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DEVELOPMENT OF ANTICANDIDAL DRESSINGS TREATED WITH FUNGAL CHITOSAN/ZNO NANOCONJUGATES

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Abstract:
The potentiality of employing nanotechnology to overcome pathogenic yeast infections was investigated. Chitosan was achieved from grown mycelial biomass of Aspergillus niger, characterized and transformed to its nano-form to have fungal chitosan nanoparticles (NCT), which was further conjugated with ZnO nanoparticles (Zn-NPs) and these individual compounds were loaded onto cotton textiles and evaluated as anticandidal agents against Candida albicans and C. parapsilosis. Fungal chitosan had a deacetylation degree of 89.1 % and a 90.18 KDa molecular weight. The FTIR analysis of composited Zn/NCT indicated strong cross linkage between their particles. The Zn/NCT NPs had a mean particles' size of 136 nm with hemispherical figure and well distribution of NPs. Both Zn-NPs, NCT and Zn/NCT nanocomposites exhibited potent anticandidal efficacy against pathogenic yeast strains, either in emulsion form or after loading onto cotton fabrics. The scanning micrographs of exposed Candida strains to Zn/NCT nanocomposites indicated complete lyses of yeast cells after 12 h of exposure. The synthesis of fungal NCT and its combination with Zn-NPs and loading them onto textiles is extremely recommended for generating bioactive anticandidal textiles.
***Key words**: Antimicrobial; Candida sp.; Characterization; Health care; Nanotechnology*

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1- INTRODUCTION:

Candida albicans is the most common etiological agent for Candidiasis and has the capability to cause most of clinical mycoses, it could be located on human skin and mucous membranes such as the mouth, rectum or vagina. It is usually a superficial but inconvenient infection may occur in intertriginous areas, nails and adjacent tissues (Tayel et al., 2013). This fungus can also transfer through the blood stream and distress the throat, intestines, bronchopulmonary system, kidney and heart valves. Disseminated candidiasis could be sometimes a serious disease, which repeatedly resulted in death (Shokohi et al., 2014).

Immune-compromised patients greatly suffered from various fungal pathogens attack; *C. albicans* was recorded in the majority of their systemic infections with 50% to 100% mortality rates. Among the systemic mycoses and fungal opportunistic infections' causal agents, *C. albicans* pose serious problems for the physicians who are limited with a very short list of antifungal agents for treating it (Badiee and Hashemizadeh, 2014). These limitations arise mainly from the fact that both the host and fungal pathogen are eukaryotic, thus it is hard to have an antifungal agent with a selective toxicity to systemic mycoses because it also binds to similar targets of host cells.

Chitosan (Cts) is a derived biopolymer from chitin, which have numerous applications in environmental, nutritional, biotechnological and health care fields (Honarkar et al., 2009). The Cts antimicrobial and biomedical characteristics, including regenerative medicine, were discussed and confirmed from many studies (Shi et al., 2006; Hein et al., 2008).

The antimicrobial activity of chitosan has been studied both in terms of bacteriostatic and bactericidal activity and has been demonstrated to inhibit the growth of a wide variety of bacteria. Moreover, chitosan has several advantages over other chemical disinfectants since it possesses a stronger antimicrobial activity, a broader spectrum of activity, a higher killing rate and a lower toxicity towards mammalian cells (Lim and Hudson, 2003; Rabea et al., 2003).

Nowadays, *Aspergillus niger* is almost exclusively used for industrial scale production of citric acid. More than 600,000 metric tons are produced annually worldwide (Anastassiadia et al., 2002). The fungal mycelia produced as byproducts of fermentation industries could be considered as a promising potential source for the isolation of chitin and /or chitosan.
Due to its biocompatibility, antimicrobial and intrinsic hemostatic properties, Cts was recommended as an attractive biomaterial for inclusion in wound care dressings (Ong et al., 2008).
Cts was recurrently applied, as a skin substitute biomaterial, in dermal tissue engineering because of its hemostasis properties, which support tissues regeneration and motivate the collagen fibroblasts synthesis (Ueno et al., 1999; Anitha et al., 2014).

The "smart" fabrics was suggested to overcome the potential dermal infections/harms; the main bases for biomaterials applications, eg. Cg and Cts, as wound dressings are their ideal characteristics to supply the skin interface with moist environment, function as microbial barriers, with gaseous exchange allowance and excess exudates removal (Tayel et al., 2011a; 2018). They should have also a non-allergenic, non-toxic and non-adherent attributes, and preferably made from a minimally-processed biomaterials that have wound healing and antimicrobial potentialities (Jayakumar et al., 2011).

Currently, metal oxide nanomaterials are among the most highly produced nanomaterials; their available applications include catalysis, sensors, environmental remediation, and personal care products (Kumar, 2006). Metal oxide nanomaterials have proven to be effective in treating hazardous substances such as chlorinated solvents, microbes, pesticides. ZnO and CuO nanomaterials are incorporated into a variety of coatings due to their antimicrobial and/or antifungal properties. ZnO nanomaterials have been added as antimicrobials to wallpaper for use in hospitals, TiO_2 nanomaterials inhibit cancer cell growth and iron oxide nanomaterials have the potential to be used as site-specific delivery carriers for drugs and diagnostic agents (Bennet and Schuurbiers, 2005; Gupta and Gupta, 2005).

Metal oxides such as ZnO have received increasing attention as antibacterial materials in recent years because of their stability under harsh processing conditions, and also because they are generally regarded as safe materials for human beings and animals (Stoimenov et al., 2002, Tayel et al., 2011b).
Cotton textiles have generally a poor resistance to microorganisms, thus antimicrobial finishing of cotton fabrics is an essential approach to prevent their harm to the human body (Seventekin and Ucarci, 1993). Many chemical compounds have been employed to impart antimicrobial activity to textile fabrics (Vigo,

1983, Tayel et al., 2013), most of these chemicals, however, are toxic to humans and could not easily be degraded in the environment. The textile industry continues to search for eco-friendly agents that substitute for toxic textile chemicals, in this viewpoint, chitosan could be proposed as an excellent candidate (Lim and Hudson, 2004).

This study was intended to extract of fungal Cts from *A. niger* mycelia and transforming it to nanoparticles and combining them with nano ZnO and loading them onto cotton textiles, then to evaluate them as anticandidal agents against pathogenic *Candida* sp.

2- MATERIALS AND METHODS:

2.1 Chitosan extraction from fungi

2.1.1. Fungal culture

Aspergillus niger ATCC 9642 stock culture was reactivated and cultivated on potato dextrose agar (PDA) plates and incubated at 25°C for 5 days.

2.1.3. Extraction and characterization of fungal chitosan

The harvested *A. niger* mycelia, by centrifugation at 8000 x *g* for 20 min, were washed with deionized water (DW), re-centrifuged and dried with forced hot air at 48 ºC for 36 h. Cts extraction was performed through immersion and homogenization of fungal biomass in 30 folds of 15 M NaOH, then heating at 95 ºC for 100 min. The insoluble materials were harvested by centrifugation (6000 x*g* for 15 min), repeatedly washed with DW and re-centrifuged until having a neutral pH. After that, harvests were immersed and agitated in 30 folds of 8 % (v/v) acetic acid solution, using a shaked water bath, at 60 ºC for 7 h, then acid insoluble materials discarded with centrifugation at 7000 x*g* for 20 min. The pH of supernatant was adjusted to 9.0 using 4 M NaOH, then centrifuged to separate precipitated chitosan, which was then respectively washed with DW, ethanol and acetone, and dried through 60 ºC forced air (Tayel et al., 2011a).

The Cts deacetylation degree (DD) was calculated from their plotted infra-red spectra using FTIR (FTS 45, Biorad, Germany), using Cts absorbance values at wavenumbers of 1655 and 3450 cm^{-1} (Niamsa and Baimark, 2009).

2.2. Zinc oxide (ZnO) suspension preparation

ZnO powder (~ 5 µm) and nano-scaled (≤ 50 nm) particle sizes (PS) were purchased from Sigma - Aldrich Co., St. Louis, MO, USA. Equal weights from ZnO powder and nanoparticles (8.1 g) were initially sterilized at 160C for 3 h then were dispersed in ultrapure water (Milli-Q®), vigorously vortexed for 10 min and additionally sonicated for 30 min to avoid aggregation and deposition of particles. The resulting suspensions (100 mL with concentration of 1 M) were considered as stock solution to be diluted and used for bacterial susceptibility evaluation.

2.3. Synthesis of nanochitosan and Zn /chitosan nano-composite

Sodium (penta) tripolyphosphate (STPP) was used as crosslinking agent for synthesizing of chitosan nanoparticles (NCT) through ionic gelation method (Gan and Wang, 2007).

Firstly, 100 mg of Cts were dissolved in 10 ml of diluted acetic acid (1% v/v) and continuously sonicated for 60 min. The pH was adjusted to 5.0 ± 0.2 of by NaOH (1 M), then a solution containing 100 mg of dissolved STPP in 10 ml DW were added to Cts dispersion and finely added drop wise (0.3 ml/min) and stirred for 60 min at 750 rpm, to form chitosan nanoparticles (NCT). Additionally, a dissolving solution containing 1% from STPP and Zn NPs was prepared and dropped into Cts to form Zn/NCT nanocomposite. The formed nanoparticles were precipitated by centrifugation at 14000 x *g* for 30 min, then lyophilized in presence of cryoprotectant (0.1% sucrose).

2.4. Nanoparticles characterization

FTIR Spectroscopy was operated to define potential Zn-NCT chemical interactions (Maya et al., 2012). Lyophilized powders from pure Cts and synthesized Zn /NCT nanocomposite, were analyzed using FTIR Spectroscopy (Shimadzu Co., Tokyo, Japan), with infrared spectra in range of 450–4000 cm^{-1}. The zeta potential, of synthesized CFT/NCT nanocomposite, were also evaluated thru Malvern Zeta nano-sizer, USA. Transmitted electron microscopy (TEM; Hitachi H-800, Japan) was employed for Zn/NCT nanocomposite imaging for achieving the quantitative attributes of particle distribution, morphology and size (Kiruthika et al., 2015); droplets of 5 µl from Zn/NCT suspensions were put into a copper grid, coated with carbon, and allowed to dry before TEM analysis.

2.5. Treatment of cotton fabrics with nanopartic

Scoured and rinsed cotton (Style S/400, 106 g/m2 plain weave) were obtained from TESTEX, Germany. The pad-dry-cure method was applied according to **El-Shafei et al. (2008)**. The experimental conditions were adopted as follows: the cotton fabric pieces of 2 cm^2 were cut and immersed in the nanoparticles solutions (containing 1 % from Zn NPs, NCT or Zn/NCT

composite) at pH 6 ± 0.3, with continual stirring for 2h at 60°C, padded and then squeezed between two nips and dips to a wet pick up 100%. The cotton fabrics were dried at 80°C for 3 min, and then cured at 120°C for 10 min. At the end cotton fabrics were washed several times with water at 40°C, and finally dried at the surrounded laboratory conditions.

2.6. Anticandidal activity

2.6.1. Yeast strains

A standard pathogenic yeast strains, *Candida albicans* (ATCC-10231) and *Candida parapsilosis* (ATCC – 22019), were used in current investigation. Yeast strains were grown and maintained on Sabouraud Dextrose Agar (SDA, Merck, Darmstadt, Germany). Inoculums for the assays were prepared by diluting scraped cell mass in 0.85% NaCl solution and counting using Haemocytometer, cell number was then adjusted to 10^5 cell/ml using above-mentioned saline solution.

2.6.2. Yeast inhibition zone (ZOI) assay

For the disc diffusion, sterile discs of Whatman No.1 paper (6 mm diameter) were positioned onto the surface of inoculated SDA with yeast cells, then 20 µl from NCT, Zn NPs or Zn/NCT solutions (1 % each) were pipetted into the discs. Inoculated plates were incubated for 26 h at 37 °C, then appeared inhibition zones diameters were precisely measured in mm.

For the anticandidal fabrics, loaded textile pieces with nanoparticles were applied as illustrated above.

Triplicated experiments were conducted and the means of ZOI were calculated.

2.6.3. Morphological test of the bacterial cells

Scanning electron microscope (SEM; S-500, Hitachi, Tokyo, Japan) was used to examine morphological changes of *Candida* sp. cells before and after treatment with Zn/NCT nanocomposite (Marrie and Costerton, 1984). First, cells were fixed with a primary fixative buffer (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M Na-Cacodylate buffer, pH 7.35) for 30 min. The samples were then rinsed thrice with ultrapure water, followed by dehydration with a series of ethanol solutions (10%, 30%, 50%, 70%, 90% and 100%). The dehydrated samples were dried immediately by critical point dryer (Auto-Samdri-815 Automatic Critical Point Dryer; Tousimis, Rockville, MD, USA), followed by mounting onto SEM stubs and sputter-coated with gold/palladium using a cool-sputter coater (E5100 II, Polaron Instruments Inc., Hatfield, PA, USA) . Sections were then observed under SEM at 8 kV each hour after treatment. Captured areas were selected according to the alteration in the morphology of treated cells.

3- RESULTS:

The grown fungus *A. niger* mycelia was a good source for Cts production; the mycelial biomass dry weight was 7.42 g/L and the extracted Cts from them was 1.38 g with a yield of 18.6 %. The extracted fungal Cts had a DD of 89.1 % and its MW was recorded as 90.18 KDa.

The spectrophotometric infrared (FTIR) analysis of produced fungal Cts and NCT indicates that they have matching spectra with similar characteristic peaks. The FTIR spectrum of NCT is presented in **Fig (1-NCT)**. The specified absorbance beaks, at wavelengths of 1565 cm^{-1} and 1651 cm^{-1}, are corresponding to the N-H primary amine bending and to C=O secondary amide stretch, respectively.

The observed broad band around 3412 cm^{-1}, is ordinarily attributed to the (-H) intermolecular bands, whereas the (-OH) vibrations are observed from at 653 cm^{-1}, and the ($–CH_2$) vibrations are detectable from the peak at 1418 cm^{-1}. The (C-O) structural stretching, is indicated at 1027 cm^{-1} of the absorbance peaks.

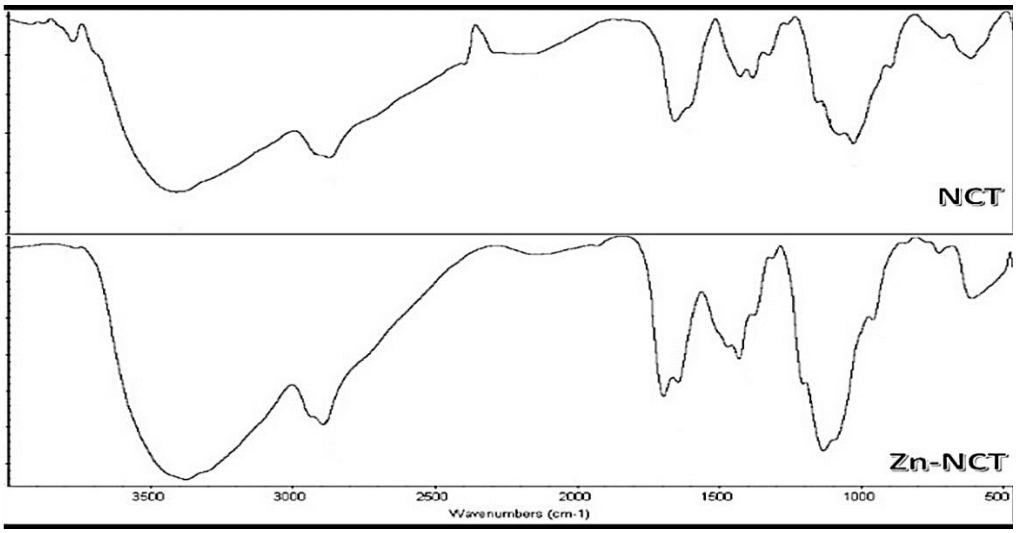

Fig 1: FTIR spectra of nano fungal chitosan alone (NCT) and after its crosslinking with ZnO nanoparticles (Zn-NCT).

The FTIR spectrum of Zn/CFT nanocomposite (**Fig 1- Zn/Cf)** indicated the appearance of characteristic peaks of Zn in addition to NCT. These indicative bands are observable at 1104 cm^{-1} (breathing mode of aromatic ring vibration), 1431 cm^{-1} (stretching C=N), 1556 cm^{-1} (aromatic ring), 1744 cm^{-1} (stretching COO), 2841 cm^{-1} (stretching C-H) and 3442 cm^{-1} (stretching N-H).

The micrographs of scanning microscope for the nano-composite of Zn/NCT indicated particle size range of 104-176 nm, with a mean particle size of 136 nm; the nanoparticles had a hemispherical shape with well particles' distribution (**Fig 2**).

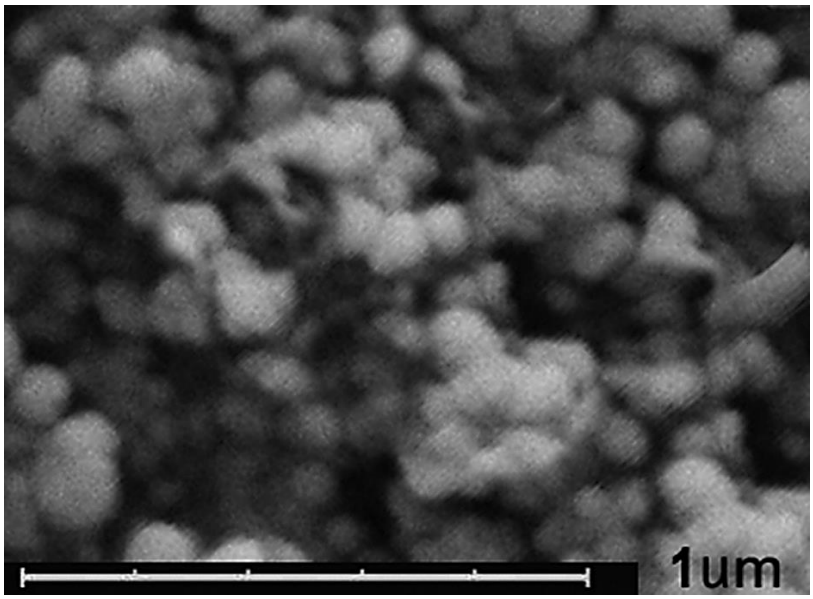

Fig 2: Scanning micrograph of synthesized nanocomposite from fungal chitosan and ZnO

The anticandidal activity of emulsions and treated dressings with ZnO, fungal chitosan (NCT) nanoparticles and their combination, against *C. albicans* and *C. parapsilosis*, was evidenced through the appeared ZOI in yeast growth (**Table 1**). All treated nanoparticles had powerful anticandidal activity toward pathogenic yeast strains; the individual NPs of Zn and NCT could inhibit yeast growth and the effect of their combination was much forceful (**Fig 2**). Generally, the yeast *C. parapsilosis* was more sensitive to the anticandidal activity of examined nanoparticles.

Table 1: Anticandidal activity of emulsions and treated dressings with ZnO, fungal chitosan (NCT) nanoparticles and their combination against *Candida albicans* and *Candida parapsilosis*

Treatment form	Agent Nanoparticle	Inhibition zone (mm) in pathogens growth*	
		Candida albicans	*Candida parapsilosis*
Nano-emulsion	ZnO	21.9 ± 1.1	23.5 ± 1.5
	NCT	24.1 ± 1.4	27.3 ± 1.6
	Zn/NCT	28.6 ± 1.5	31.6 ± 1.9
Cotton dressing	ZnO	36.3 ± 1.9	38.7 ± 2.3
	NCT	38.2 ± 1.9	39.2 ± 2.2
	Zn/NCT	46.4 ± 2.2	49.3 ± 2.4

*** Inhibition zones included 6 mm of assay disc for the nano-emulsions and 20 mm of textile pieces for cotton dressing**

The anticandidal activity of treated cotton dressing with ZnO nanoparticles (Z) and Zn/chitosan nanocomposite (ZC) against *Candida albicans* is shown in Fig.3.

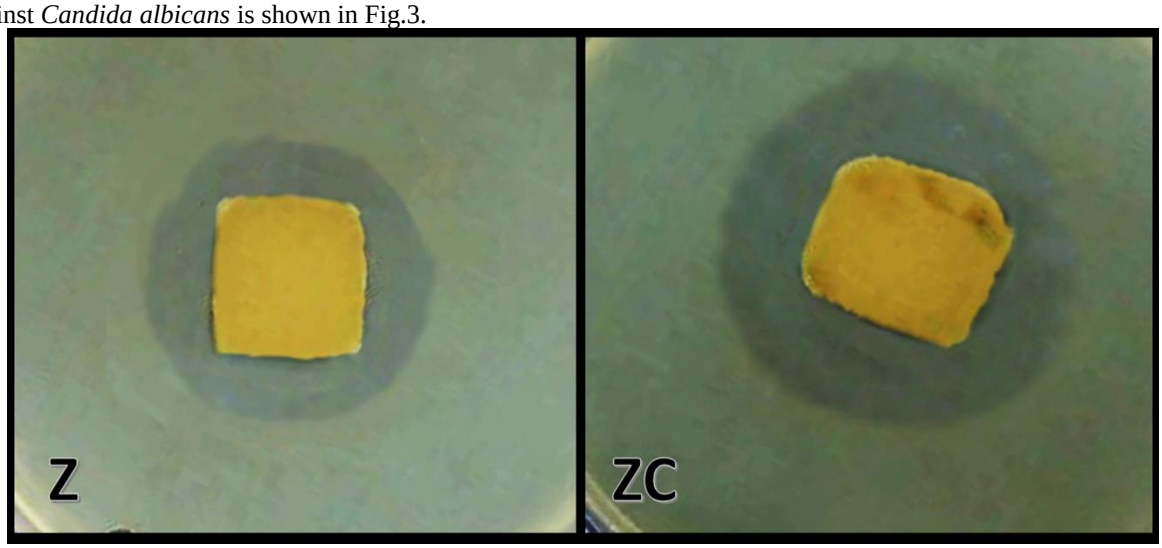

Fig 3: Anticandidal activity of treated cotton dressing with ZnO nanoparticles (Z) and Zn/chitosan nanocomposite (ZC) against *Candida albicans*

The anticandidal consequences of nano Zn/NCT treatment, on *Candida* sp. exposed cells, were tracked and proved via SEM imaging (**Fig 4**). The control (untreated) cells appeared with regular features, i.e. smooth, round, contacted cells with no attached nanoparticles. After 6 h from *Candida* strains exposure to Zn/NCT, irregular shapes in cell walls were appeared with a notable signs of lyses; nanoparticles could be detected at this phase adhering to the exposed cells. At the termination of exposure period, after 12 h, the *Candida* sp. cells were completely lysed, exploded and lost any confirmative shape for contact cells; the only observable matters in this phase are matrix from the intracellular components and cell wall residues combined with the nanoparticles.

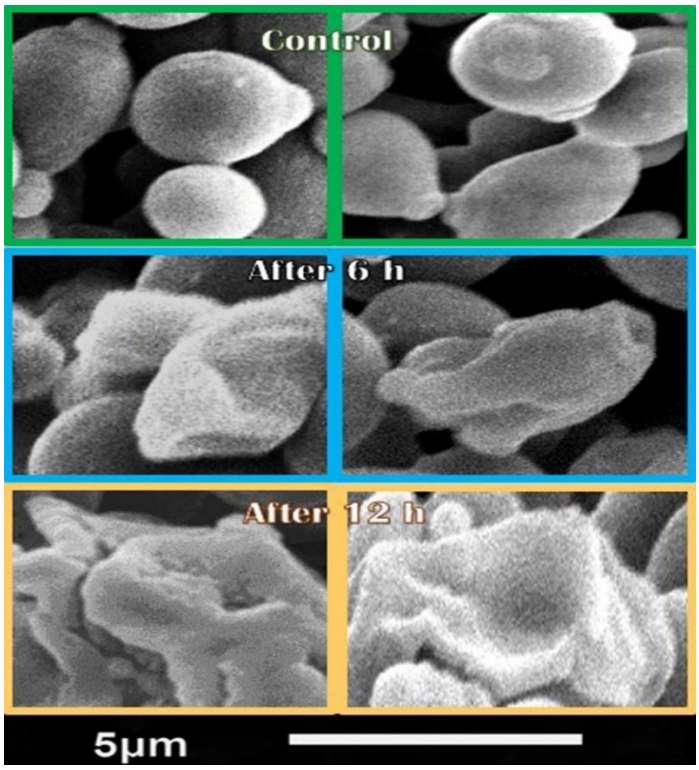

Fig 4: Scanning micrographs of treated *Candida albicans* (left column) and *Candida parapsilosis* (right column) with Zn/chitosan nanocomposite for 6 and 12 h compared to control untreated cells

4- DISCUSSION:

The FTIR spectra and biochemical bonds of fungal Cts are regularly correspondent to those of standard commercial Cts and this was recurrently reported (Tayel et al., 2011a). The FTIR analysis of fungal NCT and Zn/NCT composite indicated the strong cross-linkage between them, which was proven by the appearance of their combined characteristic beaks in their composite spectra (Fig 1 – Zn/NCT).

The characterization of Zn/NCT nano-composites indicates that the resulting nanoparticles from the applied procedure had a relatively small size (mean diameter = 136 nm) that increased their combined anticandidal activity. It was recommended for NCT delivery systems, to efficiently interact with cells, stimulate intracellular delivery, maximize cellular binding/internalization and colloidal stability, to have high zeta potential positivity and low particle size (Nasti et al., 2009), which was achieved in our synthesized NCT.

Metal oxide nanomaterials increased cell death with increasing concentrations, affected mitochondrial function, induced lactate dehydrogenase (LDH) leakage, and generated abnormal cell morphology at concentrations as low as 50-100 µg/L (Hussain, 2005; Jeng and Swanson, 2006). The generation of hydrogen peroxide presents another elucidation of the antibacterial activity of ZnO; H_2O_2 generates from the surface of ZnO is considering as an effective mean for the inhibition of bacterial growth (Yamamoto, 2001). It can be assumed that the concentration of H_2O_2 generated from the surface increases with decreasing particle size, because the number of ZnO powder particles per unit volume of powder slurry increases with particle size decreasing. Another possible mechanism for ZnO antibacterial activity is that the release of Zn^{2+} ions. It is well known that ZnO normally be unstable in the solution, and when H_2O_2 is produced the Zn^{2+} ions concentration is increased as a result of ZnO decomposition (Zhang et al., 2007).

The suggested Zn/NCT anticandidal actions could be the potentiality of NCT to attach bacterial cells and interact with their outer membranes, thus inhibiting cell wall synthesis and leading to cells' lyses and death (Sarwar et al., 2014; O'Callaghan and Kerry, 2016)

The formulated Zn/NCT nanocomposite was extremely superior in its anticandidal effect toward yeast strains; this apparently because of its enhanced cellular uptake via adsorptive endocytosis and to the successive release of entrapped/adsorbed nanoparticles. Endocytic uptake of drugs system could increase its effectiveness, which also influence membrane-impermeative of cell (Couvreur et al., 1991). Additionally, phagocytic cells/cytoplasmic

delivery targeting was supposed through nanoparticle technology (Keck and Muller, 2013). The augmentation of Zn/NCT anticandidal potentiality is seemingly attributed to the consequence of Cts, which is stated to act as absorption enhancers of drugs (Davis and Illum, 2003).

Some possible mechanisms could be proposed to explain this anticandidal activity of NCT. In one mechanism, the polycationic nature of chitosan based on the amino groups present in its molecules in addition to the positive charge from the cotton fabrics, both interact with the negatively charged microbial cell wall and lead to alteration of the cell wall permeability, and consequently, NCT molecules may interfere with the microbial metabolism and result in the death of cells (El-Shafei et al., 2008).

The impact of nanomaterials on living cells, including yeast, can also be elucidated by the interactions between the nanomaterial and the individual cell components. The first interaction between a material and a cell is at the membrane interface, some nanoparticles were suggested to embed themselves in the cell membrane (Jang et al, 2003).

Liu et al. (2009) indicated that ZnO NP may distort and damage bacterial cell membrane, resulting in a leakage of intracellular contents and eventually the death of microbial cells.

However, there is still a current lack of exact information regarding the interaction of NPs with the yeast cell wall and possible permeation of the NPs into the yeast cells (Jiang et al, 2009).
To the best of our knowledge, current study could be considered as the first to apply NCT from *A. niger* mycelia, and its nanocomposite with Zn NPs, as anticandidal textile finishing agent.

Zn/NCT showed a powerful anticandidal action against both yeast strains used, therefore, we can suggest, basing on the promising obtained results, the potential fabrication of Zn/NCT anticandidal dressings as hospital and surgery coats, hygienic diapers, wound dressings, medical bandages and bed covers.

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