ppb )**

### Table (4-22): HPLC Ochratoxin A concentration in *A.niger* and *A.ochraceus*

| No. | Con ( ppb ) | No. | Con (ppb) |
|---|---|---|---|
| ***1. A.niger*** | **3.50** | ***4. A.niger*** | **2.25** |
| ***2. A.niger*** | **8.14** | ***5. A.Ochraceus*** | **98.3** |
| ***3. A.niger*** | **2.42** | ***6. A.Ochraceus*** | **124.9** |

Table (4-22) shows the concentration of OTA extracted from *A.niger* and *A.ochraceus* in wheat and maize grains obtained from HPLC . The OTA extracted from *A.ochraceus* in maize grains recorded the highest concentration than OTA extract from *A.niger* in wheat and maize grains and the concentration of OTA from *A.niger* in wheat grains was higher than maize grains.

### Table (4-23):HPLC detection of Ochratoxin A in *A.niger* from Wheat (no.1)

| No | Reten. Time [min ] | Area [mAU.s] | Height [mAU] | Area [%] | Height [%] | W05 [min] | Sample name |
|---|---|---|---|---|---|---|---|
| 1 | 1.367 | 124.571 | 16.488 | 15.6 | 15.5 | 0.13 | ***A.niger*** |
| 2 | 1.753 | 561.959 | 75.103 | 70.6 | 70.7 | 0.13 | |
| 3 | 3.900 | 71.043 | 8.352 | 8.9 | 7.9 | 0.16 | |
| 4 | 8.513 | 38.703 | 6.245 | 4.9 | 5.9 | 0.07 | |
| | Total | 796.276 | 106.187 | 100.0 | 100.0 | | |

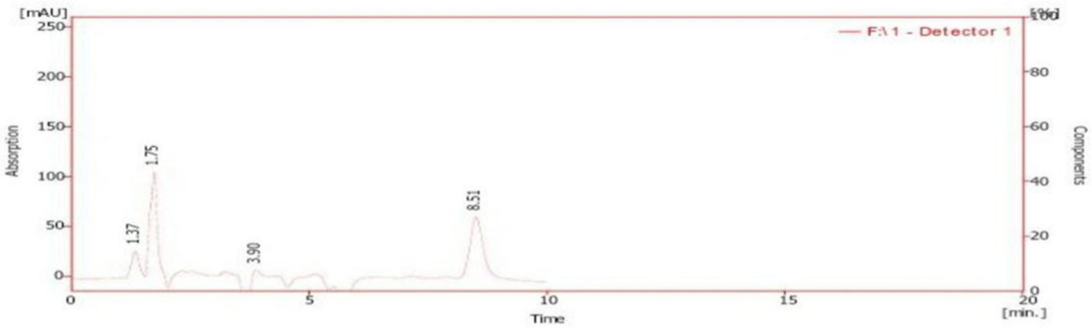


**Figure (4-27):HPLC Chromatogram of Ochratoxin A in *A.niger* from Wheat (no.1)**

Appropriate chromatographic conditions from the stationary phase and a step gradient polarity system in the mobile phase were used to detect the presence and concentration of OTA in *A.niger* from wheat grain. Table (4-23) and figure (4-27) show that the OTA extract had a retention time of 8.513 minutes and area of 38.703 as opposed to the OTA standard retention time and area of 8.517 and.302.176. This result indicate the presence of OTA in *A.niger* in concentration 3.50 ppb.

**Table (4-24):HPLC detection of Ochratoxin A in *A.niger* from wheat (no.2)**

| No | Reten. Time [min] | Area [mAU.s] | Height [mAU ] | Area [%] | Height [%] | W05 [min] | Sample name |
|---|---|---|---|---|---|---|---|
| 1 | 0.133 | 0.810 | 0.130 | 0.2 | 0.2 | 0.03 | *A.niger* |
| 2 | 1.367 | 122.391 | 15.716 | 34.8 | 26.7 | 0.13 | |
| 3 | 1.753 | 102.218 | 24.592 | 29.0 | 41.8 | 0.06 | |
| 4 | 3.900 | 25.605 | 3.929 | 7.3 | 6.7 | 0.10 | |
| 5 | 8.513 | 101.063 | 14.409 | 28.7 | 24.5 | 0.13 | |
| | Total | 352.088 | 58.776 | 100.0 | 100.0 | | |

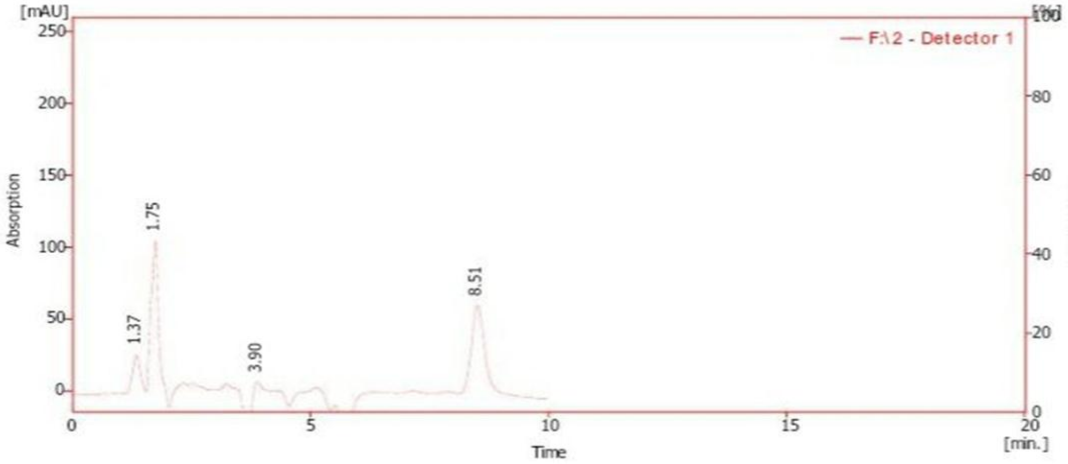


**Figure (4-28):HPLC chromatogram of Ochratoxin A in *A.niger* from Wheat (no.2)**

In wheat grains, the retention time of OTA extract *A.niger* was 8.513 min and the area was 101.063, as shown in table (4-24) and figure (4-28), compared to OTA standard retention time and area of 8.517 and 302.176, respectively. This indicates that OTA is present in *A.niger* at a concentration of 8.14 ppb.

**Table (4-25) :HPLC detection of Ochratoxin A in *A.niger* from Maize (no.3)**

| No | Reten. Time [min] | Area [mAU.s] | Height [mAU] | Area [%] | Height [%] | W05 [min] | Sample name |
|---|---|---|---|---|---|---|---|
| 1 | 1.367 | 41.445 | 8.336 | 8.6 | 13.1 | 0.09 | *A.niger* |
| 2 | 1.753 | 135.110 | 26.375 | 28.0 | 41.4 | 0.06 | |
| 3 | 3.900 | 46.205 | 5.983 | 9.6 | 9.4 | 0.13 | |
| 4 | 5.127 | 51.326 | 2.524 | 10.6 | 4.0 | 0.13 | |
| 5 | 8.513 | 209.079 | 20.523 | 43.3 | 32.2 | 0.15 | |
| | Total | 483.164 | 63.742 | 100.0 | 100.0 | | |

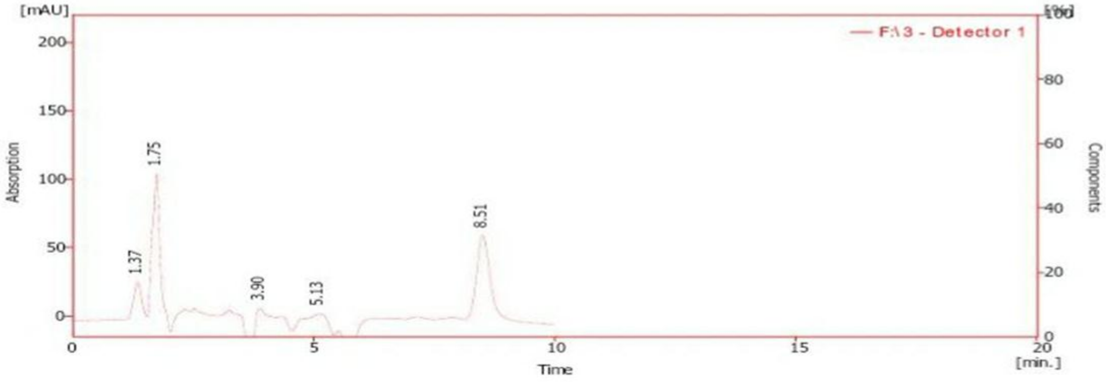


**Figure (4-29): HPLC Chromatogram of Ochratoxin A in *A.niger* from Maize (no.3)**

In maize, the retention time of OTA extract from *A.niger* was 8.513 min and the area was 209.079, compared to OTA standard retention time and area of 8.517 and 302.176, respectively. This indicates that OTA is present in *A.niger* at a concentration of 2.42 ppb. table (4-25) and figure(4-29).

**Table (4-26): HPLC detection of Ochratoxin A in *A.niger* from Maize (no.4)**

| No | Reten. Time [min] | Area [mAU.s] | Height [mAU] | Area [%] | Height [%] | W05 [min] | Sample name |
|---|---|---|---|---|---|---|---|
| 1 | 1.367 | 91.772 | 12.853 | 10.4 | 11.1 | 0.12 | *A.niger* |
| 2 | 1.753 | 440.768 | 66.578 | 49.8 | 57.4 | 0.12 | |
| 3 | 3.900 | 100.200 | 10.827 | 11.3 | 9.3 | 0.22 | |
| 4 | 8.513 | 252.696 | 25.808 | 28.5 | 22.2 | 0.17 | |
| | Total | 885.435 | 116.066 | 100.0 | 100.0 | | |

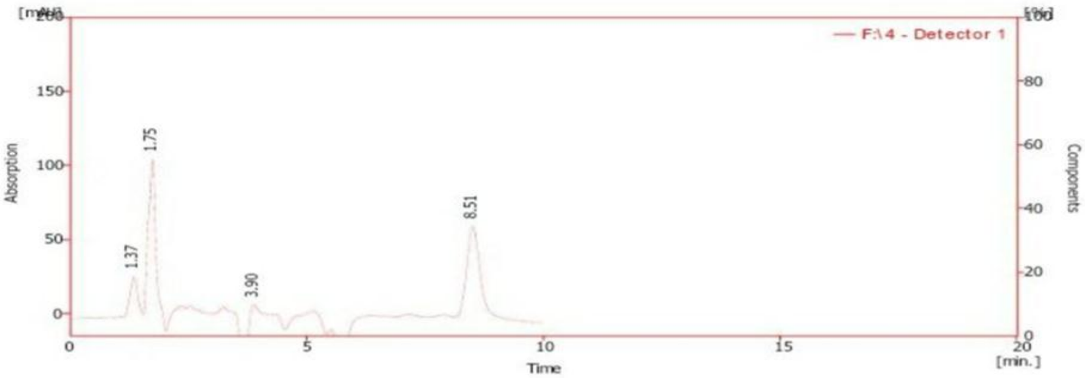


**Figure (4-30): HPLC Chromatogram of Ochratoxin A in *A.niger* from Maize (no.4)**

As shown in table (4-26) and figure(4-30) , the retention time of OTA extract OTA in *A.niger* from maize grain was 8.513 min and area was 252.696 in compared with OTA standard retention time and area which was respectively 8.517 and 302.176. This result indicate the presence of OTA in *A.niger* in concentration 2.25 ppb.

**Table (4-27): HPLC detection of Ochratoxin A in *A.ochraceus* from Maize (no.1)**

| No | Reten. Time [min] | Area [mAU.s] | Height [mAU] | Area [%] | Height [%] | W05 [min] | Sample name |
|---|---|---|---|---|---|---|---|
| 1 | 1.367 | 176.530 | 20.199 | 11.4 | 12.8 | 0.15 | ***A.ochraceus*** |
| 2 | 1.753 | 724.367 | 84.010 | 46.8 | 53.0 | 0.15 | |
| 3 | 3.900 | 121.075 | 12.491 | 7.8 | 7.9 | 0.21 | |
| 4 | 5.127 | 120.245 | 6.963 | 7.8 | 4.4 | 0.36 | |
| 5 | 8.513 | 406.747 | 34.759 | 26.3 | 21.9 | 0.20 | |
| | Total | 1548.964 | 158.422 | 100.0 | 100.0 | | |

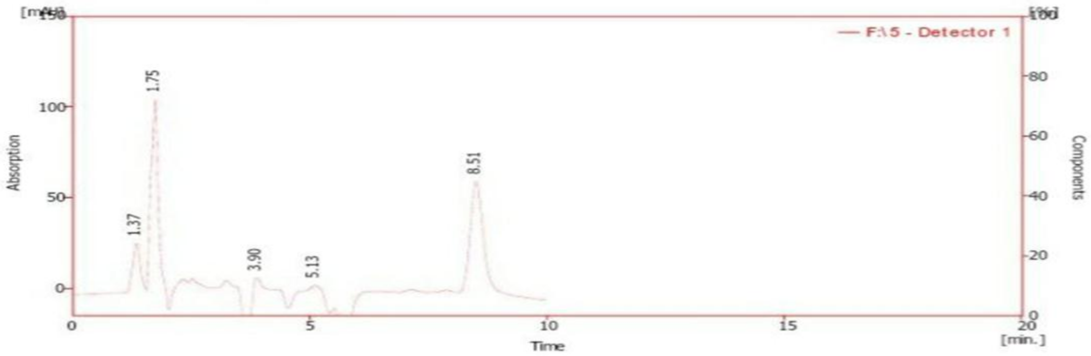


**Figure (4-31): HPLC Chromatogram of Ochratoxin A in *A.ochraceus* from Maize (no.1)**

The retention time of OTA in *A.ochraceus* from maize grain extract was 8.513 min and the area was 406.747, as shown in table (4-27) and figure (4-31), compared to the OTA normal retention time and area of 8.517 and 302.176, respectively. This indicates that OTA is present in *A.ochraceus* at a concentration of 98.3 ppb.

**Table (4-28): HPLC detection of Ochratoxin A in *A.ochraceus* from Maize (no.2).**

| No | Reten. Time [min] | Area [mAU.s] | Height [mAU] | Area [%] | Height [%] | W05 [min] | Sample name |
|---|---|---|---|---|---|---|---|
| 1 | 1.367 | 179.218 | 20.192 | 11.3 | 12.6 | 0.15 | *A.ochraceus* |
| 2 | 1.753 | 553.891 | 75.216 | 34.9 | 47.0 | 0.13 | |
| 3 | 3.250 | 24.433 | 3.242 | 1.5 | 2.0 | 0.13 | |
| 4 | 3.900 | 100.200 | 10.827 | 6.3 | 6.8 | 0.22 | |
| 5 | 5.127 | 116.521 | 6.270 | 7.3 | 3.9 | 0.27 | |
| 6 | 8.513 | 614.021 | 44.331 | 38.7 | 27.7 | 0.24 | |
| | Total | 1588.285 | 160.078 | 100.0 | 100.0 | | |

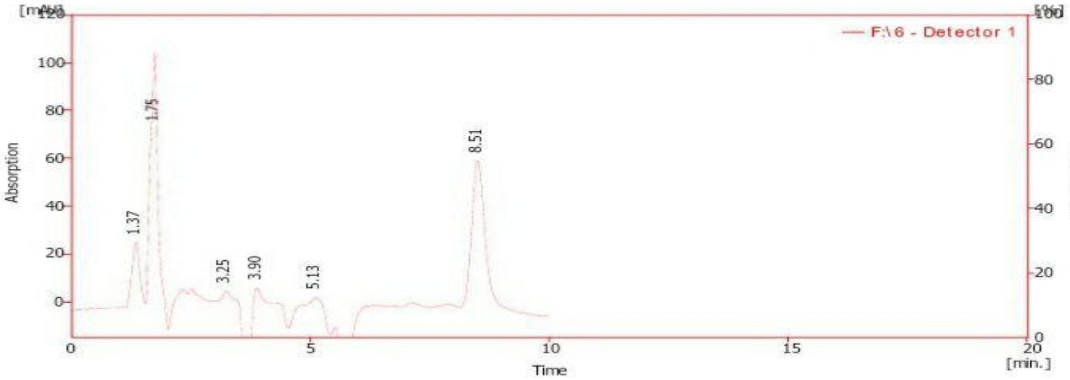


**Figure (4-32):HPLC Chromatogram of Ochratoxin A in *A.ochraceus* from Maize (no.2).**

Table (4-28) and figure(4-32),of OTA in *A.ochraceus* from maize grain where the retention time of OTA extract was 8.513 min and area was 406.747 in compared with OTA standard retention time and area which was respectively 8.513 and 614.021. This result indicate the presence of OTA in *A.ochraceus* in concentration 98.3 ppb. There are many studies which identify the presence of OTA in wheat and maize grains, Limay-Rios, Miller and Schaafsma, (2017) identify levels of OTA in wheat grains retailing and found to be lower than in stored wheat . 75% of *A. ochraceus* and *A. carbonarius* isolates produced OTA in a study of Brazilian maize grains, whereas only 3% of *A. niger* isolates were toxigenic . Durguti *et al*. (2014) analyzed 54 wine samples by HPLC-FLD after sample cleanup using immuneafnity columns. The OTA levels were below the EU limit of 2ng/ml, and the LOD and LOQ were 0.05 and 0.1ng/ml, respectively.

Kupski and Badiale-Furlong (2015) standardized the HPTLC method using hexane, ethyl acetate, and acetic acid (18:4:1.5) as mobile phase. The typical fuoresecent spots of mycotoxin were observed under UV light plate after the development of plate with aluminum chloride in

15% methanol. The addition of dried magnesium sulfate and sodium chloride as salts to the extraction solvent acetonitrile/water (2:1) resulted in improved recovery of the toxin. The developed method could detect contamination of OTA from 0.22 to 0.85µg/kg in 20 wheat flour samples. OTA in wines was analyzed with HPTLC and HPLC after a dispersive liquid–liquid microextraction .A linearity of 0.03–1.00 µ g/L was established for HPTLC with a high correlation with the HPLC method. Additionally, the HPTLC method achieved the simultaneous analysis of different wine samples and standard of OTA on the same plate (Antep and Merdivan 2012). Yazdanpanah *et al*, (2013) analyzed AFB1in rice, bread, puffed corn snack, wheat flour, and peanut samples with a HPLC method and obtained an average recovery of 94.4–100%. The LOD was 0.01ng/g. Among 90 samples collected from Tehran retail market in June 2005, the bread and wheat flour samples were not contaminated, while rice, puffed corn snack, and peanut samples showed AFB1 level below 5ng/g, the maximum tolerated level (MTL) in Iran.

### 4.4: Quantitative Reverse Transcription Real-Time PCR in *A.niger* and *A.ochraceus.*

The data which were revealed by figure (4-33) and table (4-29) for 6 isolates of *A.niger* 3 isolated from wheat and 3 from maize grains showed the presence of the gene that responsible for the production of OTA, figure (4-34) and table (4-30) for 6 isolates of *A.ohraceus* isolated from maize grains showed the presence of the gene that responsible for the production of OTA. DNA-based techniques such as real-time PCR (RT-PCR) are providing new tools for fungal detection and quantification by detecting and quantifying their DNA. RT-PCR can be performed using different chemistries, such as SYBR® Green I dye

(González-Salgado *et al*.,2009). Quantitative real-time PCR systems may be useful for determining associations between detection of a gene at critical control points in food production and quantification of the mycotoxin contamination in the final product (Rodríguez *et al*., 2012). Though quantitative real-time PCR has been applied to monitor expression of mycotoxin biosynthetic genes, it is expensive. The rapid and accurate differentiation of toxigenic and atoxigenic isolates was evaluated with PCR and real-time PCR in 22 A. *flavus* isolates from peanut kernels (Mahmoud, 2015). The PCR amplification of genes did not correlate with aflatoxin production capability. Among the four aflatoxin biosynthetic pathway genes (aD, aM, aP, and aQ), the expression of aD and aQ was a good marker for differentiating the toxigenic from atoxigenic isolates. The real-time PCR and agar culture method for detection of *A. flavus* presence had 95% agreement. Aflatoxin production by these strains was confirmed by HPLC with 72% of isolates producing the toxin. Rodríguez *et al*. (2012) proposed real-time PCR for the early detection and quantification of aflatoxin in peanut, spices, and sausages. The sensitivity and specificity of real-time quantitative PCR (qPCR)-based on SYBR Green and TaqMan were evaluated using the o-methyltransferase gene (omt-1) aflatoxin biosynthetic gene. Both qPCR methods gave a good linear correlation from 4 to 1 log cfu/g per reaction and a detection limit of 1–2 log cfu/g in the different food matrices tested. The ochratoxin-producing fungus *Aspergillus carbonarius* is often associated with grapes and wine contamination

RT- PCR quantification of *A. carbonarius* in grapes is clearly the best alternative to conventional methods in order to investigate the relation between OTA producers and OTA content. With regards to food safety, Atoui *et al*. (2007) established, according to their correlation, that

A. carbonarius DNA content has to be lower than 10 ng DNA g−1 grape berry to fulfill the maximum OTA permitted levels in the European Union (Commission regulation No. 123/2005 amending Regulation No. 67 446/2001 as regards to ochratoxin A). For this reason Real time PCR technology provides an insight into the mycotoxigenic status of food sample as well as it has the power to estimate its mycotoxin content.

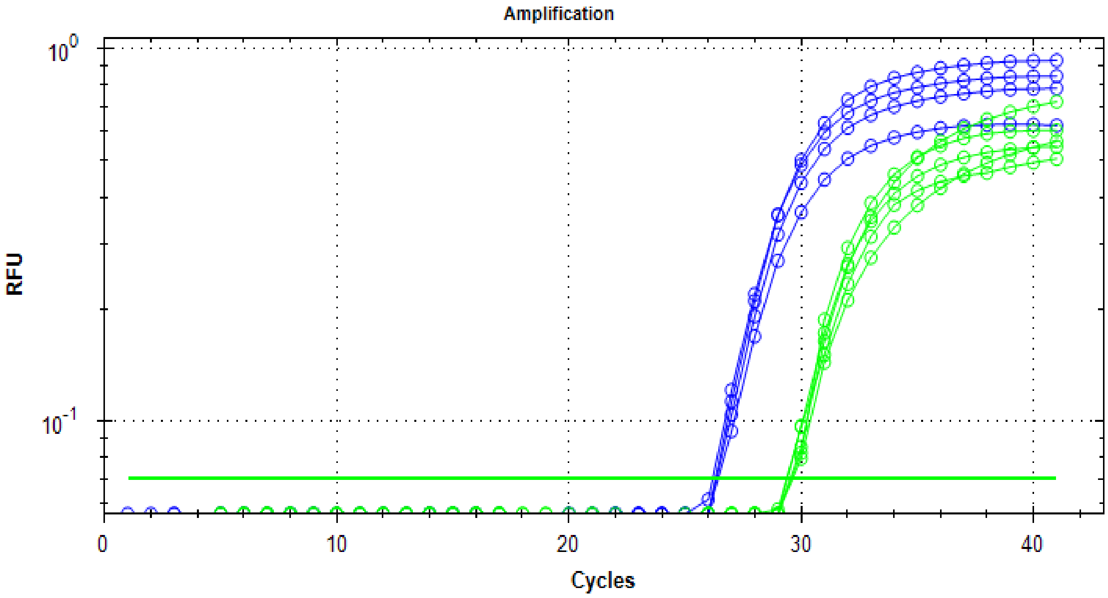


**Figure (4-33): Real Time PCR amplification plot for target gene (ochratoxin biosynthesis protein as blue plots and housekeeping gene as Green plots**

**Table (4-29): Gene expression analysis for ochratoxin A in *A.niger* isolated from wheat and maize grains**

| Gene expression | | | | |
|---|---|---|---|---|
| **No. sample** | **CT (Ochratoxin A)** | **CT (actin)** | **ΔCT** | **Fold change (2^ΔCT)** |
| **1-Wheat** | **26.35** | **29.67** | **3.32** | **9.987** |
| **2-wheat** | **26.26** | **29.79** | **3.53** | **11.551** |
| **3- Wheat** | **26.49** | **29.32** | **2.83** | **7.111** |
| **4-Maize** | **27.23** | **29.71** | **2.48** | **5.579** |
| **5-Maize** | **26.26** | **29.43** | **3.17** | **9.000** |
| **6-Maize** | **27.49** | **29.27** | **1.78** | **3.434** |

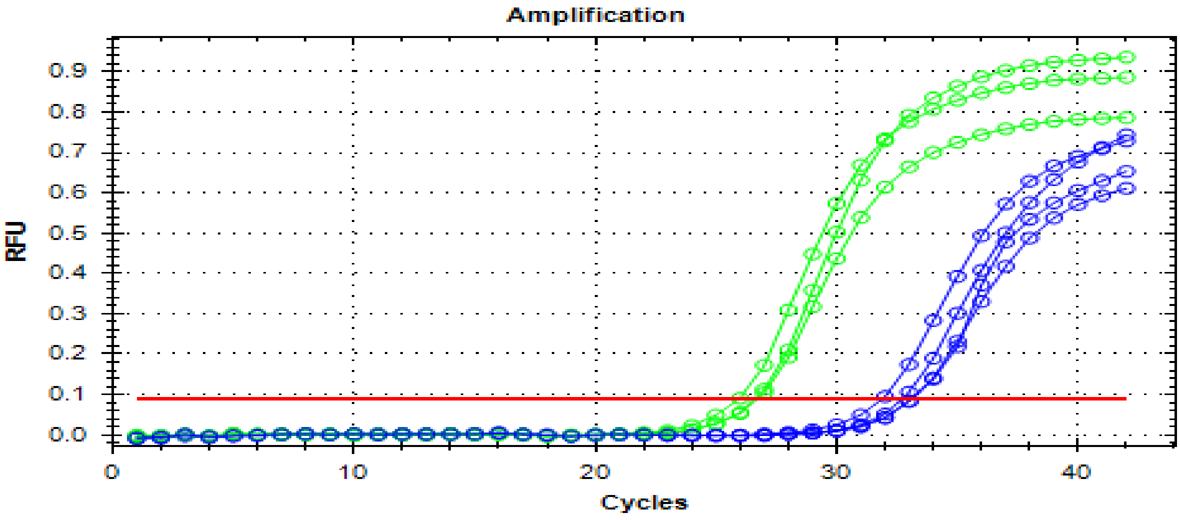


**Figure (4-34): Real Time PCR amplification plot for target gene (ochratoxin biosynthesis protein gene)as blue plots and housekeeping gene as Green plots.**

**Table(4-30): Gene expression analysis for ochratoxin A in *A.ochraceus* isolated From Maize grains**

| Gene expression | | | | |
|---|---|---|---|---|
| **No. sample** | **CT (Ochratoxin A)** | **CT (actin)** | **ΔCT** | **Fold change (2^ΔCT)** |
| **1** | **28.43** | **30.54** | **2.11** | **4.317** |
| **2** | **25.87** | **30.66** | **4.79** | **27.656** |
| **3** | **26.55** | **30.19** | **3.64** | **12.467** |
| **4** | **28.45** | **30.58** | **2.13** | **4.377** |
| **5** | **27.43** | **30.3** | **2.87** | **7.311** |
| **6** | **28.43** | **30.14** | **1.71** | **3.272** |

## 4.5: Secondary Metabolites Analysis

### 4.5.1: Qualitative Secondary Metabolites Tests

Tables (4-31) (4-32) respectively, represent the secondary metabolites constituents that present in *O. europaea L.* leaves and *C.Spinosa* flowers by using different solvents (water, methanol). The qualitative analysis of leaves extract of *O.europaea* and *C.spinosa* flowers appear the presence of phenols, alkaloid, tannins, terpenes, and glycoside by using all solvents that used in this study.

This results agree with results of Olmo-Garcia, *et al*., (2018)which found the presence of alkaloids, tannins, glycosides, flavonoids, saponin, phenols, terpene in *O.europaea L.* leaves .*O.*

*europaea L.* methanolic extract contains polyphenols and flavonoids (Vogel *et al*., 2015) . (Akkari *et a*l.,2016) found the presence of alkaloids, tannins, glycosides, flavonoids, saponin, phenols, terpene in *C. Spinosa L.* flowers. Zhang and Feei Ma (2018), found the presence of phenols in *C.spinosa L.*.

**Table (4-31): Secondary metabolites tests for *Olea europea L.* leaves ( cold water, methanol extracts)**

| No. | Secondary Metabolites | Test Name | Methanolic extract | Aqueous extract |
|---|---|---|---|---|
| 1 | **Alkaloids** | **Dragendroff** | _ | + |
| | | **Mayer** | + | _ |
| | | **Hager** | + | + |
| 2 | **Phenols** | **Lead acetate** | + | + |
| 3 | **Tanins** | **Ferric chloride** | + | + |
| | | **Gelatin** | + | + |
| 4 | **Glycosides** | **Fehling** | + | + |
| 5 | **Saponin** | **Foam** | + | + |
| 6 | **Flavonoids** | **KOH** | + | + |
| 7 | **Terpenes** | **Sulfuric acid** | + | + |

**Table (4-32): Secondary metabolites tests for *Capparis spinosa* flowers ( cold water, methanol extracts)**

| No. | Secondary Metabolites | Test Name | Methanolic extract | Aqueous extract |
|---|---|---|---|---|
| 1 | Alkaloids | Dragendroff | + | + |
| | | Mayer | + | _ |
| | | Hager | + | + |
| 2 | Phenols | Lead acetate | + | + |
| 3 | Tanins | Ferric chloride | + | + |
| | | Gelatin | + | + |
| 4 | Glycosides | Fehling | + | + |
| 5 | Saponin | Foam | + | + |
| 6 | Flavonoids | KOH | + | + |
| 7 | Terpenes | Sulfuric acid | + | + |

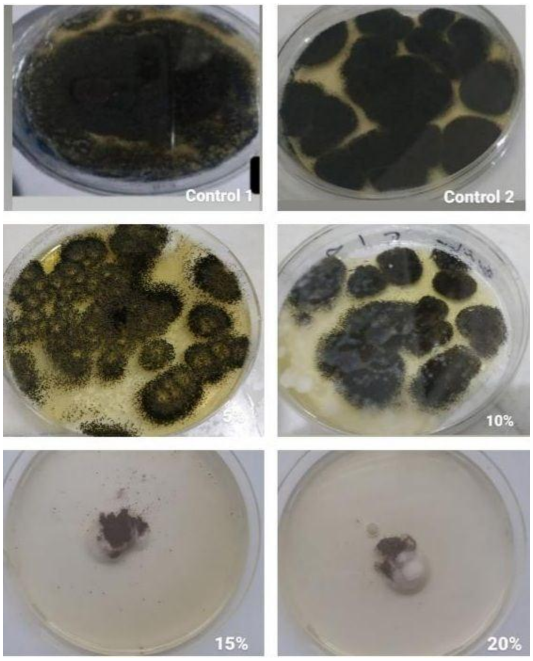
### 4.5.2: Minimum Inhibitory Concentration (MIC)

The MIC was determined only for the test organisms that had shown heist growth inhibition according to the extracts. The MIC values of different extracts of *O. europaea L.* leaves and *C. spinosa L.* flowers were shown in tables (4-33) (4-34), figures (4-35), (4-36) respectively. Inhibitory effects of *A.niger and A.ochraceus* growth by the extracts from *O. europaea L.* leaves and *C. spinosa L.* flowers (5 ,10, 15, 20%) expressed as MIC values. The concentration 20% was enough to prevent growth of *A.niger and A.ochraceus* by aqueous extract and methanol extract of *O.europaea* leaves and *C.spinosa* flowers respectively . the minimum inhibitory concentration (MIC) reached 5% when using the aqueous extract of olive leaves was 29.91%, while the concentration was 26.44% for the alcoholic extract of the same plant. The two extracts recorded a minimum inhibition concentration of 92.45% for a concentration of 20% aqueous extract and 92.66% for an alcoholic extract of the same concentration and for the same plant. As for the flowers of *C.spinosa,* the minimum inhibitory concentration (MIC) at the concentration reached 20% 92.16% and 92.89% for the aqueous and alcoholic extracts, respectively. Differences in MIC values can be explained by different concentrations of antifungal components in the extracts (Kosalec *et al*., 2005).

Markin *et al*. (2003) reported that aqueous extract of olive leaf (15%) killed C. albicans within 24 h. These differences are likely to occur because of the different olive cultivars (Ranalli *et al*., 2006), the content of the antimicrobial agents present in the extracts, or the sample preparation methods use Antimicrobial effects of these *O.europaea L*. methanolic and aqoueus extacts were tested against *Saccharomyces cerevisiae* ATCC 9763,*Schizosaccharomyces pombe, Saccharomyces uvarum, Candida oleophila, Metschnikowia fructicola* and*Kloeckera apiculata*. The antifungal activities of these extracts were tested by the disc diffusion assay, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). All extracts showed various degrees of antifungal effects with 10–28 μg/ml MIC, 20–48 μg/ml MFC and 1.5–9.3 mm inhibitory zone values against yeasts utilised, except water (Korukluoglu *et al*.,2006) .

The petroleum ether extracts of *C.spinosa* are effective to low concentration (6.25mg/ml) on S. Epidermidis. This effect is due to the petroleum root extracts contains the nonpolar compounds in its constituents, such as fatty acids or its derivatives andsteroids compounds that have antibacterial activity against a broad range of gram-positive and negative bacteria (chanda *et al*.,2018). Six different antibiotics discs were used as references. According to results *Capparis spinosa* leaves and bark extracts with different solvents revealed variable in – vitro growth inhibition activity against tested standard strains and clinical isolates with highly significant differences; $P< 0.01$. The higher plant activities were obtained from chloroform bark extract and methanol leaves extract with equal minimum inhibitory concentration of 100mg/ml against both standard *Staphylococcus aureus* ATCC 25923 and clinical methicillin resistant *Staphylococcus aureus*, and these activities may due to the high presence of the phytochemical constituents such as flavonoids, tannins, steroids and triterpines. Chloroform bark extract very effective against standard and clinical isolates of *Staphylococcus aureus* and methanol leaves extract is effective against the standard organism( Eltawaty,2018)

The spread of multidrug-resistant strains of pathogenic fungi and the reduced number of drugs available makes it necessary to discover new classes of antifungal agents that overcome these resistant mechanisms. This led to search for therapeutic alternatives, particularly among medicinal plants and compounds isolated from them used empirically for their antifungal properties. Plant extracts are potential sources of novel antimicrobial compounds against fungi pathogens (Bajpai *et al*., 2008).



**Figure(4-35): Effect of methanol extract of *Olea europaea L.* leaves on fungi growth for 7 days in 25˚c**

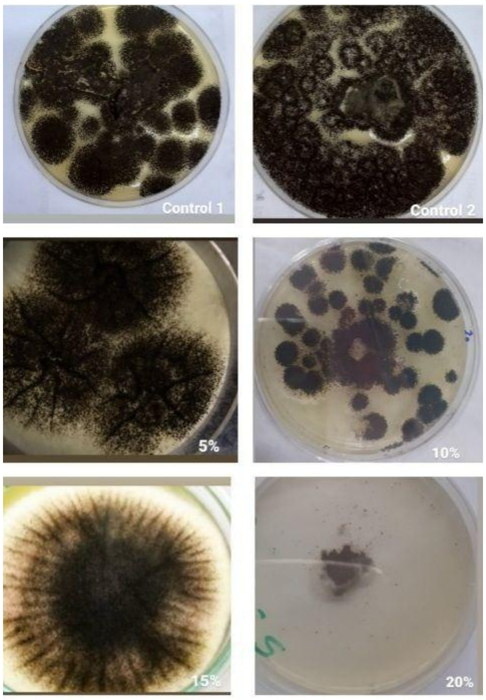


**Figure (4-36): Effect of methanolic extract of *Capparis spinosa* flowers on fungi growth for 7 days in 25˚c**

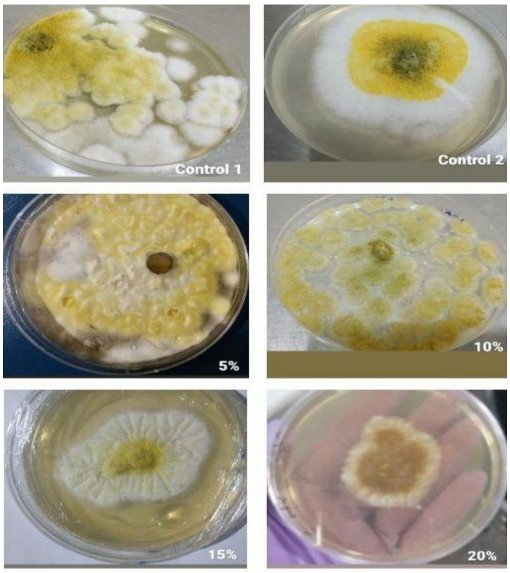


**Figure (4-37): Effect of methanol extract of *Olea europaea L.* leaves on *A.ochraceus* growth for 7 days in 25°c**

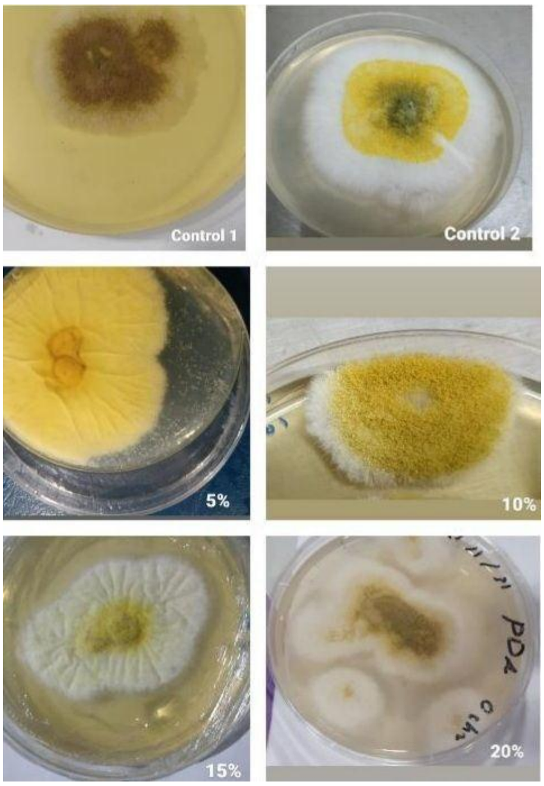


**Figure (4-38): Effect of methanolic extract of *Capparis spinosa* flowers on *A.ochraceus* growth for 7 days in 25°c**

**Table (4-33) Effect of different concentration of aqueous extract of *Olea europaea L.* leaves on fungi growth by MIC methods**

| Type of extract and concentration | Type of fungi Growth inhibition (mm) | | mean± SD |
|---|---|---|---|
| | *A. niger* | *A. ochraceus* | |
| Control 1 | 0±0 | 0±0 | 0±0 |
| Control 2 | 0.92±1.29 | 1.29±1.29 | 1.11±1.25 |
| 5% | 31.33±5.006 | 28.5±2.34 | 29.91±4.01 |
| 10% | 45.5±7.63 | 47.83±5.74 | 46.66±6.55 |
| 15% | 78.78±4.21 | 76.05±4.88 | 77.41±4.57 |
| 20% | 94.5±6.05 | 90.41±2.41 | 92.45±4.88 |
| mean± SD | 41.84±36.57 | 40.68±35.1 | 41.26±35.59 |
| LSD(P<0.05) | 1.970 | | 3.411 |
| LSD for Interaction | 4.824 | | |

Values represent mean ± Standard deviation for 6 batches.

**Table (4-34) Effect of different concentration of methanolic extract of *Olea europaea L.* leaves on fungi growth by MIC methods**

| Type of extract and concentration | Type of fungi Growth inhibition (mm) | | mean± SD |
|---|---|---|---|
| | *A.niger* | *A.ochraceus* | |
| Control 1 | 0±0 | 0±0 | 0±0 |
| Control 2 | 0.55±0.6 | 0.74±0.9 | 0.64±0.74 |
| 5% | 26.88±7.32 | 26±4.97 | 26.44±5.98 |
| 10% | 41±6.48 | 38.33±4.96 | 39.66±5.67 |
| 15% | 66.33±9.87 | 71.18±4.34 | 68.75±7.69 |
| 20% | 93.66±5.08 | 91.66±4.92 | 92.66±4.88 |
| mean± SD | 38.07±34.7 | 37.98±34.72 | 38.03±34.51 |
| LSD(P<0.05) | 2.407 | | 4.169 |
| LSD for Interaction | 5.896 | | |

### Table (4-35) Effect of different concentration of aqueous extract of *Capparis spinosa* flowers on fungi growth by MIC methods

| Type of extract and concentration | Type of fungi Growth inhibition (mm) | | mean± SD |
|---|---|---|---|
| | *A.niger* | *A.ochraceus* | |
| Control 1 | 0±0 | 0±0 | 0±0 |
| Control 2 | 1.11±0.99 | 0.92±0.83 | 1.01±0.88 |
| 5% | 31.45±3.29 | 21.16±3.65 | 26.3±6.31 |
| 10% | 49.33±9.11 | 49.66±2.8 | 49.5±6.43 |
| 15% | 73.66±3.2 | 67.05±3.71 | 70.35±4.78 |
| 20% | 95±5.51 | 89.33±7.31 | 92.16±6.84 |
| mean± SD | 41.76±35.91 | 38.02±34.12 | 39.89±34.83 |
| LSD(P<0.05) | 2.040 | | 3.534 |
| LSD for Interaction | 4.998 | | |

**Table (4-36) Effect of different concentration of methanolic extract of *Capparis spinosa* flowers on fungi growth by MIC methods**

| Type of extract and concentration | Type of fungi Growth inhibition (mm) | | mean± SD |
|---|---|---|---|
| | *A.niger* | *A.ochraceus* | |
| Control 1 | 0±0 | 0±0 | 0±0 |
| Control 2 | 0.92±0.83 | 0.74±0.9 | 0.83±0.83 |
| 5% | 23.33±4.76 | 25.03±4.98 | 24.18±4.72 |
| 10% | 36.66±7.47 | 41.33±4.27 | 39±6.29 |
| 15% | 67.66±2.87 | 67.5±9.73 | 67.58±6.84 |
| 20% | 94±6.69 | 91.78±4.96 | 92.89±5.73 |
| mean± SD | 37.09±35.02 | 37.73±34.41 | 37.41±34.47 |
| LSD(P<0.05) | 2.340 | | 4.053 |
| LSD for Interaction | 5.732 | | |

# Chapter One

# Introduction

# Chapter Two

# Literatures Review

# Chapter Three

# Material

# and

# Methods

# Chapter Four

# Results

# and

# Discussion

# Conclusion

# and

# Recommendation

# Summary

# References

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