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Antifungal activity of silver nanoparticles on some of fungi

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Abstract

In this study, we investigated the antifungal effects of silver nanoparticles (Ag-Nps) on *Candida albicans* (ATCC 5027), *Saccharomyces cerevisiae* (ATCC 5027). Investigating method by using Minimum Inhibitory Concentration (MIC) technique, some of drugs including Amphotericin B, Fluconazole and synthesized Ag-Nps have been obtained on the fungi and the changes on membrane reactions of yeasts have been elucidated by Scanning Electron Microscopy (SEM). The present study indicates Ag-Nps has considerable antifungal activity comparison with other antifungal drugs, so deserving further investigation for clinical applications.

Keywords: *Silvernanoparticles (Ag-Nps), Antifungal activity, Minimum Inhibitory Concentration (MIC).*

1. Introduction

Nowadays fungal infections resulted from opportunistic fungi have been common especially in people talented in being affected by special conditions like immune weakness , pregnancy and diseases like HIV and the candidates of affection to theses infections. Antifungal drug therapy is no exception; resistance too many of the antifungal agents now in use has emerged. Although antifungal drug resistance does not seem to be as much of a problem as resistance to antibacterial agents in bacteria, one long-term concern is that the number of fundamentally different types of antifungal agents that are available for treatment remains extremely limited. This is because fungi are eukaryotic organisms with a structure and metabolism that are similar to those of eukaryotic hosts. Therefore, there is an inevitable and urgent medical need for antibiotics with novel antimicrobial mechanisms [1, 2].

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In particular, because of the recent advances in research on metal nanoparticles, Ag-Nps have received special attention as a possible antimicrobial agent [1, 3-7, 16].It has been known that silver and its compounds have strong inhibitory and bactericidal effects as well as a broad spectrum of antimicrobial activities for bacteria, fungi, and virus since ancient times [1, 7-8]. Compared with other metals, silver exhibits higher toxicity to microorganisms while it exhibits lower toxicity to mammalian cells [9].Lately, the recent advances in researches on metal nanoparticles appear to revive the use of Ag-Nps for antimicrobial applications. It has been shown that Ag-Nps prepared with a variety of synthetic methods have effective antimicrobial activity [7-13].Hence, Ag-Nps have been applied to a wide range of healthcare products, such as burn dressings, scaffold, water purification systems, and medical devices [14, 15]. The toxic effects of silver on bacteria have been investigated for more than 60 years [16]. And the acting mechanism of silver has been known in some extent [17].Therefore, the preparation of uniform nanosized silver particles with specific requirements in terms of size, shape, and physical and chemical properties is of great interest in the formulation of new pharmaceutical products [18, 19].

2. Materials and methods

2.1. Materials

Amphotericin B powder, Fluconazole have been provided by sigma & Serva Company, dimethyl sulfoxide (DMSO) solution from Merck Company has been a transformer in all experiments, physiological serum, synthesized Ag-Nps was prepared using chemical reduction method.

2.2. Synthesis of Ag-Nps

Ag-Nps were synthesized by reduction of silver nitrate in the presence of PVP. The procedure is briefly described as follows: Firstly, 5 ml $NaBH_4$ was refluxed in a three-necked round-bottom flask at 80 °C for 2h, then 5 ml $NaBH_4$ solution of 0.02 M silver nitrate and 5 ml $NaBH_4$ solution of 0.05mM PVP were simultaneously injected dropwise. When the first drops of silver nitrate and PVP/$NaBH_4$ solutions were added, the mixture turned yellow immediately. Continuing the injection, the solution became opaque gradually. By finishing the injection, the solution turned turbid with a grey color in about 15 min indicating the appearance of Ag-NPs. The reaction was continued at 80 °C for 24 h. After finishing the reaction and removal of the supernatant, a pink precipitate remained.

The Powder X-ray diffraction (XRD) pattern was recorded on a Seisert Argon 3003 PTC using nickel-filtered XD-3a Cu K radiations (D 1:5418 Å). Absorbance mode was recorded on a Hitachi spectrophotometer model U-2101 PC. The solution form of the sample was prepared by suspending a small amount of powder in ethanol. TEM was performed on a Philips EM208 and microscope operated at 100 kV. Sample was prepared by dispersing the powder in ethanol. Imaging was enabled by depositing a few drops of suspension on a carbon coated 400 mesh Cu grid. The solvent was allowed to evaporate before imaging [12].

2.3. Mechanism of Ag-Nps

Ag-Nps attach to cell membrane and penetrate in the fungi then produce a site witch little molecular weight in center of fungi, and then Ag-Nps attach to respiratory sequence and finally cell

division stop lead to cell death, Ag-Nps release silver ion in fungal cell which increase antifungal function as result [13].

2.4. Microorganisms and culture conditions

Candida albicans (ATCC 5027), *Saccharomyces cerevisiae* (ATCC 5027) have been provided from Iran researching organization collection and in all steps of experiment, we culture SCC (sabouraud dextrose agar containing Chloramphenicol and cycloheximide), SDB (sabouraud dextrose broth) to have new yeasts.

2.5. Determination of antifungal susceptibility

Fungal cells in MIC inoculated SCC related to these organisms have been determined by a double attenuating method, of experimental components that were done due to the standards of national association of laboratory and micro dilution method. Various concentrations of Ag-Nps and Fluconazole have been provided (highest concentration has been 256 mg/ml and the least one is 0.25 mg/ml) and different concentrations of Amphotericin B have been provided (highest t concentration has been 32 mg/ml and the least one is 3.25 mg/ml). After 48 hour of incubation in 37 $^{\circ}C$, the least concentrations of the studied components have been determined, that stopped the process of growth for 50 to 90%. The process of growth is controlled and evaluated through the culture. The used culture is SCC.

2.6. Scanning Electron Microscopy (SEM)

The morphological changes of *Candida albicans, Saccharomyces cerevisiae* by Ag-NPs were observed with a scanning electron microscope (SEM). Strains were prepared by cutting the agar, fixed for a minimum of 3 h in 2.5% (v/v) glutaraldehyde (100mM phosphate buffer solution, pH 7.2), and then fixed in1% (w/v) osmium tetra oxide for 1 h. The agar blocks were dehydrated through a graded series of ethanol (30, 50, 60, 70, 80, 90, 95, and 100%; each level was applied twice for 15 min each time) and ethanol: isoamyl acetate (3:1, 1:1, 1:3, and 100% isoamyl acetate twice for 30 min). The agar blocks on grid were dried with a critical-point drier using liquid CO2 and coated with gold-coater for 5 min. The coated samples were observed under JSM-5600LV with accelerating voltage of10 kV.

2.7. Measurement of Minimum Inhibitory Concentration (MIC)

Different concentration of Ag-NPs (0, 5, 10, 50, 100 ppm) was added in LB medium. Each fungal culture (*Candida albicans, Saccharomyces cerevisiae*) was incubated at $35^{\circ C}$. To establish the antimicrobial activity of Ag- NPs on the fungal growth, the MIC of Ag-NPs for *Candida albicans, Saccharomyces cerevisiae* was determined by optical density of the fungal culture solution containing different concentration of each Ag-NP after 24 h.

3. Results

The antifungal activity of Ag-NPs against Candida albicans, Saccharomyces cerevisiae as models for fungi was investigated and Ag-NPs has been used as a comparable antifungal drug by antifungal drugs like Amphotericin B, Fluconazole. Ag-NPs exhibited a potent antifungal activity against fungal strains tested. To investigate growth inhibition effect of Ag-NPs against Candida albicans, Saccharomyces cerevisiae, we measured the MIC.MIC50 of Amphotericin B and

Fluconazole, Ag-NPs on Candida albicans were 1mg/ml, 8mg/ml, 0.5 mg/ml respectively, MIC90 of Amphotericin B and Fluconazole, Ag-NPs on Candida albicans were 4mg/ml, 16mg/ml, 2 mg/ml Respectively (Table1). MIC50 of Amphotericin B and Fluconazole, Ag-NPs on Saccharomyces cerevisiae were 16mg/ml, 64mg/ml, 4 mg/ml respectively, MIC90 of Amphotericin B and Fluconazole, Ag-NPs on Saccharomyces cerevisiae were 32 mg/ml, 256mg/ml, 32 mg/ml Respectively (Table 1).

Table.1. MIC50 and MIC90 of Amphotericin B, Fluconazole, Ag-NPs on *Candida albicans and Saccharomyces cerevisiae*

Sample:	Candida albicans			Saccharomyces cerevisiae		
	MIC50 mg/ml	MIC90mg/ml	RANGE	MIC50 mg/ml	MIC90mg/ml	RANGE
AmphotericinB	1	4	0.01325 - 32	16	32	-
Fluconazole	8	16	0.25 - 64	64	256	-
Ag-NPs	0.5	2	-	4	32	-

(In) According to this result, we understood the effect of Amphotericin B is more than to Fluconazole, and Ag-NPs have more potent effect than to Amphotericin B and Fluconazole, on Saccharomyces cerevisiae, Candida albicans. This fact is true about both MIC_{50} and MIC_{90}, because when the number of MIC is low, the drugs have more lethal characteristics. So we can substitute the chemical drugs with Ag-NPs to treat disease. This comparison is shown in SEM graphs. When we used Saccharomyces cerevisiae as model for test we observed it such other healthy yeast with spherical shape and smooth cell wall and completely cell membrane because we didn't use any drug (Figure 1.A). But when we used 4 mg/ml (MIC90) concentration of Ag-NPs we observed membrane damage and some pits that have been created cause inter cellular components leakage and finally cell death (Figure 1.B). Also we influenced fungi cell by 32 mg/ml (MIC50) concentration of Ag-NPs, we observed the same result as above result (Figure 1.C).

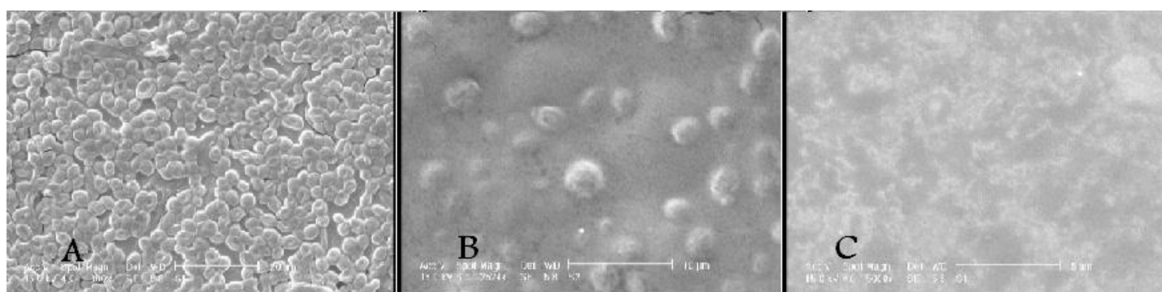

Fig.1. Scanning electron microscopy images of Saccharomyces cerevisiae. Normal cells (A) and cells influence by 4 mg/ml (MIC90) concentration of Ag-NPs (B) and cells influence by 32 mg/ml (MIC50) concentration of Ag-NPs (C) (10 ppm).

Finally using Candida albicans as our second fungi model and not using any drug, we observed healthy fungi cell under SEM (Figure 2.A) but when we used 0.5 mg/ml (MIC50) concentration of Ag-NPs (fig.2.B) and cells influenced by2 mg/ml (MIC90) Concentration of Ag-NPs (Figure 2.A), we observed destroy fungal cell with pore in their cell membrane. The result of Scanning Electron Microscopy (SEM) on two model yeast (Saccharomyces cerevisiae, Candida

albicans) show reciprocal correlation between Ag-NPs and membrane structure, cause damage and then death of fungal cell. SEM has been used for evaluating Ag-NPs capability in destroying surface membrane structure of the fungus.

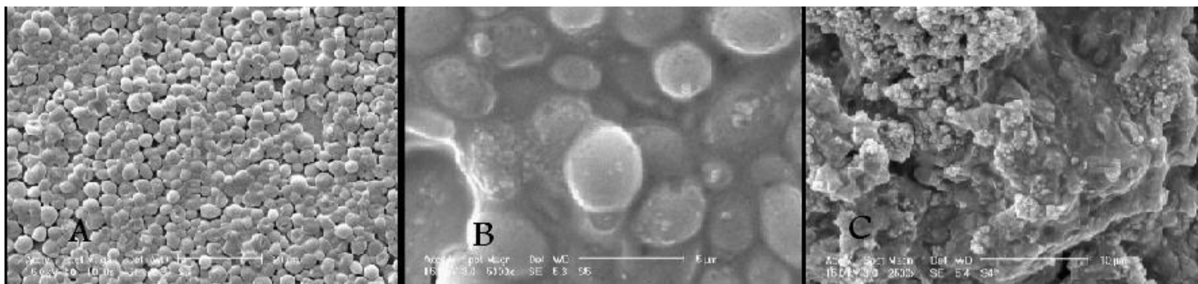

Fig.2. Scanning electron microscopy images of Candida albicans. Normal cells (A) and cells influence by 0.5mg/ml (MIC50) concentration of Ag-NPs (B) and cells influence by 2 mg/ml (MIC90) concentration of Ag-NPs (C) (10 ppm).

These results indicated that Ag-NPs have remarkable potential as an antifungal agent in treating fungal infectious diseases.

4. Discussion

In this study, the antifungal activity of Ag-NPs toward *C. albicans* and *Saccharomyces cerevisiae* as models for fungi was investigated. Ag-NPs exhibited a potent antifungal activity against fungal strains tested, similar to the antifungal activity in the MIC values of Amphotericin B and Fluconazole, which were used as a positive control. These results indicated that Ag-NPs have remarkable potential as an antifungal agent in treating fungal infectious diseases. To provide information on the mode of action of Ag-NPs, its ability to dissipate the membrane potential of *C. albicans* and *Saccharomyces cerevisiae* were investigated (Figure 2).

Many antimicrobial agents are limited in clinical applications, because of their much complications, for example Amphotericin B has bad affect on kidneys and kidney renal failure, fever, tremble, nausea, diarrhea have seen after using these drugs. The Azoles family like Fluconazole, cause liver toxicity and stopping in testosterone synthesis. So that requiring new drugs with less complications components in hosts is considered for knowing Ag-NPs function, its ability in destroying yeasts potential and membrane function have been investigated. Fungal cells by having ergosterol in the membrane and by making various gradients between cytoplasmic membranes can keep their membrane potential ability. As a result of using antifungal drugs and Ag-NPs, theses gradients and membrane conformity have been destroyed and it causes cell death. Additionally, SEM analysis confirms the interaction between Ag-NPs and the membrane structure of *C. albicans* and *Saccharomyces cerevisiae* cells, during Ag-NPs exposure, show significant changes to their membranes, which are recognized by the formation of ‘‘pits’’ on their surfaces, and finally, result in the formation of pores and cell death (Figure 2). Endo *et al.* have reported that the inhibition of bud growth correlates with membrane damage [9]. This report suggests that Ag-NPs inhibit the normal budding process, probably through the destruction of membrane integrity.
Finally, Ag-NPs exhibited potent antifungal effects on fungi tested, probably through destruction of membrane integrity; therefore, it was concluded that Ag-NPs has considerable antifungal activity, deserving further investigation for clinical applications.

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