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Myco-biosynthesis of silver nanoparticles by *Aspergillus niger* and evaluation of theirs antifungal activity in Dermatophytes spp. and *Candida albicans*

A Thesis
Submitted to the Council of the College of Medicine/University of Al-Qadisiyah in Partial Fulfillment of the Requirements for the Degree of Doctorate of Philosophy of Science in Medical Microbiology

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قَالُواْ سُبۡحَٰنَكَ لَا عِلۡمَ لَنَآ إِلَّا مَا عَلَّمۡتَنَآۖ
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Dedication

To

My Mother

A strong and gentle soul who taught me to trust in Allah, believe in hard work and that so much could be done with little

My Father

For earing an honest living for us and for supporting and encouraging me to believe in myself

My dearest husband

who leads me through the valley of darkness with light of hope and support

My beloved kids

Noor Al-Zahraa, Tamara

whom I can't force myself to stop loving

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Summary

Nanotechnology and nanoparticle (NPs) research has attracted a lot of interest in recent decades, and there is growing attention to find more effective ways for their synthesis. The use of biological organism as bionanofactories provides a clean and promising alternative process for the fabrication of silver nanoparticles. This study was aimed to biosynthesized silver nanoparticles (SNPs) by using cell free filtrate of the fungus *Aspergillus niger* and evaluate the antifungal effects of biosynthesized silver.

The study was conducted in the period between March /2016 and January/ 2017 in the microbiology laboratory of the Medicine College / University of Al-Qadisiyah.

A reference strain of *Aspergillus niger* was used in present study for biosynthesis of SNPs. While the reference strains of dermatophytes namely, *Trichophyton rubrum, Trichophyton mentagrophytes ,Trichophyton interdigitale, Epidermophyton floccosum, Microsporum canis* and *Candida albicans* were used for testing their susceptibility to bio-prepared SNPs.

The biosynthesized SNPs were characterized by means of several techniques. The UV-visible spectrophotometric showed characteristic absorption peak at 420 nm. The presence of proteins as viable reducing agents for the formation and stability of SNPs was recorded using Fourier transform infrared spectroscopic analysis (FTIR). Further scanning electron microscopy (SEM) and transmission electron microscopy (TEM) micrographs showed the formation of spherical, mono-dispersed nanoparticles with size ranging between 15 and 60 nm in diameter.

Optimization results showed that the optimum condition for biosynthesis of SNPs were the use of Potato dextrose broth medium at pH 9, 30°C for 120 h with 1mM silver nitrate.

The MIC and MFC recorded 0.313 and 2.5 µg/mL in the case of **Trichophyton rubrum** (the most susceptible species), 5 and 14.14 µg/ml for **Microsporum canis** (the most tolerant species), respectively. The biosynthesized SNPs displayed nearly similar antifungal effect to that obtained by Miconazole, while it exhibited a higher effect in comparison to Fluconazole. SNPs had pronounced and significant ($P < 0.05$) spore germination inhibition effect on all the tested dermatophytes but this effect was variable depending on the fungal isolates. A decrease in keratinase activity in the presence of SNPs was also reported. *Epidermophyton floccosum* showed higher enzyme reduction in the presence of SNPs (47.41%) compared to other dermatophytes **.**While the effect of Miconazole on it was 28.30 %. However Fluconazole had no effect on the tested dermatophytes except on *Trichophyton interdigitale.*

The biosynthesized SNPs exerted pronounced morphological alteration in the fungal mycelia; Main morphological changes include swelling, presence of large vacuoles, wall disorganization and loss of integrity of their biological membranes indicating extensive cellular death.

SNPs significantly reduced the adhesion and biofilm formation of *C.albicans* in a dose-dependent manner. The highest reduction was observed at 2 X MIC which reached to 51% and 36.1% for adherence and biofilm formation, respectively. Whereas the Miconazole had lower effect with reduction percentage of 43% and 31.1% for adherence and biofilm formation, respectively.

SNPs were completely prevented the development of germ tube with a percentage of inhibition 100%. Similar results were obtained with Miconazole

The antifungal activities of Fluconazole and Miconazole were increased in the presence of SNPs. The highest enhancing effect was observed for Fluconazole against all resistant strains with fold increase 100%. An antagonism effect was observed when a combination of SNPs and Miconazolre was applied against *Microsporum canis.*

In conclusion, the obtained results established the fact that SNPs can be synthesized in inexpensive and promising technique by fungal isolate of *A.niger*, and the qualities of these NPs. can be controlled by monitoring the environmental factors. In addition biosynthesized SNPs have considerable antifungal activity comparison with other antifungal drugs.

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List of Abbreviations

Abbreviation	*Meaning*
^{0}C	Degree of Celsius
Cfu	Colony forming unit
CT	**computed tomography**
CVS	Crystal Violet Stain
DMSO	Dimethyl sulfoxide
FCZ	Fluconazole
FTIR	Fourier Transform Infrared Spectrometer
M	Molarity
Mag	Magnification
MCZ	Miconazole
mg	Milligram
min	Minute
mL	Milliliter
mM	Mili Molarity
mm	Millimeter
MRI	Magnetic resonance imaging
N	Normality
nm	nanometer
NPs	Nanoparticles
OD	Optical density
PBS	Phosphate buffer solution
PET	**positron emission tomography**
rpm	Revolutions per minute
SEM	Scanning Electron Microscopy
SERS	**Surface-enhanced Raman spectroscopy**
SNPs	Silver nanoparticles
SPR	Surface plasmon resonance
TCP	Tissue Culture Plate

TEM	Transmission Electron Microscopy
UV	Ultra Violet
μg	Microgram
μL	Micro liter

Introduction
and
Literature Review

1. Introduction and Literatures Review

1.1 Introduction

Dermatophytes are the most common cause of fungal infections worldwide, resulting in treatment costs of close to half a billion dollars annually. The World Health Organization estimates global prevalence of dermatomycoses to be approaching 20% (Marques *et al.*,2000).

It is well-known that dermatophytes - a group of fungi which infect the keratinized outermost layer of the skin such as nail, hair and the stratum corenum of the epidermis of humans and animals. Dermatophytes including several species belong to the **Microsporum**, **Epidermophyton and Trichophyton** genera. It has the ability to invade and grow in dead keratin and exhibited variable efficiency in producing extracellular enzymes particularly keratinases which playing an important role in the virulence of this fungus. Infection is generally cutaneous and restricted to the non-living cornified layers because of the inability of the fungi to penetrate the deeper tissues or organs of hosts (Barry and Hainer,2003).

On the other hand, dermatophytosis is one of the most common infections all over the world which cause public health problem to both man and animals. Dermatophytes infection can be disfiguring, recurrent and generally need long-term treatment with antifungal agents (Ameen, 2010).

Several antifungal compounds, mainly azole, have been used to treat dermatophytosis, but fungal resistance to the azole derivatives appears very commonly. Moreover, the azole-containing medicines may have many adverse effects and drug interactions, it interfere with

activity of hepatic enzymes, the central nervous system, thyroid and sex hormones, and biosynthesis of testosterone. Therefore, there is an urgent medical need for novel antifungal agents**.** The metallic NPs are most promising antimicrobial agents as they contain superior properties due to their large surface area to volume ratio along with high fraction of surface atoms that educe elevated antimicrobial activity compared to the silver metal as a whole. Among different nanoparticles, silver nanoparticles (SNPs) have attractive the researcher interest. Compared with other metals, silver exhibits higher toxicity to microorganisms while it exhibits lower toxicity to mammalian cells (Zhao and Stevens, 1998).

The major methods used for conventional synthesis of SNPs are the physical and chemical methods. The problem with these methods was that the synthesis is energy and capital intensive and often employ toxic chemicals, as well as some chemically toxic substances being absorbed on the surface of NPs raising the toxicity issues and can hinder their usage in medical applications. Moreover, chemical synthesis of silver colloids mostly leads to aggregation as the period of storage increases. The biological methods for NPs synthesis might offer inexpensive, nontoxic, clean, and eco-friendly alternatives. There are three major sources of biological synthesis of SNPs: plant extracts, bacteria and fungi. Fungi have some distinct advantages when used as bio factories for NPs production, in comparison with bacteria, fungi can secret larger amounts of proteins which directly translate to higher productivity of NPs (Mohanpuria *et al.*,2008). Moreover, fungi have an additional advantage that downstream processing and handling of the fungal biomass would be much simpler. In addition, the biotransformation of NPs by bacteria involves the use of complicated

equipment for obtaining clear filtrates from the colloidal broths, while fungal broths can be easily filtered by simple equipment, therefore saving considerable investment costs (Korbekandi *et al.*,2013).

The physiochemical parameters impact the growth and developments of an organism in its environment. Thus the metabolic activity of an organism is influenced by these external circumferences. In case of extracellular synthesis of SNPs by fungi, the production of enzyme is highly affected by the condition in which the fungus is cultivated. Therefore, the qualities of NPs can be controlled by monitoring the environmental factors

Based on the mentioned facts, and in the view of the studies published in Iraq, it found that most of them focused on utilizing the chemically or physically synthesized nanoparticles and even those used green synthesis for production of nanoparticles, testing its effectiveness on different types of bacteria and few spp. of fungi. It did not find any study dealing with the impact of the nanoparticles on pathogenic fungi, especially the dermatophytes, or revealed the extent of this impact on the various virulent factors of fungi in particular the keratinase activity, spore germination , dimorphic transition or hyphal morphology; therefore the present study was conducted.

Aim of study

1- Bio-preparation of SNPs by using the fungus, *Aspergillus niger* as a reducing agents to silver nitrate.

2- Characterization of the biosynthesized silver nanoparticles by using different spectrospectral analysis.

3- Optimization the physioculture conditions such as culture medium, pH, incubation temperature, substrate concentration and time to improve the yield of SNPs production.

4- Evaluation the antimicrobial activity of the biosynthesized SNPs against selected strains of dermatophytes and *Candida albicans*

5- Testing the effect of SNPs on spore germination, keratinase activity and mycelia development of dermatophytes; as well as adherence, biofilm formation and dimorphic transition of *Candida albicans*.

6- Employment the unique properties of nanoparticles in reducing the resistance of different strains of fungi against conventional antifungal drugs through drug combination to get synergistic advantage.

1.2. Literatures Review

1.2.1. Dermatophytes

Dermatophytes are defined as a cluster of fungi that are belonging to three genera namely (*Tichophyton, Epidermophyton and Microsporum*). The genus* Epidermophyton *is represented by a single pathogenic species (*Epidermophyton floccosum*), the genera* Trichophyton *and* Microsporum *are complex and includes several recognized species (Baldo et al., 2012). Dermatophytes are keratinophilic that infect the superficial keratinized tissue such as skin, nail and hair. They cause cutaneous mycosis Known as dermatophytosis (tinea or ringworm) (Sahai and Mishra, 2012).

The infection of dermatomycosis may also be caused by the members of the genus Candida and non-dermatophytes moulds such as* Aspergillus, Fusarium *and* Scopulariopsis *(Naveed et al.,2009).

The dermatophytes species are divided into three ecological groups depending on their natural habitat (Baldo et al.,2012) as follow:

***(i) Anthropophilic species, which infect human e.g.* Trichophyton rubrum.**

***(ii) Zoophilic species, which infect the animals e.g.* Microsporum canis.**

***(iii) Geophilic species whose natural habitat is soil e.g.* Microsporum nanum**

Responses to dermatophytes infection may extent from mild to severe because of the host's reactions to the metabolic products of the fungus, the virulence of the infecting strain or species, the anatomic location of the infection and local environmental factors (Ameen, 2010).

Genus Epidermophyton:

The genus *Epidermophyton* includes only two known species to date, and only *E. floccosum* was pathogenic. This genus of **fungus** causing superficial and cutaneous **mycoses**, such as **tinea corporis**, **tinea cruris** (jock itch), **tinea pedis** , and **tinea unguium**. Like all dermatophytes, **E. floccosum** contains keratinase giving it the ability to

breakdown keratin, a protein commonly found within the skin and nails. **E. floccosum** is spread by direct contact with the fungus where people aggregate and share inanimate objects such as towels in a gym setting**. E. floccosum is differentiated from Microsporum and Trichophyton by the absence of microconidia. (**Kanwar **et al., 2001).**

***Genus Microsporum* :**

A **genus** of **dermatophytes** that causes **tinea corporis**, **tinea capitis**, and other **dermatophytoses** The genus includes 19 species, of which 5 are primarily isolated from humans and 7 primarily from animals. *Microsporum* infects the skin and the hair, but seldom infects nails, *Microsporum* is distinguished from *Trichophyton* and *Epidermophyton* by its fusiform macroconidia with rough to echinulate walls. Most infections in humans are acquired from infected dogs or cats (De Hoog *et al.*,2017).

***Genus Trichophyton* :**

Trichophyton is a **genus** of **dermatophytes**, which includes the **parasitic** varieties that cause **tinea**, including **athlete's foot**, **jock itch**, and similar infections of the nail, skin, beard and scalp. *Trichophyton* genus includes 28 species and characterized by the development of both smooth-walled macro- and **microconidia** (De Hoog *et al.*,2017**).**

Trichophyton interdigitale is a common cause of tinea pedis, particularly the vesicular type, tinea corporis, and sometimes superficial nail plate invasion in humans. It is not known to invade hair *in vivo* but produces hair perforations *in vitro*. Distribution is worldwide. This species may be regarded as a clonal off shoot of the zoophilic *T. mentagrophytes* (De Hoog *et al.*,2017).

The fungi can easily spread to other areas of the body as well and to the host's home environs, their spores are extremely difficult to eliminate, and spread everywhere. When the hyphae of the fungi burrow into the skin and release enzymes to digest keratin, they may irritate nerve endings and cause the host to itch, which may elicit the scratch reflex, which directs the host to scratch. Scratching directly transfers fungi and dead skin particles that are infested with the fungi to the fingers and under the finger nails. From there they can be transmitted to other parts of the host's body. Scratching also damages skin layers, making it easier for the fungi to spread at the site of the infection. If the fungi and infested debris are not washed from the fingers and fingernails soon enough, the fungi can also infect the skin of the fingers (tinea manuum), and burrow underneath and into the material of the fingernails (tinea unguium). If left untreated, the fungi continue to developed and spread.

1.2.1.1.Virulence Factors Involved in Pathogenicity of Dermatophytes

Dermatophytes produce virulence factors such as keratinases and cellulase to penetrate stratum corneum of host tissues and produce disease. This is a common opportunistic pathogen that uses several kinds of virulence factors for infection. The common virulence factors involved in pathogenicity of dermatophytes are briefly described here.

(i) Adherence to the host tissue

The first step of dermatophytes infection involves contact and adherence of the infectious elements from dermatophytes. Dermatophytes adhere to the surface of the keratinized tissue to reach the epidermis by germination of arthroconidium and then the hypha enters the stratum corneum. Increase the number of adhering spores is time dependence.

Aljabre *et al*. (1993) found that adherence of arthroconidia from *Trichophyton mentagrophytes* in stripped sheets of stratum corneum or separate keratinocytes requires approximately 6 hours and germination of the conidia begins by 4 hours. Duek *et al*., (2004) showed that polymeric material mediating between microconidia and stratum corneum cells that probably play an important role in the attachment of spores to skin

(ii) Invasion

After adherence of dermatophytes to the keratinized tissue, the spores must germinate followed by penetration to the stratum corneum. The ability of dermatophytes to degrade keratin is considered a major virulence attribute (Achterman and White, 2012). During penetration, dermatophytes produce a variety of virulence factors for infection that include both enzymes and non-enzymes.

Dermatophytes secrete a variety of virulence enzymes that have different substrate specificities; such as protease, cellulase and lipase. Dermatophytes secrete many enzymes to obtain the nutrients necessary for their developing and surviving. Among the wide kinds of enzymes secreted by dermatophytes, protease enzymes are the major type of the virulence factors from dermatophytes involved in invasion and utilization of the stratum corneum of the host (Liu *et al*.,2014). It has been suggested that dermatophytes secrete proteases in response to the presence of the components of the skin during invasion. Some authors suggest that dermatophytes secrete proteases to facilitate an efficient adhesion of these pathogens to the host tissue (Peres *et al*., 2010). Keratinases belong to the group of serine hydrolases that are capable of degrading keratin, a fibrous and insoluble structural protein extensively cross-linked with disulfide, hydrogen and hydrophobic bonds. The keratinases secreted by dermatophytes catalyze the degration of the host keratin into

oligopeptides or aminoacids which may be then assimilated by the fungus. However, these enzymes cannot act before the prior reduction of disulfide bridges inside the compact keratin network that constitutes keratinized tissues. It has been recently reported that this reduction depends on a sulfite efflux pump, codified by the *Tru Ssu1* gene, belonging to the tellurite-resistance/dicarboxylate transporter (TDT) family. Sulfite secretion by this transporter allows the cleavage of the cystine and S-sulfocysteine present in keratin, making it accessible to the action of endo and exoproteases (Lechenne *et al*., 2007)

1.2.2. *Candida albicans*

C. albicans is a type of **yeast** that is commonly referred to as a polymorphic fungus since it can grow in several different forms, as a primarily yeast, hyphae or pseudohyphae. This type of yeast consider an opportunistic fungal pathogen, It is usually a **commensal** organism (form a part of normal flora of the mucous membrane of upper respiratory tract, female genital tract and gastrointestinal tract), but can become **pathogenic** under a variety of conditions. *C. albicans* differs from other medically important fungi such as *Aspergillus fumigatus*, *Cryptococcus. neoformans and Histoplasma capsulatum,* in rarely being isolated from soil. Thus, infections are categorized as endogenous and not exogenous as with others (Odds,1988).

C. albicans are the fourth most common cause of hospital-acquired systemic infections in the USA with mortality rates reached up to 50% (Pfaller and Diekema, 2010). It can cause two major types of infections in humans: superficial infections, such as oral or vaginal candidiasis, and life-threatening systemic infections.

The oral infections with *Candida* spp. are termed "oral candidiasis". This infection is predominantly caused by *C. albicans* and can affect the oropharynx or the esophagus of immunocompromized person (Pappas *et al*., 2009). It is estimated that approximately 75% of all women suffer at least once in their lifetime from vulvovaginal candidiasis. Predisposing factors for vulvovaginal Candidiasis are including diabetes mellitus, oral contraception, use of antibiotics, hormone therapy and pregnancy (Fidel, 2004).

Cutaneous Candidiasis is a fairly common infection of skin and nails (body folds). It can involve almost any skin on the body, but most often it occurs in warm, moist areas such as the axillary folds and groin.

In contrast, the systemic Candidiasis is associated with a high mortality rate, even with first line antifungal therapy. Both damage of the gastrointestinal mucosa and neutropenia are the risk factors for the development of systemic (disseminated) Candidiasis (Koh *et al.*, 2008). Other important risk factors include central venous catheters, which allow direct access of the yeast to the bloodstream, about 8%–15% of nosocomial blood stream infections are confirmed to be caused by *Candida* spp. (Pfaller and Diekema 2010).

During both systemic and superficial infection, **C. albicans** depend on a combination of virulence factors.

1.2.2.1.Virulence Factors Involved in Pathogenicity of *Candida albicans*

Like other fungal pathogens, *C. albicans* utilizing several kinds of virulence factors. Some of them are briefly described below.

(i) Adhesion

C. albicans has a specialized set of proteins called adhesins which mediate adherence to host cells, to other **C. albicans** cells, to other microorganisms and to abiotic surfaces (Verstrepen and Klis, 2006). Adhesins expressed on morphogenetically changing cell surfaces. But the striking feature of *Candida* cells is the formation of biofilms in host tissue, resulting in enhanced adherence. Well-known adhesins are the agglutinin-like sequence (ALS) proteins which form a family consisting of eight glycosylated proteins. Of the eight ALS proteins, the hypha-associated adhesin ALS 3 is especially important for adhesion (Murciano *et al.*, 2012). Another important adhesin of **C. albicans** is Hwp1 (hyphal wall protein), which is a hypha-associated glycosylphosphatidylinositol - linked protein. This adhesins serves as a substrate for mammalian transglutaminases and this reaction may covalently link **C. albicans** hyphae to host cells (Zordan and Cormack.,2012). Morphology-independent proteins can also contribute to adhesion. These include GPI-linked proteins (Eap1, Iff4 and Ecm33), non-covalent wall-associated proteins (Mp65, a putative β-glucanase, and Phr1, a β-1,3 glucanosyl transferase), cell-surface associated proteases (Sap9 and Sap10) and the integrin-like surface protein Int1 (Naglik *et al.*,2011).

(ii) Biofilm formation

One of the most important virulence factors of **C. albicans** is its ability to form biofilms on abiotic or biotic surfaces. Biofilm is the organized structures involving microbial communities that are attached to some inanimate surfaces or tissues and circumvented in a matrix of exopolymeric materials. Biofilm formation is initiated by irreversible adherence of microbial cells to tissues or devices and followed by growth and maturation to form a mesh of cells with altered phenotype, growth rate, and gene expression compared to planktonic cells (Finkel and

Mitchell, 2011). Mature biofilms are much more resistant to antimicrobial agents and host immune factors in comparison to planktonic cells. The factors responsible for heightened resistance include the complex architecture of biofilms, the biofilm matrix, increased expression of drug efflux pumps and metabolic plasticity (Fanning and Mitchell, 2012). The ability of *Candida* to form biofilms on catheters, endotracheal tubes and other prosthetic devices has contributed to its predominant prevalence in nosocomial infections (Ramage *et al.* 2005). Such devices, in addition to providing a platform for candidal cells to form biofilm, grow and develop, provide a route through host barrier defenses for dissemination. During weakened immunity, hematogenous dissemination of candidal cells from biofilms to deep-seated organs could occur, resulting in candidemia and septicemia. Recent studies have confirmed biofilm growth in the majority of diseases caused by *Candida* spp (Chandra *et al*. 2005).

(iii) Dimorphic transition

Dimorphism defined as transition from unicellular yeast form to filamentous form (hyphae or pseudohyphae). Of all the species only *C. albicans* and *C. dubliniensis* are able to undergo such morphogenesis. A number of conditions, including starvation, near-neutral pH, the presence of serum or N-acetylglucosamine, temperature of 37ºC–40ºC and CO_2 promote the transition from yeast to hyphal form (Sudbery,2011). It has been proposed that both growth forms are important for pathogenicity. Yeast forms are more suited for dissemination in tissues and to other hosts, whereas hyphal forms are required for tissue damage and invasion (Saville *et al*.,2003). Hypha formation is linked to the expression of a subset of proteins. Such hypha-associated proteins include Hwp1, Als3 (the agglutinin-like sequence protein), the secreted aspartic

proteases Sap4, Sap5 and Sap6 and the hypha-associated proteins Ece1 and Hyr1. Deletion of *HGC1*(which encodes a hypha-specific G1 cyclin-related protein) results in cells that grow normally in the yeast form but fail to produce hyphae. Nevertheless, the *hgc1*Δ/Δ mutant cells still express at least four hypha-associated genes (*HWP1*, *ECE1*, *HYR1* and *ALS3*) (Almeida *et al.*, 2008).

Additional virulence attributes, such as phenotypic switching, environmental stress response, thigmotropism and other hydrolytic enzymes such as Phospholipase and proteinase have been identified in *C. albicans*. All of these factors may be adapted to distinct stages or types of infection and are postulated to act in concert to facilitate fungal survival during the course of infections (Cutler,1991).

1.2.3. Antifungal Resistance and New Strategies to Control Fungal Infections

Regardless of the improvement of antifungal therapies over the last 30 years, the phenomenon of antifungal resistance is still of major problem in clinical practice. However, antifungal resistance is still relatively uncommon, but the trouble will likely continue to evolve unless more is done to prevent further resistance from developing and prevent the spread of these infections (Kanafani and Perfect,2008).

Several species of **Candida** and dermatophytes are becoming increasingly resistant to first-line and second-line of antifungal agents, namely, Fluconazole and Echinocandins (Caspofungin , Anidulafungin, and Micafungin). Approximately 7% of all **Candida** bloodstream isolates are resistant to Fluconazole (Vallabhaneni *et al.*,2015). CDC's (Centre for Diseases Control) surveillance data indicate that the proportion

of **Candida** isolates that are resistant to Fluconazole has remained fairly constant over the past twenty years (Lockhart *et al.*, 2012).

There are several mechanisms by which *Candida* and dermatophytes develop drug resistance. These include increased drug efflux, decrease in drug uptake, overexpression of the target molecule and structural alterations in target site. The resistance to azoles has been attributed predominantly to increased drug efflux and development of bypass pathways. Another important mechanism in *Candida* is formation of biofilm. Biofilm engages several stress response pathways that impair the activity of antifungal drugs. The persistence or progression of infection despite adequate antifungal therapy indicates clinical resistance.

Overall, the cellular targets for antifungal action are limited because both human and fungi are eukaryotic organisms (Martinez-Rossi *et al.*, 2008) Hence, the strategy to control or prevent of drug resistance is critical. Lastly, an overview of ongoing research undertaken to develop new therapeutic strategies to fight against fungal infections will be exposed. Considering the mechanisms of drug resistance, new drug targets have been identified. The new drug should act efficiently against wide range of fungi with no or minimal toxicities to host. The cellular target should be essential and conserved in variety of fungi and more importantly its counterpart should not be present in the host (Odds, 2005). Combination therapy using drugs with more than one mechanism is essential for the development of structural modifications in the antifungal drugs and may be considered to prevent drug resistance.

The use of nanoparticles is a growing new approach against drug-resistant, biofilm-mediated, and device centered infections. For anti-infection applications, various nanomaterials are being developed. This

valuable object will be further reviewed here to understand their superior properties and potential applications.

1.2.4 Nanotechnology:

1.2.4.1 Concept and history of nanotechnology

The prefix nano derived from a Greek word meaning dwarf. This term in size, comparing a single nanometer to a meter is like comparing a marble to the entire Earth. "Nano" is now a prevalent label for different modern science, and many “nano-“words have laterally appeared in dictionaries, including: nanoscale, nanometer, nanoscience, nanostructure, nanorobot, nanotube, nanowire and nanotechnology (Buzea, *et al*.,2007)

Nanotechnology is broad term that include manipulation of different matters at a scale of less than 100 nanometers which results in supernatural effects (Narayanan and Sakthivel, 2010).

Nanotechnology considered a modern science has a history dating belong to the 9^{th} century. The gold and silver nanoparticles were firstly used by the artisans of Mesopotamia to create a glistening effect to pots. The idea that seeded nanotechnology came from the renowned physicist Richard Feynman (1959) in his famous lecture titled "There's a plenty of room at the bottom" when he discussed the conception of the nanomaterial and imagined the entire Encyclopedia Britannica could be written on the head of pin(Gribbin & Gribbin,1997). The term "nanotechnology" was first applied in 1974 by the Japanese scientist called Norio Taniguchi in Tokyo University of Science in a conference (Taniguchi, 1974). In the early 1980s, two important breakthroughs boost the evolution of nanotechnology as an emerging science. First; the invention of the scanning tunneling microscope, which provides imaging

of individual atoms and bonds (Binnig and Rohrer,1986). Second; the advances in the interface and colloidal science which led to discovery of fullerenes in 1985(Adams and Baughman,2005).

Currently, the nanotechnology undergoing explosive development and provide an excellent platform for promising applications in various fields of life like medicine, food industries, communication technique, manufacturing ….etc. (Jaidev and Narasimha, 2010).

1.2.4.2: Nanoparticles (NPs)

Nanoparticles are clusters of atoms between 1-100 nm in size. In nanotechnology, the particle is defined as a small matter that comport as a whole unit regard to its transport and properties (Dubchak *et al*.,2010). NPs found in the natural world and also created as a result of human activities. Nanomaterials have unique and extremely changed chemical, physical and biological properties in comparison to their macro-scaled counterparts (Li *et al*., 2007). The submicroscopic size of NPs makes it unique material with superior Characteristics. NPs are of great interest as they are considering a link between bulk materials and molecular or atomic structures. Currently, the metallic NPs are area of intense in scientific researches as they show good antimicrobial effect resulting from their large surface area to volume ratio, which is hoped to limit the increasing microbial resistance against the antibiotics, metal ions and the development of resistant strains (Gong *et al* .,2007).

1.2.4.2.1.Classification of nanoparticles:

Nanoparticles can be broadly classified into two main groups namely: organic and inorganic NPs.

The **organic** NPs include the carbon NPs which defined as s solid particles consist from organic compounds (lipids or polymeric), their

diameter ranging from 10nm to 1 μm (Kreuter,1994). The dendrimers, liposome and carbon nanotube are an examples for the organic NPs. At the past decades, the organic NPs met a considerable expansion and a great investigation due to the wide potentialities of this type of NPs ranging from photonic, electronic, conducting materials, biotechnology, medicine and so forth (Geckeler &Nishide,2010; Grimsdale *et al.*, 2009; Pinto *et al.*, 2006).

The **inorganic** NPs, among the different nanomaterials, are highly important in modern technologies; they can be readily and inexpensively synthesized and mass produced. For this reason, they can be easily integrated into various applications (Altavilla & Ciliberto, 2010). The inorganic NPs include magnetic NPs which can be manipulated by using magnetic field such as ferrites (iron oxide NPs). Inorganic NPs also include noble metal NPs (like gold & silver) as well as the semiconductor NPs (like zinc oxide & titanium dioxide) (Marin *et al.*,2014). Currently, metallic NPs are heavily utilized in biomedical sciences and engineering. There is a focus of interest on the metallic NPs as they provide a superior properties and huge potential in nanotechnology. These materials can be synthesized with a various chemical functional group which make them conjugatable with different ligands, antibodies and drugs. This opened a wide range of applications in magnetic separation, biotechnology, drug delivery and more importantly diagnostic imaging such as MRI, PET, CT, ultrasound and SERS (Cheon &Horace, 2009)

1.2.4.2.2. Unique properties of NPs:

At the critical size of NPs, considerably less than 100 nm, a number of new physical and chemical phenomena become more pronounced. The critical size is hardly associated to the exponential excess in the atoms

number localized at the surface as the size decrease. The smaller NPs have a unique size-dependent properties which are drastically differ from their bulk material, certain phenomena might be not come into play when the system moves from macro to micro level, but may be more significant at the Nano scale (Moreels *et al.*, 2007).

The important question is why the material behaves differently at the nanoscale level? Firstly, extremely small particle has a larger surface area in contrast to the equal amount of material in a larger mass (for example: grains of sand able to cover a surface area bigger than the same amount of sand compressed into a stone). In chemical reaction, the larger surface area makes the material more reactive. Secondly, when the material reach to the nanoscale level, all the different law of physics shift and become more significant, particularly for the sizes less than 20 nm .The material at a nanoscale size characterized by: high surface energy, large fraction of surface atoms, spatial confinement and reduced imperfections (Lue,2007).

1: Optical properties:

Metal NPs excrete very motivating optical properties due to the collective oscillation of conduction electrons, when interacting with electromagnetic radiation.

Surface plasmon resonance (SPR) represents a relevant property. SPR is the collective oscillation of the conduction electrons in resonance with the light field. SPR occur when polarized light strikes an electrically

conducting surface at the interface of atoms media. This generates electron charge density waves called plasmon, reducing the intensity of reflected light at a specific angle called resonance angle, in proportion to the mass on a sensor surface. The SPR frequency depends on the type of material, size, shape and the dielectrical properties of the surrounding medium. The origin of the color of nanomaterials attributed to the SPR phenomena, the gold NPs give deep red to black color in solution (Jain *et al*., 2007).

2: Magnetic properties

One of the important size- dependent properties is superparamagnetism in magnetic NPs, it occur when the material composed of very small particles, with a size range from 1 to 10 nm (Lu *et al*.,2007). In the Superparamagnetism NPs, the magnetization can randomly change direction when the temperature is above the blocking temperature. In this state, the NPs possess a large magnetic moment and behave like a gaint paramegnetic atom with a fast response to applied field with unassuming remanence and coercivity (Obaidat *et al*.,2015).

The ultrasensitive analytical instruments and MRI are applications of magnetic property of nanomaterials.

3: Mechanical properties

NPs display mechanical properties different from those of the microparticles or bulk materials. The mechanical properties of material depend on the bonds between the atoms, it have many basics such as elastic modulus, hardness, interfacial adhesion and friction as well as their size dependent effects (Guo *et al*. 2014). At the nanoscale, the material tends to be single crystals and its elastic modulli reduced

dramatically. This provide more influential options for the surface modification of many devices in the mechanical strength or provide improvement of the quality of nanofabrication/ nanomanufacturing..etc. the mechanical property of NPs can reinforce composite coating (Hussain *et al* .2006).

4: Melting points

Melting temperature is one of the essential properties of materials, it doesn't size dependent in bulk materials. However, when the dimension of material lowering towards the nanoscale and approach the atomic level, the melting temperature decreases with the material dimensions. This phenomenon called melting- points depression, it is very distinguishable in nanoscale materials, which melt at a temperature less than bulk material by hundreds of degrees (Jiang *et al.*,2007).

It is noticeable that the surface atoms are not completely enclitic by other atoms, but existent exposed and unbound surfaces. Thus, it is reasonable to expect that exposed atoms would display different behavior and reactivity than the embedded bulk atoms. For example: the gold NPs at 2-4 nm size have a reduced melting point by ~300C compared to bulk gold, 1nm of gold NPs be liquid phase at room temperature (Roduner,2006).

1.2.4.4.3: Application of nanoparticles.

The research of NPs is an area of scientific interest due to broad range of potential applications in various fields such as optical, electronic, alternative Energy, Soil/Water Remediation, cosmetics, catalytical and material industries. A complete list of the potential applications of nanotechnology is too vast and diverse to discuss in detail but Without any doubt, biomedical and pharmaceuticals application are the most

important applications affecting human life. Because of the high uses of metallic NPs in biomedical domains, a constant development is feasible in this area. Having numerous viewpoints to improve the diagnosis and treatment of human sicknesses, nano medicine is a growing field of study (Fadeel and Garcia-Bennett, 2010). The publication of numerous reviews and articles investigating the uses of NPs in biomedicine has been reported (Richhariya *et al*.,2015; McNarnara andTofail, 2017). Since the domain of biosynthesized NPs is somewhat novel, investigators have already began to explore their use in different areas of biomedical applications. To show these applications, a number of examples are given in this review.

1: Delivery of drugs, peptides and Nucleic acids.

Drug delivery describes the method and approach to delivering drugs or pharmaceuticals and other xenobiotic to their site of action within an organism, with the goal of achieving a therapeutic outcome. It is obvious that the nanotechnology offer platform for targeted **drug delivery** which is expected to increase the efficacy of the drug and diminish possible side-effects, through the reduction of the dose and targeting the drug to the desired site of action. Nanocrystals offer the advantage of high drug loading which makes them very efficient in transporting drug to or into cells, reaching at high therapeutic concentration for the pharmacological effect. The aims for NPs entrapment of drugs are either enhanced delivery to, or uptake by, target cells and/or a reduction in the toxicity of the free drug to non-target organs (De Jong and Borm,2008).

The brain is perhaps one of the smallest approachable organs for the drug delivery due to the presence of blood brain barrier that determine the transfer of endogenous and exogenous compounds, and thus providing the neuroprotective function. The drugs that are unable to cross blood

brain barrier may be delivered to the brain after binding to the surface modified polybutyl cyanoacrylate nanoparticles **(**Richhariya *et al*.,2015). Functionalized gold NPs represent highly attractive and promising candidates in the applications of drug delivery owing to their unique dimensions (Han *et al* .,2007). Large surface to volume ratio of gold NPs offers a large number of drug molecules being carried by these NPs **(**Daraee *et al.*, 2016). Fluconazole loaded NPs were prepared and characterized for different parameters. The data obtained from *in vitro* and *in vivo* experiments revealed that using of the NPs provided a good skin targeting effect and might be a promising carrier for topical delivery of Fluconazole offering the sustained release and maintain the localized effect, resulting in an effective treatment of a life-threatening cutaneous fungal infection (Gupta and Vyas, 2012).Other study has been found that Amphotericin B nanotubes has shown enhanced drug delivery to the interior of cells, increased antifungal efficacy and reduced toxicity to mammalian cells when compared to amphotericin B administration without nanotubes (Prato *et al*.,2008).

Iron oxide NPs (IONPs) have emerged as particularly promising nanocarriers because of their biodegradability, ability to be guided magnetically to sites of pathology, mediation of hyperthermic therapy, and imaging capabilities

2: Anticancer NPs

Numerous studies have focused their attention on the promising use of nanomaterials. Cytotoxicology effect of different NPs against different cancer cell lines have been reported. Gold NP biosynthesized via the supernatant, live cell filtrate and biomass of the fungus *Penicillium brevicompactum* were investigated against mouse mayo blast cancer C2C12 cells (Mishra *et al*., 2011).

He *et al*., (2016) suggest that green-synthesized silver NPs may act as potential beneficial molecules in lung cancer chemoprevention and chemotherapy, especially for early-stage intervention. Similarly, the biomolecule coated silver NPs exposure showed potential hepatoprotective effect against liver cancer and could be used as an effective anticancer nanodrug (Prasannaraj & Venkatachalam ,2017).

3: Medical Imaging

Biomedical imaging has received enormous attention in view of its capacity to aid analysis and diagnosis through images at the molecular and cellular levels. . For example, MRI, CT, PET and SPECT (Single-photon emission computed tomography) which have high spatial resolution and are able to provide detailed anatomical information (James & Gambhir,2012).

4: Antimicrobial activity

Recently, with the outbreak and augmentation of the resistance of microorganisms to numerous antibiotics, there has been an emphasis on NPs-based antimicrobial agents. Nanotechnology may be particularly advantageous in treating bacterial infections. Examples include the utilization of NPs in antibacterial coatings for implantable devices and medicinal materials to prevent infection and promote wound healing (Wang *et al*.,2017). NPs in particular have demonstrated broad-spectrum antibacterial properties against both Gram-positive and Gram-negative bacteria. For example, ZnO NPs were found to inhibit **Staphylococcus aureus**, and silver NPs exhibit concentration-dependent antimicrobial activity against **Escherichia coli** and **Pseudomonas aeruginosa** (Ramalingam *et al.,* 2017). The combination of nanosilver and a mixture of poly vinyl alcohol and chitosan have been studied in this context, and the resulting fiber mat can be used in wound healing. The high specific

surface area of nanosilver results in good contact with bacteria, significantly inhibiting their growth and increasing the rate of wound healing. (Li *et al.,* 2013). A concentration of nano silver as low as 0.05% can significantly reduce the number of arthroplasty surgery-related infections, including methicillin–resistant **Staphyloccocus aureus** (MRSA), **Staphyloccocus aureus**, **Staphyloccocus, epidermidis**, and **Acinetobacter baumannii** infections **(** Kose *et al*.,2016). Use of NPs is among the most promising strategies to overcome microbial drug resistance. It can overcome existing drug resistance mechanisms, including decreased uptake and increased efflux of drug from the microbial cell, biofilm formation, and intracellular bacteria. Finally, NPs can target antimicrobial agents to the site of infection, so that higher doses of drug are given at the infected site, thereby overcoming resistance (Pelgrift and Friedman ,2013)

Silver NPs (SNPs) have proven to exert anti-HIV activity at an early stage of viral replication, most likely as a virucidal agent or as an inhibitor of viral entry. SNPs bind to gp120 in a manner that prevents CD4-dependent virion binding, fusion, and infectivity, acting as an effective virucidal agent against cell-free and cell-associated virus. Besides, SNPs inhibit post-entry stages of the HIV-1 life cycle (Lara *et al.,* 2010). Several studies showing that metal NPs can be effective antiviral agents against hepatitis B virus (Lu *et al*., 2008), respiratory syncytial virus (Sun *et al*., 2008), herpes simplex virus type 1 (Baram-Pinto *et al*., 2010) and influenza virus (Papp *et al*.,2010) .

The search for new fungicides and alternatives is of paramount importance to combat newly emerging resistant strains of fungal pathogens (Kanhed *et al.*, 2014). In particular, because of the recent advances in research on metal NPs, SNPs have received special attention

as a possible antifungal agent. Compared with other metals, silver exhibits higher toxicity to microorganisms while it exhibits lower toxicity to mammalian cells (Zhao and Stevens, 1998). The antifungal effects of SNPs have been studied by different researchers. Teimoori *et al*., (2017) described the antifungal properties of biosynthesized NPs against *Macrophomina phaseolina, Alternaria alternata* and *Fusarium oxysporum.* SNPS obtained by green synthesis showed high activity against **Candida** spp. and could represent an alternative for fungal infection treatment (Mallmann *et al*., 2015). In another study, SNPs had a significant inhibitory effect on the growth of **Trichosporon asahii**. It obviously damaged the cell wall, cell membrane, mitochondria, chromatin, and ribosome (Xia *et al*., 2016). SNPs showed a high antifungal activity against **C. albicans** , **C. krusei** , **C. glabrata** , **C. parapsilosis** , **C.tropicalis** , **C. neoformans** and **Cryptococcus gattii**. Morphological alterations of **Cryptococcus neoformans** treated with SNPs were observed such as disruption of the cell wall and cytoplasmic membrane and loss of the cytoplasm content (Ishida *et al*., 2014).

Many studies have shown the potential antimicrobial effects of NPs against fungal pathogens of the skin including some dermatophytes spp. Acute dermal toxicity studies on SNP gel formulation in rats showed complete safety for topical application. These results clearly indicate that SNPs could provide a safer alternative to conventional antimicrobial agents in the form of a topical antimicrobial formulation (Jain *et al*., 2009)

1.2.4.3. Silver nanoparticles (SNPs)

SNPs are Metal particles in the nanometer size range (1-100 nm) exhibit physical properties that are different from both the ion and the bulk material. Nanosilver, is one of the nanomaterials with the highest

degree of commercialization, as 30% of all products currently registered in nano-product databases claim to contain SNPs (Sharma *et al*.,2009). Numerous shapes of NPs can be constructed depending on the application at hand. Commonly used are spherical SNPs but diamond, octagonal and thin sheets are also popular.(Christina *et al* 2003). Silver has been in use since time immemorial in the form of metallic silver, silver nitrate, silver sulfadiazine for the treatment of burns, wounds and several bacterial infections. But due to the emergence of several antibiotics the use of these silver compounds has been declined remarkably (Rai *et al.* ,2009). Metallic silver in the form of SNPs have unique optical, electrical, and thermal properties and are being incorporated into several applications. SNPs have been commonly used in different pharmacy applications and drug delivery systems due to their inert nature, stability, high disparity, non-cytotoxicity, and biocompatibility (Alaqad and Saleh, 2016).

1.2.4.4. Biosynthesis of silver nanoparticles.

The major methods used for conventional synthesis of SNPs are the physical and chemical methods. The problem with these methods is that the synthesis is energy and capital intensive, employ toxic chemicals, and often yield particles in non-polar organic solutions, as well as some chemically toxic substances being absorbed on the surface and can hinder their usage in medical applications(Parashar *et al*.,2009). Moreover, chemical synthesis of silver colloids mostly leads to aggregation as the period of storage increases (Abou El-Nour *et al.*,2010).

The growing need to develop environmentally friendly and economically feasible technologies for material synthesis led to the search for biomimetic methods of synthesis. There are three major sources of biological synthesis of SNPs: plant extracts, bacteria and

fungi. Biosynthesis of SNPs is a bottom-up approach that mostly involves reduction/oxidation reactions. It is majorly the microbial enzymes or the plant phytochemicals with antioxidant or reducing properties that act on the respective compounds and give the desired NPs. The three major components involved in the preparation of NPs using biological methods are the solvent medium for synthesis, the environmentally friendly reducing agent, and a nontoxic stabilizing agent. (Prabhu and Poulose 2012). Biological methods for NPs synthesis would help circumvent many of the detrimental features by enabling synthesis at mild pH, pressure and temperature and at a substantially lower cost.

A number of micro-organisms have been found to be capable of synthesizing inorganic nanocomposites either intra- or extracellularly. Microbial source to produce the SNPs shows the great interest towards the precipitation of NPs due to its metabolic activity. Of course the precipitation of NPs in external environment of a cell, it shows the extracellular activity of organism. Extracellular synthesis of NPs using cell filtrate could be beneficial over intracellular synthesis, the fungi being extremely good candidates for extracellular process and also environmental friendly.(Natarajan *et al* .,2010).

1.2.4.5. Fungal-Derived Synthesis of SNPs

When SNPs are produced by chemical synthesis, three main components are needed: a silver salt (usually $AgNO_3$), a reducing agent (i.e. ethylene glycol) and a stabilizer or capping agent (i.e. PVP) to control the growth of the NPs and prevent them from aggregating. In case of the fungal biosynthesis of SNPs, the reducing agent and the stabilizer are replaced by molecules produced by living fungi. (Sintubin *et al*., 2012). Fungi have some distinct advantages when used as bio factories

for NP production (Figure 1-1), in comparison with bacteria; fungi can produce larger amounts of NPs because they can secret larger amounts of proteins which directly translate to higher productivity of NPs (Mohanpuria *et al.*, 2008). Fungi have attracted more attention regarding the research on biological production of metallic NPs due to their toleration and metal bioaccumulation capability (Sastry *et al*.,2003), The easiness of fungi scale-up is a separate privilege of utilizing them in NPs synthesis (e.g., utilizing a thin solid substrate fermentation technique). Economic livability and facility of employing biomass is another merit for the utilization of green approach mediated by fungai to synthesize metallic NPs. Moreover, a number of species grow fast and therefore culturing and keeping them in the laboratory are very simple (Castro-Longoria *et al.*, 2011). Fungi are able to produce metal NPs and nanostructure via reducing enzyme intracellularly or extracellularly.

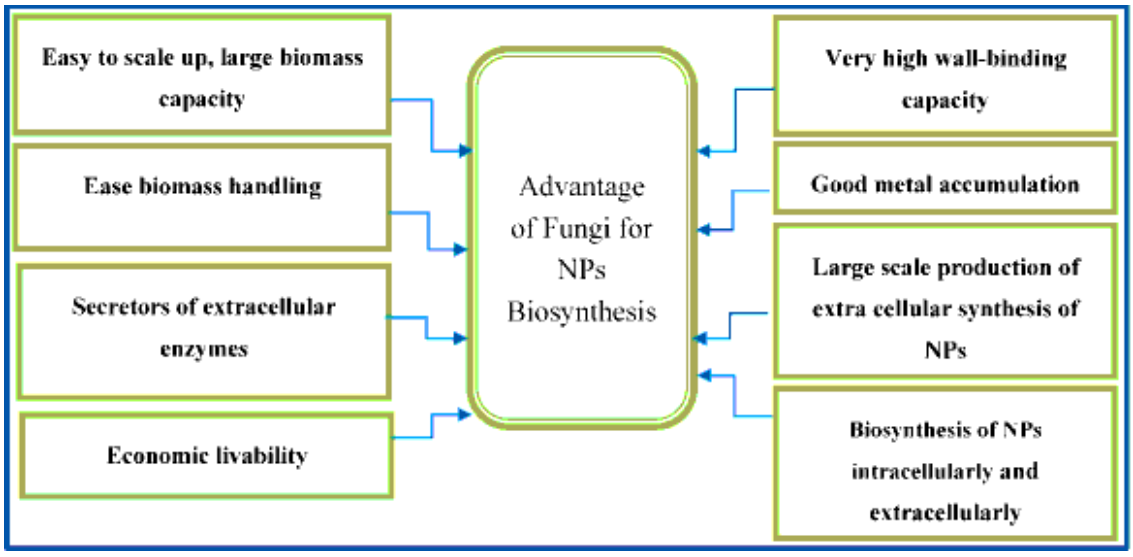


Figure (1-1). Fungi have some distinct advantages when used as bio factories for NP production (Moghaddam *et al*., 2015).

Different fungal species are utilized; and diverse NPs are formed under the similar experimental circumstances. In this review, we provide

a short summary of the study focused on the utilization of fungi in the biosynthesis of SNPs (Appendix).

1.2.4.6. The mechanistic aspects of NPs formation

Though there are many studies reporting the biosynthesis of various NPs by different microorganisms, there is very little information available regarding the mechanistic aspects of their production.

The most widely accepted mechanism of silver biosynthesis is the presence of the nitrate reductase enzyme. The enzyme converts nitrate into nitrite. In *in vitro* synthesis of silver using bacteria, the presence of alpha-nicotinamide adenine dinucleotide phosphate reduced form (NADPH) - dependent nitrate reductase would remove the downstream processing step that is required in other cases. During the reduction, nitrate is converted into nitrite and the electron is transferred to the silver ion; hence, the silver ion is reduced to silver (Ag^{+} to Ag^{0}) (Vaidyanathan *et al*., 2010). The mechanism was further confirmed by using purified nitrate reductase from *Fusarium oxysporum* and silver nitrate along with NADPH in a test tube, and the change in the color of the reaction mixture to brown and further analysis confirmed that SNPs were obtained (Kumar *et al* ., 2007). The mechanism of SNPs production by fungi is said to follow the following steps: trapping of Ag^{+} ions at the surface of the fungal cells and the subsequent reduction of the silver ions by the enzymes present in the fungal system (Mukherjee *et al*., 2001). The extracellular enzymes like naphthoquinones and anthraquinones are said to facilitate the reduction. Considering the example of *F. oxysporum*, it is believed that the NADPH dependent nitrate reductase and a shuttle quinine extracellular process are responsible for NPs formation (Ahmad *et al.*, 2003). Though the exact mechanism involved in SNPs production by fungi is not fully deciphered, it is believed that the above mentioned

phenomenon is responsible for the process. Jain *et al.* (2011) indicated that SNPs synthesis for *A. flavus* occurs initially by a "33kDa" protein followed by a protein (cystein and free amine groups) **electrostatic attraction** which stabilizes the NPs by forming a capping agent. Intracellular SNPs synthesis is not fully understood but similar fungal cell wall surface electrostatic attraction, reduction, and accumulation has been proposed (Ahmad *et al.*,2002). **Zomorodian** *et al.*(2016) investigated the relationship between nitrate reductase activity and the efficiency of *Aspergillus* species in the production of SNPs.

Aspergillus fumigatus was the most efficient species had the highest nitrate reductase activity among the studied fungi. It produced greater amount of SNPs with smaller size and higher monodispersity in comparison with other species. On the other hand, *Aspergillus flavus* exhibited the lowest capacity in the production of SNPs which was in agreement with its low nitrate reductase activity. Hence, the difference in the biosynthesis of SNPs between the studied *Aspergillus* species might be related to their different ability in the production of nitrate reductase enzyme.

1.2.4.7. Possible mechanisms for SNPs antimicrobial action.

The precise mechanism of their mode of antimicrobial action is not fully understood yet; however there are several theories may explain the mechanistic aspects of antimicrobial action of SNPs (Figure 1-2):

1. Because of their extremely large surface area, SNPs get attached to the microbial cell wall and subsequently penetrate it after causing structural changes which leads to cell lysis (Rai *et al.*, 2009).

2. SNPs bind to the sulphur and phosphorous containing bases of the DNA of microorganisms perhaps inhibiting their function (Matsumura *et al.*, 2003).

3. They release silver ions which interact with the thiol groups of many vital respiratory enzymes and inactivates them resulting in microbial cell death (Sondi and Sondi, 2004).

4. They get accumulated inside the microbial cells and have a sustained release of Ag^+ inside the cells which may create free radicals and induce oxidative stress, thus further enhancing their killing activity (Song *et al.*, 2006)

5-Considering the well-documented crucial importance of the transmembrane proton gradient in overall microbial metabolism, it seems inevitable that the elimination of proton motive force should result in cell death.

6- It was found that the NPs dephosphorylate the peptide substrates on tyrosine residues, which leads to signal transduction inhibition and thus the stoppage of growth (Shrivastava ,2008).

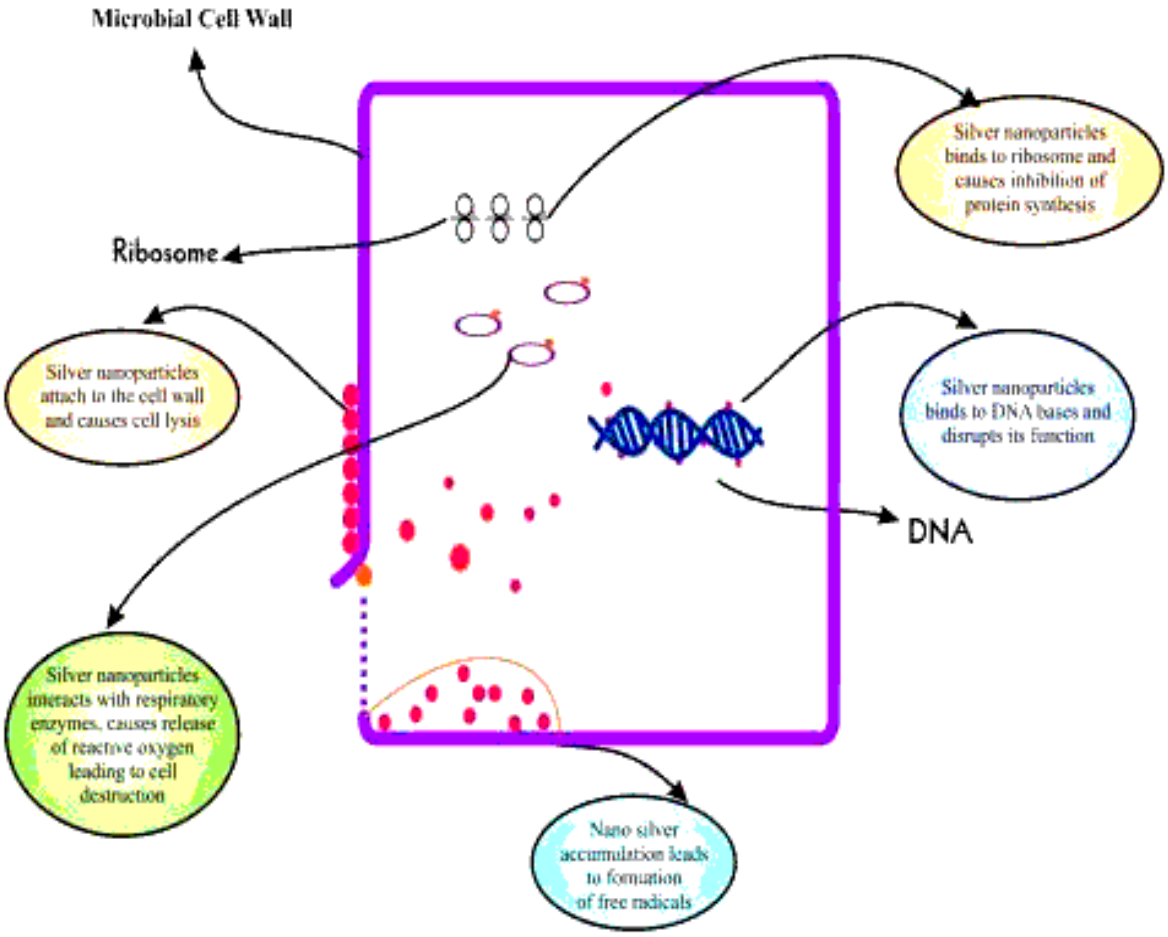


Figure 1-2:Mechanisms of antimicrobial action of SNPs on microbial cell.(Prabhu and Poulose, 2012)

1.2.5. Previous studies on the biosynthesis of nanoparticles in Iraq

There has been considerable significant research in Iraq in the field of bio- synthesis of NPs. More research has been found to be concentrated in the area of biosynthesis using plants. It has been observed that use of different plant extract are capable of reducing silver nitrate to silver NPs (Al-Kalifawi,2016; Saliem *et al*.,2016).

Recently, scientists in Iraq have reported the green synthesis of different kinds of NPs using different species of fungi. Muhsin and Hachim (2014) have reported the green synthesis of SNPs using the fungus *Curvularia tuberculata* isolated from soil samples, Particles in the size range of 10-50nm were obtained.

Fusarium solani isolated from soil has been used for the synthesis of gold NPs; particles were spherical and in the range of 18-24 nm without significant agglomeration (Mezeel,2016).

There are several literatures supporting the antibacterial activity of biosynthesized NPs. The synthesized SNPs by using the fungus *Nigrospora sphaerica* exhibited a growth inhibition activity against five human pathogenic strains of bacteria namely *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Staphylococcus aureus* and *Salmonella typhi* . SNPs were also biosynthesized extracellularly using the mold *Fusarium graminaerum* which isolated from poultry feed; the produced SNPs have antimicrobial activity against *Salmonella* spp., *Pseudomonas aeruginosa*, *Candida albicans* and against *E.coli* (Shafiq *et al*.,2016).

However, the use of biosynthesized NPs as antifungal agent against pathogenic fungi especially dermatophytes and their virulence factors, is still a largely unexplored area.

Materials and Methods

2. Materials and Methods

2.1. Materials

2.1.1. Equipments and Instruments:

Table (2-1) : **Equipments and instruments used in this study:**

Equipments and Instruments	Company
Autoclave	Sturdy (Taiwan)
Cold centrifuge	Hettich (Germany)
Electric incubater	Memmert (Germany)
Electric oven	Memmert (Germany)
Fourier Transform Infrared Spectrometer (FTIR)	Bruker Tensor 27 (Germany)
Hot plate with magnetic stirrer	Heidolph (Germany)
laminar flow cabinet	Shin Saeng (South Korea)
Light Microscope	Olympus (Japan)
Micropippetes (in different size)	Eppendorf (germany)
Microplate reader	DNM9602 (Germany)
PH-meter	Jenway (UK)
Scanning Electron Microscope (SEM)	Inspect S50/FEI (Netherland)
Sensitive balance	Sartorius (Germany)
Shaking incubator	Lab Tech (India)
Slides and cover slides	Superstar (India)
Sterile cotton swabs	china
Sterile syringes	china
Tissue culture plates (TCP)	China
UV. visible spectrophotometer	SPEKOL 1300 (Germany)
Vortex	Stuart (UK)
Water bath	Kottermann (Germany)
Water distillatory	Lab Tech (India)

2.1.2. Chemicals and Biological Materials

Table (2-2): Chemicals and biological materials used in this study:

Chemicals &Biological Materials	Manufacturers Name
Acetone	BDH (England)
Calcium chloride (CaCl2)	Sigma (USA)
Chloroform	BDH (England)
Crystal violet	Sorachim (Switzerland)
Cyclohexamide	Fluka (Germany)
Deionized water	Bioneer (korea)
Dextrose	Sigma (USA)
Dimethyl sulfoxide (DMSO)	Sigma (USA)
Dipotassium hydrogen phosphate($K2HPO_4$)	Hazard (UK)
Ethanol (96%)	BDH (England)
Glucose	Sigma (USA)
Glycerol	Fluka (Germany)
Human Serum	Al Diwaniya teaching hospital
Iron sulfate ($FeSO_4$)	Sigma (USA)
Iron Sulfate Heptahydrate ($FeSO_4.7H_2O$)	Fluka (Germany)
Lactophenol cotton blue	BHD (England)
Magnesium sulfate ($MgSO_4$)	Hazard (UK)
Magnesium Sulfate Heptahydrate (MgSO4.7H2O)	Sigma (USA)
Malt extract	Fluka (Germany)
Methanol	BDH (England)
Normal saline	china
Pepton	Oxoid (England)
Phosphate buffer	BioBasic (Canada)
Potassium bromide (KBr)	Bruker (Germany)
Potassium chloride (KCl)	Sigma (USA)
Silver nitrate ($AgNO_3$)	Sigma (USA)
Sodium chloride (NaCl)	BDH (England)
Sodium hydroxide (NaOH)	Fluka (Germany)
Sodium nitrate ($NaNO_{3)}$	Sigma (USA)

Sucrose	Sigma (USA)
Yeast extract	Sigma (USA)
Zinc sulfate ($ZnSO_4$)	Sigma (USA)

2.1.3. Antibiotics

Antibiotics and their description used in the present study are listed in Table (2-3).

Table (2-3): Antibiotic and their remarks:

Antibiotics Class	Antibiotic subclass	Antibiotic Name	Symbol	Manufactures Company
Azole	Triazoles	Fluconazole	FCZ	Sigma (USA)
	Imidazoles	Miconazole	MCZ	Sigma (USA)

2.1.4. Culture Media

Table (2-4): Culture media used in this study:

Media	Origin / State
Czapek broth	Prepared in lab.
Glucose Peptone Yeast Broth (GPYB)	Prepared in lab.
Keratin induction medium	Prepared in lab.
Potato Dextrose Agar (PDA)	Himedia (India)
Potato Dextrose Broth (PDB)	Prepared in lab.
Sabouraud Dextrose Broth (SDB)	BDH (England)
Sabouraud's Dextrose Agar	Himedia (India)
Sabouraud's Dextrose Agar with Chloramphenicol & Cycloheximide	Prepared in lab.

Malt Yeast Peptone Glucose (MYPG)	Prepared in lab.

2.2 Methods

2.2.1: Prepartion of culture media

2.2.1.1. Potato Dextrose Broth (PDB)

Composition

Ingredients	**Gms / Litre**
Potatoes, infusion from	200
Dextrose	20
Chloramphenicol	0.050
Final pH (at 25°C)	5.6±0.2

Directions: Suspend the ingredient in 1000 ml distilled water. Heated until boiling to dissolve the component completely. Sterilized by autoclaving at 15lbs pressure (121°C) for 15 minutes.

Potato Dextrose Broth is used for biomass production of *Aspergillus niger*, stimulating sporulation and maintaining stock cultures of certain dermatophytes. PDB with chloramphenicol is used to inhibit the bacterial growth (Wehr and Fran, 2004).

2.2.1.2. Sabouraud's Dextrose Agar with Chloramphenicol and Cycloheximide (SDA). (Scognamiglio *et al.*,2010)

Composition

Ingredients	**Gms / Litre**
Mycological peptone	10
Dextrose	20

Chloramphenicol	0.05
Cycloheximide	0.5
Agar	15
Final pH (at 25°C)	6.8±0.2

Directions: Suspend the ingredients in 1000 ml distilled water. Heated until boiling to dissolve the components completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mixed well and poured into sterile Petri plates.

This medium was used for cultivation and maintenance of fungi.

2.2.1.3. Czapek Broth

Composition

Ingredients	**Gms / Litre**
Sucrose	30
Sodium nitrate	3
Dipotassium phosphate	1
Magnesium sulphate	0.5
Potassium chloride	0.5
Ferrous sulphate	0.01
Final pH	7.3±0.2

Directions: Suspended the medium in one liter of distilled water. Mixed well and dissolved by heating with frequent agitation. Boiled for one minute until complete dissolution. Dispensed into appropriate containers and sterilized in autoclave at 121°C for 15 minutes.

Czapek medium produces luxuriant growth of most saprophytic Aspergilli causing the organisms to produce characteristic mycelia and conidia. (Hurst *et al*.,2002)

2.2.1.4. Glucose Peptone Yeast Broth Medium (GPYB). (Birla *et al.*, 2013)

Composition

Ingredients	**Gms / Litre**
Glucose	50
Peptone	10
Yeast extract	10
Final pH	7

Direction: Suspended the components in 1000 ml distilled water and boiled, then sterilized by autoclaving at 15 lbs pressure (121 ºC) for 15 minutes. For the best results the medium used on the day of preparation.

This medium was used for optimization of the SNPs synthesis by *A.niger*.

2.2.1.5.Malt Yeast Peptone Glucose Broth (MYPG).(Birla *et al.*, 2013)

Composition

Ingredients	**Gms / Litre**
Malt extract	3
Yeast extract	3
Peptone	5
Glucose	20
Final pH	7

Direction: Suspended the components in 1000 ml distilled water and boiled, then sterilized by autoclaving at 15 lbs pressure (121 ºC) for 15 minutes. For the best results the medium used on the day of preparation.

This medium was also used for optimization of the SNPs synthesis by *A.niger*.

2.2.1.6. Keratin Induction Medium. (Wawrzkiewicz *et al.*, 1991)

Composition

Ingredients	**Gms / Litre**
$K2HPO_4$	1.5
$CaCl_2$	0.025
$MgSO_4.7H_2O$	0.05
$ZnSO_4.7H_2O$	0.005
$FeSO_4.7H_2O$	0.015
Final pH	7

Direction:, Five hundred mg of keratin source were added to this mineral salt medium. Fifty ml of prepared medium was transferred to 250 ml capacity flasks and autoclaved at 121ºC for 15 min.

This medium was used for the induction of the crude keratinase enzyme production by dermatophytes .

2.2.2: Fungal isolates and their maintenance:

The reference fungal isolates of *Aspergillus niger* was kindly obtained from fungal research laboratory of Prof. Dr. Majeed M. Dewan / Agriculture College/ Al-Kufa University that it was previously isolated from soil sample and diagnosed by PCR technique as non-toxigenic isolate. The fungus was subcultured on Potato Dextrose Agar (PDA) at 28ºC for 96 hours and then refrigerated at 4ºC until used for biosynthesis of SNPs.

Five reference isolates of dermatophytes were used in this study for detection of their sensitivity to synthesized SNPs, namely, *Trichophyton mentagrophytes, Trichophyton rubrum, Trichophyton interdigitale, Microsporum canis* and *Epidermophyton floccosum* , in addition to one strain of *Candida albicans*. All were obtained from laboratory of Microbiology/ College of Medicine/ Al-Qadisiyah University, it was kindly supplied and diagnosed by Prof. Dr. Adnan H. Al-Hamdani . The isolates were maintained on Sabouroud Dextrose Agar (SDA) slant at 4ºC and subcultured regularly.

2.2.3: Fungal biomass production

To prepare the fungal biomass for NPs biosynthesis, the *Aspergillus niger* was cultured aerobically in Potato Dextrose Broth media (a common mycological media) in 1000 ml Erlenmeyer flask .The broth was supplemented with chloramphenicol (250 mg/L) as an antibacterial agent and the pH was adjusted to 6.5 .The flasks containing above media were incubated at 28 ºC for 7 days in shaking incubator then agitated at 100 rpm.

After 7 days of incubation , fungal mycelia were separated from broth by filtration with sterile Whatmann filter paper No.1 and the settled mycelia were washed thrice with sterile distilled water to remove any medium components from the biomass that might interact with metal ions. Twenty grams of fungal biomass were weighed and inoculated in 200 ml deionized water for 72 hours in 500 ml Erlenmeyer flask and agitated as earlier described. After the incubation, the cell filtrate was separated by filtration through Watmann filter paper No.1. The filtrate was further used for biosynthesis of NPs (Vigneshwaran *et al*.,2007)

2.2.4: Biosynthesis of nanoparticles

For biosynthesis of SNPs, 50 ml of cell –free filtrate was mixed with 50 ml of 1mM $AgNO_3$ in 250 ml Erlenmeyer flask and kept in shaking incubator at 150 rpm at 25°C for 24 hours. Simultaneously, a positive control of cell filtrate without metal salts and a negative control containing only metal salts solutions were run along with the experimental flasks (Basavaraja *et al.,* 2008). All reaction mixtures were kept in dark to avoid any photochemical reactions during the experiment. Sample of 3ml was withdrawn at fixed interval and the absorbance was taken.

2.2.5: Characterization of biosynthesized SNPs:

Various properties of the biosynthesized SNPs were investigated

2.2.5.1: UV-Vis spectroscopy analysis

The detection of SNPs was primarily carried out by visual observation of color change of the fungal filtrate after treatment with silver nitrate. Appearance of dark brown color of fungal cell filtrate indicates the formation of SNPs due to reduction of pure silver ions. Further, the

formation of SNPs were confirmed with the help of dual beam UV-Visible spectrophotometer, through sampling of $1cm^3$ of reaction solution at different time intervals and scanning the absorbance spectra in 300–700 nm range of wavelength at a resolution of 1nm. It is well known that, for monodispersed NPs, only one plasma band is obtained and the increase of its intensity is an indication of the reaction advance degree with subsequent increment in the number of particles (Birla *et al.*,2013).

2.2.5.2: Fourier Transform Infrared Spectroscopy (FTIR)

The interaction between the biosynthesized SNPs and biomolecules (which responsible for reduction, capping and stabilization of the SNPs in colloidal solution) was analyzed using FTIR spectrophotometer in the range of 500 -4000 cm^{-1}. The powder samples for FTIR analysis were prepared by centrifugation of SNPs solution at 10000 rpm for 15 minutes, the solid pellet then washed with deionized water for three times to remove any unattached molecules to the surface of SNPs. The residues then dried at 40ºC before subjecting to the FTIR analysis.

The samples were mixed with KBr (the binding agent) and put into discs at high pressure (hydraulic pressure), finally the discs were scanned to obtain the FTIR spectra (Umoren *et al.*, 2014).

2.2.5.3: Electron Microscopy studies.

The biosynthesized SNPs were also subjected to SEM and TEM analysis to evaluate their size and morphological characteristics. A drop of solution containing synthesized SNPs was placed on the carbon coated copper grids and kept until sample gets dried before loading them onto a specimen holder. The micrographs were taken by analyzing the prepared

grids at a voltage of 12.50kV at different magnifications, as indicated on the SEM and TEM images (Umoren *et al.*, 2014).

2.2.5.4: Energy dispersive X-ray spectroscopy (EDX)

Compositional analysis of the sample was carried out by the energy dispersive X-ray spectroscopy (EDS) attached with the SEM.

2.2.6: Optimization of physic-chemical conditions

Different parameters such as culture media, temperature, pH, concentration of silver nitrate and time were standardized for the optimum and maximum synthesis of SNPs. For each parameter, there was respective control. The experiment was done in triplicate for reproducibility. The UV-Visible spectrophotometer was used to monitor the resulting solutions with varying reaction parameters.

2.2.6.1: Effect of different culture media

In order to understand the effect of culture media on fungal activity and biosynthesis of NPs, five different media ,namely: Potato Dextrose Broth, Glucose Peptone Yeast Broth, Czapek broth, Malt Yeast Pepton Glucose and Sabourod Broth were tested . All the above media were evaluated for the optimum NPs synthesis. The fungal mycelia were grown for 7 days in 500 mL Erlenmeyer flasks, each containing 250 ml of test medium. The synthesis of SNPs was carried out as mentioned above.

2.2.6.2: Effect of pH

Hydrogen ion concentration has an important influence on fungal growth and enzyme production which is required for the biosynthesis of NPs. In order to study the effect of pH concentration, the biomass was

transferred to distilled water with different pH, namely, pH 5, pH 7and pH 9. To get the required pH, the distil water adjusted by 0.1 N HCl and 0.1 M NaOH and the final pH measured by using electrical pH meter before incubation for 72 hours at 28 ºC.

2.2.6.3: Effect of temperature

Temperature is an essential factor affecting NPs production. The effect of different temperatures on SNPs production by *Aspergillus niger* is carried out by suspending the fungal biomass in de-ionized water and incubated at different temperature 25, 30, 35, 40 and 45º C for 3 days. Further fungal filtrate was treated with $AgNo_3$ for the production of SNPs. The biosynthesis of NPs at different temperature was analyzed by UV. Visible spectrophotometer.

2.2.6.4: Effect of different concentration of AgNo3.

In biotransformation, one of the major factors making the reaction more economical and effective is finding out the maximum concentration of substrate which could be turn into the final product. Therefore, we investigated different concentrations of silver nitrate (1mM, 2mM, 3mM, 4mM and 5mM) in the reaction solutions in order to obtain the optimum concentration of the substrate for SNPs production. The resulting solutions were characterized by UV. Visible spectrophotometer

2.2.6.5: Effect of incubation period

The effect of incubation time on the biosynthesis of SNPs was carried out. The fungal filtrate was obtained and treating with 1mM AgNo3 for preparation of SNPs as prescribed. Then it monitored at different duration by UV.visible spectroscopy for synthesis of SNPs (after 1, 24, 48, 72, 120 hours and 2 months).

2.2.7: Determination the antifungal activity of SNPs:

2.2.7.1: Antidermatophytic effect of biosynthesized SNPs:

2.2.7.1.1: Tested microorganisms:

Five types of dermatophytes were used in our study to evaluate the antidermatophytic effect of biosynthesized SNPs, these fungi are: *Trichophyton mentagrophytes, Trichophyton rubrum, Trichophyton interdigitale, Microsporum canis, Epidermophyton floccosum*.

2.2.7.1.2: Inoculum preparation

Standardization of the inoculum is very essential for precise and reproducible antifungal susceptibility test. The inoculum suspensions of the tested dermatophytes were prepared accordance with Clinical Laboratory Standard Institute (CLSI) M38-A2 (2008). Fungal colonies previously grown on PDA medium for more than 7-15 days (to enhance sporulation) at 30 ºC were covered with 10 ml distilled water and then gently scraping the surface of colonies with the tip of sterile loop. The obtained mixture containing small hyphal fragments and conidia was put in sterile tubes and let to sediment the heavy particles at room temperature. The suspension then adjusted to obtain a final concentration of conidia ($1\text{-}3x10^3$cell/ml) by counting in haemocytometer (CLSI M38-A2).

2.2.7.1.3: Antifungal agents dilution and preparation of stock solutions:

Antifungal reference powders were obtained commercially: Fluconazole and Miconazole. These antifungal agents were used as positive control for comparison with biosynthesized SNPs.

The antifungal powders were weighed by using a calibrated analytical balance. The following formula was used to determine the amount of powder for standard solutions: (CLSI M38-A2,2008)

$$Weight\ (mg) = \frac{Volume\ (ml) \times Concentration\ (\mu g/ml)}{Assay\ Potency\ (\ \mu g/mg)}$$

Fluconazole and SNPs were dissolved in distilled water, while Miconazole was dissolved in 100% DMSO to obtain stock solutions. Further dilutions were performed in broth medium to yield twice the final desired strength in the test, then a twofold serial dilutions were done to obtain the final concentration range of the drugs: 0.125 to 64 µg/ml for Fluconazole, 0.03 to 16 µg/ml for Miconazole and 0.156 to 80 µg/ml for SNPs.

2.2.7.1.4: Determination of MIC value against dermatophytes:

The minimal inhibitory concentration (MIC) of the SNPs and other antifungal agents for tested dermatophytes was determined by using a broth microdilutions method, when possible, according to the guidelines of the Clinical Laboratory Standard Institute (CLSI) as described in document M38-A2 for filamentous fungi (CLSI,2002).

The assay was performed by using a sterile, plastic, disposable, 96-well microdilution plates with a U shaped- bottom that have a nominal capacity of 300 µL. Each well in the column, from 1-10, of the microdilution plates, filled with aliquots of 100 µL of the serially diluted antifungal agents (2x

final concentration), then a 100 µL of the inoculum, prepared previously, were added. Column 11 was filled with 200 µL of drug free medium which served as sterility control. Column 12 contained 200 µL of the fungal inoculum and served as a growth control. Aplastic covers were placed over plates. In order to prevent the desiccation of the broth resulted from prolonged incubation; the plates were sealed in plastic bags with a piece of moistened paper towel.

The micro plates were incubated at 35 ºC for *T.mentagrophytes*, *T.rubrum* and *T. interdigitale*, while *E.floccosum* and *M.canis* were incubated at 30ºC .

All experiments were carried out in duplicate. The optical densities were recorded by a spectrophotometer at 450 nm in a microtiter plate reader. The MIC concentration of the SNPs and antifungal agents was calculated from the following equation:

$$\%\ growth = \frac{OD\ of\ well\ containing\ the\ drug}{OD\ of\ the\ drug\ free\ well} \times 100$$

It was determined as the lowest concentration resulted in inhibition of fungal growth. (Tripathi, 2013).

2.2.7.1.5: Minimum fungicidal concentration assay:

After the reading of MIC, The Minimum fungicidal concentration (MFC) of SNPs against tested dermatophytes was performed. For comparison purpose, the fungicidal effect of Fluconazole and Miconazole were also determined. For that aliquot of the contents of all clear wells (in which no

growth was observed) was subcultured onto SDA plates, a positive control (from growth control well) and a negative control (from sterility control well) were included in this test. The plates were incubated at 35ºC until the appearance of growth in the growth control subcultures. These assays were carried out in duplicate. The MFC endpoints were recorded as the lowest concentration of the tested agents which showed no fungal growth or few colonies to obtain approximately 99-99.5% killing activity. (Espinel- Ingroff *et al.*,2002)

2.2.7.1.6: Effect of biosynthesized SNPs on spore germination of dermatophytes:

The efficiency of biosynthesized SNPs to inhibit spore germination of tested dermatophytes was evaluated by microscopic method. The conidia were obtained by growing the isolates on PDA for 10-14 days (according to the dermatophytes genera) as described previously (2-6-1-2). The resulting suspension was filtered through multiple layers of gauze to remove the mycelial fragments. The spore suspension of each isolate of dermatophytes was adjusted to contain at least 40-60 spore/ microscopic field. One ml of this suspension was mixed with one ml of SNPs (at MIC value) in a test tube. So, the final concentration of spores was 20-30 spore /microscopic field (Wani and Shah, 2012). The tubes were then incubated at 35ºC (*Trichophyton spp*.) or at 30º C (*Microsporum and Epidermophyton spp*.) in shaking incubator (60r/min) in order to disperse the SNPs throughout the mixture.

Daily microscopic examination was done to investigate the spore germination. The spore was considered to be germinated when the germ tube length was 1.5 times the spore diameter (Plascencia-Jatomea *et al.*, 2003).

The SNPs replaced by antifungal drugs and sterile broth for positive and negative controls respectively. All treatments were replicated 3 times.

The percentage of spore germination inhibition was recorded according to the following formula:

% spore germination inhibition = $\frac{SC-CT}{SC} \times 100$

*SC: Average of germinated spores in control.

CT: average of germinated spore in treatment.

2.2.7.1.7: Determination of the inhibitory effect of biosynthesized SNPs on the keratinase activity

It is of interest to study the effect of NPs on enzyme activity of dermatophytes especially the keratinase, as this enzyme involved in the pathogenesis process and plays an important role in the breakdown of keratin substrate (Lechenne *et al*.,2007).

1: The source and preparation of keratin substrate

Chicken feathers were used as a keratin substrate, it were obtained from local white chicken. The feathers were washed with tap water for several times and cut to small pieces, then these pieces were defatted by soaking them into chloroform –methanol mixture (1:1 v/v) for 2 days, then transferred to chloroform :acetone :methanol (4:1:3 v/v/v) for further 2 days. After that, the feathers were washed with tap water for several times to

remove any solvent residual. Finally they were autoclaved and dried in an oven at 50 ºC. The dried feathers were grinded and maintained in a sterile container until used (Tork *et al*., 2010).

2: Keratinase production: (Wawrzkiewicz *et al*., 1991)

The production of crude keratinase enzyme was performed by growing the dermatophytes on a liquid keratin induction medium. The 250 ml flask was inoculated with 1×10^5 spore/ml of corresponding spore suspension. The flasks were incubated at 30ºC for 14 days in shaking incubator. The experiment was done in triplicate.

After the incubation time, the fungal mycelium and feathers were removed from culture media by filtering through multiple layers of gauze. The resulted cell free filtrate centrifuged at 4000 rpm for 5 min and the supernatant were used as the crude enzymes (Singh , 2014).

3: Keratinase activity assay:

The keratinase activity of each isolate was done by using Muhsin & Aubaid (2001) method with slight modification. Briefly, 0.5 ml of cell free supernatant, 50 mg of chicken feathers were mixed in 5 ml of phosphate buffer (0.03 M). The mixture then incubated at 37º C for 2 hours with gentle shaking. The negative control samples were done in the same manner except that the enzyme was boiled at 100 ºC for 15 min.(Samples without SNPs were used for identification of reduction in activity of enzyme)

The effect of biosynthesized SNPs on the activity of keratinase enzyme was tested by incubating the enzyme with SNPs at concentration of MIC value for each isolate. The Miconazole and Fluconazole were used for comparison. At the end of incubation (2h.), the reaction was stopped by keeping it in an ice for 10 min. Then, the feathers were separated from mixture by filtration using Whatmann filter papers No.1. The keratinase activity was determined by reading the absorbance of the resulted filtrate spectrophotometrically at 280nm using UV.visible spectrophotometer. An increase in absorbance value 0.1 was considered as equivalents to 1 unite enzyme activity (KU)/ml.

2.2.7.1.8: Effect of biosynthesized SNPs on the mycelia development of dermatophytes in the broth media:

The experiment started by supplementation of 50 ml growth medium (SDB) with different concentration of SNPs (SNPs used at sub-MIC concentration in order to allow some fungal growth) then a two millimeters discs of 7 days old fungal culture of dermatophytes spp. on PDA were transferred into each flask. A broth medium without SNPs was inoculated and considered as control. The inoculated flasks were incubated for 7 days at 30 ºC in a shaking incubator. All experiments have been performed in duplicate.

For documentation, a sample from each flask was withdrawn and observed under light microscope at 100x objective lenses and a picture of the mycelium was taken.

2.2.7.2: Antifungal effect of biosynthesized SNPs on *Candida albicans*:

2.2.7.2.1: Determination of MIC for *Candida albicans*:

The MIC of SNPs, Fluconazole and Miconazole against *Candida albicans* were determined using broth microdilution method based on the CLSI guidelines as defined in document M27-A3 (2008) with slight modification.

1- Microplates preparation:

One hundred µL aliquots from broth medium were placed into all wells of 96-well microtiter plates with U-shaped bottoms, after this a 100 µl of antifungal agents solutions with 3x final concentration were suspended in the wells of column 1 and mixed with the medium, previously added, by sucking up and down for 6-7 times, then a 100 µl was transferred from column 1 to column 2, this make column 2 a twofold dilution of the column 1, this step was repeated down to column 10 and the final 100 µl was discarded (M27-A3 ,2008).

2- Inoculum preparation:

Inoculum was prepared by suspending yeast colonies (≥ 1mm in diameter), from 24 hours cultures on SDA incubated at 37 ºC , in 5 ml of sterile normal saline and shaking vigorously by using vortex mixer for about 15 sec.

The turbidity of the resulting suspension was adjusted to 0.5 McFarland's standards. These suspension were further diluted to obtained a final concentration of inoculum (1-5 $\times 10^3$ cfu/ml) as described by the CLSI document M27-A3.

3-Inoculation of plates:

The Microdilution plates were inoculated within approximately 30 min from inoculum preparation to maintain the viable cell concentration. Each well in the column 1-10 were inoculated with 100 µL of the 1-5 $\times 10^3$ cfu/ml suspension. This will give the desired final concentration for antifungal agents as well as the inoculum density (0.5-2.5$\times 10^3$ cfu/ml). The growth control wells (column 11) that contained a sterile drug-free medium were also inoculated, while the column 12 of the microdilution plates was left without inoculation and regarded as a sterility control and blank for the plate reader (M27-A3,2008).

The plates were incubated at 37ºC and evaluated at 24 h. The MIC endpoint was determined as the lowest antifungal concentration at which the organism was inhibited ≥ 50 % by comparing the turbidity in the treated wells with the drug-free growth control wells.

2.2.7.2.2: Determination of MFC for *Candida albicans*:

The *in vitro* fungicidal activities were evaluated for each antifungal agent against *C.albicans* as described by Cantón *et al*., (2003). Briefly, an aliquot of 100 µL from all clear wells (in which no visible growth was observed) and from the growth control well, were subcultured onto SDA plates. After the drying of the plates, it was streaked uniformly to separate cells and remove them from the drug source. The plates were incubated at 37 ºC until the appearance of growth in the growth control subculture. The MFC endpoint was defined as the lowest concentration of antifungal agents that completely inhibited the growth of the yeast, or permitted to less than three colonies to grow (resulted in 99.5% killing activity).

2.2.7.2.3: Effect of SNPs on the adherence of the tested *Candida albicans* strain on plastic surface:

The biosynthesized SNPs were evaluated for inhibition of adherence of *C.albicans* to plastic surface of tissue culture plate (TCP). The yeast cell suspension was prepared by growing the *C.albicans* in YPD (1% yeast extract, 2% pepton, 2% D-glucose) for 24 h. Then the culture was diluted in SDB medium to an optical density of 0.1 (about 106 cells/ml). An aliquot of 100 µl of prepared suspension was added to each well of a sterile, polysterine, 96-well, flat bottomed microtiter plate and incubated for 2 h. (adhesion time) at 37ºC. After the incubation period , the supernatants were totally aspirated off and each well was gently rinsed twice with 200 µl of PBS to remove non- or- loosely adhered cells (planktonic cells) (Monteiro *et al*.,2011). Then 200 µL of SDB containing SNPs in different concentration (MIC, above MIC and under the MIC concentration) was added to each well (adhered yeast cells). Miconazole was used in identical manner for comparison, while the Fluconazole was not included in this experiment because it has high MIC (larger than the tested concentration). For control, a column of plates was handled in an identical way except that no SNPs or Miconazole was added. The plates then further incubated for 24 h. at 37º C. The experiment was performed in duplicate.

2.2.7.2.4: Effect of biosynthesized SNPs on the pre-formed biofilm of the tested *Candida albicans* strain.

Mature biofilm of *C.albicans* was performed in the sterile 96-well microtiter plates using the methodology described by (Yigit *et al*.,2011). 200 µL of yeast suspension was inoculated into wells of the TCP and incubated at 37 ºC for 48h. At the end of incubation period, the supernatants were aspirated and the non- adhered cells were secluded by washing each well twice with sterile PBS without disrupting the biofilm in the bottom of the wells. 200 µl of fresh SDB containing 0.5, 1 and 2 X MIC concentration of biosynthesized SNPs were added to each well of the TCP that contained the mature biofilm. The plates were incubated for another 24 h. at 37ºC. The Miconazole activity against pre-formed biofilm was also included in this assay. Medium devoid of SNPs or Miconazole was added to the wells containing biofilm and consider as control. After the period of incubation the wells were washed, stained and read. All assays were carried out in duplicate.

Biofilm quantification:

Biomass of the tested *C. albicans* biofilm treated with biosynthesized SNPs was evaluated by the crystal violet staining method (CVS) according to O'Toole (2011). The CVS assay was used to quantify the biofilm formation or inhibition through measurement of the total biomass of biofilm, including yeast cells as well as the extracellular polymeric substances matrix (Musleh and Jebur, 2014).

The crystal violet stain method starts by adding 200 µl of 99% methanol to the biofilm for 15 minutes (to allow cell fixation). After that, the methanol was removed and they were air dried. The adherent yeast stained with 200 µl of CV stain (1% v/v), after 5 min. The excess CV solution were removed from the plates, and the later were rinsed with deionized water and air dried.

Thereafter, a 200 µl of acetic acid (33% v/v) was added to each well of the plates in order to dissolve the CV stain (Monteiro, 2012.)

Finally, the OD of microtiter plates was measured by ELISA reader and the percentage of adherence and biofilm inhibition were evaluated using the following equation:

[1-(A490 of cell treated with SNPs/A490 of non-treated control) × 100] (Wei *et al.*, 2006)

2.2.7.2.5: Inhibitory effect of biosynthesized SNPs on the dimorphic transition:

The dimorphic transition (germ tube formation) from yeast form to mycelial form, is one of the most important virulence factors of *C. albicans* and is responsible for its pathogenicity (Rocha *et al*., 2001). So, in this assay we investigate the inhibitory effect of biosynthesized SNPs on this important virulence factor. For this purpose, human serum was used to induce the germ tube formation (Aryal,2015) by inoculating a small yeast colony in one ml of human serum containing different concentration of SNPs (0.5,1 and 2X MIC) and incubated at 37ºC for more than 4 h. To study the effect of Miconazole on the dimorphic transition, the same method was used except the existences of Miconazole in the serum instead of SNPs. Tubes without treatment were kept as a negative control. The assays were done in triplicate. At the end of incubation time, an aliquot of serum were visualized under light microscope using 100 x objective lens and photographed. The percentage of germ tube formation was calculated according to following formula:

No. of germ tubes in treatment/ No. of germ tubes in control ×100 (Monteiro *et al*., 2015).

2.2.8: Evaluation of synergistic potential of biosynthesized SNPs in combination with antifungal agents:

In this experiment, the enhancement of antifungal activity of Fluconazole and Miconazole with biosynthesized SNPs was investigated against dermatophytes spp. as well as *C.albicans*, using well diffusion method (Hassan *et al* ., 2015). 25 ml of sterile SDA medium was poured into sterilized petri dishes and allowed to cool at room temperature. Once the medium was solidified, the fungal strain was swabbed on the surface of plates by using swabs dipped in the inoculum suspensions. Each inoculated plates were then dried before well of 5 mm in diameter were created on agar surface with the help of sterilized pasture pipette end. The wells were loaded with 100 µL of freshly prepared SNPs, 100 µL of Fluconazole with a potency of 25 µg, 100 µL of Miconazole with a potency of 10 µg and 100 µL of each antifungal drugs in combination with SNPs (50 µL of antifungal agents + 50µL of freshly prepared SNPs solution). Wells contained D.W. and DMSO were considered as control. All assays were performed in triplicate.

1: Incubation and reporting of the results:

The inoculated plates were incubated at 30-35 ºC for 7-10 days (for dermatophytes), and at 37º C for 24h (for *C.albicans*). At the end of incubation time, the diameters of inhibition zone around the wells were measured and the mean value was expressed in millimeters.

Criteria of sensitivity and resistance of antifungal agents (Table 2-6) was recorded according to Pakshir *et al*., (2009)

Table (2-6): Criteria of sensitivity and resistance of antifungal agents.

Antifungal drugs	potency	zone diameter in mm		
		Sensitive	Intermediate	Resistance
Fluconazole	25µg	≥22	21-15	≤14
Miconazole	10µg	≥20	19-12	≤11

2: Estimation of increase in fold area:

Increase in fold area was evaluated by calculating the mean surface area of the inhibition zone produced by the antifungal agent alone and in combination with biosynthesized SNPs. The fold increase area calculated by the following equation: (Fayaz *et al*., 2010)

$$\boldsymbol{Fold\ increase}\ \% = (\boldsymbol{b} - \boldsymbol{a})/\boldsymbol{a} \times \mathbf{100}$$

Where **a**: refers to the zone of inhibition for antifungal drug alone

b: refers to the zone of inhibition for antifungal drug with SNPs.

2.2.9: Statistical analysis

Statistical analysis was performed by Social Science Statistics and the Statistical Package for Social Sciences version 19 for Windows Software and Microsoft Excel 2010.

Continuous random variables that are normally distributed are described by mean, SD (standard deviation) and the parametric statistical tests of significant. ANOVA test are used to analysis the statistical significance of difference in mean between more than 2 groups and when ANOVA model shows statistically significant differences, additional exploration of the statistical significance of difference in mean between each 2 groups was assessed by Bonferonni t-test. Descriptive analysis was done by calculating frequencies and approximate percentages (Sheskin, 2004)

The Pearson correlation (r) is used to measure the strength of a linear association between absorbance and UV at different growth factors while the statistical significance of the effect of antifungal agents on dermatophytes was examined by the Fisher Exact Probability Test or Chi-Square test (χ^2) that used to measure the strength of association between categorical variables. All these statistical tests considered that *P*- value less than the 0.05 level was statistically significant (Viera, 2008; Kumari *et al*., 2016).

Results

3. Results

3.1: Characterization of biosynthesized SNPs:

1. Visual inspection:

At the end of growth period of *A. niger*, the cell-free filtrate was used for biosynthesis of SNPs. The filtrate was initially pale yellow in color. When the filtrate challenged with $AgNO_3$, the color of the mixture was turned to yellowish brown at first and then the intensity of the color was increased with the period of incubation, so the color was changed to dark brown on completion of the reaction with Ag+ ions. Color change was noticed only in the test flask and it a clear indication for the formation of SNPs in the reaction mixture. The remaining two control flasks i.e., aqueous solution of $AgNO_3$ and fungal filtrate without $AgNO_3$ showed no change in color when incubated in the same condition (Figure 3-1).

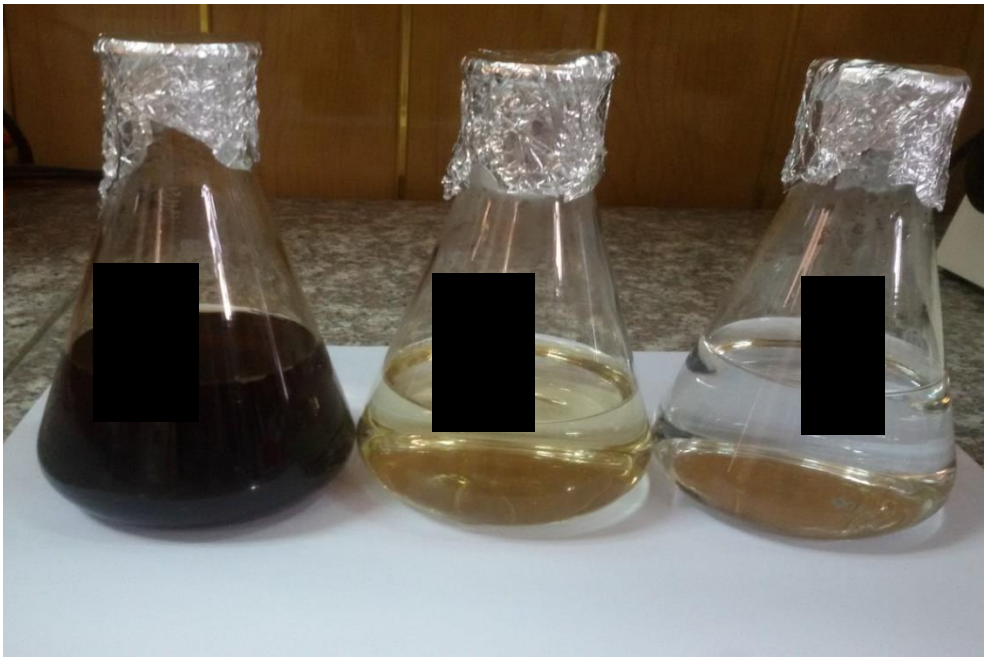


Figure 3-1: Culture flasks containing (A) 1mM $AgNo_3$ solution, (B) Fungal cell-free filtrate and (C) Mixture of fungal cell-free filtrate with 1mM $AgNO_3$.

2. UV-Vis spectroscopy

The formation and stability of the reduced SNPs in colloidal solution were detected and monitored by using UV-visible absorption spectrum scanning in the range of 300-700 nm (Figure 3-2). The analysis was evaluated at different times after the start of the reaction. The λ max 420 nm was observed only in the test flask which confirmed the production and indicating the specific surface Plasmon resonance of SNPs. The scanning was continued and absorbance was recorded every 24 hours. The absorbance intensity gradually increased with time, this indicating a continuous reduction of $AgNO_3$ and consequently, increase in SNPs concentration. Since in $AgNO_3$ aqueous solution and fungal cell-free filtrate no peaks were observed, further scanning was not continued for these solutions.

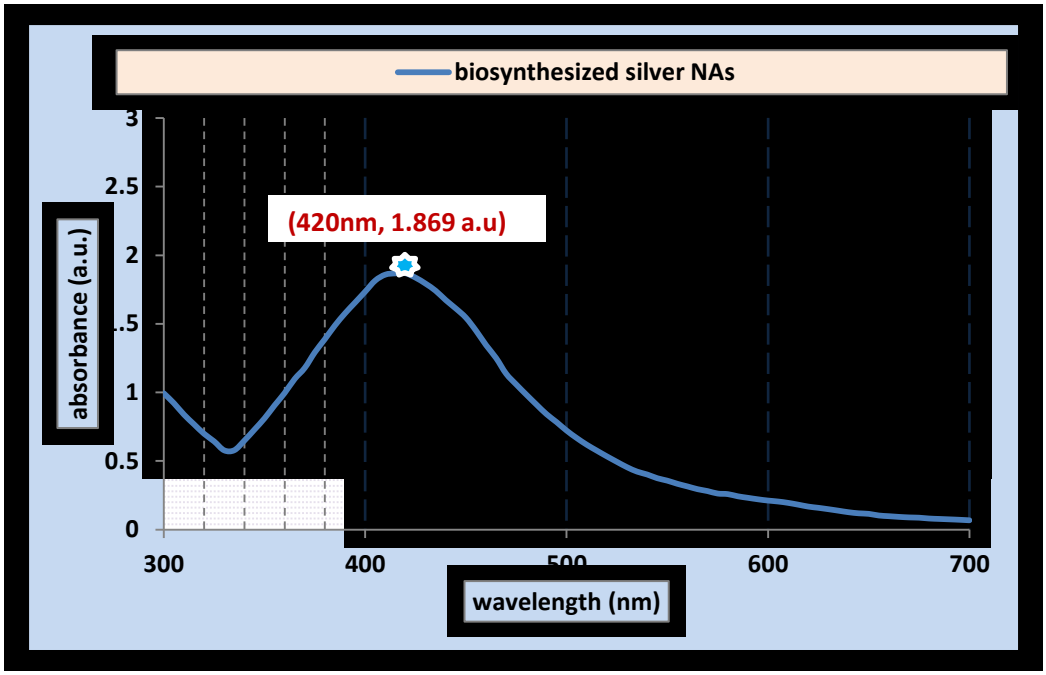


Figure (3-2): UV-Vis spectrophotometer analysis of biologically synthesized silver nanoparticles.

3: Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR measurement of the dried and powdered sample of the SNPs was performed to provide information about the chemical bonds and molecular structures of a possible material that could have a role in the reduction of silver ions and capping for SNPs. FTIR spectrum of biosynthesized SNPs revealed the presence of different distinct peaks located at 3421, 2962, 2926, 2854,1638,1554,1428, 1410, 1333, 1276, 1256,1239,1073,1048,782, 467 cm^{-1} (Figure 3-3). The peak at 3421cm^{-1} is ascribed to the stretching vibration of O-H bond of alcohol, phenols and N-H stretch vibration of primary amides of protein. The peak at 2926 cm^{-1} and 2961 cm^{-1}could be due to the C-H stretch of the methylene

groups of protein and to N-H stretching of amine salt. The absorption peak at 2854 cm^{-1}may be assigned to the C-H symmetrical stretch vibration of alkenes. The peak at 1638 cm^{-1} corresponds to the presence of amide I and amide II, which arises due to the carbonyl stretch and N-H stretch vibration while the band at 1554 cm^{-1} refers to C=C stretch corresponding to an aromatic ring. Peaks located at 1410 cm^{-1} and 1428 cm^{-1} may be related to COO^{-} symmetrical stretch from carboxyl groups of the amino acids residues. The peak at 1333 cm^{-1} corresponds to carbon hydrogen (CH_3) bending vibration. The peak located at 1276, 1256 and 1239 cm^{-1}represent C-O stretching of primary alcohol and P=O stretching respectively. The bands at 1073cm^{-1} and 1048 cm^{-1} refer to C-O bonds of aliphatic amines while the peaks at 782cm^{-1} and 467 cm^{-1} can be assigned to the aromatic C-H out of plane bending vibration of aromatic primary amines.

FTIR results revealed that secondary structure of proteins have not been affected as a consequence of reaction with silver ions or binding with SNPs. In addition, IR spectroscopic study has confirmed that amino acid and peptides have formed a coat covering the SNPs to prevent agglomeration.

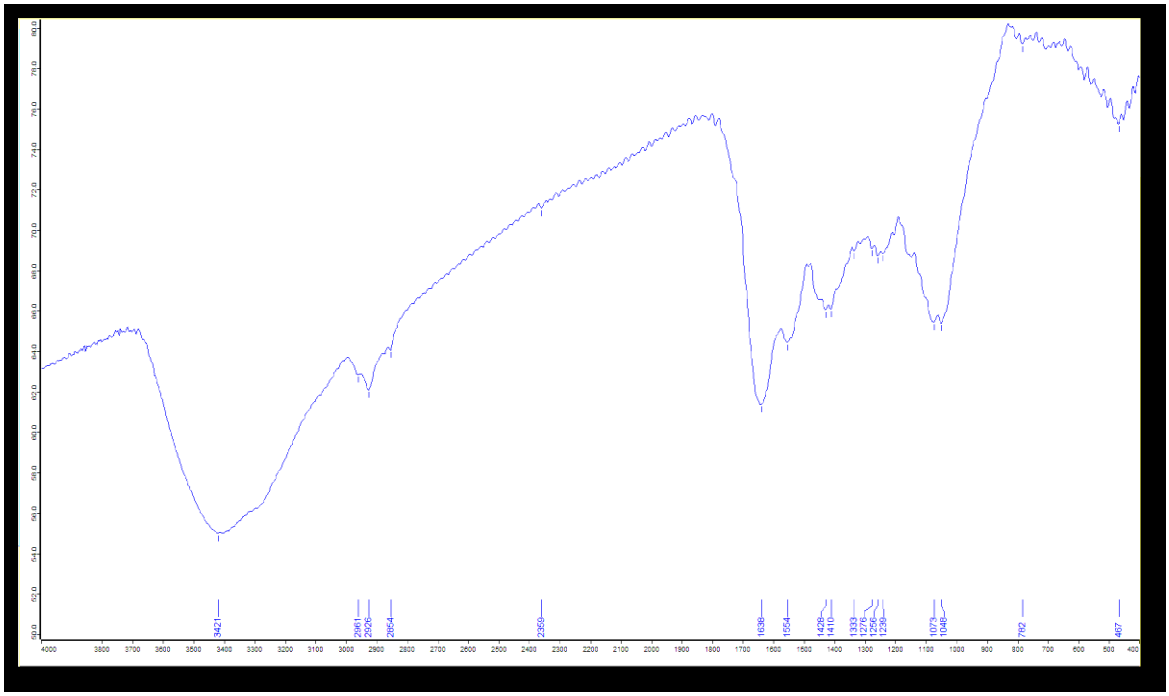


Figure (3-3): FTIR spectrum of SNPs, synthesized by *Aspergillus niger*, with distinct peaks.

4: Electron Microscopy (EM) analysis.

Electron microscopy has been employed to determine the shape, size and morphology of biosynthesized SNPs. Figure (3-4) reveals typical EM micrograph of SNPs obtained by the reduction of $AgNO_3$ solution with cell-free filtrate of *A.niger*. The morphology of NPs was spherical in shape, uniformly (monodispersed) without significant aggregation. The particle size was ranged from 15-60 nm.

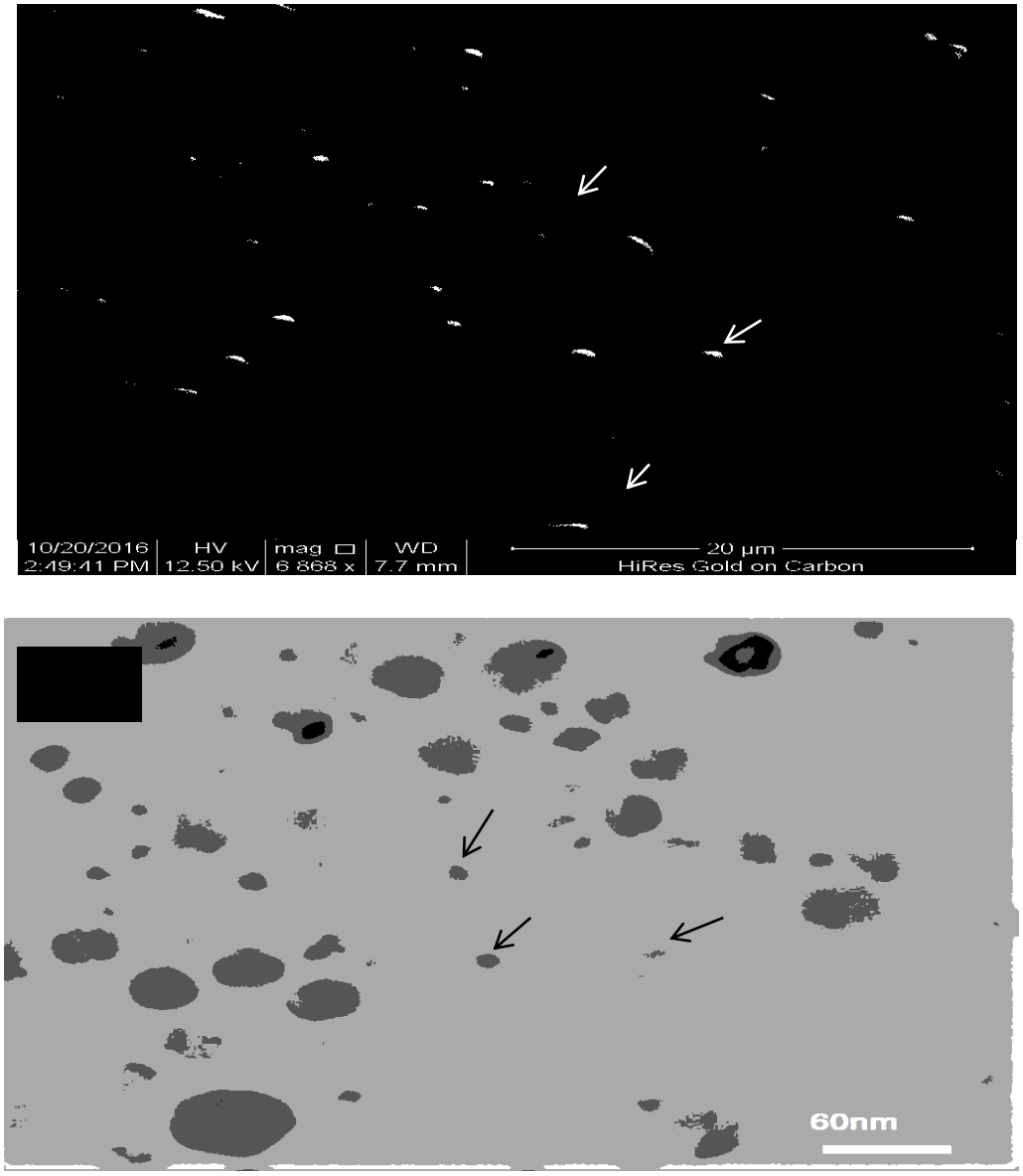


Figure (3-4): EM micrograph of biosynthesized SNPs. The image shows size and spherical shape of monodisperse SNPs.(A):SEM. (B):TEM.

5. Energy dispersive x-ray spectroscopy (EDX)

The EDX analysis was established to detect the elements that may be involved in the formation of SNPs. Figure (3-5) reveals a strong signals for metallic NPs. Powerful signals from Ag atoms in the NPs were observed. The optical absorption peak was noticed at approximately 3 KeV, which is typical for the absorption of mineral silver nanocrystalites. In addition, another peak for oxygen was observed on the left part of the spectrum at ~0.5 KeV.

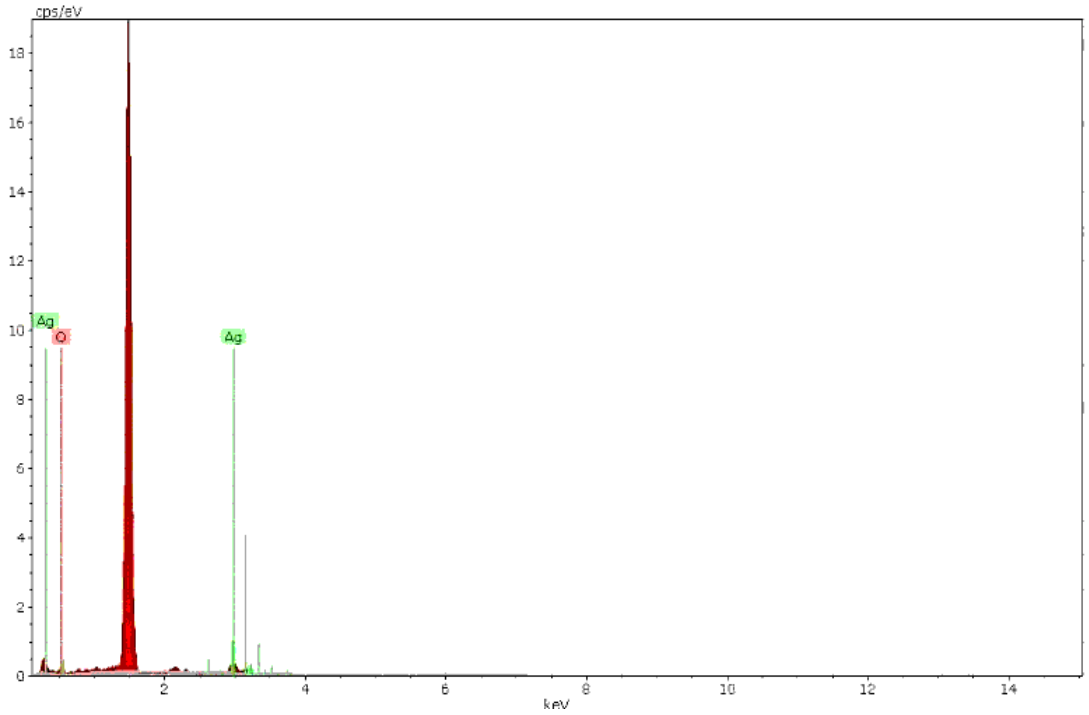


Figure (3-5): EDS analysis of extracellularly biosynthesized SNPs by *A.niger*. The vertical axis shows the number of X-ray counts, whereas the horizontal axis shows the energy in KeV.

It is clear from Table (3-1) that the weight percentage of silver is 62.49 %.

Table (3-1): The elements composition in biosynthesized SNPs solution of EDX spectra.

Spectrum: Acquisition					
Element	Series	unn.(wt.%)	C norm.(wt.%)	C Atom.(at.%)	Sigma(wt.%)
Oxygen	K-series	4.78	37.51	80.19	2.07
Silver	L-Series	7.96	62.49	19.81	0.56
Total		12.75	100.00	100.00	

3.2: Optimization of physic-chemical Conditions

3.2.1: Effect of Different culture Media

To evaluate the effect of different media for enzyme secretion and their effect on biosynthesis of SNPs, the fungus grew on five different media (PDB, MYPGB, GPYB, Czapek B and SDB) for 7 days. The cell-free filtrates were used for synthesis of SNPs. It is evident from Figure (3-6 B) that the highest SNPs production was recorded in PDB medium (1.832 a.u.) followed by MYPGB medium (1.678 a.u.). Samples obtained from these culture media have high absorption peaks at 435nm, 440 nm respectively, resulting from high levels of reduced silver ions; in addition the sharp and smooth curves represent small and uniform size distribution (Figure 3-6 A). Symmetry in graph indicates the monodispersity and stability in synthesized NPs. The GPYB medium showed minimum intensity (0.647 a.u.) with peak at 445nm, whereas Czapek B and SDB medium showed absorbance intensity reached to 1.231(a.u.) and 0.994 (a.u.) respectively, with peaks at 440 nm and 445 nm respectively. All GPYB, Czapek B and SDB media gave low surface Plasmon intensity with broad peaks but symmetry in spectrum indicates the monodispersity of SNPs

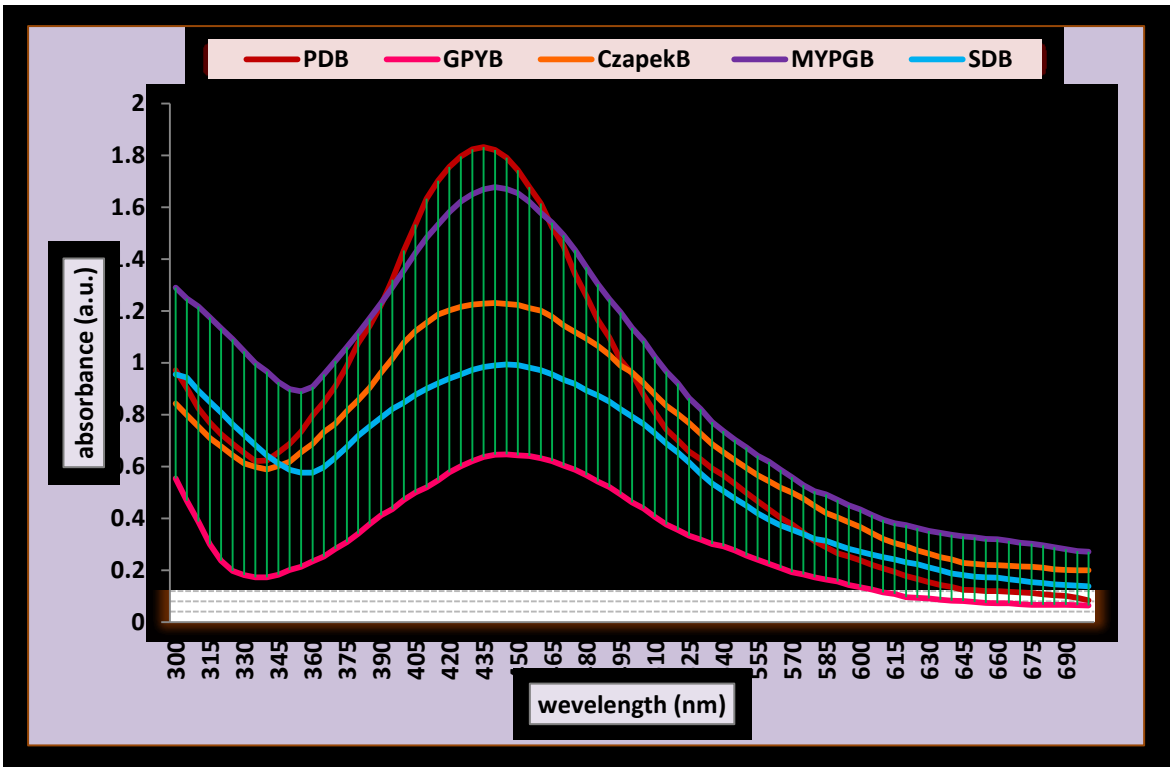


Figure (3-6 A): UV-Vis absorption spectra of SNPs using different culture media ($p < 0.05$) (r= 0.662, 0.569, 0.675, 0.767 & 0.816 for PDB, GPYB, Czapek B, MYPGB & SDB respectively)

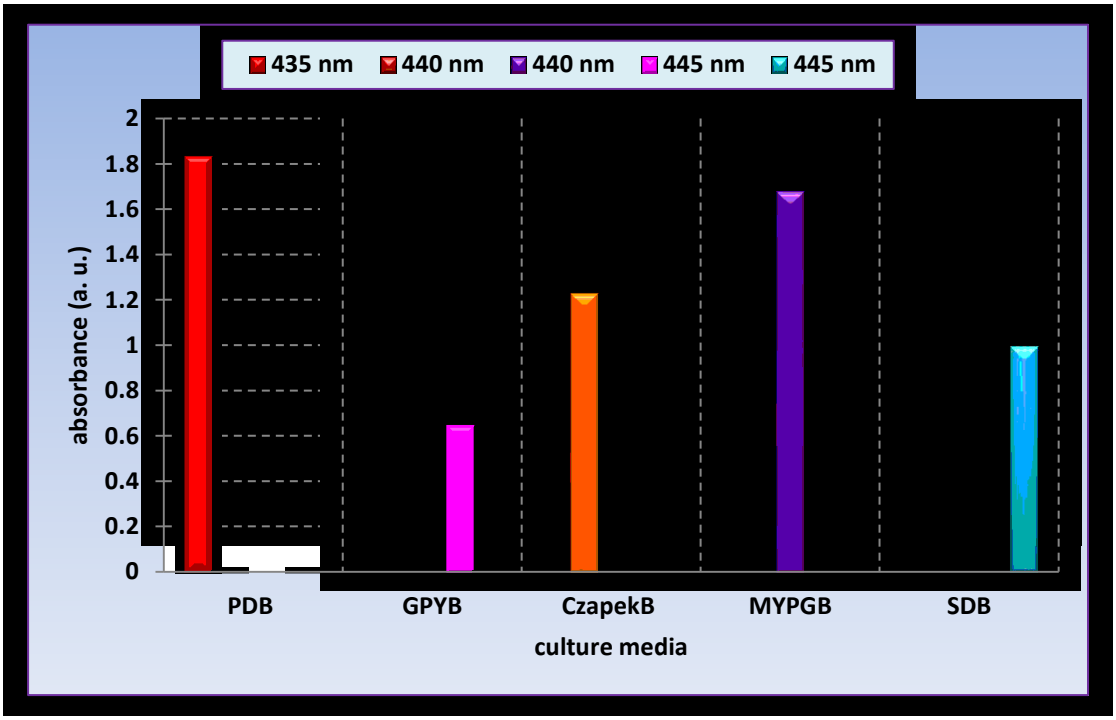


Figure (3-6) B: Comparison the absorbance of SNPs obtained with different culture media ($\chi^2 = 0.768$, $P(\chi^2 > 0.768) = 0.9427$)

3.2.2: Effect of pH

The majority of enzymes normally exhibit a strong dependence of activity on the pH of the medium, thus it is important to optimize the pH of the reaction medium in which NPs synthesizes.

In order to standardize the pH of the reaction mixture of extracellular enzyme with precursor compounds, the biosynthesis process carried out at various pH levels ranging between 5 and 9 with an increment of 2. When the fungal filtrate was challenged with 1mM $AgNO_3$ at different PH, all the test flasks showed variable change in color of reaction mixture from pale yellow to brown color and the intensity of Plasmon surface resonance bands have been varied as depicted in figure (3-7 A) by UV-visible absorption spectra. pH 9 was found to provide optimal conditions for maximal biosynthesis of SNPs with absorbance intensity 1.643 a.u. and maximum peak at 425 nm which indicate the presence of SNPs with size range between 10-100nm. On the other hand, absorbance decreased with decreasing in pH value. (Fig 3- 7 B) show the relationship between the maximum absorption and the pH value which is almost directly proportional. Particles aggregation were observed in acidic pH after few days from reaction, while there is no evidence for any aggregation in alkaline pH up to the end of experimental period suggesting that an alkaline environment was more suitable for SNPs biosynthesis.

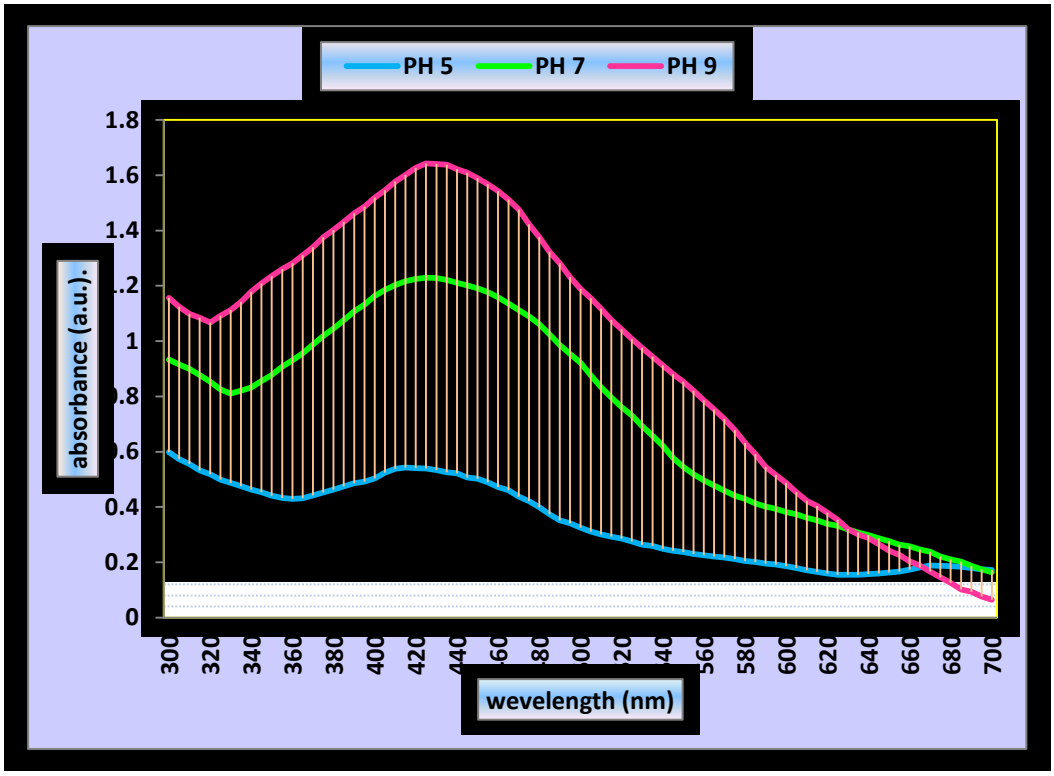


Figure (3-7 A): UV-Vis spectrum of biosynthesized SNPs at different pH (p = 0.01).

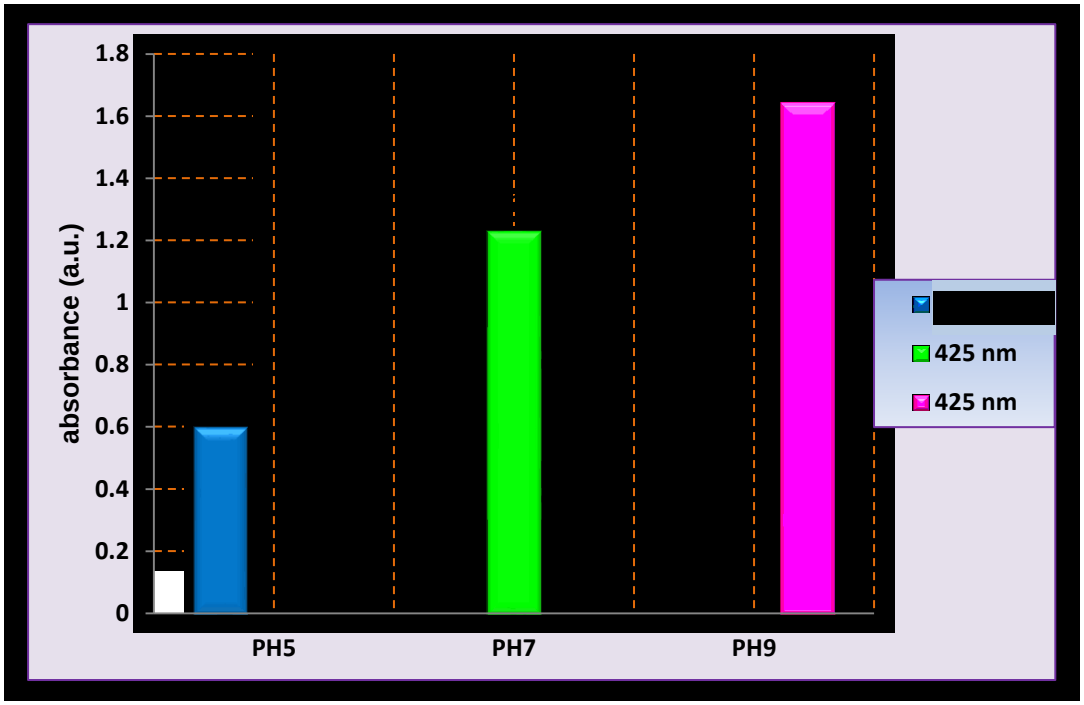


Figure (3-7 B) UV-vis spectrum showing maximum SNPs synthesis at pH 9. (χ^2 = 0.203, P(χ^2 > 0.203) = 0.9036

3.2.3: Effect of temperature

Temperature is an essential factor affecting NPs production. Thus, the effect of varying temperature on the SNPs production by the fungus *A.niger* was carried out at different temperature from 25Cº- 45Cº with a difference of 5ºC. Figure (3-8 A) detected significant differences in absorbance among different temperature degrees. The optimal temperature for silver bioreduction was 30ºC as demonstrated by color change and absorbance measurements by UV-visible spectroscopy (1.864 a.u.) with a sharp peak at 420nm, followed by 25ºC (1.61 a.u.). As we increased the temperature from 35 ºC -45 ºC, the absorbance intensity of surface Plasmon resonance was decreased (Figure 3-8 B). This result clearly indicates the reduction in SNPs biosynthesis with shifting in temperature of the reaction solution for 30ºC.

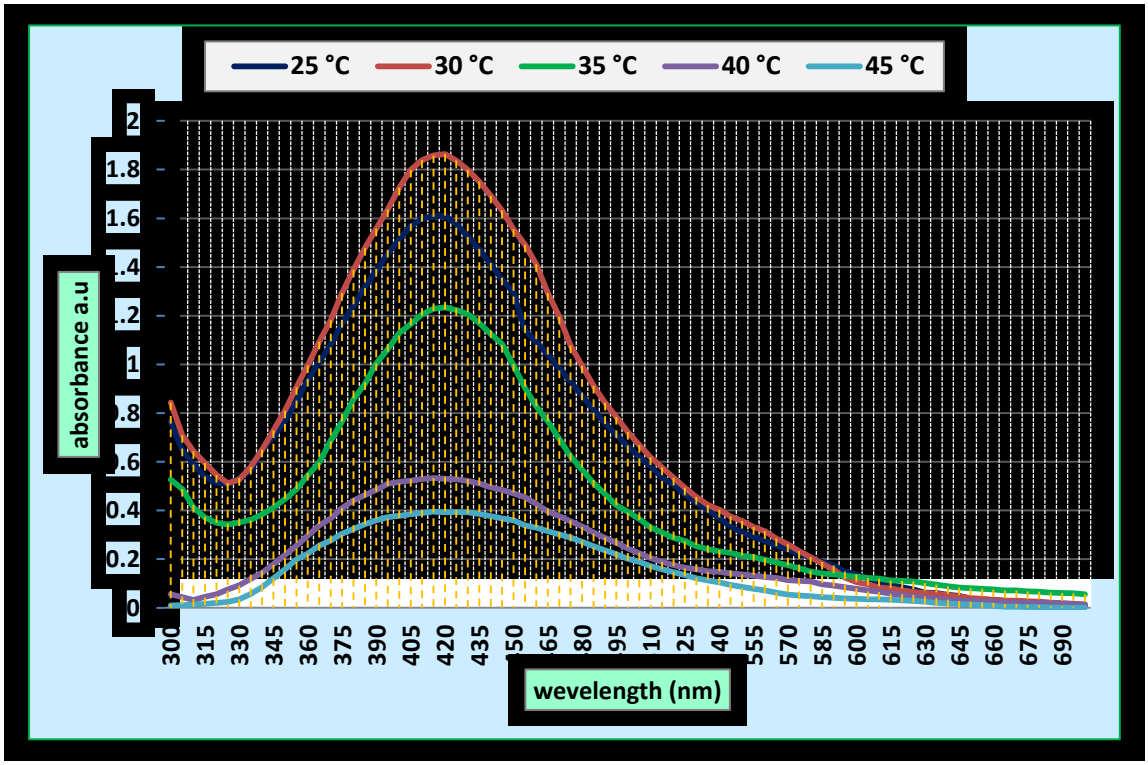


Figure (3- 8 A): UV- Vis spectrum of biosynthesized SNPs at different temperature (p = 0.044) (r= 0.729, 0.697, 0.653, 0.590 & 0.518 for 25 ºC, 30 ºC, 35 ºC, 40 ºC & 45 ºC respectively).

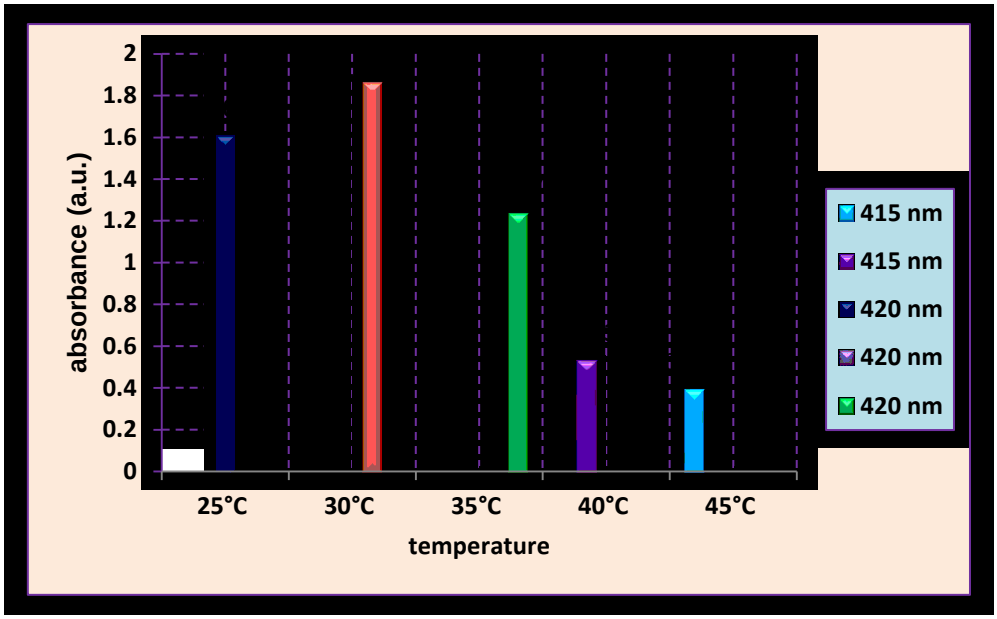


Figure (3-8 B) : Higher absorption at 30 °C indicate the optimum temperature for synthesis of SNPs. (χ^2 = 1.454, P(χ^2 > 1.454) = 0.8347

3.2.4: Effect of different concentration of $AgNO_3$.

The results exhibited that the concentration of $AgNO_3$ added strongly affects the reactions. As shown in figure (3-9 A and B) the absorbance intensity decreases with increase in concentration as recorded by UV-vis. Spectroscopy. A general trend is that the surface plasmon resonance peak shifts toward the short wavelength region as well as becomes narrower when the concentration value decreases. The concentration of 1mM showed maximum absorbance (1.188 a.u.) with a characteristic SPR band around 420nm indicating efficient production of SNPs. The smooth and Symmetry curves represent small and uniform size distribution, in addition indicates the monodispersity and stability of biosynthesized NPs. From the graph, it is obvious that the yield of SNPs decreased by gradual increase in the concentration of $AgNO_3$, the highest concentration (5mM) showed the least bio-reduction of silver ions to NPs and the reaction mixture appear unstable with aggregation and precipitation at the bottom

of flasks. The results clearly indicate that the relationship between the formation of SNPs and concentration value is inversely proportional.

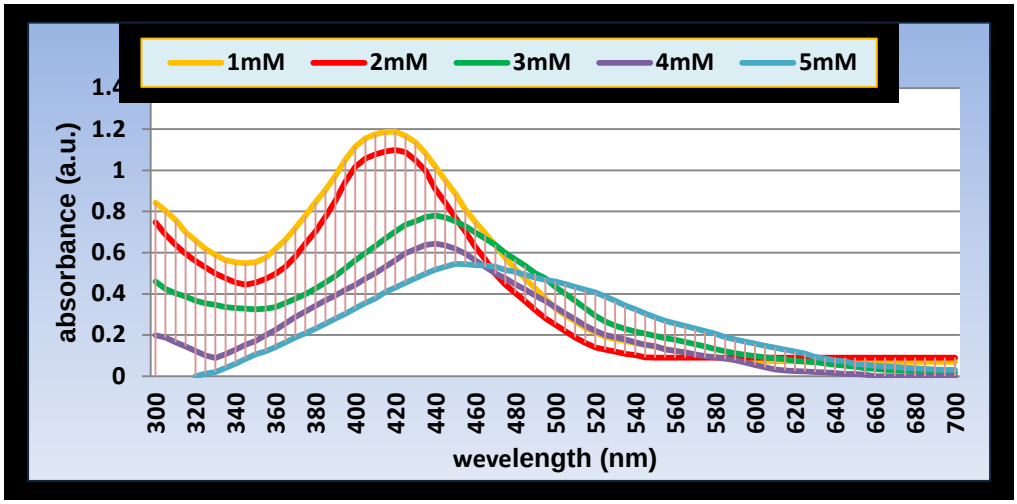


Figure (3-9 A): UV-Vis spectra of SNPs obtained with different concentration of AgNO3 solution ($p < 0.05$) ($r = 0.801, 0.754, 0.708, 0.546$ & 0.176 for 1mM, 2mM, 3mM, 4mM & 5mM respectively)

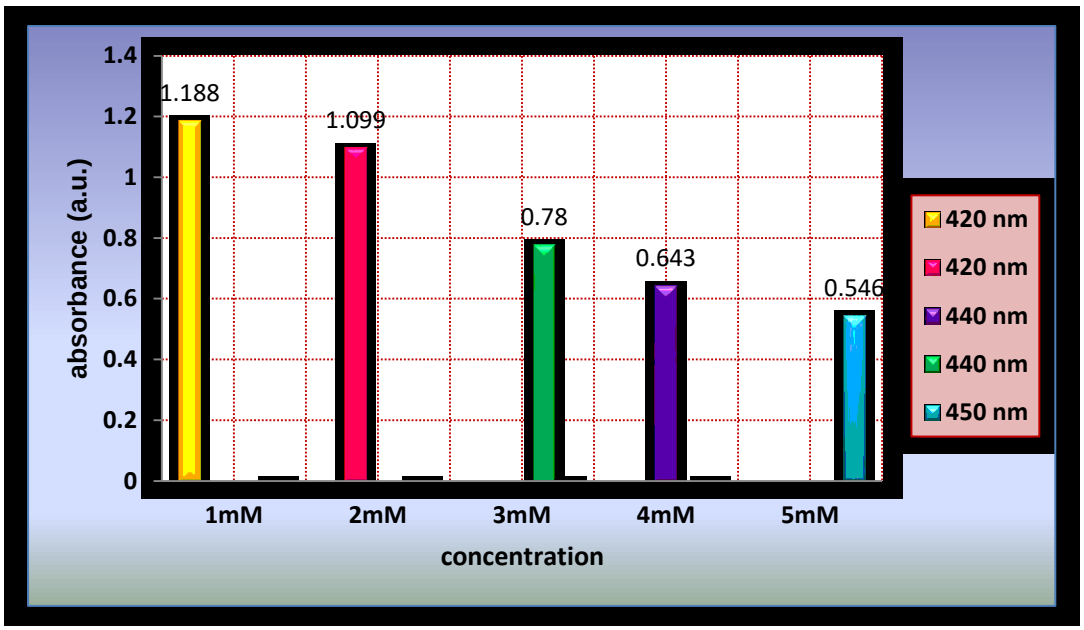


Figure (3-9 B): UV-Vis spectrum of SNPs synthesized at various $AgNO_3$ concentration. ($\chi^2 = 0.445$, $P(\chi^2 > 0.445) = 0.9787$)

3.2.5: Effect of incubation period:

Time is considerd a major factor in the biosynthesis of NPs. The fungal filtrate of *A.niger* was incubated with $AgNO_3$ for 1,24,48,72,120 h and 2 months. At the end of incubation period, the effect of time analyzed

through UV-vis. spectrophotometer. The change in color of reaction solution from pale yellow to dark brown was detected only after 24h of incubation. It was also observed that the biosynthesis rate increased with increase in time (figure 3-10 A). The highest absorbance value was recorded after 2 months (1.994 a.u.) with broad peak at 440nm. The increase in the absorbance indicates the increase in the number of NPs or increase in size of individual SNPs. Whereas after 120 h of incubation, the peak was recorded at 420 nm. Figure (3-10 B) showing a positive relationships between time and absorbance at different wavelengths.

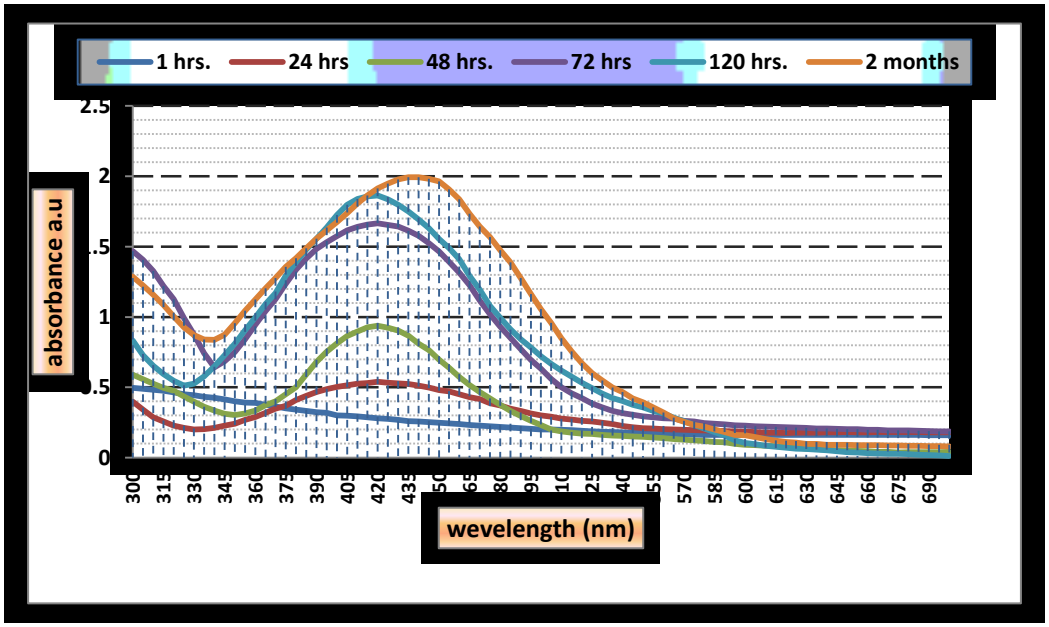


Figure (3-10 A): UV – Vis spectrum of biosynthesized SNPs at different time duration (p< 0.038)(r= 0.915, 0.58, 0.733, 0.797, 0.697 & 0.753 for 1hrs, 24hrs, 48hrs, 72hr, 120hrs & 2 months respectively).

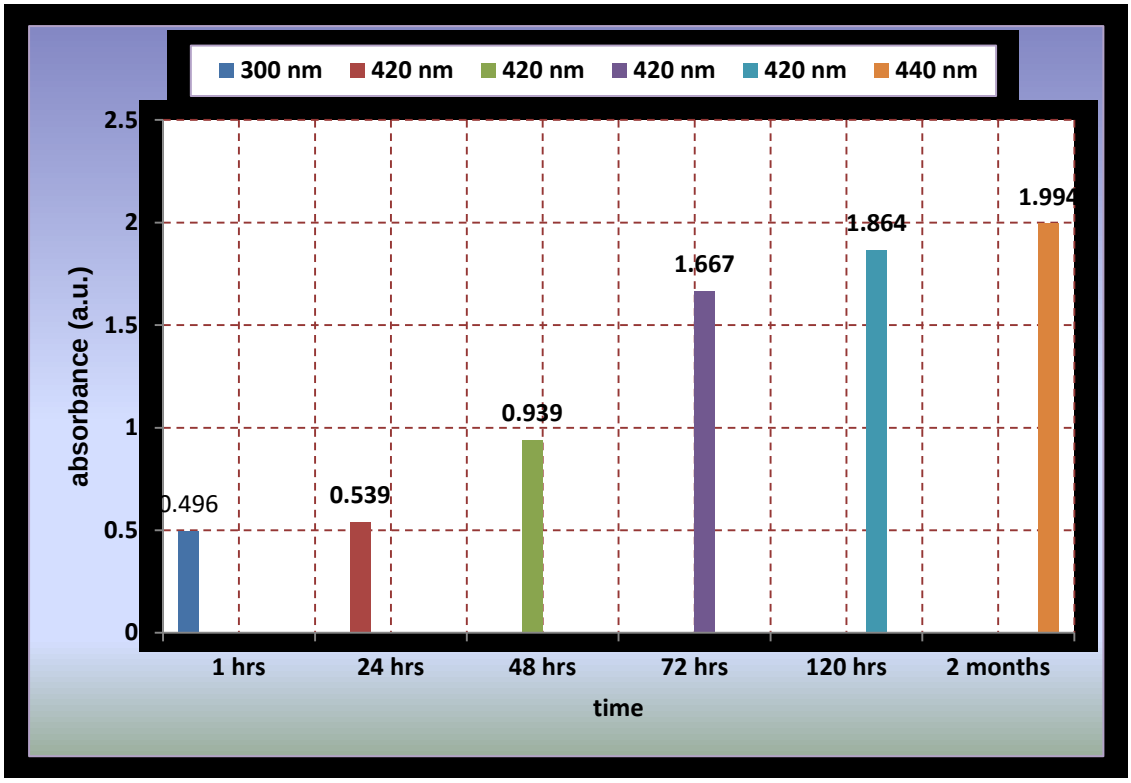


Figure (3-10 B): UV-Vis absorption spectra of biosynthesized SNPs at different time intervals (χ^2 = 1.383, P(χ^2 > 1.383) = 0.9261)

3.3: Determination the antifungal activity of SNPs:

3.3.1: Antidermatophytic effect of biosynthesized SNPs:

3.3.1.1. Determination of MIC and MFC value against dermatophytes:

The antifungal effect of biosynthesized SNPs was investigated against different dermatophytes spp. namely, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Trichophyton interdigitale*, *Microsporum canis* and *Epidermophyton floccosum*. The reference antifungal drugs (Fluconazole and Miconazole), that are widely used against many fungal infections, were used as a positive control for comparison with activity of SNPs. The MIC was determined to investigate the growth inhibition effect of SNPs against the mentioned fungi by mean of broth microdilution method. MIC values for the tested agents are presented in

Table 3-2. The obtained results revealed that the SNPs, in the range of 0.156 to 80 µg/ml, showed antifungal activity against all tested dermatophytes, the later exert significant variation in their susceptibility depending on fungal species. *T.rubrum* was the most sensitive dermatophytes to SNPs, with lowest MIC value of 0.313 µg/ml followed by *E. foloccosum* with MIC value recorded as 0.625 µg/ml. On the other hand, *M.canis* was the most resistant to biosynthesized SNPs; it exhibited the highest MICs among all the tested fungi (5µg/ml). SNPs showed antifungal activity against *T. interdigitale* with MIC value at 1.250 µg/ml, while the strain of *T. mentagrophytes* showed slightly higher value than *T. interdigitale* with inhibitory concentration at 1.77 µg/ml.

Fluconazole, with an MIC range of 0.125 to 64 µg/ml , only exhibited an antifungal activity against *T. interdigitale* and *T.rubrum* with relatively high MIC value about 8 µg/ml, 32 µg/ml respectively. However, no antifungal activity recorded for other tested dermatophytes.

The Miconazole, with an MIC in the range of 0.03 to 16µg/ml, displayed similar antifungal effect to that obtained by the biosynthesized SNPs. The statistical analysis showed that there is no significant difference between them. The lowest MIC of Miconazole, at the concentration of 0.250µg/ml, was obtained against *M.canis*. In comparison to other dermatophytes species, *E.foccosum* was less sensitive to this antifungal agent, with MIC value reaching 4 µg/ml.

Table 3-2: Comparative MIC value of biosynthesized SNPs, Fluconazole and Miconazole against dermatophytes strains.

Tested fungal strains	MIC/ Mean (µg/ml)					
	SNPs (0.156-80)	FCZ (0.125-64)	p-value	SNPs (0.156-80)	MCZ (0.03-16)	p-value
T.interdigitale	1.250	8	0.0513	1.250	1	>0.05(Ns)
T.rubrum	0.313	32	<0.001	0.313	0.5	>0.05(Ns)
T.mentagrophytes	1.77*	>64	<0.001	1.77*	0.5	>0.05(Ns)
E.floccosum	0.625	>64	<0.001	0.625	4	>0.05(Ns)
M.canis	5	>64	<0.001	5	0.250	>0.05(Ns)

FCZ: fluconazole; MCZ: miconazole; MIC: minimum inhibitory concentration.
***The value represents the mean of 2 replicates**

Simultaneously with the minimum inhibitory concentration of SNPs and standard antifungal drugs, their minimum fungicidal activities against the tested dermatophytes were assessed. As shown from Figure (3-11) to Figure (3-15), the obtained MFCs are considerably higher in comparison to MICs.

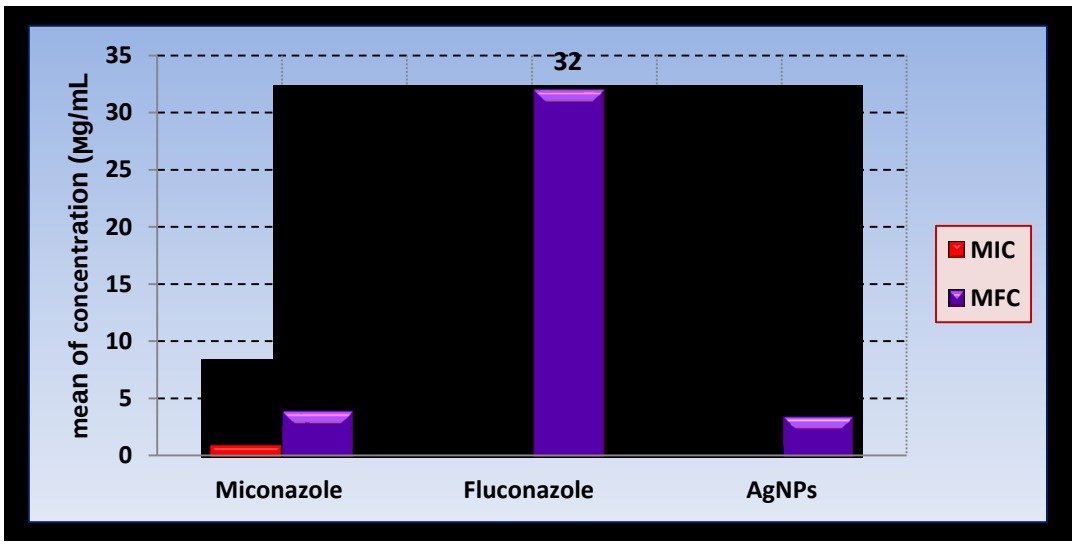


Figure 3-11: Sensitivity profile of *T. interdigitale* to the antifungal agents

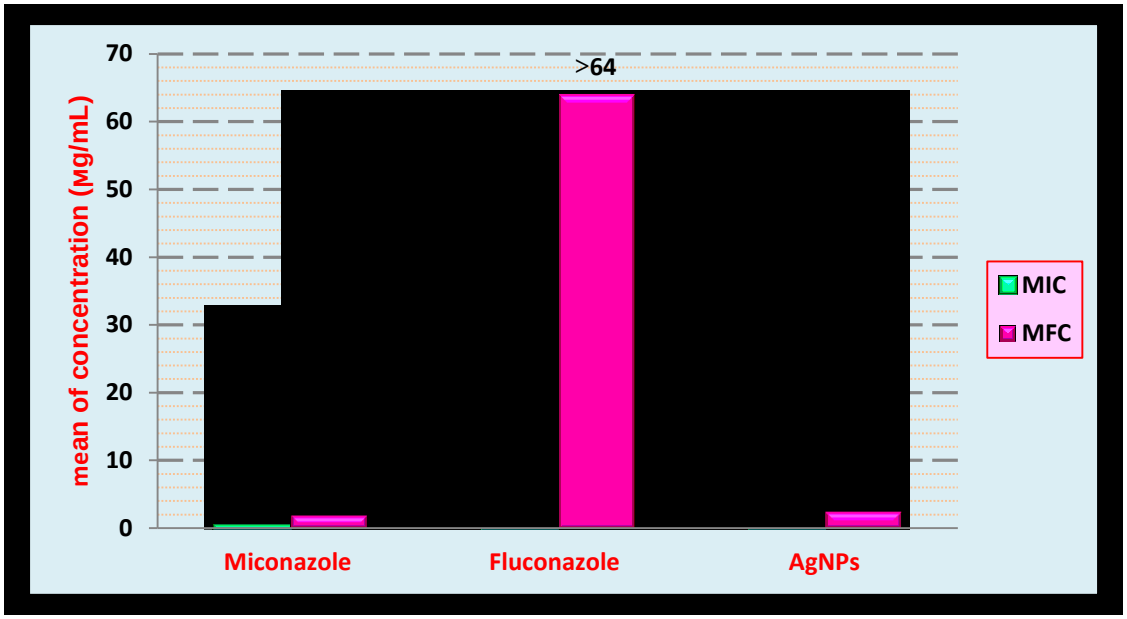


Figure 3-12 : Sensitivity profile of *T. rubrum* to the antifungal agents

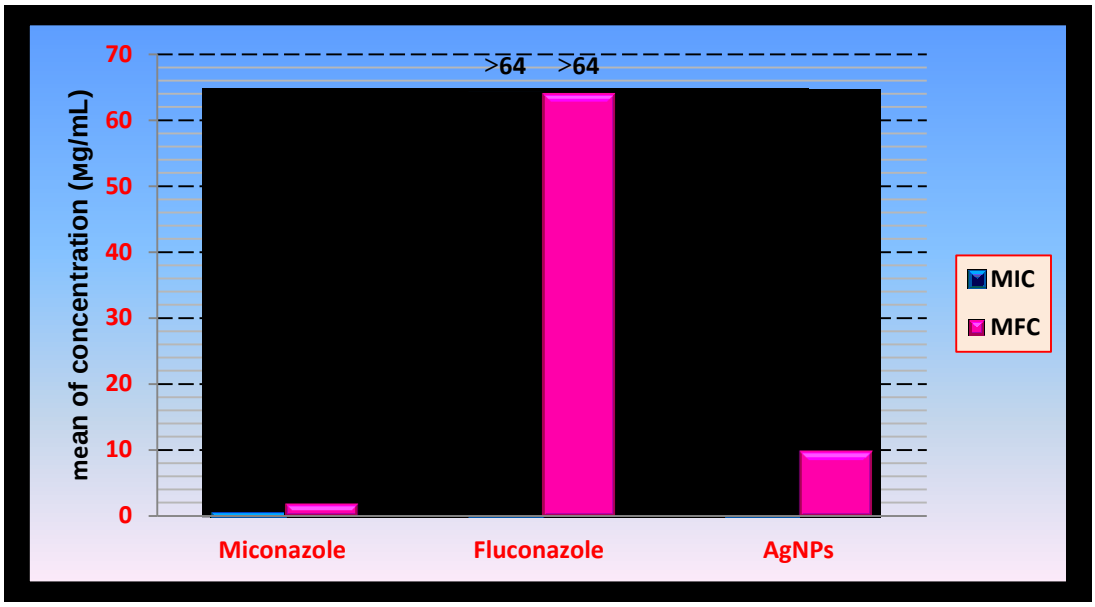


Figure 3-13 : Sensitivity profile of *T.mentagrophytes* to the antifungal agents

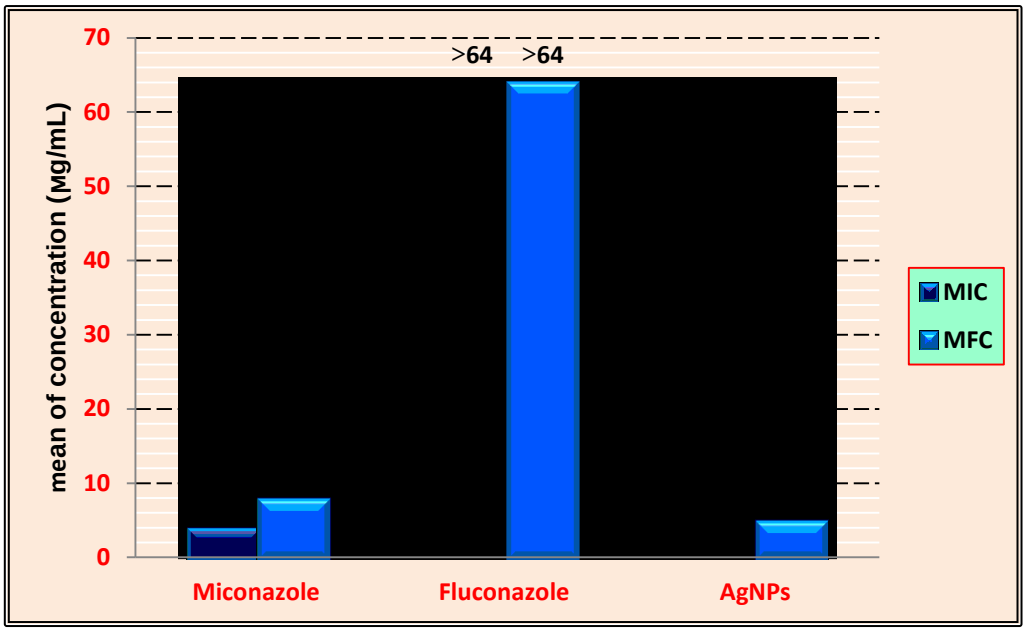


Figure 3-14 : Sensitivity profile of *E. floccosum* to the antifungal agents

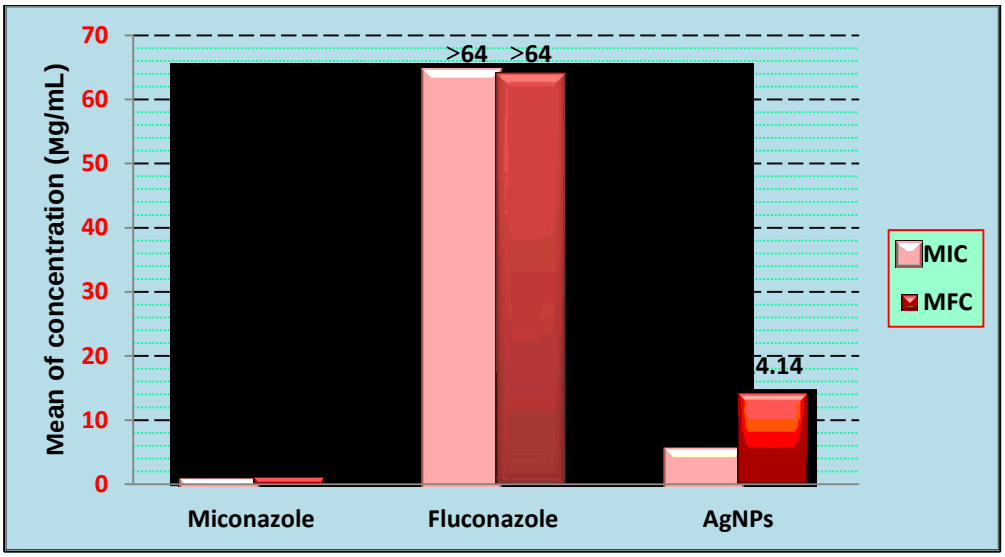


Figure 3-15 : Sensitivity profile of *M. canis* to the antifungal agents

SNPs killed the tested dermatophytes at variable concentration (from 2.5-14.14 µg/ml) depending on the species of dermatophytes. (Table 3- 3)

Table (3-3):Comparative MIC value of biosynthesized SNPs, Fluconazole and Miconazole against dermatophytes strains

Tested fungal strains	MFC/ Mean (µg/ml)					
	SNPs (0.156-80)	FCZ (0.125-64)	p-value	SNPs (0.156-80)	MCZ (0.03-16)	p-value
T.interdigitale	3.54	32	< 0.001	3.54*	4	>0.05(Ns)
T.rubrum	2.5	>64	<0.001	2.5	2	>0.05(Ns)
T.mentagrophytes	10	>64	<0.001	10	2	0.0383
E.floccosum	5	>64	<0.001	5	8	>0.05(Ns)
M.canis	14.14	>64	<0.001	14.14*	1	0.0210

FCZ: fluconazole; MCZ: Miconazole; MFC: Minimum Fungicidal Concentration
***The value represents the mean of 2 replicates**

The common antifungal drug, Fluconazole, had no fungicidal effect on the tested dermatophytes except *T. interdigitale* with value of 32 µg/ml whereas for others dermatophytes the MFC value were greater than the highest tested concentration (64 µg/ml), indicating that these isolates were resistant to this common antifungal drug.

The lowest minimum fungicidal concentration of SNPs was observed against *T.rubrum* (2.5 µg/ml) while the MFC of Miconazole was 2 µg/ml. Moreover, SNPs show excellent fungicidal activity against *T. interdigitale* and *E. floccosum*, with low MFC value 3.54 µg/ml, 5 µg/ml, respectively compared with those obtained by standard antifungal drug, Miconazole, 4 µg/ml and 8 µg/ml respectively.

T. mentagrophytes and *M.canis* killed with a concentration of 10µg/ml & 14.14 µg/ml for SNPs, respectively; while the MFC of Miconazole was 2 µg/ml and 1 µg/ml, respectively.

3.3.1.2: Effect of biosynthesized SNPs on spore germination of dermatophytes:

The effect of biosynthesized SNPs on spore germination of different dermatophytes is presented in Table (3-4).

Table (3-4): Effect of biosynthesized SNPs on spore germination of the tested dermatophytes as compared with Miconazole and Fluconazole as reference antifungal drugs.

Tested fungal strains	Mean percentage of spore germination inhibition					
	SNPs	FCZ	p-value	SNPs	MCZ	p-value
T.interdigitale	84.2	66.6	0.0221	84.2	63.6	0.021
T.rubrum	100	0	<0.0001	100	91.3	>0.05
T.mentagrophytes	78.9	0	<0.0001	78.9	86.3	0.0478
E.floccosum	88.8	0	<0.0001	88.8	57.6	0.0022
M.canis	64.7	0	<0.0001	64.7	100	0.0001

SNPs had pronounced and significant spore germination inhibition effect ($P < 0.05$) on all the tested dermatophytes but this effect was variable depending on the fungal strains (Figure 3-16).

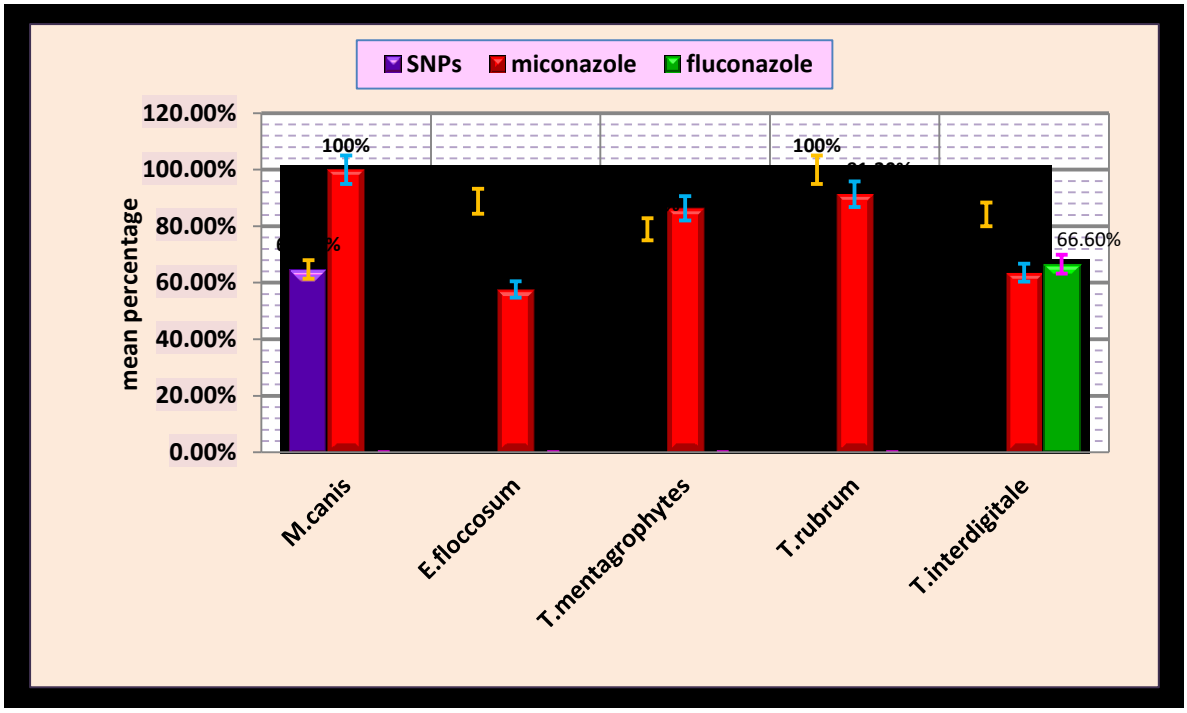


Figure (3-16): Effect of biosynthesized SNPs and antifungal drugs on spore germination of the tested dermatophytes.

The spores of *T. rubrum* incubated with SNPs showed complete inhibition of germination (100%) compared to that in negative control (sterile broth without SNPs) (Figure 3-17), while Miconazole showed potent but not complete effect on the spores germination of *T. rubrum*, the inhibition of germination reached to 91.3%.

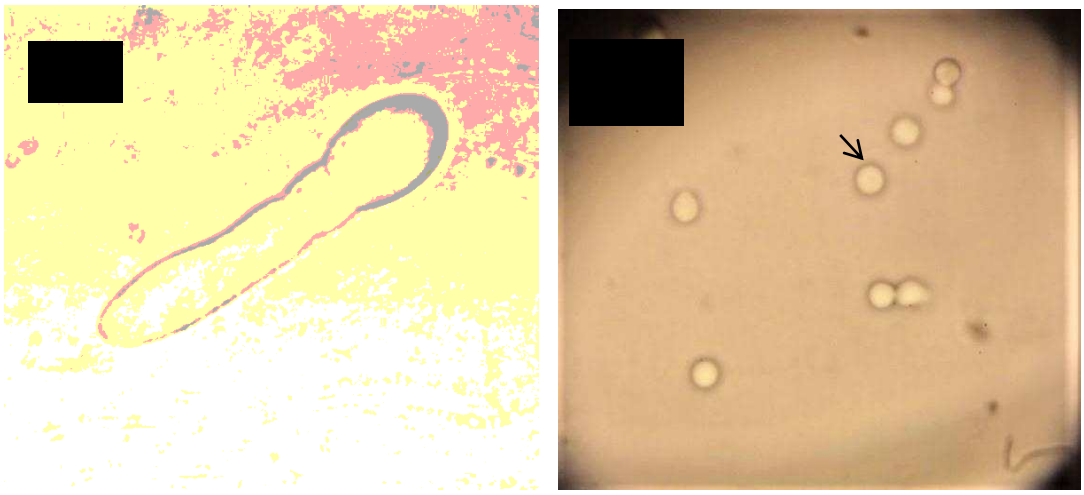


Figure (3-17): Effect of biosynthesized SNPs on spores germination of *T.rubrum* (A): control without SNPs. (B): spores treated with SNPs (100 % inhibited).(Mag:1000x)

Spore germination of the *E. floccosum* and *T. interdigitale* fungi were more affected, but still not completely inhibited, when incubated with SNPs at MIC concentration. The percentages of germination inhibition for these fungi were 88.8% (Figure 3- 18) and 84.2% (Figure 3-19), respectively**.** The Miconazole had significantly lower effect on the spore germination (57.6 % for *E. floccosum* and 63.6% for *T. interdigitale*) compared to that get by SNPs.

The only fungal spores that have been shown to be affected by Fluconazole are the spores of *T. interdigitale* as it inhibited by 66.6%.

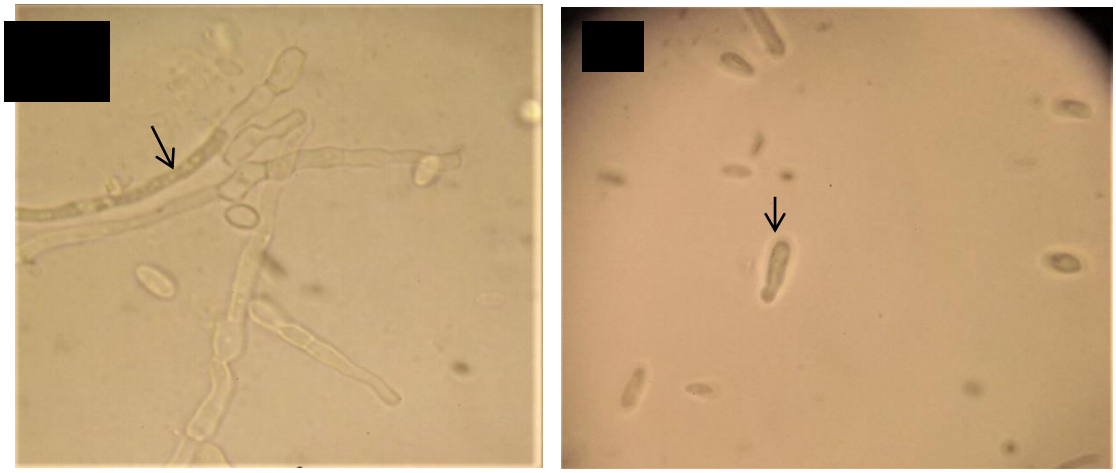


Figure (3-18): Effect of biosynthesized SNPs on Spore's germination of *E.floccosum* (A):control without SNPs.(B):spores treated with SNPs (88.8% inhibited) .(Mag:1000x)

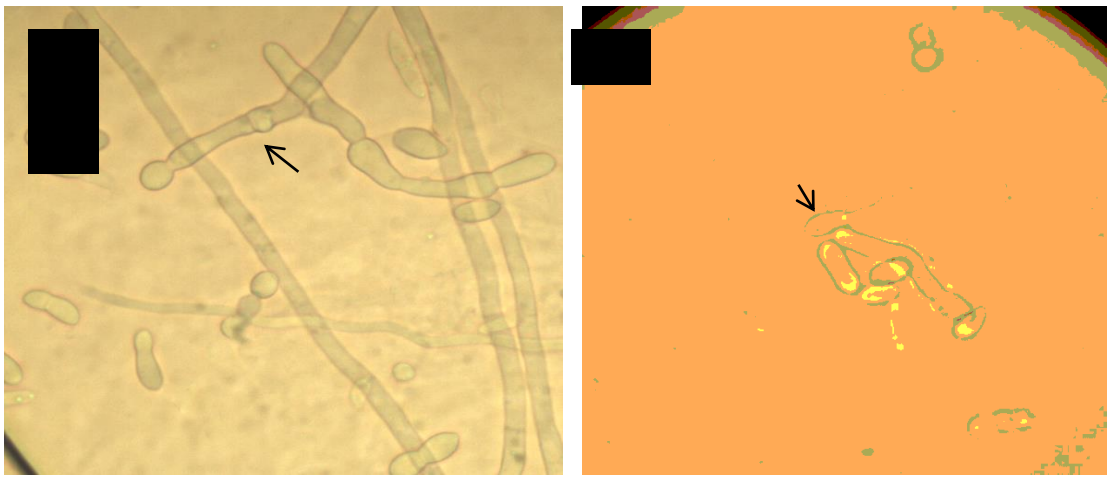


Figure (3-19): Effect of biosynthesized SNPs on Spore's germination of *T.interdigitale* (A): control without SNPs. (B) after treatment with SNPs (84.2 % inhibited). (Mag:1000x)

SNPs showed notable inhibition of *T. mentagrophytes* spore germination (Figure 3-20), the percentage was 78.9%, while the effect of Miconazole on the same spore was higher than the effect of SNPs and it reached to 86.3%.

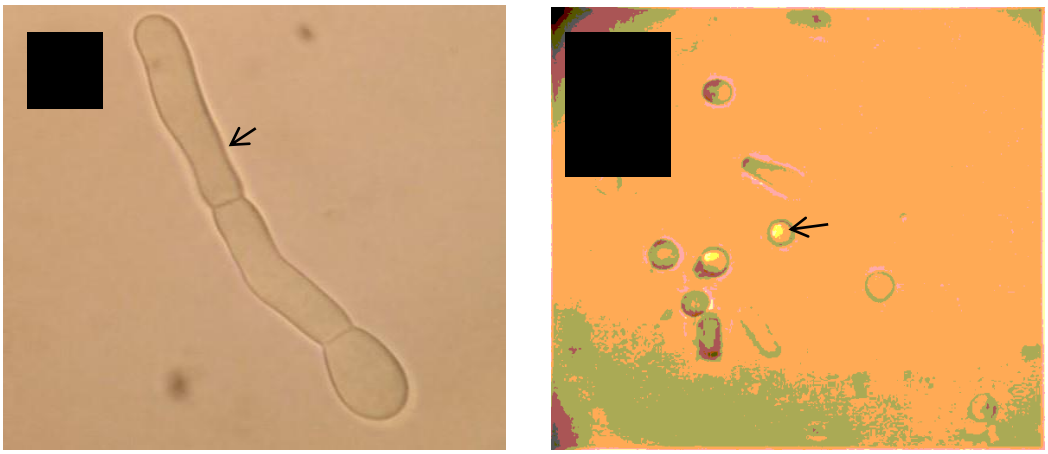


Figure (3-20): Effect of biosynthesized SNPs on spore germination of *T.mentagrophytes*. (A): control without SNPs. (B): spores treated with SNPs (78.9 % inhibited). (Mag:1000x)

M.canis spores were the least affected by SNPs among the rest of the tested dermatophytes (Figure 3-21), the spore germination inhibition (64.7%) was significantly lower compared to those obtained by Miconazole (100%).

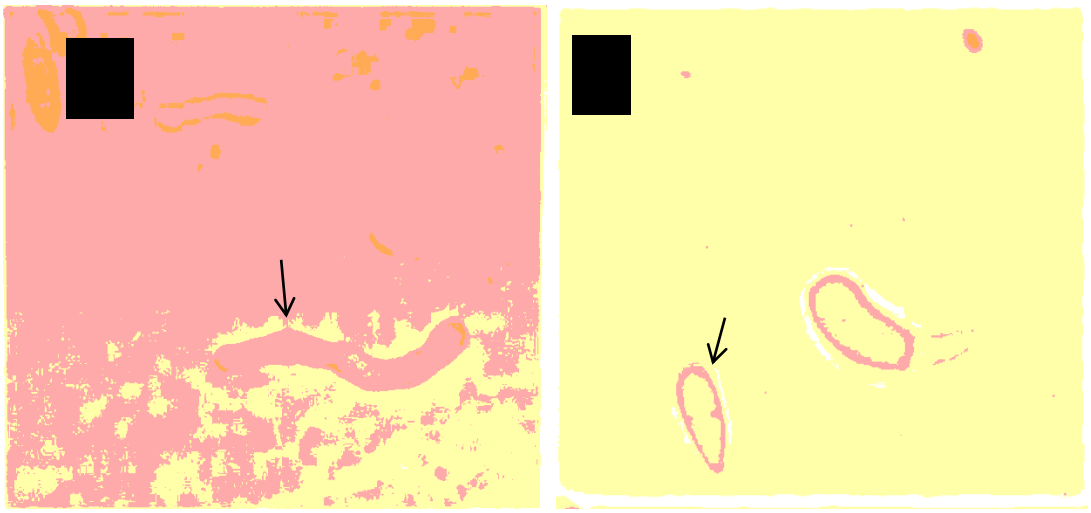


Figure (3-21): Effect of biosynthesized SNPs on spores germination of *M.canis* (A): control without SNPs. (B): spores treated with SNPs (64.7 % inhibited). (Mag:1000x)

In general, the SNPs had a notable impact on fungal spore morphology. In control sample (without SNPS), after germination, small elongated particles are formed, through the formation of a singular germ tube with a tubular shape. While the presence of SNPs produce visible morphological changes in the germinating spores. These changes represented as abnormal germination tube, pronounced enlargement or abnormal swelling of the conidia like chlamydospores and in some cases there was no hyphae grew from it (Figure 3- 22).

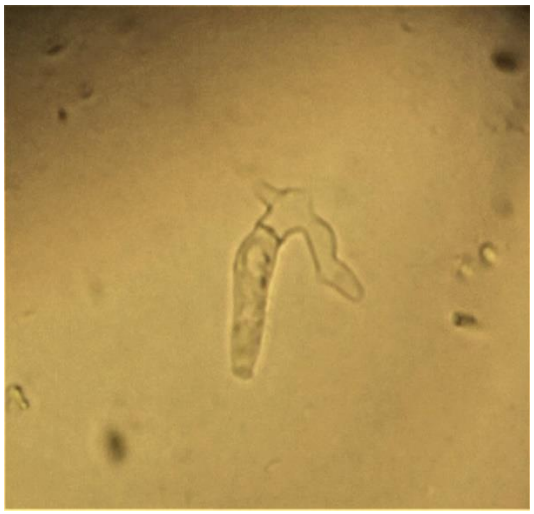


Figure (3-22): Deformities in spore germination when incubated with SNPs. (Mag:1000x)

3.3.1.3: Determination of the inhibitory effect of biosynthesized SNPs on the keratinase activity

Keratinase is the key enzyme in fungal invasion of skin and its appendages. So the effect of biosynthesized SNPs on the keratinase activity of the tested dermatophytes were performed under *in vitro* laboratory conditions in the liquid mineral medium incorporated with keratin. The results indicated that the SNPs applied at MIC value caused significant and differential reduction in keratinase activity depending on the tested species. The effects of SNPs on keratinase enzyme activity are mentioned in Table (3-5) .

Table 3-5: Effect of biosynthesized SNPs on keratinase activity of the tested dermatophytes as compared with Miconazole and Fluconazole as reference antifungal drugs.

Dermatophytes	Keratinase activity (KU)						
	Control (Mean ± SD)	SNPs		Miconazole		Fluconazole	
		Activity	Reduction %	Activity (Mean ± SD)	Reduction %	Activity (Mean ± SD)	Reduction %
T.interdigitale	9.47± 0.65	6.267± 1.25	33.82	7.77± 1.24	17.97	6.6 ± 0.656	30.23
T.rubrum	37.53±3.82	26.57± 1.32*	29.22	22.63± 0.86*	39.7	0	0
T.mentagrophytes	11.67±1.67	9.93± 0.862	14.83	8.367± 0.71	28.3	0	0
E.floccosum	11.03±2.15	5.83± 1.457*	47.41	8.7 ±0.67	26.01	0	0
M.canis	19.37±2.32	11.73± 2.074*	39.41	15.67± 2.46*	19.11	0	0

***= Significant ($p < 0.05$) compared with control**

The maximum reduction in activity was recorded with *E.floccosum* (47.41%) which had significant values compared with control at $P< 0.05$, while treatment with Miconazole resulted in lower enzyme reduction (26.01%). The keratinase activity of *M. canis* was significantly affected in the presence of either SNPs or Miconazole (39.41 %, 19.11% respectively).

Although the SNPs inhibited the keratinase activity of *T. interdigitale* (33.82%), the effect was insignificant; a similar effect was demonstrated by both Miconazole and Fluconazole (17.97%, 30.23% respectively).

T. rubrum keratinase activity was significantly affected when treated with SNPs or Miconazole with areduction value reached to 29.22% and 39.7%, respectively.

T. mentagrophytes showed slightly lower enzyme reduction in the presence of SNPs (14.83%) compared to other dermatophytes (Figure 3-23).While the effect of Miconazole on it was 28.30 %.

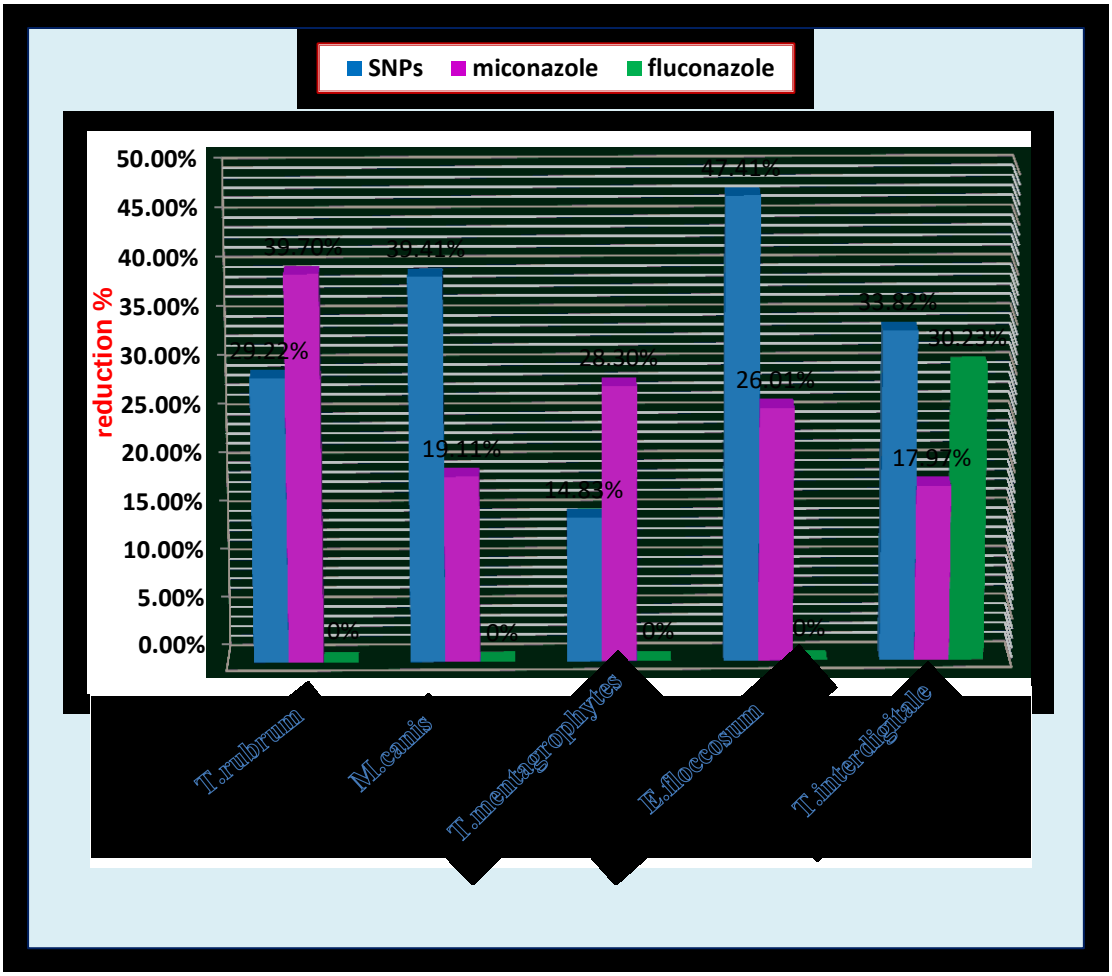


Figure (3-23): Effect of biosynthesized SNPs on keratinase reduction of the tested dermatophytes as compared with FCZ and MCZ as reference antifungal drugs

3.3.1.4: Effect of biosynthesized SNPs on the mycelia development of dermatophytes in the broth media

Our results showed that fungal exposure to biosynthesized SNPs caused drastic changes in the mycelial morphology of all strains of dermatophytes compared with untreated controls, and most of induced morphological abnormalities were similar in all the tested dermatophytes.

The microscopic observation at 1000X magnification showed that mycelia in the control samples (untreated cells) had typical net structure, regular cell, long clear and smooth hyphae with homogenous cytoplasm (Figure 3-24 A). In addition, abundant conidiation with micro and macroconidia are present, while the chlamydospores were completely absent. In contrast, SNPs clearly damaged the hyphae with sever distortion. It could be clearly seen that SNPs either attached to the mycelium wall or penetrated the mycelial filaments which can be visualized as black aggregations. The mycelium had shown slightly darker than the control. Moreover, the brown color of the liquid media, where the fungi grew, that resulting from surface Plasmon resonance was diminished or disappeared in some cases. Furthermore, quite a few hyphae had a significant amount of aborted lateral branching as in *M.canis* (Figure 3-24 B) and the higher magnification showed that some hyphal tips had dichotomous branching . The vast majority of hyphae were swelling and often appeared wider than normal hyphae with presence of large vacuoles inside them (Figure 3-24 C &D).

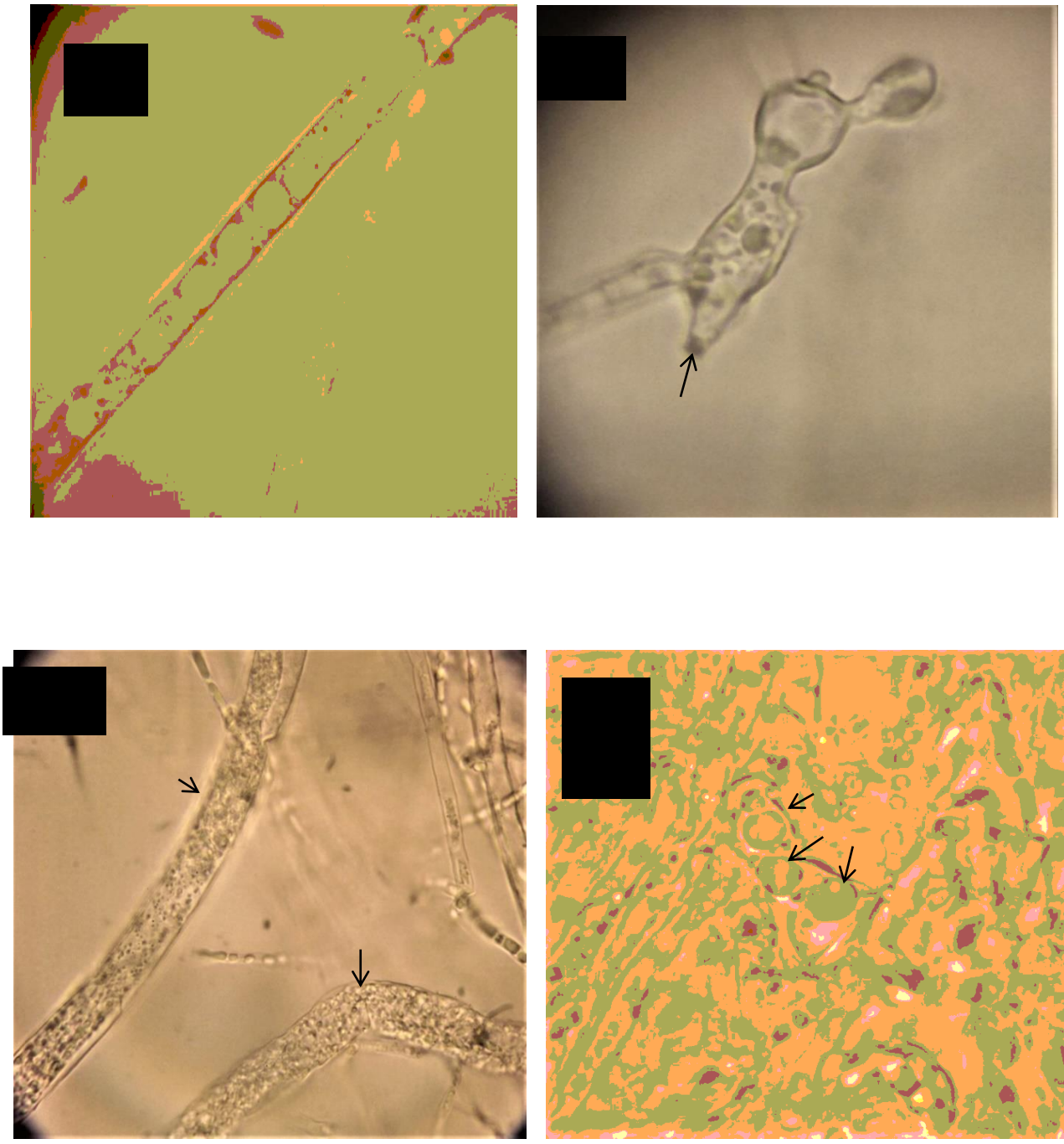


Figure (3-24) : *M.canis* mycelium (A) control. (B) incubated with SNPs the hyphae showed aborted lateral branching (C) Swelling hypha of *M.canis* which appear wider than the normal hypha.(D) The arrows refer to large vacuoles inside the hyphae. (Mag:1000x).

Some hyphae showed ballon–like enlargement of individual cells which located either in the middle of hyphae or apically, as in case of *M.canis* (Figure 3-25) and *T.rubrum* (Figure 3-26).

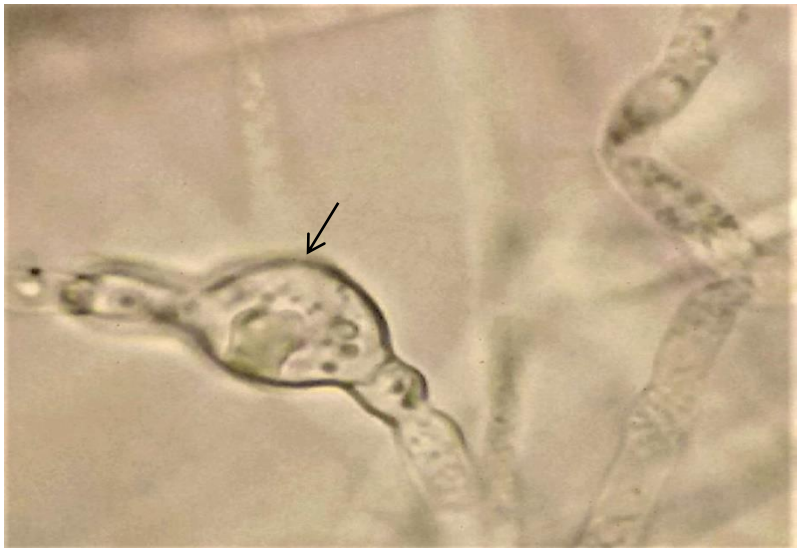


Figure 3-25 : *M.canis* incubated with SNPs .The arrow indicates the presence of balloon like cell. (Mag:1000x)

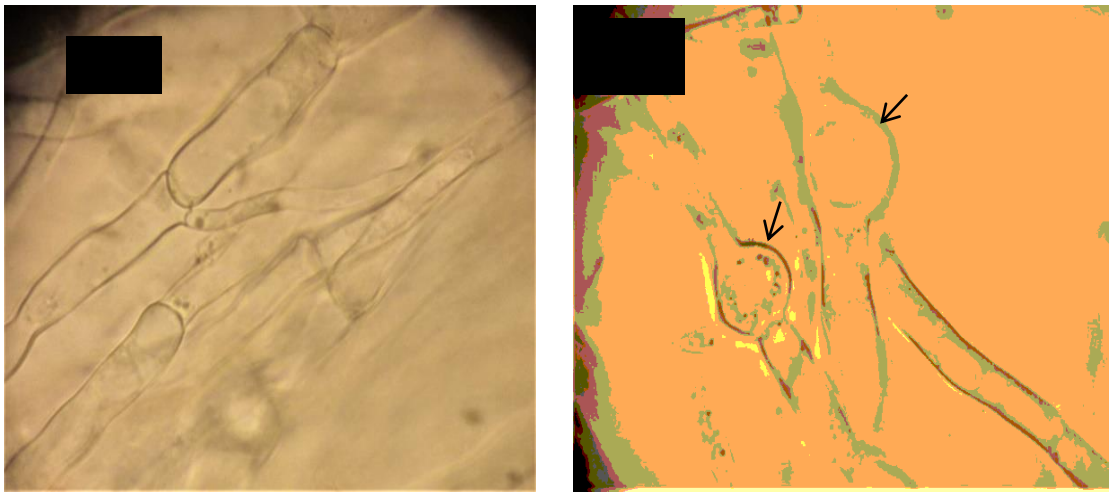


Figure 3-26 : *T.rubrum* hyphae (A) Control (B) incubated with SNPs with evident swollen cells like balloon shape. (Mag:1000x)

Observation of *T. interdigitale* under light microscope, after exposure to SNPs, showed collapsed hyphae, wall disorganization and loss of integrity of their biological membranes indicating extensive cellular death (Figure 3-27).

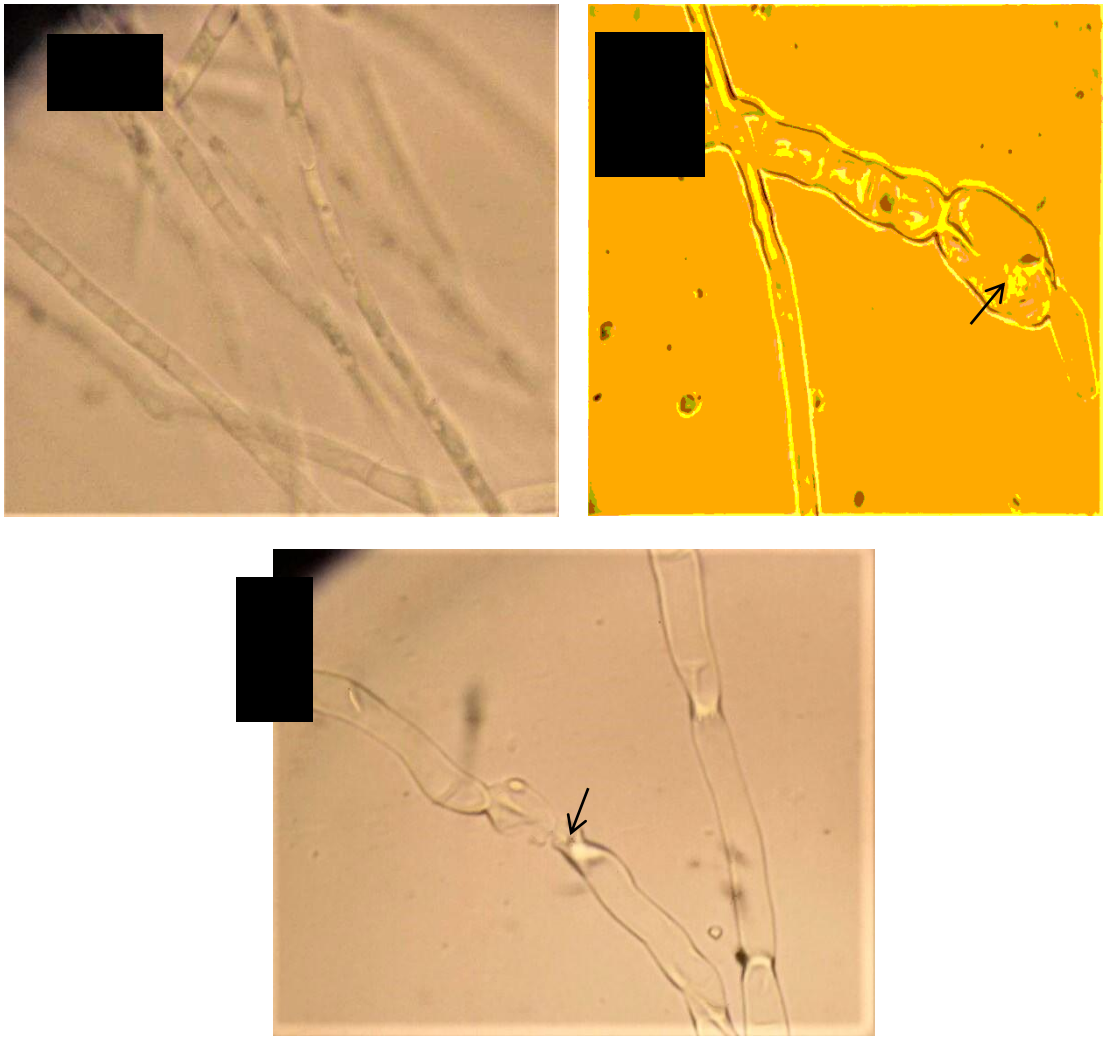


Figure 3-27: Effect of SNPs on mycelial growth of *T.interdigitale* (A) Normal mycelia in control sample (B) deformed hyphae with obvious cracks on the cells wall.(C) Damaged hyphae. (Mag:1000x)

The presence of chlamydospores was abundant in all the tested dermatophytes (Figure3-28 B and 3-29 B).

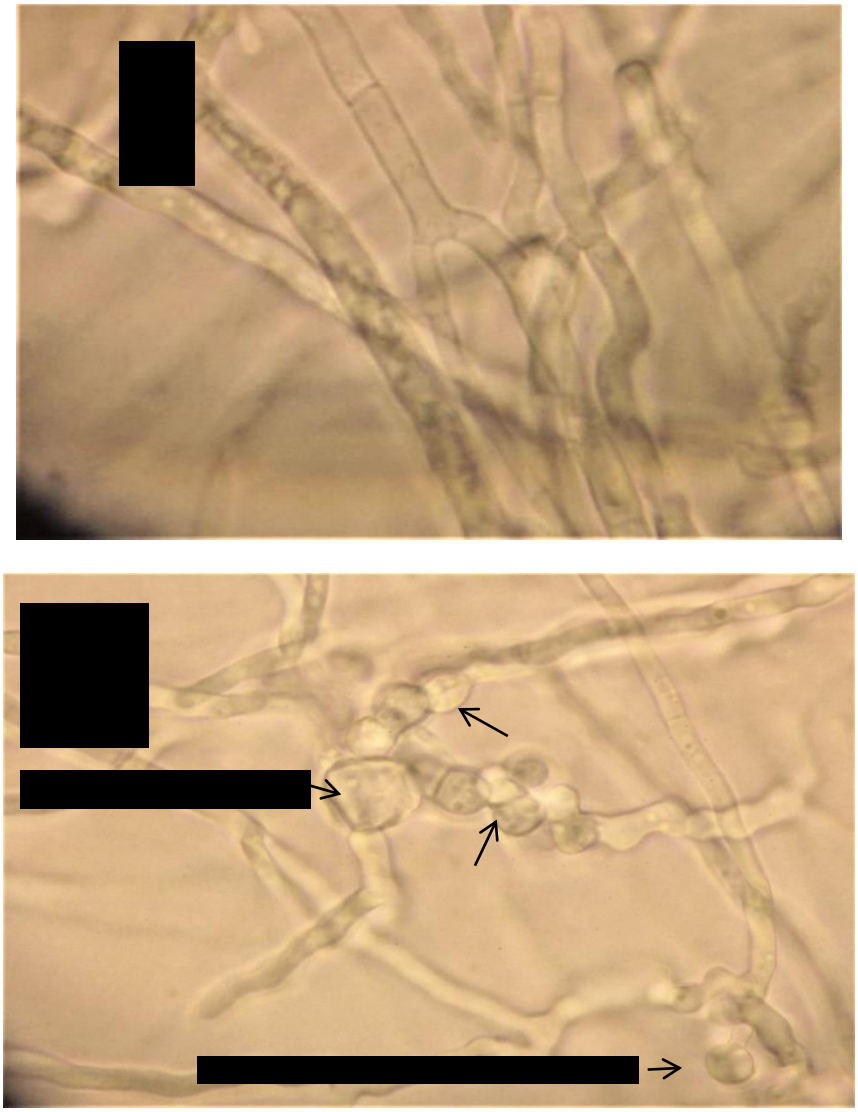


Figure (3-28): Effect of SNPs on mycelial growth of *E.floccosum* (A) normal mycelia in control sample (without treatment). (B) Distorted mycelium due to effect of SNPs, It exhibited a terminal and an intercalary chlamydospores in the middle of hyphae (or perhaps the hyphae itself running through it). (Mag:1000x)

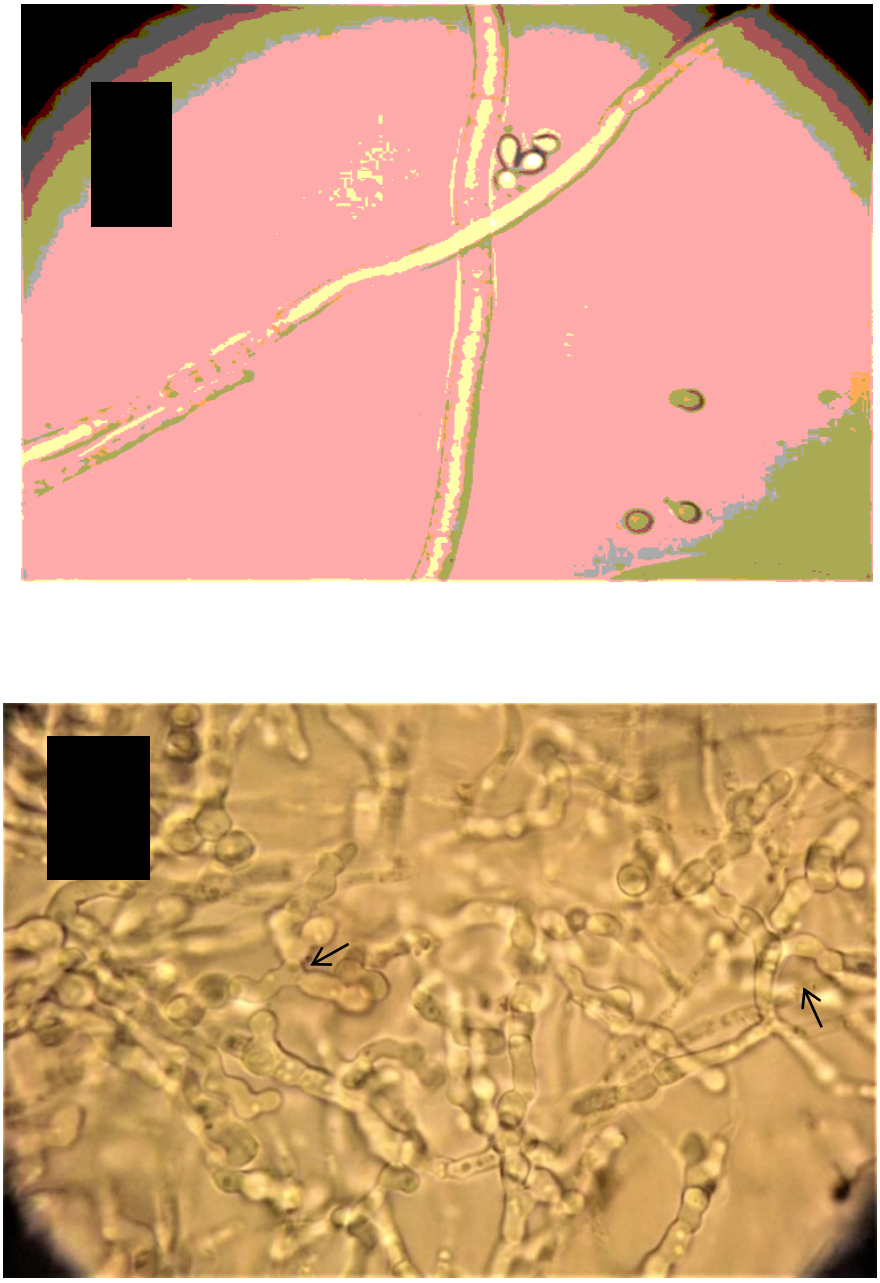


Figure (3-29): Effect of SNPs on growth of *T.mentagrophytes* (A) normal hyphae with obvious conidiation in control sample (B) Mycelia incubated with SNPs. The arrows indicate dichotomous tip branching as well as chlamydospores. (Mag:1000x)

3.4.2: Antifungal effect of biosynthesized SNPs on *Candida albicans*:

3.4.2.1: Determination of MIC and MFC values for *Candida albicans*:

Candida albicans was chosen as a model for yeast to study the effect of biosynthesized SNPs. MIC against *C. albicans* was determined by broth microdilution technique in accordance with CLSI guidelines.

According to the Table (3 –6), the SNPs had a significant inhibitory effect on *C. albicans*. When 2.5 µg of SNPs was added, the growth rate was inhibited.

Table (3-6): Minimum inhibitory concentration (MIC) of *Candida albicans* to SNPs, Fluconazole and Miconazole as proposed by CLSI (broth microdilution method).

Tested fungal strains	MIC			MFC			p-value
	Mean (µg/ml)			Mean (µg/ml)			
	SNPs (0.156-80)	FCZ (0.125-64)	MCZ (0.03-16)	SNPs (0.156-80)	FCZ (0.125-64)	MCZ (0.03-16)	
Candida albicans	2.5	>64	1	10	>64	2	<0.001

In comparison to the standard antifungal agents which are frequently used in the treatment of candidiasis, such as Fluconazole and Miconazole, the SNPs exhibited variable effects . No significant differences were demonstrated between SNPs and Miconazole; this implies that the two agents have approximately similar effect. While the MIC of SNPs was significantly lower than Fluconazole. All concentration of Fluconazole used in our study did not cause any inhibition against *C. albicans* indicating that this strain was resistant to this common antifungal drugs.

The MFCs of the SNPs, as well as the standard antifungal agents, against *C. albicans* were examined. As clear from the Table (3-6), the fungicidal activity of SNPs needs higher concentration in comparison to the MIC reached to 4x MIC. The tested strains were highly sensitive to Miconazole, it was generally fungicidal at concentration close to 2µg/mL. While SNPs exhibited a more moderate fungicidal ability in that it was fungicidal at 10µg/ml (Figure 3-30). However, when Fluconazole was tested against *C. albicans*, it was inactive even in high concentration as described previously.

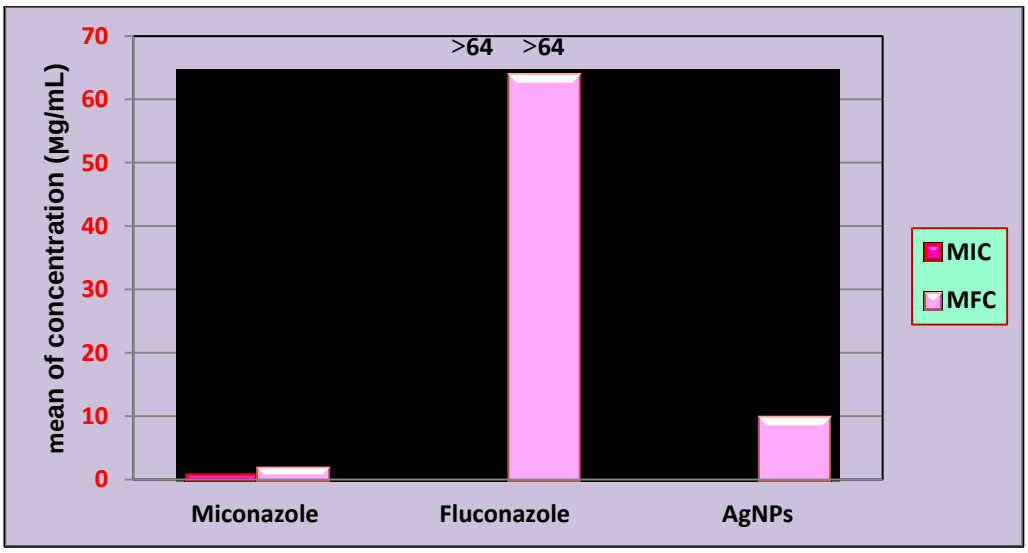


Figure (3-30) : Sensitivity profile of *C. albicans* to the antifungal agents

3.4.2.2: Effect of SNPs on the adherence of the tested *Candida albicans* strain on plastic surface:

Attachment of *C. albicans* to plastic materials of medical devices seems to be a critical event in the initiation of colonization and biofilm development. In this study, we investigate the ability of SNPs to block the adhesion of *C. albicans* to plastic surface.

The results showed that biosynthesized SNPs at different concentration were able to significantly ($P < 0.001$ & $P < 0.05$) inhibit the

adherence capacity of the tested strain of *C. albicans* that were resistant to Fluconazole in vitro.

SNPs at 0.5 MIC and MIC concentration (1.250µg/mL and 2.5µg/ml, respectively) produce a significant reduction in the *C. albicans* adherence to plastic surface of 26% and 43% ($P < 0.05$), respectively. While at higher concentration of 2X MIC (5µg/mL) produce a greater effect reached to 51% ($P < 0.001$) compared to the negative control (Table 3 - 7).

Table (3-7): Effect of biosynthesized SNPs at the sub-MIC, MIC and above MIC concentration on the adherence of *C.albicans*.

Concentration	Optical Density		% of biofilm inhibition
	Mean	± SD	
0.5 MIC	0.303*	0.006	26 %
MIC	0.277*	0.009	43 %
2X MIC	0.259**	0.008	51%
Control	0.369	0.0007	-

** **Significant ($p < 0.001$)** in compared with control, *= **Significant ($p < 0.05$)** compared with control

The study also demonstrated the anti-adherent properties of Miconazole against the Fluconazole-resistant *C. albicans*. As illustrated in Table (3-8), all the tested concentration used exhibited significant decreases in the yeast adherence ranging from 22% ($P < 0.05$) for 0.5 MIC value (0.5µg/ml) to 43% ($P < 0.001$) for 2X MIC value (2 µg/ml).

Table (3-8): Effect of Miconazole at the sub-MIC, MIC and above MIC concentration on the adherence of *C.albicans*

Concentration	Optical Density		% of biofilm inhibition
	Mean	± SD	
0.5 MIC	0.319*	0.0057	22 %
MIC	0.295**	0.002	35 %
2X MIC	0.281**	0.0021	43%
Control	0.369	0.0007	-

** **Significant (p < 0.001)** in compared with control, *= **Significant (p < 0.05)** in compared with control

Figure (3-31) display the comparison between the anti-adherent activities of biosynthesized SNP and the standard antifungal agent, Miconazole. It is evident that the SNPs have a greater effect than the Miconazole in all tested concentration. The superiority of biosynthesized SNPs in preventing adherence of *C. albicans* to plastic surface indicates that this agent could be a good candidate in the prevention of the early stage of biofilm formation and so in the prevention of candidiasis related to medical devices.

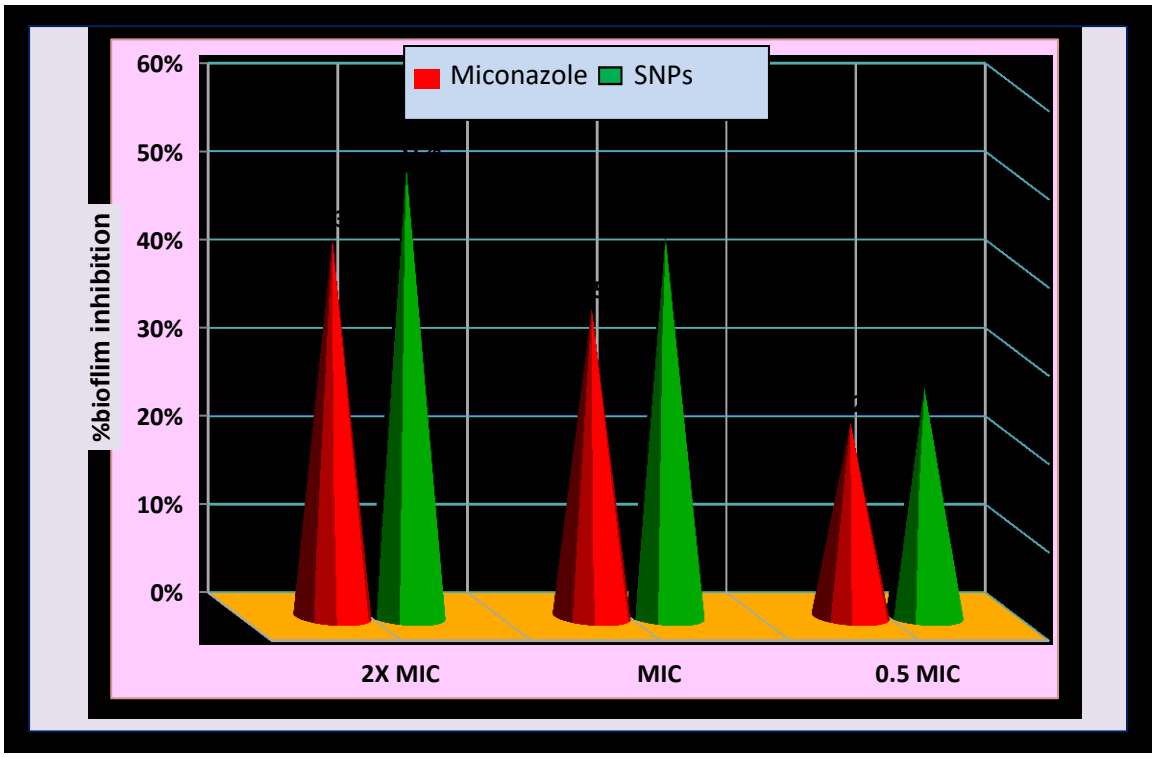


Figure (3-31): inhibition effect of biosynthesized SNPs & MCZ at sub MIC, MIC and above MIC concentration on adherence of *C. albicans*

3.4.2.3: Effect of biosynthesized SNPs on the pre-formed biofilm of the tested *Candida albicans* strain.

To know the antifungal efficacy of biosynthesized SNPs against *C. albicans* mature biofilms, it performed susceptibility testing against preformed biofilms. The SNPs demonstrated a dose-dependent inhibitory effect when tested against pre-formed *C. albicans* biofilms (Table 3-9) resulting in a significant biomass reduction of 13.8% and 22.8% ($P < 0.05$) for 0.5 MIC and MIC, respectively. Moreover, further increase in the SNPs concentration to 2X MIC had effectively reduced the established biofilms to 36.1% ($P < 0.001$).

Table (3-9): Effect of biosynthesized SNPs at sub MIC, MIC and above MIC concentration on pre-formed biofilm of *C. albicans*

Concentration	Optical Density		% of biofilm inhibition
	Mean	± SD	
0.5 MIC	0.362*	0.0113	13.8 %
MIC	0.324*	0.006	22.8 %
2X MIC	0.268**	0.0042	36.1%
Control	0.420	0.001	-

** **Significant (p < 0.001)** in compared with control, *= **Significant (p < 0.05)** in compared with control

Similarly, the Miconazole was significantly decreased the total biomass of *C. albicans* in dose-dependent manner in all concentration used compared to control (Table 3-10). Their ability to reduce the pre-formed biofilm was ranged from 9.5% (P < 0.05) to 31.1 % (P <0.001).

Table (3-10): Effect of Miconazole at sub MIC, MIC and above MIC concentration on pre-formed biofilm of *C. albicans*

Concentration	Optical Density		% of biofilm inhibition
	Mean	± SD	
0.5 MIC	0.381*	0.0049	9.5 %
MIC	0.3445*	0.016	18.09 %
2X MIC	0.289**	0.0014	31.1%
Control	0.420	0.001	-

** **Significant (p < 0.001)** in compared with control, *= **Significant (p < 0.05)** in compared with control

However, the anti-biofilm effect of Miconazole was lesser than that produced by biosynthesized SNPs (Figure 3-32). Thus, it can be expected

that biosynthesized SNPs may have potency as antifungal agent to prevent biofilm-associated diseases.

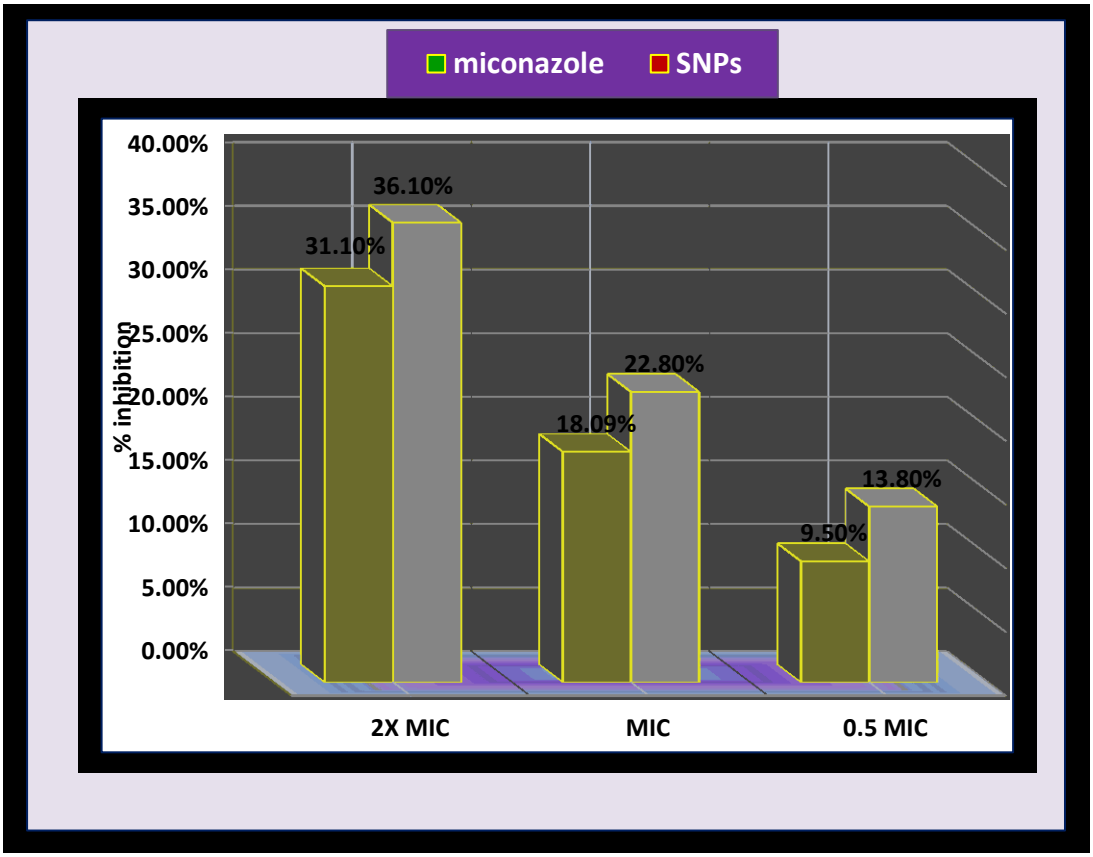


Figure (3-32): Inhibitory effect of biosynthesized SNPs & MCZ at sub MIC, MIC and above MIC concentration on pre-formed biofilm of *C. albicans*

3.4.2.4: Inhibitory effect of biosynthesized SNPs on the dimorphic transition:

The effect of biosynthesized SNPs on mycelial formation of *C. albicans* was further examined. Hyphal growth assay was performed in presence of different concentration of SNPs in hypha inducer (human serum) at 37 °C. After 4 h of incubation, aliquots of the cells were microscopically visualized. In control samples (Figure 3-33), clear *C. albicans* hyphae were observed. Each individual yeast cell had formed elongated germ tube with a length about one-half or more the diameter of their mother cell. Then, each germ tube grew into long multinucleated mycelium.

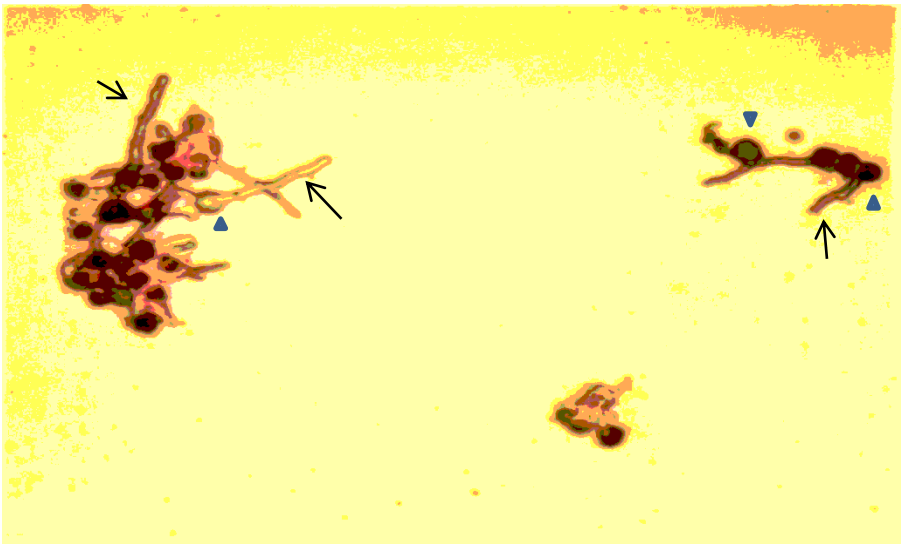


Figure (3-33): Germ tube formation in *C. albicans* incubated with human serum. Filled triangles refer to mother cells while the arrows refer to germ tube. (Mag:1000 x).

In contrast, the presence of SNPs completely suppressed the formation of mycelia (Figure 3- 34).

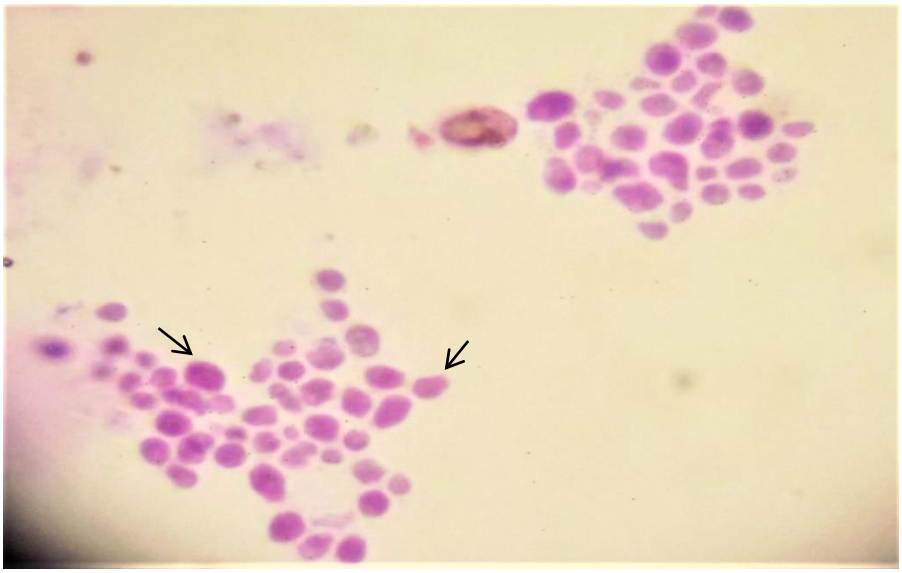


Figure (3-34): Light microscopy image of *C. albicans* incubated with human serum in the presence of SNPs. reveals complete absence of germ tubes. (Mag:1000 X)

As shown in Table (3-11), all concentrations of SNPs used in this experiment were completely prevented the development of germ tube with a percentage of inhibition 100% and the *C. albicans* remains in yeast form.

Table (3-11): Percentage of inhibitory effect of biosynthesized SNPs on the dimorphic transition of tested *C.albicans*

Concentration	SNPs (% of inhibition)	Miconazole (% of inhibition)
0.5 MIC	100*	94.3*
MIC	100*	100*
2X MIC	100*	100*

*** Significant ($p < 0.001$) in compared with control.**

Similar results were obtained for Miconazole; it showed a significant effect on the germ tube formation in *C. albicans*. However, the inhibition percentage was 94.3% at 0.5 MIC concentrations, indicating concentration dependent inhibition of germ tube development by Miconazole (Figure 3-35).

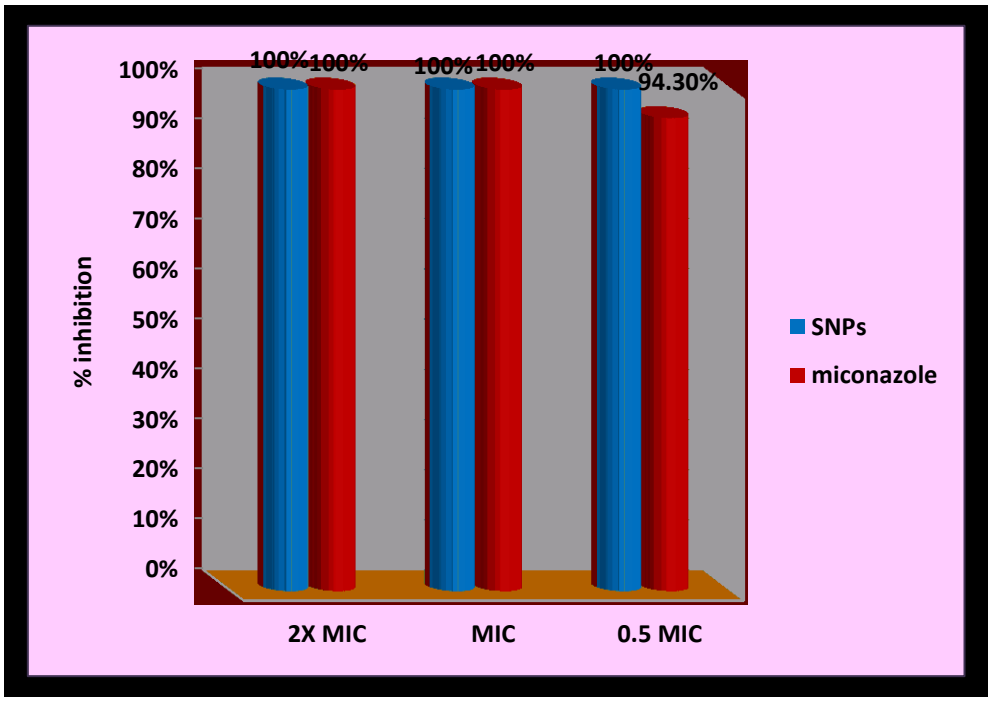


Figure (3-35): Inhibitory effect of biosynthesized SNPs & MCZ on the dimorphic transition of the tested *C. albicans*

The above findings indicate that exposure of *C. albicans* to relatively low concentration of biosynthesized SNPs can modulate the candidal germ tube formation, and thereby interference with the organisms pathogenicity.

3.4.3: Evaluation of synergistic potential of biosynthesized SNPs in combination with antifungal agents:

The synergistic effect of mycosynthesized SNPs combined with two conventional antifungal drugs against both dermatophytes, namely *T.interdigitale, T.rubrum, T.mentagrophytes, M.canis and E.floccosum* as well as *C. albicans*, was investigated using standard well diffusion method and the effect was evaluated by measuring the diameter around the inhibition zone and estimation of increase in fold area.

According to the results obtained by the susceptibility test of the standard antibiotic alone, all strains were resistant to Fluconazole except *T.interdigitale*. Whereas, the Miconazole showed variable degrees of effect. Table (3-12) summarizes the susceptibility of the tested fungi to the antibiotic alone.

Table (3-12): Susceptibilities of dermatophytes and *Candida albicans* to the antifungal agents.

Fungal strain	Fluconazole (25µg)	Miconazole (10µg)
T.interdigitalis	**S**	**I**
T.rubrum	**R**	**S**
T.mentagrophytes	**R**	**S**
E.floccosum	**R**	**I**
M.canis	**R**	**S**
C.albicans	**R**	**I**

S= sensitive, I= intermediate, R= Resistant. (The result interpreted according to Criteria of sensitivity & resistance of antifungal agents mentioned previously)

No inhibition zones were observed when the fungal cell-free filtrate alone was used; similar observation was recorded in case of $AgNO_3$ for all test fungi. The biosynthesized SNPs alone exerted significant activity against all tested fungi, resulted in formation of varying zone of inhibition depending on the fungal strain used. The maximum inhibition zone was observed with *T.rubrum* which was about 30.77mm in diameter, whereas the minimum is found against *C. albicans* which showed an inhibitory zone diameter of 17.67mm. *T.interdigitale, T.mentagrophytes, E.floccosum* and *M.canis* were also showed zones of inhibition about 23.33 mm, 22.1mm, 24.2mm and 18.67mm in diameter, respectively.

The synergistic activities of SNPs in the presence of conventional antibiotics showed effectiveness against most of the fungal strains and mostly enhanced the antifungal effect of the antibiotics.

A very surprising and unexpected finding was observed in the case of the synergistic effect of Fluconazole combined with SNPs. Fluconazole alone showed no antifungal activity against most of the tested fungi. The synergistic effect had shown enhanced zone of inhibition of 22.53mm with 2.4% fold increase against *T.interdigitale* (figure 3-36), 27.3mm inhibition zone with 100% fold increase against *T.rubrum* (Figure 3- 37), 26.87mm inhibition zone with 100% fold increase against *T. mentagrophytes* (Figure 3-38), 19.87 mm zone of inhibition with 100% fold increase against *E.floccosum* (Figure 3-39), 14.63mm with 100% fold increase against *M.canis* (Figure 3-40) and finally 19.33mm inhibition zone with 100 % fold increase against *C. albicans* (Figure 3-41). It is evident from the obtained results that combination of Fluconazole with SNPs restores its antifungal activity, so a low - concentration of silver is sufficient to restore the susceptibility of fungi to Fluconazole.

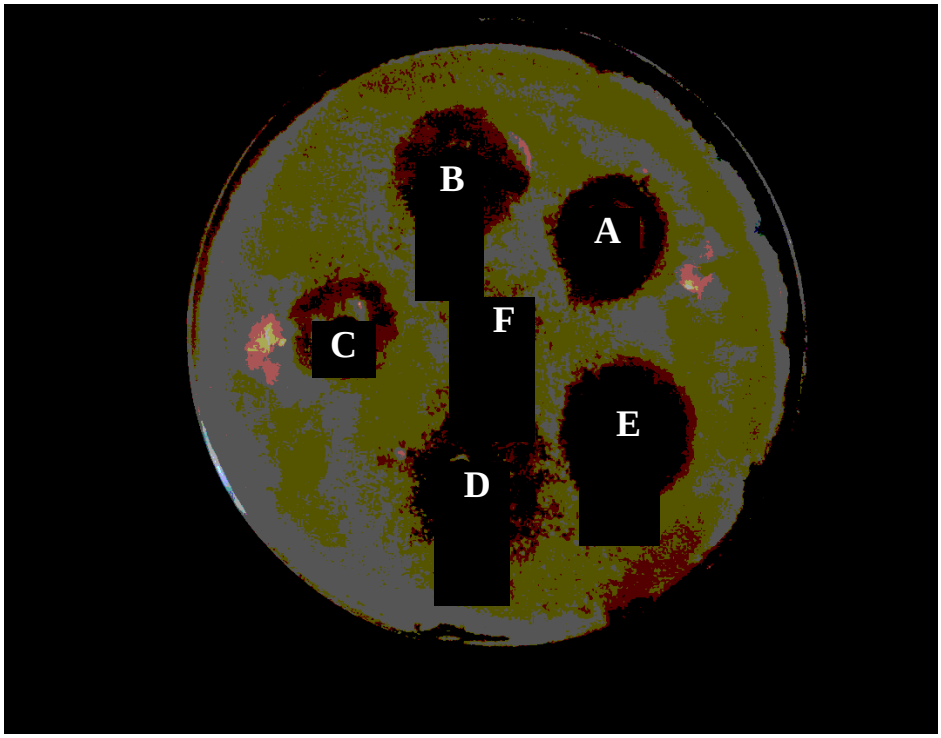


Figure (3-36) : Antifungal activity of SNPs, in comparison with antibiotics, against *T.interdigitale* and their synergistic effect (A) SNPs alone (B) FCZ alone(C) MCZ alone (D) SNPs + FCZ (E) SNPs +MCZ (F) Control.

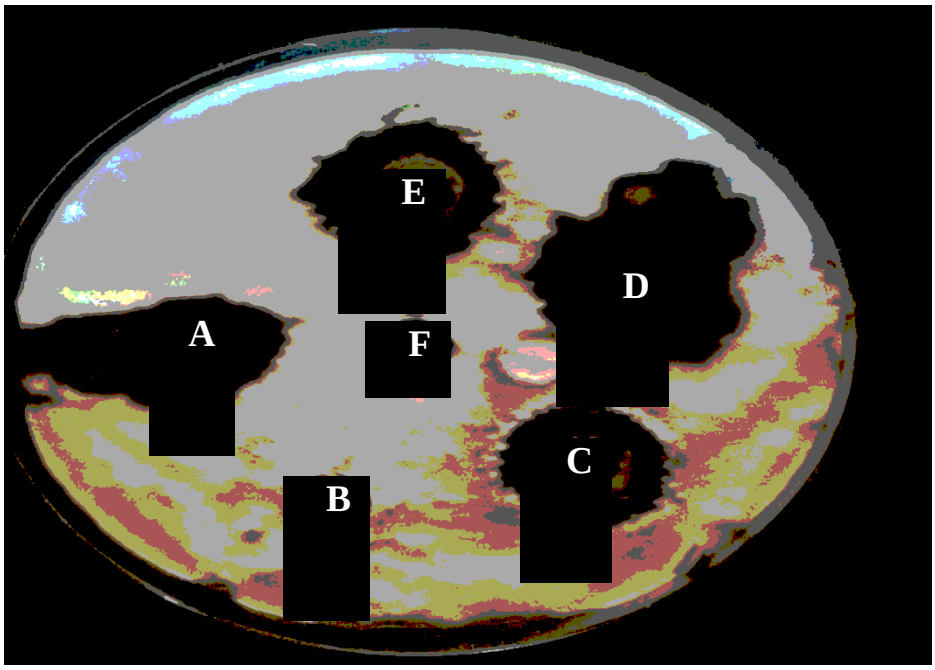


Figure (3-37) : Antifungal activity of SNPs, in comparison with antibiotics, against *T.rubrum* and their synergistic effect (A) SNPs alone (B) FCZ alone(C) MCZ alone (D) SNPs + FCZ (E) SNPs +MCZ (F) Control.

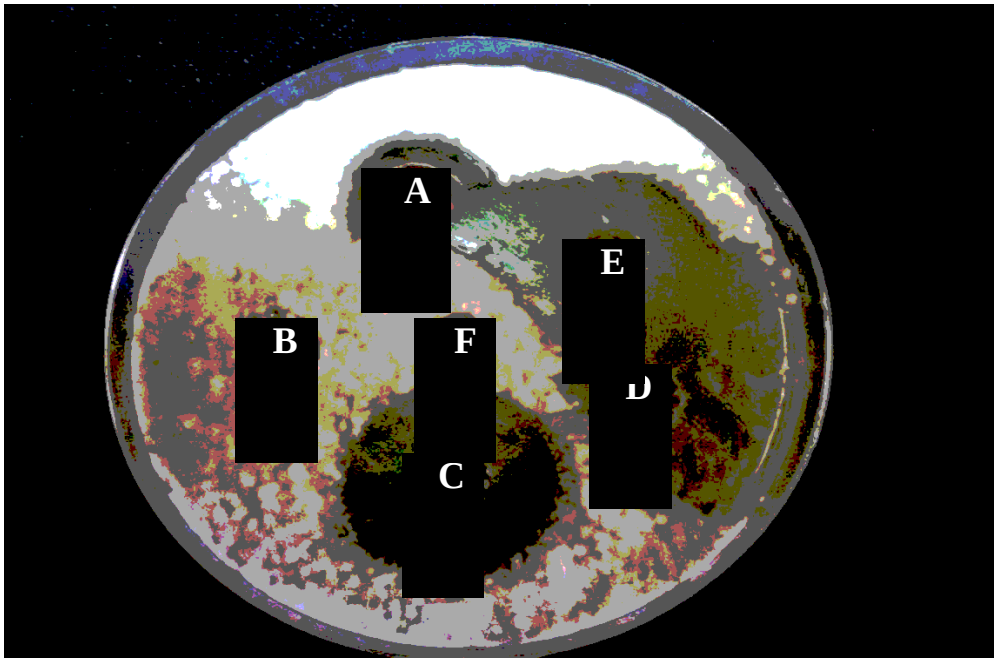


Figure (3-38) : Antifungal activity of SNPs, in comparison with antibiotics, against *T.mentagrophytes* and their synergistic effect (A) SNPs alone (B) FCZ alone(C) MCZ alone (D) SNPs + FCZ (E) SNPs +MCZ (F) Control.

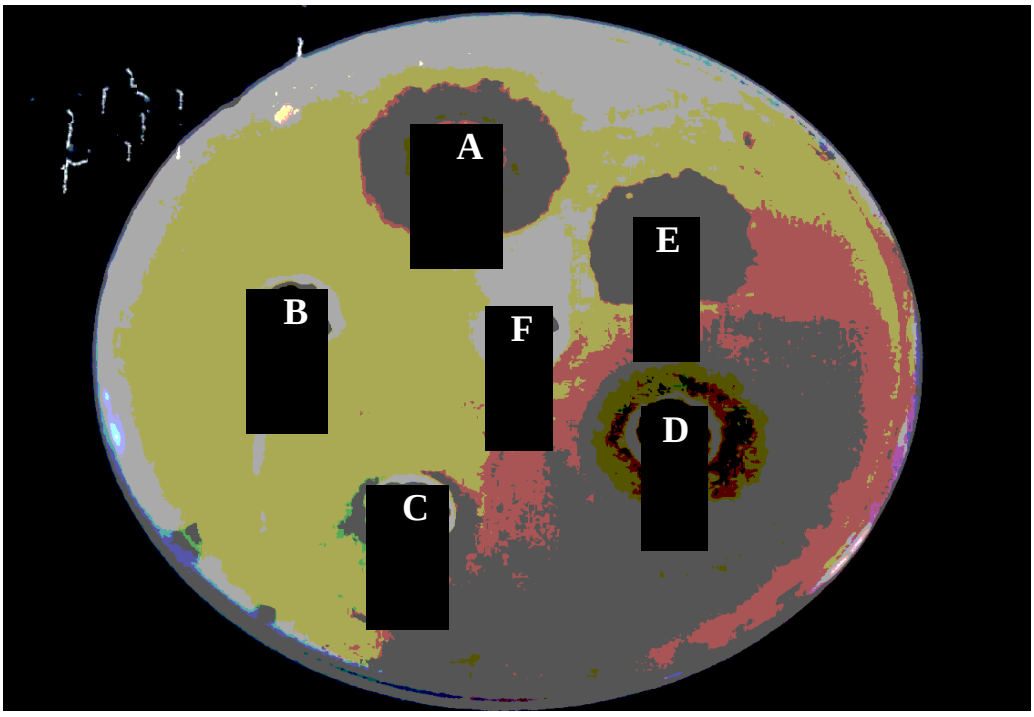


Figure (3-39): Antifungal activity of SNPs, in comparison with antibiotics, against *E.floccosum* and their synergistic effect (A) SNPs alone (B) FCZ alone(C) MCZ alone (D) SNPs + FCZ (E) SNPs +MCZ (F) Control.

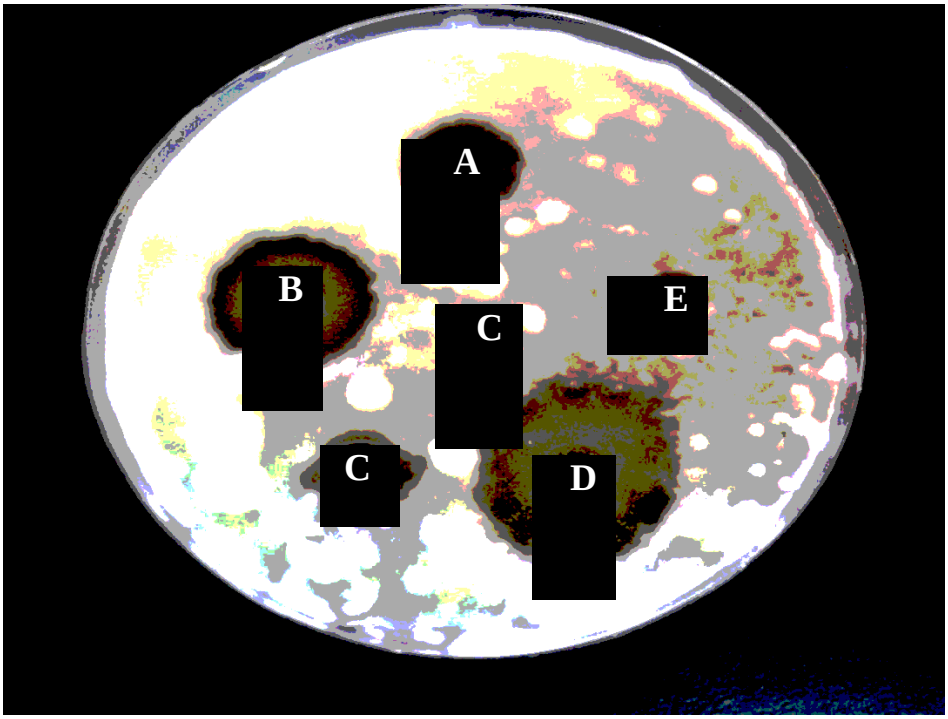


Figure (3-40) : Antifungal activity of SNPs, in comparison with antibiotics, against *M.canis* and their synergistic effect (A) SNPs alone (B) FCZ alone(C) MCZ alone (D) SNPs + FCZ (E) SNPs +MCZ (F) Control.

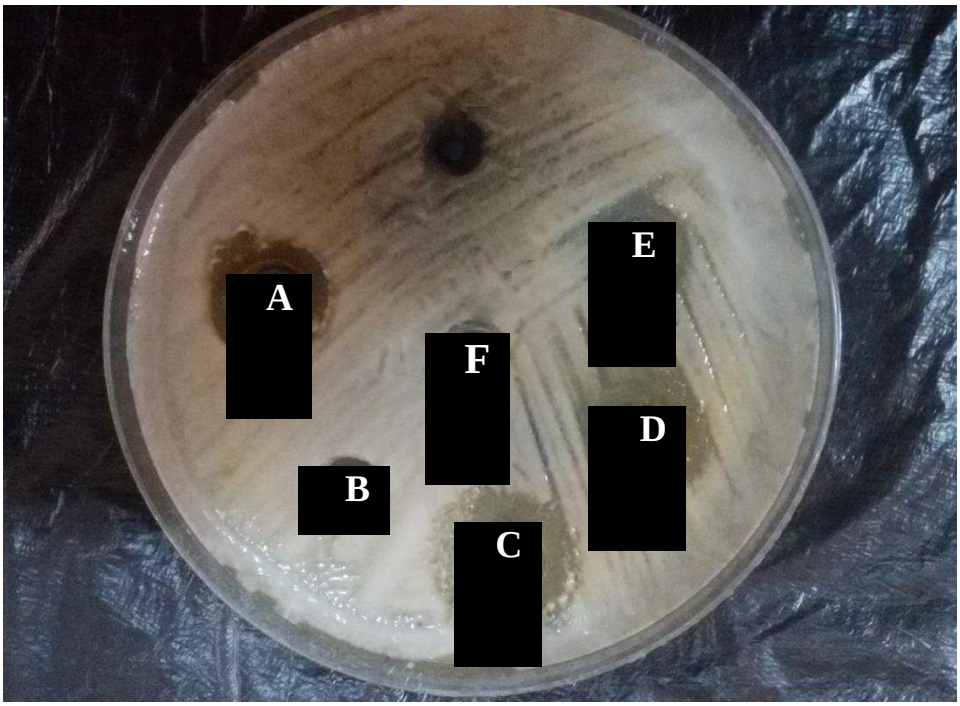


Figure (3-41): Antifungal activity of SNPs, in comparison with antibiotics, against *C. albicans* and their synergistic effect (A) SNPs alone (B) FCZ alone(C) MCZ alone (D) SNPs + FCZ (E) SNPs +MCZ (F) Control.

In the case of Miconazole, the biosynthesized SNPs together with Miconazole exhibited an enhanced synergistic activity against different pathogens. It shows 22.63mm inhibition zone against *T.interdigitale* with fold increase 47.61 % and 28.63mm with fold increase of 37.18% against *T.rubrum*, while against *T. mentagrophytes* the diameter of inhibition zone reached to 31.53 mm with 35.32% fold increase. The synergistic effect also had shown enhanced zone of inhibition in *E.floccosum* from 12.73mm to 18.2 mm in diameter with 42.96% fold increase. Maximum enhancement was found against *C. albicans*, it was showing 24mm inhibition zone with 75.56% fold increase. This study clearly indicated that combination of Miconazole with biosynthesized SNPs provide better synergistic activity. But it was observed in case of *M.canis* that Miconazole in combination with SNPs bring down the inhibitory effect of antibiotics activity (Table 3-13).

Table 3-13: Synergistic activity of Fluconazole & Miconazole combined with SNPs against different pathogenic fungi.

Microorganism	Zone of inhibition in mm			Fold increase (%)	Zone of inhibition in mm		Fold increase (%)
	SNPS alone	FCZ alone	FCZ+SNPs		MCZ alone	MCZ+SNPs	
T.interdigitale	23.33	22	22.53	2.4	15.33	22.63	47.61
T.rubrum	30.77	0	27.3	100	20.87	28.63	37.18
T.mentagrophytes	22.1	0	26.87	100	23.3	31.53	35.32
E.floccosum	24.2	0	19.87	100	12.73	18.2	42.96
M.canis	18.67	0	14.63	100	25.73	24.97	− 2.95
C.albicans	17.67	0	19.33	100	13.67	24	75.56

The comparison between the inhibitory effects of individual substance and combination against dermatophytes spp. and *C. albicans* as ZOI was depicted in the Figure (3-42)

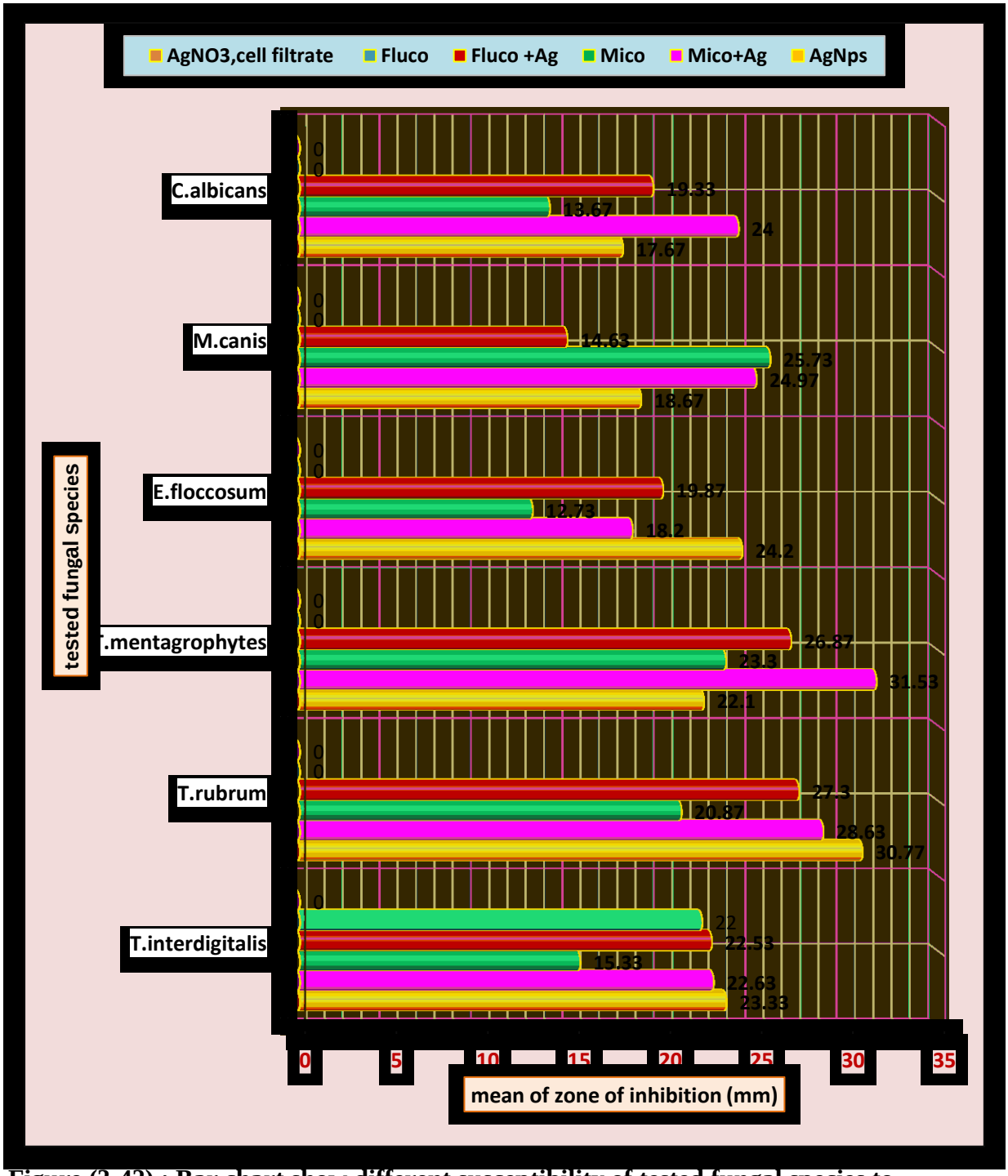


Figure (3-42) : Bar chart show different susceptibility of tested fungal species to anti-fungal agents (X^2 = 109.5, p< 0.0001).

[redacted]

Discussions

4. **Discussion**

4.1. Extracellular biosynthesis of Silver nanoparticles and characterization of it .

Silver nanoparticles had been utilized in various aspects like energy production, optical receptors, polarizing filters, consumer product, tissue engineering, catalysts in chemical reaction, biolabelling and antimicrobial agents **(**Jaidev *et al.,* 2010**).**

Application of SNPs in these fields is dependent on the ability to synthesize particles with different chemical composition, shape, size and mono-dispersity. Development of simple and ecofriendly method would help in developing further interest in the synthesis and application of metallic NPs. In this respect, nature has provided exciting possibilities of utilizing biological systems for this purpose. This comes from the fact that microorganisms while interacting with metal ions have shown to reduce the ions into metallic particles. Microorganism can synthesize metal NPs through metal bioreduction to remove soluble metals from the surrounding environment, thus decreasing their toxicity and bioavailability. Microorganisms that capable of metal bioreduction can colonize metal-contaminated environments. Among microorganisms fungi would be advantageous **(**Sastry,2003**).** In general, fungi tolerate higher metal concentrations than bacteria and secrete abundant extracellular redox proteins to reduce soluble metal ions to their insoluble form and eventually to nanocrystals. Fungi harbour untapped biological diversity and may provide novel metal reductases for metal detoxification and bioreduction (Kitching *et al*.,2015). Therefore, we have successfully demonstrated an easy, rapid and efficient route for extracellular synthesis of SNPs by employing the cell- free filtrate of *A. niger*. This type of

synthesis has been preferred on the intracellular synthesis as the later demands an additional steps of releasing the NPs from the biomass by certain chemical methods or ultrasound treatment and purification of it. It was observed that upon addition of the silver ion (1 mM) into the flask containing the cell filtrate, the color of the medium changed from pale yellow to brown (Figure 3-1), which indicates the formation of colloidal SNPs in the medium. The brown color of the medium could be due to the excitation of surface Plasmon vibrations, typical of the SNPs (Li *et al.*,2012). Surface Plasmon resonance is attributed to the collective oscillation of electrons on the surface of metal NPs which excited by external energy source or light (Kumari *et al.,* 2016). It is well known that the reduction of silver ions into SNPs is commonly followed by a colour change so that the formation of SNPs can be visually observed. Therefore, the change in colour was visually monitored to check the formation of SNPs and there was no additional change in the solution colour when the reaction was stopped (Abdel-Hafez *et al*., 2016).

1. UV-Vis spectroscopy:

The progress of reduction of silver nitrate to SNPs can be easily evaluated using a UV-Visible spectrophotometer. This is because SNPs can absorb light in the visible region due to the surface Plasmon resonance phenomenon based on their size and shape (Rahimi *et al*., 2016). The results showed strong and characteristic surface Plasmon resonance centered at 420 nm (Figure 3-2). This spectroscopic pattern results from interactions of free electrons limited to tiny metallic spherical objects with episode electromagnetic wave. Many studies confirmed that the fungal cell filtrate treated with $AgNO_3$ solution gave a peak around 420 nm (Xue *et al* .,2016; Abdel-Rahim *et al*.,2017), which

supports our finding of the absorbance peak at 420 nm and indicating the biosynthesis of SNPs by *A.niger*.

2. FTIR spectroscopy

Fourier transform infrared spectrum indicted that *A.niger* cell-free filtrate contain active biomolecules which may be responsible for the biotransformation of silver ions to SNPs. This revealed by distinct peaks located at two IR spectrum regions: functional group region and fingerprint region (Figure 3-3). The organic compound show absorbance bands in functional group region, while the metal normally gives absorption bands in fingerprints region resulting from the atomic vibration of molecule. The IR spectra reveals presence of NH group as well as the carbonyl group which attributed to the peptide linkage of the fungal filtrate and many other functional groups most of them resulted from amino acid residue and protein. Thus, The presence of the signature peaks of amino acids supports the presence of proteins in cell-free filtrate and revealed that secondary structure of proteins have not been affected as a consequence of reaction with silver ions or binding with SNPs. In addition, these results confirmed that amino acid residues and peptides of proteins has a stronger ability to bind with metal, so that the proteins could most possibly form a coat covering the metal NPs, that is, capping of SNPs to prevent agglomeration of the particles and stabilizing in the medium (Basavaraja *et al*., 2008). This finding resembles the results of Gole *et al*., (2001) who reported that proteins can bind to NPs either through free amine groups or cystein residues or through the electrostatic attraction of negatively charged carboxylate groups in enzymes , and stabilization of SNPs may be due to the surface bound protein (Gopinath *et al*., 2012) . From above results we conclude that the presence of protein in reaction medium provide reducing agent and coat covering the

biosynthesized SNPs known as capping proteins. Capping protein prevents undesirable particles agglomeration in the medium and responsible for forming high stable SNPs. capping protein provides advantage over polymer and surfactant (widely used as capping agent in preparation of SNPs) as it is coast effective, safe, ecofriendly and does not need special conditions. Another advantage of capping protein, when compared to surfactant and polymer, is it acts as the anchoring layer for drug or genetic materials to be transported into human cells (Hu *et al.*, 2011). The presence of a nontoxic protein cap also increases uptake and retention inside human cells (Rodriguez *et al*., 2013). Finally, the release of proteins in the fungal filtrate probably had a role in the formation and stabilization of SNPs.

3. Electron Microscopy (SEM and TEM) analysis.

The biosynthesis of SNPs was further characterized for its shape, size, morphology and surface chemistry by EM analysis. The nanostructural studies of EM micrograph showed SNPs to be spherical in shape and are uniformly distributed (mono dispersed) without significant agglomeration (Figure 3-4). The monodispersity of NPs attributed to the capping agents which provide stability of NPs and prevent agglomeration of it.

Electron Microscopy results confirm that surface Plasmon peak around 420 nm indicate that the SNPs have spherical form (Abdel-Rahim *et al.*, 2017). The spherical shape is one of the desired qualities of NPs, because the shape and size can affect the NPs function, cellular internalization and affects the particle contact area with the cell membrane. Chithrani and Chan, (2007) reported that spherical gold NPs have a higher propensity to be internalized *in vitro* by HeLa cells compared to rod-shaped particles of similar dimensions. Moreover, spherical NPs were taken up by cells 375–

500% more compared to rod-shaped particles (Jiang *et al.*, 2008). These results were compatible with Elgorban *et al*., (2016) who obtained spherical SNPs by extracellular synthesis using *Aspergillus versicolor.* Al juraifani and Ghazwani (2015) also reported well distributed spherical shape SNPs by the *Aspergillus niger, Fusarium oxysporum and Alternaria solani.*

4. **Energy dispersive X-ray spectroscopy (EDX)**

The elemental structure of powdered specimen was evaluated using SEM equipped with an EDS detector. The energy dispersive X-ray analysis displayed the strong signal at about 3 keV of the Ag regions (Figure 3-5). In general, metallic silver nanocrystals show a typical optical absorption peak at approximately 3keV due to their surface Plasmon resonance (Ibrahim, 2015).Thus this result reveals the presence of pure metallic SNPs along with the O signatures. Identification bands for the main emission energies for Ag are shown and are compatible with peaks in the spectrum, which gives confidence that the Ag was identified correctly. Same result of EDS optical absorption peak at 3kev was also reported by Elgorban *et al.*, (2016).

The results from UV–visible absorption spectroscopy, FTIR, EDX, and EM all demonstrate that SNPs were biosynthesized from extracellular filtrate. Therefore, we used a simple process to complete biosynthesis of SNPs, which required very little complex equipment compared with chemical and physical methods. Although the exact mechanism of NPs biosynthesis by fungi is not yet clearly defined but several hypothesis have been proposed by many research scientists. It is stated that certain extracellular proteins released into the filtrate by the organism could play a role in the synthesis and stability of the S NPs. The biosynthesis of

SNPs may be attributed to the reductase enzyme, one of the most important extracellular enzymes produced by endophytic fungi like A.niger. The nitrate reductase enzyme is produced aerobically and released in the solution, so it present in the culture filtrate (Korbekandi *et al*., 2013). This enzyme is induced by nitrate ions and reduces silver ions to metallic SNPs. Zomorodian et al., (2016) showed a reasonable relationship between nitrate reductase activity and the efficiency of A. niger in the production of SNPs. The reduction mediated by the nitrate reductase enzyme consider the widely accepted mechanism for the synthesis of SNPs (Kumar et al.,2007). Another possible mechanism that may involve in the reduction of silver ions is the electron shuttle enzymatic metal reduction process; NADH and NADH-dependent reductase were probably the key factors for the biosynthesis of SNPs. In organism, NADH is a widespread reduced coenzyme involved in redox reaction, and can be used as a reducing agent by many enzymes *in vivo* (Dudev and Lim, 2010). In the process, NADH acted as an electron carrier, and the silver ions obtained electrons from NADH via the NADH-dependent reductase, and then were reduced to Ag (Li *et al*., 2012; Kathiresan *et al*., 2009). Besides these extracellular enzymes, several naphthoquinones and anthraquinones with excellent redox properties have been reported in some fungi that could act as electron shuttle in metal reductions (Gericke and Pinches, 2006). Jain *et al*., (2011) indicated that SNPs synthesis for *A. flavus* occurs initially by a "33kDa" protein followed by a protein (cystein and free amine groups) electrostatic attraction which stabilizes the NPs by forming a capping agent. Some chemical functional groups, such as the hydroxyl and phenolic groups, act as reducing agents, while carboxyl groups can possess shape-directing functionality (Choi *et al*., 2014). Secretory carbohydrates are abundant with oxygen-bearing functional

groups, such as hydroxyl, carboxylic, carbonyl and phenolic groups. Silver ions have a strong affinity to these functional groups via electrostatic or coordination interactions. These interactions allow electrons to pass to the Ag^{+} ions resulting in nucleation and growth of SNPs: Commonly, reduction of Ag^{+} ions occurs via oxidation of hydroxyl groups to carboxyl (Ebrahiminezhad *et al*.,2016) . Chiang et al., (2011) found that the spore pigment (melanin) and its precursor (naphtha-gamma-pyrones) commonly found in significant quantity in A.niger culture extract. Melanin may play a role in the reduction of Ag^{+} to Ag^{0} NPs. However, experiments with melanin reduction of silver ions have not been reported. Therefore, it is not certain if reduction of silver ions by melanin is a plausible pathway, but similar experiment proved that melanin could mediate the reduction of metal salts to their elemental forms as nanostructures while being oxidized to its quinone form (Apte *et al*., 2013). Finally, *A. niger* represents a promising candidate for large-scale production of SNPs.

4.2. Optimization of reaction conditions for biosynthesis of SNPs

Size, shape, highest yield and mono-dispersity of NPs depend on the physical and chemical factors. The optimum metal ion concentration, pH, culture media and temperature of reaction mixture play key role in NPs biosynthesis. . In this context, optimization study was conducted for biosynthesis of SNPs by *A.niger*.

It is well known that in different culture media compositions and conditions microbial cell responds differently and as a result it secretes different metabolites and different kinds of proteins. In addition, the biological synthesis of SNPs is mainly enzymes catalyzed reaction (Kumar *et al*., 2007). For maximum production of SNPs, fungi should

secrete specific metabolites or enzymes which are responsible for reduction of silver ions to SNPs. In the present study, fungal biomass grown in PDB has shown enhanced SNPs synthesis (Figure 3-6 A). This may be due to presence of ingredients in PDB stimulating better growth of fungi and help in producing augmented level of reducing agent responsible for silver ion reduction and hence enhance the synthesis of SNPs (Birla *et al*., 2013). Ikechi – Nwogu and Elenwo (2015) reported that among 7 fungal species, *A. niger* had the best growth in PDB. There are a few reports on the effects of culture media on the biosynthesis of metal NPs. Vaidyanathan *et al*., (2010) have investigated the effects of medium on the biosynthesis of SNPs by *F. oxysporum*, they showed that MGYP medium may promote the extracellular nitrate reductase secretion and enhance the synthesis of SNPs. Similar study by Saxena *et al.*, (2016) revealed that PDB was the optimum medium for biosynthesis of SNPs by *Sclerotinia sclerotiorum* . Our finding indicates that PDB provide high growth rate and low cost requirement for biosynthesis of SNPs by *A.niger*.

In the biosynthesis of SNPs, concentration of hydrogen ion of the reaction medium plays an important role for NPs formation. Result clearly indicated that absorbance increased with pH (Figure 3-7 B), suggesting that an alkaline environment was more suitable for SNPs biosynthesis. Moreover, there was no evidence for aggregation of particles at alkaline pH which indicates monodispersity and stability of the NPs at alkaline pH, whereas at acidic pH aggregates were appear within few days from reaction. Birla *et al.*, (2013) showed the formation of larger particles at acidic pH due to aggregation, while at alkaline (pH 9 and pH 11) monodispersed and stable SNPs of mode size less than 30 nm were found. Several reports suggest pH plays role in formation as well as

shape and size control in NPs synthesis. Singh *et al*., (2014) and Banu and Rathod (2011) showed that the maximum absorbance peaks for biosynthesized SNPs at neutral PH (PH 7). Another report proposed increase in absorption with decrease in pH and indicated the production of bigger particles with decrease in pH (Namita and Prakash 2011). Our results correlate with Xue *et al*., (2016) who reported that the alkaline pH was more favorable for production of SNPs by the fungus *Arthroderma fulvum* . The availability of OH^- ions in alkaline medium is very important to reduction of metal ions. Alqadi *et al*., (2014) also revealed the relationship between the maximum absorption and the pH value is almost directly proportional, while the relationship between the size of SNPs and the pH value is inversely proportional. In addition they proposed that the SNPs prepared under high pH (10 and 11) are smaller and more regular compared to the samples prepared at lower pH values.

At lower pH, protein structure gets affected and the protein becomes denatured and loses its activity (Banu and Rathod, 2011). The extracellular enzyme catalyzing the synthesis is probably deactivated as the conditions become acidic, and this may be the reason for reduced synthesis which is observed at lower pH values. Birla *et al*., (2013) found that zeta potential value of SNPs at alkaline pH was quite higher, and particles were relatively stable due to the electrostatic repulsion which might be due to the adsorption of OH^- on SNPs, while at acidic pH aggregates were formed due to unavailability of OH^- ions and show low value of zeta potential.

The temperature is one of the important factors in any chemical and biological reaction as it affects the rate of reaction. The maximum production of SNps was recorded at 30°C (Figure 3-8 A) by rapid change in color compared with other temperatures and also detected by UV-

visible absorption spectra. This may be due to the 30°C was the optimum temperature for enzymatic activity while high temperature resulting in denaturation or inactivation of enzymes and active molecules which are involved in biogenesis of SNPs. This result was in contrast with the results of many workers such as Saxena *et al*., (2016) who reported that maximum synthesis of SNPs by *Sclerotinia sclerotiorum* was observed at 80 °C. This may be attributed to the difference in biosynthesis mechanism between the two fungi. While our finding is in complete correlation with the work reported based on exracellular biosynthesis of SNPs by *Chrysosporium tropicum* and *Fusarium oxysporum* , where the maximum rate of formation was recorded at 30°C (Soni and prakash 2011). Quite similar results were reported by Magdi *et al*., (2014) who indicated that the rate of synthesis was increase with an increase in reaction temperature up to 40°C, which showed maximum synthesis after which a decline in the synthesis was observed and that may be due to deviations from the optimized parameters resulted in an increase in size and poly dispersity of SNPs. The broadening peaks obtained at low and high temperature show formation of large sized NPs and the narrow peak obtained at moderate temperature, indicates the NPs synthesized are smaller in size (Vanaja *et al*., 2013). This increase in SNPs size is due to low activity of enzymes involved in biogenesis as a result of unsuitable temperature.

The results of present study clearly indicate that the relationship between the biosynthesis of SNPs and concentration value of $AgNO_3$ is inversely proportional. The maximum synthesis of NPs occurred at 1 mM $AgNO_3$ in the reaction mixture (Figure 3-9 A), and the production was reduced with increase in the concentration (Figure 3-9 B). This can be interpreted on the basis of enzyme-substrate kinetics; i.e. the active site in

the key biomolecule responsible for reduction of NPs is already saturated with the silver ions, and no site is available for excess ions to get reduced, so there is no further increase in biosynthesis of SNPs despite the addition of more salt (Singh *et al*., 2014). The shift in the surface Plasmon resonance peak with low concentration indicates a change in the size of SNPs and hence any shift of the peak toward the shorter wavelength is accompanied by a decrease in the size of the synthesized SNPs, On the other hand, the broadening of the surface Plasmon resonance peak indicates the existence of a wider range of sizes in the solution (ALqadi *et al*., 2014). Our result was agreed with a lot of researcher such as (Vanaja *et al*., 2013; Singh *et al*., 2014) where they found the maximum production of NPs at 1mM. On the other hand, it is inconsistent with other workers who indicated that the production of NPs increased with increasing in substrates concentration (Patil *et al*., 2017).

Our result showed increasing in the intensity of the absorbance peak as the reaction time increased (Figure 3-10 B), which indicated the continued reduction of the silver ions. The synthesis of SNPs by the fungus had been enhanced with the reaction time. The increase in the absorbance with the reaction time means that the concentration of biosynthesized SNPs increases. With the passage of time, the intensity of SPR band increased without any shift in peak wavelength. When the reaction time reached 2 months the absorbance increased slightly, and the λ max value was shifted to toward long wavelenght . This phenomenon indicating that the size of particles was increased which may be resulted from the aggregation of particles. Similar result was obtained by many workers (Kumari *et al*.,2016; Birla *et al.*,2013). The results of our optimization experiments were suggesting that yield and stability of SNP

biosynthesis can be affected by several different parameters. Thus, these parameters can be manipulated to get the highest NP yield.

4.3. Antifungal effect of biosynthesized SNPs on dermatophytes

The antimicrobial activity of SNPs on bacterial species has been reported by many authors (Balakumaran *et al*., 2016 ; Salvioni *et al.,* 2017), While the antifungal efficacies available in the literatures are very few. Recently the antifungal effect of SNPs has received a marginal attention. These inorganic NPs have a distinct advantage over conventional chemical antifungal agents because the treatment of the fungal disease with the current therapeutic agents can result in the damage of host tissues due to the similarity between fungi structure and human's eukaryotic cells, emergence of multi drug resistance fungal strains, and treatment failures (Pakshir *et al*., 2009). Therefore, an alternative way to overcome the drug resistance of various microorganisms is needed in medical devices desperately (Jun Sung *et al.,* 2007).

In the present study, the MIC of the SNPs on 5 isolates of dermatophyte species was investigated (Table 3-2). MIC is important in diagnostic laboratories to confirm susceptibility of microorganism to an antimicrobial agent. In addition, it monitors the activity of new antimicrobial agents. The results obtained revealed that the MIC and MFC values of biosynthesized SNPs ranged from 0.313 to 5 and 2.5 to 14.14 µg/ml, respectively, depending on fungal species. The difference in MIC values of SNPs between the fungal species (even with the same species) has been documented by Ouf *et al.*, (2015). Several authors obtained higher or lower MIC and MFC values than those reported in the present study. Mishra *et al.,* (2016) indicated potent antifungal effect of SNPs against *T. rubrum* and *E. floccosum* with MIC values of 6.68 µg/ml

and 11.3 μg/ml, respectively; while the MFC 12.5 μg/ml was evaluated to be consistent for both test pathogens . Ouf *et al.,* (2015) have been found that SNPs exhibit highest growth inhibition against *E. floccosum*, *M.canis*, *T. mentagrophytes* and *T. rubrum* with MIC value 5,9,12 and 16 μg/ml, respectively. While the MFC value were 15, 21, 25 and 32 μg/ml respectively. This variation in SNP toxicity level found for the fungal species in this research compared with the same species demonstrated by other authors may be attributed to the difference in susceptibility pattern of the strains or may be explained by differences in the method used for NPs synthesis and subsequent stabilization (Monteiro *et al.,* 2011).

There is no available study on the effect of SNPs on the fungus *T.interdigitalis*, Thus such study was carried out for the first time.

All tested isolates of dermatophytes were resistant to Fluconazole except *T.interdigitalis* and all isolates were sensitive to Miconazole. There are several studies proved that Fluconazole had less antifungal activity against dermatophytes (Pakshir *et al.,* 2009; Singh *et al*., 2007). Our data was in agreement with those reports. The azole antifungal agents such as Fluconazole are frequently used in treatment of fungal infection especially candidiasis, prolonged usage of these azole as well as emergence of fungal spp that have decreased susceptibility or intrinsic to these agents have resulted in increased resistance and treatment failure. Moreover and unfortunately, the over prescription of this drug by physicians for prophylaxis and treatment of fungal infection led to an increase in resistance to azole drugs (Vandeputte *et al*., 2012).

Although the antimicrobial mechanism of action of SNPs has not been fully bring to light yet and remains controversial, evidence of their effect in the inhibition of the enzyme respiratory system, in the cell wall

degradation and in the alteration of the microbial DNA has been provided (Jung *et al*., 2008). The SNPs exhibit efficient antimicrobial property due to their extremely large surface area, which enable better contact with microorganisms (Mahendra *et al.*, 2009). It is suppose that fungi carry a negative charge while the nanomaterials release ions carry a positive charge. Thus, an electrostatic attraction between the NPs and microbe will be created. As a result, the microbe will oxidize and killed (Abbaszadegan *et al.*,2015). Hassan *et al* .,(2013) observed the interaction between SNPs and the membrane structure of *T. mentagrophytes* cells by SEM and detected a significant changes in their membranes such as the formation of ‘‘pits’’ on their surfaces, and finally, result in the formation of pores and cell death. Ouf *et al*., (2015) have also supported this observation; they reported that treatment of dermatophytes with SNPs induced a significant increase in cellular leakage due to loss of membrane integrity of the investigated fungi. Another hypotheses have been proposed to give some insights on the antimicrobial action of SNPs is inactivation of thiol-groups in the fungal cell wall and disruption of the potential membrane-bound enzymes and lipids, which lead to cell lysis (Fatima *et al.*, 2015).

4.3.1. Effect of biosynthesized SNPs on spore germination of dermatophytes

Fungal growth involves germination and then hyphal extension. Thus, germination can be considered as the main step to be focused on. The percentage of germinated spores can be considered as the probability of a single spore to germinate (Dantigny *et al*., 2002). The present study revealed that biosynthesized SNPs had pronounced and significant spore germination inhibition effect on all the tested dermatophytes but this effect was variable depending on the fungal strains (Table 3-4). In

addition morphological changes had been shown in most of the germinated spores such as enlargement of spores. Such changes coincide closely with the fungistatic effect of SNPs. It is therefore supposed that the mechanism of fungistasis by SNPs may involve alteration in osmotic and permeability of the spores. Khalil (2013) was supported this speculation when she investigated the effects of the biogenic SNPs on spores of *A. fumigatus* via TEM analysis. The main abnormalities noted via TEM study was the alterations in the morphology and complete collapse of the spores after exposure to the biogenic SNPs. She also noted that parts of the cell wall, plasma membrane and the inner constituents of the spores were obviously damaged which may be due to the toxic effect of the SNPs. While Nychas (1995) attributed the effect of sporocidal or sporostatic agents on sporulation to denaturation or interference with amino acid or enzymes responsible for spore germination**.** Different workers investigated the impact of SNPs on spore's germination of different pathogenic fungi. Kasprowicz *et al*., (2010) reported that *Fusarium culmorum* spore germination was significantly reduced in a 2.5 ppm solution of SNPs compared to the control.

4.3.2. Determination of the inhibitory effect of biosynthesized SNPs on the keratinase activity

Keratinases are key proteolytic enzymes produced by dermaptophytes; they hydrolyze both soft and hard keratins (Karthikeyan *et al.*, 2007). Hence, in the past few decades, a number of research projects have focused on the activities of keratinases and their role in the virulence of dermatophytes such as *Trichophyton* (Muhsin and Aubaid , 2001). Chicken feathers used as a primary source of energy, carbon, nitrogen and sulfur. It was clear that presence of SNPs in the growth medium act as an enzyme inhibitor, and their activity was decreased significantly in

some of tested dermatophytes (Table 3-5). This finding agree with Ouf *et al.*, (2015) who found that treatment a number of dermatophytes with 10 µg/ml SNPs produced variable reduction in keratinase activity of 36.8%, 40.9%, 27.1% and 23.3 % for *E. floccosum, M. canis, T. mentagrophytes and T. rubrum*, respectively. A number of hypotheses have been proposed to explain the mechanism of keratinase inhibition by SNPs; it has been suggested that silver ions (Ag+), which released from NPs, can interact with sulfur-containing proteins; or may attach to the sensing surface of C-terminal residue of amines in keratinase enzyme, all these events lead to modification and enzyme inactivation (Matsumura *et al.*, 2003; Brandelli *et al*., 2010). It was also proposed that SNPs can attach or adsorbed to the enzyme surface (Fischer *et al*., 2002) which induce changes in configuration of enzyme and possibly limit the access of substrate to the active site of enzyme (Bhinder & Dadra, 2009). The present experminets indicate a possibility of using the biosynthesized SNPs as a very useful agent to reduce the keratinolytic activity of dermatophytes.

4.3.3. Effect of biosynthesized SNPs on the mycelia development of dermatophytes

Good fungal growth of *dermatophytes especially Trichophyton* species, produce hyphae which can penetrate the innermost layer of skin and aggravate the damage in the host (Gupta *et al*.,2003). Therefore, some researchers are investigating the NPs potential in inhibiting mycelial growth of pathogenic fungi due to their importance in the mycosis development. The biosynthesized SNPs induced considerable morphological changes in the hyphae of all the tested dermatophytes. As previously mentioned in results chapter, the characteristic dark brown color of the medium containing SNPs, resulted from surface Plasmon resonance, was decreased to a great degree. This phenomenon can be

explained by attachment of SNPs to the surface of fungal mycelium or in some cases internalization into the fungal cells due to their small size, which lead to elimination of silver particles from the medium. The result obtained by Kotzybik *et al.*,(2016) supports our findings, where they reported that the SNPs were attached to the mycelial cell surface of *Penicillium verrucosum* and could also penetrate the fungal filaments resulting in silver agglomerates localized within the cytoplasm. They also observed the persistence of a typical coloration of SNPs In cases were the fungus could not grow or totally inhibited. Morphological alterations also include dichotomous tip branching (Figure 3-29 B) and alterations of branch emergence, Harris and Momany (2004) described the dichotomous branching as phenocopies of filamentous fungal mutants in which establishment and maintenance of polarity are altered**.** Polar growth requires the recruitment of the morphogenetic machinery to specific sites for localized cell wall deposition. Swelling of hyphal cells, as determined by microscopical examination (Figure 3-24 C), is indicative of alterations in the cell wall structure. The presence of large vacuoles inside hyphae were detected in most of the tested fungi (Figure 3-24 D), altered vacuolar morphology and physiology have been associated with impairment of hyphal growth and virulence in different human pathogens such as *C. albicans* (Veses *et al*., 2008).

It is evident that chlamydospores were abundant in all SNPs treated dermatophytes (Figure 3-28 B). A fungus can survive unfavorable environmental conditions by forming chlamydospores (Eyal *et al.*, 1997). Therefore, the excessive production of chlamydospores was induced by the presence of SNPs in the growth medium. Such phenomenon may be important for these dermatophytes to take their growth in the existence of this adverse component. Observation under light microscope revealed

that most hyphae undergo wall disorganization and loss of integrity of their biological membranes indicating extensive cellular death after exposure to SNPs. Nalwade and Jadhav (2013) observed that such morphological changes become evident upon interaction between SNPs and negatively charged cell membrane of the microorganisms and can be characterized by shrinkage of the cytoplasm and membrane detachment which lead finally to rupture of cell wall . Similarly, silver ions released by NPs can also alter transport and the release of potassium (K+) ions from the fungal cells. Besides affecting the transport system, the alteration in membrane permeability may have more pronounced effects such as loss of cellular contents by leakage, including ions, proteins, reducing sugars and sometimes cellular energy reservoir, ATP (Lok *et al*., 2006; Kim *et al.*, 2011; Li *et al*., 2013). Finally, all these modifications in the cytological structure of fungi may be related to the interference of the SNPs with the vital events responsible for synthesis or maintenance of fungal cell. These finding demonstrated that biosynthesized SNPs was effective in restricting the fungal growth of filamentous fungi.

4.4. Antifungal effect of biosynthesized SNPs on *Candida albicans*:

Candida albicans is the most representative model of pathogenic yeasts, it is responsible for a number of major fungal diseases in human (Sardi *et al.*2013*),* which means that there is considerable encouragement for finding novel antifungal agents against it. The present study investigates the effect of biosynthesized SNPs against this type of fungi. The obtained results showed that SNPs had a remarkable anti-candida effect with relatively low MIC and MFC values (Table 3-6). However, the Miconazole had better antifungal effect than SNPs, while the later displayed a broader antifungal spectrum than Fluconazole. Although the

Fluconazole is the first choice drug for systemic fungal infection; but studies proved the increase of resistance of *Candida* strains to this agents (Abrantes *et al.*,2014). The antifungal activity of SNPs against *C. albicans* was confirmed in some other studies (Bonilla *et al.,* 2017; Carline *et al.,* 2016), but the reported values were different from ones that obtained in this work. Such differences can probably be explained by the differences in the yeast strains tested, or may be attributed to the differences in the NPs synthesis method and nature of NPs used such as size, shape and stabilizing agents (El-Sayed, 2001). While the Xue *et al*., (2016) obtained MIC value exactly identical to what we got in our work.

It was reported that SNPs inhibit the normal budding process, probably through the destruction of membrane integrity (Kim *et al.,* 2009). This report found a correlation between the inhibition of bud growth and membrane damage. Keuk-Jun *et al*., (2009) found that SNPs could be break down the cell membrane permeability barrier of *C.albicans*, it is possible that SNPs disorganize the membrane lipid bilayers, leading to the leakage of ions and other materials as well as forming pits and dispersing the electrical potential of the membrane .Furthermore, the production of reactive oxygen species and apoptosis has been demonstrated (Hwang *et al.,* 2012). Phosphorus-containing elements like DNA considered preferential sites for SNPs binding, because silver has a high tendency to react with these compounds (Panáček *et al*., 2006).

4.4.1. Effect of biosynthesized SNPs on some virulence factors of *Candida albicans*.

Adherence and colonization of planktonic cells on host tissues and on medical devices initiates the biofilms formation (Finkel *et al.,* 2012) *Candida* biofilm lifestyle leads to higher levels of resistance to the most

commonly used antifungal drugs compared to their planktonic counterparts (Taff *et al*., 2013). In present study, adherence of *C. albicans* to the plastic surface in the presence of different concentration of biosynthesized SNPs was significantly lower than that of control. Increasing the concentration of SNPs enhanced anti-adherence activity (Table 3-7). These findings are in agreement with several previous studies examining the possibility of using SNPs to prevent the adherence of *C. albicans* to host tissues and different surface of implanted medical devices .Kurt *et al*., (2017) found that the addition of SNPs to the denture base material significantly reduced the adherence of *C. albicans* to the surface and hence prevents oral candidiasis. Monteiro *et al*.,(2011) verified that SNPs were highly effective on adhered *C. glabrata and C.albicans* and this capacity was dependent on the silver concentration. The SEM observation done by Kamikawa *et al*., (2014) for *C. albicans* and *C. glabrata* on the surface of the SNPs treated Acron piece, showed remarkably deformed, swollen cells with shriveled cell walls and leakage of cellular components, the authors attributed the loss in ability of yeast cells to adhere to the host cells to these effects. *Candida* adherence is mediated by cell wall proteins through different types of chemical bonds (Dranginis *et al* ., 2007), depending on the fact that SNPs generate reactive oxygen species interact with, and potentially disturb the functioning of biomolecules such as proteins, the inhibition of adhesion may be belong to this interactions. This indicates that biosynthesized SNPs could be a good candidate in the prevention of the early stage of *C. albicans* biofilm development and so in the prevention of candidiasis related to different medical devices.

Biofilm is an organized community of cells, embedded in a matrix of exopolymeric substances. An important reason for the failure of

current antifungal agents is attributed to formation of biofilms which are inherently resistant to most of the antifungal drugs (Finkel & Mitchell, 2011). SNPs has been reported to exhibit a good inhibitory effects on bacterial biofilm formation (Panácek *et al.*,2006) , as well as candidal biofilm formation (Monteiro *et al.*, 2011). However, the antimicrobial activity of SNPs against pre-formed *Candida* biofilms has received a greater attention. Consequently, the present work evaluated the antimicrobial effects of biosynthesized SNPs and Miconazole on pre-formed *C.albicans* biofilms. Results revealed that both SNPs and Miconazole significantly inhibited *C. albicans* biofilms in terms of total biomass (Figure 3-32). It was found that antibiofilm effect need higher concentration as compared to the MIC or adherent cell, indicating that matured biofilm is moderately resistant towards SNPs as compared to planktonic cells. Such results are in good agreement with those reported by Chandra *et al*., (2001) who found that the progression of drug resistance in *C. albicans* biofilms was associated with the accompanying the increase in metabolic activity of developing biofilms. When compared to young and mature *Candida* biofilms of 24–48 h, the adhesion phase contains a lower cell mass due to the primary production of the extracellular matrix. Nevertheless, adhesion phase cells seem to be in a metabolically excited state compared with their older counterparts in well-established biofilms. However, the effective concentration of SNPs found in our study was lower than in previous reports concerning the toxic concentration of SNPs on human dermal fibroblasts and epidermal keratinocytes (Galandáková *et al*.,2016), this study confirmed that SNPs are more suitable for the intended application as a topical agent up to the concentration 25 µg/mL.

Previous investigations have identified the antibiolfilm effects of SNPs against different biofilm forming microorganisms, Yuan *et al.,* (2017) reported the antibiofilm activity of SNPs against different strains of Multi-drug resistance bacteria, indicating that the inhibitory effect of SNPs against biofilm is likely to be broad spectrum. In another study the antifungal effect of SNPs against the formation of biofilm in *C. albicans* and *C. glabrata* was explored and it was indicated that SNPs in the range of 3.3µg/ml to 54µg/ml had a significant reducing effect (Kim *et al.*,2008).

Several hypotheses have been proposed to explain the mechanism of antifungal effect of SNPs against *Candida* biofilms. At least one of the important hypotheses of antibiofilm activity of the SNPs was attributed to their surface-modifying properties. According to Kim *et al*., (2009), SNPs affect yeast cells by disrupting the membrane potential and formation of pores on the membrane surfaces of *C. albicans* and subsequent cell death. The extracellular polysaccharide of the biofilm is a recognized inhibitor/barrier to diffusion of antimicrobial agents with an ability to bind to certain ions as they diffuse through the biofilm, thereby effectively reducing bio-availability (Williams and Lewis, 2011). In the same context, Kalishwaralal *et al*., (2010) suggest that the effect of SNPs on pre-formed biofilms could be due to the presence of water channels throughout the biofilm. Since water channels are present in all biofilms for nutrient transportation, SNPs can directly diffuse inside the matrix layer and impart an antifungal function.

Another interesting observation in the present study, is that the hyphal growth was significantly inhibited by biosynthesized SNPs, as will be mentioned later. Many authors revealed a closely relationship between the hyphal transition and biofilm formation in *C. albicans*. The

morphogenic transition of yeast to hyphae has been shown to play an important role in biofilm formation in (Samaranayake *et al.*, 2005). It is not surprising that genes required for hyphal growth are also necessary for proper biofilm formation (Nobile *et al.*, 2012). Probably, hyphal elements facilitate the exuberant architecture of *C. albicans* biofilms, making them more difficult to eliminate. Thus, SNPs may induce changes in the structure of *C. albicans* biofilms by blocking or eliciting the yeast to hyphal transition. This explanation is in agreement with San Milla!n *et al*., (2000) who showed that adhesion of *C. albicans* to polystyrene is related to germ tube induction since adhesion increased in parallel with germination. Previously they published reports showing that the hyphae of *C. albicans* adhere more readily to plastic materials and buccal epithelial cells than yeast cells (San Milla! n *et al.*, 1996).

One of the major virulence factors of *C. albicans*, is its ability to switch between yeast and filamentous form. Thus, the inhibition of hyphal growth by biosynthesized SNPs is a significant finding. Beside the importance of hyphae in biofilm formation, it mediate the dissemination of *C.albicans* to the host tissue through invasion (Sudbery, 2011). It was reported that virulence of *C. albicans* is reduced to a great extent in hypha deficient mutants (Berman & Sudbery, 2002). The inhibition of dimorphic transition at all concentration of SNPs used in this experiment (Table 3-11), even in the presence of serum (hypha inducing agents), emphasizing the significant role of SNPs in the inhibition of hyphal growth. Keuk-Jun *et al*.,(2009) reported the potential effect of SNPs on dimorphic transition of *C. albicans* at higher concentration (2 mg/ml) than that we used in this work , this may be attributed to the difference in the methods used for synthesis of SNPs. Since, they used chemical method. Lara *et al.*,(2015) also showed that treatment of *C.albicans* with

SNPs was able to inhibit filamentation. Finally, the effects of SNPs can interfere with the organisms pathogenicity by blocking the dimorphic transition.

4.5. Evaluation of synergistic potential of biosynthesized SNPs in combination with antifungal agents

The discovery rate of new antifungal agents is in decline, while resistance in pathogens is rapidly increasing. Overcoming this issue can be achieved by using antifungal agents with multimode action. SNPs are one of the well-known antimicrobial substances showing such multimode action. In recent years conjugation of NPs with antibiotics has significant impact, because such conjugation would prevent development of resistance by microbes and enhance the antimicrobial property of the antibiotic. The interaction between antibiotics and NPs depends on the electrostatic forces resulting in functionalized NPS in a reversible manner based on the opposite charges. As NPS possess unique properties which form a base toward developing hybridization with the biomolecules resulting in a specific activity (Bagwea *et al.*, 2003). Synergistic effect may be occurs by the bonding reaction between SNPs and antibiotic. As the antibiotic molecules contain many active groups such as hydroxyl and amino groups, which react easily with SNPs by chelation, as a result, SNPs-antibiotic conjugate is formed in which a SNP core is surrounded by antibiotic molecules. Thus, the antimicrobial concentration is increased at the focal site, which leads to increased destruction of microorganism (Yang and Webster, 2009). Buszewski *et al.*, (2016) revealed the binding of the SNP to antibiotic molecules functional groups, including carboxyl, therefore, in addition to its antimicrobial activity, the S NPs probably operate as an antibiotic carrier.

Enhancement of antifungal potential of biosynthesized SNPs after combination with two antifungal drugs belonging to Azole is reported in the present study even against resistant strains of dermatophytes and *C.albicans* (Figure 3-42). Strong synergistic effects were shown for both tested drugs combined with SNPs at very low concentrations of antibiotics. Moreover, a very low amount of silver is needed for sufficient antifungal effect of the antibiotics, which represents an important finding for potential medical applications due to the negligible cytotoxic effect of SNPs towards human cells at these concentration levels. It may be assumed that prevention or treatment of infections would be more effective when synergistic effects of antibiotics combined with SNPs occurred at very low concentrations of both antimicrobial substances, this will minimizing the risk of side toxic effects (Paná˘cek *et al*., 2016). In addition, our results clearly demonstrate that antibiotic-resistant fungi become susceptible again when an antibiotic is combined with biosynthesized SNPs as proved in case of the synergistic effect of Fluconazole with SNPs aginst Fluconazole –resistant strains.

Azoles are class of antifungal drugs inhibiting ergosterol biosynthesis through targeting lanosterol 14α-demethylase, which is a core enzyme encoded by *ERG11* in ergosterol biosynthesis (Lupetti *et al.,* 2002)

The main known mechanisms of resistance to azoles in *C. albicans* and dermatophytes reported in previous studies are: 1) Mutation in the ERG11 gene, resulting in structure alteration of Lanosterol 14α-demethylase, the substrate to which azoles bind, thereby rendering the azole ineffective by preventing or reducing the binding of azoles to the enzymatic site (Löffler *et al*., 1997). 2) Overexpression of the efflux pump, results in decreased drug concentrations at the site of action through ejection of the drug out of the cytosol (Kanafan *et al.,* 2008).

3) The third resistance mechanism is overexpression of the altered target enzyme (Lanosterol 14 α- demethylase), this may lead to targets not binding well with the azole drugs and inhibiting ergosterol synthesis. On the other hand, the mechanisms leading to enhancement in fungal susceptibility towards drugs combined with SNPs or even to restoration of sensitivity of fungi originally resistant to antibiotics can be of various nature taking into account the multiple mode of action of SNPs. SNPs and antifungal agents inhibiting synthesis of a cell wall can cooperate together promoting disturbance or damage of the cell wall. One of the most important mechanisms of action of antifungal agents that the drug enters the fungal cell for efficient killing, as described above, an important antifungal mechanism of SNPs is the enhancement of permeability, so it appears entirely reasonable that the interaction of antifungal drugs and SNPs should be synergistic rather than simply additive

A bonding reaction between antibiotics and SNPs by chelation ultimately increases the concentration of antimicrobial agents at specific points on the cell membrane. This may be attributed due to the selective approach of SNPs towards the cell membrane that consists of phospholipids and glycoprotein. Therefore, SNPs facilitate the transport of antibiotics to the cell surface acting as a drug carrier. More recently, it was shown that silver chelation prevents unwinding of DNA (Batarseh, 2004). Inhibition of the activity of fungal enzymes responsible for fungal resistance could be another possible mechanism responsible for restoring of antifungal activity of antibiotics. Enzymatic activity can be also inhibited by ionic silver released from SNPs (Paná˘cek *et al.*, 2016).

The synergy between NPs and various antimicrobial agents has been already carried out in several works. Brown *et al.*, (2012) showed a

synergistic effect of SNPs and gold NPs with ampicillin, even against multi-resistant strains such as multiple-antibiotic-resistant isolates of *Enterobacter aerogenes ,Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus*. Kulkarni *et al.,* (2016) found that combination of SNPs with standard antibiotic (ketoconazole) has enhanced the antifungal effect against *M.gypseum, C.tropicalis* and *T.rubrum* with increased in fold area indicating that synergistic effect has more effect than SNPs or antibiotic alone. The combination between Fluconazole and SNPs showed maximum inhibitory effect against *C.albicans, Phoma glomerata* and *Trichoderma* spp.(Higa *et al*.,2013) . Gajbhiye *et al.,(*2009) evaluated the increasing antifungal activity of Fluconazole in combination with SNPs against *P.glomerata, Pleospora herbarum, F. semitectum, Trichoderma spp.* and *C.albicans*. They showed that Fluconazole in combination with SNPs showed maximum inhibition against *C.albicans*. The efficacy of the antifungal drug Miconazole was increased by conjugation with SNPs and revealed efficient fungicidal activity due to the inhibition of ergosterol biosynthesis and also biofilm inhibition by increasing ROS level (Kumar and poornachandra, 2015). Singh *et al*., (2013) explained that antifungal activity of Fluconazole and itraconazole was enhanced against pathogenic fungi in the presence of SNPs and confirmed from the increase in fold area of inhibition. Noorbakhsh (2011) found that combination of Fluconazole with SNPs reduced its effective concentration against *T.rubrum* from 40 to 10 µg/ml

The other combinational effect (SNPs- Miconazole) was found as antagonistic activity against *M.canis.* Antagonistic interactions of SNPs were detected with amoxicillin and oxacillin in a methicillin-resistant *Staphylococcus aureus* strain (Sousa *et al.,* 2006) and with

chloramphenicol in *Pseudomonas aeruginosa* (Jaya *et al.*, 2009). We did not find an appropriate explanation for this phenomenon. However, as we observed in our result, the *M.canis* was the less sensitive dermatophytes to biosynthesized SNPs, it had the higher concentration for MIC and MFC in comparison with Miconazole. Therefore, the effectiveness of Miconazole as antifungal agents was altered when they are combined with SNPs. A higher concentration may be needed to obtain a benefit of drug combination.

Herein, we present the first detailed study of the synergistic potential of biogenic SNPs in combination with Miconazole against the dermatophytes spp. and *C.albicans*. Finally, the unique property of biosynthesized SNPs can be therapeutically valuable for fabrication of innovative hybrid drugs for enhancing effectiveness of antibiotics to kill these types of organisms.

Conclusions

1- Silver naboparticles (SNPs) synthesized in inexpensive and promising technique by using cell-free extract of *A. niger*. And monodispersed, spherical NPs with range of size of 15-60 nm were obtained.

2- The qualities of the biosynthesized NPs were controlled by monitoring the environmental factors.The optimum condition for biosynthesis of SNPs by *A.niger* were the use of Potato dextrose broth medium at pH 9, 30°C for 120 h with 1mM silver nitrate.

3- The SNPs have been successfully inhibited five dermatophytes spp. as well as *C.albicans*. It has potent activity against different virulence factors of these organisms such as spore germination, keratinase activity, mycelium development in dermatophyes and adherence, biofilm formation and dimorphism in *C.albicans*. Thus it promises to be an alternative to the classic medicines used for treatment of diseases caused by these pathogens.

4- The SNPs in combination with standard antifungal agents has shown enhanced and good antifungal activity even against resistant strains indicating that synergistic effect has more effect than SNPs or antibiotic alone.

Recommendation

1-Further studies should be done to test various fungal species for biosynthesize SNPs to find a more non-toxic and economical method of synthesis.

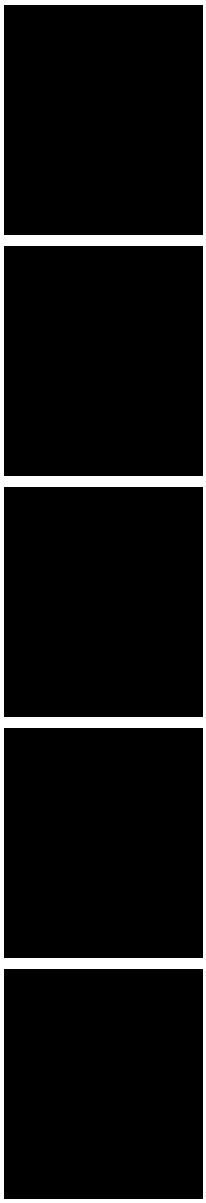
2- Further investigations should be performed about the antifungal effects of the combination of SNPs with other conventional antifungal agents**.**

3- Complete knowledge of the molecular mechanisms involved in the fungal synthesis of NPs is nesseccory.

4-Characterization of enzymes involved in the biosynthesis of NPs is also required

5- Further studies should be carried out to understand the toxicity of biosynthesized SNPs before developing them for clinical applications.

6- Future researches about the role of SNPs on cellular functions of dermatophyte pathogens may help us to acquire more information concerning the using of these NPs against cutaneous fungal infections



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الخلاصة

جذبت أبحاث التكنولوجيا النانوية والجسيمات النانوية الكثير من الاهتمام في العقود الأخيرة. وازداد الاهتمام بإيجاد طرق أكثر فعالية لتخليقها. و يُعد التصنيع الحيوي من افضل الطرق البديلة والواعدة لانتاج جزيئات الفضة النانوية. وقد هدفت الدراسة الحالية إلى تصنيع جسيمات الفضة النانوية باستخدام الراشح الخالي من الخلايا للفطر *Aspergillus niger* وتقييم كفاءة جزيئات الفضة المصنعة حيوياً كعوامل مضادة للفطريات.

أجريت الدراسة في الفترة ما بين آذار / 2016 و كانون الثاني / 2017 في مختبر الاحياء المجهرية في كلية الطب / جامعة القادسية.

استخدمت عزلات مرجعية للفطر *Aspergillus niger* في عملية التخليق الحيوي لجزيئات الفضة ،في حين استخدمت عزلات مرجعية مختلفة من الفطريات الجلدية والتي شملت: *Trichophyton rubrum, Trichophyton mentagrophytes ,Trichophyton interdigitale, Epidermophyton floccosum and Microsporum canis.* اضافة الى خميرة المبيضاء البيضاء في اختبار حساسيتها لجزيئات الفضة المحضرة.

تم توصيف الجزيئات المنتجة باستخدام عدة تقنيات.حيث اظهرت نتائج التحليل الطيفي للاشعة فوق البنفسجية ان اعلى ذروة للامتصاص كانت عند الطول الموجي 420 نانومتر. تم قياس وجود البروتينات كعوامل حيوية مختزلة مسؤولة عن عملية التصنيع الحيوي و ثباتية الجسيمات المصنعة وذلك باستخدام التحليل الطيفي للاشعة تحت الحمراء. وبينت نتائج الفحص باستخدام المجهر الالكتروني الماسح و النافذ تكوين جسيمات نانوية كروية مفردة وبحجم يتراوح بين 15-60 نانومتر بالقطر.

اثبتت النتائج ان االظروف المثلى لعملية التصنيع الحيوي هي استخدام وسط مرق البطاطا و الدكستروز وبدرجة حامضية 9 لمدة 120 ساعة في 30 °م مع وجود نترات الفضة بتركيز 1 ملي مول .

بلغت قيمة كلاً من التركيز المثبط الأدنى (MIC) و التركيز القاتل الادنى(MFC) 0.313 و 2.5 مايكروغرام /مل على التوالي على فطر *T.rubrum* (النوع الاكثر تحسساً) ، 5 و 14.4 مايكروغرام/مل على التوالي على فطر *M.canis* (النوع الاكثر تحملاً).

اظهرت جزيئات الفضة النانوية المحضرة بايولوجياً فعالية تثبيطية مقاربة لما ابداه المضاد الحيوي المايكونازول بينما اظهرت فعالية عالية مقارنة بالمضاد الفطري الفلوكونازول. و امتلكت دقائق الفضة المختبرة تأثيرا واضحاً و معنويا ($P < 0.05$) في تثبيط انبات ابواغ جميع الفطريات الجلدية المختبرة ولكن هذا التأثير كان متبايناً اعتمادا على نوع العزلات الفطرية. كما لوحظ كبح فعالية انزيم الكيراتين بوجود جسيمات الفضة النانوية. اذ سجل فطر *E. floccosum* اعلى نسبة في تثبيط الانزيم وصلت الى 47.41% مقارنة ببقية الفطريات ، بينما بلغ تأثيرالمضاد الفطري المايكونازول على نفس الفطر المذكور 28.30 % . اما الفلوكونازول فلم يكن له أي تأثير على الفطريات المختبرة ما عدا *T.interdigitale* .

اظهرت خلايا الفطريات المعالجة بالجسيمات النانوية لعنصر الفضة تغيرات شكلية وكانت ابرزهذه التغيرات هي: الانتفاخ ، وجود فجوات كبيرة، وفقدان سلامة الأغشية البيولوجية التي تشير إلى موت خلوي واسع النطاق.

بينت النتائج تثبيط قابلية الخميرة على الالتصاق و تكوين الاغشية الخلوية لخميرة المبيضات البيضاء في وجود حسيمات الفضة المحضرة، واعتمد التأثير على الجرعة المستخدمة أذ كانت اعلى نسبة للتثبيط عند التركيز فوق المثبط الادنى (2X MIC) والتي وصلت الى 51 % و 36.1 % للالتصاق و تكوين الغشاء الحيوي ، على التوالي. فيما اظهر المايكونازول تأثيرا اقل بلغ 43% و 31.1 % للالتصاق و تكوين الغشاء الحيوي ، على التوالي.

تسببت جزيئات الفضة النانوية المحضرة في منع تطوير الأنبوب الجرثومي بشكل تام مع نسبة تثبيط 100٪. تم الحصول على نتائج مماثلة مع الميكونازول.

أثبتت النتائج ان الأنشطة المضادة للفطريات لكل من الفلوكونازول والميكونازول قد ازدادت في وجود جسيمات الفضة النانوية. وقد لوحظ أعلى تأثيراً تآزرياً للفلوكونازول ضد جميع السلالات المقاومة مع زيادة 100٪. في حين ظهر تأثيراً تضادياً عند مزج جزيئات الفضة مع المايكونازول للفطر *M.canis* .

استنتجت الدراسة الحالية انتاج جسيمات الفضة النانوية بواسطة تقنية واعدة و فعالة من حيث التكلفة و الامان وذلك باستخدام راشح فطر (*Aspergillus niger*)، كما ان صفات الجزيئات المنتجة يمكن التحكم بها من خلال السيطرة على الظروف البيئية اثناء عملية التصنيع

الحيوي. اضافة لذلك فقد اظهرت جزيئات الفضة المصنعة تأثيراً كبيراً ضد الفطريات المختبرة مقارنةً بالمضادات الحيوية المستخدمة.

وزارة التعليم العالي و البحث العلمي
جامعة القادسية
كلية الطب
فرع الاحياء المجهرية

التصنيع الحيوي الفطري لجزيئات الفضة النانوية بواسطة الفطر *Aspergillus niger* و تقييم نشاطها المضاد للفطريات في الفطريات الجلدية و خميرة المبيضات البيضاء

اطروحة مقدمة
الى مجلس كلية الطب/جامعة القادسية
و هي جزء من متطلبات نيل شهادة دكتوراه فلسفة في علم الاحياء المجهرية الطبية

من قبل

بلسم ميري مزهر المحنة
بكالوريوس طب و جراحة بيطرية - 1999
ماجستير احياء مجهرية – 2005

باشراف

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صفر / 1438 هـ تشرين الاول / 2017 م