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The impact of silver and chitosan nanoparticles on *Aspergillus fumigatus* isolates from Ras cheese and sputum of respiratory patients

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Abstract

This work was designed to study the incidence of *Aspergillus fumigatus* (*A. fumigatus*) in Ras cheese sold in different markets at Sohag city, Egypt and also in sputum samples of respiratory diseases patients admitted to Sohag University Hospital, as well as study the effect of silver (SNPs) and chitosan (CNPs) nanoparticles against the *A. fumigatus* isolates. Potato dextrose agar (PDA) was used for mycological culture of samples and the antifungal effect of nanoparticles (with different concentrations) using well diffusion method. High resolution transmission electron microscope (HRTEM) was used for detection of nanoparticles size, and scanning electron microscope (SEM) was used for investigation of nanoparticles effect on *A.fumigatus* morphology. The isolation rate of *A. fumigatus* was 22.11 % (21/95), 32.17 % (37/115) from Ras cheese and sputum samples, respectively. SNPs had inhibitory effect at concentrations 1 and 2 µg/ml, and complete growth inhibition with concentrations 4, 8, and 16 µg/ ml. While CNPs had no effect in concentrations 1 and 2 µg/ ml, but at concentrations 4, 8 and 16 µg/ ml represented inhibitory effect with mean inhibition zones 5±0.21, 9±0.41 mm and 11 ±0.77mm, respectively. SNPs and CNPs by SEM patently damaged the *A. fumigatus* cell structure. Using nanoparticles against *A. fumigatus* from food and human sources is important to stop food spoilage and protect human health. So, further studies are necessary for the investigation of suitable application methods, and the expected hazards which may occur after nanoparticles usage.

Keywords: Ras cheese, *A. fumigatus*, respiratory patients, silver nanoparticles, chitosan nanoparticles, scanning electron microscope.

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INTRODUCTION

Aspergillus species resulted in severe public health hazards, either production of toxins in food, or a pathogen releasing spores in outdoor environment (soil, air, animal feed, plant, water, and decayed vegetation) and indoor environment (Bennett, 2010; Mead et al., 2019). It causes a wide range of infections as fungus ball, allergy, keratitis, skin infection, osteomyelitis, and endocarditis (Muñoz et al., 2006; Lamoth, 2016)

The presence of fungi in food leads to its deterioration and responsible for severe health hazards to Humans (Mc Sweeney and Sousa, 2000; Vivek-Ananth et al., 2018; Sass et al., 2019). Ras cheese is a hard cheese produced by artisan workers using raw milk without addition of starter; it is stored for up to six months to be sharp in flavor (Elramly et al., 2019). Fungal contamination of Ras cheese may occur during manufacturing, storage, marketing, upon using raw materials and environmental contamination (Delavenne et al., 2011).

Fungal infection was increased in recent years especially those resulted in invasive infection as *A. fumigatus* especially in immune-compromised patients (Kami et al., 2000). Antifungal drugs are degraded in blood and not directed to the infected site, which lowers its viability. So, using nanoparticles as drug delivery system may upgrade its effect (Barnard, 2016). Nanotechnology is a scientific field generating new materials at nanoscale called nanomaterials which applied in different areas as water treatment, medicine, solar energy and provide solutions to environmental and technological challenges (Dahl et al., 2007; Saifuddin et al., 2009; Elsherif and Ali, 2019). From several metal nanomaterials (zinc, copper, magnesium, and platinum); SNPs have special attention as one of the most effective antimicrobial agents with wide spectrum activity and nontoxic effect to human in low concentration (Gajbhiye et al., 2009; Rai et al., 2009; Xia et al., 2016; Elsherif et al., 2020). Also, the organic CNPs which were derived from chitin are more desirable as they are non-toxic, biodegradable with massive antimicrobial activity and widely used in different fields (Cheung et al. 2015). Therefore, owing to their antimicrobial effect, using nanoparticles in nano medicine and agriculture is important and need several researches for suitable application. This study detects *A. fumigatus* in Ras cheese and sputum samples and describes the effect of SNPs and CNPs on *A. fumigatus* using well diffusion method and SEM.

MATERIALS AND METHODS

Samples collection and mycological examination

From September 2019 through February 2020; 95 of Ras cheese samples were purchased from dairy markets and supermarkets in different localities in Sohag city, Egypt and 115 sputum samples were collected from patients admitted to Sohag University Hospital with respiratory diseases as chronic bronchitis, chronic obstructive pulmonary disease (COPD), asthma and pneumonia in sterile cups. All samples were sent to the laboratory for mycological examination. Samples were cultured on PDA with antibiotics for seven days at 25˚C (Kim *et al.,* 2012). Fungal identification was done macroscopically, microscopically, and then confirmed by SEM (Joel-Japan) in Electron Microscope Unit in Assiut University (Wang *et al.,* 2015).

Silver and chitosan nanoparticles preparation

SNPs were synthesized as reported by Ranoszek-Soliwoda *et al.,* (2017) using silver nitrate crystal (Sigma Aldrich, USA) and sodium citrate (Oxoid) with molar ratio of 1:7. Under reflux; heating of silver nitrate solution to boiling and then added sodium citrate, followed by further heating for 15 min then cooled. While CNPs were prepared by dissolving chitosan (Oxford, India) in 0.05 mg/ml acetic acid and stirred for 24 hours, then adjusted pH at 5.5 and diluted to the required concentrations using deionized water. TTP (Sodium Tripolyphosphate) was prepared by dissolving 0.25 mg/ml in deionized water, then added to chitosan solution drop by drop (0.03 ml/ min) under vigorous magnetic stirring and left 30 min to jellified (Calvo *et al.,* 1997). The size of SNPs and CNPs was measured by HRTEM (JEOL-Japan) in the Electron Microscopy Unit, Assiut University, Egypt.

SNPs and CNPs effect

All *A. fumigatus* isolates from Ras cheese and sputum samples were cultured in potato dextrose liquid (PDL) medium at 28ºC for 6 days, then 1×10^6 cfu/ ml were grown on PDA containing 100 µl of different concentrations of previously prepared nanoparticles (1, 2, 4, 6, 8, and 16 µg/ml) on each well and incubated at 28ºC for 6 days. Nanoparticles-free PDA plates were used as control. The antifungal effect was detected by well diffusion technique and the sizes of the inhibition zones were measured (NCCLS, 2002 and Klerk *et al.*, 2016). Also, SEM was used for identification of nanoparticles effect on fungal ultrastructure.

Statistical analysis

Mean and standard error was described by SPSS 18 for evaluation of the effect of CNPs and SNPs. Least significant differences were used at $P < 0.05$.

Ethical approval

Ethical approval was obtained from Sohag University ethical committee. Patients' participation was optional, and collection of samples was done after their consent.

RESULTS

A. fumigatus was detected in 21 (22.11%) out of 95 Ras cheese samples and 37 (32. 17%) out of 115 sputum samples (Table 1).

Table 1. Incidence of *A. fumigatus* in Ras cheese and sputum of respiratory patients

Fungal species	Ras cheese n=95		Respiratory patients n=115	
A.fumigatus	**No**	**%**	**No**	**%**
	21	22.11	37	32.17

SNPs had inhibitory effect on *A. fumigatus* in concentrations 1 µg/ml and 2 µg/ ml, with mean inhibition zone 10±0.62 mm and 28±0.87 mm respectively. Complete inhibition (no fungal growth) was detected in concentrations of 4 µg/ml, 8 µg/ ml and 16 µg/ ml.

Table 2. Inhibitory effect of SNPs and CNPs on *A. fumigates* isolates from Ras cheese and sputum samples

Concentration µg/ml	Inhibition zone by mm		P value
	SNPs	CNPs	
	Mean ± SdE	Mean ± SdE	
1 µg/ml	10±0.62	NZ*	0.05
2 µg/ml	28±0.87	NZ*	
4 µg/ml	No growth	5±0.21	
8 µg/ml	No growth	9±0.41	
16 µg/ml	No growth	11±0.77	

Mean ± SdE: mean± standard error
*NZ: no zone present

While CNPs did not affect the fungal growth with concentrations 1 µg/ml and 2 µg/ ml, and their inhibitory effect was reported in concentrations 4 µg/ml with mean inhibition zone 5±0.21 mm, 8 µg/ ml with mean inhibition zone 9±0.41 mm and 16 µg/ml with mean inhibition zone 11±0.77 mm. Increased concentration for SNPs and CNPs significantly increased its antifungal effect (P value< 0.05) (Table 2). SEM described the damage of *A. fumigatus* ultrastructure due to the effect of SNPs and CNPs (Figure 1).

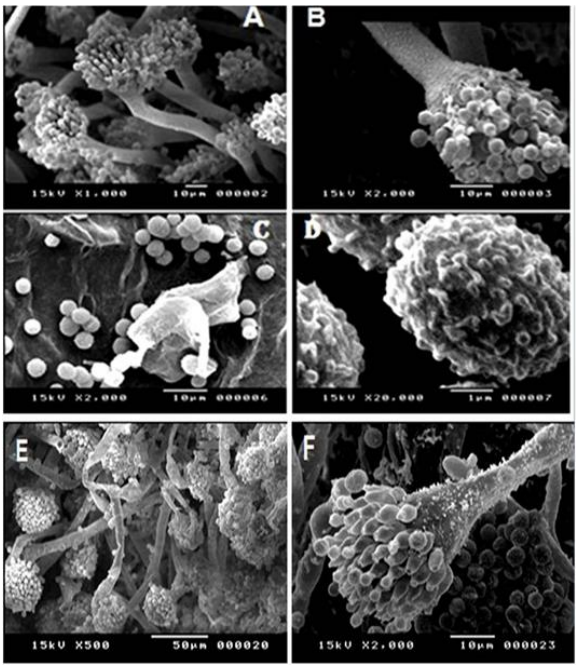

Figure 1. Showing SEM for untreated *A. fumigatus* (A and B); effect of SNPs at 4 µg/ml on *A. fumigatus* (C and D) and effect of CNPs at 4 µg/ml on *A. fumigatus* (E and F).

DISCUSSION

A. fumigatus is associated with food spoilage, human respiratory diseases, invasive infection and mycotoxicosis. Its source diversity required several researches to find safe, effectiveand readily applied antifungal substances.

The mycological examination of Ras cheese samples detected *A. fumigatus* in a percentage of 22.11% (Table 1), this result is higher than that mentioned by Elramly *et al.,* (2019), and nearly similar to that reported by Seddek *et al.,* (2016). The presence of *A. fumigatus* in Ras cheese may be related mostly to the long storage period in wood shelves, which lasts for several months for pungent flavor and texture development. Sprinkling of cheese with salt allows the water drip to infiltrate from cheese blocks into the wood shelves which forms a suitable humid media for growth of diversity of microorganisms (Elramly *et al.,* 2019). As Ras cheese is widely consumed by the Egyptian people especially at breakfast and dinner, its microbial load possesses a risk for human health.

A. fumigatus was detected in 32.17% of respiratory patient (Table 1), this is nearly similar to that obtained by Bafadhel *et al.,* (2014), and higher than Everaerts *et al.,* (2018), and lower than that mentioned by Shahi *et al.,* (2015). *A. fumigatus* is more frequent in COPD, asthma and bronchiectasis patients (Everaerts *et al.,* 2017). Fungal colonization has a major role in chronic respiratory disease exacerbation especially in immune-compromised patients, unlike healthy people in which bronchial epithelial cells and macrophage in lower and upper respiratory airways hinder its mechanism (Fukuda *et al.,* 2018). Due to the eukaryotic nature of fungi, it is difficult to develop effective antifungal drugs. Also, raise of antifungal resistance against the existing drugs urgently need new antifungal agents (Seneviratne and Rosa, 2016).

Impact of nanoparticles

SNPs and CNPs were applied in concentrations 1, 2, 4, 8, and 16 µg /ml with an average size 18.3 nm and 17.9 nm, respectively (Figure 2).

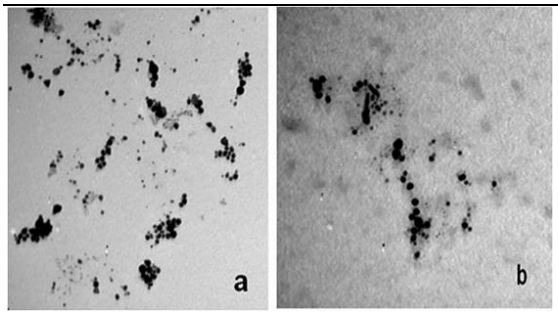

Figure 2. showing (a) SNPs with average size 18.3 nm , (b) CNPs with average size 17.9nm.

SNPs represented inhibitory effect on *A. fumigatus* isolates at concentrations of 1 and 2 µg/ ml, unlike CNPs in which no zone of inhibition was detected in the same concentrations. However, increased concentration of both types of nanoparticles stopped the fungal growth completely in case of SNPs, and inhibited the growth with CNPs at concentrations 4, 8, and 16 µg/ ml (Table 2). Increased concentration of SNPs resulted in its saturation which allowed it to attach the fungal hyphae and destroy it by stopping of DNA replication and inactivation of ribosomal protein and enzymes which needed for ATP production (Ranoszek-Soliwoda *et al.,* 2017)

CNPs have three inhibition mechanisms; firstly, its positive charge combines with phospholipid component in fungal membrane, which is negatively charging, resulting in increased membrane permeability, cellular content leakage and cell death. Secondly, due to the chelating action of chitosan, it binds to the trace elements which make the necessary nutrients unfit for fungal growth. Thirdly, penetration of fungal cell wall by chitosan and binding to DNA causing stop mRNA synthesis and essential enzymes and proteins production. Therefore, is considered a safe, natural and powerful antifungal agent (Ing *et al.*, 2012).

SEM describes the *A. fumigatus* morphology surface and assesses the effect of SNPs and CNPs (Figure 1). The untreated fungal hyphae appear strong and vigorously grew with attached conidia. Treatment with SNPs caused severe damage of fungal hyphae and fallout of conidia. Also, the fungal conidia were shrinked and deformed. While fungal hyphae with CNPs appears distorted and fractured with weak conidia. Therefore, SNPs can be applied as a probable antifungal agent (Xia *et al.,* 2016) with attention to SNPs cytotoxicity to humans (Foldbjerg *et al.,* 2011; Elsherif *et al.,* 2020). However, SNPs at low concentrations have no cytotoxic effect on human cells, but increased concentration or doses may be cytotoxic (Borase *et al.,* 2013; Pauksch *et al.,* 2014). Further researches related to application of nanoparticles in different fields as food technology and human medicine is important to make them applicable without adverse effect.

CONCLUSION

SNPs and CNPs demonstrated an excellent antifungal activity against *A. fumigatus*. However, the CNPs at higher concentrations give complete inhibition for fungal growth but as a natural nanoparticle considered safer than metal nanoparticles (SNPs). So, the toxicity of SNPs to human and animals require additional evaluation to be viable for various medical fields and antimicrobial systems.

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CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest.

REFERENCES

Bafadhel M, Mckenna S, Agbetile J, Fairs A, Desai D, Mistry V, Morley J, Pancholi M, Pavord I, Wardlaw A, Pashley C and Brightling, C (2014). *Aspergillus fumigatus* during stable state and exacerbations of COPD. Euro Respir J, 43: 64-71.

Barnard A S (2016). Challenges in modelling nanoparticles for drug delivery. J Phys Condens Matter, 28 (2): 023002.

Bennett JW (2010). An overview of the genus *Aspergillus*. In: Machida M, Gomi K, editors. *Aspergillus* molecular biology and genomics. UK: Caister Academic Press, 1-17.

Borase HP, Patil CD, Sauter IP, Rott MB and Patil SV (2013). Amoebicidal activity of phytosynthesized silver nanoparticles and their in vitro cytotoxicity to human cells. FEMS Microbiol Lett, 345:127e31.

Calvo P, Remunan-Lopez R, Vila-Jato CJL and Alonso MJ (1997). Chitosan and chi-tosan/ethylene oxide–propylene oxide block copolymer nanoparticles as novel carriers for proteins and vaccines. Pharm Res, 14: 1431–1436.

Cheung RCF, Ng TB, Wong JH and Chan WY (2015). Chitosan: An update on potential biomedical and pharmaceutical applications. Marine Drugs, 13: 5156-5186.

Dahl JA, Maddux BL and Hutchison JM (2007). Toward greener nanosynthesis. Chem Rev,107: 2228−2269.

Delavenne E, Mounier J, Asmani K, Jany JL, Barbier G and Le Blay G (2011). Fungal diversity in cow, goat and ewe milk. Int J Food Microbiol, 151: 247–51.

Elramly MH, El-Leboudy AA and Al-Ansary M (2019). Mycological evaluation of Egyptian Ras cheese with special reference to mycotoxins. Alexandria Journal of Veterinary Sciences, 63 (2): 33-38.

Elsherif MW and Ali DN (2019). Antibacterial effect of silver nanoparticles on antibiotic resistant *E. coli o157:H7* isolated from some dairy products. Bulg J Vet Med. Available: http://tru.uni-sz.bg/bjvm/bjvm-2019-0027%20OnFirst.pdf

Elsherif MW, El Hendy AHM, Elnisr NA and Zakaria MI (2020). Ameliorative effect of zeolite packaging on shelf life of milk. J Pack Tech Res. Available: https://doi.org/10.1007/s41783-020-00093-x

Everaerts S, Lagrou K, Dubbeldam A, Lorent N, Vermeersch K, Hoeyveld E, Bossuyt X, Dupont L, Vanaudenaerde B and Janssens W (2017). Sensitization to *Aspergillus fumigatus* as a risk factor for bronchiectasis in COPD. Int J COPD, 12: 2629–2638.

Everaerts S, Lagrou K, Vermeersch K, Dupont LJ, Vanaudenaerde BM and Janssens W (2018). *Aspergillus fumigatus* detection and risk factors

in patients with COPD–bronchiectasis overlap. Int J Mol Sci, 19: 523.

Foldbjerg R, Dang DA and Autrup H (2011). Cytotoxicity and genotoxicity of silver nanoparticles in the human lung cancer cell line, A549. Arch Toxicol, 85:743e50.

Fukuda Y, Homma T, Suzuki S, Takuma T, Tanaka A, Yokoe T, Ohnishi T, Niki Y and Sagara H (2018). High burden of *Aspergillus fumigatus* infection among chronic respiratory diseases. Chronic Respiratory Disease, 15(3): 279–285.

Gajbhiye M, Kesharwani J, Ingle A, Gade A and Rai M (2009). Fungus-mediated synthesis of silver nanoparticles and their activity against pathogenic fungi in combination with fluconazole. Nanomedicine, 5: 382-6.

Ing LY, Zin NM, Sarwar A and Katas H (2012). Antifungal activity of chitosan nanoparticles and correlation with their physical properties. Int J Biomater, 2012: 1-9.

Kami M, Fukui T, Ogawa S, Kazuyama Y, Machida U, Tanaka Y, Kanda Y, Kashima T, Yamazaki Y, Hamaki T, Mori S, Akiyama H, Stevens DA, Kan VL, Judson MA, Morrison VA, Dummer S, Denning DW, Bennett JE, Walsh TJ, Patterson TF and Pankey GA (2000). Practice guidelines for diseases caused by *Aspergillus.* Infect Dis Soc Am Clin Infect Dis, 30: 696-709.

Kim S, Jung J, Lamsal K, Kim Y, Min J and Lee Y (2012). Antifungal effects of silver nanoparticles (AgNPs) against various plant pathogenic fungi. Mycobiology, 40(1): 53-58.

Klerk C, Fosso-Kankeu E and Waanders F (2016). Antifungal activities of chitosan and nanoparticle derivatives under various pH conditions. Inter Conference on Adv Sci Eng Technol and Natural Res. (ICASETNR-16) Nov: 24-25.

Lamoth F (2016). *Aspergillus fumigatus*-related species in clinical practice. Front Microbiol, 7: 683.

Mc Sweene PLH and Sousa MJ (2000). Biochemical path-ways for the production of flavor compounds in cheese during ripening. Le Lait, 80 (3): 293 – 324.

Mead ME, Knowles SL, Raja HA, Beattie SR, Kowalski CH, Jacob LS, Lilian PS, Jessica C, Laure NA, Gustavo HG, Robert AC, Nicholas HO and Antonis R (2019). Characterizing the pathogenic, genomic, and chemical traits of *Aspergillus fischeri*, a close relative of the major human fungal pathogen *Aspergillus fumigatus*. J Ecological and Evolutionary Science, 4 (1): 1-18.

Muñoz P, Guinea J and Bouza E (2006). Update on invasive aspergillosis: clinical and diagnostic aspects. Clin Microbiol Infect, 12 (7): 24-39.

NCCLS "National Committee for Clinical Laboratory Standards" (2002). Reference method for broth

dilution antifungal susceptibility testing of yeasts. Approved standard M27eA2. Wayne, PA: Clinical and Laboratory Standards Institute.

Pauksch L, Hartmann S, Rohnke M, Szalay G, Alt V, Schnettler R and Lips K (2014). Biocompatibility of silver nanoparticles and silver ions in primary human mesenchymal stem cells and osteoblasts. Acta Biomater, 10(1): 439-449.

Rai M, Yadav A and Gade A (2009). Silver nanoparticles as a new generation of antimicrobials. Biotechnol Adv, 27: 76-83.

Ranoszek-Soliwoda K, Tomaszewska E, Socha E and Krzyczmonik P (2017). The role of tannic acid and sodium citrate in the synthesis of silver nanoparticles. J Nanopart Res,19: 273.

Saifuddin N, Vong CW and Nur Yasumira AA (2009). Rapid biosynthesis of silver nanoparticles using culture supernatant of bacteria with microwave irradiation. E-Journal of Chemistry, 6(1): 61-70.

Sass G, Ansari SR, Dietl AM, De´ziel E, Haas H and Stevens DA (2019). Intermicrobial interaction: *Aspergillus fumigatus* siderophores protect against competition by Pseudomonas aeruginosa. PLoS ONE, 14 (5): e0216085.

Seddek NH, Gomah NH and Osman DM (2016). Fungal flora contaminating Egyptian Ras Cheese with reference to their toxins and enzymes. Food Science and Technology, 4(4): 64-68.

Seneviratne CJ and Rosa EAR (2016). Editorial: antifungal drug discovery: new theories and new therapies. Front Microbiol, 7: 728.

Shahi M, Mousavi SA, Nabili M, Aliyali M, Khodavaisy S and Badali H (2015). *Aspergillus* colonization in patients with chronic obstructive pulmonary disease. Curr Med Mycol, 1(3): 45–51.

Vivek-Ananth RP, Mohanraj K, Vandanashree M, Jhingran A, James PC and Areejit S (2018). Comparative systems analysis of the secretome of the opportunistic pathogen *Aspergillus fumigatus* and other *Aspergillus species*. Scientific Reports, 8: 6617.

Wang S, Lian Z, Wang L, Yang X and Liu Y (2015). Preliminary investigations on a polygalacturonase from *Aspergillus fumigatus* in Chinese Pu'er tea fermentation. Bioresources and Bioprocessing, (2015) 2:33.

Xia ZK, Ma QH, Li SY, Zhang DQ, Lin C, Tian YL and Yang RY (2016). The antifungal effect of silver nanoparticles on *Trichosporon asahii*. J Microbiology, Immunology and Infection, 49: 182-188.