Biosynthesis of silver nanoparticles (Ag-Nps) (a model of metals) by *Candida albicans* and its antifungal activity on Some fungal pathogens *(Trichophyton mentagrophytes and Candida albicans).*

Atef, A. Hassan[ab] ; Mogda, K. Mansour[b] and H.H. Mahmoud[c]

[a]Animal Health Research Institute, Doki, Giza, Egypt [b] Dep. of Mycology and Mycotoxins & Biochemistry
[c]Central Laboratory of Elemental and Isotopic Analysis, Nuclear Research Centre, Atomic Energy Authority, Egypt
Atifhassan2000@yahoo.com

Abstract: The silver nanoparticles (AgNPs) (as a model for other metals) is of utmost significance in various applied medical sciences. The chemical synthesis of Nano particles of metals particularly Ag-NPs is reported to be harmful when used in medical preparations. So, the silver nanoparticles were synthesized biologically using fungi as Candida *albicanse,* after treating silver nitrate with fungus mycelia. The biosynthesis of AgNPs were identified though the culture mycelia and further confirmed with UV-visible spectroscopy and scanning electron microscope (SEM). The prepared Ag-NPs were evaluated for the antifungal effects on *Candida albicans and T. mentagrophytes*. This effect was observed by measuring Minimum Inhibitory Concentration (MIC) using well diffusion technique in comparison with some drugs including Grisofulvin and Itraconazole have been obtained on the fungi and the changes on membrane reactions of treated fungi have been detected by Scanning Electron Microscopy. The obtained results revealed the MIC50 of Grisofulvin and Itraconazole and Ag-NPs on *Candida albicans and T. mentagrophytes* which were 4±0.25 ug/ml, 8±0.18 ug/ml and 2±0.10 ug/ml respectively, on *Candida albicans*. Whereas, the results of the same effects on *T. mentagrophytes* were 5±0.35 ug/ml, 12±1.5 ug/ml and 2±0.13 ug/ ml, respectively. However, the MIC100 of the tested antifungal Grisofulvin and Itraconazole and Ag-NPs were relatively required higher concentrations, 7±1.2 ug/ml, 13±3.0 ug/ml and 4±2.0 ug/ml for antifungal effect against *C.albicans*, respectively. Whereas, the concentrations of 8±1.5 mg/ml, 17±2.5 ug/ml and 5±1.0 ug/ml were required for MIC100 for inhibition the growth of *T. mentagrophytes*. In case of *Candida albicans* as model for other fungi, when we used (MIC50) concentration of Ag-NPs we observed membrane damage and some pits that have been caused inter cellular components leakage and finally cell death. Whereas, the using of (MIC100) concentration of Ag-NPs, we observed destruction of fungal cell with pore in their cell membrane. Therefore, Silver nanoparticles as a model for other metals could be used in the field of human and veterinary medicine as successful treatment of microbial diseases of human and animal particularly fungal diseases. Also, the fungi also could be used for the production of these nanoparticles which is highly biocompatible, cheap and environmental friendly.

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1. Introduction

Up to date the elimination of environmental pollution become the national aim of scientist. Microbial pollutants were the most dangerous factors and a wide range of diseases resulted from fungal contamination and their toxins which constituted the major problem for animal and human health. However, many reports detected the association of fungi with diseases outbreak in animal.The correlation between the environmental factors, mycosis and mycotoxicosis in animals and the role of these environmental factors in initiation of food born infections had been reported by [1,2] The fungal infections particularly infections by *C. albicanse and dermatophytes* represent the widest spread and most prevalent mycotic diseases of man and animal [3, 2]. The incidence and prevalence of these serious mycoses continue to be a public health problem. Despite aggressive treatment with new or more established licensed antifungal agents, these infections are important causes of morbidity and mortality, especially in immunocompromised patients, [4]. Transmission of mycotic infection from man to man or animal to man has not been documented. Occasionally, there has been a known or presumed exposure to weathered contaminated environmental sources which often contains the yeast including soil, air, feeds and skin likely to be contaminated with fungi very infrequently [5].

Also, in recent years, a rapid increase in microbes that are resistant to conventional antibiotics has been observed by many authors [6,7, 1,2, 8].

Especially, the frequency of infections provoked by opportunistic fungal strains has increased

dramatically. Even though, the majority of invasive fungal infections are still due to the *Aspergillus* or *Candida* species, the spectrum of fungal pathogens has changed and diversified [9-12]. Azoles that inhibit sterol formation and polyenes that bind to mature membrane sterols have been the mainstays regarding antifungal therapy for several decades [13,14]. Nowadays, resistance to many of the in use antifungal agents has emerged and 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 [15,16]. However, not only the emergence of chemical drugs and herbs Resistance among different pathogenic strains but also the high toxicity of amphotericin B., [17; 18, 2, 8] has prompted research on new antifungal agents [19] Bio-nanotechnology has emerged for developing biosynthesis and environmental-friendly technology for synthesis of nano-materials. Among them, the metallic nanoparticles are most promising as they contain remarkable antibacterial properties due to their large surface area to volume ratio, which is of interest to researchers due to the growing microbial resistance against metal ions, antibiotics, and the development of resistant strains [20,21]Different types of nanomaterials like copper, zinc, titanium [22,23] magnesium, gold [24-26] and silver have been developed but silver nanoparticles (Nano-Ag) have proved to be most effective as they exhibit potent antimicrobial efficacy against bacteria, viruses and fungi. However, Nano-Ag used as a disinfectant drug also has some risks as the exposure to silver can cause argyrosis or argyria; it can be toxic to mammalian cells [21]. The recent studies investigated the production of silver nanoparticles which is highly biocompatible, cheap and environmental friendly. It was reported that the cell biosynthesis is associated with silver nanoparticles and these methods can be divided into three categories depending on the place where nanoparticles are created; intra, extra cellular [27,28]. The use of eukaryotic organisms such as fungi holds promise for large scale metal nanoparticles production as the enzymes secreted by fungi is an essential element for the biosynthesis of metal nanoparticles [29] Different fungi such as *Verticillium*, *Fusarium oxysporum* and *Colletotrchum sp.* have been reported to synthesize metal nanoparticles [30-34].

Therefore, the aim of the present work was undertaken to evaluated the antifungal properties and mechanism of actions of Nano-Ag against some pathogenic fungal strains and study the therapeutic potential of Nano-Ag for treating fungal diseases.

2. Material and Methods

Fungal strains and culture conditions

A total of 40 strains of fungal species were used in this study, 20 isolates of each of *Candida albicans*, *Trichophyton mentagrophyte*. The used isolates were clinically recovered from samples of feed, food and skin scraping which were obtained from farms encountered animal diseases and identified in the Department of Mycology, Animal Health Research Institute, Ministry of Agriculture, Egypt. All tested strains of were cultured on a Sabouraud's dextrose agar (SDA) and/or potato dextrose agar (PDA) at 30-35 °C, respectively.

Preparation of Mycelium and Supernatants of fungi for preparation of silver Nanoparticles [35].

The spore suspension of fungus of *Candida albicanse*(10^5/ml) inoculated into 250 ml Erlenmeyer flasks, each containing 50mL of semi defined medium (SDM) composed of KH_2PO_4 (7g/L), K_2HPO_4 (2g/L), $MgSO_4.7H_2O$ (0.1g/L), $(NH_4)_2SO_4$ (0.1 g/L), yeast extract (0.6g/L), and glucose (10g/L) at 30°C under shaking condition (200 rpm) for 96 hrs. After 96 hrs of cultivation, mycelia were separated from the culture broth by centrifugation at 4500 rpm, 10°C, for 15 min. The settled mycelia were washed thrice with de-ionized water.

Biosynthesis of Silver Nanoparticles [35,36].

The 1% of washed mycelia of fungus was inoculated into aqueous silver nitrate solution (10-3M). The mixtures were thereafter incubated in a rotary shaker at 200 rpm in the dark at 30°C. The bioreduction of silver nitrate into AgNPs were monitored periodically by visual inspection and in a UV-visible spectrophotometer. The culture supernatants were inoculated into AgNO3 and cultured using similar condition. The Particle sizes and morphology of Nano-Ag distributions of these samples were also obtained using scanning electron microscope (SEM)(Joe, JSM-5600LV, Japan).

Antifungal susceptibility testing by *measurement of Minimum Inhibitory Concentration (MIC)* [1].

The minimum inhibitory concentration (MIC) for *Candida* spp. and *T. mentagrophytes* was determined by a well diffusion method. The medium of SDA was poured to palates containing spore suspension for each of the tested fungal culture separately (*Candida albicans,* 2.5 x 10^3 cells/ml) *and T. mentagrophytes (*5 x 10^4 cells/ml*)*, shaken over the tables on rotary manner and remained too solidified.

After solidification, the medium plates were pored to 5 pores. Different concentration of Ag-NPs (2, 4, 6, 8, 10, 12 – 16 ug/ml) was added to separate pores of medium plates and it was incubated at 35◦C for 28 hrs. To establish the antimicrobial activity of Ag- NPs on the fungal growth, the MIC of Ag-NPs for *Candida albicans* and *T. mentagrophytes* was determined by optical density of the fungal culture solution containing different concentration of each Ag-NP after 24 h, 48, and 96 h. The inhibitory concentration was defined as the lowest concentration that inhibited the growth as determined by a comparison with the growth in the control wells. Amphotericin B and fluconazole were used as a positive control toward fungi; amphotericin B is a fungicidal agent widely used in treating serious systemic infections, [37], and fluconazole is used in the treatment of superficial skin infections caused by dermatophytes and *Candida* species [38].

Scanning Electron Microscopy (SEM) [21].

The morphological changes of *Candida albicans and T. mentagrophytes* 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.

3. Results and discussion

The progressive increased population in the world requires a parallel raise in the production of food. The recent researches reported that the majority of this food may carry the dangerous factors for human and animal health. However, the fungal infections could be resulted from polluted water, food, manure and silage, so gain access to the human, animals and birds during eating, drinking, breathing in a such mixed form and the medication with antibiotics does not eliminate these infections but cause the flourishment of fungi, also steroid treatment, cytotoxic drugs and treatment with surgical operations as well as mal nutrition, pregnancy, endemic diseases, adverse environmental conditions as bad hygienic measures and over crowdiness in both animal and bird farms may predispose to infection [39].

Many of the recent reports indicated 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, [15,40; 41, 26]. Compared with other metals, silver exhibits higher toxicity to microorganisms while it exhibits lower toxicity to mammalian cells [42]. 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, [41-46]Hence, Ag-Nps have been applied to a wide range of healthcare products, such as burn dressings, scaffold, water purification systems, and medical devices, [47-49]. The toxic effects of silver on bacteria have been investigated for more than 60 years [50]. And the acting mechanism of silver has been known in some extent, [20]. 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 [51,52] In the present study, the biological synthesis of AgNPs by fungal strains of *Candida albicanse* was investigated. The appearance of cloudy light-grey colour in the Erlenmeyer flask indicated a reduction of silver ion and the formation of silver nanoparticles has taken place. The colour changes were observed immediately when the tested fungus was transferred into the flask containing silver nitrate. Bioreduction indicates the presences of reducing agent which served as electron shuttle in this reduction reaction and it was also reported that, fungus reduction was most probably either by reductase action or by electron shuttle quinones or both [53-54]. The presence of hydrogenanse and nitrate reductase [53,55] is the essential element for metal reduction. In the present work, the UV-visible spectroscopy and scanning electron microscope can be used to observe the size and morphology of nanoparticles by electron charge oscillation principle in silver nanoparticles that exhibit by light Fig. 1 as stated by [56; 57]. Using UV-visible spectrophotometer, the, wavelength of 370 nm is selected for identification of optical density of AgNPs production.The optical density of Ag-NPS which produced extracelluler in culture broth of *C. albicanse* was 0.20, but, Ag-NPS which produced in the intracellular of *C. albicanse* showed OD of 0.65 Fig. 1. Similar results were obtained by, [54] who found that the OD of *Schizophyllum commune, Lentinus sajor caju* and *Pycnoporus sanguineus* were in the order of increasing OD at 0.190 > 0.05 > 0.02, respectively. They added that the intracellular Ag-NPS for all the tested fungus showed instantaneous production of AgNPs, with OD of 0.733 (*S. commune*), 0.624 (*L. sajor caju*), and 0.576 (*P. sanguineus*). Whereas the estimated size of the silver

nano particles for the extracellular secretion of silver nano particles diameter sizes was 80.5±3.0 nm. However, the intracellular Ag-NPS- diameter size was 62.1±1.5 nm which was relatively lower than the size of produced Ag-NPS (Fig.2). These findings were confirmed the results of, [54] who detected that culture broth of fungus *Lentinus sajor caju* could produce nanoparticles with average diameter of 53 nm while the extracellular secretion of, *Lentinus sajar caju* observed that nano-diameter sizes were 89.76 nm and the intracellular secretion analysis, nano-diameter size identified were consistent at about 50-60 nm. Whereas, [58] demonstrated the size of silver particles produced by the filamentous fungus Phoma sp.3.2883 via under transmission electron microscope and their estimated size was 71.06 ± 3.46 nm.

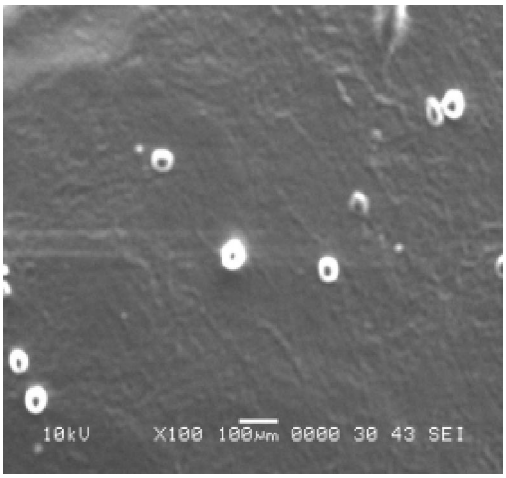

Fig. 1 The micrograph of tiny Ag Nano- particles (The black dots) under SEM. (x 20 000).

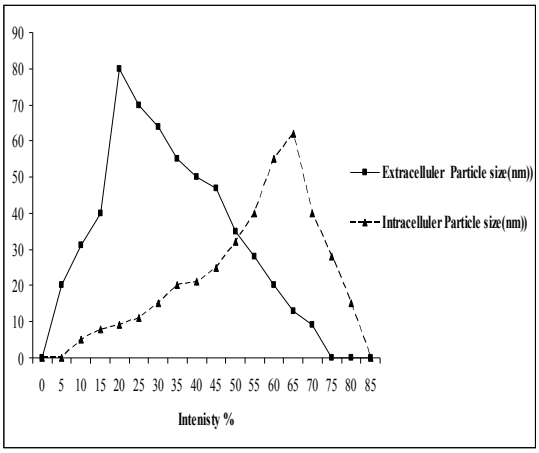

Fig. (1): The particles size(nm) of the Ag-NPs produced using *C.albicanse* after 48 hr

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The antifungal activity of Ag-NPs against *Candida albicans and T. mentagrophytes* as models for pathogenic fungi was investigated and Ag-NPs has been used as a comparable to known antifungal drugs like Grisofulvin and Itraconazole Ag-NPs exhibited a potent antifungal activity against fungal strains tested. To investigate growth inhibition effect of Ag-NPs against Candida albicans and *T. mentagrophytes* we measured the MIC. The MIC50 of Grisofulvin and Itraconazole and Ag-NPs on *Candida albicans,* were in 4±0.25 ug/ml, 8±0.18 ug/ml and 2±0.10 ug/ml respectively. Whereas, the results of the same effects on *T. mentagrophytes* were 5±0.35 ug/ml, 12±1.5 ug/ml and 2±0.13 ug/ml, respectively Table 1. However, the MIC100 of the tested antifungal Grisofulvin and Itraconazole and Ag-NPs were relatively required higher concentrations, 7±1.2 ug/ml, 13±3.0 ug/ml and 4±2.0 ug/ml for antifungal effect against *C. albicans*, respectively. Whereas, the concentrations of 8± 1.5 ug/ml, 17±2.5 ug/ml and 5±1.0 ug/ml were required for MIC100 for inhibition the growth of *T. mentagrophytes* Table 2and Fig. 3.

Table(1)Antifungal activity of Nano-Ag., Grisofulvine and Itraconazole(MIC_{50} ug/ml)

Fungal strains (no. of strains)	MIC50 (ug/ml)		
	Grisofulvine	Itraconazole	Nano-Ag
C. albicans (20)	4±0.25	8±0.18	2±0.10
T.mentagrophytes (20)	5±0.35	12±0.15	2±0.13

Table (2) Antifungal activity of Nano-Ag., Grisofulvine and Itraconazole ($MIC10_{100}$ ug/ml)

Fungal strains (no. of strains)	MIC 100 (ug/ml)		
	Grisofulvine	Itraconazole	Nano-Ag
C. albicans (20)	7±1.2	13±3.0	4 ±2.0
T. mentagrophytes (20)	8-±1.5	17±2.5	5±1.0

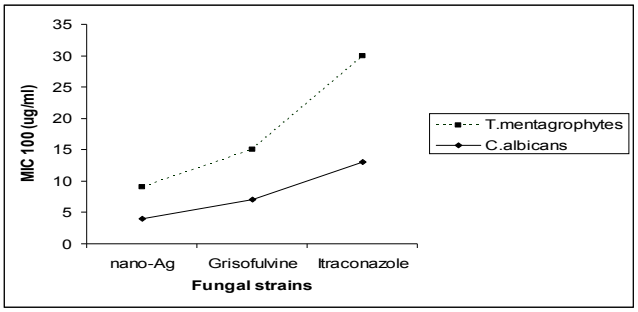

Fig.3; Antifungal activity MIC of Nano-Ag., Grisofulvine and Itraconazole (MIC_{100} ug/ml)

It is clear that the effect of Grisofulvin and Ag-NPs have more potent effect than to Itraconazole on *Candida albicans and T. mentagrophytes*. This fact is true about both MIC50 and MIC100, because when the number of MIC is low, the drugs have more lethal characteristics. So we can substitute the chemical drugs with nanoparticles of metal elements as Ag-NPs to treat fungal disease. This comparison is shown in SEM graphs. When we used *Candida albicans* which in the normal condition have a spherical shape and smooth cell wall and intact cell membrane Fig.4. 1. But when we used 2±0.10 ug/ml (MIC50) concentration of Ag-NPs we observed membrane damage and some pits that have been created caused inter cellular components leakage and finally cell death Fig. 4.2. Whereas, the using of 4±2.0 ug/ml (MIC100) concentration of Ag-NPs, we observed destroy fungal cell with pore in their cell membrane Fig. 4.3. Similar findings were observed in case of T. *mentagrophytes*, where the damage of cell membranes of normal cells Fig. 5.1. Showed when Ag-NPs used at concentration of 2±0.13 ug/ml (MIC50) (Figure, 5.2). However, the using 5±0.10 ug/ml (MIC100) concentration of Ag-NPs caused destruction and death of fungal cells occurred Fig. 5.3.

Fig.4. 1. Fig. 4.2. Fig. 4.3

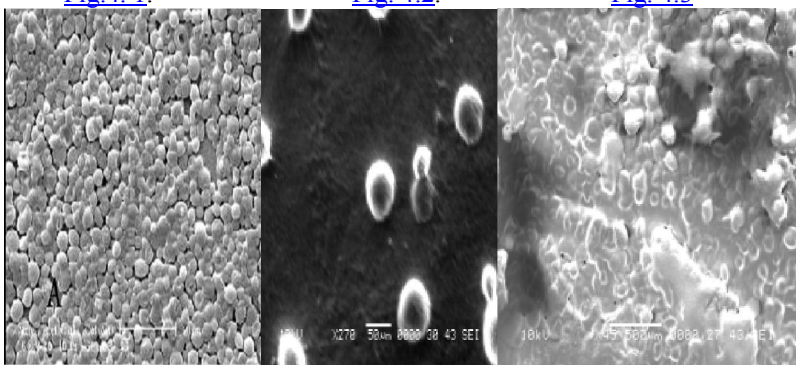

Fig.4. 1-3. Scanning electron microscopy images of Candida albicans. Control Normal cells (1) and cells treated by 2 mg/ml (MIC50) Concentration of Ag-NPs (2) Cells treated by 4 mg/ml (MIC100) concentration of Ag-NPs (3).

Fig.5. 1. Fig. 5.2. Fig. 5.3.

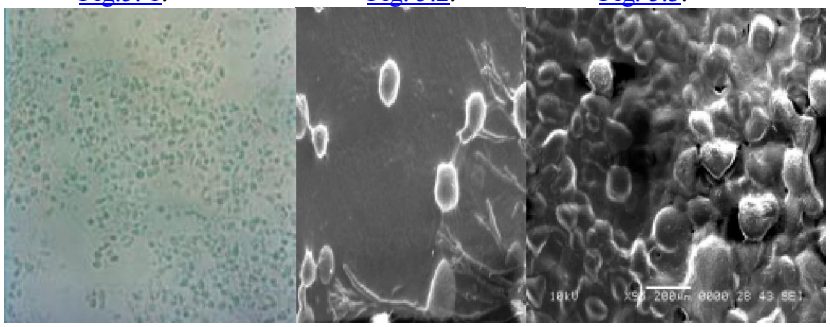

Fig.5. 1-3; Scanning electron microscopy images of T. mentagrophytes. Normal cells (1) and cells influence by 2 mg/ml (MIC50) Concentration of Ag-NPs (2) and cells influence by 5 mg/ml (MIC100) concentration of Ag-NPs (3).

Many antimicrobial agents are limited in clinical applications, because of their cumulative and side effects on the internal organs which resulted much complications as Amphotericin B and Grisofulvine has bad affect on kidneys and kidney renal failure, fever, tremble, nausea, diarrhea have seen after using these drugs and the Fluconazole, cause liver cirrhosis and toxicity and stopping in testosterone synthesis [4]. Hence, the continuous investigation for new drugs with less complications become urgent requirement in hosts is considered for knowing Ag-NPs function, its ability in destroying fungal potential and membrane function. Fungal cells by having ergosterol in the membrane and by making various gradients between cytoplasmic membranes can keep their membrane potential ability [27]. However, in the present study the scanning by electron microscopy analysis observed the interaction between Ag-NPs and the membrane structure of *Candida albicans and T. mentagrophytes* cells by detection a 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 due to Ag-Nps which release silver ion in fungal cell and increased antifungal function. These results came in accord with [46] who reported that SEM has been used for evaluating Ag-NPs capability in destroying surface membrane structure of the fungus. The Ag-Nps attached to cell membrane and penetrate it 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 its antifungal function. These results indicated that Ag-NPs have remarkable potential as an antifungal agent in treating fungal infectious diseases. Also [42,48], had reported that the inhibition of bud growth correlates with membrane damage. 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.

In Conclusion: In general, fungi were reported as potential pathogens and caused different diseases conditions in human and animals, particularly after prolonged exposure to adverse environmental condition. [2]. The dangers of mould and yeast besides caused animal mycosis, they produced fungal metabolites such as mycotoxins, such mycotoxins produced under adverse effect of environmental conditions. These mycotxoins residues in food and feed causes carcinogenic, teratogenic, haemorrhagic and immunosuppression effect to human and animal health [1, 2]. Therefore, the essential significance of this study is the indication that Silver nanoparticles as a model for other metals nanopartricles as (zinc, selenium, iron, manganese, cupper …etc) can inhibit the growth of *Dermatophytes and Yeast of C.albicanse,* which cause superficial or deep fungal infections and could be used in the field of human and veterinary medicine as a bactericide, fungicide and antiviral in successful treatment of microbial diseases of human and animal. This is from the first studies that apply Nano- particles of elements successfully to dermatophytes and pathogenic fungal strains. Also, the fact that preparation method of Nano-Ag described here is cost-effective, environmentally friendly and non infectious for industrial worker are also of importance. Therefore, it can be expected that Nano-Ag as a model for nano-particles of other metals may have potential as an anti-infective agent for human and animal fungal diseases.

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