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Influence of two different nanomaterials on *Aspergillus fumigatus* isolated from salted fish

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Abstract

This study detecting *Aspergillus fumigatus* in salted fish as Feseakh and Moloha that is being sold in Sohag city markets using culture methods and scanning electron microscope (SEM). In addition, study the effect of chitosan nanoparticles (CNP) and carvacrol nanoemulsion (CNE) with five concentrations on the isolated strains of *A. fumigatus* using well diffusion method (WDM) and SEM. Results showed that *A. fumigatus* was detected in 11(14.7%) and 14 (18.7%) of Feseakh and Moloha (75 each), respectively. CNP has no effect on *A. fumigatus* growth at concentrations 0.5%, 1% and 2% and has inhibitory effect at concentrations 5% and 7 %. CNE inhibits *A. fumigatus* growth at concentrations 0.5%, 1% and 2 and stops its growth at concentrations 5% and 7 %. The effect of both nanomaterials had increased with high concentration. SEM shows degenerative changes on fungus ultrastructure. Therefore, CNP and CNE as antifungal are suitable for food industry to enhance the food safety.

Keywords:

Aspergillus fumigatus, Carvacrol nanoemulsion, Chitosan nanoparticles, Feseakh, Moloha.

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Introduction

Feseakh and Moloha are un-eviscerated salt-cured fish products made in Egypt by wet salting method. Feseakh is prepared from *Mugil cephelus* brackish water fish by fermenting method, while Moloha is prepared from *Hydrocymus fumskallii* freshwater fish (Huss et al., 2003). Salting and smoking are traditional methods for preservation of fish all over the world, while in Arabic region salting is more common (Suleiman et al., 2014).

Several pathogens as fungi and bacteria were identified in different types of fish. They can produce their toxic metabolites in fish flesh even after salting causing serious public health hazards (Dima and Todd, 2020). In Egypt, human outbreaks with food poisoning symptoms were reported after the consumption of Feseakh and Moloha especially in some Egyptian holidays. Fungi may contaminate these products before processing or during preparation and transportation (AbdEl-Razik et al., 2020).

A. fumigatus is widely spread in nature especially on decaying organic matters and disseminates spores in the environment and can produce mycotoxins. It causes various hazards to human health, ranged from allergic to chronic lung infection and invasive infection in immunocompromised patients (Fang and Latgé, 2018).

Nanoparticles (NP) have gained interest as sensitive and specific materials in controlling many viral, fungal, and bacterial diseases in aquaculture (Shaalan et al., 2016). They have special chemical and physical properties, which significantly differ from their conventional characters especially as antifungal agent (Dina, 2018). Chitosan is a natural material that has several biological properties as biodegradability, biosafety and biocompatibility. Therefore, it can be used in diverse applications as a food preservative due to its low side effects, and food additive to improving immune system, and inhibition of intestinal pathogen (Abdel-Ghany and Salem, 2020). Carvacrol nanoemulsion (CNE) is a phenolic compound found in the essential oils of thyme, oregano, marjoram, pepperwort and Alaskan yellow cedar. It acts as antimicrobial agent and widely used as a food additive (Burt, 2014). Therefore, multiple researches were required for the best methods for chitosan nanoparticles (CNP) and CNE application in different fields, especially food industry. The present work aimed to isolate and identify *A. fumigatus* from salted fish (Feseakh and Moloha) by using mycological culture as well as detecting the effect of CNP and CNE on the isolated *A. fumigatus* growth by Well diffusion method and the fungus ultrastructure by SEM.

Materials and methods

Samples collection and mycological examination

A total of 150 salted fish samples were collected from different markets and supermarkets in Sohag city, the samples include 75 Feseakh (*Mugil cephelus*) and 75 Moloha (*Hydrocymus fumskallii*). Based on Knox et al., 2016, muscles samples were cultured on Potato dextrose agar (Himedia, India) with antibiotics such as chloramphinicol and gentamycin for 7 days, then identification of *A. fumigatus* was done macroscopically and microscopically followed by SEM (Joel-Japan) in Assiut University in Electron Microscope Unit.

Preparation of chitosan nanoparticles (CNP) and carvacrol nanoemulsion (CNE)

Chitosan nanoparticles was prepared by dissolving chitosan powder (Oxford) in 1% acetic acid and stirring for 60 min. Preparing of Sodium tripolyphosphate (STTP) by adding of 0.05 mg/mL in deionized water then dissolving. Under magnetic stirring and room temperature; 1ml of STTP was added drop by drop to 100 ml of chitosan solution. Then, adjust pH to 4.7 by sodium hydroxide. After 20 min of stirring, the solution was centrifuged at 10.000 rpm for 20 min. The precipitate was suspended in distilled water and centrifuged for removing of the residual sodium hydroxide (Hassanien and Shaker, 2020). While CNE was prepared by dissolving Tween 80 2v/v% in double distilled water and shaking for 10 min using magnetic stirrer to form a homogenous solution, then carvacrol oil was added slowly and mixed for 15 min (Moradi and Barati, 2019). The resulting curde emulsion was sonicated by ultrasonic homogenizer (Daihan). Size of both types of nanoparticles was measured by transmission electron microscope (TEM) in in Assiut University Electron Microscopy Unit.

Effect of CNP and CNE

Culture of *A. fumigatus* isolates in liquid potato dextrose medium at 25˚ C for 6 days, then 1×10^6 CFU/ ml were cultured on PDA containing the prepared nanoparticles and nanoemulsion with concentrations (0.5%, 1%, 2%, 5% and 7%) using well diffusion method (Rajeshkumar and Malarkodi, 2014),with 3 replicates, and 4mm of well diameter. The effect of nanoparticles was detected by growth of inhibition zone and SEM to identify the ultrastructure of fungi.

Statistical analysis

SPSS 14 software was used for detecting the effect of CNP and CNE. All values are presented as Mean ± standard error (SE).

Results

Feseakh and Moloha muscles harbor *A. fumigatus* with percentage of 14.7% and 18.7%, respectively (Table 1). TEM shows that the size of CNP is 65.7 nm and CNE is 52.8 nm (Fig. 1). CNP has no effect on the isolated strains at concentrations 0.5 %, 1% and 2%, and has inhibitory effect at concentrations 5% and 7% with mean inhibition zones 8±0.56 and 20±0.78 mm, respectively. CNE has inhibitory effect in concentrations of 0.5, 1 and 2%, with mean inhibition zones 9±0.41, 17±0.69 and 31.052 mm, respectively. CNE stops the fungal growth at concentrations 5% and 7%. Increased concentration of both nanomaterials significantly increased its effect (Table 2). SEM detected the normal structure of *A. fumigatus* and the degenerative effect of CNP and CNE on the ultrastructure of the fungus (Fig. 2).

Table 1: Incidence of *A. fumigatus* in salted fish

Fungal species/Salted fish	Feseakh (n=75)		Moloha (n=75)	
A. fumigatus	**No**	**%**	**No**	**%**
	11	14.7	14	18.7

Table 2: Effect of CNP and CNE on *A. fumigates* isolated from salted fish

Concentrations (%)	Inhibition zone (mm)		
	CNP	CNE	*P* value
	Mean ± SE	Mean ± SE	
0.5 %	Nil	9±0.41	0.05
1 %	Nil	17±0.69	
2 %	Nil	31±0.52	
5 %	8±0.56	No growth	
7 %	20±0.78	No growth	

Mean ± SE: mean± standard error
Nil: no zone present

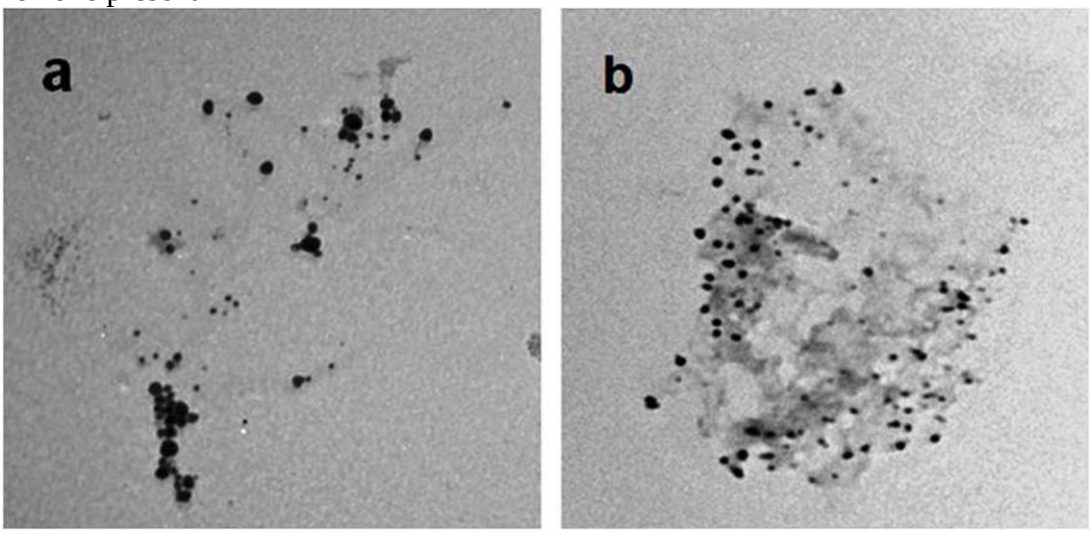

Fig. 1. Transmission electron microscope (TEM) showing (a) Chitosan nanoparticles (CNP) with average size 65.7 nm. (b) Carvacrol nanoemulsion (CNE) with average size 52.8 nm

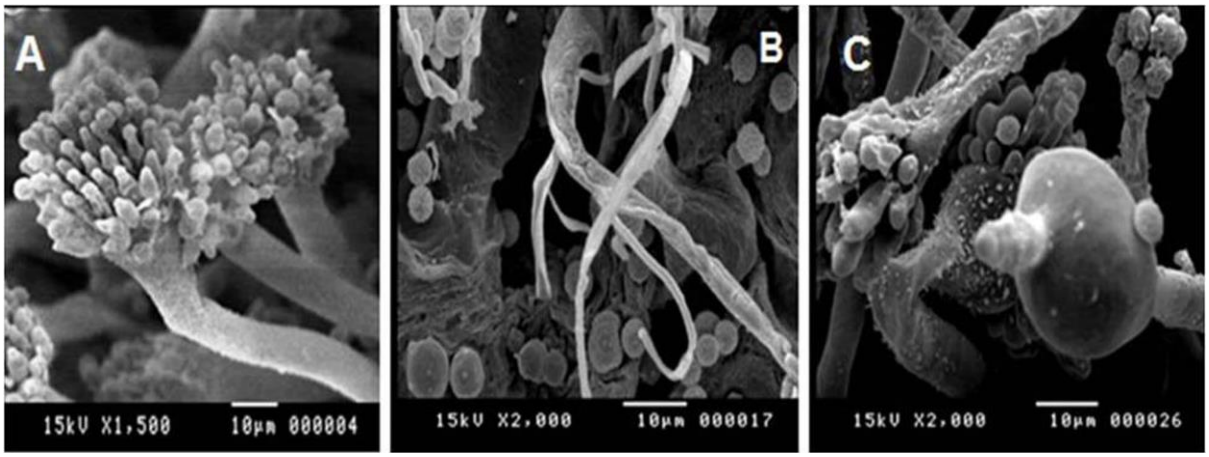

Fig. 2. Scanning electron microscope (SEM) for (A) *A. fumigatus* without treatment, (B) effect of Chitosan nanoparticles (CNP) at 5% on *A. fumigatus* and (C) effect of Carvacrol nanoemulsion (CNE) at 2% on *A. fumigate*.

Discussion

The present findings in Table 1 show that *A. fumigatus* was identified in 11.8% and 14.8% of Feseakh and Moloha, respectively. Lower results were reported by Youssef et al., 2003. Presence of *A. fumigatus* in salted fish may be resulted from long storage in humid atmosphere

which enables the fungal growth, insufficient time of salting, using low quality or raw materials, salting fish in unhygienic conditions obtained by workers, and using contaminated equipment and utensils (Susanti et al., 2019). The risk of *A. fumigatus* existence in fish products lies in production of mycotoxins which has hepatotoxic and carcinogenic effect causing human health risks (Pietsch, 2019). Also, it lowers the quality of food and causes food spoilage (Snyder et al., 2019).

Two natural nanomaterials were used against the isolated *A. fumigates* strains from Feseakh and Moloha with average size 65.7 and 52.8 nm for CNP and CNE, respectively (Fig. 1). CNP shows resistant effect on *A. fumigatus* growth at concentrations 0.5 %, 1% and 2%, while concentrations 5% and 7% inhibit its growth with mean inhibition zones 8±0.56 and 20±0.78, respectively. Different results were reported by CNE which have an inhibitory effect at concentration 0.5 %, 1% and 2%, and complete stop of the fungal growth at concentrations 5% and 7%. The inhibitory effect of both CNP and CNE was increased with high concentrations (Table 2). Similar results were reported by Shaker et al., 2021, who mentioned that CNP inhibit *A. fumigatus* growth. These results spotlight on using these nanomaterials in food industry as a natural antifungal agent to keep the food safe for human consumption during long storage.

The inhibition mechanisms of CNPs are firstly, the cell death which occurs by the combination of the positive charge of CNP with the negatively charged phospholipid component of the fungal membrane causes leakage of the cellular content. Secondly, it makes the important nutrients unfit as it binds to the trace elements which hinders the fungal growth. Thirdly, it binds to the fungal DNA and prevents mRNA synthesis. Therefore, it is considered a powerful and natural antifungal agent (Lo et al., 2020).

The volatile compounds have antimicrobial activity because they can invade the cell membrane and disrupt the intracellular mechanism, also their lipophilicity increase the membrane permeability, disrupt membrane proteins, inhibit respiration, induce ions leakage and alter the process of ion transportation in the fungal cells (Zhang et al., 2019).

SEM described the difference between the untreated *A. fumigatus* structure and the treated isolates with CNP and CNE (Fig. 2). *A. fumigatus* without treatment had normal, strong and regular hyphae and attached conidia. After treatment with CNP and CNE, the conidia were detached and fallout with deformed, shrunken and distorted hyphea. These observations explained the fungicidal activity of CNP and CNE. The changes in the fungus morphology due to the CNP and CNE effect will interfere with the synthesis of the cell wall causing inhibition of the fungus growth (Hassanien et al., 2021). From these results, CNP and CNE can be used as a safe food preservative and /or food packaging to protect food from spoilage especially with their natural source that don't harm the human health (Cheba, 2020, Sharifi-Rad et al.,2021).

Conclusion

A. fumigatus was detected in two types of salted fish as Feseakh and Moloha. CNP and CNE have antifungal activity through inhibition and/or stop of the fungal growth and effect on the ultrastructure of fungi. Additional researches are needed for the application methods and effective doses of CNP and CNE in food industry.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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