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**RESEARCH ARTICLE**

# The suppuration impact of β-glucan biopolymer and *Saccharomyces cerevisiae* against gliotoxin secretion by *Aspergillus fumigatus*

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## *Abstract*

Feeds and their raw materials are susceptible to attack from many fungi that may affect them in terms of quality and economic value. Most of these fungi have the ability to produce numerous mycotoxins. In this respect two hundred and thirty five fungal isolates were isolated from random collected samples of wheat, corn, soybeans, feeds poultry and livestock from different Saudi markets in the summer of 2012. These fungal isolates belong to 22 species and/or 11 genera. The isolated fungi included 8 isolates of *Aspergillus fumigatus*. The fungal isolates were identified by classic method and BIOLOG Inc. System. The *Aspergillus fumigatus* re-identified using nucleotide sequences namely ITS1 and ITS4. The resulting sequences are matched with national center for biotechnology information (NCBI) database. To inhibit the toxic effect of *A. fumigatus* on feed materials, the baker's yeast strain *Saccharomyces cerevisiae* and β- D- glucan were used to reduce on the growth of *A. fumigatus* on MEA medium. β- D- glucan biopolymer treatment led to reduced growth between 65.43-85.6%, while the yeast reduced the growth of *A. fumigatus* within 33.3 to 50.8%. Scanning electron microscopes examination showed deformation in the mycelium, swelling, many branching and short as a result of β- D- glucan biopolymer treatment, while the effect of the yeast alone was less. β - D- glucan biopolymer treatment led to reduce the level of gliotoxin to 53% in the feed of ruminant, poultry feed (48.3%), corn (44%), wheat (43.7%) and soybean (42.3%) while, the results of yeast not exceed than 18.1%. The gliotoxin reduction measurements conducted by HPLC instrument. The investigation aims to find safety solution to reduce mycotoxins contamination in feed. This alternative protocol has not any effect on animal health and its productivity as well as away from pesticides that may contaminate soil, water, air and environment in general.



## INTRODUCTION

Molds can produce other secondary metabolites such as antibiotics and mycotoxins. Mycotoxins are not essential to maintaining the life of the mold cell in a primary way such as obtaining energy or synthesizing structural components, informational molecules or enzymes. They are products whose function seems to be to give molds a competitive advantage over other mold species and bacteria. Mycotoxins are nearly all cytotoxic, disrupting various cellular structures such as membranes, and interfering with vital cellular processes such as protein, RNA and DNA synthesis. They are also toxic to the cells of animals, humans and higher plants. Not all molds produce mycotoxins,

but numerous species do. Toxigenic molds vary in their mycotoxin production depending on the substrate on which they grow (Jarvis, 1990; Burge, 1990 and Yang, 1995).

*Aspergillus fumigatus* is one of the most common *Aspergillus* species to cause disease in individuals with an immuno-deficiency. *A. fumigatus*, a saprotrophic widespread in nature, is typically found in soil and decaying organic matter, such as compost heaps. Colonies of the fungus produce from conidiophores thousands of minute grey-green conidia (2–3 μm) that readily become airborne. The fungus is capable of growth at 37 °C (normal human body temperature), and can grow at temperatures up to 50 °C, with conidia surviving at 70 °C. Its spores are ubiquitous in the atmosphere, and it is estimated that everybody inhales several hundred spores each day; typically these are quickly eliminated by the immune system in healthy individuals. *A. fumigatus* grown on certain building materials can produce genotoxic and cytotoxic mycotoxins, such as gliotoxin. (Cui, et al., 1996; Jean-Paul, 1999; Nieminen et al., 2002 and O'Gorman et al., 2008).

Gliotoxin is a sulfur-containing mycotoxin produced by several species of fungi, including pathogens of humans such as *A. fumigatus.* Gliotoxin possesses immuno-suppressive properties as it may suppress and cause apoptosis in certain types of cells of the immune-system, including neutrophils, eosinophils, granulocytes, macrophages, and thymocytes. It also acts as an inhibitor of farnesyltransferase. It noncompetitively inhibits the chymotrypsin-like activity of the 20S proteasome (Scharf et al., 2012).

Many strains of yeasts as *Saccharomyces cerevisiae* were found to inhibit mycelia growth and ochratoxin production in YES medium. Moreover, a decrease in *pk* transcription was observed in the presence of living cells Cubaiu et al., 2012. *S. cerevisiae* inhibited mold growth only weakly at the highest inoculum level (Petersson and Schnurer, 1995).

*S. cerevisiae* show potential mycotoxins binders beside its nutritional value. Using only yeast cell walls instead of whole cells, the adsorption of mycotoxins can be enhanced. The cell walls harboring polysaccharide (glucan, mannan), proteins, and lipid exhibit numerous different and easily accessible adsorption centers as well as different binding mechanisms, e.g. Hydrogen bonds, ionic, or hydrophobic interactions (Huwig et al., 2001). β-D-glucan fraction of yeast cell wall is directly involved in binding process with mycotoxins and that the structural organization of β-D- glucan modulates the binding strength. (Yiannkouris et al.,2004a , Yiannkouris et al.,2004b and Jouany, 2007). It appears that carbohydrate components are common sites for binding, with different toxins having different binding sites. It is also known that toxin binding in *saccharomyces cerevisiae* is strain dependent. (Shetty and Jespersen, 2006). Soluble fraction of fungal membrane binding protein that modulates the biosynthesis of β-D-glucan of fungal cell walls and probably has a major role in the regulation of cell wall morphogenesis (Kang and Cabib, 1986).

This investigation aims to reduce contaminants such as mycotoxins using natural products do not have any side effect affect the quality of feed and the vitality and productivity of animal and human intake

## MATERIALS AND METHODS:

**Fungal isolation:**

*Aspergillus fumigatus* and other fungi isolates were isolated from different row materials feed grains as yellow corn, Wheat, soybean and fabricated feed pellets. The samples of grains, poultry and ruminant feed pellet collected randomly from Saudi markets in Riyadh at summer 2012 and all experiments were conducted by represented samples. Isolation trials were carried out for subsample (approximately 250 g.) after surface sterilization with (1%) sodium hypochlorite and incubated for 7 days on malt extract agar (MEA) at 25±°C according to Pacin et al., (2002). Fungal cultures were maintained on slants of malt extract agar (MEA) medium at 4°C.

**Fungal isolates identification:**

Fungal isolates were pre-identified according to the method of Samson and van Reenen-Hoekstra, 1988. Identification for all fungal isolates was re-confirmed by the Microloge system (Biolog, Inc., Hayward, CA). The pure fungal isolates were propagated onto Biolog Universal Agar (BUY) medium (Biolog, Inc.) and incubated at 25°C even the appearance of growth and sporulation (approximately 3-5 days). The filamentous fungi (FF) tubes are inoculated with loopful of tested isolate to form fungal spore suspension. The fungal spore suspension was quantified

with a turbidimeter. 100 ml of the fungal suspension solution were pipetted into each of the 96 wells in the Biolog microplates. the plates were incubated at 25°C for 5 days maximum and then read with an automated plate reader (Biolog, Inc.), assessed visually and identified to genus or species level.(Grizzle , 2006 and Sing, 2009)

Isolates of *Aspergillus fumigatus* were identified through DNA extraction, PCR techniques, sequencing and phylogenetic analysis of 18S (ITS; ITS-1; 5´-TCC GTA GGT GAA CCT GCG G-3´ and ITS-4; 5´-TCC TCC GCT TAT TGA TAT GC-3´) and 28S (LSU; LR0R (F) (ACC CGC TGA ACT TA AGC) and TW13(R) (GGT CCG TGT TTC AAG ACG) with the help of methods already described in Khan et al. (2011). The sequences of these isolates were already matching to NCBI GenBank.

**Microorganisms:**

*Saccharomyces cerevisiae* (baker's yeast strain) was a gift from Research Center Lab, Grain Silos and Flour Mills Organization, Saudi Arabia.

β- D- glucan biopolymer consisted of 45.0% inactivated yeast (*Saccharomyces cerevisiae*), 43.0% high adsorbent clay minerals, 2.0% calcium propionate and 10% plant derivatives, antioxidant premix, ethoxyquin and citric acid. The recommended applicable dose is 2.5 g/ kg or 2.5 g/L for liquid media. β- D- glucan binder manufactured by Innovad Company, Belgium

**Inocula:**

Yeast suspensions were prepared by inoculating 100 ml of a yeast extract-malt extract-sucrose broth (0.2 g of yeast extract,1.5 g of malt extract and 1.0 g of sucrose in 1.0 liter of distilled water) with a loopful of *Saccharomyces cerevisiae* cells from a culture stored on MEA media at 4°C. After incubation on a rotary shaker (100 rpm) at 30°C for 24 h, the number of yeast cells was enumerated with a hemocytometer. The spore suspension of *Aspergillus fumigatus* was prepared by collecting spores from 5-day-old colonies (grown on MEA at 25°C) in peptone-water (2 g of peptone per liter of distilled water) with 0.015% Tween 80 added to assist in the dispersal of conidia. The spore concentration was enumerated with a hemocytometer.

**Inhibitory effect of *Saccharomyces cerevisiae* and β- D- glucan biopolymer on different *A. fumigatus* isolates on agar plates:**

*Saccharomyces cerevisiae* spore suspensions and previously isolated different isolates of *A. fumigatus* were prepared as described above. The agar medium in which inhibition experiments was performed as MEA. A top agar was prepared by mixing 6 ml of agar and 1 ml of yeast suspension with either $10^4$ or $10^8$ cells per ml. The agar-yeast suspension was poured into petri dishes that contained 15 to 20 ml of the same agar medium according to (Petersson and Schnürer, 1995).

Once the agar had set, 10 ml portions of *A. fumigatus* suspension with $10^5$spores/ml were inoculated at three spots on each plate, with three replicates for each isolate. The plates were inoculated as spots to make it possible to measure the radial extension rates of colonies. Plates were incubated at 25±2°C for 7 days. Plates not inoculated with any of yeasts used in this study served as controls. The degree of inhibition was determined as linear growth (mm).

β- D- glucan biopolymer had been used the same steps mentioned above with replace *Saccharomyces cerevisiae* with 2.5 g per liter of β- D- glucan polymer under aseptic conditions.

**Scanning electron microscope examination:**

The inhibitory effect of β- D- glucan biopolymer and/or *Saccharomyces cerevisiae* on *A. fumigatus* was studied anatomically by Scanning electron microscope. The fungal examination prepared on sodium alginate according to Rajaonarivony et al, (1993).

The samples were spread over spread holder and dried under vacuum at room temperature (25ºC). The sample was shadowed in a cathodic evaporator with a gold layer 20nm thick. The diameters of all field were calculated using a JSM-6400. Scanning electron microscope (Tokyo, Japan) in Central Lab., King Saud University, Saudi Arabia according to Grant et al., (1973).

**Gliotoxin determination in SMKY medium**:

The isolates of *A. fumigatus* which isolated from corn, wheat, soybean, poultry feed and ruminant feed (8 isolates) were grown individually on (SKMY) broth medium (100 ml); the first group without any another inoculation as a

control, the second group inoculated with a loopful of *Saccharomyces cerevisiae* cells while the third group had a 2.5g/l of tested biopolymer. The inoculated flasks incubated for 2, 5 and 9 days at 25±2°C. Gliotoxin concentrations were analyzed in culture media in three replicate using a high performance liquid chromatography (HPLC). Culture medium was centrifuged at 140,000 x g for 4 min at 10°C. Supernatant (200 µl) plus an internal standard (3-nitrophenol) was then extracted by solid-phase extraction with a 10 kHz-cutoff filter system (Sep-Pak; Waters, Milford, MA). The elution (80 µl) was then injected in the HPLC system, which consisted of a Waters 2487 dual absorbance detector with a wavelength set to 270 nm and a 4.6- by 250mm C18 reverse column packed with 5 µm particles. The mobile phase consisted of acetonitrile and water (43:57 volume/volume) run at a flow rate of 1 ml/min. Gliotoxin concentrations were determined by interpolation from a 5 point standard curve (25 to 1,000 ng/ml) prepared using a purified gliotoxin powder (Sigma, St. Louis, Mo.). A mocker inoculated sample consisting of gliotoxin (250 to 500 ng/ml) was analyzed with each HPLC run as a positive control, according to Lewis et al., (2005).

**Effect of *S. cerevisiae* and β- D- glucan biopolymer on gliotoxin content in grain and feed materials:**

Two hundred grams of tested grains (wheat, corn, gran) and manufacturer feeds (poultry and ruminant) were inoculated individually with the most toxigenic isolate *A. fumigatus* (Fum5). Then treated with β-D-glucan biopolymer (2.5g/kg) and incubated for 9 days at 25 ±2°C and once again inoculated with suspension of *Saccharomyces cerevisiae* ($1x10^5$spore/ml). The spore suspension of *A. fumigatus* isolate was adjusted to contain approximately $10^4$spores/ml. The negative control was conducted using the same material without any treatment while the positive control was conducted using the same material after inoculated with *A. fumigatus* and incubated for 9 days at 25±2°C without any another treatment. The gliotoxin concentration was determined with HPLC protocol which former mentioned, according to Lewis et al., (2005).

**Statistical analysis**

The data obtained were statistically analyzed using ANOVA with the MSTAT-C statistical package. The least significant difference procedure was used at the 0.05 level of probability (Fisher, 1948).

## RESULTS

**Isolation and identification:**

Two hundred thirty five fungal isolates were isolated from wheat, corn, soybean, poultry feed and ruminant feed (Table 1). These fungal isolates belong to 22 species and 11 fungal genera. The samples collected randomly from the Saudi market in summer 2012. The most isolated fungi were from corn (66 isolated) followed by wheat (52 isolates), soybeans (51 isolates) and ruminant feeds (39 isolates) while, the lower number of isolated fungi was from poultry feed (27 isolates only). Fungal isolates varied in species and their frequencies percentage according to samples. The highest frequency in wheat grains was 7.69% for *Aspergillus flavus* and *Fusarium verticillioides*, the most frequency in corn was 12.12% for *A. flavus*, while the highest one in soybean was *F. oxysporum* and *Rhizoctonia solani* with 7.84%. The isolates of *A. flavus, A. niger, A. parasiticus, F. proliferatum* and *F. verticillioides* were the highest frequency (7.41%) in poultry feed, while in ruminant feed the most frequency was *A. niger, A. parasiticus* with 10.26%.

The fungal isolates identified by classic techniques and confirmed with Biolog Inc., system. Meanwhile the *A. fumigatus* identified by nucleotide sequence to detect the similarity between *A. fumigatus*. Eight isolates of *A. fumigatus* were isolated from all tested samples. Two isolates were isolated from each wheat, corn, soybean and only one isolate from both poultry and ruminant feed by frequency 3.85, 3.03, 3.92, 3.70 and 2.56%, respectively.

Nucleotide sequences analysis of eight tested isolates of *A. fumigatus* were conducted by using DNA STAR program (Fig.1). The isolates sequences were divided to two main groups; the similarity among them arrived to 78%. The first group was contained Asp2 (Fum2), Asp5 (Fum5), Asp7 (Fum7) whereas, the Asp5, Asp7formed individual branched group. The second group had five isolates; Asp4 (Fum4) and Asp8 (Fum8) sequences formed individual branched group while, the sequences of Asp1 (Fum1), Asp3 (Fum3) and Asp6 (Fum6) formed Single-shunt separately within this group.

**Table (1): The occurrence and frequencies of fungi associated with wheat, corn, soy bean, poultry feed and ruminant feed from Saudi market in summer 2012 on Malt extract agar media and incubated for 7 days at 25±°C.**

| Fungal isolates | Wheat grains | | Corn grains | | Soybean seeds | | Poultry feed | | Ruminant feed | |
|---|---|---|---|---|---|---|---|---|---|---|
| | No. isolates | % Fr* | No. isolates | % Fr | No. isolates | % Fr | No. isolates | % Fr | No. isolates | % Fr |
| *Alternaria* spp. | 0 | 0.00 | 1 | 1.52 | 3 | 5.88 | 1 | 3.70 | 0 | 0.00 |
| *Aspergillus flavus* | 4 | 7.69 | 8 | 12.12 | 3 | 5.88 | 2 | 7.41 | 3 | 7.69 |
| *A. fumigatus* | 2 | 3.85 | 2 | 3.03 | 2 | 3.92 | 1 | 3.70 | 1 | 2.56 |
| *A. niger* | 3 | 5.77 | 5 | 7.58 | 2 | 3.92 | 2 | 7.41 | 4 | 10.26 |
| *A. ochraceus* | 2 | 3.85 | 1 | 1.52 | 2 | 3.92 | 0 | 0.00 | 1 | 2.56 |
| *A. oryze* | 1 | 1.92 | 2 | 3.03 | 1 | 1.96 | 0 | 0.00 | 2 | 5.13 |
| *A. parasiticus* | 2 | 3.85 | 3 | 4.55 | 1 | 1.96 | 2 | 7.41 | 4 | 10.26 |
| *Chaetomium* sp. | 1 | 1.92 | 0 | 0.00 | 2 | 3.92 | 1 | 3.70 | 0 | 0.00 |
| *Cladosporium* spp. | 1 | 1.92 | 0 | 0.00 | 2 | 3.92 | 0 | 0.00 | 0 | 0.00 |
| *Fusarium chlamdosporium* | 2 | 3.85 | 1 | 1.52 | 1 | 1.96 | 0 | 0.00 | 1 | 2.56 |
| *F. culmorum* | 1 | 1.92 | 2 | 3.03 | 0 | 0.00 | 0 | 0.00 | 0 | 0.00 |
| *F. gramineaum* | 1 | 1.92 | 4 | 6.06 | 0 | 0.00 | 1 | 3.70 | 2 | 5.13 |
| *F. oxysporum* | 2 | 3.85 | 2 | 3.03 | 4 | 7.84 | 0 | 0.00 | 1 | 2.56 |
| *F. proliferatum* | 3 | 5.77 | 5 | 7.58 | 3 | 5.88 | 2 | 7.41 | 1 | 2.56 |
| *F. verticillioides* | 4 | 7.69 | 6 | 9.09 | 3 | 5.88 | 2 | 7.41 | 2 | 5.13 |
| *F. equiseti* | 2 | 3.85 | 0 | 0.00 | 1 | 1.96 | 1 | 3.70 | 0 | 0.00 |
| *Macrophomina phaseolina* | 0 | 0.00 | 0 | 0.00 | 2 | 3.92 | 0 | 0.00 | 0 | 0.00 |
| *Mucor* spp. | 5 | 9.62 | 4 | 6.06 | 2 | 3.92 | 3 | 11.11 | 4 | 10.26 |
| *Nigrospora* sp. | 3 | 5.77 | 1 | 1.52 | 2 | 3.92 | 0 | 0.00 | 0 | 0.00 |
| *Penicillium* spp. | 9 | 17.31 | 12 | 18.18 | 8 | 15.69 | 5 | 18.52 | 9 | 23.08 |
| *Rhizoctonia solani* | 0 | 0.00 | 1 | 1.52 | 4 | 7.84 | 0 | 0.00 | 1 | 2.56 |
| *Rhizopus* spp. | 4 | 7.69 | 6 | 9.09 | 3 | 5.88 | 4 | 14.81 | 3 | 7.69 |
| Total | 52 | | 66 | | 51 | | 27 | | 39 | |

% Fr*: % Frequency of isolates

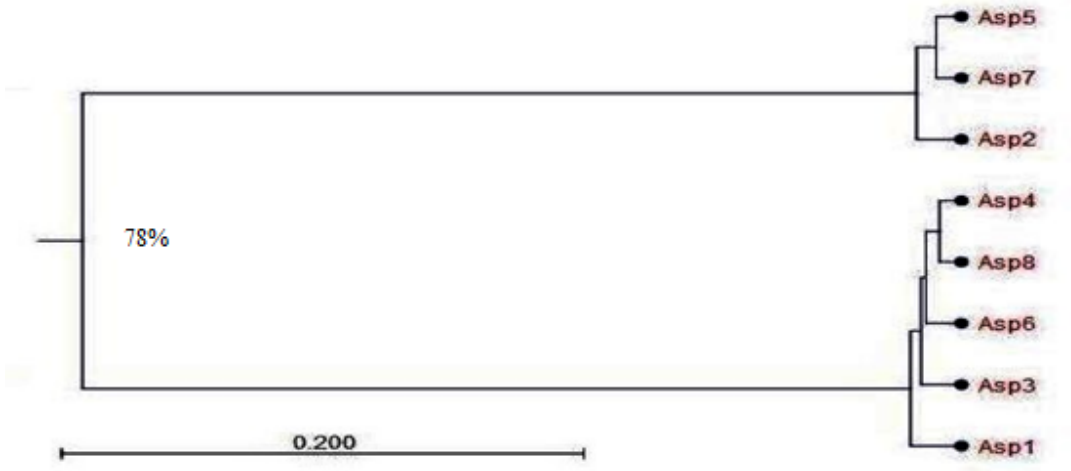


**Fig. (1): Similarity diagram between nucleotide sequences of 8 isolates of *A. fumigatus* Isolated from wheat (Asp1 and Asp2), corn (Asp3 and Asp4), soybean (Asp5 and Asp6), poultry feed (Asp7), and ruminant (Asp8). ITS-1; 5´-TCC GTA GGT GAA CCT GCG G-3´ and ITS-4; 5´-TCC TCC GCT TAT TGA TAT GC-3´).**

**Inhibitory effect of *Saccharomyces cerevisiae* and β- D- glucan biopolymer on different *A. fumigatus* isolates on agar plates:**

The qualitative differences of linear growth between tested isolates of *A. fumigatus* were studied on malt extract Agar (MEA) media under optimum condition in the presence of yeast (*Saccharomyces cerevisiae*), β-D glucan polymer and control, (Table2 and Fig.2). In control, there is no significant different between isolates Fum8, Fum4 and Fum1; (6.5, 6.6 and 6.8 mm, respectively). There is no significant difference among isolates Fum2 and Fum3 and their values which were 7.1 and 7.3 mm. Similarity also with isolates Fum5 and Fum6 where there is no significant difference between them; 8.1 and 7.9 mm, respectively.

The treatment of *Aspergillus fumigatus* with yeast (*Saccharomyces cerevisiae*) led to a decrease in linear growth rates of all Aspergillus isolates. The highest decrease was found with Fum1 isolate followed by Fum4 and Fum8 (50.8, 47.0 and 42.6 %, respectively), while the isolates no. Fum5 was the less reduction (33.3%). The isolates nos. Fum3, Fum6 and Fum7 were almost equal rates in reduction 35.6, 35.4 and 35.5%, respectively.

The treatment usingβ-D glucan polymer led to a clear reduction in the growth of all tested *A. fumigatus* isolates (Table2 and Fig.2). The rates of reduction varied with the tested isolates, the highest reduction was occurred with isolate no. Fum1 while the less one was occurred with isolate no. Fum5 (83.08 and 65.43, respectively). The significant difference between all isolates was found after treated with β-D glucan polymer.

**Scanning electron microscope examination:**

Apparent decrease in the rate of linear growth of all *A. fumig*atus isolate as a result of treated with *Saccharomyces cerevisiae* and β- D- glucan polymer led to the study and exanimate the mycelium anatomically using Scanning electron microscope.

The mycelium of *Aspergillus* fumigates were examined by electron-microscope after treated withβ- D-glucan biopolymer and *Saccharomyces cerevisiae* on sodium algenate. The treated hyphae of *A. fumigatus* in Fig (3, B) become abnormal, very short, swelling and had many number of smooth branching as well as small colonies when *Saccharomyces* surrounding *A. fumigates* mycelium. The degree of hyphae malformation was higher in β- D-glucan biopolymer than *Saccharomyces cerevisiae*. In control treatment, the mycelium was natural without any changed, cylindrical, filled and haven't any degradation signs Fig. (3, A).

**Effect of *S. cerevisiae* and β- D- glucan biopolymer on gliotoxin content in fungal broth:**

The impact of both yeast and β- D- glucan biopolymer on gliotoxin secretion by *A. fumigatus* has been studied on three periods of incubation, (Table 3). Liquid SMKY broth which inoculated with tested *A. fumigatus* isolates re-inoculated individually with both yeast and β- D- glucan biopolymer for two days at 25 ℃ and found that, the concentration of gliotoxin down with both yeast and β- D- glucan polymer compared to control. In control treatment the highest concentration of gliotoxin was occurred with isolates no. Fum5 (4 ppb) while the greatest reduction with yeast was occurred with isolate Fum2 (2.9 ppb) with reduction rate 19.4%. The highest reduction with β- D- glucan biopolymer in this period was done with isolate no. Fum5 (2.1 ppb) with 47.5% reduction.

After 4 days incubation the most gliotoxin concentration in control was 12.7 ppb with isolate no. Fum5. The highest reduction of toxin with yeast was occurred by isolate no. Fum8 (9.5 ppb) and the reduction ratio was 14.4% while, with β- D- glucan biopolymer was 25.6% with isolate no.Fum1 (6.4 ppb).

After 9 days of incubation, isolate no. Fum5 secreted the greatest amount of gliotoxin (19.4 ppb) in control comparing to another tested *A. fumigatus* isolates. The toxin reduced in presence of *Saccharomyces cerevisiae* to 14.8 ppb by isolate no. Fum6 and reduction ratio was 12.9%. β- D- glucan biopolymer led to toxin reduction with all tested isolates and the highest reduction was occurred with no. Fum3 (10.3 ppb) with rate 43.7%.

**Effect of *S. cerevisiae* and β- D- glucan biopolymer on gliotoxin content in grain and feed materials:**

The impact of both yeast (*Saccharomyces cerevisiae*) and β- D- glucan biopolymer to reduce the secretion of gliotoxin in grain and some manufactured feeds was examined by artificial inoculation with the most toxigenic isolate (Fum5) for 9 days at 25±2℃ in Table (4) and Fig (4).

**Table (2): The average of linear growth (mm) of *A. fumigatus* isolates and their percentage reduction after treated with Streptomyces cerevisiae and β-D glucan polymer for 7 days, on MEA media at 25±2°C**

| As. fumigatus isolates | The average of linear growth of *A. fumigatus* isolates and their % reduction with Streptomyces cerevisiae and β-D glucan polymer for 7 days, on MEA media at 25±2°C | | | | |
|---|---|---|---|---|---|
| | Control | *Saccharomyces cerevisiae* | Reduction (%) | β-D glucan polymer | Reduction (%) |
| Fum1 | $6.5^{f}$ | $3.2^{f}$ | 50.8 | $1.1^{f}$ | 83.08 |
| Fum2 | $7.1^{de}$ | $4.3^{cd}$ | 39.4 | $1.6^{cde}$ | 77.46 |
| Fum3 | $7.3^{cd}$ | $4.7^{bc}$ | 35.6 | $1.8^{bcd}$ | 75.34 |
| Fum4 | $6.6^{f}$ | $3.5^{ef}$ | 47.0 | $1.2^{ef}$ | 81.82 |
| Fum5 | $8.1^{a}$ | $5.4^{a}$ | 33.3 | $2.8^{a}$ | 65.43 |
| Fum6 | $7.9^{ab}$ | $5.1^{ab}$ | 35.4 | $2.1^{b}$ | 73.42 |
| Fum7 | $7.6^{bc}$ | $4.9^{b}$ | 35.5 | $2^{bc}$ | 73.68 |
| Fum8 | $6.8^{ef}$ | $3.9^{de}$ | 42.6 | $1.3^{def}$ | 80.88 |
| LAD at 0.05% | 0.41 | 0.46 | | 0.46 | |

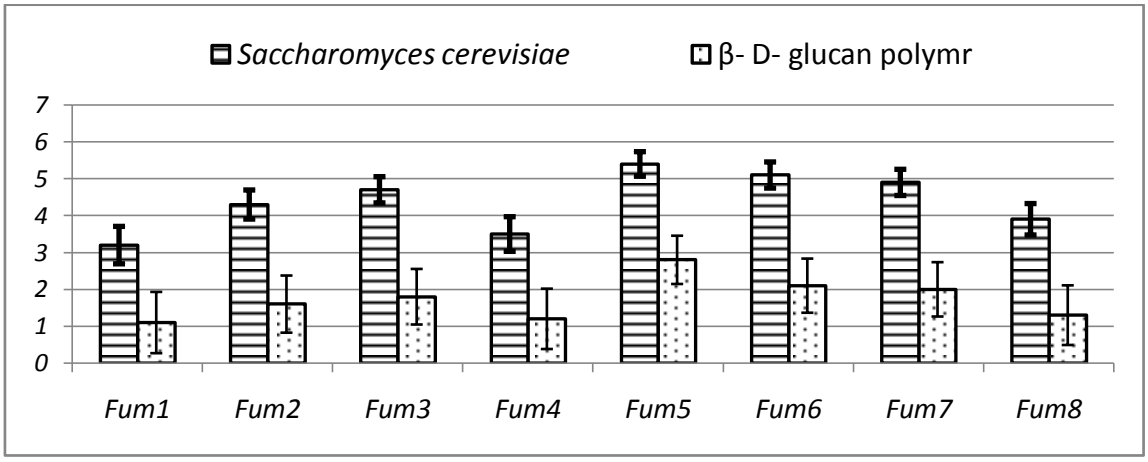


**Fig.(2): The reduction percentage of linear growth of *A. fumigatus* isolates after treated with *Saccharomyces cerevisiae* and β-D glucan polymer for 7 days, on MEA media at 25±2°C.**

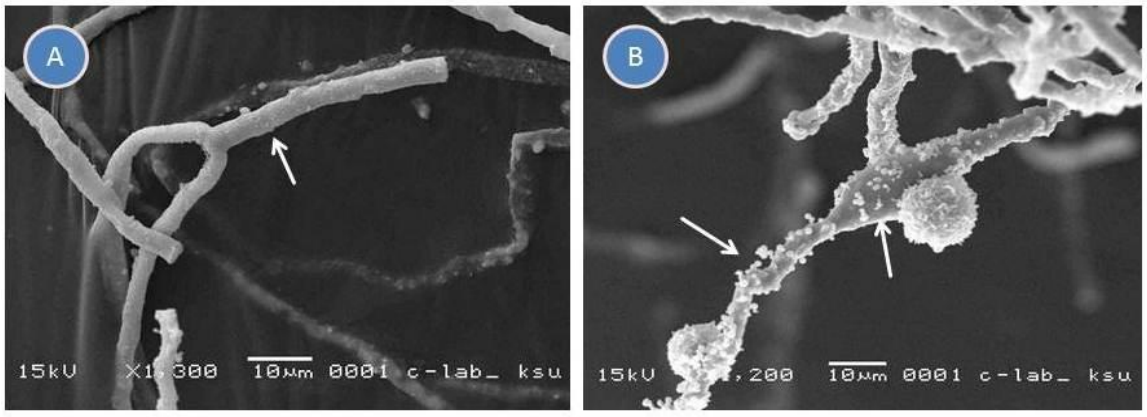


**Fig. (3): Scanning electron microscope examination for *A. fumigatus* mycelia A: control treatment and the mycelium appear as a natural without any changed, cylindrical, filled and haven't any degradation signs. B: The mycelia of *A. fumigatus* after treated with β- D- glucan biopolymer and the hyphae become abnormal, very short, swelling and had many number of smooth branching as well as small colonies if Saccharomyces around the mycelium.**

**Table (3): Gliotoxin determination for 8 different isolates of *A. fumiga*tus in SMKY culture broth after treated with *Saccharomyces cerevisiae* and β- D-glucan biopolymer and incubated for 2, 4 and 9 days on 25±2°C.**

| *A. fumigatus* isolates | Gliotoxin (ppb) for 8 isolates of *A. fumigatus* in SMKY culture broth after treated with *Saccharomyces cerevisiae* and β- D- glucan biopolymer and incubated for 2, 4 and 9 days on 25±2°C. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2 days | | | 4 days | | | 9 days | | |
| | Control | S. cerevisiae | Biopolymer | Control | S. cerevisiae | Biopolymer | Control | S. cerevisiae | Biopolymer |
| Fum1 | $2.7^{c}$± 0.03 | $2.2^{d}$ ±0.09 | $1.7^{a}$ ±0.49 | $8.6^{e}$±0.03 | $7.6^{e}$±0.43 | $6.4^{d}$±0.03 | $13.2^{f}$±0.12 | $11.6^{e}$±0.03 | $9.8^{c}$±0.12 |
| Fum2 | $3.6^{ab}$± 0.26 | $2.9^{abc}$ ±0.15 | $2.3^{a}$ ±0.15 | $11.5^{b}$±0.32 | $9.9^{bc}$±0.38 | $8.7^{abc}$±0.35 | $17.5^{bc}$±0.32 | $15.3^{bc}$±0.03 | $11.2^{b}$±0.38 |
| Fum3 | $3.5^{ab}$± 0.17 | $2.9^{abc}$ ±0.29 | $2.3^{a}$ ±0.20 | $11.3^{bc}$±0.26 | $10.1^{bc}$±0.03 | $8.6^{bc}$±0.20 | $16.8^{c}$±0.35 | $14.8^{c}$±0.23 | $11.3^{b}$±0.52 |
| Fum4 | $3.1^{bc}$± 0.03 | $2.6^{cd}$ ±0.03 | $2.0^{a}$ ±0.23 | $10.1^{d}$±0.32 | $8.9^{d}$±0.23 | $7.7^{c}$±0.15 | $14.7^{e}$±0.09 | $13.0^{d}$±0.43 | $10.6^{bc}$±0.40 |
| Fum5 | $4.0^{a}$± 0.26 | $3.4^{a}$ ±0.20 | $2.1^{a}$ ±0.23 | $12.7^{a}$±0.20 | $11.0^{a}$±0.03 | $9.7^{a}$±0.55 | $19.4^{a}$±0.32 | $17.1^{a}$±0.40 | $12.4^{a}$±0.23 |
| Fum6 | $3.5^{ab}$± 0.20 | $3.1^{abc}$ ±0.23 | $2.2^{a}$ ±0.20 | $11.1^{bcd}$±0.32 | $9.5^{cd}$±0.03 | $8.4^{bc}$±0.09 | $17.0^{c}$±0.20 | $14.8^{c}$±0.49 | $11.2^{b}$±0.26 |
| Fum7 | $3.8^{a}$± 0.15 | $3.2^{ab}$ ±0.20 | $2.3^{a}$ ±0.26 | $11.9^{ab}$±0.61 | $10.4^{ab}$±0.26 | $9.0^{ab}$±0.52 | $18.3^{b}$±0.38 | $16.2^{ab}$±0.40 | $10.3^{bc}$±0.38 |
| Fum8 | $3.2^{bc}$± 0.09 | $2.7^{bcd}$ ±0.03 | $1.8^{a}$ ±0.03 | $10.4^{cd}$±0.26 | $9.4^{cd}$±0.15 | $7.9^{c}$±0.01 | $15.5^{d}$±0.23 | $13.7^{d}$±0.35 | $9.8^{c}$±0.26 |
| LSD at 0.5% | 0.52 | 0.53 | 0.76 | 0.98 | 0.73 | 0.93 | 0.81 | 1.02 | 1.01 |

In negative control samples (sample without any treatment), the gliotoxin ranged from 1.7 in poultry to 3.1 µg/ kg in wheat while, after artificial inoculation with toxigenic isolate and incubated the treated samples for 9 days the gliotoxin was ranged from 16.8 in poultry to 24.6 ppb in positive control of soybean.

After treated with yeast the gliotoxin concentration decreased in all tested samples but the most reduction recorded with poultry feed, ruminate feed, corn, wheat and then soybean (29.1, 28.8, 23.8, 23 and 20.2,%) , respectively. The greatest reduction of gliotoxin concentration was occrued with ruminate feed (53%) followed by poultry feed (48.3%), corn (44%), wheat (43.7%) and then soybean (42.3%) after artificial inoculated with toxigenic *A. fumigatus* (Fum5) and treated with β- D- glucan biopolymer for 9 days at 25±2°C.

## DISCUSSION

Many species of fungi were isolated from corn, wheat, soybeans, manufacturers feed, which varied in frequency. However three genera of fungi; *Aspergillus*, *Penicillium*, and *Fusarium* were most frequently isolated. These results were matching with those recorded by Miller, 1995, Pacin et al., 2002 ; and Chukunda et al. 2013. The total numbers of isolated fungi from grains were more than manufacturers feed which may be exposure to high temperature ranged from 85-90°C and steaming through feed processing. Also the fungal frequency in poultry feed less than ruminate feed. This may be due to the effect of some antibiotic which add to poultry feed against *Coccedia* pathogen. Also, fungal frequency in starchy grains more than which associated with oily seeds.

*Aspergillus fumigatus* isolated from wheat grains with frequency 3.85% while, the isolation frequencies in corn and soybean were3.03 and 3.92%, respectively. These result were in a harmony with those recorded by Traczyk et al. (2001) but they mentation that, *A. fumigatus* is dominant constituent of fungal flora which isolated from wheat grains and its frequency was (76.5%).Also, in our study *A. fumigatus* was isolated from both poultry and ruminant feed and the result was compatible with Pena et al., (2010) records.

The linear growth of all tested isolates of *A. fumigatus* (8 isolates) was inhibited by *Saccharomyces cerevisiae* in different proportions. Inhibition by yeast was also, mentioned by Muñoz et al. (2010) and Cubaiu et al. (2012).The differences in inhibition ratio between isolates may be due to the interaction between Saccharomyces cerevisiae and *A. fumigatus* isolates as well as the physiological and genetic characteristics of each. 1,3- β-D- glucan biopolymer inhibited the radial growth of all tested *A. fumigatus* despite the different inhibition ratio between the isolates and these results were in agreement with Tentler et al.,(1997) who recorded that, 1,3-β-D-glucan to a corresponding decrease in growth of the filamentous fungi as *Neurospora crassa* isolates. *A. fumigatus* isolates were tested for their ability to secrete gliotoxin on three time periods (2, 4 and 9 days).

**Table (4): Gliotoxin determination in wheat, corn, soybean, poultry, ruminate feed after inoculated with *A. fumigatus* isolate (Fum5),treated with *Saccharomyces cerevisiae* and β- D- glucan biopolymer and incubated for 9 days on 25±2°C.**

| Treatment | Gliotoxin (ng/kg) for in wheat, corn, soybean, poultry, ruminate feed after inoculated with *A. fumigatus* isolate (Fum5) for 9 days on 25±2°C | | | | |
|---|---|---|---|---|---|
| | Wheat | Corn | Soybean | Poultry | Ruminate |
| Control -* | $3.1^{d}$ ±0.1 | $2.1^{d}$ ±0.3 | $2.9^{d}$ ±0.5 | $1.7^{d}$ ±0.6 | $2.7^{d}$ ±0.1 |
| Control +** | $19.3^{a}$ ±0.7 | $22.4^{a}$±0.7 | $24.6^{a}$±0.8 | $16.8^{a}$ ±0.7 | $18.3^{a}$±0.4 |
| *S. cerevisiae* | $14.9^{b}$ ±0.2 | $17.1^{b}$±0.4 | $19.6^{b}$ ±0.9 | $11.9^{b}$ ±0.6 | $13.1^{b}$ ±0.4 |
| % of reduction | 23.0 | 23.8 | 20.2 | 29.1 | 28.8 |
| Biopolymer | $10.9^{c}$ ±0.4 | $12.5^{c}$±0.5 | $14.2^{c}$ ±0.1 | $7.9^{c}$ ±0.6 | $8.7^{c}$ ±0.5 |
| % of reduction | 43.7 | 44 | 42.3 | 48.3 | 53.0 |
| LSD at 0.5% | 2.94 | 3.63 | 2.12 | 2.23 | 2.54 |

Control -*: The sample without any artificial inoculation

Control +**: The sample inoculated with toxigenic As. fumigates (fum5) isolate

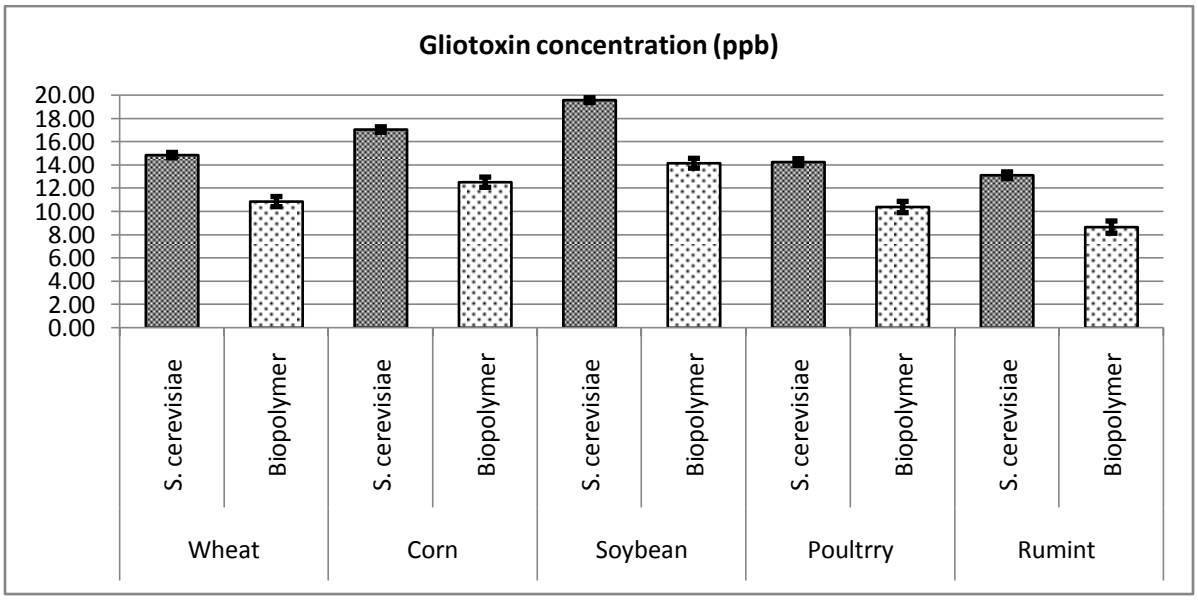


**Fig.(4): Gliotoxin reduction (%) in wheat, corn, soybean, poultry, ruminate feed after inoculated with most toxigenic *A. fumigatus* isolate(Fum5),treated with *Saccharomyces cerevisiae* and β- D- glucan biopolymer and incubated for 9 days on 25±2°C.**

The rate of gliotoxin secretion was the most higher value after 9 days incubation followed by 4 days while the less secretion of gliotoxin occurred after two days and this results may be compatible with those mentioned by Kosalecet al., (2005) where he proved the gliotoxin could be detected after three days of incubation at concentrations 4.06 mg $mL^{-1}$ (at 37 °C).The isolates no. Fum1, Fum 4 and Fum 8werethe most gliotoxin excretion while, isolates no. Fum5, Fum3 and Fum2 were less secretion in all incubation periods. The toxicological results can be linked with DNA sequences similarity between *A. fumigatus* isolates. The sequence similarity of tested isolates was in two main groups and the similarity reached to 79%. The most toxigenic isolates Fum 5, Fum 7 and Fum2 were in sub-group while, the isolates were less toxicity (Fum4 and Fum8) were also in sub-group except Fum1 which was in independent one. These results were consistent with those recorded by Mohankumar et al., (2010) who stated that highly toxigenic and moderate or less-toxigenic isolates of A. flavus were belonged to group V which consisted of 2 sub-groups while, the results was disagree with Toth et al. (2004) who, reported that no correlation could be observed between clustering of the isolates of *Fusarium culmorum* based on RAPD and mycotoxin-producing abilities or aggressiveness.

In the presence of Saccharomyces cerevisiae, the most reduction of radial growth of *A. fumigatus* was with isolate no. Fum5 (33.3%), despite being the most toxic isolate. Fum1 was less affected by yeast isolate (50.8%) despite being one of the most gliotoxin producers. This trend was the same with β-D- glucan biopolymer; Fum5 was the most isolate affected by biopolymer although Fum1 was less one (65.43 and 83.08, respectively). Can be inferred that there is a semi inverse relationship between gliotoxin secretion and linear growth of *A. fumigatus* isolates in the presence of both yeast and β-D- glucan biopolymer.

The soybean was the most contaminate with gliotoxin followed by corn, wheat, poultry feed and ruminant feed after artificial inoculation with the most toxigenic isolate of *A. fumigatus* (Fum5). The treatment with yeast was antipyretic for gliotoxin; the highest reduction value was in poultry feed (29.1%), while the lowest one was in soybean (20.20%) and this result was similar with those state by Stanley et al. (1993). The β-D- glucan biopolymer treatment was more effect than yeast, the reduction percentage with soybean reached to 42.3%, while with ruminant feed was 53% and these results were in harmony with those recoded by Yiannikouris, et al. (2004), Diaz et al. (2004) and El-Naggar and Thabit (2014).

The scanning electron microscope examination for *A. fumigatus* mycelia showed that abnormal, very short, swelling hyphae, general malformation and the mycelium had many number of smooth branching due to treated

with β- D- glucan biopolymer and *Saccharomyces cerevisiae*. The mycelium malformation appearance after treated with Saccharomyces cerevisiae was less than 3- β-D- glucan biopolymer.

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**Disclosure Statement**

**No competing financial interests exist.**

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