

Control for Mycotoxigenic Fungi by Nanosilver Synthesised by Wild Plants Extracts

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Abstract

The biosynthesis of silver nanoparticles (AgNPs) by plant extracts is a preferred method because it is an environmentally safe, fast and low cost method. Therefore, this study aimed to use aqueous extracts from (*Sonchus oleraceus* and *Cichorium pumilum*) to synthesize AgNPs, and their evaluation antifungal activity and inhibitory impact on the production of aflatoxin B₁ (AFB₁) produced by the new strain *Aspergillus aflatoxiformans*, and *A. ochraceus* that produce ochratoxin A (OTA). AgNPs synthesized by *S. oleraceus* give size ranged between 6 and 30 nm, While AgNPs synthesized by *C. pumilum* sizes ranging from 3-36 nm. The inhibition zone with *A. aflatoxiformans* was 33.3±7.6, and 30.7±5.13mm at 400ppm from AgNPs synthesized by *S. oleraceus* and *C. pumilum*, respectively, while with *A. ochraceus* the inhibition zone increased to (35.0 ± 5.0 and 37.0 ± 4.3 mm). Finally, this study recorded for the first time, the impact of AgNPs on growth and ability of *A. aflatoxiformans* to produce AFB₁. The inhibition percentage of AFB₁ reached to 97%.

Keywords: Nanosilver particles (AgNPs) • *Aspergillus aflatoxiformans* • Aflatoxin B1 • Ochratoxin A • Anti-mycotoxins

Introduction

In recent years, nanomaterials have received immense attention from researchers due to their physical and chemical properties, which encouraged many researchers to use a lot of metals, such as gold, silver, etc., for the synthesis of nanomaterials [1,2]. Several methods have been for the synthesis of nanoparticles namely, chemical methods, physical and biological methods, but the biosynthesis of nanoparticles by the microorganisms and plant extracts. It is the favorite method because of it has advantages like simplicity, lower cost and environmental-friendly. So, it is called a green synthesis [3,4]. Silver nanoparticles (AgNPs) a biosynthesis has gotten expanding consideration in the field of nanotechnology because of their use widely in several pharmaceutical and agricultural applications, as well as it has been an antimicrobial activity, and anticancer properties, anti-inflammatory action, and antitumor activity [5,6]. Many studies reported that plant extract used for the synthesis of AgNPs may be due to containing many active compounds that act as the reducing agent for silver nitrate [7-9]. Egypt has many wild plants such as *Sonchus oleraceus* and *Cichorium pumilum* have been several bioactive compounds were identified in the leaves including alkaloids, flavonoids, tannins, terpenes, steroids and phenols [10,11]. To the best of our knowledge and literature survey, these two plants widespread in Egypt have not been used for the synthesis of AgNPs. Therefore, in this study, firstly we biosynthesized AgNPs using aqueous extracts from *S. oleraceus* and *C. pumilum*, and then evaluate its antifungal activity properties against as newly strain *Aspergillus aflatoxiformans* and *Aspergillus ochraceus* isolated from cereals, secondly in this study impact evaluated these particles as anti-mycotoxins aflatoxin B1 (AFB1) and ochratoxin A (OTA) they are produced by these two strain, respectively.

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Materials and Methods

Materials

Plant material: Fresh leaves of *Sonchus oleraceus* and *Cichorium pumilum* were collected from various cultivated fields in Qalubia governorate, Egypt. The leaves were dried by air, then ground to a fine powder and stored in plastic package.

Fungal strains: The fungal strains used in this study were *Aspergillus aflatoxiformans* and *Aspergillus ochraceus* isolated from cereals. The retrieved sequences were deposited to NCBI Gen Bank with accession # MN093924.1 and MN093933.1, respectively.

Chemicals and solvents: Silver nitrate, Potato Dextrose Agar (PDA), yeast extract agar and yeast extract were obtained from Sigma- Aldrich, Lyon, France. AFB1 and OTA standards, methanol, acetonitrile, acetic acid, chloroform, sodium chloride and sodium sulphate anhydrous were purchased from Sigma Chemical Co. (St. Louis, MO). All solvents were of HPLC grade. The water was double distilled with Millipore water purification system (Bedford, MA).

Methods

Preparation of aqueous extract: Five grams of powder plants leaves were extracted by 100 ml of distilled water at room temperature for 24 h, the mixtures were centrifuged at 3000 rpm for 15 min, and the extract was concentrated under vacuum with a rotary evaporator, and then evaporated to near dryness, it used in this study.

Synthesis of AgNPs: For the biosynthesis of AgNPs, one mL for each separate aqueous extract was added to 9 ml of 3 Mm aqueous solution of silver nitrate for reduction into Ag⁺ ions and kept at room temperature until the color changed from colorless to yellowish-brown and finally to dark brown.

Characterization of AgNPs: The optical absorption spectrum was obtained using UV-visible spectrophotometer using Lambda 2 spectrophotometer Perkin-Elmer at a resolution of 1 nm in the range 200–800 nm. The morphology of the synthesized AgNPs was observed transmission electron microscope (TEM). Dynamic light scattering (DLS) instrument (PSS, Santa Barbara, CA, USA), using the 632 nm line of aHeNe laser as the incident light with angel 90° and Zeta potential with external angel 18.9°. Nanomaterial

Investigation Lab., Central Laboratory Network (CLN), National Research Centre (NRC). To characterization of functional groups on the surface of AgNPs was performed by Fourier-transform infrared spectroscopy (FTIR 6100, Perkin-Elmer, Germany), and the spectra were scanned in the 400–4000 cm^{-1} range at a resolution of 4 cm^{-1} .

Assay of antifungal activity: The antifungal activity of synthesized AgNPs with the gradual concentrations (50 to 400 ppm) were tested on the mycotoxigenic strains *A. aflatoxiformans* and *A. ochraceus* using agar well diffusion technique. After incubation time, the plates were tested for the mycelial growth inhibitory zones around the wells [12].

Inhibition of AFB1 and OTA produce in (YES) culture medium by AgNPs: The yeast extract sucrose (YES) culture medium (2% yeast extract and 15% sucrose/liter distilled water) was used in this experiment. The culture medium was poured into 250 ml Erlenmeyer flask and autoclaved at 120°C for 15 min, cooled at room temperature and inoculated with approximately (1×10^5) spores suspension of *A. aflatoxiformans* and *A. ochraceus* both separately (control). Gradual concentrations of (50, 100, 150, 200, 250, 300, 350 and 400 ppm) from AgNPs were added to 100 ml YES medium and incubated at 28°C for 14 days. After the end of the incubation period, the AFB1 and OTA were extracted then determined using HPLC as following: toxins were extracted from YES medium by using 20 ml of chloroform (twice with 10 ml media) then homogenization for 3 min in a separation funnel. The chloroform phase was filtered through filter paper Whatman No. 3 and concentrated to dryness under a nitrogen stream then determination the quantitative for toxins by HPLC [13,14]. The percentage of inhibition was calculated as following equation. The percentage of inhibition toxin = $(C-T)/C \times 100$ where: C is concentration of toxin (AFB1 or OTA) in control sample that inoculated by spores of fungus only. T is concentration of toxin in the sample treated by AgNPs (treatment sample).

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. All statements of significance were based on the probability of P-value ≤ 0.05 was considered to be statistically significant.

Results and Discussion

Characterization of Ag NPs synthesized by wild plant extracts

The color change of the AgNO_3 after mixing with plant extracts indicating the synthesis of AgNPs; accordingly, the UV–vis spectroscopy is a critical procedure to confirm the formation of AgNPs in solution. In this study it was observed that the absorption peaks occur at 430 nm and 460 nm indicating that AgNPs were produced by extracts from *S. oleraceus* and *C. pumilum*, respectively as Figure 1. The color of AgNPs to become apparent may be due to a phenomenon known as plasmon absorbance, where the incident light occurs oscillations in the conduction electrons on the surface of the nanoparticles, and electromagnetic radiation is dispersed [15]. Concerning TEM investigation clarify that the size of AgNPs produced by aqueous extracts *S. oleraceus* and *C. pumilum* was 6–30 nm and 3–36 nm, respectively (Figure 2). On the other hand, these results have been confirmed with size particle diffraction analysis (DLS) as shown in Figure 3. It has shown the average size distribution of AgNPs in colloidal solution which was found to be (30.1 and 53.4 nm) with *S. oleraceus* and *C. pumilum*, respectively. Also, the obtained data indicated that the average zeta potential was (12.33 and -1.07 mV) with *S. oleraceus* and *C. pumilum* respectively (Figure 4). The size measured by DLS was, somewhat, larger compared with the measurements reported from TEM, which is normal as DLS measures the hydrodynamic size [16]. Whereas, the negative zeta potential value may be due to the capping agents, which mainly consisted of negatively charged groups coating the surface of AgNPs. Regarding the particle-size distribution, all the prepared AgNPs were polydisperse, this may be due to be attributed to variations in the growth rates of individual particles during the nucleation step [17,18]. The FTIR spectrum for AgNPs biosynthesized by plant extracts illustrated in Figure 5. Revealed that the absorption peaks at 3431.71, 2919.7, 1625.7, 1383.68 and 1033.66

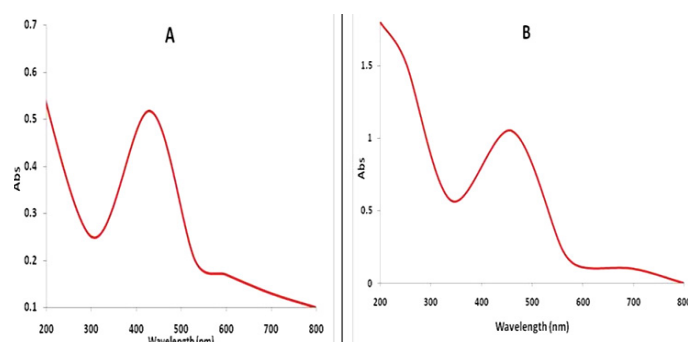


Figure 1. The UV/Vis spectrum of AgNPs synthesized by (A) *S. oleraceus* and (B) *C. pumilum* extract.

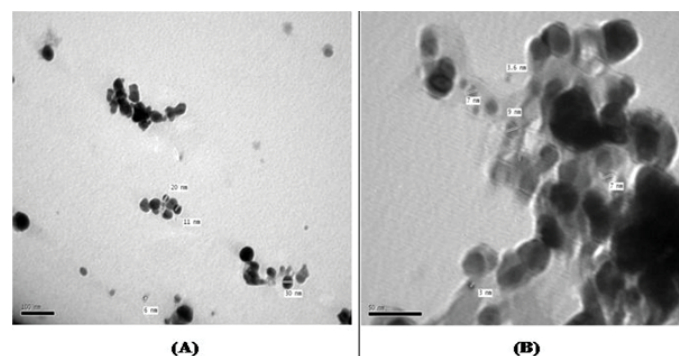


Figure 2. TEM image of AgNPs biosynthesized using extracts of (A) *S. oleraceus* and (B) *C. pumilum*.

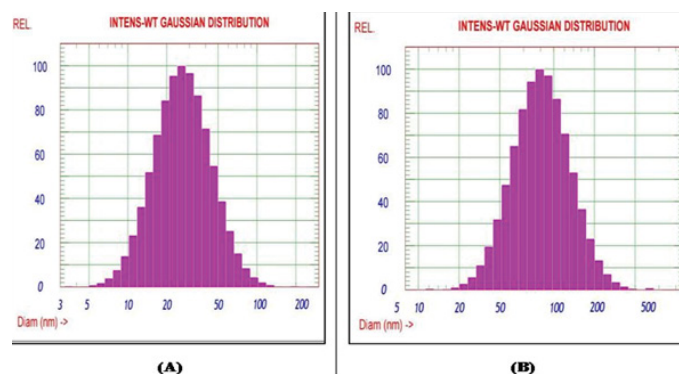


Figure 3. The distribution of size AgNPs biosynthesized using extracts of (A) *S. oleraceus* and (B) *C. pumilum*.

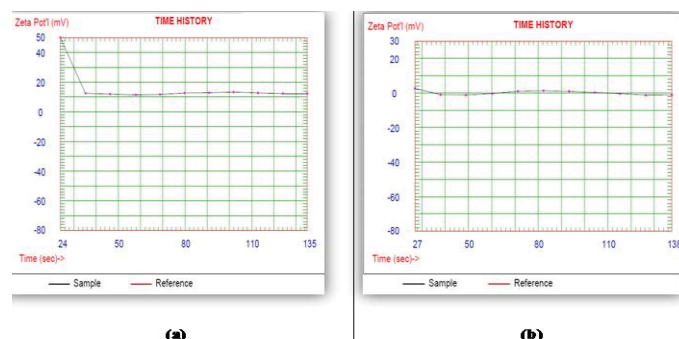


Figure 4. The zeta potential of AgNPs biosynthesized using extracts of (a) *S. oleraceus* and (b) *C. pumilum*.

cm^{-1} . The hydroxyl functional groups in phenolic compounds had shown the stretching vibrations at 1110 and 1460 cm^{-1} [19]. On the other hand, the band appeared at that 1600–1800 cm^{-1} can be ascribed to C = O functional groups [20], while peaks at 3400 to 3900 cm^{-1} correspond to stretching for bond –OH groups. The band at about 2300 to 2900 cm^{-1} can be attributed to C=N and

C-S [21,22]. All these functional groups were responsible for the decrease of Ag⁺ particles to AgNPs, which bounded along with AgNPs. So these plants were a good source of natural effective compounds that can use as reducing for metals such as silver [23,24].

Identification of fungal isolates

The two fungal strains were further identified based on the sequence of ITS regions. The amplified ITS 4/5 rDNA region of the two fungal isolates, the obtained ITS sequences of these isolates was were BLAST searched with non-redundant sequences on the NCBI database aligned on NCBI database giving *A. Aflatoxiformans* and *A. ochraceus* deposited on Gene bank with accession # MN093924.1 and MN093933.1, respectively (Figure 6a and b). It should be noted [25], the first to describe as a new species named *A. aflatoxiformans*, also added this strain closely related to *A. flavus*, and grows faster on YES media [25].

Evaluation of the sensitivity of selected *Aspergillus* strains for AgNPs

Data presented in Figures 7 and 8 showed the inhibition zone (mm) of *A. aflatoxiformans* and *A. ochraceus* after treatment with AgNPs colloids at the concentration 50, 100, 150, 200, 250, 300, 350 and 400 ppm. The results showed there is a direct correlation between concentration of the AgNPs and inhibition zone, where the inhibition zones were (33.3 ± 3.8 and 30.7 ± 4.0) mm with AgNPs synthesized from *S. oleraceus* and *C. pumilum* against *A. aflatoxiformans*, respectively. While for *A. ochraceus* the inhibition zones were (35.0 ± 4.0 and 36.3 ± 3.3 mm) with AgNPs synthesized from *S. oleraceus* and *C. pumilum*, respectively. There are many ways for inhibition of fungal growth by AgNPs, which join to the cell membrane causes structural changes, and damage vital cell functions such as transport activity, penetrability, and prompt neutralization of the surface electric charge and produce cracks and pits through which internal cell contents are effluxes [26]. In addition, Ag⁺ linked with -SH of cell membrane to produce stable S-Ag bonds or disulfide bonds (R-S-S-R). On the other hand, nanoparticles producing reactive oxide species (ROS) leads to the damage to the lipid bilayer cell membrane and the breakdown of the affected cell [27-29]. AgNPs caused imposed damage on fungal cells, preventing hyphae elongation and spore germination. Fungal hyphae treatments with AgNPs shown morphological changes damage such deformations in mycelial growth and the shape of hyphal walls, unusual bulges and ruptures [30]. The analysis of variance for the effect of AgNPs on growth *A. aflatoxiformans* and *A. ochraceus* show that treatments by AgNPs have a significant effect on growth depends significantly on the concentration of AgNPs, while types of AgNPs synthesized by extracts of *S. oleraceus* and *C. pumilum* were no significant (Tables 1 and 2).

Impact of AgNPs on the production of AFB₁ and OTA

Data presented in Figure 9 showed the percentages of inhibition of AFB₁ production after treated YES media by AgNPs at gradually concentrations ranged 50 to 400 ppm. This is the first study that examined the effect of AgNPs on the ability of *A. aflatoxiformans* to produce toxin. The results indicated that the AgNPs synthesized by *S. oleraceus* and *C. pumilum* reduced AFB₁ to 90.0 and 91.1%, respectively at 50ppm, while increased to 97.9 when treated liquid media by 400 ppm from AgNPs. According to these preliminary results, it is clear that AgNPs have a high ability to inhibit the production of the AFB₁ produced by that strain (an extended of that study, we will study

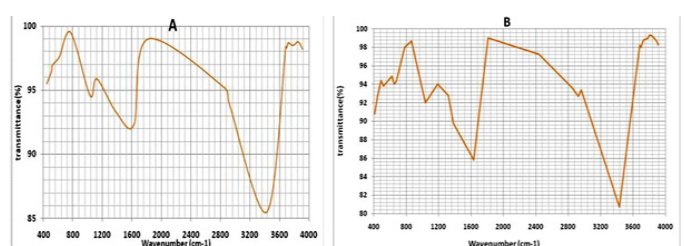
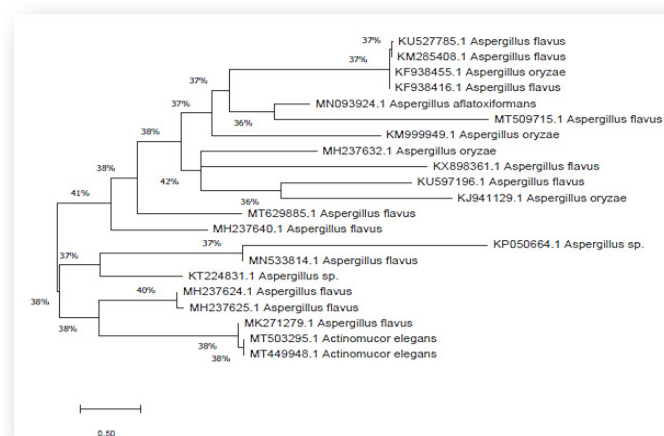
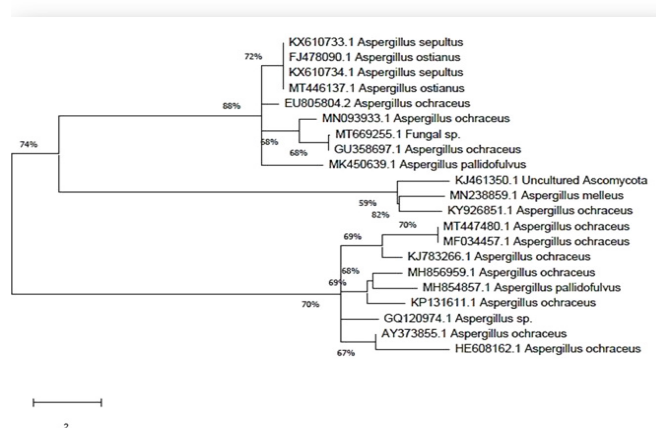


Figure 5. FTIR spectrum of AgNPs synthesized by aqueous extract from (A) *S. oleraceus* and (B) *C. pumilum*.



(a)



(b)

Figure 6. Neighbor joining tree showing phylogenetic relationship between *A. ochraceus* and their representative species from NCBI database.

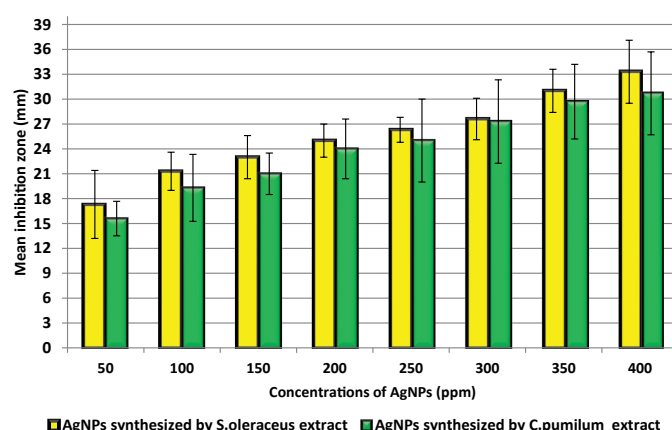


Figure 7. Effect of AgNPs synthesized by extracts of *S. oleraceus* and *C. pumilum* on growth of *A. aflatoxiformans*.

the effects AgNPs on the gene expression responsible for the production of the aflatoxins). The results displayed in Figure 10 indicated that at 50 ppm from AgNPs synthesized by extracts of *S. oleraceus* and *C. pumilum* inhibit the production of OTA to 86.7 and 95.5%, respectively. While at the high concentrate from AgNPs (400 ppm) increased the percentages of inhibition of OTA to 97.9 and 99.1%, respectively. The inhibitory effect may due to by many routes such as damage on hyphae and spores, especially *Aspergillus* reproduction based on spores. Also, adding AgNPs for YES culture media

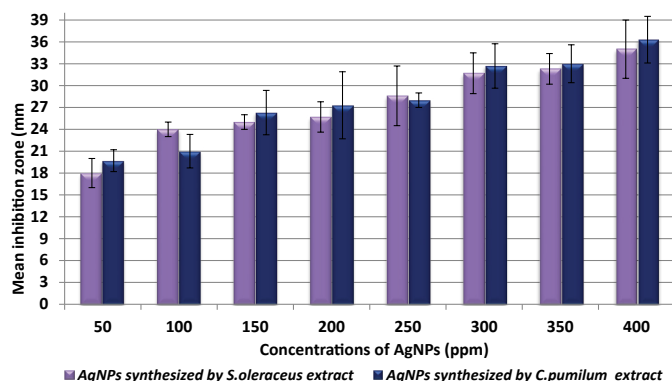


Figure 8. Effect of AgNPs synthesized by extracts of *S. oleraceus* and *C. pumilum* on growth of *A. ochraceus*.

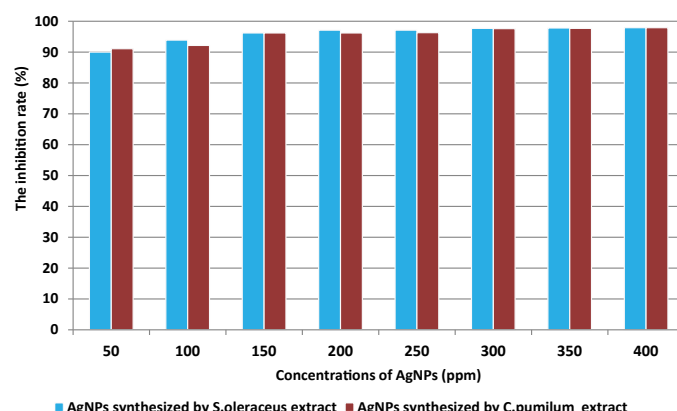


Figure 9. The percentages of inhibition of AFB1 produced by *A. aflatoxiformans* in YES media treated with different concentrations of AgNPs solutions.

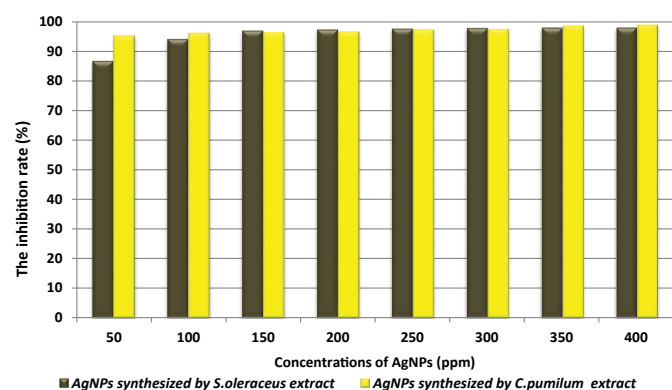


Figure 10. The percentages of inhibition of OTA produced by *A. ochraceus* in YES media treated with different concentrations of AgNPs solutions.

Table 1. ANOVA analysis of the effect of concentration and type of AgNPs on a growth *A. aflatoxiformans*.

Source	SS	df	MS	F	P
Intercept	29700.75	1	29700.75	1762.22	0.000000
Type Nano	27	1	27	1.601978	0.214761
Concentrations AgNPs	1117.917	7	159.7024	9.475543	0.000000
Type Nano * Concentrations	5	7	0.714286	0.04238	0.999878
Error	539.3333	32	16.85417		
Total	31390	48			

SS: Sum of Squares; df: degree of freedom; MS: Mean Square; P: Probability at confidence 0.95.

Table 2. ANOVA analysis of the effect of concentration and type of AgNPs on a growth *A. ochraceus*.

Source	SS	df	MS	F	P
Intercept	37129.69	1	37129.69	4629.156	0.000000
Type Nano	3.520833	1	3.520833	0.438961	0.512368
Concentrations AgNPs	1307.479	7	186.7827	23.2872	0.000000
Type Nano * Concentrations	23.64583	7	3.377976	0.42115	0.881843
Error	256.6667	32	8.020833		
Total	38721	48			

SS: Sum of Squares; df: degree of freedom; MS: Mean Square; P: Probability at confidence 0.95.

caused a change in enzymatic activities responsible for pathways of synthesis AFB₁ and OTA inside fungi [31]. On the other hand, the AgNPs absorb the AFB₁ and OTA through electrostatic reactions between functional groups [32].

Conclusion

It is concluded that aqueous extracts that wild plants *S. leraceus* and *C. pumilum* were effective to synthesis of AgNPs. These AgNPs showed inhibit growth of *A. aflatoxiformans* with lost the ability to produce AFB₁ and the same to OTA produced by *A. ochraceus* in the culture medium. Finally, the AgNPs may be useful to control mycotoxigenic fungi associated with crops in the field and grain stores.

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