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Effect of Metal Nanoparticles on the Growth of Ochratoxigenic Moulds and Ochratoxin A Production Isolated From Food and Feed

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Abstract*: The present study was undertaken to evaluate the antifungal potential of ZnO and Fe_2O_3 nanoparticles in comparison with some commercial antifungal feed additives (probiotic, propionic acid and clove oil) in inhibiting the growth of A. ochraceus and A. niger strains that were isolated from animal and poultry feeds using well and disc diffusion tests. The diameters of inhibition zones induced by metal nanoparticles for non-ochratoxigenic strains were larger than that of ochratoxigenic strains and the zone diameters increased when the concentration increased. The concentrations of metal nanoparticles 20 ug/ml did not affect the growth of all A. ochraceus and A. niger strains, whereas the zones of inhibition produced by the metal nanoparticles required lower concentration (25 ug/ml and more) than that produced by the commercial antifungal feed additives (50 ug/ml and more). The ochratoxin A production by ochratoxigenic strains in liquid medium (YES) or on yellow corn was significantly diminished in parallel with the decline parameters in colony count of the treated ochratoxigenic strains. The field application of the used nanoparticles and other drugs on commercial animal feed evidenced the availability to use ZnO and Fe_2O_3 nanoparticles only as antifungal but their antimycotoxins effect was limited to their use as feed additives during manufacture and before exposure of feeds to fungal contamination. The significance of the present results was fully discussed. It is concluded that further studies are required for investigating the synergistic effects of combined antioxidant metal nanoparticles and other commercial antimycotoxins to obtain dual synergistic actions in order to decrease the amount of used chemicals in the feed manufacture and to study the availability of its use in vivo.*

Keywords: *Zinc oxide nanoparticles (ZnO-NPs); Biosynthesis; Iron oxide nanoparticles (Fe_2O_3-NPs); A. ochraceus and A. niger ; ochratoxin A; Antimycotoxins.*

1. INTRODUCTION

The environmental pollutions and their elimination become a worldwide problem and gain attention of all authorities. Microbial pollutants are the most dangerous factors and there are a wide range of diseases caused by fungi and their toxins and constitute a major problem for animal and human health. The correlation between the environmental factors, mycosis and mycotoxicosis in animals and the role of these environmental factors in initiation of food born infections had been reported by [**1, 2**).

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 [**2, 3, and 4**]. In addition, outbreaks of food borne pathogens continue to draw public attention to food safety. Therefore, there is a need to develop new antimicrobials to ensure food safety and extend shelf life. The use of antimicrobial agents directly added to foods or through antimicrobial packaging is one effective approach. In recent years, the use of inorganic antimicrobial agents in non-food applications has attracted interest for the control of microbes [**5**]**.** Among these agents, nanoparticles of Fe_2O_3 and ZnO exhibited strong antimicrobial activity [**6, 7**]. Currently, Fe_2O_3 and ZnO NPs were proved able to inhibit the mycelia growth of aflatoxigenic moulds, particularly *A. flavus* and to prevent aflatoxin production **[8, 9].** The ability of ZnO –NPs to inhibit the growth of all types of mycotoxigenic moulds and to prevent production of their respective mycotoxins on liquid medium (YES) was illustrated **[10].** On the other hand, few data demonstrate antimicrobial efficacy of ZnO in foods. In addition,

different types of nanomaterials like copper, zinc, iron (**9, 10, 11, 12, 13]** magnesium and gold **[14]** were reported to have antimicrobial effect. Moreover, iron oxide nanoparticles have shown great potential in many biological and biomedical applications such as targeted drug delivery, magnetic fluid hyperthermia, magnetic resonance imaging, tissue engineering and antimicrobial effects against bacterial and fungal causes of skin affection of cattle **[7, 15]**. The resistance to many of the antifungal agents now in use has emerged and seems to create a huge problem ,while the number of fundamentally different types of antifungal agents that are available for treatment remains extremely limited.

Therefore, there is an inevitable and urgent medical need for antibiotics with novel antimicrobial mechanisms **[16, 17].** This has prompted a renewed interest in the use of metals as antifungal agents. The present work was undertaken to investigate the antifungal potential of zinc oxide and iron oxide nanoparticles in comparison with some commercial antifungal as (Probiotic, propionic acid and clove oil) on the growth of *A. ochraceus and A. niger* and ochratoxin A production in animal feeds. The field application of the obtained results on commercial highly contaminated feeds with mould and ochratoxins was investigated.

2. Material and Methods

2.1. Fungal Strains and Culture Conditions:

A total of 34 strains of *A. ochraceus and A. niger,* including 24 ochratoxigenic strains (12 of each of *A. ochraceus and A.niger)* and 10 non- ochratoxigenic strains (5 of each of *A. ochraceus and A. niger*) were used in this study. The used isolates were recovered from samples of animal and poultry feeds obtained from farms that suffered from diseases in the Department of Mycology, Animal Health Research Institute, Ministry of Agriculture, Egypt. All tested strains were cultured on Sabouraud dextrose agar (SDA) and /or potato dextrose agar (PDA) and incubated at 25-28°C for 3-5 days. Then, the cultures were preserved at 8-10 °C until used.

2.2. Antifungal Agents:

***2.2.1. Metal Nanoparticles*:**

***2.2.1.1. Zinc Oxide Nanoparticles*:**

Biosynthesis and characterization of ZnO-NPs were kindly done by fund of Dr. Hazem H. Mansour, Chairman of The Central Laboratory of Elemental and Isotopic Analysis, Nuclear Research Centre, Atomic Energy Authority, Egypt.

***2.2.1.2. Iron oxide nanoparticles*:**

Iron oxide nanoparticles were purchased from Sigma Chemical Company USA. They were characterized and identified by the company and their particle size is 50 nm.

2.2.2. Commercial Antifungal Feed Additives

2.2.2.1. Propionic Acid: It was purchased from Sigma Chemical Company.

***2.2.2.2. Probiotic* :** Each vial contained 1gm of powder which consisted of: *Lactobacillus plantarum* $1X10^8$ CFU, *Lactobacillus acidophilus* $1X10^8$ CFU *Saccharomyces cerevisiae* $1X10^7$ CFU , Carrier-skim milk up to 0.5gm.

***2.2.2.3. Hydrated Sodium Calcium Aluminium Silicate (HSCAS)* :**

Commercial toxin adsorbent used as feed additives was purchased from El gomhorya Drugs Company.

2.3. Methods

***2.3.1. Preparation of C. Albicans Cells Culture [18]*:**

The spore suspension of *Candida albicans* (10^5/ml) of 2-5 days age cultures was inoculated into 250 ml Erlenmeyer flasks, each containing 50 mL of semi defined medium (SDM) composed of KH_2PO_4 (7g/L), K_2HPO_4 (2g/L), $MgSO_4.7H_2O$ (0.1g/L), $(NH_4)_2SO_4$ (0.1 g/L), yeast extract (0.6g/L), and glucose (10g/L) at 30°C under shaking condition (200 rpm) for 96 hrs. After 96 hrs of cultivation, mycelia were separated from the culture broth by centrifugation at 4500 rpm, 10°C, for 15 min. The settled mycelia were washed with deionized water. 1% of the washed C. *albicans* cells was inoculated into flasks containing 100 ml of Sabouraud broth medium for 24 hours at 30 C and treated with 1.0% NaCl.

2.3.2. Biosynthesis of Zinc Oxide Nanoparticles and Their Identification and Characterization **[19, 20, 21]:** Twenty-five ml of above prepared culture were taken in separate sterilized flask and twenty ml aqueous solution of 1 mM zinc oxide were added to the culture broth and the flask was kept at 30 ^{o}C for 24 h until white deposition started to appear at the bottom of the flask, indicating the initiation of transformation of zinc oxide to zinc oxide nanoparticles. The culture solution was cooled and incubated at room temperature in the laboratory ambience. After 12-15 hours, white clusters deposited at the bottom of the flask. The reaction mixture was subjected to centrifugation for 15 min. The sediment was collected, washed by di-ionized water and filtrated through What-man filter paper No. 1 and the filtrate was discarded. While, the obtained powder in the filter paper was dried at room temperature or in hot oven at 50-60 ^{o}C for 2-3 days. The prepared ZnO nanoparticles were characterized for their optical and structural properties by using a UV-Vis spectrophotometer (Lamda-25; PerkinElmer; Waltham, Massachusetts) and the particle sizes and morphology were observed and measured under Transmission electron microscope (TEM) and Scanning electron microscope (SEM) (Joe, JSM-5600LV, Japan).

2.3.3. Preparation of Plant Oil : The small pieces of clove plant materials (300 g) were pressed in a commercial mill without any heat treatment and the pure oil was collected, dispensed into dark bottles, and stored at 4°C until used **[22].**

***2.3.4. Production And Estimation of Ochratoxin A by Tested Strains* of *A. ochraceus and A. niger* in liquid and on yellow corn [23]:** The strains of *A. ochraceus and A. niger* that were recovered from animal feeds were screened for ochratoxin A production on liquid media (YES) and on yellow corn. The estimation of prepared ochratoxin A was measured qualitatively by TLC **[24]** and the positive samples for aflatoxins were measured quantitatively by fluoroumeteric method using specific FGis Ochra test standards **[25].**

2.3.5. Evaluation the Effect of Metal Nanoparticles and Commercial Antifungal Feed Additives on the Growth of A. ochraceus and A. niger using Diffusion Tests[13, 26] :

2.3.5.1. Well Diffusion Technique:

One ml of 10^5 spore suspensions of tested fungus was incorporated with the SDA medium plates. Wells of 5 mm in Φ were made on the SDA surface and 100 ul of the gradual concentration of (0, 25, 50, 100, 150, 200, 250 ug/ ml) of metal nanoparticles (ZnO-NPs & Fe_2O_3 -NPs), probiotic, propionic acid and clove oil were added. Then, the plates were incubated at 28-30°C for 24 hrs. After incubation the plates were tested for the growth inhibitory zones around wells.

2.3.5.2. Disc Diffusion Technique, [27]:

A-Preparation of paper discs of commercial antifungal feed additives: Filter paper discs, What-man of 5 mm Φ, were impregnated for 10 minutes with different concentration of (0, 25, 50, 100, 150, 200, 250 ug/ ml) of metal nanoparticles (ZnO-NPs & Fe_2O_3 -NPs), probiotic , propionic acid and clove oil**.** The prepared discs were dried by heating at 40- 50°C for one hour.

B-Placement the discs of tested commercial antifungal feed additives: One ml of 10^5 spore suspensions was added to sterile plates and over-layered with SDA. The plates were rotated to mix the content and allowed to solidify at room temperature. On the surface of plates, the paper discs of drugs were pressed firmly for complete contact with the agar. The discs were distributed evenly in a manner such as to be not closer to each other, 15 mm from edges of dishes, 20 mm between each 2 discs and 24 mm from center of plates. The plates were incubated at 25°C for 2-5 days.

C-Reading the results (inhibitory zone):After the end of incubation period, the sensitivity of fungi to the tested drug was determined by measuring the diameter of the growth inhibition zone in mm.

2.3.6. Evaluation the Effect of Metal Nanoparticles(Zno-Nps & Fe_2O_3 -Nps) and Commercial Antifungal Feed Additives (Propionic Acid and Clove Oil And Probiotic) on the Growth Of A. ochraceus And A. niger and Ochratoxin A Production Using Synthetic Medium (YES) [27, 28]:

A. **Metal Nanoparticles:** gradual concentrations of ZnO-NPs and Fe_2O_3- NPs (0, 50, 100, 150, 200, 250 ug/ml) were added to a set of one liter flasks containing 200 ml of YES broth.

B. **Commercial Antifungal Feed Additives:** The used concentrations (0, 50, 100, 150, 200, 250 ug/ ml) of probiotic , propionic acid and clove oil were added to a set of one liter flasks containing 200 ml of YES broth.

C. The flasks were autoclaved for 20 minutes at 120 psi, cooled at room temperature overnight and inoculated with one ml spore suspension (10^5 spore/ml) of tested strains.

D. These procedures were repeated for each kind of toxigenic and non - toxigenic strains of (*A. ochraceus and A. niger)* and all the inoculated flasks were incubated in dark place at room temperature (22-25°C) for 20 days.

E. After the end of incubation period, the content of each flask was filtrated, where the filtrates were subjected for measurement of the levels and of mycotoxins. The mycelia mat was used for further colony count and detection of the effect of metal nanoparticles using scanning electron microscope with accelerating voltage of 10kV.

2.3.7. Scanning Electron Microscopy (Sem) [29]:
The mycelia mat obtained after filtration of YES or surrounding agar of diffusion zones were subjected to a scanning electron microscope (SEM), where the morphological changes of mycotoxigenic isolates by Metal-NPs were observed.

2.3.8. Evaluation the Effect of Metal Nano particles(Zno-Nps, Fe_2O_3 Nps) and Commercial Antifungal Feed Additives (Propionic Acid, Clove Oil And Probiotic) on the Growth of A. ochraceus and A. niger and Ochratoxin A Production using Yellow Corn [27, 30] :

One hundred grams of finely ground yellow corn were added in sterile one liter glass flask and different concentrations of the tested zinc oxide and iron oxide nanoparticles , probiotic, propionic and clove oil (0, 50, 100, 150, 200, 250 ug / ml) were added, sterilized by autoclaving , then 15 ml of sterilized distilled water were added and one ml spore suspension (10^5 spore/ml) from each of tested isolates was inoculated to each flask. All flasks were incubated at 30°C for 1 month. Each test was repeated 3 times.

2.3.9. Reisolation of Tested Fungus and Detection of Ochratoxin A After Treatment of YES And Yellow Corn:

From each flask, one gram of corn or I ml of YES was subjected to re-isolation of inoculated strains according to **[31].**

2.3.10. Detection of Mycotoxins Residues in Treated Corn: This was done according to the method of *[25].*

2.3.11. Field Application of Tested Commercial Antifungal Feed Additives [27] :

The same procedures as in (2.3.7 and 2.3.8) were repeated using 20 commercial contaminated animal feed. The total colony count of fungal contamination was evaluated before and after treatment as recommended by **[31]**. On the other hand, other 20 samples of poultry feed were autoclaved and 70 ppb of ochratoxin A were added to these samples at different concentrations of drugs and HCAS (see 2.3.7.) and incubated at 28-30°C in dark place. After one week, the residues of ochratoxin A were measured.

2.3.12. Statistical Analysis:
The obtained data were computerized and analyzed for significance. Calculation of standard error and variance according to **[32].**

3. RESULTS AND DISCUSSION

The fungal pollution of animal and poultry feeds and human food by mycotyoxigenic fungi contributes a major problem to their health and are responsible for high economical losses in animal production due to decrease in milk and meat production and may be transmitted to human through consumption of contaminated food of animal origin .

Such contamination constitutes a public health hazard due to production of mycotoxins, which cause some degree of acute toxicity when consumed in high amounts and are potential carcinogen. In developing countries; it appears that there is a direct correlation between dietary aflatoxins intake and the incidence of liver cancer **[3, 4, 31]**. In addition, the outbreaks of food borne pathogens continue to draw public attention to food safety.

Aspergillus flavus and *A. ochraceus were recovered* from 100 samples of air, water supply and feeds including tibn, hay and processed feeds. Most of isolates *A. ochraceus* from animal feeds in diseased farms produced significant levels of aflatoxins and ochratoxins, respectively. The isolated *A. ochraceus* from tibn yielded higher mean levels of ochratoxins (3250±2.5 ppb) **[33]**. The ochratoxigenic moulds and ochratoxins were also detected in samples of feed and the maximum levels of ochratoxins produced by isolated *A.ochraceus* were at the mean levels of (45.00±0.30; ppb) **[1]**.

In the present study, the *A. ochraceus and A. niger* isolated from animal and poultry feeds were screened for ochratoxin A production on synthetic medium and yellow corn. The results revealed that the ochratoxigenic *A. ochraceus* strains were capable to produce significant levels of ochratoxin A that reached to (40±3.0 ppb) in synthetic medium of YES and to (70±5.4 ppb) on yellow corn. Whereas, the ochratoxigenic *A. niger* strains were able to produce levels of ochratoxin A reached to (27±2.5 ppb) on liquid medium of YES and reached to (34±6.5 ppb) on yellow corn.

Similar findings were reported by **[12],** who detected significant levels of ochratoxin A production by *A. ochraceus* recovered from raw milk samples at the mean levels of (6.5±0.35 ppb) and cattle feeds (18.5±0.55 ppb).

Up to date it is difficult to control such microbial affections by traditional antibiotics due to the subsequent resistance developed in the successive generations of microbial agents and to overcome this resistance, it is important to explore novel antimicrobial agents, which may replace current control strategies **[34].** However, not only the emergence of chemical drugs and herbs resistance among different pathogenic strains but also their high toxicity**,** had prompted research on new antifungal agents **[33, 35]**.

Recently, nanoparticles (NPs) materials have received increasing attention due to their unique physical and chemical properties, which differ significantly from their conventional counterparts **[36]**.

Nowadays, the metals of zinc and iron are used as antioxidant feed additives for animals and human. Bio-nanotechnology has emerged for developing biosynthesis and environmental-friendly technology for synthesis of nano-materials. Among nano-materials, ZnO and Fe_2O_3 have gained more attention due to their special properties and less hazard to environment, in addition to their toxic effect on organisms, which can be used as antibacterial, antifungal and antiviral agents **[2, 7, 10, 12, 15, and 30]**.

In the present study, the biosynthesis of ZnO nanoparticles by fungal strains of *Candida albicans* was investigated. The appearance of white clusters deposited at the bottom of the flask indicated a reduction of metal ion and the formation of nanoparticles has taken place. Bio-reduction indicates the presences of reducing agent which served as electron shuttle in this reduction reaction and it was also reported that, fungus reduction was most probably either by reductase action or by electron shuttle quinones or both [**1, 9, 10, 12, 37] .**

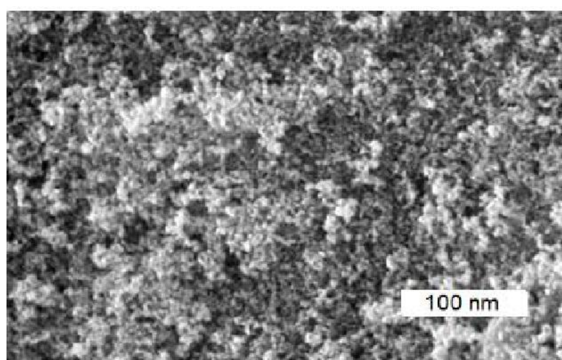

Fig.(1): *The TEM image of the size and distribution of the particles of zinc oxide (100 nm)(x 20 000).*

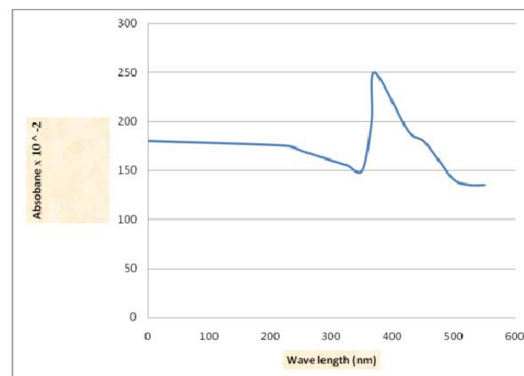

Fig. (2): *The UV-VIS absorbance spectra of ZNO-NPS(the optimal W.L was 370 nm)*

The prepared ZnO-NPs were identified and characterized by visual inspection; in a UV-visible spectrophotometer and Scanning b y Transmission electron microscope (TEM) and scanning electron microscope (SEM) for detection of their particle size and the purity of the prepared powder. The particle size of prepared ZnO nanoparticles was 100 nm (Fig.1). It was reported that the characterized absorption peak of ZnO-NPs is detected at 370 nm due to electron transition from valence band to conduction band (Fig 2). The results revealed that ZnO-NPs with spherical and granular morphology had uniform distribution. Because of their unique properties and large number of applications, zinc oxide nanostructures are one of the main subjects of the nowadays research. ZnO nanoparticles are durable, free from affecting the soil fertility in comparison to traditional antifungal agents **[8, 10, 12, 38].**

The purchased iron oxide nanoparticles were characterized and identified by Sigma chemical company (USA) and the particles size was 50 nm in size.

Several studies evaluated the antimicrobial activity of NPs of metal oxide particularly ZnO powder against fungi in culture media. Metal oxides nanoparticles are characterized by very high surface area to volume particularly ZnO –NPs which is of the least hazards to the environment **[9, 12, 39].** ZnO is one of five zinc compounds that are currently listed as generally recognized as safe (GRAS) by the U.S. Food and Drug Administration. Zinc salt has been used for the treatment of zinc deficiency **[40].** With regard to the antifungal activities of ZnO, the yeast of *C. albicans* was more sensitive for relatively lower concentrations (100 ug/ml). While, *A. niger and A. ochraceus* required higher concentration of ZnO NPs to inhibit their growth. The diameters of of inhibition zones of ZnO NPs (MIC) against *A. flavus and A. ochraceus* were 7 and 15 mm at the concentration of 300 ug/ml using (WD) test, whereas, A. niger required relatively lower concentration (200 ug/ml) of nanoparticles to inhibit their growth [**2**]. Other study by [**30]** reported that the MIC of ZnO against Aspergillus spp. and *C. albicans* was 1.013-296 µg/ml and for SDS and Fluconazole were 0.001-0.56 and 0.062-128 µg/ml, respectively. They added that lower concentrations of zinc oxide particles were most effective as anti-fungal and antibacterial. Furthermore, different studies conducted in different laboratories showed that the antimicrobial activity is influenced by not only nanoparticles concentration but also by the size of the ZnO particles **[38].**While, significant levels of ochratoxin A were detected in raw milk samples (6.5±0.35 ppb) and cattle feeds (18.5±0.55 ppb R), that were positive for *A. ochraceus and A. niger* [12] . The antifungal potential of ZnO-NPs on the growth and mycotoxins production by mycotoxigenic moulds and the levels of produced mycotoxins were decreased when the concentration of ZnO-NPs increased. The growth of ochratoxin A producing moulds and mycotoxins production were inhibited by addition of 10 µg/ml of ZnO-NPs to tested medium **[12].**

In the present work, the authors detected the higher efficiency of well diffusion test than disc diffusion test in evaluation of antimicrobial potential of ZnO-NPs, and Fe_2O_3 NPs, where they found that it was essential for ZnO molecules to contact or penetrate into microbial cells to express their antibacterial activities. Similar findings to our results were reported that this might be interpreted as a requirement for interaction of ZnO with the microbial cell wall or membrane for expression of antimicrobial activity **[2, 9, 37, 41].**

On the other hand, the antimicrobial effect of ZnO-NPs was reported to occur by 2 ways. The first is the formation of H_2O_2 on the surface of ZnO-NPs due to the possible formation of hydrogen bond between hydroxyl group of cellulose molecules of fungi with oxygen atom of ZnO-NPs leading to inhibition of the microbial growth, while the second is the release of Zn_2+ that causes damages of cell membrane and interacts with intraocular contents **[42].** Several natural and engineered nanomaterials have demonstrated strong antimicrobial properties through diverse mechanisms including photocatalytic production of reactive oxygen species that damage cell components and viruses (as ZnO), compromising the cell envelope (e.g. peptides, chitosan carboxyfullerene, carbon nanotubes, ZnO and interruption of energy transduction **[38, 43].**

In the present work, the antifungal potential of prepared ZnO and Fe_20_3 nanoparticles was evaluated against ochratoxigenic and non- ochratoxigenic *A. ochraceus and A.niger* that were recovered from animal and poultry feeds associated with animal diseases using well and disc diffusion tests. The zones diameter of inhibition of non- ochratoxigenic strains were larger than in ochratoxigenic strains. The concentrations of nanoparticles of 20 ug/ml did not affect the growth of all tested strains of *A. ochraceus and A. niger.* The use of well diffusion test in studying of antifungal potential of nanoparticales was more efficient than disc diffusion test (Table, 1, 2 and Fig.3-6). Similar findings were also obtained when traditional antifungals were used as probiotic, propionic acids and clove oils. It is interesting to report here that the zone of *A. ochraceus* and *A. niger* growth inhibition appeared at lower concentrations (25 ug/ml) of ZnO and Fe_20_3 nanoparticles, whereas, similar effects in traditional antifungals required relatively higher concentration (50-150 ug/ml). In addition, it was reported that the antifungal effects of clove oil against *A. ochraceus* and *A. niger,* showed comparatively lower antifungal effects than other used drugs in these study (Table, 1, 2).

Table (1): *Mean growth inhibition zones (mm) of Aspergillus ochraceus induced by metal nanoparticles and commercial antifungals and commercial antifungals*

Anti-fungal agents	*A. ochra-ceus* isolates	Diameter of inhibition zones at different concentrations (ug/ml)													
		20		25		50		100		150		200		250	
		W	D	WD	DD	WD	DD	WD	DD	WD	DD	WD	DD	WD	DD
ZnO-NPS	*Toxic 12*	0	0	4±0.9	6±1	6.5±1	8±1.8	8.5±2	9±2	10±2.4	10.6±2	12±3	12±2	15±3.	13±3
	Non-Toxic 5	0	0	7±0.3	10±2	9.4±0	12±2	11±0	13±3	15±0.7	15±3.7	16.±1	17±4	20±1	19±4.5
Fe_2O_3 NPS	*Toxic 12*	0	0	0	0	5±0.1	0±0	8±0.2	5±0. 4	9±0.5	6±0.1	10±1	7±.1	13±0.8	9±0.2
	Non-Toxic 5	0	0	0	0	10±1	0±0	11±1	7±0.8	12±2	9.5±.1	13±2	10±.3	18±2.8	15±0.7
Probiotic	*Toxic 12*	0	0	0	0	7±0.5	7±0.4	8±0.5	8±0.5	8±0.6	8±0.5	9±0.7	9±.6	14±0.8	14±0.8
	Non-Toxic 5	0	0	0	0	14±2	15±07	16±2	17±0.9	18±3	19.5±1	19±3.	20±1	24±4	26±1
Propionic Acid	*Toxic 12*	0	0	0	0	15±0.3	14±0.7	18±0.7	14±0.7	21± .8	15±0.7	25 ±1	17±.8	27±1.2	19±0.9
	Non-Toxic 5	0	0	0	0	15±0.8	14±0.4	20±1.1	15±0.7	24±1.8	16±0.8	27±2	17±.9	30±2.5	20 ±1
Clove oil	*Toxic 12*	0	0	0	0	0	0	0	0	9.0±.2	8 ±0.4	13±.4	15±.7	17±0.8	16±0.5
	Non-Toxic .5	0	0	0	0	0	7±0.4	10±1	7±0.2	14±0.3	10±0.4	15±.5	16±.1	20±0.5	17±0.3

WD: Well diffusion test, DD: Disc diffusion test

Table (2): *Mean growth inhibition zones (mm) of Aspergillus niger induced by metal nanoparticles and commercial antifungals*

Anti-fungal agents	*A. niger* isolates	Diameter of inhibition zones at different concentrations (ug/ml)													
		20		25		50		100		150		200		250	
		W	D	WD	DD	WD	DD	WD	DD	WD	DD	WD	DD	WD	DD
ZnO-NPS	*Toxic 12*	0	0	2±. 4	3±.5	4±1	6±1.	8±1	9±1	11±2	10±1	15±2	13±1	17±3	15±2
	Non-Toxic 5	0	0	4±.7	6±.7	7±1	9±1.	9±1	12±1	14±2	15±2	16±2	18±2	19±3	20±2
Fe_2O_3 NPS	*Toxic 12*	0	0	8±.5	7±.5	8±.5	7±.5	8±.6	8±. 6	8±.5	9±.7	9±7	10±.8	9±.9	12±.9
	Non-Toxic 5	0	0	8±.6	8±.6	10±.8	8±.7	11±0	9± .7	12±.9	10±.8	13±1	10±.8	15±1	11±.9
Probiotic	*Toxic 12*	0	0	0	0	7±.5	4±.3	10±.6	5±.5	12±.8	7±.6	14±.9	9±.8	17±1	12±.9
	Non-Toxic 5	0	0	0	0	10±.5	6±.2	12±.6	8±.2	13±.7	10±.3	15±.7	13±.5	18±.9	20±.7
Propionic Acid	*Toxic 12*	0	0	0	0	14±1	14±.7	17±1	15±.9	18±2.1	16±1	21±2	22±2	25±3	25±.3
	Non-Toxic 5	0	0	0	0	15±2	14±.8	19±2	16±.8	22±2.7	19±.9	25±3	23±1	27±3	25 ±1
Clove oil	*Toxic 12*	0	0	0	0	0	0	0	0	11±0.2	10±.5	14±.3	13±.6	16±.4	15±.7
	Non-Toxic 5	0	0	0	0	0	7±.4	12±. 5	9±.2	16±.3	11±.4	18± .6	15±. 6	22±.8	19 ±.7

WD: Well diffusion test, DD: Disc diffusion test

Fig.(3): Zone of inhibition of *A. ochraceus* by ZnO NPS using well D.T.

Fig.(4): Zone of inhibition of *A. ochraceus* by $\mathbf{Fe_2O_3}$ NPS using well D.T.

Fig. (5): Zone of inhibition of *A. niger* by ZnO- NPS Using well D.T.

Fig. (6): Zone of inhibition of *A. niger* by . ZnO- NPS NPS using disc D.T

The ochratoxin A production by toxigenic strains of *A. ochraceus* and *A. niger* on synthetic or natural medium was affected by all used nanoparticles and other fungal inhibitors when added before growth of fungus (Table 3, 4). Significant correlation between growth of *A. ochraceus* and *A. niger* and ochratoxins production was clearly observed, where, levels of ochratoxin declined as the number of fungal colonies decreased till complete inhibition of both (Table 3 , 4).

Table (3): *Effect on ochratoxin production by A. ochraceus (12 strains) after addition of metal nanoparticles and commercial antifungals to synthetic (Yeast Extract Sucrose-YES) or natural media (yellow corn-Y.C.) .*

Antifungal agent	Media	Levels of mycotoxins produced(ppb) (ug/l) and colony count after treatment with different concentrations (ug/ml) of tested chemical ±SE											
		٠	CC	25	CC	50	CC	100	CC	150	CC	200	CC
ZnO- NPS	YES	40±3.0	$5x10^5$	15.2±2.	$4x10^3$	0		0	0	0	0	0	0
	Y.C.	70±5.4	$7x10^4$	14±0.9	$4x10^3$	0	0	0	0	0	0	0	0
Fe_2O_3- NPS	YES	40±3.0	$5x10^5$	12.±.١	$3x10^3$	0	0	0	0	0	0	0	0
	Y.C.	70±5.4	$7x10^4$	12±0.8	$1x10^4$	6±0.4	2x10	0	0	0	0	0	0
Probiotic	YES	40±3.0	$5x10^5$	0	0	٠	٠	٠	٠	٠	٠	٠	٠
	Y.C.	70±5.4	$7x10^4$	١٠±0.9	$2x10^4$	9±1	$9x10^3$	6±0.8	$7x10^3$	4±0.6	0	0	0
Propionic Acid.	YES	40±3.0	$5x10^5$	11±2.3	$6x10^2$	0	0	0	0	0	0	0	0
	Y.C.	70±5.4	$7x10^4$	12±0.6	$4x10^3$	0	0	0	0	00	0	0	0
Clove oil	YES	40±3.0	$5x10^5$	22±6.0	$6x10^4$	16±2	$8x10^3$	9±1	$2x10^2$	2±0.6	5x10	0	0
	Y.C.	70±5.4	$7x10^4$	20±2	$1x10^4$	15±1	$4x10^3$	10±0.9	$4x10^2$	6±0.6	5x10	0	0

CC: colony count

Table (4): *Effect on ochratoxin production by A. niger (12 strains) after addition of metal nanoparticles and commercial antifungals to synthetic (Yeast Extract Sucrose-YES) or natural media (yellow corn-YC)*

Anti-fungal agent	Media	Levels of mycotoxins produced(ppb) (ug/l) and colony count after treatment with different concentrations (ug/ml) of tested chemical ±SE											
		0	CC	25	CC	50	CC	100	CC	150	CC	200	CC
ZnO- NPS	YES	27±2.5	$3x10^5$	8±0.9	$2x10^3$	0	0	0	0	0	0	0	0
	Y. C.	34±6.5	$4x10^4$	7±0.5	$2x10^3$	0	0	0	0	0	0	0	0
Fe_2O_3- NPS	YES	27±2.5	$3x10^5$	4±0.9	$2x10^2$	0	0	0	0	0	0	0	0
	Y. C.	34±6.5	$4x10^4$	10±0.7	$8x10^3$	5±0.4	$7x10^2$	0	0	0	0	0	0
Probiotic	YES	27±2.5	$3x10^5$	7±2	$2x10^3$	4±2.5	$3x10^2$	0	0	0	0	0	0
	Y. C.	34±6.5	$4x10^4$	4±0.5	$5x10^3$	2±0.2	$2x10^2$	1±0	7x10	0	0	0	0
Propionic Acid	YES	27±2.5	$3x10^5$	9±1.5	$4x10^2$	0	0	0	0	0	0	0	0
	Y. C.	34±6.5	$4x10^4$	7±0.3	$2x10^3$	0	0	0	0	0	0	0	0
Clove oil	YES	27±2.5	$3x10^5$	16±2	$4x10^4$	12±3	$2x10^3$	4±0.9	7x10	2±0.2	3x10	0	0
	Y. C.	34±6.5	$4x10^4$	17±1	$8x10^3$	12±1	$3x10^3$	7±0.8	$2x10^2$	2±0.1	2x10	0	0

Several studies were reported similar findings to our results as **[44],** who used *Rhamnus cathtica* plant extract, **[45]** used clove , onion and garlic oils and **[46, 47],** who used clove oil and all these studies revealed that these plants extracts were possessed antifungal activities against *A. ochraceus* and *A. niger* . On the other hand, propionic acid and probiotic drugs showed a significant mould inhibition of *A. ochraceus* and *A. niger* growth and declined their potential for ochratoxins production (Tables 1, 2, 3, 4). These findings are similar to that reported by **[48]** and **[49],** who detected significant effects of chemicals as propionic acid in inhibition of fungal growth. So that, when the probiotics were administered in adequate amounts as antimicrobial agents , resulted in a huge benefit on the animals and human health **[50, 51, 52] .**

When the treated fungi were subjected to SEM, the damage and rupture of their cell wall were detected in the area surrounding growth. The normal conidial cell of *A. ochraceus* and *A. niger* has a spherical shape and smooth cell wall and intact cell membrane. The effect of high concentration of ZnO or Fe_20_3 nanoparticles on the treated fungi was observed as membrane damage of cells and some pits that have been caused in inter cellular components, leading to leakage and finally cell death. (Fig.7-10). Similar findings were also reported by **[2, 15, 37, and 38].**

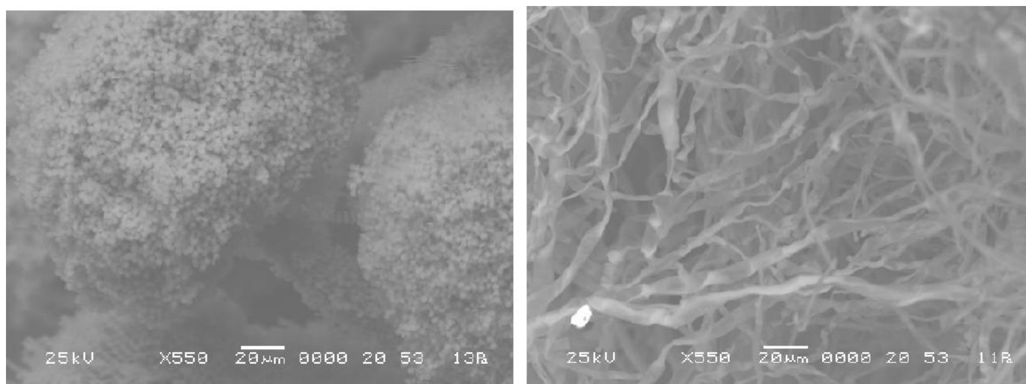

Fig. (7): *(***L***) Scanning Electron Microscopy (SEM) of normal A.ochraceus conidia (***R***): Scanning Electron Microscopy (SEM) of A.ochraceus conidia after treatment with ZnO- NPS.*

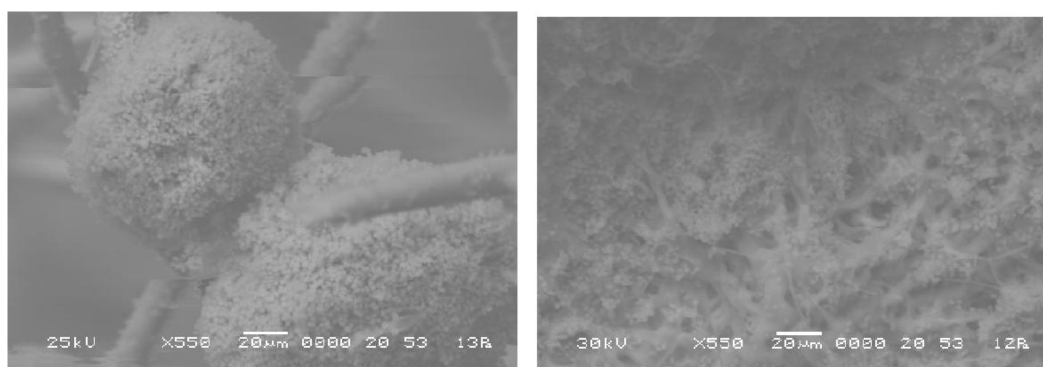

Fig. (8): *(***L***) Scanning Electron Microscopy (SEM) of normal A.ochraceus conidia (***R***): Scanning Electron Microscopy (SEM) of A.ochraceus conidia after treatment with Fe_20_3- NPS.*

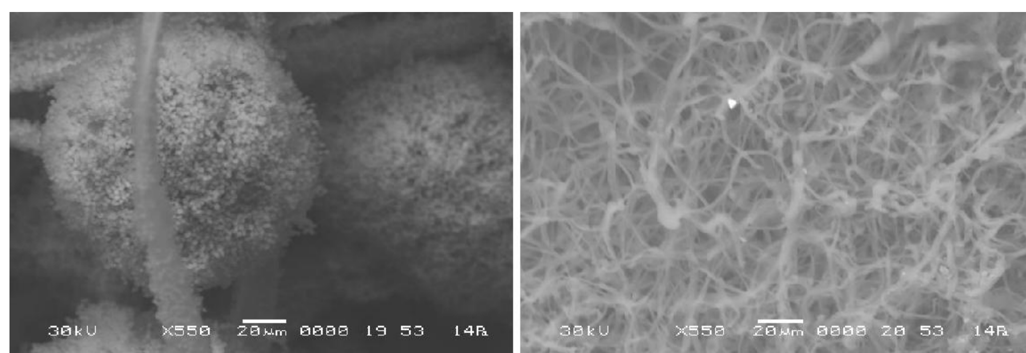

Fig. (9): *(***L***) Scanning Electron Microscopy (SEM) of normal A. niger conidia (***R***): Scanning Electron Microscopy (SEM) of A.niger conidia after treatment with ZnO- NPS.*

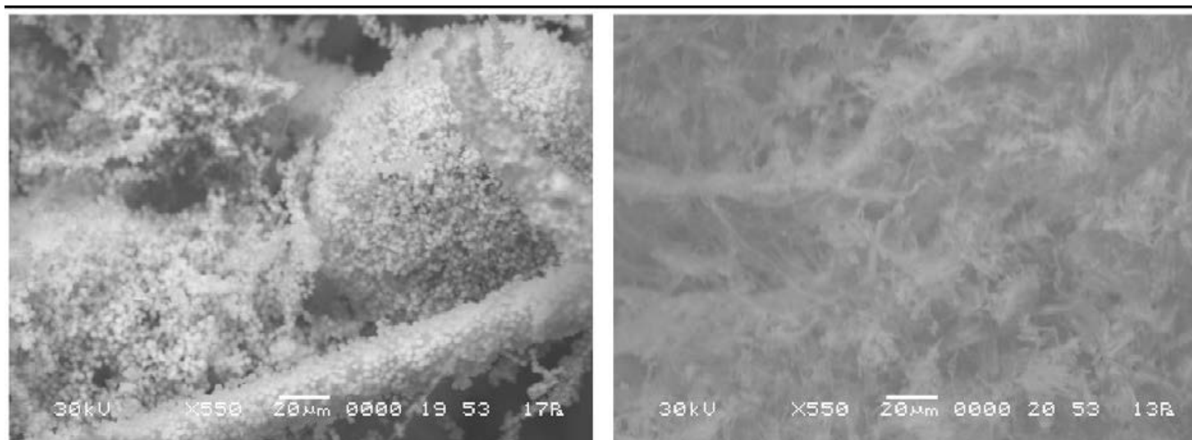

Fig. (10) :(L) Scanning Electron Microscopy (SEM) of normal *A.niger conidia* (**R**): Scanning Electron Microscopy (SEM) of *A.niger conidia* after treatment with Fe_2O_3- NPS.

As the first steps in this direction, the authors here applied the obtained results in this work on commercial poultry feeds that highly contaminated with different moulds. The obtained results evidenced also higher antifungal potential effects of ZnO or Fe_2O_3nanoparticles in comparison with other commercial antifungals (Table, 5). Whereas, the evaluation of these antifungal agents in detoxification of ochratoxin in commercial feed indicated that the metal nanoparticles and herbs as clove oils had no effect on the existed ochratoxin in feeds. On the other hand, the commercial sorbent as HSCAS; biological preparations as probiotic and propionic acid were able to eliminate the ochratoxin content in feeds (Tables, 6). Similar results to our work were obtained by **[7, 12,13,44, 46, 47],** who detected the efficacy of propionic acid and probiotic preparation in detoxification of ochratoxin in feeds and no effect of clove oils and metal nanoparticles when added to the ochratoxicated feeds. However, hydrated sodium calcium aluminosilicate (HSCAS) is perhaps the most studied mycotoxin-sequestering agent among the mineral clays and adsorbent compounds obtained from natural zeolite, which has demonstrated an ability to adsorb mycotoxins. A high affinity addition of these compound to feedstuffs contaminated with ochratoxin has been shown to have a protective effect against the development of mycotoxicosis in farm animals **[52, 53, 54].** The major advantages of these adsorbents include expense, safety and easy administration through addition to animal feeds. However, not all adsorbents are equally effective in protecting poultry against the toxic effects of mycotoxins and several adsorbents have been shown to impair nutrient utilization. Generally, until now the metals as zinc, iron, cadmium, selenium and cupper are used as feed additive for their antioxidant and growth promoters for animals and poultry **[55].**

Table (5): *Influence of total colony counts of commercial poultry feeds (20 sample) after treatment with nanoparticle and antifungals*

Types of antifungals	T.C.C. BEFOR TREATMENT	TCC after treatment with different concentrations (ug/ml) of tested chemical ±SE					
		50	100	150	200	250	300
Zinc oxide nanoparticles	$1x10^7$±0.2	$2.5x10^5$±0.15	$5x10^3$±0.4	$2x10^2$±0.1	00	00	00
Iron oxide nanoparticles	$1x10^7$±0.2	$5.5x10^5$±0.13	$4x10^4$±0.2	$1x10^7$±0.25	$1x10^2$±0.32	00	00
Propionic acid	$1x10^7$±0.2	$8x10^4$±0.33	$2x10^3$±0.2	7x10±0.0	00	00	00
Probiotic preparation	$1x10^7$±0.2	$7x10^4$±0.3	$8x10^3$±0.5	4x10±0.4	00	00	00
Clove oil	$1x10^7$±0.2	$2x10^5$±0.3	$6x10^4$±0.5	$7x10^3$±0.2	5x10±0.12	00	00

The concentrations below 50 ug/ml not affected the growth of and its potential for o production

Conc. : Concentration of tested chemical CC : Colony count

Table (6): *Levels of mycotoxins in commercial poultry feeds after treatment with antifungals and anti-mycotoxins.*

Types of antifungals and Anti-mycotoxins	Levels of ochratoxin Before treatment (ppb)	Levels of mycotoxins (ppb) after treatment with different concentrations (ug/ml) of tested chemical ±SE					
		50	100	150	200	250	300
Zinc oxide	**70±5.4**	**70±5.4**	**70±5.4**	**70±5.4**	**70±5.4**	**70±5.4**	**70±5.4**
Iron oxide	**70±5.4**	**70±5.4**	**70±5.4**	**70±5.4**	**70±5.4**	**70±5.4**	**70±5.4**
Propionic acid	**70±5.4**	**30±2.7**	**13±1.0**	**3±0.5**	**00**	**00**	**00**
Clove oil	**70±5.4**	70	70	70	70	70	70
Probiotic	**70±5.4**	**32±3.5**	**10±0.5**	**2.5±0.4**	**00**	00	**00**
Silicate Compound (HSCAS)	**70±5.4**	**18±3.5**	**5±3.0**	**00**	**00**	00	00

HSCAS = Hydrated Sodium Calcium Aluminosilicate Conc.: Concentration of tested chemical CC : Colony count

The recent reports illustrated the efficacy of nanoparticles of metal oxides as most potent antifungal and antibacterial agents against all fungal and bacterial animal pathogens **[2, 10, 12, 13, 15]**

4. Conclusion and Future Recommendation

This study is one of the first steps in direction about the use of metal nanoparticls in laboratory and field control of fungal contamination of feeds with ochratoxigenic moulds and their potential for ochratoxins production. The antimycotoxin effects of nanoparticles were limited to their addition to food and feed during processing preparation to prevent fungal growth and their potential for mycotoxins production and even toxicities and could be used in the field of veterinary medicine as fungicides in successful treatment of microbial diseases. In addition, the biosynthesis of nanoparticles by saprophytic fungi is cost-effective, environmentally friendly and non- infectious for industrial workers. Hence, advanced and further investigations are required for direct treatment of farm animals by metal nanoparticles considering their toxic doses to avoid health hazard which may result from misusing of nanoparticles. Up to date the use of nanoparticles of these metals will have dual synergistic effects of antioxidant and antifungal which will be significantly reflected in improving animal health.

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