Ministry of Higher Education
and Scientific Research
University of Al-Qadisiyah
College of Pharmacy

Biosynthesis of Silver Nano Particles using Leaves Green Tea Extract and its effect on *Cryptococcus neoformans* growth

A Research

Submitted to the Council of the

College of Pharmacy/ University of Al-Qadisiyah

in Partial Fulfillment of the Requirements for

The Degree of Bachelor in Pharmacy

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2017 A.D. **1438 A.H.**

بسم الله الرحمن الرحيم

"قَالُواْ سُبْحَانَكَ لاَ عِلْمَ لَنَا
إِلاَّ مَا عَلَّمْتَنَا إِنَّكَ أَنتَ
الْعَلِيمُ الْحَكِيمُ"
صدق الله العلي العظيم
سورة البقرة (٣٢)

ACKNOWLEDGMENTS

We would like to express our gratitude for the god of this universe who kept showing us the light till the end of the tunnel and provided us with the necessary strength to keep working till the very end of the path of this project

Thanks also goes for Prof.Dr.Neeran Obied Jasim who has been guiding and supervising us during the project as well as our dean Prof.Dr Basim Irahim for his support.

Special thanks for our families the ones without them we would not be where we are, and the cause of our existence to this life and our top supporters in the long life struggle

In the end of this acknowledgement no words can express or describe our gratefulness for those who are standing in the frontal lines of battles to protect our homelands , those who are standing in front of the bullets and shields sacrificing their lives to save ours, the words "THANK YOU " are just not enough at all.

DEDICATION

To our families

To our supervisor prof.

To our professors

To ours self's

Summary:

This study has been carried out to synthesis of silver nanoparticles using green synthesis by aqueous leaves extract of green tea and Investigation the antifungal activity of it. Results shown ,that silver nanoparticles were synthesized using leaf extract of green tea in present of silver nitrate and the color change to brown .UV-Vis spectrophotometer appear peak in wavelength at 420nm which is prescribed for silver nanoparticles .Also, microscope images by SEM ,showing the size of Ag nanoparticles in range 10-50nm.Results of antifungal activity explain the ability of biosynthesis silver nanoparticles to inhibition the growth of yeast ,*C.neoformans* with zone of inhibition 7mm compare with leaves extract in diameter 2.7mm.

Chapter One
Introduction

1-Introduction:

The development of toxicity free metal nanoparticles has become a great challenge in recent times. The primary challenge in this focal area is the maximization of the usage of environmental friendly materials in the generation of metal nanoparticles. Silver nanoparticles (Ag-NPs) are important materials that have been studied extensively. Such nano scale materials possess unique electrical, optical, as well as biological properties and are thus applied in catalysis, bio sensing, imaging, drug delivery, nano device fabrication and medicine (Smith *et al*., 2010). Due to strong antimicrobial activity, Ag-NPs are also used in clothing, the food industry, sunscreens and cosmetics (Kokura *et al*., 2010).

Nanoparticles possess a very high surface to volume ratio. This can be utilized in areas where high surface areas are critical for success. This could for example be in the catalytic industry; some nanoparticles actually have proven to be good catalysts. Although different techniques such as ultraviolet irradiation, aerosol technologies, lithography, laser ablation, ultrasonic fields and photochemical reduction have been used successfully to produce metal NPs, they remain expensive and sometimes involve the use of hazardous chemicals (Butkus *et al*., 2003; Ashby *et al*., 2009). Consequently, green synthesis of NPs has received increasing attention due to the growing need to develop an environmentally benign technology in nanoparticle synthesis. Several biological systems including bacteria, fungi, yeast and plants have been used in this regard (Nabikhan *et al*., 2010; Shivaji *et al*., 2011; Zaki *et al*., 2011). Although the green synthesis of Ag-NPs by various plants has been reported, the potential of plants as biological materials for the synthesis of NPs is yet to be explored fully. In addition, information on the biological response of human cells to green synthesized Ag-NPs is also very limited. (Sundrarajan and Gowri, 2011).

Green synthesis is superior to chemical and physical methods as it is environment friendly, cost-effective, easily scaled up for large-scale synthesis and there is no need to use high pressure, energy, temperature or hazardous chemicals (Singh *et al*., 2010) .therefore ,the present study explain the biological synthesis of silver nanoparticles by using plant extract ,and including the following steps :

1-green synthesis of silver nanoparticles by using aqueous extract of leaves green tea in present of $AgNO_3$.

2-study the properties these particles by using two techniques ,UV-Vis spectroscopy and SEM..

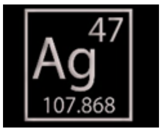
3-Study the effective of nanoparticles that synthesis against of pathogenic yeast ,*C.neoformans*.,using disc diffusion method.

Chapter Two Literature Review

2-LITERATURE REVIEW

2- 1. Background

2-1-1 silver



Silver is a white lustrous transitional metallic element found widely in the human environment. Low concentrations of silver are present in the human body through inhalation of particles in the air and contamination of the diet and drinking water (Lansdown, 2006). Silver is in the group 11 of the periodic table and is the most reactive of the precious metal elements. It has an atomic number of 47, an atomic mass of 108, three main oxidation states (0, +1, and +2). Silver had been used for in traditional folk medicine for centuries. People used it as an antimicrobial agent to reduce bioburden and prevent infection. Its usage diminished when antibiotics were introduced but remained one of the most attractive agents for wound infections, especially in burned patients (Edwards-Jones, 2009). To date, Ag is the most widely studied oligodynamic material due to its wide range of microbicidal effectiveness, low toxicity and ease of incorporation on various substrates in a host of dynamic disinfection applications. Furthermore, the systems supported with nano-metallic Ag particles are effective in reducing the presence of target microorganisms in a wide variety of water disinfection applications. The main known adverse health effect from Ag is argyria, which is an irreversible darkening of the skin and mucous membrane resulting from overexposure to ionic silver (Ag(I), Ag+) (Butkus *et al.*, 2005). Silver-containing treatments are popular and used in wound treatments to combat a broad spectrum of pathogens (Storm-*Versloot* et al., 2010). In 2013 Finley et al. reported improved healing and reduced bioburden in normal wounds with silver dressings.

2-1-2 *Cryptococcus neoformans*

Cryptococcus neoformans is an encapsulated fungal organism (Figure 1) that can cause disease,e.g. pulmonary cryptococcosis, in apparently immunocompetent, as well as immunocompromised hosts. Patients with T-cell deficiency tend to be most susceptible to infection caused by C. neoformans. No reports indicate that they are part of the normal microbial flora of human, C. neoformans is only transiently isolated from persons with no pathologic features.

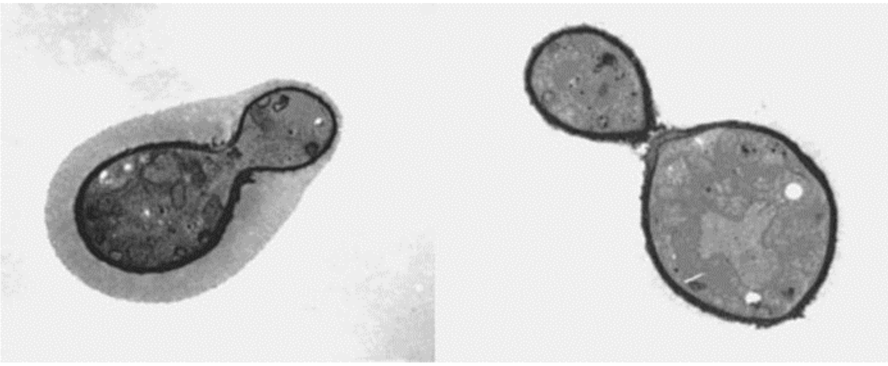


Figure 1. Transmission electron micrograph C. neoformans showing the characteristic polysaccharide capsule. (Buchanan et al.,. 1998)

It is accepted that the organism enters the host by the respiratory route in the form of a dehydrated haploid yeast or as basidiospores. After some time in the lungs, the organism hematogenous spreads to extrapulmonary tissues. Infected persons usually contract meningoencephalitis since these organisms have the preference to infect the brain; therefore meningoencephaliti is one of the usual manifestations. If untreated, cryptococcal meningoencephalitis is 100% fatal. (Buchanan *et al*., 1998) There for there is a pressing need to develop new and more efficient antifungal agent suitable for therapeutic use.

2-1-3 Green Tea (Camellia sinensis)

Tea is one of the most widely consumed beverages in the world, second only to water, and its medicinal properties have been extensively explored(Alschuler 1998).

Black, oolong, and green tea are produced from its leaves. It is an evergreen shrub or tree, and the leaves are dark green, alternate and oval, with serrated edges, and the blossoms are white, fragrant, and appear in clusters or singly.

Figure 2. *Camellia sinensis* plant, with cross-section of the flower (lower left) and seeds (lower right)

Unlike black and oolong tea, green tea production does not involve oxidation of young tea leaves. Green tea is extracted from steaming fresh leaves at high temperatures, thereby inactivating the oxidizing enzymes and leaving the polyphenol content intact. The chemical components of tea leaves include polyphenols (catechins and flavonoids), alkaloids (caffeine, theobromine, theophylline, etc.), volatile oils, polysaccharides, amino acids, lipids, vitamins (e.g., vitamin C), inorganic elements (e.g., aluminum, fluorine, and manganese), etc. However, the polyphenols are primarily responsible for the beneficial healthful properties of tea (Sharangi, 2009). The flavonoids have antioxidant, anti-inflammatory, antiallergic and anti-microbial effects.

Green tea contains six primary catechin compounds namely catechin, gallocatechin, epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate (EGCG), the later being the most active component.

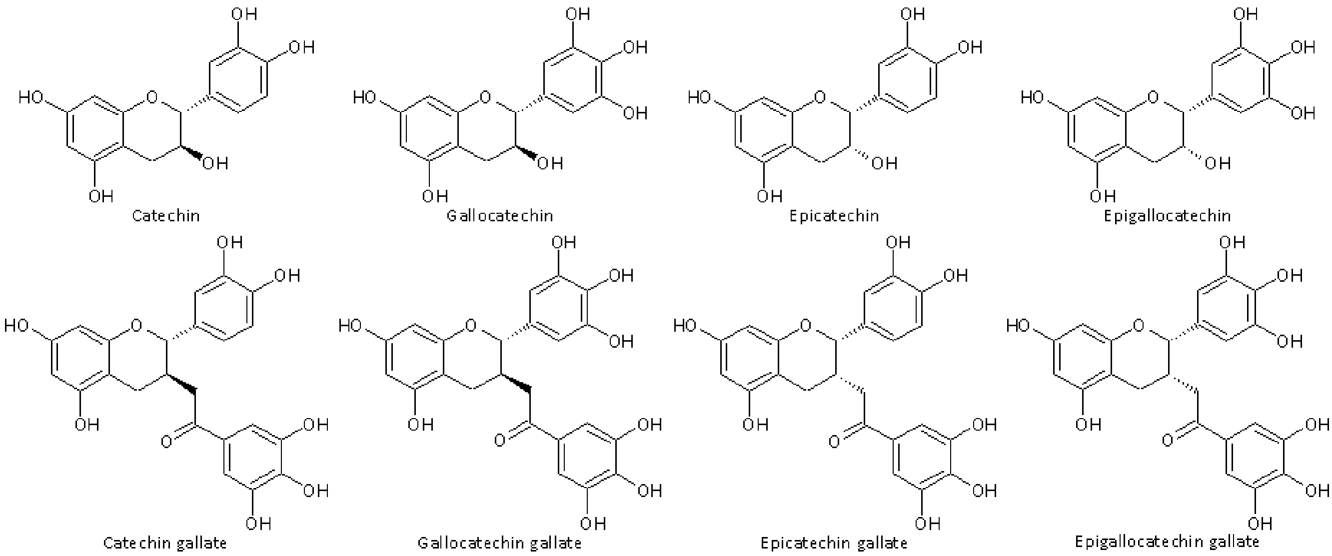


Figure 3. Chemical-structures of catechin and its derivatives

Catechins are important and promising compounds which their antioxidative roles and free radicals scavenging activity of have been confirmed in vitro and in vivo (Matsuzaki and Hara, 1985; Mukhtar and Ahmad, 2000; Frei and Higdon, 2003). Also, many studies have shown that tea catechins could suppress the genotoxic activity of various carcinogens with both in vitro and in vivo systems (Kuroda, 1996; Sinha et al., 2005; Isbrucker et al., 2006). As a result, those compound are now highly focused by researchers. They are also the compound that is thought to work as bio-reducers in the green synthesis of metal nanoparticles.

The polyphenols content of green tea and black tea varies from 30% to 40% and 3% to 10%, respectively.(Alschuler 1998, Graham HN 1992)

2-2- Green biosynthesis of Nanosilver

2-2-1 Nanotechnology

The word “nanotechnology” was first introduced in the late 1970s. While many definitions for nanotechnology exist, we can define it as it is the ability to observe, measure, manipulate, and manufacture things at the nanometer scale. A nanometer (nm) is an SI (Syst`eme International d’Unit´es) unit of length 10−9 or a distance of one-billionth of a meter. That is minuscule. At this scale, you are talking about the size of atoms and molecules.

At the nanoscale, fundamental mechanical, electrical, optical and other properties can differ significantly from their bulk material counterparts, and materials can self-assemble spontaneously into ordered structures. Nanostructured materials also have enormous surface areas per unit weight or volume, so that vastly more surface area is available for interactions with the other materials around them. That is useful because many important chemical and electrical reactions occur only at surfaces and are sensitive to the shape and texture of a surface as well as its chemical composition (Van Hove, 2006; Ashby et al., 2009).

The Nanomaterial's bear the promise of revolutionizing the development of biomaterials for the medical sciences and biosensing. However, prior to safe and efficacious translational applications of such materials in the clinic, comprehension of the nature of nanoparticles and the properties they impart to the materials that they are incorporated into them, is necessary. Hence, multidisciplinary collaboration amongst biologists, chemists, engineers, physicists, and clinicians is critical for designing the next generation of nanomaterials with improved biological activity and regenerative properties, and for moving these along the translational pipeline from “bench to bedside.”(Emilio *et al*) . Metal nanoparticles such as gold, silver, and copper also display unique and interesting optical and electronic properties. The unusual optical properties have driven a surge of research interest over the past couple of decades for applications in molecular sensing (Rosi *et al*. 2005), and biomedicine for targeting and killing cancer cells (Lewinski *et al.* 2008).

2-2-2 Nanosilver

The past few decades have experienced the resurgence in the use of silver nanoparticles (AgNP) in biomedical applications(Alexander *et al*. 2009).

Due to their unique physical and chemical properties, silver nanoparticles (AgNPs) are extensively used in electronics, catalysts, biosensors, medical areas, and large commercial antibacterial products(Sujuan *et al*.). Like other metal nanoparticles, nano silver also displays unique and interesting optical and electronic properties which make them a target for many new and interesting medical and non-medical applications The optical properties of AgNP depend on characteristics such as size, shape, and capping-coating.

However Silver nanoparticles (AgNP) are not only the future, but they are also the present as they are already part of many commercial products, being present in clothes (e.g. in socks); household and personal care products, mainly due to their antimicrobial properties. (Rogers, *et al*. 2012, Alarcon, *et al*. 2012). This causes a further need to investigate nanosilver to explore its full benefit and also the risk .

2-2-3 synthesis of nanoparticles

Different approaches used in the synthesis of nanomaterials and nanodevices can accommodate solid, liquid, and/or gaseous precursor materials. In general, most of these techniques can be classified as bottom-up and top-down approaches (Figure 4) and strategies that have elements of both.

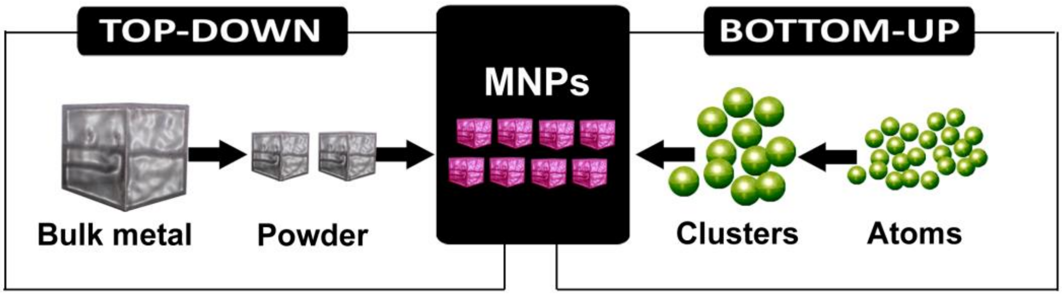


Figure 5. Schematic representation of Top down and bottom up approaches in nanotechnology

The top-down approaches start with a bulk material and then break it into smaller pieces using mechanical, chemical, or any other form of energy.

The bottom-up approach, on the other hand, is to synthesize the nanomaterials from atomic or molecular species via chemical reactions or self-assembly, allowing for the precursor particles to grow or gradually assembling the atomic or molecular precursors until desired structure is achieved. (O. Masala, Seshadri, 2004).

Both approaches can be performed in the gas or liquid phases, supercritical fluids the solid state, or under vacuum. The most important aspect of any method lies in its ability to control the particle size, particle shape, size distribution, particle composition, and the degree of particle agglomeration. In both approaches of nanomaterial fabrication, two fundamental requisites are control of manufacturing conditions (e.g., the energy of electron beam) and control of environmental conditions (e.g., dust, contaminations).

Nanotechnologies use highly sophisticated fabrication tools that are mostly operated in a vacuum in clean room laboratories. Liquid- and gas-phase processes are based on the assembly of nanoparticles from single atoms or molecules and, thus, allow good control of particle size, morphology, and sometimes size distribution. The above mentioned two techniques contain three submethods of synthesis:

a) Physical synthesis method

b) Chemical reduction method

c) Biological synthesis method

Green routes involving plant saps/microbes and biomimetic processes of self-organization and self-assembly have also been suggested for nano synthesis.

Green synthesis or Biomimetic Approaches

Green technologies have been around since the first public health projects were set up in cities to provide people with clean drinking water. With the development of science and technology, a growing number of researchers are merging green chemistry and green engineering with nanotechnology, and these researchers see a bright future for a new field known as ‘green nano.'

Biomimetics refers to applying biological principles for materials formation. Initially, bacteria were used to synthesize nanoparticles, and this was later succeeded with the use of fungi, actinomycetes, and more recently plants and specific peptides (M. Sarikaya et al. 2003). One of the basic steps in the biosynthesis of nanoparticles is bioreduction. Phytochemicals with antioxidant or reducing properties and microbial enzymes are usually responsible for the preparation of metal nanoparticles (Sundrarajan and Gowri, 2011).

The major implication of this biological approach is its relative simplicity in the synthesis of nanoparticles, and it is less time-consuming. In addition to this, the high yield, low toxicity, low cost and its bio compatibility adds to its value (Kalimuthu et al., 2010). Also, the size of the nanoparticles synthesized can also be controlled easily by various parameters like pH and temperature (Gurunathan et al., 2009a,b). The use of stabilizers to prevent aggregation is not required as the proteins in the system act as stabilizers (Kalishwaralal et al., 2010).

Plants

The use of plant extracts for nanoparticle synthesis obtained from neem, Camellia sinensis, Coriandrum sativum, Nelumbo nucifera, Ocimum sanctum and several other species has been studied. The phytochemicals responsible for the synthesis of nanoparticles are mainly flavonoids, terpenoids, ketones, aldehydes and amides (Sundrarajan and Gowri, 2011). Green tea, Camellia sinensis, the extract was used as a reducing and stabilizing agent to produce gold nanoparticles and silver nanostructures in aqueous solution at ambient conditions (Vilchis-Nestor et al. 2008). Generally, plant extract contains both a reducing agent and a stabilizing polymer. For these reasons, plant extracts are responsible for the nanoparticle synthesis .

Table.1. A survey of literature for the use of plants in nanoparticles biosynthesis

S. No	Plant species	Parts/extracts	Nanoparticle synthesized	Reference
1	*Cinnamomum camphora*	Leaf extracts	Au-NPs	Kumar et al., 2011
2	*Emblica officianalis*	Whole-plant extracts	Au-NPs	Kumar et al., 2011
3	*Hibiscus rosa-sinensis*	Whole-plant extracts	Au-NPs	Kumar et al., 2011
4	*Catharanthus roseus*	leaves	Ag-NPs	
5	*Acalypha indica*	leaves	Ag-NPs	Krishnaraj et al,.
6	*Ocimum sanctum*	leaves	Ag-NPs	Philip and Unni, 2011

7	*Ocimum sanctum*	leaves	Au-NPs	Philip and Unni, 2011
8	*Iresine herbstii*	leaves	Ag-NPs	Dipankar et al., 2012
9	*Azadirachta indica*	leaves	Ag-NPs	Shankar, et al 2004
10	*Rosa rugosa*	leaves	Ag-NPs	Dubey et al., 2010
11	*Rosa rugosa*	leaves	Au-NPs	Dubey et al., 2010

The advantage of using plants for the synthesis of nanoparticles is that they are readily available, safe to handle, and possess a broad variability of metabolites that may aid in reduction. A number of plants have been investigated for their role in the synthesis of nanoparticles While fungi and bacteria require a comparatively longer incubation time for the reduction of metal ions, water-soluble phytochemicals do it in a much lesser time. Also using plant extracts can be advantageous over other biological processes because it eliminates the elaborate process of maintaining cell cultures and can be suitably scaled up for large-scale production under non-aseptic environments. Therefore, plants are better candidates for the synthesis of nanoparticles when compared to that of bacteria and fungi. Biomimetic Green synthesis provides other advancements over chemical methods as it is environment-friendly, cost-effective, and easily scaled up for large-scale synthesis. The green synthesis method involves three main steps, (1) solvent medium selection, (2) environmental benign reducing agent selection, and (3) non-toxic substances for nanoparticles stability selection

Phenolic acid-type biomolecules (e.g., caffeine and theophylline) present in the C. sinensis extract seemed to be responsible for the formation and stabilization of silver NPs. Black tea leaf extracts were also used in the production of silver NPs (Begum NA et al. 2009). The NPs were stable and had different shapes, such as spheres, trapezoids, prisms, and rods. Polyphenols and flavonoids seemed to be responsible for the biosynthesis of these NPs.

2-3 silver and nanosilver as antimicrobial

2-3-1 metallic silver

The antibacterial activity of silver has long been known and has found a variety of applications because its toxicity to human cells is considerably lower than to bacteria. The most widely documented uses are the prophylactic treatment of burns and water disinfection.(Clement and Penelope 1994). Silver is a noble metal, i.e. chemically, it is inert. However, its interaction with moisture on the skin surface and with wound fluids leads to the release of silver ions, which have anti-microbial effects (Chernousova *et al*. 2012). In biomedical applications, today, metallic silver has been used or tested in dental alloys (also as silver amalgam) and prosthetic implants such as “mega-endoprostheses” that are grafted after the removal of bone tumors (Butany *et al*. 2002). In bone prosthesis, incorporation of silver appears to have resulted in a reduction of the infection rate with no adverse side effects.

2-3-2 silver salts (e.g., silver nitrate) and ionic silver

Silver salts such as silver nitrate (AgNO3) are one of the best-known sources of ionic silver. Two of the primary biomedical uses for ionic silver were as prophylactic eyedrops for newborns against gonorrhea and other bacterial/fungal infections and in the so-called ‘silver-baths,' that were standard practice in burn units to prevent biofilm formation. Colloidal silver was hailed as the “magic bullet” when Erlich began treating syphilis with silver-salvarsan in 1910, and this became the treatment of choice (Hasitha de Alwis Weerasekera *et al*. 2015). Silver in form of AgNO3 has also been combined with antibiotics, such as sulfonamide in 1968, to produce silver sulfadiazine (SSD) cream that resulted in a broad spectrum silver-based antibacterial that continued to be prescribed mostly for the management of burns.(Liau, *et al*,1997; Fox ' 1968). The mechanism of the antimicrobial action of silver ions is closely related to their interaction with thiol (sulfhydryl) groups (Belly *et al* 1982., Bragg PD, Rainnie DJ 1974 , Fuhrmann GF, Rothstein A 1968 , Furr 1994), although other target sites remain a possibility (Richards et al. 1984 , Thurman et al. 1989). Amino acids, such as cysteine, and other compounds containing thiol groups, such as sodium thioglycolate, neutralized the activity of silver against bacteria (Liau SY et al. 1997). By contrast, disulfide bond-containing amino acids, non-sulfur-containing amino acids, and sulfur-containing compounds, such as cystathione, cysteic acid, l-methionine, taurine, sodium bisulfate, and sodium thiosulfate, were all unable to neutralize the activity of silver ions. These and other findings imply that the interaction of silver ions with thiol groups in enzymes and proteins plays an essential role in its antimicrobial action, although other cellular components, like hydrogen bonding, may also be involved (Furr 1994). Silver was also proposed to act by binding to key functional groups of enzymes. Silver ions cause the release of K+ ions from bacteria; thus, the bacterial plasma or cytoplasmic membrane, which is associated with many important enzymes, is an important target site for silver ions (Fuhrmann GF, Rothstein A 1968, Miller 1957, Schreurs WJ, Rosenberg H-J)

2-3-3 nanosilver

The antimicrobial mechanism for AgNP remains not fully understood Yet many believe that examining the antimicrobial activity of ionic silver is of great importance to finding the answer . some studies have employed transmission electron microscopy (TEM) as a tool to discern AgNP accumulation within the cell. In 2010, Li et al. evaluated the antimicrobial mechanism and activity of silver nanoparticles in E. coli, analyzing the growth, permeability, and ultrastructure of bacterial cells after treatment with 5 nm AgNP. Their study provided evidence of membrane-disruption and changed permeability as cause of inhibition and death in E. coli. While A study by Kelly Ishida et al 2014 investigated the antifungal effect of silver nanoparticles on Candida albicans andCryptococcus neoformans reported their susceptibility to theilver nanoparticles as their growth was inhibited. Also, SNPs exhibited fungicidal activity (with MFC values up to 4 times the MIC value) against the species tested.The study of ultrastructural Morphological alterations of C. neoformans treated with SNPs exhibited a disrupted Cell Wall and several invaginations in the cytoplasmic membrane. In addition, increased CW thickness was observed

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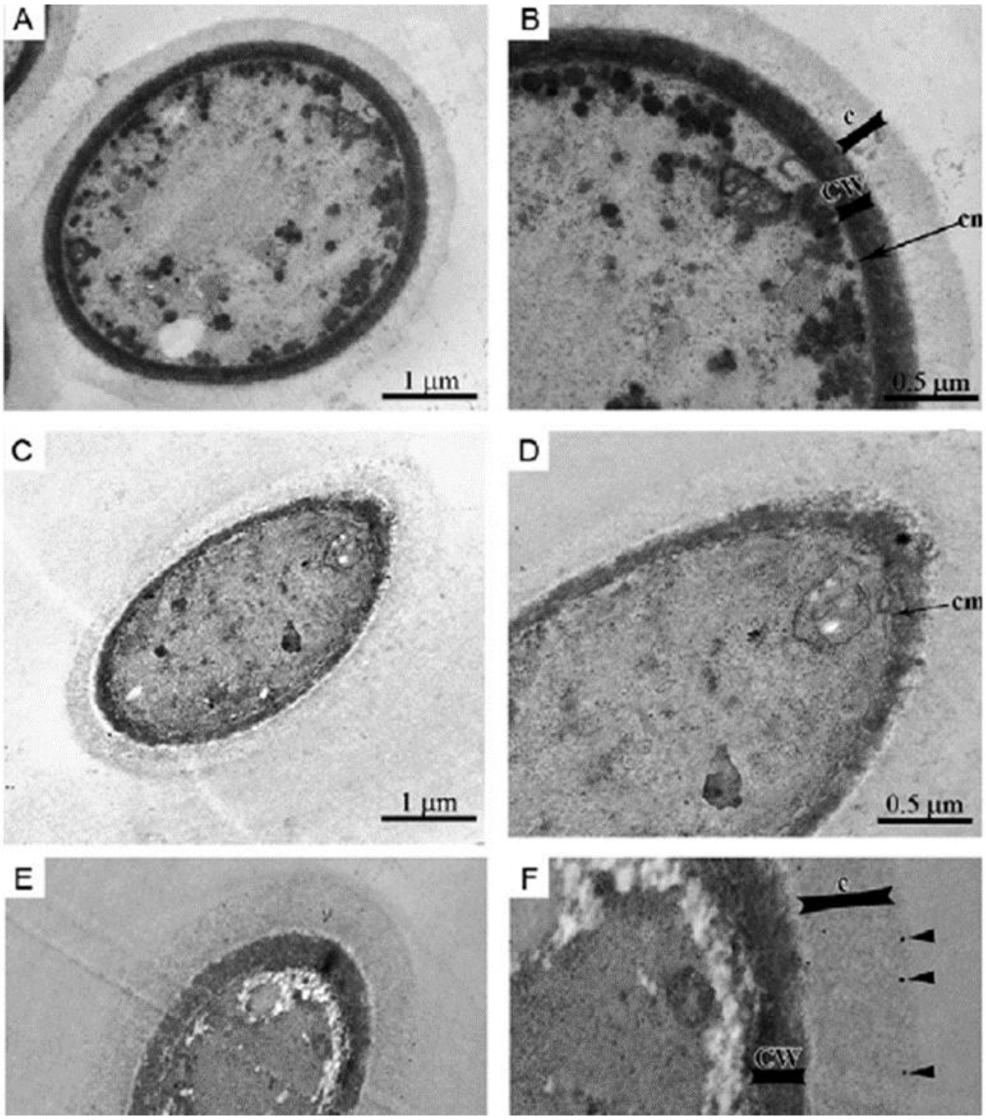

Figure 5. Morphological alterations of *Cryptococcus neoformans* treated with sub-inhibitory concentrations of silver nanoparticles (SNPs) (0.21 µg/mL) for 72 h at 35ºC. The untreated yeast exhibit a compact cell wall (CW), continuous cytoplasmic membrane (cm), homogeneous and electron-dense cytoplasm and a polysaccharide capsule (c) surrounding the cell (A, B). By contrast, yeasts treated with SNPs had a disrupted cytoplasmic membrane and CW (asterisk) and increased cell wall thickness (CW) (C, D). The SNPs appear to be retained in the polysaccharide capsule (F, black arrowhead) (Ishida K. et al 2014)

2-4 relationship between the antimicrobial effect of nanosilver and their size and shape

The wide range of methods that have been used for the synthesis of silver nanoparticles have given rise to variable particle morphologies including spheres, cubes, rods, wires and multifaceted. Silver nanoparticles of different shapes and sizes show unique interactions with bacteria and. As AgNP of smaller size have higher surface area to volume ratios, the relative rates of silver release may be higher for the smaller sized particles. Baker and colleagues also showed that AgNP antimicrobial properties were directly related to the total surface area of the nanoparticles(Baker et al. 2005). The antimicrobial efficacy of silver nanoparticles has been confirmed to be shape dependent in studies that utilized differentially shaped nanoparticles and measured the inhibition of bacterial growth suggested that the most efficient geometry is truncated triangular silver nanoparticles. Also, AgNP manufactured using different synthetic techniques and for different purposes may vary in their physicochemical properties, which can lead to significant differences in their biological activity. If we also take into consideration possible coatings, stabilizers and/or other hybridized materials it is clear that these additions may lead to modified cellular uptake and altered interactions with biological macromolecules. Consequently, not all AgNP should be considered the same and it is important to understand that adverse reactions that would not be seen in other silver species can arise. Taken together, it appears that the killing efficacy is directly dependent on the rate and location where Ag(I) is being released from the nanoparticles. This release is in turn reliant on the shape and size of the nanoparticle as well as the physicochemical nature of the surroundings.

2-4 application

Many of the most important biomedical uses and future applications of silver nanoparticles are due to their antimicrobial effect and base on this; the following are some of those uses:

1. Dental Material: Studies have shown that AgNP infused dental filling cement have remarkable bactericidal effects. Adding AgNP to orthodontic adhesives has also demonstrated notable antimicrobial properties, which in turn prevents enamel demineralization without jeopardizing the physical attributes of the adhesive in the process.

2. Wound Dressings Recent studies have shown that wound dressings containing AgNP can be used to inhibit infection and accelerate the healing process as a result of the reduced bacteria colonization ActicoatTM was the first commercially available product utilized for wound dressing containing AgNP. This product was clinically tested for skin wounds including burns, Steven-Johnson syndrome, chronic ulcers, and pemphigus

3. Medical Catheters The antibacterial effect of AgNP on polyurethane, one of the most common polymers used in manufacturing medical catheters, has been widely studied in the past decade as an application that inhibits biofilm formation and prevents catheter indwelling on central venous and neurosurgical catheters. In vitro, in vivo and clinical studies have shown the effective inhibition of biofilm development along with excellent biocompatibility.

4. Bone Cement AgNP integrated poly (methyl methacrylate( )PMMA) based bone cement used for the secure attachment of artificial joint replacements have demonstrated notable prophylactic effects along with exceptional biocompatibility.(Alwis Weerasekera *et al*. 2015)

2-5 toxicity

Three routes of exposure to silver nanoparticles are present inhalation, oral ingestion, and dermal contact. The most important route is the oral one as silver nanoparticles are found in canned food, dietary supplements and also in animal feed to replace antibiotics. Although the toxicity of the silver compound is known the toxicity of silver nanoparticles is still controversial and requires further studies.

The main toxicity features, which were investigated and reported in a review by Gaillet and Rouanet 2015, are weight loss, altered liver enzymes, altered blood biochemistry values. There is evidence suggesting that the effects induced by AgNPs are mediated via silver ions that are released from the particle surface. From an arbitrary point of view, AgNPs can produce ROS and cause oxidative stress in cells, inducing oxidative damage to cell membranes and organelles (lysosomes, mitochondria) and the nucleus, directly causing apoptosis or necrosis. This is the dominant factor mediating their toxicity. Oxidative stress caused by AgNPs can trigger inflammatory reactions. Several factors such as dose, exposure time, temperature, size, shape, surface coating, surface charge and cell types play important roles in influencing the toxicity of AgNPs and in mediating cellular responses. Excretion occurs via the bile and urine. There is evidence suggesting that the effects induced by AgNPs are mediated via silver ions that are released from the particle surface. They also concluded that from a mechanistic point of view, AgNPs could produce ROS and cause oxidative stress in cells, inducing oxidative damage to cell membranes and organelles (lysosomes, mitochondria) and the nucleus, directly causing apoptosis or necrosis. This is the dominant factor mediating their toxicity. Oxidative stress caused by AgNPs can trigger inflammatory reactions. Several factors such as dose, exposure time, temperature, size, shape, surface coating, surface charge and cell types play important roles in influencing the toxicity of AgNPs and in mediating cellular responses. This indicates that risk assessments should be conducted on a case-by–case basis.

Chapter Three Material & Methodes

3-1-Materials and Methods

3-1-1-Equipments & Chemical materials:

Table (3-1): Equipment's & Chemical materials used in the laboratory experiments

No.	Equipment	Company	source
1	Incubator	Gallenkamp	(England)
2	Sensitive balance	Gallenkamp	(England)
3	autoclave	Gallenkamp	(England)
4	Hot plate with magnetic stirrer	Gallenkamp	(England)
5	Vortex	Electrothermal	(England)
6	UV Spectrophotometer	Memmert	germany
6	Whitman(No.1)filter paper	Gallenkamp	(England)
7	Centrifuge	Gallenkamp	(England)
8	Silver nitrate	BDH	England
9	Leaves of green tea		original
11	Sabuobaud's Dextrose Agar	BDH	(England)

3-2-Methods:

Prepration of Sabouraud dextrose Agar (SDA):

Culture media were prepared according to the instructions of the manufacture companies, the culture media were sterilized by autoclave at 121C under pressure 15 pound /cubic inch for 20 minutes .

Fungal Strain*:*

Cryptococcus neoformans which obtain from laboratory of microbiology of science college /university of AL-Qadisiyah.

Synthesis of silver nanoparticles :

2 g. of leaves tea put in beaker containing 20ml of DW .the mixture was thoroughly agitated over night by using a magnetic stirrer .Than the mixture was filtered to obtain brown liquor .The liquor was centrifuged and again filtered to remove the impurities .1M of $AgNO_3$ solution was prepared in DW. Silver nanoparticles was prepared by mixed 0.2 ml of $AgNO_3$ solution ,0.1 ml of tea extract and 3.7 ml DW .Then it was kept overnight in dark to stabilize .Change in color was observed on the next day .(Lipi *et.al.*,2014)

UV –spectrophotometer analysis :

Optical characters of the synthesized silver nanoparticles and $AgNO_3$ were studied by analyzing the UV-Vis specter using spectrophotometer at room temperature .

Scanning Electron Microscopy (SEM):

We used Scanning Electron Microscopy (SEM) technique for measuring nanoparticle size of silver.

Antifungal activity :

The Antifungal activity of the extracts was carried out by disc diffusion method(Kim *et.al.*,1995) Circular discs of 5 mm diameter were made from the Whatman No.1 filter paper and sterilized by autoclaving at 15lb/inch2 for 15 minutes. The sterile discs were impregnated with equal volume (100µg/ml) of tea leaf extracts and silver nanoparticles The discs containing each of 25µl samples were aseptically placed on plates containing SDA medium after being spreaded with each of the test pathogens, . The plates were incubated at 37 ºC for 24 hours and the zone of inhibition was measured (in mm diameter).

Chapter Four
Results & Discussion

4- Results &Discussion

4-1- Synthesis of silver nanoparticles:

Results showed that the silver nanoparticles were synthesized by using leaf extract of green tea from silver nitrate. The change in color from yellow to brown in mixture of reaction after 24h indicated the reduction in Ag^+ to Ag^0. These color changes arise because of the excitation of surface Plasmon vibrations with the silver nanoparticles.(Mulvaney,1996).Li *et.al.*,2011 reported that producing of nanoparticles by plant extracts with solution of the metal salt at room temperature complete in few minutes.

4-2- U-V-Vis Spectroscopy :

UV results are obtained from this test shown in Fig(1,2) ,its represented the UV-vis spectrum of the silver nano sample, and AgNO3 Respectively .The absorption peak obtained for these samples are in rang of 400- 420 nm for silver nanoparticles .This results emphasizes the existence of silver nanoparticles in the solution .This results coordinated with Praphulla *et.al.*(2014) that found the wavelength of silver nanoparticles in rang 400-430 nm. Also, the absorbance in the range of 420-450 nm has been used as an indicator to the reduction of Ag^+ to metallic Ag(Karuppiah &Rajmohan,2013).

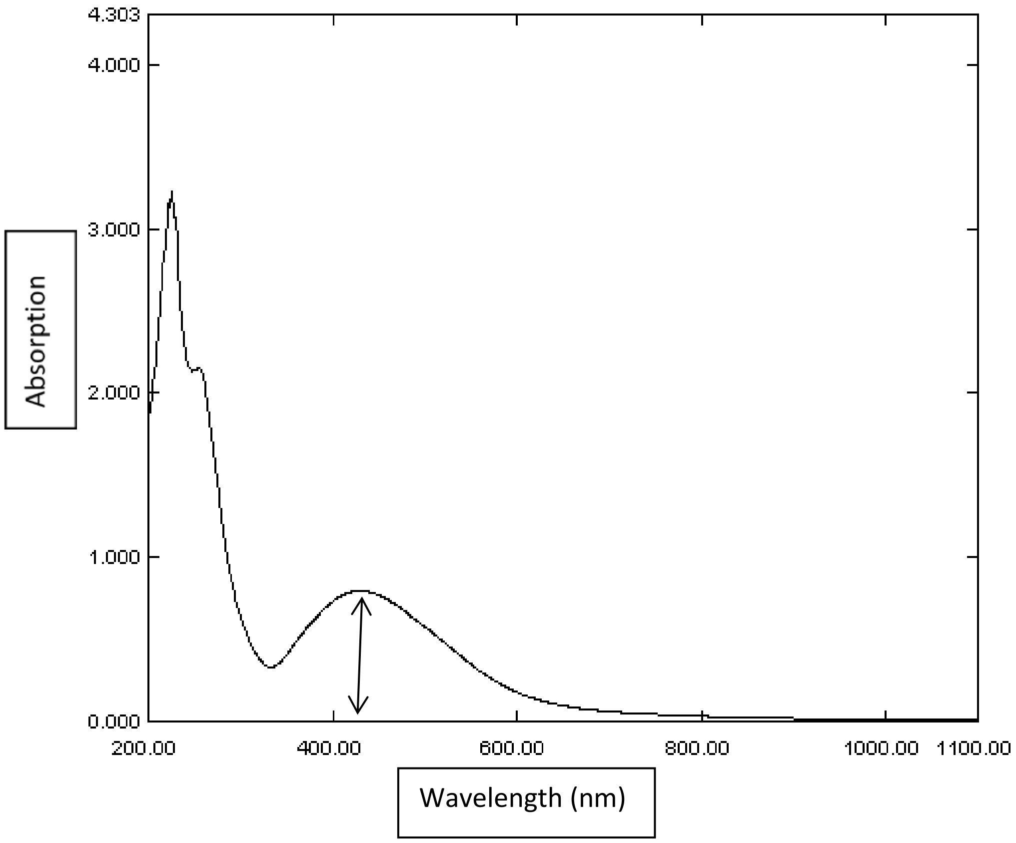

Fig.(1) VIS absorption spectra of Ag nanoparticles

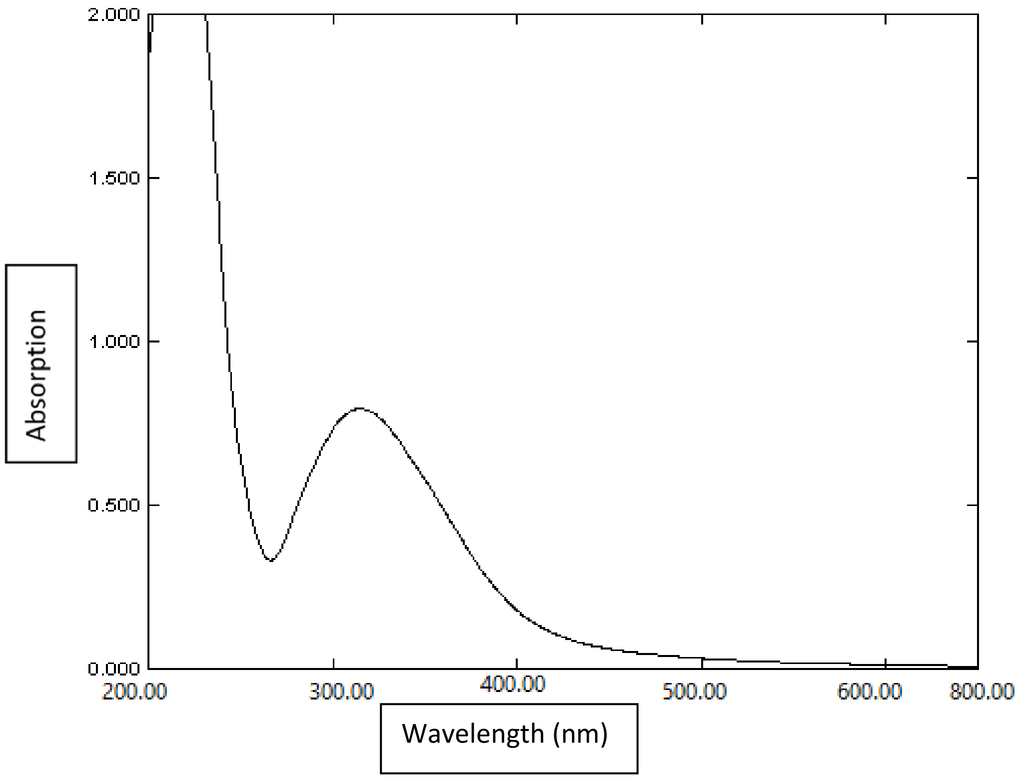

Fig.(2) VIS absorption spectra of $AgNO_3$

4-3- SEM Images:

The results by SEM show in fig.(3,4,5) ,and this results indicated the present of silver nanoparticles with varies size ranging 10-50nm . The images show that most of the particles are spherical in shape with a smooth surface.

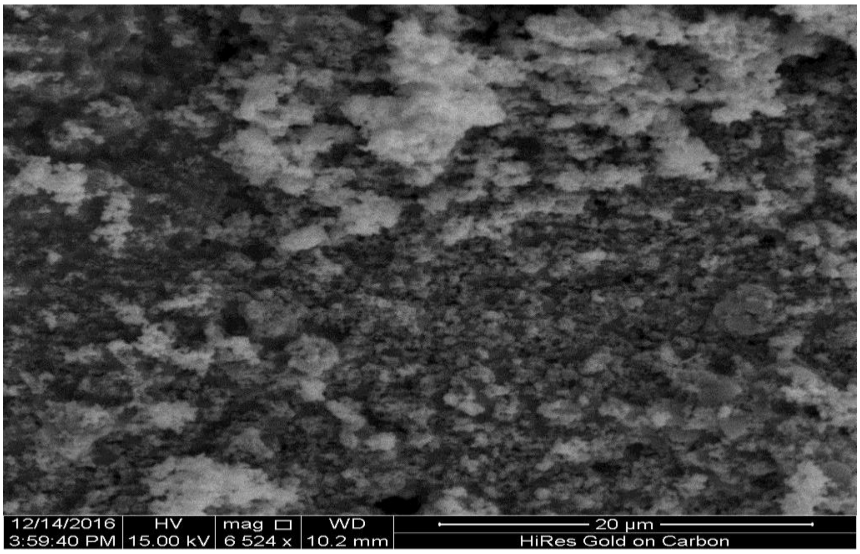

Fig.(3) ESM sections of the biosynthesis of AgNps with size (20nm)

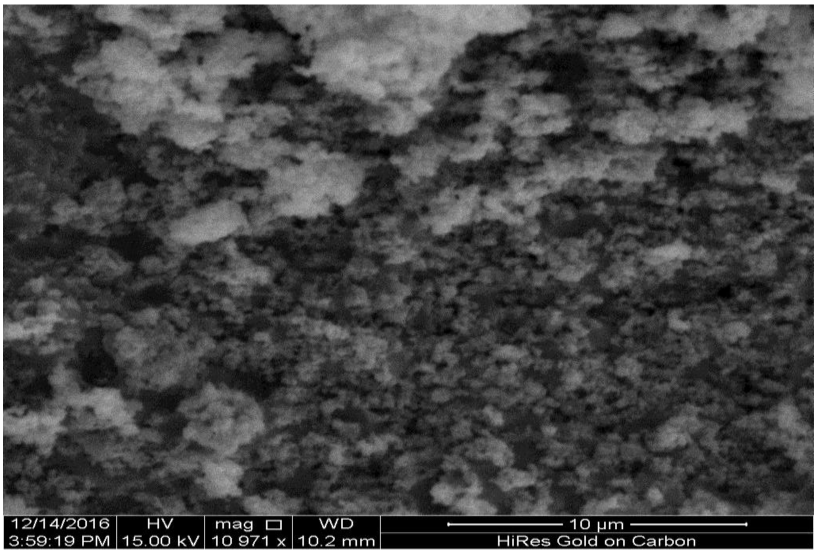

Fig.(4) ESM sections of the biosynthesis of AgNps with size (10nm)

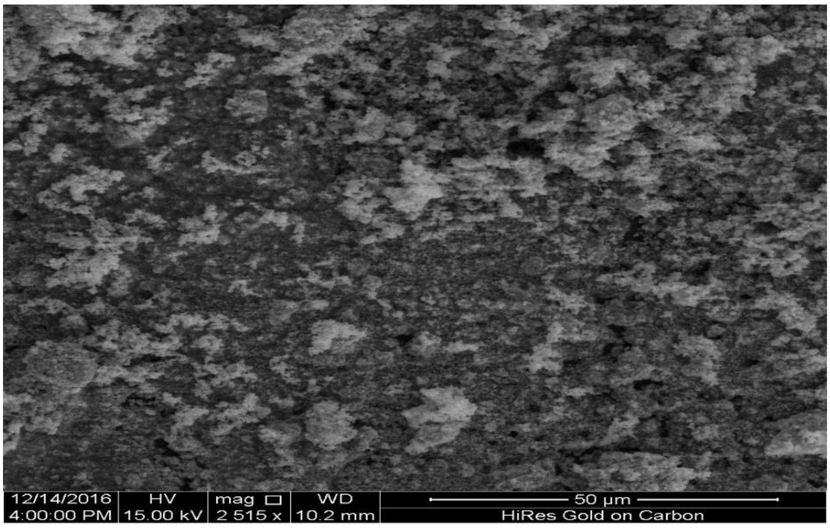

Fig.(5) ESM sections of the biosynthesis of AgNps with size (50nm)

The present study has shown that the leaf extract of green tea has the capability of reducing Ag from respective ion sources. This agreement with many study that green synthesis of Ag Nps from leaf extract of plant , Babu and Prabu (2011)synthesized 35 nm silver nanoparticles using a flower extract of *Calotropis procera*. Kouvaris *et al*. (2012) used a leaf extract of *Arbutus unedo* to produce nanoparticles with a narrow size distribution .Baskaralingam *et al*. (2012) used a leaf extract of *Calotropis gigantean* to produce silver nanoparticles.

4-4 -Antifungal activity:

The antifungal effect of biologically silver nanoparticles was investigated against *Cryptococcus neoformans* ,that causing many types of infections, by disc diffusion method .Growth inhibition of the yeast by silver nanoparticles was compared with An aqueous extract of leaf green tea .Results showed that the green synthetic Ag nanoparticles had effect on *C.neoformans* with rate of inhibition zone 7mm.Fig.(6,7).This results do not agree with Sarah *et.al*.(2014) which found that green synthesis of Ag nanoparticles by water extract of *Aloe vera* had no effect on two fungi(*Candida albicans &Aspergillus niger*) . Size and shape of nanoparticles are more effect on the antifungal activity.(Prashanth *et.al*.,2011).While the results of this study agreement with Khalil(2013) that reported the Ag Nps inhibition of the forming of conidia in *A.fumigatus*.

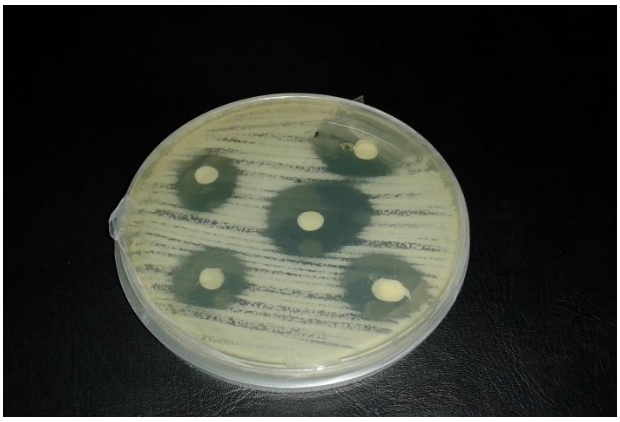

Fig.(6) Effect of biosynthesis of Ag Nps on *C.neoformans*

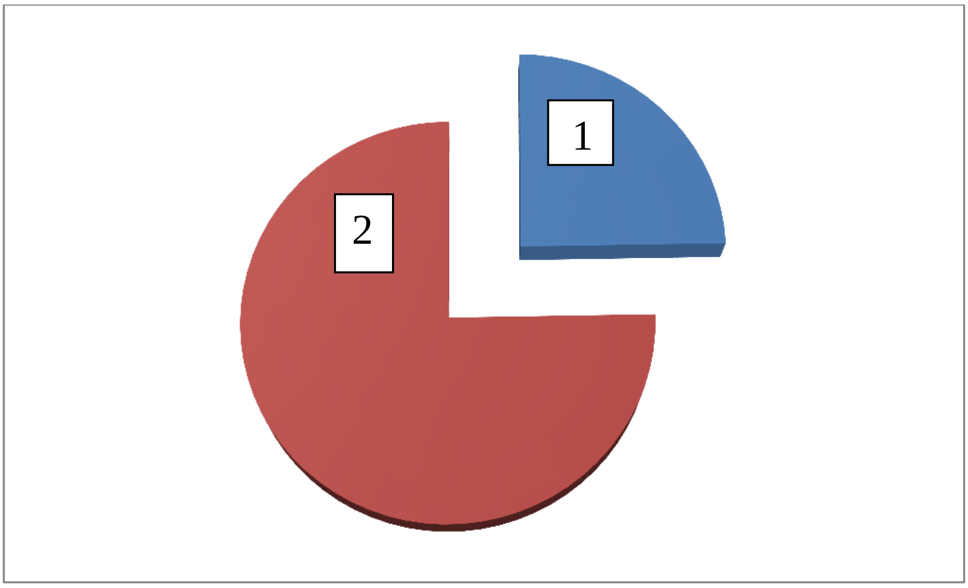

Fig.(7) Effect of biosynthesis of Ag Nps on *C.neoformans*

1= An aqueous extract of leaf green tea

2= The biosynthesis Ag nanoparticles

The reason in the inhibitory effects of silver nanoparticles its wide surface area for size and this lead to reaction of this particles with cell wall of microbe to make an imbalance in the cellular permeability .(Kanhed ,*et.al*.,2014) .

Conclusion & Recommendation

Conclusions :

1-Silver nanoparticles are produced by reduction of silver ions ,in this study leaf extract of green tea was used as reduced agent .

2-The biosynthesis method consider an easily ,eco-friendly and Cost effective for prepare nanoparticles.

3-UV-spectrophotometer and SEM technique are usefully for identification of biosynthesis Ag nanoparticles.

4- Ag nanoparticles which were synthesized by aqueous leaves extract in present silver nitrate have effective for reducing the growth of yeast ,*C. neoformans.*

Recommendations:

1-Posibility to biosynthesis of silver nanoparticles from aqueous leaves green tea extract.

2-Study other plant extract for biosynthesis of silver nanoparticles especially that contain reducing agent .

3-Study the effect of biosynthesized silver nanoparticles against *C.neofomans* in vivo using animals lab.

4-Study the effect of biosynthesized silver nanoparticles against other fungi ,including pathogenic filamentous fungi.

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الخلاصـــــــــة:

أجريت هذه الدراسة لتصنيع جزيئات الفضة باستخدام التخليق الحيوي باستخدام المستخلص المائي لاوراق الشاي الأخضر والتحري عن الفعالية المضادة للفطريات .وأظهرت النتائج أن جزيئات الفضة تم تخليقهاا باستخدام مستخرج أوراق الشاي الأخضر بوجود نترات الفضة وتغير اللون إلى اللون البني .. قياس الطيف باستخدام مطياف الاشعة فوق البنفسجية اظهر الذروة في الطول الموجي في ٤٢٠ nmالذي يوصف للجسيمات النانوية الفضة. أيضا، صور المجهر الالكتروني الماسح والتي تبين حجم الجسيمات النانوية للفضة في نطاق ١٠-٥٠.nm .نتائج النشاط المضاد للفطريات تفسر قدرة الجسيمات النانوية للفضة لتثبيط نمو الخميرة، *C.neoformans*مع منطقة تثبيط(٧) ملم مقارنة مع مستخلص الأوراق في قطر(٢,٧)**ملم.**