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Efficiency of biologically and locally manufactured silver nanoparticles from *Aspergillus niger* in preventing *Aspergillus flavus* to produce aflatoxin B_1 on the stored maize grains

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ABSTRACT

This study was conducted in the mycotoxins laboratory, College of Agricultural Engineering Sciences, University of Baghdad, Iraq to evaluate the efficiency of silver nanoparticles manufactured locally and biologically by *Aspergillus niger* in preventing *A. flavus* to produce aflatoxin B_1 (AFB_1). The results of laboratory isolation showed that the companion of fungi genera were *Aspergillus* spp., *Fusarium* spp., *Penicillum* spp. and *Rhizopus* spp. at rates of 5.66, 14.91, 21.18 and 38.86% respectively. The highest frequency of *A. flavus* was 19.32%. The results of the TLC test showed that all isolates produced AFB_1 in varying rates (%), and the Baghdad / Al-Youssifia isolate was the most productive of AFB_1, since it has a largest spot area and most intense fluorescence under the chromatographic plate, hence given a symbolic name AFBY7. The results of HPLC showed that the toxin concentration in the AFBY7 isolate was 124.167 ppb. Also, the results showed the high efficiency of A. *niger* in the manufacture of silver nanoparticles, as the colour of solution changed from yellow to dark brown. On the other hand, the results of using locally and biologically manufactured silver nanoparticles in the storage experiment to prevent the fungus from producing toxin showed superiority of treatments (T) 0.4, 0.6 and 0.8 mg L^{-1}, since AFB_1 was 0.0 ppb compared to $T_{0.2}$ (3.990 ppb). In addition, the results showed the efficiency of locally and biologically manufactured silver nanoparticles used in reducing AFB_1 in the storage experiment of maize grains stored. So that, $T_{0.6}$ and $T_{0.8}$ were superior in reducing the AFB_1 to 0.0, compared to $T_{0.2}$ and $T_{0.4}$, leading AFB_1 to reach 10.230 and 5.180 ppb respectively.

Keywords: Silver nanoparticles, *Aspergillus niger*, *Aspergillus flavus*, Aflatoxin.
Article type: Research Article.

INTRODUCTION

Humans are exposed to mycotoxins through contaminated agricultural products such as grains and maize or by consuming foods of animal sources such as milk and eggs (Flores *et al.* 2015). AFB_1 is one of the most toxic mycotoxins, since chronic exposure to AFB_1 causes liver cancer and many other diseases. It has been classified as a carcinogen by the International Agency for Research on Cancer (Ostry *et al.* 2017). In order to reduce fungal infections and their toxins, many methods were used to provide storage conditions that prevent infection with fungi and their by-products, including chemical methods, such as treatment with ammonium hydroxide (Al-Ibrahimi 1994), and physical methods. So that, Al-Humairi (2020) reported the effectiveness of ozone gas in reducing AFB_1 in grains of rice. Recently, the trend began to resist fungi and reduce their toxins using nanotechnology, which has become the focus of scientists' attention, and this technology is a great leap in all sciences (Keiper 2003). Nanotechnology is a new technology derived from nanomaterials (Pissuwan *et al.* 2011). Nanomaterial is manufactured by several methods, however, bio-manufacturing is the safest method, since living organisms are used for biosynthesis of nanoparticles, and fungi are among the most important organisms used in

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biosynthesis due to their very effective secretion outside the cell (Castro *et al.* 2012). Fungi have many advantages compared to other living organisms, since they produce large amounts of proteins and enzymes, part of which can be used in the rapid manufacturing of silver nanoparticles (Vahabi *et al.* 2011; Naser Al-Isawi 2022). For example, *Penicillium expansm* as a fungus produced spherical silver nanoparticles with a size of 20 nm (Dubey *et al.* 2012). Raudabaugh *et al.* (2013) produced silver nanoparticles by Rhizoctonia (a fungus). Many experiments were conducted on the *Aspergillus* spp. fungi, and the results proved the ability of these fungi to produce silver nanoparticles. Ninganagouda *et al.* (2013) used *A. niger* and produced round silver nanoparticles with a size of 20-55 nm. Also, silver nanoparticles with a size ranging between 10-18 nm were produced from *A. terrus*, which proved effective in reducing ochratoxin (Ammar & El-Desouky 2016). There are many reports about nanoparticles in the world (Bagherzadeh Lakani *et al.* 2016; Bozorgpanah Kharat *et al.* 2018) Many recent studies have been conducted to estimate the properties and characteristics of silver nanoparticles as an anti-fungal substance when used and treated with a number of pathogens with it (Kim *et al.* 2012).

MATERIALS AND METHODS

Isolation and identification of fungi

The *Aspergillus flavus* and *A. niger* were isolated from stored maize by growing them on a medium of potato dextrose agar (PDA). This medium was prepared according to the instructions of the supplied company (HIMEDIA), by dissolving 39 g of the prepared medium in a 1 litre of distilled water, sterilizing with a autoclave at a 121 °C and a pressure of 1.5 kg cm^{-1} for 20 min, followed by cooling medium to 45°C and adding the antibiotic tetracycline at a concentration of 250 mg L^{-1} to medium. This medium was used in the cultivation of maize grains to isolate the associated fungi and development of *A. flavus* and *A. niger*. In addition, it was used in the laboratory experiments to inhibit the growth of *A. flavus*. A total of 25 ± 2 fungi were diagnosed according to their phenotypic traits based on the approved taxonomic keys (Pitt & Honcking 1985; pitt 2009).

Detection of AFB_1 using Thin Layer Chromatography (TLC)

Sheets silica gel 60F254 (20 cm × 20 cm, thickness 0.25 mm) produced by Sigma chemicals was used. The silica sheets were activated at a 120 °C for 15 min before use. Then, 10 µL of each sample (32 samples) was placed by micro syringe on the TLC plate at a distance of 2 cm from the edge of the plate, and at a distance of 1.5 cm between samples. The standard AFB_1 supplied by Sigma chemicals was added at 5 µL to each spot at one end of the plate. The mobile phase consisting of chloroform-methanol (97:3) was well mixed and placed in a glass transfer container (Goldblatt 1969). The plate was placed in the transfer container and left until the mobile phase reached a distance of 2 cm from the top edge of the TLC plate. Theresfter, the plate was lifted, dried at laboratory temperature and then examined under ultraviolet rays at a wavelength of 366 nm using a UV viewing cabinet. The isolates producing AFB_1 were determined according to their fluorescence intensity, spot area, and the most productive isolates were selected to obtain the biomass of *A. niger*. The biomass of *A. niger* was obtained by growing it in petri dishes containing PDA medium for 7 days. Afterward, two pieces of 5-8 mm size were taken by cork piercing and placed inside the culture medium PDB sterile liquid in a volume of 200 mL per glass flask. The flasks were closed and placed in the incubator at 25 °C for 7 days to obtain biomass of fungal hyphae of *A. niger* (Sadowski 2010).

Local and biological preparation of silver nanoparticles from *A. niger*

The biomass of *A. niger* was filtered by filter paper (Whatman No. 1) and washed at least three times with sterile distilled water to get rid of the liquid media residue, then $AgNO_3$ was added to an amount of 100 g of *A. niger* biomass and placed in the dark for 96 h at 37 °C (Sadowski 2010).

Characterization of silver nanoparticles

Atomic force microscope (AFM)

This examination was carried out at the Department of Chemistry, College of Science - University of Baghdad by preparing the sample to be examined using an atomic force microscope by dropping a few drops of biologically silver nanoparticles on a glass slide. The sample was dried at 50 °C in a desiccator. Atomic force microscope is used to find out the fine details of the topography of the crystalline surface, along with the surface roughness rate and the grain size rate.

Evaluation the efficiency of silver nanoparticles manufactured locally and biologically by *A. niger* and commercial in preventing the *A. flavus* from producing AFB_1 on stored maize grains

Silver nanoparticles and commercial silver were tested to prevent the isolate of the most toxin-producing *A. flavus* on maize grains. The grains were soaked with water for 2 h, then filtered and placed in 100 mL glass vials at a 50 g $tube^{-1}$. Thereafter, 25 mL deionized water was added to it and then sterilized with autoclave at 121 °C and a pressure of 1.5 kg cm^{-1} for 20 min followed by re-sterilizing after 24 h to fully kill the microbial content. The prepared grains of maize were contaminated with *A. flavus* isolate by 4 discs with a diameter of 10 mm taken from the colony with a cork piercing for each container, followed by treating with commercial silver and nanoparticles separately at the concentrations (treatments) of 0.2, 0.4, 0.6 and 0.8 mg L^{-1} ($T_{0.2}$, $T_{0.4}$, $T_{0.6}$ and $T_{0.8}$) then shaken well to ensure homogeneous distribution of spores and incubated at 25 °C for 21 days.

Evaluation the efficiency of silver nanoparticles manufactured locally and biologically by *A. niger* and commercial in preventing the *A .flavus*in reduction AFB_1 on stored maize grains

The prepared grains of maize were contaminated and incubated at a 25 °C for 21 days, sterilized with autoclave at 121°C and a pressure of 1.5 kg cm^{-1} followed by treating with biologically and commercially manufactured silver nanoparticles at the concentrations (treatments) of 0.2, 0.4, 0.6 and 0.8 mg L^{-1} ($T_{0.2}$, $T_{0.4}$, $T_{0.6}$ and $T_{0.8}$) then incubated at a 25 °C for 21 days. The treatments were dried in an electric oven, and 25 g of each treatment was crushed to detect and estimate AFB_1 in it using HPLC technology. The toxin reduction ratio was calculated by the following equation:

$$\text{Reduction} = \frac{\text{Concentration of AFB1 in control} - \text{Concentration of AFB1 in treatment}}{\text{Concentration of AFB1 in control}} \times 100$$

Extraction of AFB_1 from the solid medium of maize grains

AFB_1 was extracted from prepared maize grains according to the AOAC (2005) method, which is a highly efficient method for extraction, as follows:

1. 25 g of the ground repeater was weighed, including the control repeats, and placed in a glass beaker. Then 25 mL methanol and 25 mL chloroform were added and placed on the shaker for 1 hour until the mixture was well homogenized.
2. The samples were filtered by filter paper (Whatman No. 1) and 25 mL methanol (90%) was added to it and then separated by a separating funnel.
3. The filtrate was transferred to the separating funnel again, then 25 mL hexane and 25 mL methanol (90%) were added to it until the two layers were separated. The lower layer containing methanol was taken into glass flasks and placed in a water bath to be dried at 50 °C.

After the samples were dried, chloroform and distilled water were added to it at a 25:25 (v:v) and placed in a separating funnel followed by dipping twice by distilled water with the funnel being shaken and fixing until the two layers were separated. The upper layer was discarded, while the lower layer passed through filter paper. Then 5 g anhydrous sodium sulphate was added to it. The fungal filtrate was taken into small glass tubes, covered with sterile screw caps and dried in a water bath at a 50 °C, then kept in the freezer for further examinations.

RESULTS AND DISCUSSION

Isolation and diagnosis of fungi companion of maize grains

The results of isolation and diagnosis showed that the maize grains samples collected from different governorates in Iraq were infected by many fungal genera, including *Aspergillus* spp., *Fusarium* spp., *Penicillum* spp. and *Rhizopus* spp. with frequency rates of 38.86, 21.18, 14.91 and 5.66% respectively (Table 1). The *Aspergillus* exhibited a highest frequency rate. Salumi (2007) and Al Ramahi (2020) reported the contamination of the maize grains with these fungal species. These results were in agreement with Khosravi *et al.* (2007) who reported the dominance of the *Aspergillus* spp., *Fusarium* spp. and *Penicillium* spp. in varying proportions in their study on the fungi isolated from maize grains.

Detection of AFB_1 using TCL

The results of the detection of AFB_1 using TLC for *A. flavus* isolates companion stored maize grains showed that all isolates produced AFB_1 (Table 2) at a different concentrations according to the storage area, since the isolate that produced the most toxin was from Al-Youssifia area and it was determined based on the size of spot and fluorescence intensity on the TLC plate when exposed to ultraviolet ray (Fig. 1).

Table 1. Frequency rates of fungi companion of maize grains.

Fungi	Frequency rates (%)
A. flavus	19.32
Aspergillus spp.	38.86
Fusarium spp.	21.18
Penicillum spp.	14.91
Rhizopus spp.	5.66

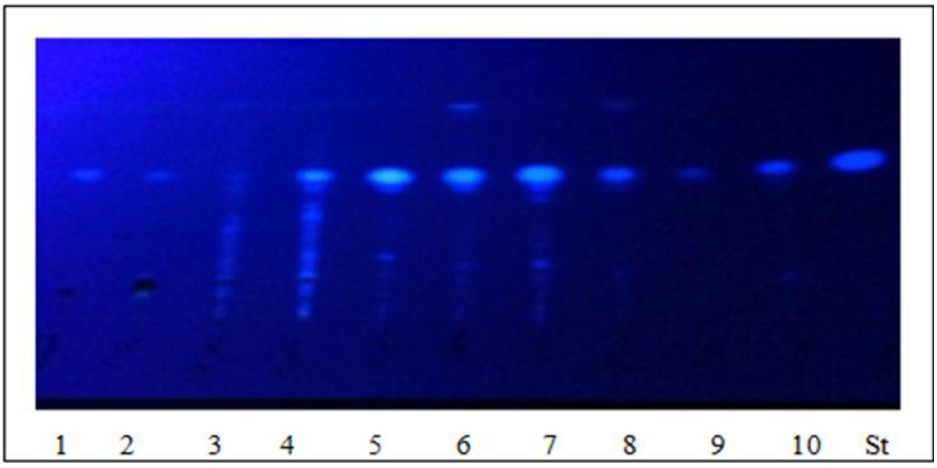

Fig. 1. The ability of *Aspergillus flavus* isolates to produce AFB_1 on TLC plate.

Thus, all isolates of *A. flavus* were aflatoxin-producing. These results are consistent with Fakruddin *et al.* (2015) who showed that the maize grains have an outer shell that protects them from infection with fungi. However, exposing these grains to mechanical damage, insect damage, or environmental damage such as high temperatures, rain and stress facilitate the presence of outlets that allow the entry of fungi and the production of toxin inside them. High temperatures cause cracks in the cover, allowing the entry of *Aspergillus* and secretion of the toxin. Most of the fungi diagnosed were of the species belonging to the *Aspergillus* genus, and these species are of great danger to humans and animals due to their production of mycotoxins. The isolate 7 from Al-Youssifia area produces higher toxin based on the fluorescence of the spots colour under ultraviolet ray as well as the Rf value compared to the standard toxin spot (Green & Diekman 1992). The results of quantitative estimation of AFB_1 produced by *A. flavus* isolates, grown on liquid yeast and sucrose medium, showed that the toxin concentration was 124.167 ppb, which displayed the highest fluorescence intensity on TLC plate (++++++) compared to AFB_1. This isolate was chosen for subsequent tests including the effects of some treatments on the growth of *A. flavus* and the reduction of AFB_1. The reason for differences in the secretion of toxin amounts from *A. flavus* isolates may be due to several factors, the most important including the type of strain, temperature, humidity and pH (WHO 1990).

Biological manufacturing of silver nanoparticles

After mixing the biomass of *A. niger* with silver nitrate solution, and after 96 hours of placing the mixture in the vibrating incubator in the dark, the colour of the mixture changed from yellow to brown compared to the control solution containing the biomass leachate of the fungus without adding nitrate solution, which was placed in the same conditions, no change was observed in its colour and it remained yellow. The colour change is a preliminary evidence for the formation of silver nanoparticles (Fig. 2) by the presence of surface plasmons, and the colour variation is caused by the difference in the electronic density of nanoparticles due to the difference in nano-size. This result is consistent with Ali *et al.* (2014) and Netala *et al.* (2015).

Evaluating the efficiency of biologically and locally manufactured silver nanoparticles from *A. niger* and commercial silver in preventing the *A. flavus* from producing AFB_1 in the stored maize grains

The results of examining locally manufactured silver nanoparticles with atomic force microscope (AFM) showed that the size of silver nanoparticles is 55.49 nm (Fig. 3).

Table 2. Qualitative estimation of the ability of *A. flavus* isolates according to the intensity of fluorescence of spots and plates.

No.	isolates	Toxin presence	No.	isolates	Toxin presence
1	Kut / Azizia	+	21	Baghdad / Kadhimiya	+
2	Kut / Azizia	+	22	Baghdad / Kadhimiya	++
3	Kut / Azizia	+	23	Baghdad / Kadhimiya	++
4	Kut / Azizia	+++	24	Wasit / Kut	+
5	Baghdad / Youssifia	++++	25	Wasit / Kut	+
6	Baghdad / Youssifia	++++	26	Wasit / Kut	+
7	Baghdad / Youssifia	+++++	27	Nasiriyah / Shatrah	+
8	Maysan / Omara	+++	28	Nasiriyah / Shatrah	+
9	Maysan / Omara	+++	29	Nasiriyah / Shatrah	+
10	Maysan / Omara	+++	30	Nasiriyah / Shatrah	++
11	Babylon / Hilla	+	31	Nasiriyah / Shatrah	+
12	Babylon / Hilla	++	32	Babylon / Hilla	++
13	Babylon / Hilla	+++	33	Baghdad / Taji	++
14	Baghdad / Tarmiyah	+	34	Baghdad / Taji	+
15	Baghdad / Tarmiyah	+	35	Baghdad / Taji	+++
16	Baghdad / Tarmiyah	+++	36	Baghdad / Taji	+
17	Baghdad / Abu Ghraib	+	37	Baghdad / Taji	++
18	Baghdad / Abu Ghraib	+	38	Nasiriyah / Shatrah	+
19	Baghdad / Abu Ghraib	+	39	Nasiriyah / Shatrah	+
20	Baghdad / Abu Ghraib	+	40	Karbala	++

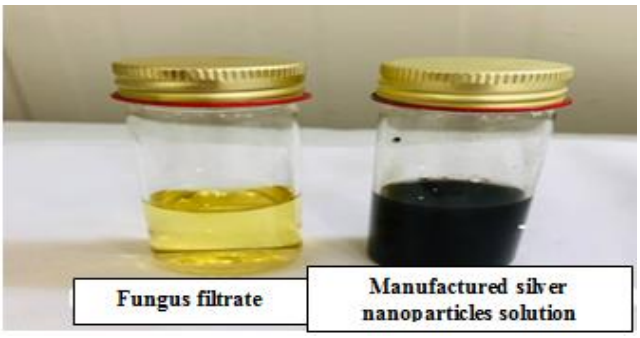

Fig. 2. Manufactured silver nanoparticles and fungus filtrate.

The results in Table 3 showed the efficiency of biologically and locally manufactured silver nanoparticles from *A. niger* in reducing AFB_1 in the stored maize grains. These treatments ($T_{0.2}$, $T_{0.4}$, $T_{0.6}$ and $T_{0.8}$) recorded 3.99, 0.00, 0.00 and 0.00 ppb at a reduction rate of 94.61, 100, 100, 100 and 100% respectively. These concentrations were in comparison with control treatment recording 124.67 ppb and in deionized water recording 74.003 ppb. This result is in agreement with Gibson *et al.* (2010) who reported the efficiency of nanoparticles in reducing aflatoxin. Also, it is in line with Al-Othman *et al.* (2014). They reported that using silver nanoparticles at 50 and 100 ppb led to the reduction of mycotoxins at a rates ranging between 46.1-82.2% and 48.2- 61.8% for both concentrations respectively.

Table 3. Efficiency of biologically and locally manufactured silver nanoparticles by *A. niger* and commercial silver for the most productive isolate of AFB1 by HPLC.

Treatment	Concentration	Toxin concentration	Reduction rate
Control		124.167	----
Deionized water		74.003	----
Manufactured silver nanoparticles	0.2	3.990	94.61%
	0.4	0.000	100%
	0.6	0.000	100%
	0.8	0.000	100%
Commercial silver	0.2	1.250	98.31%
	0.4	1.050	99.58%
	0.6	0.000	100%

	0.8	0.000	100%
LSD 0.05		1.185**	0.08044**

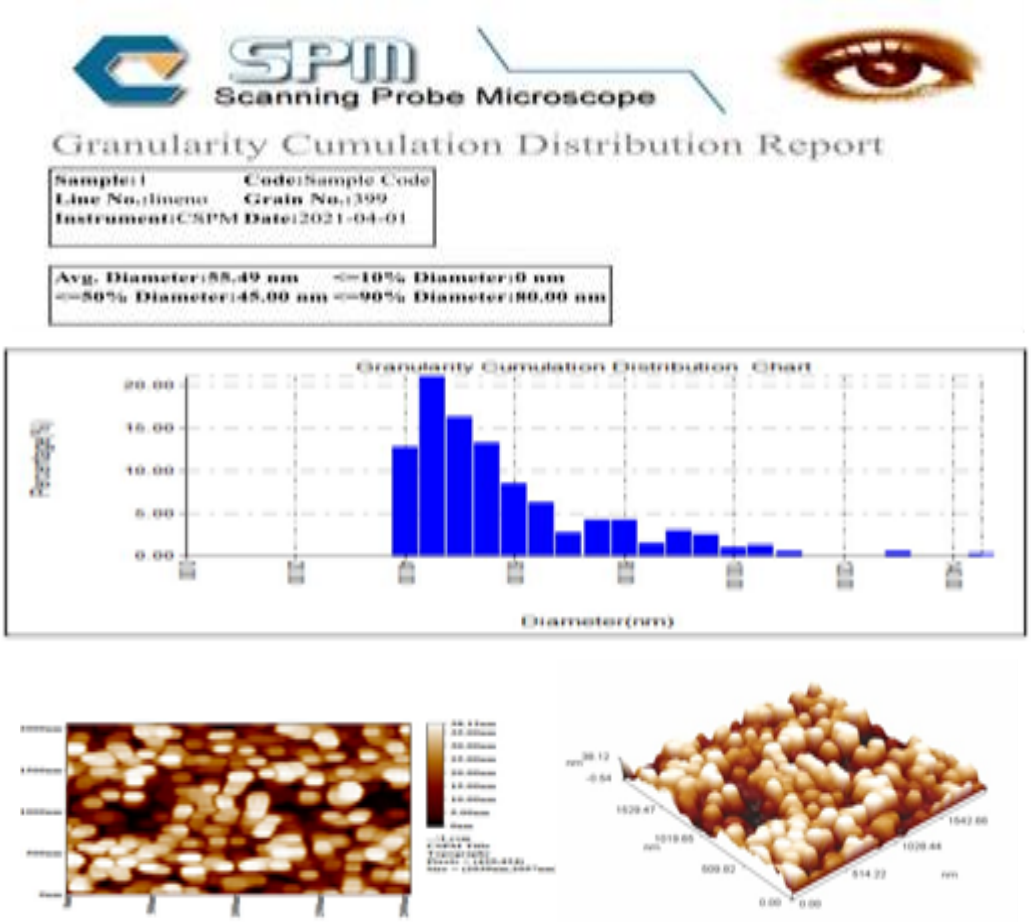

Fig. 3. The results of the nano-size examination of the biologically and locally manufactured silver nanoparticles from *A. niger*.

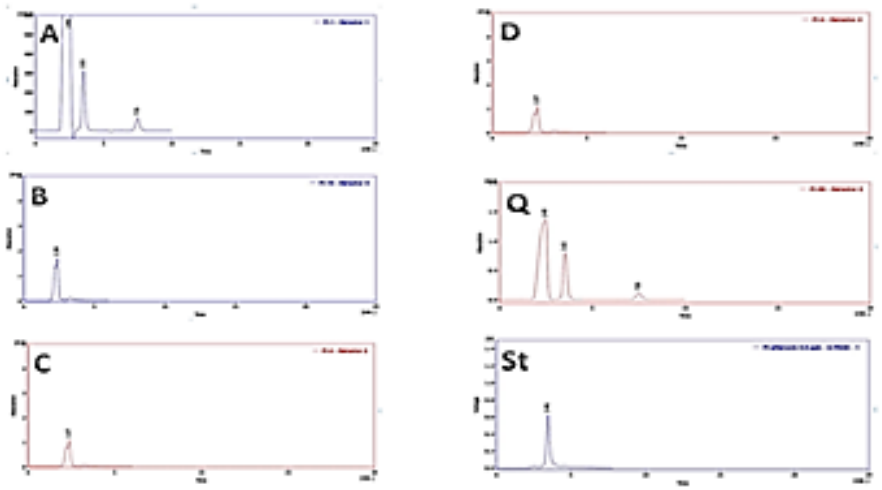

Fig. 3. Effects of biologically and locally manufactured silver nanoparticles from *A. niger* on AFB_1 production in the stored maize grains.

A-C = 0.2-0.6 B = 0.4 Q-D = 0.8- Control Conc. St = AFB_1 Standard Conc.

Evaluating the efficiency of biologically and locally manufactured silver nanoparticles from *A. niger* and commercial silver in reducing AFB_1 by HPLC

The results showed the high efficiency of biologically and locally manufactured silver nanoparticles in reducing AFB_1 in the stored maize grains with significant differences (Table 4). The concentrations of toxin were 10.23, 5.18, 0.0 and 0.0 ppb in $T_{0.2}$, $T_{0.4}$, $T_{0.6}$ and $T_{0.8}$ respectively. This may be attributed to the silver nanoparticles capable of reducing and destroying the toxin (Horky *et al.* 2018). These results are in agreement with Al-Rawi (2017) who reported that using silver nanoparticles inhibits fungi and also reduces mycotoxins along with the risks of toxins.

Table 4. Efficiency of biologically and locally manufactured silver nanoparticles by *A. niger* and commercial silver in reducing AFB_1 by HPLC.

Treatment	Concentration	Toxin concentration	Reduction rate
Control		124.167	----
Deionized water		74.003	----
Manufactured silver nanoparticles	0.2	10.230	86.18%
	0.4	5.180	93.00%
	0.6	0.000	100%
	0.8	0.000	100%
Commercial silver	0.2	12.587	83.00%
	0.4	10.693	85.56
	0.6	8.220	88.8%
	0.8	0.000	100%
LSD 0.05		1.182**	0.3226**

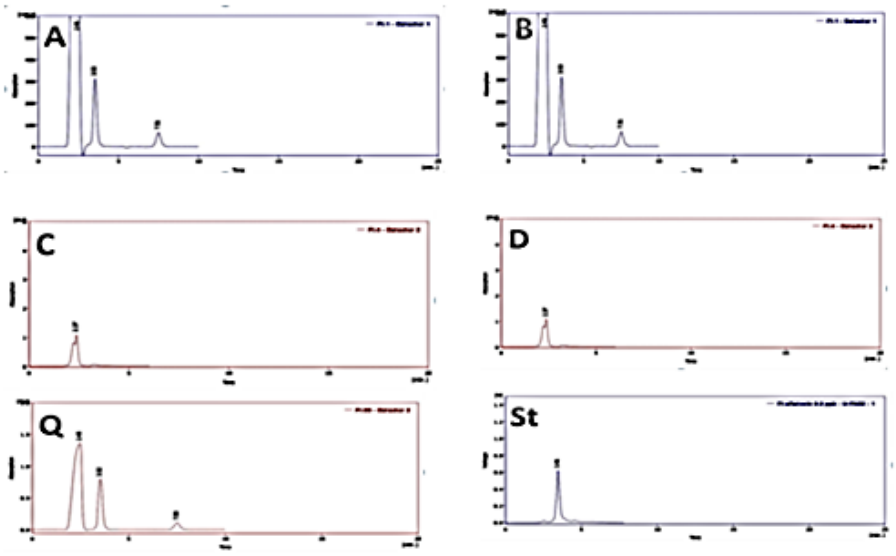

Fig. 4. Effects of biologically and locally manufactured silver nanoparticles from *A. niger* on AFB_1 reduction in the stored maize grains.

A-C = 0.2-0.6 B = 0.4 Q-D = 0.8- Control Conc. St = AFB_1 Standard Conc.

CONCLUSION

The study suggested the efficiency of the *A. niger* in the biologically and locally manufactured silver nanoparticles and also its high efficiency in preventing *A. flavus* from producing and reducing AFB_1.

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