The effectiveness of Pleurotus ostreatus extract and two types of bacteria Staphylococcus aureus and Escherichia coll in inhibiting aflatoxin B1 on local or imported zea mays in Kirkuk city / Iraq

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Abstract

This study was conducted at the University of Kirkuk | College of Education for Pure Sciences | Department of biology, for the period from 3-11-2022 to 12-30-2022. The study included the use of two types of bacteria Staphylococcus aureus and Escherichia coli and an extract of Pleurotus ostreatus in the inhibition of aflatoxin B1 on local and imported maize corn grains. It included collecting about 3 kilograms of local and imported corn in the city of Kirkuk /Iraq. The contaminated fungi were isolated from the local and imported zea mays and were diagnosed morphologically and partially.

The results of the quantitative assessment of aflatoxin B1 in local and imported maize corn grains were shown by the high-performance liquid chromatography technique, as it showed the results of the concentration of aflatoxin in local corn 18.99 ppb, which exceeded the permissible limit according to the instructions of the American Food and Drug Administration (FDA) and the World Health Organization (WHO). As for the imported, the percentage of aflatoxin B1 was 6.28, and it is from within the permissible limit.

The results of examining 31 isolates of the fungus showed their ability to produce 18 isolates to produce aflatoxin about 60%, which came from zea mays grains the results of the ammonia vapor test showed that the isolates flaves Aspergillus and Aspergillus niger were among the most producing isolates of aflatoxin B1 as they appeared in a walnut red . The results of the alcoholic and aqueous extract of Pleurotus ostreatus showed effectiveness in inhibiting the radial growth of the fungus Aspergillus flORvus the concentrations of the alcoholic extract about %200_%100_%50_%25 as it led to inhibition of 20mm - 16mm _10mm – 0mm, respectively. As for the aqueous extract, its concentrations were %20_%15_%10_%5 which led to inhibition of 20mm – 18mm – 10mm – 8mm, respectively. As for the isolation of Aspergillus niger it did not respond to the aqueous and alcoholic extract of Pleurotus ostreatus. The results of some types of bacteria were shown which are Staphylococcus aureus at concentrically and 0.5 Mcfarland (1.5 x 10) cfu/ ml 0.5 Mefarland (3x10) cfu/ml and Escherichia coli bacteria Mcfarland(1.5 0.5 10) cfu and 0.5 Mefarland (3x10) cfu/ml as it is through experiments that conducted in the laboratory between these two types of bacteria have no effect in inhibiting the radial growth of fungal colonies A. Flanvas and A.niger

Keywords: *local corn, imported corn, Pleurotus ostreatus, Staphylococcus aureus bacteria and Escherichia coli bacteria.*

INTRODUCTION

Zea mays occupies the third rank after rice and grain in terms of production and cultivated area in the world, as it is considered one of the most important food crops for animals and human (FAO, 2021; FAO, 1992) zea mays crop faces many problems during harvesting and after storage. One of the most important innate problems, the Aspergillus flavus which is considered one of the first fungal species that cause and attack maize crops, thats due to its high ability to produce large quantities of spores which are carried in the air and then reach the crop, whether in the field or store (Cocker et al., 1984). The grains of maize corns are contaminated with fungi and mycotoxins, so they go through two phases: the first represents the growth and development of the crop due to physiological stress, and the second: when the crop is exposed to high humidity and warm temperatures as well as rain after the crop is ripe before or after harvest during storage or transportation at any time that is consumed (Cotty, 2001). Maize corn is one of the most fungal-infected crops compared to wheat and rice (Saleh et al, 2009), and these grains represent a good nutrient medium that allows the development and growth of pathogens, in addition to the environmental conditions of the maize corn crop whether in the store or the in the field, which will help to pollute corn with toxins (, Chulze, 2010) and that fungi have damages, including causing damage to food, fodder, and grains and not only that, but also secrete toxic metabolites, so it is necessary to search for safer methods, as biological methods are considered one of the most important methods used in control

(Blagojev et al,2012) that mycotoxins exceeded 400 compounds (Zain, 2011) and one of the most common toxins present in food and feed which is considered the most dangerous to the health of humans and animals, is Aflatoxins, Ochratoxin, Fumonisin, Zearlenone, Deoxynivalenol (Richard 2007) and that among the most dangerous types of mycotoxins are aflatoxin which are secondary metabolites that are produced mainly by fungi Aspergills Flavus, Aspergills Parasiticus (Levin, 2012) contamination of foodstuffs and feed with toxins has harmful and carcinogenic effects on the liver (Van Egmnod & Jonker 2004) and that the harmful effects of aflatoxins many research efforts have been directed to study the means and strategies that work to reduce the growth of fungi as well as prevent the production of aflatoxins and remove pollution (Holmes et al. 2008) and that many fungi cause spoilage of grains and food, in addition to health damage to human and animals, as they change the food taste and color, in addition to a change in its smell, as well as the secretion of toxic products, so the searches for antimicrobials have become of plant origin which encourages researchers to rely on them especially the development of pathogens factors from resistance of antibiotic (Kumar et al, 2017), so it was necessary to search for several means or methods that are considered safer and including biological methods in mold control (Blagojev et al. 2012)

Studies have shown that there are many medicinal plants and microorganisms that contain various biologically active compounds such as alkaloids tannins, flavonoids, terpenoids, phenols, sapindales and glycosides and others, (Chitemerere & Mukanganyama 2020, Tukur, 2011) among the medicinal plants is Pleurotus ostreatus which represents the biological control fungus from infection with contaminated fungi and their toxins.Pleurotus ostreatus are characterized by the fact that they contain substances that activate the immune system, as well as anti-

fungal substances and mycotoxins (Daba et al. 2009), as for the medical aspect, this mushroom is considered one of the medicinal fungi that contain effective and defensive substance it is used as an anti-toxin and anti-inflammatory (Gregori et al.2007) therefore the most important objectives of this research ia as follows :

1. Estimation of aflatoxin B1 toxins exists in local and imported zea mays by HPLC.

2. Studying the ability of some types of bacteria and pleurotus ostreatus extract to inhibit aflatoxin B1 in local and imported corn.

Materials and Work Methods

Sample collection

Samples were collected randomly in the period from 1/3/2022 to 8/3/2022 from local corn from the feed store for birds and poultry in the city center of Kirkuk, as well as corn imported from separate places and shops in the city of Kirkuk, Nawras type originating in South Africa and packed in Erbil, samples were collected in paper bags and sealed then brought to the laboratory and kept in a laboratory temperature.

Quantitative determination of aflatoxin B1 by high-performance liquid chromatography. HPLC

Aflatoxins were extracted from the samples according to the following steps

1. The weight of 25 grams of the sample was taken after drying and grinding and placed in a 50 ml beaker.

2. - 25 ml of methanol and 25 ml of chloroform were added to it.

3. It placed on the vibrator for 60 minutes for the purpose of homogenization.

4. The samples were filtered with No Whatman1 filter paper and 25 ml of methanol 90% and separated by Separating Funnel.

5. Transferring the filtrate to a Separating Funnel adding 1 ml of hexane and 25 ml of methanol 90% and separating by Separating Funnel.

6. The bottom layer containing methanol is taken and dried in a water bath.

7. Take the sample and add chloroform and distilled water 25:25 ml Separating Funnel and wash twice with distilled water after shaking the funnel and leave it until the two layers separate (neglecting the upper layer).

8. The bottom layer of chloroform was passed through filter paper of 10 g of sodium sulfate anhydrous.

9. The filtrate was taken and evaporated until drying in a water bath.

Detection of mycotoxins using HPLC High-performance liquid chromatography technique:

The examination was conducted in the laboratories of the Ministry of Science and Technology – Department of Environment and Water and according to the method presented by the scientist using a high-performance liquid chromatography device (SECAM – German origin) where the carrier phase was used acetonitrile: distilled water 30-70 and the separation column was C18-ODS (25cm*4.6mm)

To separate mycotoxins and using fluorescence reagent :

(ex=365nm , em=445nm) detection of mycotoxins where the flow velocity of the carrier phase was: 0.7ml/min according to the concentration of the toxin compared to the

standard poison and according to the special equation (Liu et al., 2012)

$$\text{AFB1 concentration} = \frac{\text{standard substance concentration X area of the Model}}{\text{Standard substance area}} \text{ X } \frac{\text{Dilution factor}}{\text{Model size}}$$

Detection of Aflatoxin toxins B1 based on a ammonia hydroxide test:

In this method if the fungal isolates were grown on the center of grape suger and embraced for

Seven days and at a temperature ranging from 25_27 degrees Celsius and after the incubation period 1_2 drops of ammonia hydroxide solution were placed on the cover of the dish, the dish was turned over and placed in the incubator for 24 hours, where a change was observed in the underside of the colonies that tend to walnut red and this indicates that the colonies produce aflatoxin. While there are some colonies where no color change occur which indicates that it is not productive, where the most productive isolates that produced the most aflatoxin B1 were selected, as they were the most productive isolates are the isolates of A. Flavus and A. Niger

Aquatic extraction of effective compounds:

The active compounds were extracted in the sample and dissolved in the distilled water by a method called (Digestion) according to the source, where the fungus was weighed, which weighed 65 grams and placed in a beaker containing 600 milliliters of distilled water, the beaker is placed with its contents on a moderate heat source whose temperature does not exceed 50 degrees Celsius for two hours, with continuous stirring to ensure the extraction of most of the effective and compounds and water soluble, after that cooling then filtering with filter paper, then drying the yield by lyophilization in a way known as (lyophilization) using a special device called (Lyophilizer) and the weight of the dry yield which weighed 12 grams and is kept in a dry airtight container away from heat and moisture until the steps of statements and other analyzes are performed to the substance .

Alcoholic extraction of active compounds:

65 grams of grind mushrooms have been weighed and put ethanol alcohol (600 ml) the Soxhlet extractor was connected and operated at a temperature not exceeding 70 degrees Celsius, which is the boiling point of alcohol, the extraction process continued for 8 hours, and then the device saturated with plant matter was cooled and evaporated, this process was repeated several times to ensure all compounds of effective powdered mushrooms.

The alcohol layers from which the active compounds were extracted and evaporated by rotary evaporator were collected and the yield was weighed which is 4.5 grams, in a dry container sealed away from light, heat and moisture until carry out the steps of the disclosures and other analyzes of the substance .

1-6-1_3 Efficiency test of the effect of alcoholic and aqueous extract of Pleurotus ostreatus on isolates fungus the most aflatoxin producer A. Niger and A.favus B1

Four concentrations were chosen of the alcoholic and aquatic extract of Pleurotus ostreatus which are as shown in tables (6 and 7) to show it’s effect on the inhibition of the fungal isolates A niger and A.flavus when the Agar well method was used for diffusion by the digging method, as 1 ml of the isolates Fungs was taken

And mixed with agar and placed in a Petri dish, mixed well and left to solidify after the medium hardened a drill was made by corkscrew with a diameter of 6 ml if 100ml of extracts were added by micropipettes in each of the drills and placed in the refrigerator for 30 minutes to allow the extracts to spread well and then incubated the dishes then in the incubator at temperatures ranging from 25_27 degrees Celsius for a period of 5_7 days after that, the inhibition area is measured through a numbered ruler that appeared after the incubation period.

Percentage of inhibition % = Average colony diameter compared_Average diameter of the treated colony

_____ X 100

Average colony diameter compared

Table (6)

Fungi isolates	Effect of aqueous extract of Pleurotus ostreatus on the inhibition zone			
	C4=5%	C3=10%	C2=15%	C1=20%
(Z) A.flavus (G) A.niger	8	10	18	20
	–	–	–	–

Table (7)

Fungi isolates	Effect of alcoholic extract of Pleurotus ostreatus on the zone of inhibition			
	C4=25 %	C3=50 %	C2=100 %	C1=200 %
(Z) A.flavus (G) A.niger	0	10	16	20
	–	–	–	–

Table (8)

Fungi isolates	Effect of S.aureus bacteria on the zone of inhibition (mm)	
	C2=0.5 Mcfarland (3 x10^8CFU/ml)	C1=0.5 Mcfarland (1.5x10^8CFU/ml)
(Z) A.flavus	–	–
(G) A.niger	–	–

Table (9)

Fungi isolates	Effect of E.coli bacteria on the zone of inhibition (mm)	
	C2=0.5 Mcfarland (3x10^8CFU/ml)	C1=0.5 Mcfarland (1.5x10^8CFU/ml)
(Z) A.flavus	–	–
(G) A.niger	–	–

Statistical analysis

The data was analyzed statistically by using a computer based on SPSS program and using ANOVA according to the F test, in order to test the importance or unimportance of the groups used in the study at the level of probability ($P < 0.05$) and the level of probability ($P < 0.01$), and it was compared to Duncan's multiple range test (Al_rawi, 2000)

Results and discussion

Detection of aflatoxin B1 based on the hydroxide ammonia test:

In this method if the fungal isolates were grown on the center of grape suger and embraced for Seven days and at a temperature ranging from 25_27 degrees Celsius and after the incubation period 1_2 drops of ammonia hydroxide solution were placed on the cover of the dish, the dish was turned over and placed in the incubator for 24 hours, where a change was observed in the underside of the colonies that tend to walnut red and this indicates that the colonies produce aflatoxin. While there are some colonies where no color change occur which indicates that it is not productive, where the most productive isolates that produced the most aflatoxin B1 were selected, as they were the most productive isolates are the isolates of A.flavus and A.niger in both local and imported zea mays grains, and this study agreed with (Entizar Al-Eidani 2014) If a change appears in the rules of the colonies and the appearance of the orange color in different degrees, and this gradation indicates the ability of the isolates to produce aflatoxin, and this is shown by all (Maehida& satio, 1999), that showed that the degree of red color is attributed to the quantities producing the toxin, so the isolates that has a dark red orange colour indicates that the isolate has a large production of aflatoxin , but the isolates whose bases are light red or pink are less toxic, a test of 31 mushroom isolates showed their ability to produce 18 isolates to produce aflatoxin by 60%, which are sourced from zea mays grains, and this study is consistent with the study of (Al-Adil, 1997) if it found 59% of the toxin-producing isolates of aflatoxin, and also this result is consistent with the study of (Al-Saadi, 2012) who indicated that the highest percentage of toxin-producing fungi isolates was sourced from zea mays and beans, and also this study is consistent with Result of (Al-Worshan et al. 2002) shows the response or lack of response of the isolates to ammonia vapor.

Table (12) shows the results of isolates producing aflatoxin B1 using ammonia vapor

No.	Isolates No.	Response to ammonia vapor	No.	Isolates No.	Response to ammonia vapor
1	Local zea mays G A.niger	_	17	Local zea mays	_
2	Local zea mays A.niger G	+	18	Local zea mays E	_
3	F	_	19	Local zea mays E	_
4	Local zea mays Z A.flavus	+	20	Local zea mays Z A.flavus	+
5	Local zea mays G A.niger	_	21	Local zea mays E	+
6	Local zea mays F	_	22	Imported zea mays 3	+
7	Local zea mays Z A.flavus	+	23	Local zea mays Z A.flavus	+
8	Local zea mays F	+	24	K	+
9	Local zea mays E	_	25	I	+

10	Local zea mays G A.niger	_	26	H	+
11	F	+	27	J	+
12	E	+	28	Imported zea mays 5	_
13	G A.niger	+	29	Imported zea mays 6	+
14	Imported zea mays 4	+	30	Local zea mays G A.niger	_
15	Local zea mays Z A.flavus	+	31	E	_
16	Local zea mays G A.niger	_			

Qualitative detections of Pleurotus ostreatus extract

The extracts affecting the active compounds were investigated by using chemical reagents.

Pleurotus ostreatus appeared to contain a number of active ingredients such as Tannins, Saponins , Glucosides , phenols, Alkaloids, Resins, Flavonoid, Carbohydrates, Coumarins, etc shown in Table (13)

Results:

Tests name	**Reagent**	**Alcoholic extrac**	**Results of aqueous extract**	**Inredicaton**
Alkaloids	**a_Dragangrof b_Mayer c_Hager**	**_Ve**	**_Ve**	**a_Orange_brown ppt b_Yellow ppt c_Yellow_ppt**
Flavonoids	**a_7.5%Fecl3 Solution b_Alkaline Reagent (NaOh)**	**+Ve**	**+Ve**	**a_Dark color b_Yellow**
Polyphenolic Compounds	**a-ferric chloride 5% Solution**	**+Ve**	**+Ve**	**Brown color**
Tannins	**a-Lead acetate 1%Solution**	**++Ve**	**+Ve**	**a-creamy precipitate b-Dark color**
Saponins	**a-Foam formation b-1%Mercuric Chloride Test**	**++Ve**	**+Ve**	**a-Foam b-White ppt**
Polysaccharides Carbohydrates and glycosides	**a-nthroneTest; b-Benedict reagent C-Keller-Kiliani Test**	**+Ve**	**+Ve**	**a-Green color b-Reddish-brown ppt c-Reddish-brown ring**
Steriod Tests	**a-Sallowskis Test**	**+Ve**	**+Ve**	**No Red Color**
Proteins & aAmino Acid tests	**a-Ninhydrin b-Biuret Test**	**+Ve**	**+Ve**	**a-Blue Color b-Purple Color**
Coumarins	**10% NaOH Solution**	**+Ve**	**+Ve**	**Yellow Color**

Effect of aqueous and alcoholic extract of Pleurotus ostreaties on aflatoxin B1-secreting fungi isolated from zea mays grains:

1- The effect of a aqueous extract of Pleurotus ostreatus on the radial growth of AFBI toxin-secreting fungi

The results of treatment with the aqueous extract of Pleurotus ostreatus showed against types of the most productive fungi of aflatoxin B1 with concentrations of 20%_15%_10%_5%

Against isolated fungi which are A.flavus and A.niger, as the percentages of the concentrations of the aqueous extract against A. Flavus are 20%_15%_10%_5% where the aqueous extract led to an effect on the radial growth of the diameter of A.flavus , which reached the diameters of the fungal colonies and inhibition ratios were 20mm – 18mm – 10mm – 8mm, respectively, which led to significant changes in the radial growth. As for A. Niger diameter, it did not lead to any significant changes in the radial growth of fungal colonies, meaning that the aqueous extract of Pleurotus ostreatus did not lead to change or inhibition of A.niger isolate .

Table (14)

Fungi isolates	Effect of aqueous extract of Pleurotus ostreatus on the inhibition zone			
Focus%	C1=20%	C2=15%	C3=10%	C4=5l
	20	18	10	8
(Z) A.flavus (G) A.niger	–	–	–	–

Effect of alcoholic extract of Pleurotus oestreatus on the radial growth of AFBI toxin-secreting fungi

The results of the treatment with the alcoholic extract of Pleurotus ostreatus against the most Aflatoxin B1-producing fungi and attributed concentrations were 200%_100%_50%_25% against the fungi isolated from Zea mays grains which are A. Flavus and A.niger, as the ratios of the concentrations of the alcoholic extract against A. Flavus led to significant changes that affected the radial growth and they had diameters of -20mm, 16mm, 10mm – 0mm, respectively, as for A.niger the alcoholic extract of Pleurotus ostreatus did not leads to an effect or significant changes on the radial growth of the fungal isolate

Table (15)

Fungai isolates	Effect of alcoholic extract of Pleurotus ostreatus on the zone of inhibition			
Focus%	C1=200 %	C2=100 %	C3=50 %	C4=25 %
(Z) A.flavus (G) A.niger	20	16	10	0
	–	–	–	–

The use of some types of bacteria in the inhibition of aflatoxin B1 isolates that produce most toxins :

During our study two types of bacteria were used which are Staphylococcus aureus and Escherichia coli (Muhammad Jassem ,Mahdi, Najdat Bahjat 2022) as it was through experiments conducted in the laboratory that these two types of bacteria had no effect in inhibiting the radial growth of A.flavus and A. Niger colonies , the most productive of aflatoxin B1 .Table (16 and 17) shows the concentrations that were used in the experiment:

Isolate No.	Effect of S. Aureus inhibition zone(mm)	
	C1=0.5 Mcfarland (1.5 x 10^8CFU/ml)	C2=0.5 Mcfarland (3x10^8CFU/ml)
Z/ A.flavus	_	_
G/A.niger	_	_

Isolate No.	Effect of E.coli inhibition zone(mm)	
	C1=0.5 Mcfarland (1.5x10^8CFU/ml)	C2=0.5 Mcfarland (3 x10^8CFU/ml)
Z/A.flavus	_	_
G/A.niger	_	_

The use of Hplc technique to quantitatively estimate aflatoxin B1:

The mycotoxin aflatoxin B1 was quantitatively estimated in both local and imported maize by using a High-Performance Liquid Chromatography device (HPLC), this method is suitable and good for conducting the process of separating all types of compounds as it has many advantages including the multiplicity of columns available and used, as well as its sensitivity and the possibility of using it in quantitative analyzes with high accuracy, as well as its ability to separate non-volatile and non temperature-tolerant materials, as it was used in several fields, including health, food, and agricultural in addition to the environmental field (Stanciu et al. 2007, Hao et al. 2008) as the results of the quantitative estimate of aflatoxin B1 toxins for local and imported maize grain showed varying changes and the most prominent of which represent the type of strain, temperatures, humidity and pH (WHO , 1987)

Results of the estimate of aflatoxin B1 toxins

The results of the quantitative assessment of aflatoxin B1 for each of the local and imported zea mays grains during our study showed that they are contaminated with AFBI toxins as the concentration of Aflatoxin B1 toxins

In local maize was 18.99 ppb, which has exceeded the permissible limit for human consumption, the US Food and Drug Administration (U.S.FDA) recommended that the amount of aflatoxin in maize that is prepared for human or animal must not exceed (10) parts per billion (ppb) this is for the purpose of reducing the seriousness of these toxins. As for the amount of toxins in the imported zea mays grains, Nawras type , its origin is (Turkey – packing Erbil) the percentages of toxins estimated by Hplc device was (6.28ppb), which is less than the allowable limit set by the US Food and Drug Administration, thus it is fit for human consumption, as shown in Table (18)

The amount of aflatoxin B1 in local and imported maize grains with the HPL apparatus

No	**Name**	**(ppb) AFBI**
1	Imported zea mays	6.28
2	Local zea mays	18.99

(Broggi et al, 2010) mentioned through a study that he carried out on maize samples, that 80% of the seeds were isolated from A.flavus fungi the producer of aflatoxin which indicates the extent to which it is likely to contain aflatoxins, as (Zeina Najm El Din, Noureddine, Hadi Alwan, 2022) showed that the percentage of AFBI toxins in samples of walnuts, almonds, hazelnuts, cashews, field

pistachios, and pistachios was contaminated with AFBI toxins, where the concentration of AFB1 was in samples of field pistachio nuts, cashew nuts and almonds (148.97, 120.66, 69.58, 44.89, 41.25, 36.89) ppb respectively and all concentrations exceed the permissible limit for human consumption ppb 20 (WHO, 1978).

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