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Effect of Hybrid Nanomaterial of Copper-Chitosan against Aflatoxigenic Fungi in Poultry Feed

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ABSTRACT

In the past decades, the application of nanotechnology indicated significant improvements in animal health. In the present work, 60 samples of poultry feeds were examined, including 20 samples for each yellow corn, soya bean, and processed feed. The prevalence of total fungi was reported as 100%, 95%, and 100% in yellow corn, soya bean, and processed feed, respectively. Toxin-producing *Aspergillus flavus* represented 75% of isolates from yellow corn, 88% from soya bean meal, and 50% from processed feed. Aflatoxins were found in 88%, 60%, and 80% of yellow corn, soya bean, and processed feed with mean levels of 18.5 ± 3.216.0 ± 4.08.3 ± 1.7 ppm, respectively. The copper nanoparticles embedded with chitosan were green synthesized using an eco-friendly method, and their antifungal activity was evaluated against aflatoxigenic mold that recovered from poultry feeds. However, the molecular detection of virulent genes of *Aspergillus flavus* (*aflR* gene*)* after their exposure to high doses of copper-chitosan nanoparticles (CuCh-NPs) 150 µg/ml prevents *aflR* gene expression. The embedded chitosan with copper nanomaterial helps decrease their suspected toxicity to animals by reducing the used doses. Hence, the use of nanocomposites of nanomaterials with green benefits substances, such as chitosan, was the essential strategy of field application in veterinary.

Keywords: *Aspergillus flavus*, Chitosan, Copper nanoparticles, Nanotechnology, Poultry feed

INTRODUCTION

The use of nanotechnology is gaining more popularity in improving livestock health and productivity, especially in developing countries (Hassanen et al., 2019; Khalaf et al., 2019; Hassanen et al., 2020). Fungal infections caused by mycotoxigenic molds in food can cause significant carcinogenic effects on humans and animals (Hassan et al., 2022). Under adverse environmental conditions, aflatoxigenic molds produce aflatoxins (AFs) in food, and their consumption leads to several health problems (Adam et al., 2017; Çelik, 2020; Hassan et al., 2021). Given that the conventional methods of elimination, such as chemical antifungal, azoles, and antimycotoxins were proved inefficient, the elimination of AFs can be difficult and costly (Brunet et al., 2018; Di Mambro et al., 2019; Gintjee et al., 2020; Tiew et al., 2021). Consequently, recent studies have introduced new agents to eradicate toxin-producing pathogens (Singh et al., 2018). Earlier studies also assessed the advantages of using metal nanomaterials over chemical agents in controlling the growth and viability of pathogens (Tran and Webster, 2011; Mohd Yusof et al., 2021). In the same vein, others used copper and zinc nanoparticles (Cu-NPs and Zn-NPs, respectively) for the degradation of fungi and mycotoxins to improve the safety of food production (Castro-Mayorga et al., 2020; Agrimonti et al., 2021; Konappa et al., 2021; Hassan et al., 2022). Therefore, the present study aimed to evaluate the prevalence of AFs in feed and the effect of copper-chitosan nanoparticles (CuCh-NPs) on inhibiting the fungi and aflatoxin-regulating genes using molecular detection.

MATERIALS AND METHODS

Samples

A total of 60 samples of poultry feeds were examined, including 20 samples from yellow corn, soya bean, and processed feed. Approximately 100 g from each sample was aseptically collected from poultry farms from

June to August 2021 in sterile polyethylene stretch film followed by an ensiling process, and it was stored in a dry aerobic place.

Isolation and identification of fungi species in samples

To begin, 5 g of each feed sample was separately transferred aseptically into sterile tubes, to which 45 ml of sterile distilled water was added, and 10-fold serial dilutions were prepared. Afterward, 1 ml of the previously prepared serial dilutions was inoculated separately into sterile Petri dishes plates and mixed with Dichloran-rose Bengal chloramphenicol agar or Sabouraud's dextrose agar (SDA) medium containing 0.05 mg of chloramphenicol/ml. The plates were left to solidify and dry. They were then incubated aerobically in the incubator at 25°C ± 1°C for 5 days. The plates were read during 2-5 days of incubation. The fungal cultures were separated based on morphological characteristics, including colony size (diameter, millimeter), texture, and surface. The fungal cultures were examined periodically during the incubation period. The culture characteristics and sporulation on different culture media were recorded after 7 days of incubation at 28°C. The morphological characteristics of each fungal isolate were determined using the light microscope (OPTO-EDU, China). The microscopic examination of fungal isolates was described after the fungal colonies were sporulated on the different culture media. For this purpose, small mycelia part from the center and edge of the growing colony was mounted onto a microscope slide using distilled water and covered by a cover slip. The characteristics of vegetative and reproductive structures, such as hyphal color and structures, spore shape, as well as spore size, were determined (ISO 21527/1, 2008; Pitt and Hocking, 2009).

Copper nanoparticles

Copper nanoparticles with the size of 50 nm were prepared at Biochemistry, Toxicology, and Feed Deficiency Department, Animal Health Research Institute, Egypt, and identified at the Central Laboratory of Elemental and Isotopic Analysis, Nuclear Research Centre, Egypt.

Synthesis and characterization of chitosan-copper nanoparticles

The method was based on a study by Du et al. (2009), indicating some changes as copper ions were converted into nanosized material by mixing with a solution of acetic acid and chitosan, in which chitosan was dissolved in 1% (v/v) acetic acid to obtain a 0.3% (w/v) chitosan solution followed by refrigeration for 12 hours. The mixture was then centrifuged at 12000 rpm for 20 minutes at 4°C (Sigma Laborzentrifugen, Germany). The sediment was washed with distilled water, centrifuged again, and frozen until use. The freeze-dried CuCh-NPs were identified according to the method of Kaur et al. (2015). Their structures were detected by transmission electron microscopy (JEOL 2100F TEM instrument), and their infrared spectra were obtained using FTIR (Fourier transform infrared spectroscopy, Spectrum BX11, USA).

Preparation of tested isolates

The tested *Aspergillus flavus* (*A. flavus*) that were isolated from the present samples were subjected to Polymerase chain reaction (PCR) to identify their virulent genes. They were cultured on Sabouraud's dextrose broth medium and incubated at 25°C for 1-3 days for proper growth. The negative control was *Fusarium* species, while the positive control was standard isolates of *A. flavus*. On the other hand, the isolates of *A. flavus* were treated with CuCh-NPs under a septic condition in 100 ml flasks, then 20 ml of SD broth was added, and 0.2 ml of 10^4 spores was inoculated into the flask. The doses of treatments were leveled as low as 50 µg/ml and as high as 150 µg/ml for Cu-NPs. Then, the treated isolates were incubated at 25°C for 3 days and kept at 5-8°C until DNA extraction.

Genotypic evaluation of aflatoxigenic genes of *Aspergillus flavus*

DNA extraction and PCR amplification were performed according to Somashekar et al. (2004) and Fittipaldi et al. (2012). Genomic DNA of the strains was obtained using the genomic DNA Extraction Kit (Quick-DNA Miniprep DNA purification) following the manufacturer's instructions. DNA concentration was determined spectrophotometrically at 260/230 nm using SPECTRO star Nano BMG LABTECH. DNA was stored at -20°C until PCR amplification for the target fragments of aflatoxin-producing and control fungal genes. Invitrogen Company prepared the PCR primer used in the current study (Table 1). The PCR reaction was performed in a Gradient Thermal cycler (1000 S Thermal cycler Bio-Rad USA). The reaction mixture (total volume of 50 µl) was 25 µl Dream green PCR Mix (DreamTaq Green PCR Master Mix (2X) Thremoscientific Company, cat., No. K1081, USA), 5 µl target DNA, 2 µl of the primer

(containing 10 p mole/ µl), and the mixture was prepared by sterile Nuclease-free water to 50 µl. The PCR amplification conditions for the aflatoxin regulatory gene were 5 minutes for the initial step at 95°C, followed by 35 cycles at 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 30 seconds, and a final extension step at 72°C for 10 minutes. Amplification products were electrophoresed in agarose gels in Tris-borate-EDTA (TBE) buffer with 1 µl of ethidium bromide/gel added for visualization under UV light (1.5% w/v, Agarose, Sigma, USA), using 100 bp DNA Ladder H3 RTU (Ready-to-Use) Cat. No. DM003-R500 from Gene Direx, Inc. Company, Litwania (Isalar et al. 2021).

Measurement of the minimum inhibitory concentration of CuCh-NPs against isolated *Aspergillus flavus*

The minimum inhibitory concentration (MIC) of CuCh-NPs for aflatoxigenic *A. flavus* was detected by a broth micro-dilution method (CLSI, 2008) which starts by adding 900 µl of Sabouraud's dextrose (SD) broth in plastic test tubes, then inoculating with100 µl of *A. flavus* (10^4 spores/ml), then adding 100 µl of CuCh-NPs in 0, 50, 100, 150 µg/ml concentrations, then incubated for 2-5 days at 25-28°C. The MIC that suppressed the growth of pathogen cultures and the turbidity was checked every 24 hours. A UV-vis spectrophotometer detected the optical density of each tube content (SP-LUV759, China) set at 405 nm.

Statistical analysis

Results were expressed as mean ± SE. The statistical analysis was conducted using Statistical Package for Social Sciences Version 14, released in SPSS (2006).

Table 1. Primers for molecular identification of *Aspergillus flavus*

Virulence factor	Target virulence gene	Primers	Amplicon size (Base pair)	Annealing temperature (°C)	Reference
Aflatoxin regulatory gene (*aflR)* of *A. flavus*	*aflR*	*afl*R-F AACCGCATCCACAATCTCAT *afl*R-RAGTGCAGTTCGCTCAGAACA	800	60	Somashekar et al. (2004)

RESULTS AND DISCUSSION

The results in Table 2 revealed that the prevalence of total fungi was 100% in yellow corn, 95% in soybean, and 100% in processed feed. *Aspergillus flavus* was the most prevalent mold of *Aspergillus* spp. with a total incidence of 45%. *Aspergillus* species were *Aspergillus ochraceus, A. niger, A. candidus, A. fumigatus, and A. glaucus*. The detected molds were *Penicillium* spp., *Mucor* spp., and *Rhizopus* spp., with a total incidence of 1.6%, 35%, and 33.3%, respectively. *Candida albicans* were found in 25% of samples. Similar findings were reported by FDA (2000), Hassan et al. (2020) who detected that *A. flavus* was the predominant species isolated from the feed. This proves that it requires several methods, such as using nanomaterials to inhibit fungal growth and activity from preventing human and animal diseases.

According to Table 3, the recovered *A. flavus* was used for the production of AFs, most of which were on yellow corn with the mean levels (750 ± 5.3 ppb) followed by processed feed (600 ± 6.1 ppb) and soya bean meal (170 ± 3.5 ppb). Toxin-producing *A. flavus* represented 75% of isolates from yellow corn, 88% from soya bean meal, and 50% from processed feed.

Generally, mycotoxins cause serious health hazards, especially in humid regions in developing countries, such as Egypt, due to the presence of AFs in feed (Nayak and Sashidhar, 2010; El-Nahass et al., 2019; Monda et al., 2020). Aflatoxins were detected in feed and feedstuffs by El-Hamaky et al. (2016) at levels ranging from 170 to 750 ppb.

Table 4 shows that AFs were found in 88% of yellow corn with a mean level of 18.5 ± 3.2 ppm; 60% of soya beans with a mean level of 16.0 ± 4.0 ppm, and 80% of processed feed with a mean level of 8.3 ± 1.7 ppm. Herein, the obtained results of AFs were more than the safe permissible limits and can cause serious adverse effects on health by causing hepatic injury and cancers (FDA, 2000). The results agree with the previous findings of Frisvad et al. (2006), Nayak and Sashidhar (2010), and Hassan et al. (2020), who detected the dangerous hepatic-carcinogenic effects of AFs on rabbits and rats' livers.

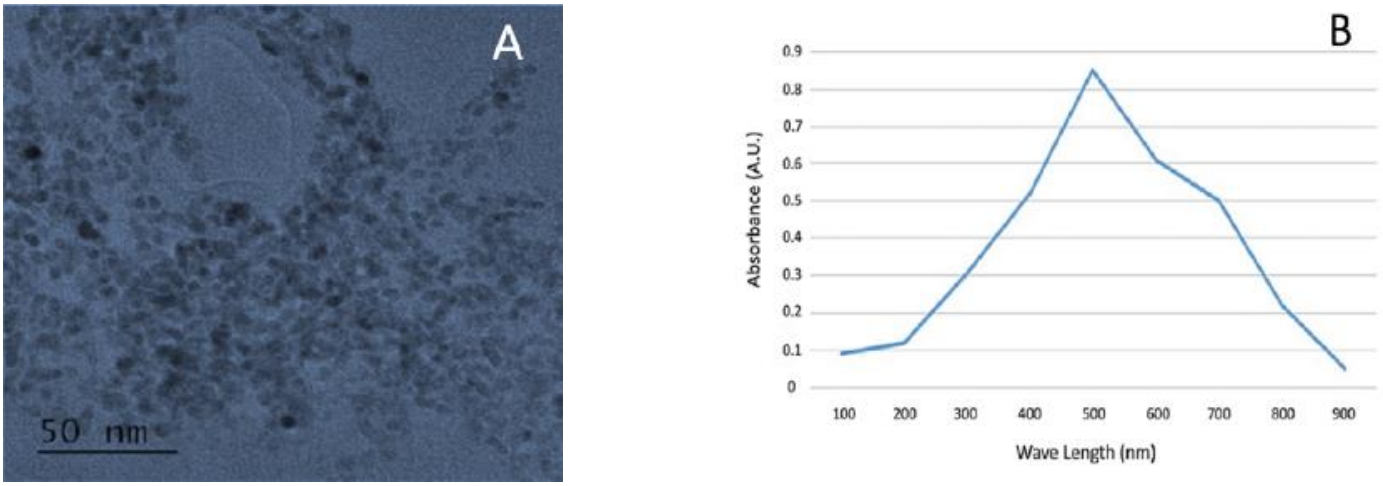

Figure 1. The morphological characters of copper-chitosan nanoparticles (CuCh-NPs) under transmission electron microscopy (50 nm in size, **A**). UV–visible spectrophotometry of CuCh-NPs (the peak was at a range of 500–600 nm, **B**).

These results in Figure 1 are similar to the finding of Vanti et al. (2020). Due to the emergence of multidrug-resistant bacteria, conventional antibiotics are rendered ineffective (Hassan et al., 2020a), which led to the use of nanosized materials as an alternative to the traditional antimicrobial drug (Abinaya et al., 2016; Munir et al., 2020). Furthermore, nanosized particles were more effective than crude metals (El-Sayed and Kamel, 2020).

Today, several microbial infections, such as fungal and bacterial infections, have a multidrug resistance to conventional antibiotics, increasing the severity of the conditions (Hassan et al., 2020a). Hence, the search for new effective antimicrobial agents is required, and the nanosized materials showed significant success in this purpose as Zn-NPs and Cu-NPs (Sharma et al., 2018; Zakaria et al., 2020). The nanomaterials are more effective than crude materials and can be used for antibiotic and disease diagnosis (El-Sayed and Kamel, 2020). They may be supplemented in drinking water and feeds of broiler chickens to improve their health and immune status (Hassanen et al., 2020; Hassan et al., 2022).

As can be seen in Table 5, the increase in the CuCh-NPs concentration led to a decrease in optical density (OD), degree of turbidity (DT), and growth after treatment (GT). Therefore, the minimum inhibitory concentration (MIC) of CuCh-NPs against *A. flavus* was determined to be 150 µg/ml. Nanomaterials can inhibit microbes by penetrating them and damaging their protein and DNA synthesis (Rudramurthy et al., 2016; Huang et al., 2020).

PCR detected the toxic gene (*aflR*) in *A. flavus* strains found in the samples. The efficacy of CuCh-NPs was evaluated the inhibiting that gene. Previous studies have also successfully detected *aflR* gene in *A. flavus* recovered from feed (Scherm et al., 2005; Cruz and Buttner, 2008; El-Hamaky et al., 2016). The exposure of *A. flavus* to high doses (150 µg/ml) of CuCh-NPs significantly decreased the *aflR* gene expression (the efficiency percentage, the molecular weight of DNA, and the cycle threshold of the gene declined). Currently, the *aflR* gene expression in the case of treatment with a low dose (50 ug/ml) also resulted in similar activity but lower than the exposure to high doses of CuCh-NPs (Table 6). Hence, the exposure of virulent genes of toxigenic fungus to high amounts of nanomaterials resulted in the complete removal of genes and prevented drug resistance. The present study indicated the high efficiency of CuCh-NPs nanocomposites in suppressing the viability and growth of aflatoxigenic *A. flavus*. Several studies reported that Cu-NPs have antimicrobial potential against isolated fungi from clinical cases of animal disease and feeds (Sharma et al., 2018; Zakaria et al., 2020; Hassan et al., 2022). Currently, the composites of metals nanomaterials with green benefits materials, such as the chitosan effect, decrease the used dose of nanomaterials and overcome its suspected ecotoxicity. Hence, nanotechnology has significant progressive advancements in biotechnology and biomedicine related to human and animal science as it increases the safety of their health and production (Contera et al., 2020).

Table 2. Incidence of fungi species in poultry feeds

Fungal species	Yellow corn (20)		Soya bean meal (20)		Processed feed (20)		Total (60)	
	No. +ve	%	No. +ve	%	No. +ve	%	No. +ve	%
Total fungi	20	100	19	95	20	100	59	98.3
Aspergillus species	17	85	14	70	15	75	46	76.6
Aspergillus flavus	8	40	9	45	10	50	27	45
Aspergillus ochraceus	3	15	2	10	4	20	9	15
Aspergillus niger	6	30	5	25	7	35	18	30
Aspergillus candidus	4	20	2	10	2	10	8	13.3
Aspergillus fumigatus	3	15	1	5	1	5	5	8.3
Aspergillus glaucus	1	5	0	0	0	0	1	1.6
Penicillium species	0	0	0	0	1	5	1	1.6
Mucor species	7	35	5	25	9	45	21	35
Rhizopus species	5	25	8	40	6	30	20	33.3
Geotrichum species	1	5	1	5	0	0	2	3.2
Candida albicans	5	25	6	30	4	20	15	25

No +ve: Number of positive

Table 3. Production of aflatoxins B_1 by *Aspergillus flavus* isolated from poultry diets

Source of *Aspergillus flavus* isolates	Incidence of toxigenic *Aspergillus flavus* isolated from poultry feeds			Produced aflatoxins (ppb)	
	Total tested	No. +ve	Percentage	Mean levels	Types
Yellow corn	8	6	75	750 ± 5.3	B^1,B^2
Soya bean meal	9	5	88	170 ± 3.5	B^1, B^2, G^1 , G^2
Processed feed	10	5	50	600 ± 6.1	B^1
Total	27	16	59.2		

The permissible limits of AFB_1 were 15 ppb (WHO, 2002) and 20 ppb (FAO, 2004). No +ve: Number of positive.

Table 4. Levels of aflatoxins in poultry feeds

Feed types	Incidence of aflatoxins		Aflatoxins in samples (ppm)			Types of aflatoxins
	No. +ve	Percentage	Maximum	Minimum	Mean ± SE	
Yellow corn	22	88	30.0	9.5	18.5 ± 3.2	B_1, B_2, G_1,G_2
Soya bean meal	15	60	23.0	10.2	16.0 ± 4.0	B_1, B_2, G_1, G_2
Processed feed	20	80	3.2	1.6	8.3 ± 1.7	B_1, B_2, G_1, G_2

The permissible levels of aflatoxin, according to WHO (2002) 15 ppb and FAO (2004) 20 ppb. SE: Standard Error, No +ve: Number of positive

Table 5. Optical density and degree of turbidity of treated *Aspergillus flavus* at a gradual concentration of CuCh-NPs

CuCh-NPs concentrations (µg/ml)	*Aspergillus flavus*	
	OD (a.u)	DT and GT
0	2.27	4+
50	1.65	3+
75	1.38	2+
100	1.08	1+
125	1.00	1+
150	0.60	0

Control antifungal: Fluconazole 20 µg (Its OD: zero and turbidity: zero), OD: Optical density of treated spores at wavelength 405 nm, DT: Degree of turbidity of treated suspension, GT: Growth after Treatment. (a.u): absorbance unit

Table 6. Detection of *aflR* regulatory gene expression of *Aspergillus flavus* before and after treatment with CuCh-NPs

Aspergillus flavus	*aflR* gene expression at different doses of treatment					
	Eff. (%)		Mole		C.T	
	Low dose	High dose	Low dose	High dose	Low dose	High dose
Untreated controls	94.42		3.45		26.33	
Treated with CuCh-NPs	32.46	13.63	0.01983	0.0198	26.62	23.26

Eff: Efficacy of *aflR* gene expression, Mole: Molecular weight of DNA (µg/ml), CT: Cycle Threshold CuCh-NPs: 50 µg/ml (Low dose), 150 µg/ml (High dose)

CONCLUSION

The presence of aflatoxigenic molds in animal feeds can produce AFs. The essential preventive and therapeutic activities of Cu-NPs embedded with chitosan have been evaluated against the aflatoxigenic mold. Additionally, CuCh-NPs could remove *aflR* genes of *A. flavus* when a higher dose of 150 µg/ml was used. The conjugation of Cu-NPs with chitosan reduced the used dosages of metal nanoparticles and avoided the toxic hazard of copper nanoparticles. Therefore, more studies are needed to evaluate the effects of copper chitosan nanoparticles at different doses in poultry diets.

DECLARATIONS

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Authors' contributions

Noha Oraby, Rasha Sayed-ElAhl designed the research. All authors analyzed the data. Atef Hassan wrote the draft of the manuscript. Noha Oraby, Rasha Sayed-ElAhl and Manal El-mesalamy have revised the manuscript. All authors read and approved the last version of the manuscript for publishing in the present journal.

Competing interests

The authors declare that they have no competing interests.

Ethical consideration

The authors investigated ethical issues such as plagiarism, permission to publish, malfeasance, data falsification and/or fabrication, double publishing and/or submission, and redundancies.

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