Dual synergistic actions of silver nanoparticles with natural products on Ochratoxin A production

Abdel Ghany TM[1,2], Kasem W T[1,2], M.A.Nabih[1], Ahmed S. Mabrouk[1]

[1]Biology Department, Faculty of Science, Jazan University, KSA.
[2] Botany and Microbiology Department, Faculty of Science, AL-Azhar University, Cairo, Egypt
Corresponding Email: tabdelghany@yahoo.com

Abstract: Recently, the utilization of fungi has emerged as a novel method for the synthesis of nanoparticles. In this study we report the extracellular biosynthesis of silver nanoparticles (AgNPs) by using *Alternaria alternata* as an alternative to chemical procedures and to evaluate its antimycotoxigenic activity. Synergistic effects of combined AgNPs and plant extract as well as cinnamon oil to obtain dual synergistic actions in order to decrease the Ochratoxin A (OTA) production. Production of OTA has been decreased with increasing applied AgNPs concentration in growth medium. Different concentrations of *Juniperus procera* extract were added to 50 ppm of AgNPs. Complete inhibition of OTA was observed at high concentration of *J. procera* extract with50 ppm of AgNPs.On the other hand using low concentration of *Adenium obesum*, inhibition of OTA production not affected but the addition of AgNPs showed better enhancing for antiochratoxigenic productivity. Cinnamon essential oil showed inhibitory action toward OTA in production and their action was enhanced with the addition of AgNPs. Hence, advanced and further investigations are required for direct treatment grains and feeds by AgNPs with plant extract or essential oil considering their toxic doses to avoid health hazard.
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1. Introduction

Mycotoxins are natural products and highly toxic secondary metabolites are produced by filamentous fungi found in cereals, dried fruits and nuts (Sekar*et al.,* 2008). They are found in many food and food-products, dry-fruits and nuts. These are responsible for infection in animals, plants and human beings (mycotoxicoses) (Jogee *et al*., 2012). Moulds were recorded to produce several mycotoxins such as aflatoxins, ochratoxins, patulin and zearalenone. These compounds cause some degree of acute toxicity when consumed in high amounts and are potential carcinogens. In developing countries, it appears that there is a direct correlation between dietary mytcotoxins intake and the incidence of liver cancer (Bahtnager and Ehrlich 2002; Hassan *et al.,* 2014; Abdel Ghany *et al.,* 2016). Among the most important mycotoxins are aflatoxins and ochratoxins (*Aspergillus*), trichothecenes and fumonisins (*Fusarium*), alternariol and tenuazonic acid (*Alternaria*) and in case of *Penicillium* ochratoxins, patulin and citrinin (Bennett and Klich 2003; El-Taher *et al*., 2012). *Penicillium verrucosum*, which has been used throughout this study, represents a worldwide distributed fungal species. Furthermore *P. verrucosum* is known as wheat contaminant producing the mycotoxins OTA and citrinin.

The biosynthesis of nanoparticles has been proposed as a cost effective and environmental friendly alternative to chemical and physical methods (Anju *et al.,* 2015). A number of microorganisms such as bacteria, fungus, yeasts and plants either intra or extracellular which are of higher production yields and with low expenses have been discovery to be capable of synthesizing nanoparticles (Vardhana and Kathiravan, 2015). Fungi are ideal candidates in the synthesis of metal nanoparticles with different sizes, because of their ability to secrete large amount of enzymes (Abdel Ghany 2013; Abdel Ghany *et al.,* 2013, Vardhana and Kathiravan, 2015). Biological synthesis of AgNPs using the fungus *Pestaloptiopsis pauciseta* was studied (Vardhana and Kathiravan, 2015). *Candida albicans* was tested for AgNPs biosynthesis. Endophytic fungus Fusarium sp. was subjected for the extracellular biosynthesis of AgNPs (Ashish *et al.,* 2015), and the TEM revealed the formation of small sized spherical shaped AgNps ranging 12-20 nm. According to Abd El-Aziz (2015) production of AgNPs as safe and economically viable by successfully synthesized using culture filtrates of Fusarium solani with high stability. The cell filtrate of *Trichoderma harzianum* was used as a producer of AgNPs, resulting the formation of it within 3 hours and the TEM analysis showed polydisperse spherical and occasionally ellipsoid nanoparticles in the size range from 19-63 nm and average size 34.77 nm (Gitanjali and Ashok, 2015). Three endophytic fungi *Aspergillus tamarii*, *Aspergillus niger* and *Penicllium ochrochloron* isolated from an ethno-medicinal plant *Potentilla fulgens* L. were used for the biosynthesis of

AgNPs (Lamabam and Joshi 2015). The AgNPs synthesized by *T. viride* were observed (Abdallah *et al.,* 2016), in sizes ranging from 1 to 50 nm. The biogenic AgNPs significantly inhibited the growth of all tested pathogenic bacteria. Biosynthesis of AgNPs (with the average size of 15.5 nm) using extracellular filtrate of *Aspergillus versicolor* ENT7 as reducing agent has been reported and exhibited a very good antioxidant and antimicrobial activity (Netala*et al.,* 2016). Many species of *Aspergillus* were tested for AgNPs synthesis (Kamiar *et al.,* 2016). Juhi et al. (2016) utilize plant pathogenic fungi *Sclerotinia sclerotiorum* MTCC 8785 strain for synthesis and optimization of AgNPs production as well as evaluation of antibacterial properties. Antimicrobial activity of AgNPs it may be due to lysis of AgNPs to the degradation of Deoxyribonucleic acid (Duran *et al.,* 2015). Mycosynthesis of AgNPs with size ranging from 20 to 50 nm was achieved by endophytic Colletotrichum sp. DNA of E. coli. treated with AgNPs synthesized by *Colletotrichum* sp showed deformed and damage of Deoxyribonucleic acid indicating the action of AgNPs (Pasha *et al.,* 2016). AL-Othman *et al.,* (2014) have investigated the effect of AgNPs biosynthesized by *Aspergillus terreus* (KC462061) on growth and aflatoxin production by A. flavus isolates. They found that The inhibition of AFB1 production was 48.2 -61.8%, at 50 ppm, 46.1-82.2% at 100 ppm and 100% at 150 ppm AgNPs, while the inhibition of fungal growth was 100% at the concentration of 150 ppm AgNPs.

Exploration of naturally occurring antimicrobials compounds for grains and food protection receives increasing attention because consumer awareness of natural products (Schuenzel and Harrison, 2002 Abdel Ghany 2015; Marei et al, 2016). Numerous studies indicated that, secondary metabolites of plants and plant based pesticides appear to be one of the best alternatives as they are known to have minimal environmental impact and danger to consumers in contrast to synthetic pesticides (Krishnaiah *et al.,* 2011; Abdel Ghany 2014; Abdel Ghany and Othman 2014; Abdel Ghany *et al.,* 2015; Bapat *et al.,* 2016). New researches about biological active secondary compounds present in essential oils (EOs) of plants have been seen as a potential way to control fungal contamination (Tajkarimi *et al.,* 2010). Recently, there has been increasing interest in using naturally occurring compounds, especially EOs, to control food spoilage fungi in vitro and in vivo (Tzortzakis 2009; Choudhary and Kumari, 2010). Varahalarao and Chandrashekar, (2010) tested the antimicrobial activities of *Calotropis procera* against *Alternaria alternata*, *Aspergillus flavus*, *A. niger* and *Penicillium expansum*. Also, Ennajar *et al.* (2010) evaluated the antimicrobial activities of essential oil from leaves of *Juniperus phoenicea*.

2. Material and Methods

Biosynthesis of silver nanoparticles

Alternaria alternate was inoculated on potato dextrose broth medium and incubated at 25°C for 8 days. Then, the biomass of *A. alternate* was harvested after incubation period by filtering through filter paper followed by repeated washing with distilled water to remove any medium component from the biomass for several times. Ten grams (wet weight) was brought in contact with 100 mL of sterilized double distilled water treated with aqueous 1 mM $AgNO_3$ for 72 h at 28°C in a 250 mL Erlenmeyer flask and agitated again at 120 rpm. Control (without the silver ions) was also run along with the experimental flask.

UV-visible spectroscopic analysis

The reduction of silver ions was confirmed by qualitative testing of supernatant by UV-visible spectrophotometer. One ml of sample supernatant was withdrawn after 24 hours and absorbance was measured by using UV-visible spectrophotometer between 300-800 nm at the Regional Center for Mycology and Biotechnology (RCMB) at Al- Azhar University Cairo, Egypt.

Fourier Transform Infrared Spectroscopy and Transmission Electron Microscopy analysis

The dried powder of AgNPs was subjected to Fourier Transform Infrared Spectroscopy (FTIR) analysis. Two milligrams of the sample was mixed with 200 mg KBr (FTIR grade) and pressed into a pellet. The sample pellet was placed into the sample holder and FT-IR spectra were recorded in the range 450- 4000-500 cm^{-1} in FTIR spectroscopy at a resolution of 4 cm^{-1}. Finally, the AgNPs were characterized by Transmission Electron Microscopy (C Joel Jem-1200 EX II. Acc. Voltage 120 KV.MAG-medium) at RCMB.

Plant material and preparation of extract

The vegetative system including leaves and stems of *Juniperus procera* and *Adenium obesum* were collected, Jazan region, Kingdom of Saudi Arabia (KSA). Fresh 500 g of plant samples were air-dried at room temperature under shade, and ground into powder using an electric grinder. Then extracted with methanol in a Soxhlet apparatus. The solvent was removed using rotary evaporator under reduced pressure at temperature below 50°C. The resulting crude extracts were stored at 4°C in dark until used. Essential oil of cinnamon was prepared from market.

Effect of Silver Nanoparticles, plant extracts and essential oil on Ochratoxin A production

For these experiments a volume of fungal suspension containing 1×10^7 spores/ml of *A. ochraceus* was added to 250 ml Erlenmeyer flasks

containing 50 ml of broth potato dextrose medium in the presence of different concentrations of AgNPs, plant extracts, essential oil and their combination. The culture incubated at 30°C for 10 days.

Detection of Ochratoxin A production

The filtrates of the culture media of *A. ochraceus* after incubation period were obtained and assayed for the presence of mycotoxins. Ochratoxin A was detected with using Microtitre plate enzyme-linked immunosorbent assay (ELISA) reader (automated Chem-well) in Saudi Grains Organization (SAGO), Saudi arabia. OTA kit was used to ELISA analyses. Ten ml of blended fungal broth (treated with plant extract and silver nanoparticles or untreated) has been sub-sampled with 20ml of 70% methanol and vortex for 10 min by magnetic stirrer. The extract was filtrated by Whatman number one filter paper and then diluted as 5ml filtered solution, 15ml distilled water and 0.25ml Tween 20. The solution was mixed by magnetic stirrer for 2min. 50 µl toxins (5, 10, 20, 45 ppb) standard solutions and 50 µl prepared test samples were added into separate wells of micro-titer plate. Plates were incubated at room temperature. The liquid was then removed completely from the wells, the each well was washed with 250 µl washing PBS-Tween-Buffer (pH 7.2) and this was repeated two times. Subsequently, enzyme substrate (50 µl) and Chromogen (tetramethyl-benzidine, 50 µl) were added to each well and incubated for 30min at room temperature in the dark. 100 µl of the stop reagent (1M H_2SO_4) was added and the absorbance was measured at 450nm in ELISA reader.

3. Result and Discussion

The biological synthesis of AgNPs by different fungi was investigated according to numerous studies (Abdel Ghany 2013**;** Abdel Ghany *et al.,* 2013; Honary *et al.,* 2013). Silver nitrate ($AgNO_3$), upon incubation with the *A. alternate* biomass, turned dark brown color (Figure 1A), while the positive and negative control flasks remain as such during the 72 h incubation period (Figures 1B and C). The generation of dark brown color is due to the surface plasmon resonance exhibited by the nanoparticles. Similar observation was made by several authors (Abdel Ghany 2013**;** Abdel Ghany *et al*., 2013; Honary *et al*., 2013). The UV-visible spectrum (Figure 2) showed the peak of AgNPs around 400 nm. It is well known that the size and shape of the AgNPs reflects the absorbance peak. According to Kannan *et al*. (2010) wide spectrum range around 390 to 410 nm was observed with AgNPs. For three fungi *Aspergillus niger, Alternaria solani* and *Fusarium oxysporum*, the UV-vis spectrum exhibited absorption band around 435, 445 and 440 nm respectively (Amal and Azzah 2015). The results of scanning electron microscopy (SEM) showed that AgNPs have a uniform spherical shape in solution with an average size less than 100nm (Figure 3). The detected size of AgNPs synthesized by *Alternaria alternata* may agree or relatively differ with previous scientific papers, this may be due to the species type, incubation period or environmental conditions of microbial growth. It is observed that nanoparticles size was 42.12, 89.76 and 120.6 nm with using *Schizophyllum commune, Lentinus sajar-caju* and *Pycnoporus sanguineus* respectively (Yen San and Mashitah, 2012). The FTIR spectrum of silver nanoparticles (Fig.4) indicate that the nanoparticles manifest absorption peaks which represent amide linkages groups. Furthermore, the peaks near 3401 was assigned to OH stretching. The band at 1626 cm-1 corresponds to amide due to carbonyl stretch in proteins. The peak at 1041 cm-1 corresponds to C-N stretching vibration of amine (Honary *et al.,* 2013). Aso X-Ray diffraction patterns of silver nanoparticles was assayed (Fig.5).

Figure 1. Conversion of silver nitrate to nano silver by *A. alternata* (A, silver nitrate solution inoculated with fungus biomass; B, distilled water inoculated with biomass of fungus; C, silver nitrate solution without fungus biomass).

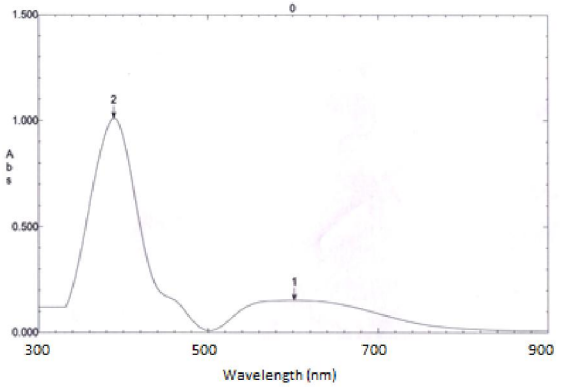

Figure 2. UV-Vis spectrum of silver nanoparticles produced by A. alternate

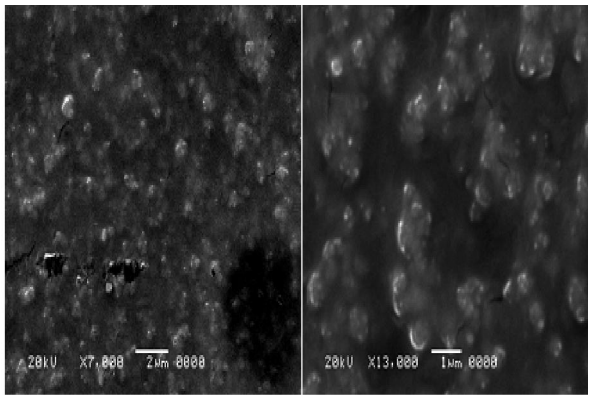

Figure 3. Characterization of AgNPs produced by A. alternate with different magnification power using Transmission Electron Microscopy

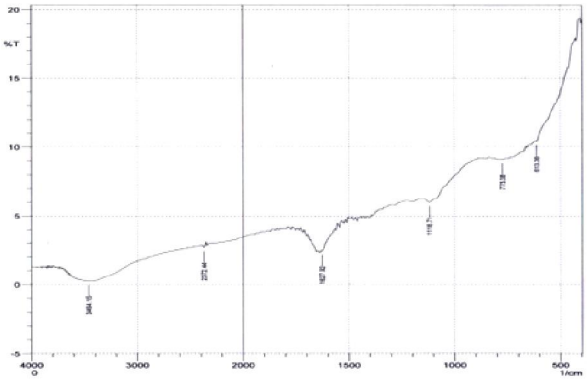

Figure 4. FTIR spectrum of silver nanoparticles produced by A. alternate

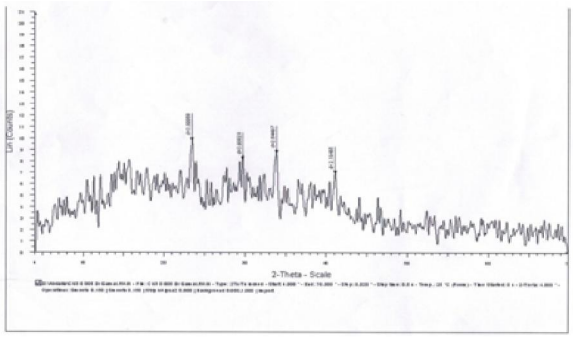

Figure 5. X-Ray Diffraction of silver nanoparticles produced by *A. alternate*

Our results show that AgNPs have significant effect on OTA production by *A. ochraceus*. Also, the concentration of OTA has been decreased with increasing AgNPs concentration, respectively (Table 1). This results agreement with numerous studies, where El-Desouky and Ammar (2016) reported that AgNPs have significant effect on aflatoxin and OTA production by *A. parasiticus* and *A. ochraceus*, respectively, and the concentration of OTA has been decreased to 45.8, 58.2 and 79.85% after the addition of 1, 2 and 3 mg AgNPs/100ml media, respectively. In another study, AgNPs effectively inhibited AFB_1 production at a concentration of 90µg/ml (Mousavi and Pourtalebi, 2015). Katarzyna *et al.* (2015) found that the addition of silver nanoparticles to the medium decreased the produced mycotoxins by 81.1–95.5%. The highest decrease of mycotoxin amount was noticed for OTA. Seyyed and Somayyeh (2015) demonstrated that a minimum inhibition concentration (MIC) equal to 180 µg/mlwas determined for AgNPs against *A. parasiticus*. The AgNPs effectively inhibited AFB_1production at a concentration of 90 µg/ml.

Table 1. Effect of silver nanoparticles and plant extract/essential oil on Ochratoxin A production

Treatment	**Concentration**	**Ochratoxin A detection**
Control	0.0	18.3 µg/kg
AgNPs	50 ppm	7.7 µg/kg
	75ppm	5.2 µg/kg
	100ppm	1.6 µg/kg
J. procera extract	100 mg	2.5 µg/kg
	200mg	6.3 µg/kg
	400mg	0.4 µg/kg
J. procera + 50 ppm AgNPs	100 mg	3.5 µg/kg
	200mg	1.8 µg/kg
	400mg	0.0 µg/kg
A. obesum extract	100 mg	18.2 µg/kg
	200mg	18.1 µg/kg
	400mg	5.2 µg/kg
Plant extract+ 50 ppm AgNPs	100 mg	17.1 µg/kg
	200mg	13.3 µg/kg
	400mg	7.9 µg/kg
Cinnamon oil	50µg/ml	6.4 µg/kg
	100 µg/ml	6.2 µg/kg
	200 µg/ml	3.0 µg/kg
Cinnamon oil+ 50 ppm AgNPs	50µg/ml	5.0 µg/kg
	100 µg/ml	2.8 µg/kg
	200 µg/ml	2.1 µg/kg

Natural antifungal agents can be potential exploited in controlling the growth of fungi consequently inhibiting mycotoxin production (Grayer and Harborne 1994; Al-Rahmah *et al.,* 2011). Addition of AgNPs to plant extracts as well as essential oils was studied in the current study. Different concentrations of *J. procera* extract were added to 50 ppm of AgNPs. Complete inhibition of OTA was observed at high concentration of *J. procera* extract with50 ppm of AgNPs. Abdel Ghany (2014) reported that *J. procera* extract inhibit the secondary metabolites production of *F. oxysporum*, including

fusaric acid and ergosterol (production inhibition was 8.36% and 73.34% respectively). The highest concentration of AgNPs gives the highest percentage reduction of OTA (El-Desouky and Ammar 2016). Cinnamon EO showed inhibitory action toward OTA production and their action was enhanced with the addition of AgNPs (Table 1). This activity of cinnamon EO is mainly due to its major component, cinnamaldehyde which is a powerful fungistatic agent (Bakkali*et al*. 2008). Bokhari and Mohammad, (2009) stated that the plants and spice extracts (saffron, ginger, cinnamon, cloves, cardamom) decrease the mycotoxigenicity by 12.5-37.5%. On the other hand using low concentration of *A. obesum*, inhibition of OTA production not affected but the addition of AgNPs showed better enhancing for antiochratoxigenic productivity (Table 1).

Conclusion and future recommendation

Biosynthesis of AgNPs by *A. alternata* is cost-effective and environmentally friendly. This study is one of the first steps in direction about the use of AgNPs to control OTA production. The extracellular biosynthesis of silver nanoparticles (AgNPs) by using *A. alternata* as an alternative to chemical procedures and to evaluate its antimycotoxigenic activity was evaluated. Also, synergistic effects of combined AgNPs and plant extract as well as cinnamon oil to obtain dual synergistic actions in order to decrease the OTA production was estimated by ELISA analysis. Hence, advanced and further investigations are required for direct treatment grains and feeds by AgNPs with plant extract or essential oil considering their toxic doses to avoid health hazard. Hence, advanced and further investigations are required for direct treatment grains and feeds by AgNPs with plant extract or essential oil considering their toxic doses to avoid health hazard.

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Corresponding Author:
Prof. Tarek M. Abdelghany
E-mail: tabdelghany@yahoo.com

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