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The inhibitory effect of *Pleurotus ostreatus* on the growth of *Aspergillus niger* and the production of Ochratoxin A. and isolated from apples

Sadeq Mohammed Ali Akram Ali Mohammed Hisham Romil Mutib

Department of Plant Protection, Faculty of Agriculture ,University of Kufa ,Republic of Iraq

Abstract

The objective of this study was to explore and diagnose the mycotoxic from apple in order to decreases their toxic effects. The results showed the two species of fungi which included , *Aspergillus niger* and *Rhizopus stolonifer*. It was found that there was a significant appearance of *A. niger*, followed by *Rhizopus stolonifer*. With a frequency of 84.21% and 15.79% respectively, while the rate Percentage of appearance was 100% and 37.5%, respectively. . The results of chemical analysis of TLC showed that 5 isolates out of the16 tested isolates of *A. niger* wrer a produced of Ochratoxin A with a production rate of 31.25%. The results also showed that *Pleurotus ostreatus* was high resistance against *A. niger* the nutritional medium Potato Sucrose Agar , Where covered the entire area of the dish and were not allowed to *A. niger* to grow, and showed a degree of contrast (1). The results showed that *Pleurotus ostreatus* was in high efficient reducting the Ochratoxin A, which was produced by *A. niger*. The efficiency of the reduction in the Spectrophotometer was varied with the treatment of nine and six tablets of the fungus 100 ml^{-1} of the PD medium significantly in the reduction of 74.75% Respectively, compared to the treatment of three tablets of the fungus 100 ml^{-1} from the center of PD with reduction rate of 61.90%.

Keywords (apple ,*Aspergillus niger*, Ochtratoxin A, *P. ostreatus*)

Introduction

Mycotoxins are an important secondary metabolites produced by fungi because they have a harmful effects on the public health, cause chronic and have a severe effects on the liver, kidneys and immune system, causing kidney failure, liver cancer and weak immune system when they are exposed to high doses of mycotoxins (22).

The most of fungi that produced mycotoxin are *Aspergillus*, *Penicillium*, *Alternaria* and *Fusarium*. These fungi grow on a number of food products and produce fungi produced mycotoxin a number of mycmtoxin . (5). has used many methods to prevent infection and contamination of various fruits with fungus-producing mycotoxin , which including physical methods by treating fruits with hot water or exposing them to various rays, as well as use some preservatives. Microorganisms, especially bacteria, were also used in the treatment of fruits by dipping those fruits with of some bacterial species that have antagonistic activity towards fungal species that produce mycotoxin and contaminate their fruits (12)

Pleurotus ostreatus is one of a fungi that has medicinal benefits because it contains effective extracts or compounds and is characterized by containing antimicrobial (19) Indication it significant and mycotoxins . has effect in reducing the cholesterol level in the serum (8). and in treating many human diseases for a long time in many countries, especially India, China and Japan, as a results importance in the evaluation of the immune system of the human body (3).this fungi has Antiviral, bacteria and fungi and their mycotoxins . such as the 1-octen-3-ol-compound, which is antibacterial and present in the body and the 4-Methoxy benzaldehyde, are present in the fungal mycelium (23). It also plays a role in the destruction of mycotoxins in the media where the fungi is grow which produces this fungus by the enzymes it is that's produced (20,14). As well as its ability to treat parasites, including worms, where it is remove metabolic toxins and then attacks these worms (7). *P. ostreatus* has appositive effect in

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treatment of cancer prevention of tumor growth (24). Species belong and the speed of growth of the mycelium on different irrigated media and production in different environmental conditions (13). At present, it is second only to *Agaricus bisporus* and 25% (18), it is unique in its high nutritional value because it is rich in protein, which accounts for 40-40% of dry weight (1)

Materials and Methods.

1. Potato Sucrose Agar (P.S.A.)

200 g of potato tubers, were cut into small pieces and boiled with 500ml distilled water for 20-30 minutes in a glass beaker. After the boiling period, then filtered content by a piece of gauze. 10 g of sucrose and 17 g of agar were dissolved in 500 ml of distilled water and then the potato leach and full size to liter. The media was distributed in flasks according to its use, and their vents were sealed with cotton bolts and sterilized with the sprinkler device at 121 ° C and 15 lb / kg for 20 minutes. After that the flasks were left to cool and then placed in the refrigerator until use. This medium was used to isolate and grow the fungi.

2. Potato Sucrose Broth (P.S.B.)

The media was prepared using the same method described above but without adding agar ,this media was used to grow and obtain its secondary metabolites .

Isolation and diagnosis of fungi associated with apples

Apple samples were brought (10 samples) from local markets and were isolated by direct culture on P.S.A. The fruit were cut into small pieces (1 cm) and sterilized with sodium hypochlorite solution (2%) for 2 minutes. After that, washed with distilled water and placed on filter papers to dried. These pieces were placed in petri-dishes with PSA (five piece in each dish were then incubated at 25C˚ for three days. After the of incubation period, growing isolates were purified. and identified according to the taxonomic characteristics cited by both (21,10).

The percentage of appearance and frequency of fungi were calculated according to the following equations:

$$\text{Percentage of appearance (\%)} = \frac{\text{Number of specimens showing sex or gender}}{\text{Total number of samples}} \times 100$$

$$\text{Percentage of frequency (\%)} = \frac{\text{Number of isolates of each species}}{\text{Total number of isolates of all fungi}} \times 100$$

Detection of Ochratoxin A using thin layer chromatography (TLC)

The procedure described by Balzer et al. (4) was used to detect of Ochratoxin A, as following:

1. *A. niger* was grow on the center of the PSA by placing a week-old tablets of fungus with 5 mm diameter at the center of each petri- dishes. the process was replicated three times for each fungal isolate. Then all petri-dishes were incubate at (30 ± 2)° C for a week . Later three dishes were taken and the media was cut by a sterile knife to small pieces. and then transferred by sterile needle to blander containing 100 ml of acetonitrile water solution (90: 10) for 10 minutes .

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2. The extract was filtered through the filter paper of Whatman No. 4.
3. The mixture was placed in 250 mL Separatory Funnel, and 25 mL of hexane was added eliminating of the fat. It was shacked gently for 30 seconds, with the accumulated gases being expelled whenever needed (twice at least) it was left on the stand for a minute to separate the two layers, neglected the upper layer and took the bottom layer and repeated the process for three times .
4. 25 mL of distilled water, 8 mL NaClO3 and 25 mL of chloroform were added to the bottom layer. Three minutes later, two upper and lower layers were separated and the lower layer was kept. The process was repeated twice.
5. extract was Transfered to a 100 mL separating funnel and 15 ml of the standard hydrochloric acid 1, and 20 ml of chloroform were added and shakers for a minute. The bottom layer was removed and 20 mL chloroform was added to the top layer and re-extracted. The bottom layers were collected and passed through a filter paper on a layer of NaOSO4 for disposal of the remaining water.
6. The mixture was taken and places in an electric oven at 40 C° until the dried and then solvent in 1 ml of chloroform. The presence of mxytoxins was detected using TLC (20×20 cm) with platelets activated at 105 ° C for an hour before use (11). Using a separation system for Ochratoxin A, which included toluene: chloroform: ethyl acetate: formic acid at volume ratio 35: 25: 25: 15.respectively A light straight line was made on a TLC plate 1.5 cm from the base of the plate and 15 microliters were taken by a capillary tube of the standard OchratoxinA toxin and placed on 2 cm from the left edge of the plate and 2 cm from the spot of the standard toxin. Samples *A niger* at the same distance and quantity equal to the amount of the standard poison, then extract was placed the spots were left to dry and then placed in the basin of the chapter containing the separation system referred to above and was monitored until the separation system reached a distance of about 2 cm from the top end of the plate, they were took off and dried at Laboratory conditions for 5 minutes and then treated with alkaline solution (sodium hydroxide) and then examined under UV radiation with 360 nm a wavelength Ochratoxin A was dected by deportation Rf coefficient and sparkle color standard for color with antivenom and deportation of extract samples Isolates for fungal from Ochratoxin A.

Preparing of *Pleurotus ostreatus*

Pure isolation of the fungus (*Pleurotus ostreatus*) was obtained from Laboratory of fungi in the Department of Plant Protection - Faculty of Agriculture, University of Kufa, and increased isolation on the culture medium P.S.A

culture media used for isolation, development and conservation of oyster fungi

Testing the antagonism *P. ostreatus* against *A.niger* on P.S.A.

A number of fungi were obtained from the fungi laboratory / Plant Protection Department. Antimicrobial activity of *P. ostreatus* against *A.niger* in culture medium P.S.A by inoculation with *P. ostreatus*, by taking 0.5 cm disk from the fungus three days, aye and inoculation with *A.niger* three days. Each inoculums in the center of half petri dishes. The experiment was carried out with three replicates. The dishes were incubated at 25 ± 1 C° for three days.

The antagonism actuate was estimated according Bell (6)

Class Specifications

1- *P. ostreatus* fungus covers the entire area of the dish without allowing the isolated fungus to grow.

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2 -*P. ostreatus* fungus covers two thirds of the area of the dish, and the isolated fungus cover the remaining third of the dishes.

3- *P. ostreatus* fungus covers half the area of the dish, and the isolated fungus cover the other half of the dishes.

4- *P. ostreatus* fungus cover one third of the area of the dish, while the isolated fungus cover the remaining two thirds of the dishes.

5- Insulated mushrooms cover the entire dishes.

The fungus is effective in terms of resistance when showing a degree of contrast (2) or less with isolated fungus species.

Effect of *P. ostreatus* on the toxin rate of Ochratoxin A

A. niger isolates produced the Ochratoxin A have been grown in 12 flasks (100 mL PSB flask $^{-1}$) for 14 days. The mixture was then filtered through a gauze piece and then inoculated by taking a 0.5 cm disc from the three-day *P. ostreatus* culture . The treatments were as follows: without adding (comparative treatment), (3, 6 and 9) tablets. With three replicates per treatments all petri dishes were incubated at 25 ± 2 C° for a week. The Ochratoxin A was extracted by taking 100 ml of each treatment and then transferred to an blander containing 100 ml of chloroform. The mixture was mixed for 10 minutes. and then filtered by filtration papers and the placed in 250 mL Separatory Funnel. And shakers gently for 30C˚ Then remove the mixture and place in a clean, sterile flask and place in an electric oven at a temperature of 30 C°. Heat 40 C° to dry, then dissolve in 5 ml of chloroform (12). (Spectrophotometric spectroscopy). This technique was based on spectroscopy, which depends on the properties of the compound to absorb light in the field of UV or IR radiation, where there is a direct correlation between absorption time and concentration of poison. During a standard curve between light absorption and concentration of poison and extract the concentration value corresponding to the reading given by the anonymous sample. The readings were compared with standard poison concentrations where the wavelength was 365 nm and the reduction ratios were calculated using of the following equation:

$$\text{Percentage of Reduction} = \frac{\text{Concentration of the control treatment - the concentration of the model}}{\text{Concentration control treatment}} \times 100$$

Statistical analysis: The data were carried out according to the complete random design as a single-factor experiment. The averages were compared using less significant difference (LSD) at 5% level of significance ($P< 0.05$) .(2).

Results and discussion

Isolation and identification of fungi isolates from apple fruit

The results of isolation and diagnosis that two species of fungi were isolated from apples fruits , *A. niger* and *R. stolonifer*.. the frequency rate was 84.21% and 15.79% to *A. niger* , *R. stolonifer* respectively, while the rate of appearance was 100% and 37.5%, respectively (Table 1). the dominance of *Aspergillus* in apples was due to its wide spread in the environment, which comes from its ability to produce a large number of spores that resistant to adverse environmental conditions, which form plankton in the air because its diameter is less than 15 nm and thus it can reach many places where it can enter the stores through the windows and other openings, as well as its growth in wide ranges of temperature and humidity, as some

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species of *Aspergillus* species are growing in ranges of temperatures ranging from 5 - 45 ° C or higher (10).

Table (1) Percentage of frequency and appearance of fungi isolated from apples

Fungal species	Frequency(%)	Appearance (%)
A. niger	84.21	100
R. stolonifer	15.79	37.5

Detection of Ochratoxin A production using thin Layer chromatography (TLC).

chemical analysis of TLC showed that 5 isolates out of 16 isolates of *A. niger*, were able to produce Ochratoxin A with a production rate of 31.25% (Table 2). However these isolates were varied in their ability production. An4 was the most productive of the toxin based on ultraviolet light intensity. Differentity of isolates in their production of Ochratoxin A may be some attributed to the genetic ability of isolate.

Table (2): Testing the susceptibility of isolates *A. niger* on production of Ochratoxin A isolated from apple fruits using thin Layer chromatography (TLC).

Fungal isolatie	ability of Ochratoxin A production	Fungal isolatie	ability of Ochratoxin A production
An1	-	An9	-
An2	-	An10	++
An3	-	An11	-
An4	+++	An12	-
An5	+	An13	+
An6	-	An14	-
An7	++	An15	-
An8	-	An16	-

(+) Isolate producer of Ochratoxin A, (-)Isolate not producer of Ochratoxin A

Testing the antagonism ability *P. ostreatus* against *A.niger* on P.S.A.

The results of this study showed that *P. ostreatus* have high antagonism rate aganist *A. niger* on the culture medium P.S.A. (1). (Figure 1) where it covered all petri dishes distance without allowing to *A. niger* to grow. These results were in agreement with Hussien (9) about the high resistance of *P. ostreatus* against fungi, where the fungus covered the entire area of the dish without allowing the fungus to grow. except. *T. harzianum*, where it covered two thirds of the dish and the fungus *P. ostreatus* covered the remaining third of the area of the dish, while the fungus *Mucor* sp and *Rhizopus* sp, covered one third of the dishes and *P. streatus* had covered two-thirds of the area of the other dishes.

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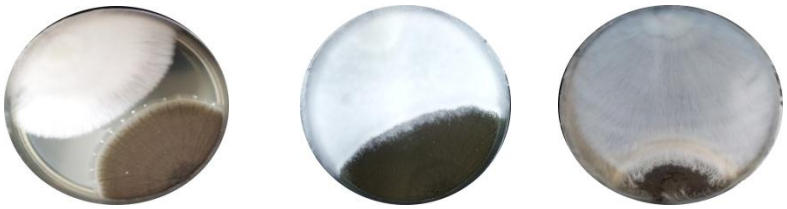

Figure (1) Antagonism ability of *P. ostreatus* against *A. niger* fungus on the culture medium P.S.A.

Effect of *P. ostreatus* on the Ochratoxin A rate production

The results in (Table 3 showed efficiency adding the of *P. ostreatus* in reducing of Ochratoxin A, which produced by *A. niger*. The efficiency of the reduction in the Spectrophotometer was varied between treatments where it was significant higher at nine and six tablets of the fungus 100 ml. 74.75% and 73.32%, respectively, compared with three tablets of the fungus 100 ml from the center of PD with reduction rate of 61.90%. (Table 3) The reason may be related to the fact that it, contain effective extracts or compounds and also antimicrobial, fungal and toxin substances (19), which reduces the toxicity of these fungi . The fungus has anti-viral, bacterial and fungal substances and its toxins, such as the 1-Octen-3-ol compound, which is present in the sporangia and the 4-Methoxy benzaldehyde, which is present in the mycilium (23). It has a role in the destruction of toxic substances in the medium where this fungi is grown by the enzymes produced by it (20,14). As well as its susceptibility to parasites, including thrombosis, as it removes metabolic toxins and attacks these worms (7). Many studies have demonstrated the efficacy of *P. ostreatus* in the adsorption of many Mycotoxin. T-2 toxin reduced by 68%, (16) and indicated the ability of the fungus to destroy the AFB1 toxin and extracted an enzyme from the fungus that had the effect of destroying the toxin.(17).

Table (3) Effect of *P. ostreatus* on the proportion of Ochratoxin A

Treatment	Amount of Ochratoxin A Microgram / 100 ml	Percentage of Reduction %
Control (without addition)	93.33	00.00
Three tablets of fungus *P. ostreatus*	29.33	61.90
six tablets of fungus *P. ostreatus*	18.70	73.32
nine tablets of fungus *P. ostreatus*	17.33	74.75
L.S.D.	9.80	4.73

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