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Effects of Quantum Dot Zinc Oxide, Nano Zinc Oxide, and Zinc Oxide on Gene Expression and Aflatoxin Production Due to *Aspergillus Flavus* Atcc50041

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A B S T R A C T

Aflatoxin, which is mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus*, is a severe problem and threat in the fields of medicine and agriculture and is classified as the first class human carcinogen due to its carcinogenic, mutagenic, teratogenic, hepatotoxicity, and immune system defects. This toxin contaminates many agricultural products around the world. Considering the high toxicity of aflatoxin and the destructive effects of this metabolite, achieving low-risk and cost-effective methods to control toxin production is of great importance. In this study, the effects of different concentrations of quantum dot zinc oxide, nano zinc oxide, and zinc oxide on the expression of important key genes (*aflR*, *aflP*, *aflM*, *aflD*) in the gene cluster of aflatoxin production and also the amount of aflatoxin production (B_1, B_2) in *Aspergillus flavus* was studied. The fungus was cultured in different concentrations of quantum dot zinc oxide, nano zinc oxide, and zinc oxide (2000, 4000, 6000 ppm) for 3 days at 30 ◦C. The results showed that quantum dot zinc oxide significantly reduced toxin production and gene expression compared to other groups, in which the concentration of aflatoxin was decreased by increasing quantum dot zinc oxide. Regarding the obtained results, nano zinc oxide was effective just at 6000 ppm, and zinc oxide is suggested because nano zinc oxide and zinc oxide cannot completely dissolve in water. Hence, nano zinc oxide and zinc oxide showed weak activity in reducing aflatoxin concentration. All three agents under study significantly caused low gene expression, but quantum-dot zinc oxide showed more activity than the others. The formulation of highly active quantum dot zinc oxide to reduce aflatoxin may be a potential feed additive for the future management of mycotoxins.

Keywords: *Quantum dot zinc oxide, Zinc oxide nanoparticle, Aflatoxin, aflR, aflP, aflM, aflD*

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1 Introduction

The filamentous fungus *Aspergillus flavus* is the most common cause of aflatoxin (AF) contamination in food and feed. Four types of aflatoxin are found in AFB_1, AFB_2, AFG_1, and AFG_2 (1, 2). It is remarked that tropical and sub-tropical countries are susceptible to aflatoxin contamination because of their climatic circumstances, which are suitable for fungus growth and mycotoxin synthesis (3). Aflatoxins represent a public health problem. Aflatoxins are more significant than other fungal toxins due to their carcinogenic effects and acute poisoning (1). AFB_1 has strong carcinogenicity potential; therefore, it has been classified by the International Agency for Research on Cancer (IARC) as a human carcinogen, Group 1(4).

Biosynthesis and genetic regulation of aflatoxin have been well characterized. It has been shown that 27 defined genes clustered within a 70-kb DNA region in the chromosome are involved in aflatoxin biosynthesis (5, 6). In the aflatoxin gene cluster, *aflR* is a positive regulatory gene for activating pathway gene transcription (7). Three structural genes,*aflM*, *aflD*, and aflP, in the cluster genes of the biosynthesis aflatoxin pathway, are coded for key enzymes in the creation of aflatoxin. Therefore, they are essential for producing aflatoxin (7).

The nanoparticles (NPs) have obtained remarkable interest because of their tiny size (1-100 nm) with the vast surface-to-volume ratio that causes diversity in their functions such as catalytic action, biological properties, melting point, and mechanical properties (8, 9). In the past years, a new type of fluorescent particle has been used as a good troth for single molecule and single particle tracking in cells and organisms, the semiconductor quantum dots (QDs) that usually range between 2-20 nm in diameter. QDs exhibit special luminescence and electronic properties and find potential uses in biological science (10, 11).

Between the metal oxide NPs/QDs, zinc oxide (ZnO) nanostructures are the spearhead of research because of their marked properties and wide-range applications like UV-detection, nano-optoelectronic devices, biomedical uses like antimicrobial activity such as antifungal and antitoxic effects (1). ZnO is 1 of 5 zinc combinations that is recorded as generally recognized as safe by the U.S. Feed and Drug Administration (FDA) (12). ZnO, an inorganic compound, has received great scientific and technological attention because of its antibacterial, antifungal, antioxidant, anticancer, antiangiogenic, and antiapoptotic activities (3). Several methods have been employed to produce metal oxide NPs/QDs However, these methods show several disadvantages related to nanostructure stability, crystal growth regulation, particle agglomeration, and particle size heterogeneity of ZnO NPs (13). So, an alternative, economical, and green chemistry was developed to synthesize ZnO QDs (14). Green chemistry reduces the employment or production of harmful materials and incorporates renewable materials in the formulation and application of products. This method highlights the advantages of cost-efficient cultivation, safety, rapid production timelines, and scalability, positioning plant systems as a significant alternative for synthesizing quantum dots compared to other biological systems (15-17).

Zinc oxide nanoparticles show up to be white powder and are insoluble in water. Zinc oxide nanoparticles have a vitality band of 3.37 eV and a holding vitality of 60 meV, which gives amazing chemical, electrical, and warm stabilities (18). Zinc oxide nanoparticles exhibit notable optical, electrical, and photocatalytic characteristics (19). Due to these properties, zinc oxide nanoparticles are used in sun-powered cells, photocatalysis, and chemical sensors (20).

Zinc oxide nanoparticles, moreover, encompass firm and unbending structures, making them valuable within the ceramic industry. One of the points of interest in utilizing zinc oxide nanoparticles within the biomedical field is that they act as an excellent surface fabric. Zinc oxide nanoparticle is generally known as a solid resistance of organisms (21). For these reasons, zinc oxide nanoparticles are broadly utilized for organic labeling, natural detection, sedate conveyance, quality conveyance, and nanomedicine (22). The nourishment and sedate organization has endorsed zinc oxide as a secure fabric. Zinc oxide can also dissolve in an acidic environment, providing the fabric with the potential to serve as multifunctional nanocarriers, facilitating drug delivery and release (23).

Although the antifungal activity of ZnO NPs has been widely studied, no effort has yet been formed to evaluate the inhibitory effect of ZnO QD against the toxigenic strain of *A.flavus*. Therefore, the objectives of the current study were to assess the anti-aflatoxigenic efficacy of Zinc oxide, ZnO NPs, and ZnO QDs against a toxigenic strain of *Aspergillus flavus* ATCC50041 in liquid culture, as well as to investigate their impact on the expression of critical genes involved in the aflatoxin biosynthesis pathway. To this end, the Real-time PCR method was used to detect the expression of three structural genes, *aflM*, *aflD*, and *aflP*, and the regulatory gene, all of the aflatoxin biosynthetic pathway.

2 Materials and Methods

2.1 Synthesis of ZnO QDs

ZnO QDs have been synthesized from the Lemon peel using a green chemistry approach and characterized by transmission electron microscopy (TEM), scanning electron microscopy (SEM), and x-ray diffraction (XRD) studies. The lemon fruit was washed well and dried. The skins were placed in a feed dehydrator for 12 hours to completely dry. Then they were ground into relatively fine powder. Then 1 gram of the powder was placed in a glass container with 50 ml of ionized water and mixed for 3 hours. The mixture was placed in a water bath at 60 °C for 60 minutes. Finally, the mixture was filtered, and the resulting extract was stored in an argon atmosphere. Zinc oxide nanoparticles were synthesized by adding 2 grams of zinc nitrate with 42.5 ml of lemon peel extract. Then, mixtures were stirred for 60 minutes and then placed in a water bath with a temperature of 60 °C until the consistency of each mixture was similar to glass caramel. The mixture was heated at 400 °C for 1 hour. Finally, the obtained sample was ground into fine white powder (mesh number 40) and stored separately (24, 25).

2.2 Synthesis of ZnO NPs

Zinc sulfate heptahydrate ($ZnSO_4$. $7H_2O$) and ammonium hydroxide (NH_4OH) are the two main materials for synthesizing ZnO NPs The preparation method of ZnO NPs through direct deposition method is briefly shown below (Figure 1). Zinc sulfate heptahydrate (2.9386 ml) was dissolved in 100 ml deionized water. A stock solution prepared from 25ml of zinc sulfate solution (0.2 M) and 50ml of distilled water, and ammonium hydroxide, was used as an alkaline solution. 25ml of alkaline solution (25%, NH_4OH) was added drop wise to the main solution at a specific rate of 5 ml/min. A heater with magnetic rotation was used at a temperature of 50-60 °C. The sediment obtained was collected through filtration, washed three times with distilled water, and collected using a centrifuge or Buchner funnel. The final deposit was obtained after drying in an oven at a temperature of 60 °C for 8 hours (24, 26). It should be noted that this nanoparticle was prepared in cooperation with Gostran Pars Nanomaterials Company (NAMAGO).

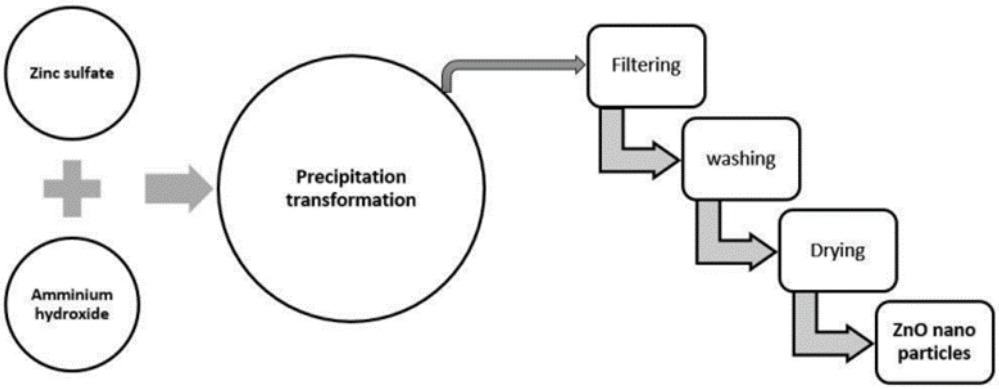


Figure 1. The preparation method of ZnO NPs through the direct deposition method

2.3 Fungal strain

Wild-type aflatoxigenic strain of *A.flavus* ATCC50041 was received from the collection of Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran. Fresh spores of *A. flavus* were yielded from a 7-day-old culture of the strain grown on Sabouraud glucose Agar slant (Merck, Germany). Suspension of the conidia was prepared in 0.5% tween 80 solution, and the number of conidia in the suspension was equaled using a hemocytometer to approximately 108 conidia/mL.

2.4 Effect of Nanostructured ZnO on Aflatoxin Production in Liquid Media

For the experiment, 1 µL of the conidia suspension was added to 500 mL round bottom flasks containing 250 mL of Sabouraud glucose broth (SGB). Different concentrations of the ZnO QDs (2000, 4000, 6000 ppm), ZnO NPs (2000, 4000, 6000 ppm), and Zinc oxide (2000, 4000, 6000 ppm) were added to the cultures and incubated for 3 days at 30 °C. At the end of the incubation period, the content of each flask was filtered through a Whatman no. 4 filter paper. HPLC

analysis and Real-Time Quantitative PCR assayed cultures for aflatoxin production.

2.5 *Aflatoxin Assay*

RP-HPLC determined aflatoxins (AFB_1, AFB_2) according to AOAC (2000). Summarily the filtrated content of each flask was mixed with 150 ml MeOH: H_2O (80:20) and 2.5 g NaCl. Each flask vortexed for 3 min. Phosphate buffer solution (PBS) was used as a liquid mobile phase to move the mixture through an immunoaffinity column (Puri-Fast AFLA IAC, Libios, France). 65 microliter of PBS was added to 10 ml of this mixture, shaken strenuously and passed through glass fiber filter. 70 ml of liquid was transferred onto the column at a flow rate of 3 ml min-1. The column was then washed with 15 ml PBS and dried by passing air gently through it, and aflatoxins were passed by adding 500 and 750 µl methanol at 1 min intervals. The elution was diluted with 1750 µl H_2O. The fractional of 200 µl was injected into the HPLC system. HPLC system was armed with a separator module (2695, Waters, USA), a Nova-Pak LC-18 column, and a fluorescence detector (474, Waters, USA). Aflatoxins were derivatized by K.B. Cell post-column derivatization system (Libios, Chemin de Plagne 69210 Bully, France) in an H_2O–MeCN–MeOH mobile phase comprising HNO_3 and KBr at a flow rate of 1 ml min-1. Aflatoxins were detected at electromagnetic radiation with an excitation wavelength of 365 nm and a fluorescence emission wavelength of 435 nm. Aflatoxins were quantified using the peak height by Millenium 32 v 4.0 software (Waters, USA). Aflatoxin standards were bought from Sigma (St. Louis, MO, USA). The detection limit of the HPLC method was 0.5 ng ml-1 for all aflatoxins, and the recovery rates were found to be 78.5%, 82.7%, 83%, and 80.9% for AFB_1 and AFB_2, respectively. The pursuant equation calculated the percent inhibition of aflatoxin production:

Inhibition of aflatoxin production % = (Ac-As)/Ac × 100

Where Ac is the amount of aflatoxin in the control sample, As is the amount of aflatoxin in the treated sample.

2.6 *Real Time PCR*

Total RNA was extracted from a strain of A. flavus following the method described by TRIsure (Bioline, Luckenwalde, Germany). RNA concentration and quality were measured by assessing the absorbance at 260/280 nm using a NanoDrop One UV-Vis Spectrophotometer. The RNA quantity was determined by (Thermo Fisher Scientific, Waltham, MA), and verified using % one gel agarose electrophoresis. Reverse transcription of total RNA was done with the BioFact™ RT Series cDNA synthesis kit. Quantitative real-time PCR was carried out using the SYBR Green method using forward and reverse primers, developed using the published sequences of three structural genes (*aflP*, *aflM*, *aflD*) and one regulatory gene (*aflR*) associated with the aflatoxin biosynthesis pathway, and *GAPDH* as detailed in Table 1 qPCR data were analyzed by the delta delta C.T. (ddCT) method and normalized to *GAPDH*.

Table 1. Primers used in this study, target gene, sequence

Gene	Primer code	Primer sequence (5´-3´)
aflP	aflP, Forward	CACGCTTTCAGAGCAGGTAA
	aflP, Reverse	TTCGGTGGAGGAGGGAGTT
aflM	aflM, Forward	GAGCCAAAGTCGTGGTGAAC
	aflM, Reverse	GCCTGGATTGCGATAGCGTC
aflD	aflD, Forward	ATGCTCCCGTCCTACTGTTT
	aflD, Reverse	ATGTTGGTGATGGTGCTGAT
aflR	aflR, Forward	CCTTTCTCACTACTCGGGTTT
	aflR, Reverse	GCAGGTAATCAATAATGTCGG
GAPDH	GAPDH, Forward	AAGGGTGGTGCTAAGCGTGT
	GAPDH, Reverse	GTGATGGCATGGACAGTGGT

2.7 *Statistical Analysis*

Statistical analysis was performed by SPSS version 25. The results were analyzed via analysis of variance (ANOVA). The Tukey Post Hoc test was used to determine the statistically significant difference in different groups. The significance level was set at $p<0.05$.

3 Results

3.1 Analysis and characterization of ZnO QDs

A transmission electron microscope was used to obtain the exact size of ZnO QDs. These nanoparticles are imaged individually with a scale of 10 nm. The results obtained from measuring the size of ZnO QDs by image software showed that each ZnO QDs average size was around 20 nm (Figure 2). The scanning electron microscope image obtained from the characterization of ZnO QDs shows that nanoparticles have been synthesized in a spherical shape (Figure 3). X-ray diffraction (XRD analysis) is one of the non-destructive methods for qualitative and quantitative analysis of crystalline materials in powder form. The analysis of ZnO QDs was carried out in the range of 10 to 70 °C in the range of 2θ angle. The peaks in the approximate range of 32, 33, 34.5, and 36 are related to the pure synthesis of ZnO QDs, respectively and according to JCPDS standards (26), it indicates the presence of the crystalline structure of ZnO QDs, which was consistent with the previous tests (Figure 4) (26).

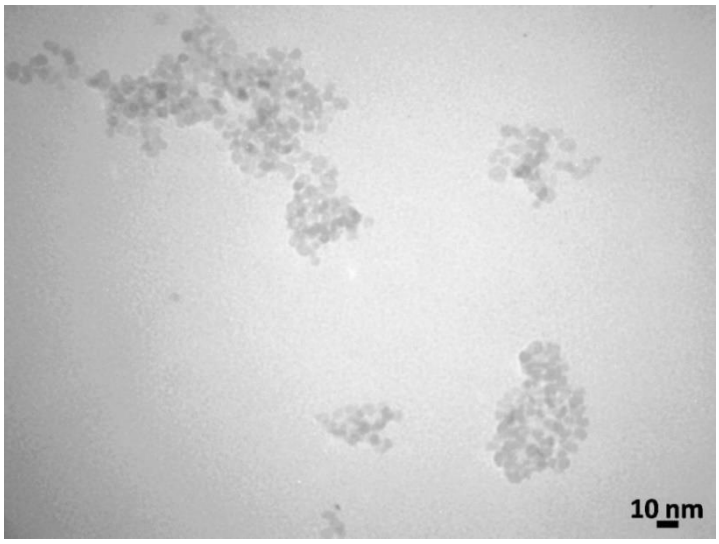


Figure 2. Transmission electron microscope image of synthesized ZnO QDs

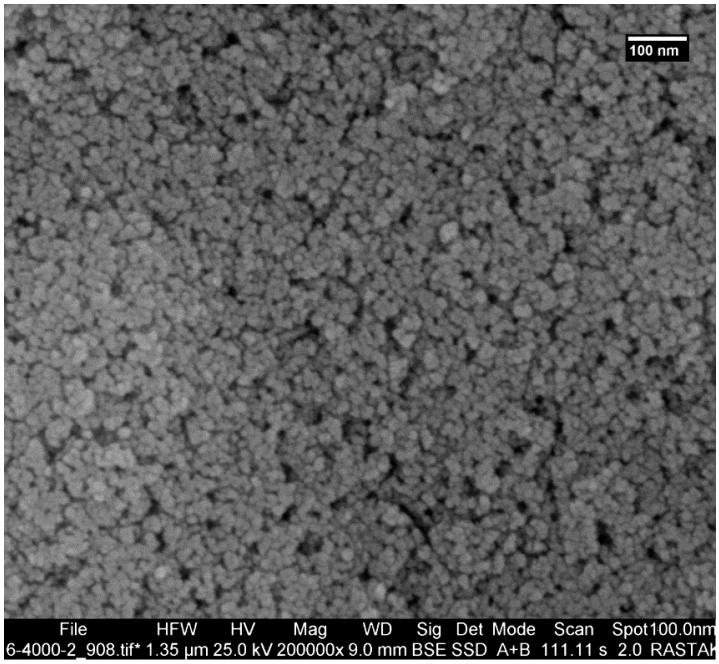


Figure 3. Scanning electron microscope image of synthesized ZnO QDs

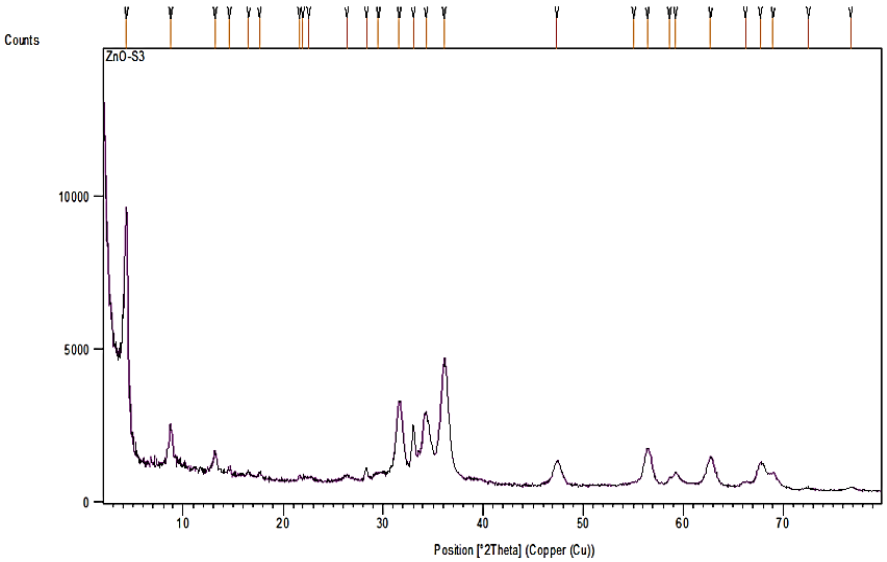


Figure 4. XRD patterns of ZnO QDs: The results of nanoparticle size measurement of samples by XRD and TEM indicate that the size of the ZnO QDs was about 20 nm.

3.2 Analysis and characterization of ZnO NPs

The nanoparticles obtained using the above method had a wurtzite structure and an average particle size of 30 nm. TEM images showed that the obtained ZnO NPs have a uniform structure and a high degree of purity (Figure 5).

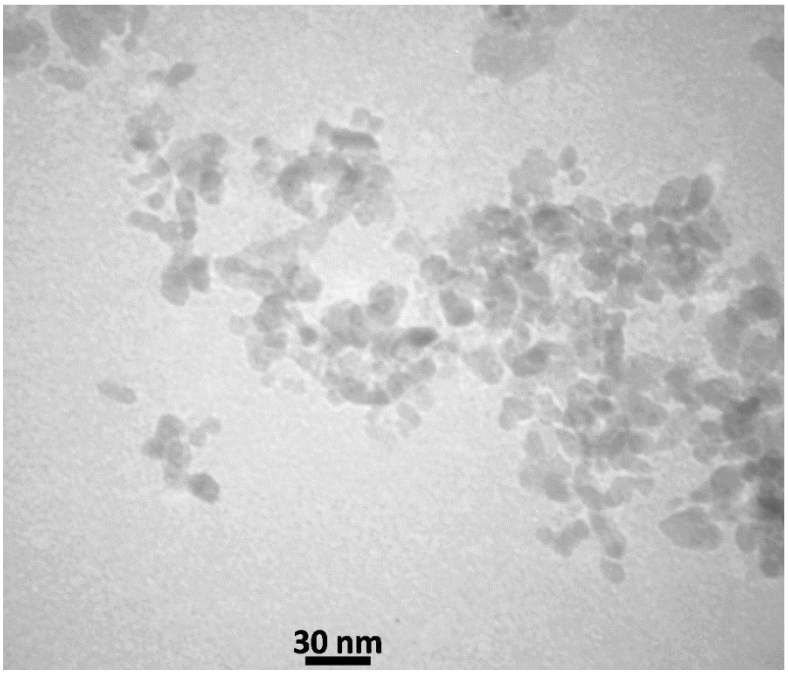


Figure 5. Transmission electron microscope image of synthesized ZnO NPs

3.3 *Effect of different particles on aflatoxin production of A.Flavus ATCC50041 on SDB*

The production of aflatoxin in different concentrations of ZnO QDs, ZnO NPs, and Zinc oxide (2000, 4000, 6000 ppm) was studied. *A.flavus* ATCC50041 was cultured on SGB in different concentrations of ZnO QDs, ZnO nanoparticles, and Zinc oxide for 3 days at 30 °C. Tables 2, 3, and 4 show the aflatoxin B_1 and B_2 production in the studied groups. In the group treated with ZnO QDs, the amount of aflatoxin B_1 decreased significantly (p<0.001). The amount of aflatoxin B_2 production showed a non-significant increase at 2000 ppm but decreased significantly in the other two concentrations (p<0.001). Total aflatoxin production decreased non-significantly at 2000 ppm and significantly at 4000 and 6000 ppm, respectively (p<0.05, 0.001). In the ZnO NPs group, two concentrations of 2000 and 4000 ppm increased the production of aflatoxin B_1 but decreased significantly at 6000 ppm (p<0.05). Aflatoxin B_2 was significantly reduced at 6000 ppm (p<0.001). Total aflatoxin production increased at 2000 and 4000 ppm, decreasing significantly at 6000 ppm (p<0.001). In the zinc oxide group, the total results showed a trace of the stimulation of aflatoxin B_1 and B_2 production in different concentrations. It seems that because nano zinc oxide and zinc oxide cannot completely dissolve in water, so nano zinc oxide and zinc oxide showed weak activity to reduce aflatoxin concentration.

Table 2. Effect of different concentrations of ZnO QDs on aflatoxin production of A.Flavus ATCC50041 on SDB

ZnO QDs (ppm)	Aflatoxins (ppb)		
	B_1	B_2	Total aflatoxin
0	38.04 ± 0.71	9.21 ± 0.91	23.62
2000	27.25 ± 0.41 ***	11.41 ± 0.92 ns	19.33 ns
4000	10.25 ± 1.32 ***	1.76 ± 0.69 ***	6.0 **
6000	1.12 ± 0.91 ***	0.14 ± 0.99 ***	0.63 ***

ns: No significant, **: ($p < 0.05$), ***: ($p < 0.001$)

Table 3. Effect of different concentrations of ZnO NPs on aflatoxin production of A.Flavus ATCC50041 on SDB

ZnO NPs (ppm)	Level of aflatoxins (ppb)		
	B_1	B_2	Total aflatoxin
0	38.04 ± 0.71	9.21 ± 0.91	23.62
2000	88.29 ± 1.05	47.12 ± 0.19	29.7
4000	66.39 ± 0.42	14.13 ± 0.88	28.26
6000	10.25 ± 0.39 **	0.95 ± 0.51**	11.6 ***

: ($p < 0.05$), *: ($p < 0.001$)

Table 4. Effect of different concentrations of Zinc oxide on aflatoxin production of A.Flavus ATCC50041 on SDB

Zinc oxide (ppm)	Aflatoxins (ppb)		
	B_1	B_2	Total aflatoxin
0	38.04 ± 0.71	9.21 ± 0.91	23.62
2000	163.45 ± 1.61	48.14 ± 0.62	31.79
4000	98.80 ± 0.09	28.51 ± 0.93	30.45
6000	72.88 ± 0.48	17.39 ± 0.81	24.13

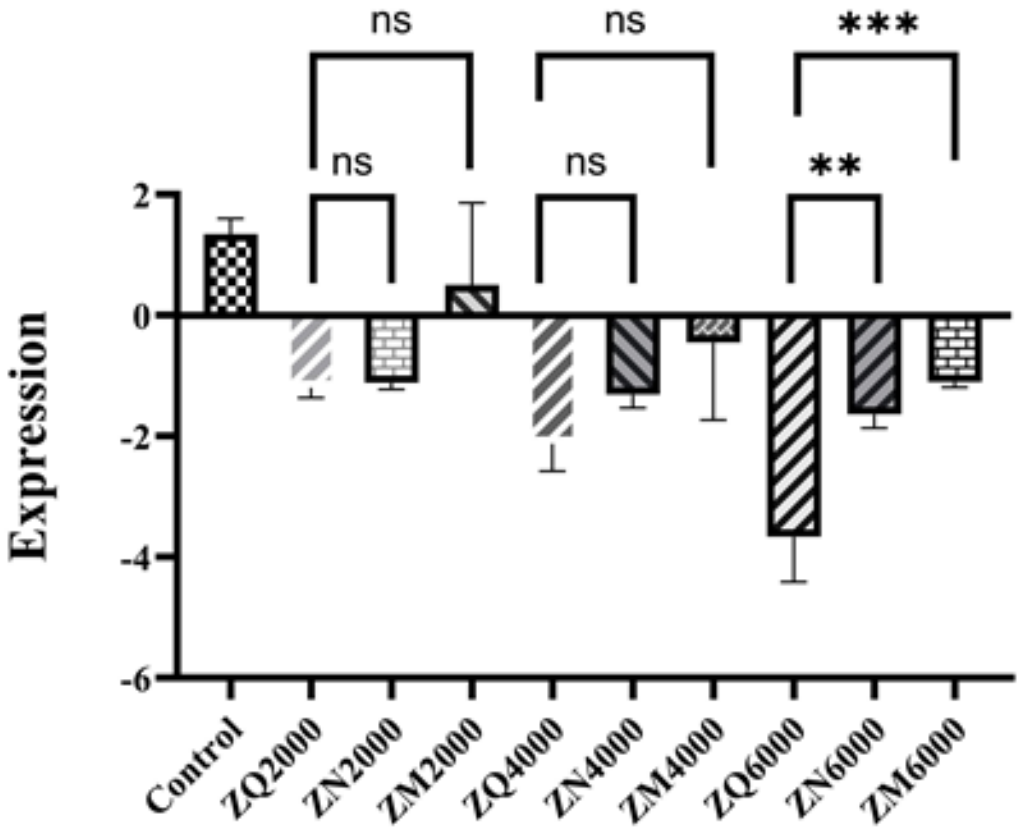
3.4 *Real Time PCR*

All the treated samples compared to the untreated samples were measured regarding the expression level of the studied genes. The most abundant expression of the *aflR* gene was related to the control group, and a significant decrease in expression was observed in the groups treated with different particles compared to the control group. The best response at the concentration of 6000 ppm was related to the ZnO QDs group. The group of ZnO QDs and ZnO NPs decreased the expression of this gene at 2000 and 4000 ppm at a non-significant level and at 6000 ppm at a significant level, respectively. (p<0.05, 0.001). Although ZnO QDs group mostly had a better response, this response was usually non-significant (Figure 6).

The most abundant expression of the *aflP* gene is related to the control group, and a significant decrease in expression was observed in the groups treated with different particles compared to the control group. The best response at the concentration of 6000 ppm corresponds to the ZnO QDs group (p<0.001). The group of ZnO QDs, ZnO NPs, and zinc oxide in all three concentrations of 2000, 4000, and 6000 ppm significantly decreased the expression of this gene, respectively (p<0.001) (Figure 7).

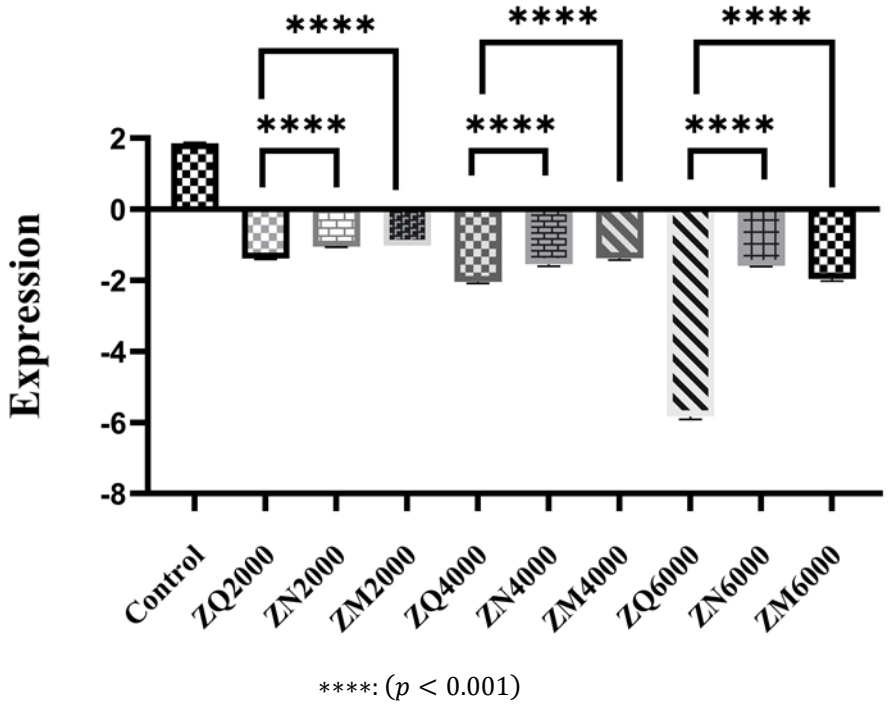
The most abundant expression of the *aflM* gene was related to the control group, and a significant decrease in expression was observed in the groups treated with different particles compared to the control group. The best response at the concentration of 6000 ppm was related to the ZnO QDs group. The group of ZnO QDs and ZnO NPs decreased the expression of this gene 6000 ppm significantly (p<0.001) and at 2000 and 4000 ppm at a non-significant level, respectively. Although ZnO QDs mostly had a better response, this response was usually non-significant. The group of ZnO QDs and zinc oxide decreased the expression of this gene at 2000 ppm (p<0.05) and 6000 ppm (p<0.001) significantly and at 4000 ppm to a non-significant level (Figure 8).

ZnO QDs at 6000 significantly decreased the expression of the *aflD* gene compared to the other two groups (p<0.001). The nano zinc oxide decreased gene expression more than zinc oxide, and in total, all three groups decreased gene expression in different concentrations compared to the control group (p<0.05, 0.001) (Figure 9).



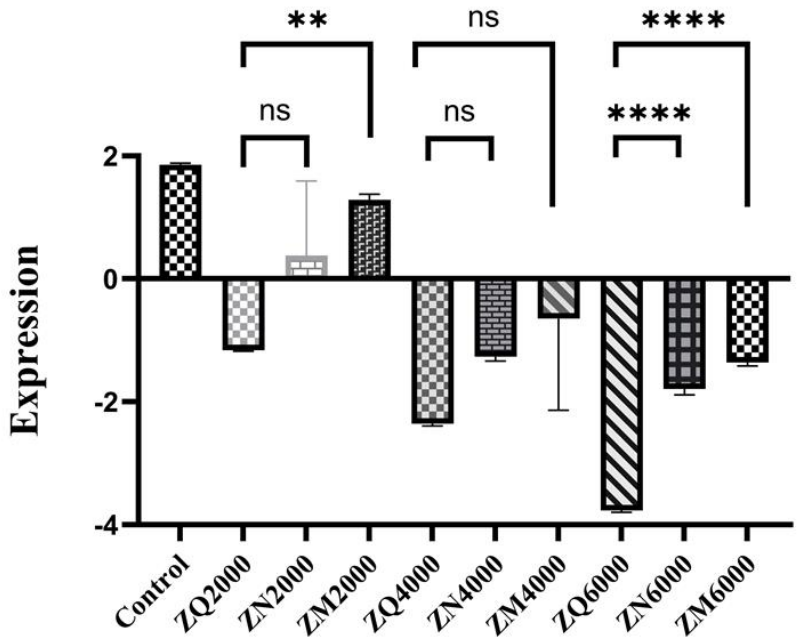
ns: No significant, **: ($p < 0.05$), ***: ($p < 0.001$)

Figure 6. Comparison of the effect of different concentrations of ZnO QDs, ZnO NPs and Zinc oxide on expression level of aflR gene in A.Flavus ATCC50041



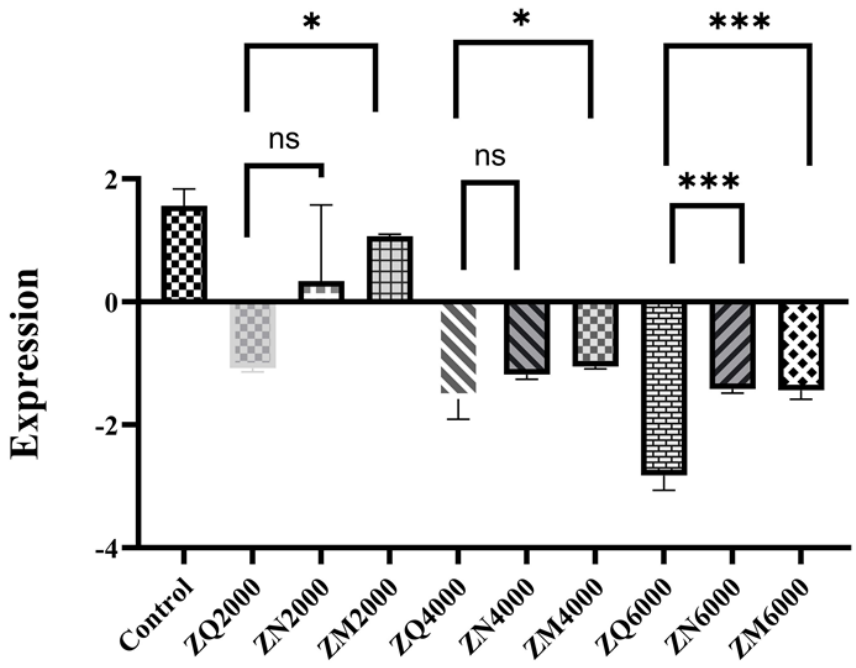
$****: (p < 0.001)$

Figure 7. Comparison of the effect of different concentrations of ZnO QDs, ZnO NPs, and Zinc oxide on the expression level of aflP gene in A.Flavus ATCC50041



ns: No significant, $**: (p < 0.05)$,$****: (p < 0.001)$

Figure 8. Comparison of the effect of different concentrations of ZnO QDs, ZnO NPs, and Zinc oxide on the expression level of aflM gene in A.Flavus ATCC50041



ns: No significant, ∗: ($p < 0.05$),∗∗∗: ($p < 0.001$)

Figure 9. Comparison of the effect of different concentrations of ZnO QDs, ZnO NPs and Zinc oxide on expression level of aflD gene in A.Flavus ATCC50041

4 Conclusions

Mycotoxins are among the most important toxic factors in human and livestock diets. Many fungi grow easily on simple or mixed feed and can spoil the feed by producing some enzymes. Some fungi can grow on feed and produce toxins under certain conditions of temperature and humidity. Among the fungal toxins, aflatoxins, which are often produced by *Aspergillus flavus* and *Aspergillus parasiticus*, cause the most frequent feed poisoning in animals, which causes death in acute poisoning and immunodeficiency and increased infection in chronic form (27).

Here, the antiaflatoxigenic (B_1 and B_2) activity of ZnO QDs, ZnO NPs, and Zinc oxide on toxigenic *A. flavus* ATCC50041 concerning reduction in expression of aflatoxin biosynthesis key genes pathway (*aflM*, *aflD*, a*flP*, and *aflR*) was studied.

According to the results obtained from the molecular RT-qPCR test, the decrease in the expression of these genes in most cases showed a significant difference with the control group, and ZnO QDs showed a more significant decrease in expression than the other two groups. According to the studies, zinc oxide has antifungal properties, especially against toxin-producing fungi, so the study of Hassan et al. showed that nano zinc oxide can inhibit the growth of *A. flavus*, *Aspergillus parasiticus*, and *Aspergillus ochraceus* (1). In the review of Hernandez et al. (2018), the antifungal and anti aflatoxigenic activities of ZnO nanostructures were evaluated using a highly toxic strain of *Aspergillus flavus*. The results showed the inhibition of the growth of the fungus, and the suppression of aflatoxin biosynthesis (99.7%) was also observed (28). In 2018, Deabes et al . conducted a study to investigate the effect of silver nanoparticles (Ag NPs) on the production of aflatoxin B_1 by evaluating the transcriptional activity of the genes of the aflatoxin biosynthesis pathway in *Aspergillus flavus*. The specific primers involved in AFB_1 biosynthesis is the *omtA* gene. AFB_1 production in YES medium was measured by high pressure liquid chromatography (HPLC). The results showed a decrease in AFB_1 production. Also, the cultivation of *A. flavus* in the presence of Ag NPs decreased the level of omt-A gene expression (29).

Here, nanoparticles were used to accelerate the drug's effect and save the agent's amount. Quantum dot method, a relatively new method, and green synthesis were also used. In the synthesis of nanoparticles by the green synthesis method, the number of chemicals used is reduced, and as a result, the amount of pollutant production is also reduced. Also, this method is cost-effective. Green synthesis of

nanoparticles uses environmentally friendly, non-toxic, and safe reagents. Among the natural components used for synthesizing zinc oxide, plants and plant extracts are very attractive as sources for the green synthesis of metal nanoparticles because they eliminate the need to use hazardous materials and the tedious process of cultivation and processing.

Considering that four different and important genes in aflatoxin biosynthesis were tested, these results show that ZnO QDs and nano zinc oxide in concentrations of 2000 to 6000 ppm have appropriate inhibitory effects on the expression of these genes. Of course, with the increase in concentration, the decrease in the expression of genes was evident; considering the acceptable level of inhibition of these genes in the concentration of 2000 ppm, this concentration can be used for this purpose.

The amount of aflatoxin B_1, B_2, and total were measured using the HPLC test. For this reason, the standard strain was incubated in SDB, flasks of the same size, and a shaker for 3 days at 30 °C; after filtration and through standard procedures, the obtained liquid was tested. The results show that ZnO QDs in different concentrations had significant inhibitory effects compared to the control and other groups under study. However, in the nano zinc oxide group, the amount of total aflatoxin in concentrations of 2000 and 4000 ppm and the zinc oxide group in all three concentrations showed an increase in total aflatoxin level. It seems that this increase, which was confirmed by repeating the experiment three times, may be due to the stimulation of the expression of some genes that were not studied because a set of gene clusters is responsible for the biosynthesis of aflatoxins also because nano zinc oxide and zinc oxide cannot completely dissolve in water. Hence, nano zinc oxide and zinc oxide showed weak activity in reducing aflatoxin concentration.

In this study, although gene expression decreased in these two groups at a 2000 ppm, other genes probably have increased expression, so other research is needed using new genes. In Khosravi et al.'s study, geranium at 0.2 µg/ml stimulated *nor1* and *ver1* genes and increased aflatoxin production, while at 0.4 µg/ml, expression (production) of aflatoxin decreased. Considering that in a review of other studies, the use of ZnO QDs for antifungal and antitoxigenic purposes was not carried out, and considering the effective results of this technique in reducing gene expression and toxin production, extensive studies in the future using this method and the use of other fungal toxins such as doxynivalenone, T2 toxin, zearalenone and ochratoxin is necessary.

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Conflict of Interest

The authors declared no conflicts of interest.

Author Contributions

All the authors contributed to all parts of this research.

Data Availability Statement

Data are available from the first author upon reasonable request.

Ethical Considerations

There is no ethical consideration.

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