Effects of Chitosan nanoparticles as antimicrobial activity and on mycotoxin production

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ABSTRACT

This research aimed to study the effect of chitosan nanoparticles (CSNPs) against many bacterial and fungal growth and on aflatoxins production. Remarkable results were obtained. CSNPs showed significant antibacterial activity against gram negative bacterial isolates as compared with Gram positive bacteria. Moderate activity against the yeast isolates used was observed, and good results were obtained against filamentous fungi. Significant reduction of aflatoxin was detected using HPLC.

Key words: Antimicrobial, Aspergillus, aflatoxin, chitosan, nanoparticles.

INTRODUCTION

Chitosan, chitin, and their derivatives belong to a family of polymers that exert bio-stimulating activities, such as acceleration of wound healing, regeneration and vascularization of tissue, and reduction of scars. The antimicrobial activities of chitosan include fungi, anascogenic yeasts and bacteria, the activity being more intense with Gram (+) bacteria than Gram (−) bacteria. Chitosan properties depend upon molecular mass, deacetylation degree, concentration, pH, and composition of the surrounding. It also exhibits good adhesion and coat forming properties, which carries the chance of providing an antibacterial coating or playing the role of a carrier for other antimicrobial substances. Such properties along with biocompatibility and biodegradability make chitosan an excellent material for uses in medicine (Malczewska et al., 2016).

Mould toxicity has gained attention, especially in the fields of agriculture and food industry. Microscopic filamentous fungi often contaminate vegetable and animal products, becoming a source of diseases in man and slaughter animals (Bennett and Klich, 2003). Mycotoxicosis is example of “poisoning by natural means” and thus is analogous to the pathologies caused by exposure to pesticides or heavy metals residues. The symptoms of mycotoxicosis depend on the type of mycotoxin, the amount and duration of the exposure, the age and health of the exposed individual, and many poorly understood the synergistic effects involving genetics, dietary status, and interaction with other toxic insults (El Ghreeb et al., 2013).

Aflatoxins are toxic metabolites produced by some species of fungi. Common aflatoxins are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), and G2 (AFG2). Aflatoxins have been shown to be hepatotoxic, carcinogenic, mutagenic and teratogenic to different species of animals (Eaton and Gallagher, 1994). AFB1 is the most prevalent and carcinogenic of the aflatoxins and the International Agency for Research on Cancer (IARC) classify AFB1 as a group I carcinogen (that is, an agent that is carcinogenic to humans). Epidemiological studies also indicate that areas in the world with high levels of aflatoxin are correlated with high incidence of liver cancer. These toxins are highly carcinogenic and elicit a wide spectrum of toxic effects when foods and feeds contaminated with aflatoxins are ingested (Lee et al., 2004).

The main objectives of this study were to evaluate the action of CSNPs, as antimicrobial potency and study its activity on aflatoxin produced by two fungal isolates.
Table 1: List of microbial isolates used and microbial code in RCMB.

<table>
<thead>
<tr>
<th>Bacterial isolates used</th>
<th>Fungal isolates used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram positive bacteria isolates</td>
<td>Fungal isolates</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Aspergillusflavus</td>
</tr>
<tr>
<td>Staphylococcus haemolyticus</td>
<td>Aspergillusparasiticus</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>Aspergillusniger</td>
</tr>
<tr>
<td>Bacillus thuringiensis</td>
<td>Syncephalastrumracemusam</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>Penicilliumchrysogenum</td>
</tr>
<tr>
<td>Gram negative bacteria isolates</td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>Candida albicans</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Candida dubliniensis</td>
</tr>
<tr>
<td>Klebsiella pneumonia</td>
<td>Cryptococcus humicola</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>Geotrichiumcandidum</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>Trichosporoncutameum</td>
</tr>
</tbody>
</table>

(Aspergillus flavus and A. parasiticus) using HPLC.

MATERIALS AND METHODS

Organisms

Twenty bacterial and fungal isolates, as listed in Table 1, obtained from the culture collection in the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University were used as antimicrobial activity.

Preparation of chitosan nanoparticle (CSNPS) according to Venkatesan et al. (2016)

An ionic gelation method has been previously used to synthesize EHP–CSNPS and EMMP–CSNPS utilizing NaTPP as a gelating agent with some modification. Briefly, chitosan (1.25 g) was dissolved in 500 mL of 1% (v/v) acetic acid along with 0.25% (1.25 mL) of Tween 80 to yield a chitosan solution at concentration of 2.5 mg/mL. The chitosan solution was continuously stirred overnight at room temperature, and the pH of the solution was adjusted to 5.5 using 1 M NaOH aqueous solution. Thereafter, NaTPP solution in water(0.25 mg mL1, 25 mL) was added dropwise to the chitosan/PL mixture under mild stirring. The isolated nanoparticles were rinsed with fresh water and then lyophilized.

Characterization of the biosynthesized nanoparticles:

The characterization of chitosan nanoparticles was carried out using different instruments and techniques. It includes visual observation, UV–Visible spectrophotometer (Spectronic Milton Roy 1201 UV), X-ray diffraction (XRD) of TEM (XRD/TEM JEOL 1010, Japan), energy-dispersive X-ray (EDX) SEM/EDX, JSM-5500 LV JEOL, SEM, Japan) of SEM, Fourier transform infra-red (FT-IR) (IRPrestige-21®, German) analysis and TEM (TEM JEOL 1010, Japan). The antimicrobial activities of CSNPs, EHP–CSNPs and EMMP–CSNPs and their mode of actions were previously studied, so that, the present study is concerned with their activity on aflatoxins production and fungal genetic exchange.

Antimicrobial Assays:

Antimicrobial potentialities against twenty microbial species were expressed as the diameter of inhibition zones; as hole-plate diffusion method was used. Six equidistant (1 cm diameter) holes were made in the agar using sterile cork borer in nutrients and Sabouraud agar sterile plates (10 × 10 cm) for bacterial and fungal growth respectively, which had previously been seeded with microbial isolates tested, were filled by 100 µL with CSNPs. Control holes were filled with chitosan solution, which were used in the extraction methods. Plates were left in a cooled incubator at 4 (±2)ºC for one hour and then incubated at 37 (±2)ºC (Abde–Kader and Seddkey, 1995). Inhibition zones developed due to active seed ingredients were measured after 24-48 h of incubation.

Determination of CSNPSeffects on the total aflatoxin production

Extraction of aflatoxins

Inoculation of 1 ml spore suspension (10^4 cells/ml) of each of A. flavus or A. parasiticus in 250 ml Erlenmeyer flasks was done, with each containing 100 ml of sterile YES broth medium mixed (1 ml) with each phenolic compound used. Three replicates for each organism were prepared and incubated at 25±2ºC for 21 days. The broth filtrates were mixed with an equal volume of chloroform in a separating funnel. The residue was re-extracted twice for complete
extraction. The chloroform extract was defatted with hexane for separate lipid layer, concentrated in a rotary evaporator. The residues were reconstituted in 1 ml methanol for further HPLC chromatographic analysis (Hermínia et al., 2003), while the mycelia of fungi were dried and weight to determine the effect of extracts on fungal growth.

**Determination of aflatoxins B1, B2, G1 and G2 by HPLC**

The samples were analysed for the quantification of aflatoxins using immunoaffinity columns supplied from Rhône-diagnostics technologies Ltd (Spain), and quantified by high performance liquid chromatography (HPLC) according to the method described by Stroka et al. (2000), which has been accepted as the official method by AOAC, with modifications in the initial extraction phase. The solvent mixture was water: methanol (8:2) instead of methanol: water (8:2,v/v). The sample extract was filtered, diluted and applied in an immunoaffinity column containing antibodies specific to aflatoxins B1, B2, G1 and G2. Standard aflatoxins (AF) B1, B2, G1 and G2 were purchased from Sigma-Aldrich (Ref.A-6636, A-9887, A-0138 and A-0263 respectively) (Quimica S.A.S-pain). The stock solution, working standards and the calibration curve were prepared and determined as described by Stroka et al. (2000).

**Statistics study**

All samples were submitted to this process, and statistical analysis of the experimental data was performed using one-way analysis of variance (ANOVA). Mean comparisons were carried out with the Tukey’s test, which retains the overall significance level at 5% (P<0.05) (Pfaller et al., 1988).

**RESULTS AND DISCUSSION**

Figure 1 show the characterization of CSNPs under TEM, FTIR, XRD and X-ray. 55 nm of CSNPs particle were detected by TEM. Arriola et al. (2017) reported nanoparticles and microcapsules (Mcapsules) of 35–40 nm, respectively.

The present study investigated the effects of chitosan nanoparticles on ten species bacterial growth (Table 2) and ten species of fungal growth (Table 3). The measurements of inhibition zones microbial growth were expressed as cm. Gram negative bacteria were observed to
be more sensitive to chitosan and chitosan nanoparticle than Gram positive bacteria. Whereas Klebsiella pneumonia showed inhibition of 3.5 cm, followed by Salmonella typhi, Escherichia coli and Proteus vulgaris with 3.0, 2.9 and 2.5 cm inhibition, respectively. Only, Staphylococcus haemolyticus showed 2.5 cm inhibition to CSNPs, which belong to the Gram positive bacterial isolates used.

With regards to the moderate potency of CSNPs as antifungal, A. parasiticus and Syncephalastrum racemusam were the most sensitive fungi to CSNPs (1.9 and 1.8 cm inhibition, respectively). This result is in agreement with those of Arriola et al. (2011) who reported that chitosan can be used as antifungal agents at high concentration and also, Venkatesan et al. (2016) who declared that CSNPs became high active when loaded with piperlongumine.

Due to the deleterious effects of the aflatoxins, numerous studies have been conducted to control their production and to prevent the growth of fungi producing them. However, the problem is more compounded based on the fact that the toxins cannot be eliminated from foodstuffs or animal feeds by ordinary processing practices. Since fungicides cannot be applied to foodstuff and animal feeds, the use of essential oils and plant extracts was the most attractive procedure (Singh and Upadhyay, 1991). Also, Mekawey (2010) used different type of honey which exhibited high reduction of aflatoxins production. A. flavus produced Afla B1 and B2, while A. parasiticus produced aflatoxin 1, 2, G1 and G2 as compareds with the standards (Figure 1). This result is in line with the findings of Accinelli et al. (2008) who reported A. flavus that typically produced aflatoxins (B1 and B2), whereas A. parasiticus produced aflatoxins (G1, G2, B1 and B2).

Mycotoxin contamination of food is considered the major source of contamination of manufactured products. Evaluation of this problem, which is very common in many developing countries such as Egypt, will help to setup control strategies. The results in Table 4 showed the mean ± SD. Previously, Golge et al. (2013) reported the level of aflatoxins in commercially used Turkish red chilli. Aflatoxin contamination were detected from all different spices, except in cumin. Fazekas et al. (2005), also reported AFB1, AFB2, AFG1, AFG2, and OTA contamination in spices from Hungary. It has been observed that CTN contamination is mainly confined to the dry ginger red chilli, coriander and black pepper samples. These samples were highly contaminated with P. citrinum or P.
Concluding remarks and future prospects

The use of natural products as drugs are preferred to synthetic compounds, which possess sever toxicity and side effects besides being very expensive. Hence, the use of natural products represents a valuable solution. In this study, Egyptian cumin, black pepper and chitosan (found in fresh shrimp head) were found to be of good quality and could act as helper in therapy or control of the aflatoxins produced by fungi, which caused serious diseases of the liver, such as hepatic necrosis, cirrhosis, and/or carcinoma. In the present study, the effect of chitosan on fungal growth was assessed, in vitro. Chitosan nanoparticles (CSNPs) were prepared. The CSNP’s successfully decreased the production of total aflatoxins. The data presented in this paper show that chitosan gives a good level of radial growth inhibition against *A. parasiticus* and *A. flavus*. Therefore, further studies regarding the determination of aflatoxin production genes and nanoparticles synthesis from natural and safe components need to be carried out.

Various analytical methods employed in analysis of aflatoxins in agricultural food crops and feeds have been evaluated. While chromatographic methods such as TLC and HPLC are considered the gold standard and are thus the most widely used techniques in aflatoxins analysis, they remain largely cumbersome, requiring extensive sample preparations, let alone very expensive equipment. This makes their routine use in analysis confined to laboratories. It is on the account of such limitations that it was necessary to develop more sensitive and better techniques for aflatoxins analyses.

**REFERENCES**


**Table 4:** Effect of cumin and black pepper extracts on aflatoxins (AFs) production by *A. flavus* and *A. parasiticus* (µg mL⁻¹)ᵃ.

<table>
<thead>
<tr>
<th>Treatments</th>
<th><em>A. flavus</em></th>
<th><em>A. parasiticus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AFB1</td>
<td>AFB2</td>
</tr>
<tr>
<td>Standards</td>
<td>86.2±2</td>
<td>88.3±2</td>
</tr>
<tr>
<td>Control</td>
<td>22.9±5</td>
<td>19.5±6</td>
</tr>
<tr>
<td>Chitosan</td>
<td>16.4±4</td>
<td>13.8±9</td>
</tr>
<tr>
<td>Chitosan nanoparticles</td>
<td>4.4±3</td>
<td>3.8±7</td>
</tr>
</tbody>
</table>

ᵃ: Mean of triplicate analyses.  b: Below detection limit.


