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Impact of Silver Nanoparticles on Gene Expression in *Aspergillus Flavus* Producer Aflatoxin B1

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Abstract

AIM: In this study, we evaluated the effect of silver nanoparticles (AgNPs) on the production of aflatoxin B_1 (AFB_1) through assessment the transcription activity of aflatoxin biosynthesis pathway genes in *Aspergillus flavus* ATCC28542.

MATERIAL AND METHODS: The mRNAs were quantitative by Real Time-polymerase chain reaction (qRT-PCR) of *A. flavus* grown in yeast extract sucrose (YES) medium containing AgNPs. Specific primers that are involved in the AFB_1 biosynthesis which highly specific to *A. flavus*, O-methyltransferase gene (*omt-A*), were designed and used to detect the fungus activity by quantitative PCR assay. The AFB_1 production (from *A. flavus* growth) which effected by AgNPs were measured in YES medium by high-pressure liquid chromatography (HPLC).

RESULTS: The AFB_1 produced by *A. flavus* have the highest reduction with 1.5 mg $^{-100\ ml}$ of AgNPs were added in media those records 88.2%, 67.7% and 83.5% reduction by using AgNP HA1N, AgNP HA2N and AgNP EH, respectively. While on mycelial growth give significantly inhibitory effect. These results have been confirmed by qRT-PCR which showed that culture of *A. flavus* with the presence of AgNPs reduced the expression levels of *omt-A* gene.

CONCLUSION: Based on the results of the present study, AgNPs inhibit growth and AFB1 produced by Aspergillus flavus ATCC28542. This was confirmed through RT-PCR approach showing the effect of AgNPs on omt-A gene involved in aflatoxin biosynthesis.

Introduction

Aflatoxins are secondary polyketide metabolites mainly produced by aflatoxigenic fungi of *Aspergillus flavus* and *Aspergillus parasiticus*. Among the aflatoxins that have been identified, aflatoxin B_1 (AFB_1) is most prevalent form, presents the highest potential toxic [1]. AFB_1 is classified as group 1 carcinogenic to mammals by the International Agency for Cancer Research (IARC) [2]. AFB_1 has potential hepatic [3], carcinogenicity [4], cytotoxicity, genotoxicity [5] [6] [7] and immunotoxicity [8].

Swallowing of AFB_1 has a toxic to the immune system works [9] and mainly reduces the function of cell-mediated immunity [8]. Biochemical pathways and gene regulation it is vital to aflatoxin (AF) also characterized. Many studied already were published in genetics about the AF biosynthesis [10] [11]. Approximately Twenty Seven enzymatic and at least 25 genes involved in the AF biosynthesis in *A. flavus*, this genes are cluster within 75 kilobytes (kb) in the fungal genome [12] [13]. Most of that seems to be co-organized by the DNA binding protein AflR, encoded by the genes aflR [14]. Yabe and Nakajima [15] demonstrated that the three genes pksA, ver-1 and *omt-A* encode enzyme proteins were involved in the AF biosynthetic pathway. On the other hand, gene aflR is a regulatory gene whose product regulates

transcription of some genes; pksA, ver-1 and *omt-A* [16]. Many strategies have been developed to reduce aflatoxins contamination, either by preventing the growth of aflatoxigenic fungi or by blocking the production of the toxin after infection [17]. Silver nanoparticles (AgNPs) is recent advancements in the field of nanotechnology were used as a novel therapeutic agent as antibacterial, antifungal, antiviral, anti-inflammatory and anti-cancer agents [18] [19]. The effect of Silver nanoparticles on growth and production of other types mycotoxins by toxigenic fungi were studied but; the effect Silver nanoparticles AgNPs on gene expression of AFs biosynthesis pathway genes still need more studies. This study aims to evaluate the effects of three types AgNPs on the production of AFB_1 through assessment the transcription activity (gene expression) of AFB_1 biosynthesis pathway genes in *Aspergillus flavus* ATCC28542.

Material and Methods

Fungal strain

Fungal Strain: a Toxigenic strain of *Aspergillus flavus* (ATCC 28542) was obtained from Microbial Research Center, Faculty of Agriculture, Ain Shams University Cairo, Egypt (MIRCEN).

Chemicals and solvents

Potato dextrose agar (PDA) and yeast extract sucrose (YES) liquid growth medium and Sodium sulphate anhydrous were obtained from Sigma-Aldrich, France. Aflatoxin B_1 (AFB_1) standard was purchased from Sigma, Chemical Co. (St. Louis, MO, U.S.A). Stock solutions and standard were prepared and assayed according to Association of Official Analytical Chemists (AOAC) [20] Method 990.33A. Methanol and acetonitrile HPLC grade were produced by BDH, England. The water was double distilled with Millipore water purification system (Bedford, M A, USA).

Synthesis and characterised of AgNPs

AgNPs in this study were synthesised by *Aspergillus terreus*HA1N (KR364880) and *Penicillium expansum* HA2N (KR269857) which isolated and identified according to Ammar and El-Desouky [21]**.** Also, the characterisation of AgNPs has been done by UV-Visible Spectrophotometer, Dynamic Light Scattering (DLS), Fourier Transform Infrared Spectroscopy (FTIR), and Transmission Electron Microscope (TEM) in the previous study [21]**.** On the other hand, in the same study, we synthesised the AgNPs by Egyptian honey (EH) preparation and production according to El-Desouky and Ammar [22].

Inhibition of A. flavus growth and aflatoxin B_1 production in the presence of silver nanoparticles

A hundred ml of YES medium were put in a 500 ml flasks and then autoclaved at 120°C for 15 min. Inoculation was carried out by adding 1 ml of a suspension of spores (10^5 spores) of a toxigenic *A. flavus* ATCC28542 strains without AgNPs (control) or with 0.5, 1.0 and 1.5 mg/100ml YES medium of one of the tested AgNPs (6a, 6b, 6c AgNps HA1N were synthesized by *Aspergillus terreus* (KR364880),3a, 3b, 3C AgNps EH were synthesized by Egyptian honey, and 1a, 1b, 1C AgNps HA2N were synthesized by *Penicillium expansum* (KR269857). The flasks were incubated in the dark for 14 days at 28°C. After the incubation period, extraction of AFB_1 from in the YES culture according to the method of Munimbazi and Bullerman [23]. Where, the mycelium of each flask contained YES medium was harvested by filtration through Whatman paper (No. 4), then extracted with 100 ml chloroform. The chloroform extract was dried by addition of anhydrous sodium sulfate. The residue was transferred to a vial and evaporated off using a stream of nitrogen at a temperature below 60°C. The dry film was used for the detection and determination of AFB_1 by (HPLC) according to (Deabes et al., [24,6] the retention time of AFB_1 standard separation is 4.061. The percentage of inhibition of AFB_1 is calculated using equation: % inhibition = (control- treatment /control) X100

Effect of AgNPs on biosynthesis pathway genes of AFB_1 in Aspergillus flavus ATCC28542

DNA extraction

DNA was extracted from 25 mg of the harvested mycelia, which was frozen in liquid N2 and ground in a mortar, according to the protocol recommended for the DNA Tissue purification mini kit (Qiagen). The genomic DNA was checked by agarose gel electrophoresis, and the concentrations of the purified total genomic DNA were determined with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and stored at -20°C for further use.

Primer design

Primers presented in Table 1 were selected according to the sequence of the *omt-A* gene of *A. flavus* from GenBank database (http://www.ncbi.nlm.nih.gov/).

Table 1: Primer used in this study for amplification of AFB_1 gene

Primer	5'-3' nucleotide sequence
omt-F	GACCAATACGCCCACACAG
omt-R	CTTTGGTAGCTGTTTCTCGC

Polymerase Chain Reaction amplification

PCR reactions were carried out in a total reaction volume of 20 µl, containing (10 µl of 2 X Go Taq master mix (Promega Corporation, Madison, WI) and 10 pmol of each primer of 50 ng template DNA. Amplification was performed in a T100-Bio-Rad Gradient Thermal cycler. The following programmer was used to amplify the DNA: 5 min at 94°C (1 cycle); 1 min at 94°C, 1 min at 59°C, and 1 min at 72°C (35 cycles); and 10 min at 72°C. A 10 µl aliquot of PCR products were separated on a 1.5% agarose gel stained with ethidium bromide (0.1 mg/l) and photographed under Gel Doc™ XR+ Gel Documentation System. Thermo Scientific GeneRuler 100 bp DNA Ladder was used as a size standard.

Gene Expression Analysis

Extraction of mRNA and cDNA synthesis

mRNA was extracted from selected *A. flavus* isolates treated with nanoparticles using mRNA Isolation Kit (Roche Applied Science).

Quantitative Real Time-PCR (qRT-PCR)

A Step One Real-Time PCR System (Applied Biosystem, USA) was used to assess the copy of the cDNA of *A.flavus* strain treated with AgNps HA1N, AgNps HA2N and AgNps EH, to detect the expression values of the tested genes according to El-Baz *et al.* [25].

Statistical analysis

All data were statistically analysed using the General Linear Model procedure of the SPSS ver. 18 (IBM Corp, NY).The significance of the differences among treatment groups was determined by Waller–Duncan k-ratio [26]. All statements of significance were based on the probability of $P < 0.05$.

Results

The present study was undertaken to investigate the antifungal activity of silver nanoparticles on *A. flavus* in vitro study. The addition of AgNPs including (AgNps HA1N, AgNps HA2 N and AgNps EH) individually to the (YES) growth medium at a level of 0.5, 1.0 and 1.5 (mg/100ml). The percentages of AFB_1 reduction with AgNPs HA1N were 22.8, 50.7 and 88.2% after treating by 0.5, 1 and 1.5 mg AgNPs /100mL medium, respectively. On the other hand, in case of AgNps HA2N AFB_1 reduced to 13.3, 37.3 and 67.7%, while AgNps EH reduced $AFB_1$to 22.1, 42.9 and 83.5% as (Fig. 1).

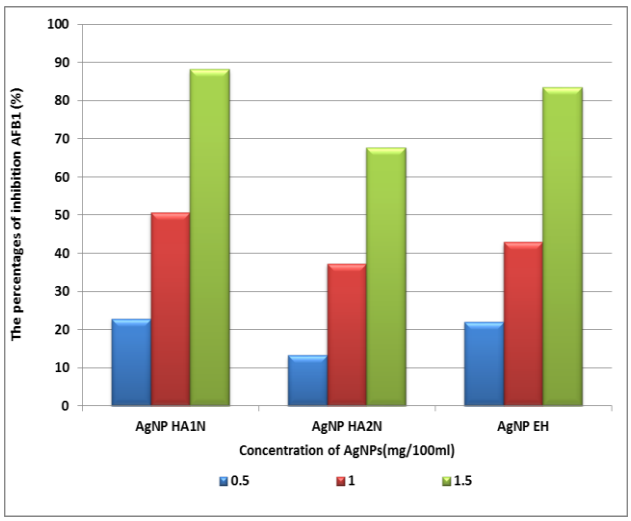

Figure 1: The percentages of inhibition of AFB1 production by Aspergillus flavus ATCC 28542 in YES medium

The results have also indicated that the AgNps HA1N at concentration 1.5 mg/100 mL medium gave the highest reduction of AFB_1 were determined by HPLC (Fig. 2).

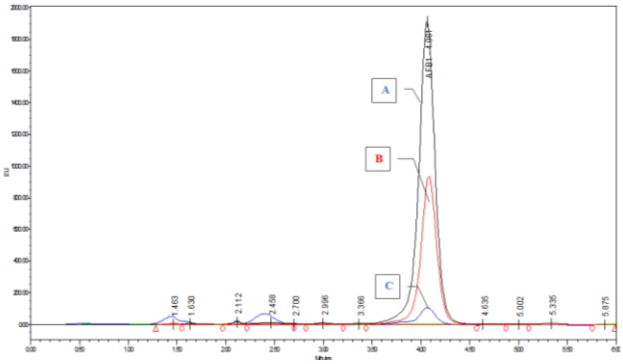

Figure 2: HPLC chromatogram of AFB1 with different concentration of AgNPs HA1N. A = AF; B1 treated by 0.5 mg AgNp HA1N/100 ml media; B = AFB1 treated by1 mg media AgNp HA1N/100 ml media; C = AFB1 treated by 1.5 mg AgNp HA1N/100 ml media

The obtained data in (Table 2) showed the AFB_1 concentrations in YES medium treated with three types of AgNPs, which showed the significant differences between the different concentrations as well as between the types of AgNPs (Table 3). On the other hand, data in (Table 4) display the effect of AgNPs on mycelial production from *A. flavus* ATCC 28542. The reduction on fungal growth and AFB_1 creation was dependent on the concentration of AgNPs. The antifungal activity of AgNPs was demonstrated by the diminution of AFB_1 production by *A. flavus.* This reduction is due to the interfering with

growth and fungal proliferation that AgNPs are altering protein activity and leads to cell death.

Table 2: Effect of AgNPs on AFB_1 produced by *Aspergillus flavus* ATCC 28542

Concentration of AgNPs (mg)/100ml media	AFB_1 (µg/100 ml media)* AgNP HA1N	AgNP HA2N	AgNP EH	Mean for concentration
0.5	0.826 ± 0.051	0.928 ± 0.041	0.833 ± 0.044	0.863 ± 0.063[c]
1.0	0.602 ± 0.010	0.746 ± 0.029	0.685 ± 0.018	0.677 ± 0.065[b]
1.5	0.126 ± 0.054	0.346 ± 0.036	0.176 ± 0.058	0.216 ± 0.109[a]
Mean for type AgNPs	0.518 ± 0.312[a]	0.673 ± 0.259[c]	0.564 ± 0.301[b]	

Control = 1.07 ± 0.11; *Mean ± SD.

This action may be attributed to the associated with the cation of silver, and it's the soluble complexes [27] [28] [29] [30]. With the result of Ionic silver (Ag+) binding to the thiol groups in NADPH enzyme, and hamper the bacterial respiratory chain creating interactive oxygen species that cause oxidative stress and cell damage [30].

Table 3: Analysis of variance of the effect of type and different concentration of AgNPs on AFB_1 produced by *Aspergillus flavus* ATCC 28542

Source	SS	df	MS	F	*P*
Intercept	9.258	1	9.258	5423.688	0.000
Type Nano	0.114	2	0.057	33.377	0.000
Con. Nan	1.996	2	0.998	584.762	0.000
Type Nano x Con. Nan	0.016	4	0.004	2.376	0.091
Error	0.031	18	0.002		
Total	11.415	27			

SS - the sum of squares; df - degree of freedom; MS - mean square; *P* - probability at confidence 0.95.

Some previous research suggests that the metabolic activity of fungi is also overlapped with AgNPs by decreasing the mycotoxins production, cytotoxicity and organic acid production. Moreover, a change in the extracellular enzyme profile is observed [31]. The antimicrobial effects of AgNPs depend on their size and silver rate release ion, and also the decrease of the size of AgNPs increased the antimicrobial activity [32] [33] [34].

Table 4: Effect of AgNPs on mycelial production from *Aspergillus flavus* ATCC 28542

Concentration of AgNPs (mg)/100ml media	The weight of mycelial production (mg/ml)* AgNP HA1N	AgNP HA2N	AgNP EH
0.5	11.7±1.25	17.2±0.76	14.5±0.5
1.0	8.5±1.32	13.2±0.77	10.6±1.27
1.5	4.4±1.15	8.6±0.87	6.5±0.51

*Control=25mg/ml; Mean ±SD

The obtained data recorded in Fig. **3** showed that the bands of the *omt_A* gene fragments which could be visualised at 300 bp. The patterns showed in all treatments isolates indicating that the presence of this structure gene, *omt-A*, enclosed in aflatoxin biosynthetic pathway which regulates the production of aflatoxin *aflR* gene [16].The results of the gene expression analysis using quantitative real-time RT-PCR are summarised in (Fig. 4). The gene encoding AFB_1 was determined in all isolates of *A. flavus* (ATCC 28542) after treatment with three types of AgNPs at 1.5 mg/100 ml media 6a AgNps HA1N, 1C AgNps EH, and 3c AgNps HA2N.

Discussion

The results found that samples of *A. flavus* treated by AgNps HA2N (1C) were very lower expression levels of AFB_1 gene than those collected from AgNps HA1N (6A) treatment and AgNps HA2N (3C). In control sample (without treated) isolate increased significantly ($P < 0.01$) the expression levels of AFB_1 gene with highest up-regulation action of the gene compared with the other treatments.

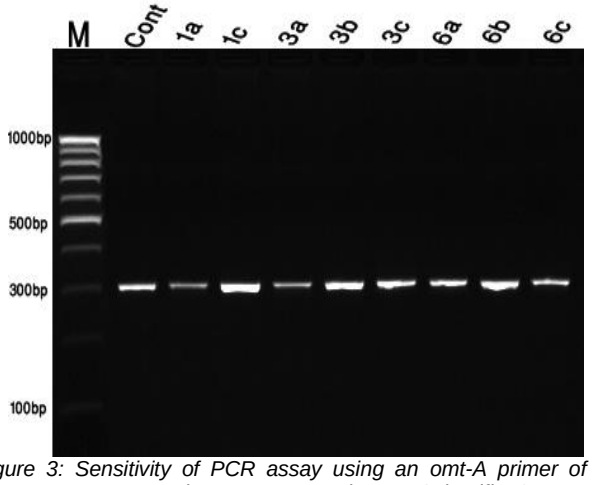

Figure 3: Sensitivity of PCR assay using an omt-A primer of A. flavus. DNA as template Lane1 M, Thermo Scientific GeneRuler 100bp DNA Ladder; lane 2 to lane8 isolates treated with nanoparticles

Moreover, samples 6a (AgNPs HA1N) aflatoxin gene compared with 1C (AgNps HA2N) and 3C (AgNps EH) samples. The RT-PCR experiment elucidated that the AgNpS consolidated expression of aflatoxins pathway *omt-A* gene [35]. On the other hand, Jing et al. [36] found that the treatment of AgNps significantly decreased the secretion of AF from *A. flavus*. Also, they explained the mechanisms of AgNps could cause the depression of AF production by *A. flavus.*

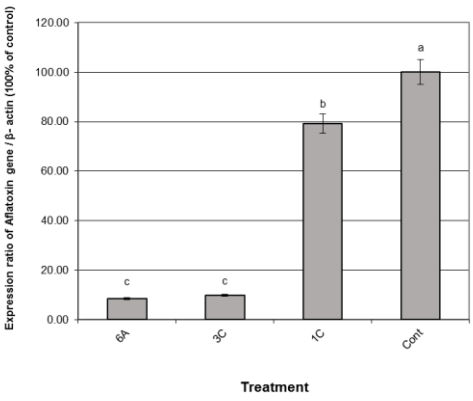

Figure 4: Expression levels of AFB1 gene in A. flavus isolates treated with 1.5 mg /100ml medium AgNPs of 6a AgNps HA1N, 3C AgNps EH and 1C AgNps HA2N. Data are presented as mean ± SEM ^{a,b,c} followed by different superscripts are significantly different ($P \leq 0.05$)

In conclusion, based on the results of the present study, AgNPs inhibit growth and AFB_1 produced by *Aspergillus flavus* ATCC28542. This was confirmed through RT-PCR approach showing the effect of AgNPs on *omt-A* gene involved in aflatoxin biosynthesis.

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