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**Research Article**

# Biosynthesis of Gold Nanoparticles by *Aspergillus Niger* And Their Role Against Pathogenic Bacteria

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## Abstract:

The study included the use of *Aspergillus niger* filtrate for the biosynthesis of gold nanoparticles (GNPs) using a reducing medium designed for this purpose. The synthesis of Nanoparticles was verified by means of detections by spectrophotometry, X-ray diffraction, FTIR, and scanning electron microscopy .Nanoparticles were used to inhibit pathogenic bacteria obtained from urinary infections patients, and they included *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* by poisoning the nutrient medium and drilling method. It was also verified that there are no toxins accompanying the biosynthesis of these nanoparticles by the ammonia method. The detections of characterization of GNPs confirmed that the particles were synthesized in spherical, oval, and crystalline shapes with sizes ranging from a few nanometers to 500 nanometers, and they gave a light absorption band at the wavelength of 520 nanometers. Concerning the effect of GNPs on pathogenic bacteria, the method of poisoning the media confirmed that the 15% concentration was significantly effective against the three bacterial species, as it gave the lowest diameter of colony growth (1.1, 1.6, 2.3) cm for *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* respectively. As for the drilling method, it also showed a high significant ability of 15% concentration to inhibit the tested bacteria by measuring the inhibition zone, which gave an area of (5.1, 5.4,6) cm for *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* respectively, with regard to the toxicity test, the study showed that the isolate used in the synthesis does not produce toxins, which confirms its suitability and dependence in the biosynthesis of nanoparticles.

**Keywords: Aspergillus niger, biosynthesis, gold nanoparticles, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus**

## Introduction

Biological methods of synthesizing nanoparticles are one of the best methods, they depend on the metabolic materials produced from living cells and use them to assemble the particles of the material, which may be gold, silver, zinc, or others, to become nanoparticles with new characteristics that differ from what the substance was before the treatment, this method is considered successful and environmentally friendly, as it has fewer harmful by-products compared to chemical and physical methods. also, the cost of biological methods is low and does not need to use complex devices and no giant factories because of their small size and the possibility of moving and controlling them easily (Pantidos & Horsfall,2014). regarding to bacteria, many species have shown the ability to convert metallic elements into nanoparticles, and they were later used in various applications, such as antivirals, antibacterials, antifungals, anti-oxidants, free radicals, cancer treatment, inhibitor of living membrane formation, joint treatment and other applications, especially *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Staphylococcus aureus* and *Escherichia coli* (Ghorbani *et al.*,2015; Vanamala *et al.*,2021). Regarding to fungi, they are among the best organisms in the production of nanoparticles of all kinds. Yeasts, molds, cumin and mushrooms have been used and they have given amazing results in many fields as they are characterized by the abundance of their enzymatic content and other secondary metabolites, for example, *Saprolegnia parasitica* has been successfully used in the production of silver nanoparticles, and it has given results that outperformed chemical drugs as antibacterial and breast cancer effects (Al-Khuzai *et al.*,2019), while many species of Aspergillus were used in the preparation of various metallic nanoparticles. Significant results of inhibiting the growth of pathogenic bacteria and fungi and reducing their virulence, as well as having effectiveness against the development of cancers (Li *et al.*,2011**;** Omran *et al.*,2020). Mushroom and quantum filtrates were used to prepare metallic nanoparticles due to the high protein content of its extract and its high ability to reduce metallic elements and convert them into nanoparticles. Nanoparticles of most metallic elements such as gold, silver, copper, lead, zinc and others, which have been used successfully in controlling pathogenic germs (Owaid *et al.*,2018). This study will help us better understand the synthesis of multimetallic nanoparticles and the substitution of

traditional antibiotics with new antimicrobial agents to kill multi drug resistant bacteria.

## Materials and Methods

### Microorganisms used in the study

An isolate of the fungus *Aspergillus niger* (1015) was obtained from the microbiology laboratory in college of education. As for the bacterial isolates, they were obtained from from patients in the Hospital with urinary infections, and they included *Escherichia coli* (25922), *Pseudomonas aeruginosa* (27853) and *Staphylococcus aureus* (ATCC 25923) .

### Preparation of gold nanoparticles

To prepare gold particles, a suitable biomass of *A.niger* must after the fungus growth under aerobic conditions on Sabouraud dextrose broth medium. The medium was sterilized by autoclaving and then cooled to 45°C, then chloramphenicol was added to prevent bacterial contamination and distributed into 250-mL glass beakers at a rate of 150 mL per beaker. Each beaker was inoculated with a disc of young culture of the fungus under study, then it was incubated at 27 °C for 7 days. After incubation, the fungal hyphae that had grown were washed by using sterile distilled water to remove any remaining medium. Precisely, 10 g (wet weight) of biomass was placed in 100 ml of sterile distilled water in erlenmeyer flasks and incubated in a rocking incubator (120 rpm) for 48 h at 27°C. Then, the fungal-free filtrate was obtained by passing it through a Whatman No. 1 filter paper. Then silver nanoparticles were prepared by adding gold chloride to the fungal filtrate at (10−3 M) and then incubated in a rocking incubator at 120 rpm for 72 minutes at 28°C while other flasks were left without addition of gold chloride for comparison and monitoring the color change of the filtrate, which is a preliminary indicator of nanoparticle formation (Balakumaran et al.,2016; Soni & Prakash,2012).

### Characterization of GNPs

The initial characterization of gold nanoparticles was done by visual observation of the change in color as the color of the solution turned from yellow to bright red.The time-dependent formation of gold nanoparticles was observed by using (UV-Vis spectrophotometer Evolution™ 201/220 -Thermo Fisher Scientific USA – Wisconsin). The innate gold nanoparticles were confirmed by taking samples of the reaction mixture at regular intervals, and the absorption spectra at a wavelength of 300-700 nm were scanned in a spectrophotometer. The optical properties of the gold nanoparticles were studied by a UV-VIS spectrophotometer (Shimadzu). The effective functional groups that make up the fungus extract and the prepared gold nanoparticles were determined, which can participate and act as reducing, encapsulating and fixing silver nanoparticles by means of Fourier transform infrared (FTIR) as well as by measuring X-ray diffraction. As for the structural properties of the formed nanoparticles in terms of the shape and size were determined by a scanning electron microscope (SEM) (Faramarzi & Forootanfar,2011; Philip,2008).

### Antibacterial effects of GNPs

#### Food Poisonings method

The bacterial suspension was prepared by taking a part of the bacterial culture of one day by loop to test tubes containing sterile PBS physiological solution and shaking the solution well with a Vortex device, then the number of cells was counted to obtain a concentration of $1 \times 10^8$ cells/ml using a hemocytometer to calculate the number of cells .Mueller-Hinton agar medium was prepared according to the instructions of the producing company, after sterilization and cooling to 45 °C, and before putting into dishes, it was distributed in 250 ml beakers in equal quantities, then concentrations of gold nanoparticles were added, which are 0%, 5%, 10%, 15% to the flasks.Three replicates for each concentration, then added to the mixture, the tested bacterial cells were suspended, then the medium was left to solidify and then incubated at a temperature of 37 °C for 24 h. The positive control was the antibiotics Gentamycin, Penicillin and Ciprofloxacin at a concentration of 3000 mg/L (Al-Khuzai *et al.*,2019).

#### Wells method

Mueller-Hinton agar was Prepared according to the instructions of the producing company and after sterilization put into plastic Petri dishes with a diameter of 9 cm and after solidification, drilling was done with a cork drill, which nano concentrations were added, which are 0%, 5%, 10%, 15%. After that, the media were inoculated with bacteria at 37 °C for 24 h, the positive control was the antibiotics Gentamycin, Penicillin, and Ciprofloxacin at a concentration of 3000 mg/L (Al-Khuzai *et al.*,2019).

#### Toxicity test

This test is used to ensure that the nanomaterial is free of mycotoxins, especially the aflatoxins produced by some fungi belonging to the genus Aspergillus, A coconut agar medium was inoculated with the fungus *A.niger* then incubated at 27 °C for one week ,when the colonies appeared, a gauze soaked with ammonia was placed under the cover of a Petri dish and the dishes were closed and incubated upside down for a week, Any change in the color of the medium to red, indicates the presence of aflatoxin (Saito & Machida,1999).

## Results and Discussion

The synthesis of gold nanoparticles was validated by visually monitoring two flasks containing only the aqueous filtrate prepared from *Aspergillus niger* fermentation, and reaction mixture.The fungal aqueous filtrate and the auric chloride solution were observed to retain their original color, whereas the auric chloride mixed supernatant turned to dark purple (ruby red) after 24 h of incubation (Fig. 1).A visible color shift in the reaction mixture indicates the creation of colloidal gold. Colloidal gold solutions are well recognized for their bright hues attributed to surface plasmon absorption via gold nanoparticles. The solution color would indicate the approximate size of the obtained particles. Color changes from red to purple, violet, and finally blue as particle size increases from 1 to 200 nm. generally, hues of pink, purple, violet, blue, red, and gold have been seen in the reaction systems during

production of gold nanoparticles, either the liquid phase or in the biomass.The emergence of such characteristic colors linked exclusively to biomass due to intracellular or cell-bound gold ions reduction.If a significant change on color was observed in the cell free filtrate / supernatant, it indicated that gold ions were reduced extracellularly (Panda & Deepa,2011).

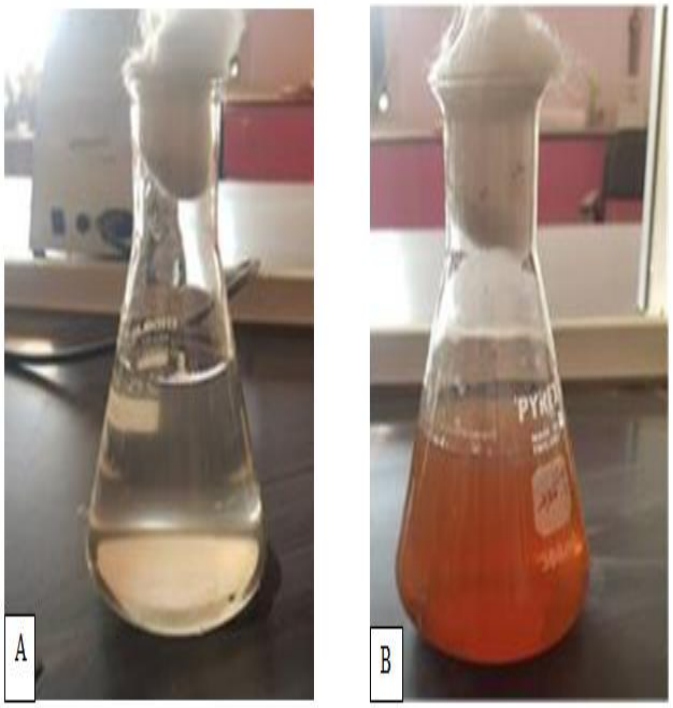


**Fig. 1. Fig (1) Visual observation of the gold nanoparticle formations: (A) aqueous filtrate control, (B) reaction mixture**

**Ultraviolet Visible Spectroscopy (UV-Vis)**

Gold nanoparticles exhibit a distinct optical feature commonly referred to as localized surface plasmon resonance (LSPR), that is, the collective oscillation of electrons in the conduction band of gold nanoparticles resonating with the specific wavelength of the incident light. The LSPR of gold nanoparticles results in a strong absorption band in the visible region (500 nm - 600 nm), which can be measured by UV-visible spectroscopy (Fig. 2) **.** The LSPR spectrum can be influenced by both the shape & size of the gold nanoparticles; the highest absorption wavelength elevates with particle diameter, and when compared to a spherical particle of the same diameter, the absorption spectrum of irregularly shaped particles, such as the gold nanoparticles, dramatically changes to the far red region of the spectrum (Pantidos & Horsfall, 2014). At 540 nm, a potent surface plasmon resonance was centered.This is one of the characteristics of colloidal gold (Mukherjee *et al.*,2001).According to the spectra, the strength of the gold nanosuspension's absorbance increased with time, showing that as the reaction progressed, more gold nanoparticles were being formed in the solutionThe gold nanoparticle peak stayed about 540 nm during a period of 96 hours, showing that the particles are thoroughly distributed in solution.Additionally Aggregation also was noted .

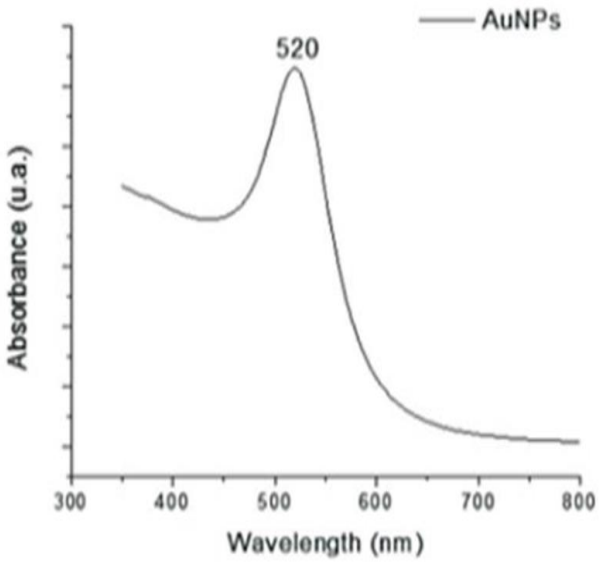


**Fig. 2. Fig (2) Ultraviolet Visible Spectroscopy (UV-Vis)**

**XRD**

The shape, size, stability (high and low), visible assembly and precipitation of nanoparticles synthesized from the fungal isolate were determined by XRD analysis. The XRD pattern of the gold nanoparticles in Fig. 2 shows intense peaks in the full spectrum of 2θ values, ranging from 20° to 80°. Strong peaks at 2θ values of 38.21°, 44.44°, 64.61°, and 77.63° correspond to (1 1 1), (2 0 0), (2 2 0), and (3 1 1) levels for gold nanoparticles. Thus, the XRD pattern clearly demonstrates that the biosynthesized gold nanoparticles are crystalline in nature (Fig. 3). XRD can be utilized to determine the chemical composition, crystalline structure, and average size of the resulting nanoparticles. based on the idea that each crystalline substance has a unique diffraction pattern that characterizes it (Mandal *et al.*,2006).The broadening of the diffracted lines, which is seen as peaks of different intensities in the XRD pattern, is caused by particle size effects (Pradeep,2007).

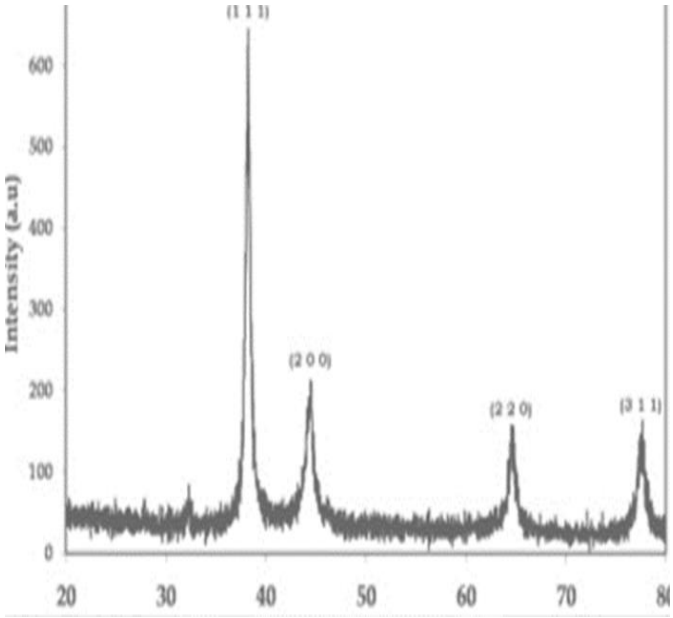


**Fig. 3. X-ray diffraction spectrum of gold nanoparticles confirms their formation in the form of crystals**

**FTIR Infrared Spectrophotometer**

The FTIR test shows packages indicating that the resulting nanoparticles contain different chemical groups, including C-O-C, C-O, C=C, and C-HO-H, which gives important information about the nature of these chemical particles and where it was confirmed that these particles formed and their composition differed from the material from which they were made (Fig. 4) . For FTIR analysis, it has been found that the presence of Au NPs causes some bands' intensities to increase and some bands' original positions to shift toward longer or shorter wavelengths. The presence of interaction between phytochemicals and Au NPs is indicated by a shifting of the band and the increasing in band intensity (Maleki *et al.*,2019).

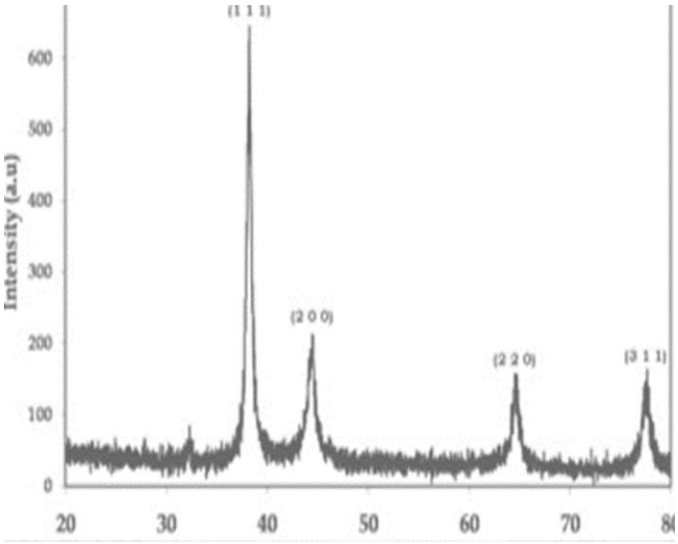


**Fig. 4. Infrared spectrum of FTIR gold nanoparticles**

**SEM (scanning electron microscope)**

The figure taken by the scanning electron microscope show that the gold nanoparticles that were deposited on the slides have shapes ranging from indefinite to crystalline and spherical, and their diameters range from a few nanometers to about 200 nanometers (Fig. 5).Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are instruments that are used to observe the nanoparticles' shape, size, and arrangement.There are many brief overviews of the organisms known to synthesis nanoparticles, features of the resulting nanoparticles like shape,size, and localization, and conditions under in which they were produced are supplied (Thakkar *et al.*,2010).The location of the nanostructures can be determined by using electron micrographs of the reaction mixture containing nanoparticles and the biomass (Wen *et al.*,2009). Cryo TEM imaging of stained specimens or TEM/SEM pictures of microtomed specimens can be used to detect the presence of nanoparticles inside the cells (Nangia *et al.*,2009).Selected-area electron diffraction (SAED) & Energy dispersive X-ray analysis (EDX) analysis, that provide details about the sample's crystal structure & elemental composition, can also be done during TEM/SEM examination respectively.In some situations, the presence of gold sulfide nanoparticles results in the sulfur peak (Lengke *et al.*,2006).If the observed SAED pattern matches that of face-centered cubic gold, the crystallinity of the gold nanoparticles can be determined (Feng *et al.*,2008).

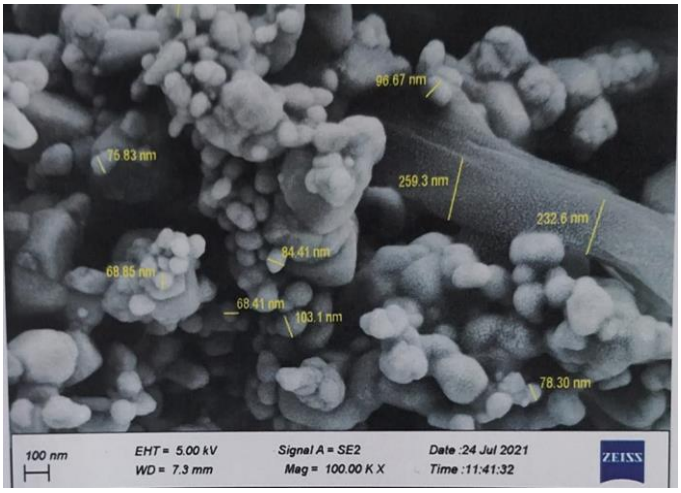


**Fig. 5. scanning electron microscope figure of gold nanoparticles**

**The effect of gold nanoparticles on pathogenic bacteria**

**Food Poisonings method**

It was observed that there were different effects of gold nanoparticles prepared by the biological method on different bacterial isolates.The greatest effect was on *E.coli*, the inhibition was directly increased with the concentrations used and with a growth diameter of (1.1, 2.7, 4.2,6) cm for the concentrations (0.5, 10, 15)%, respectively, followed by *S.aureus* with a colony diameter of (1.6, 2.3, 5.1,6.6) cm for concentrations (0.5, 10, 15)%, respectively. while *P.aeruginosa*, it gave a diameter of (2.3, 3.1, 4.5,7) cm for concentrations (0.5, 10, 15)%, respectively (Table 1) . Antibacterial effects, the reason for this may be due to the high ability of P. aeruginosa resistance to various inhibitory substances, as it is known for its multi-drug resistance (MDR) and its high tolerance to difficult conditions through resistance genes carried on plasmids.The concentrations of 10 and 15% were significantly superior to the antibiotics used, which opens new horizons in the resistance of bacterial diseases, as nanoparticles have effects on the plasma membrane of the bacterial cell, as they affect the osmosis process by making pores in the membrane and it becomes fully permeable instead of optional Permeability, which causes salts and nutrients to leave the cell and lose their essential contents, and the cell may swell and burst and die (Paidari & Ibrahim,2021).

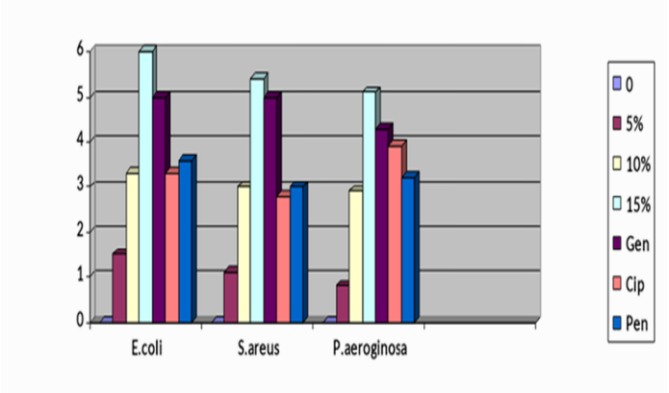


**Fig. 6. The effect of gold nanoparticles on pathogenic bacteria using the well method**

**Table 1. The effect of gold nanoparticles on the diameters of colonies of pathogenic bacteria**

| Pathogenic bacteria | Gold nano | | | | Antibiotics | | |
|---|---|---|---|---|---|---|---|
| | 0% | 5% | 10% | 15% | Gen | Cip | Pen |
| *E.coli* | 6 | 4.2 | 2.7 | 1.1 | 3.5 | 4.1 | 7 |
| *S.areus* | 6.6 | 5.1 | 2.3 | 1.6 | 2.8 | 4.6 | 5.7 |
| *P.aeruginosa* | 7 | 4.5 | 3.1 | 2.3 | 1.4 | 3.4 | 6.8 |

### Well method

It is obvious from Figure (6) that the concentration exceeded 15% on all treatments, as it caused a region of inhibition zone around the pits greater than all other treatments due to its effect on the cell membrane and making holes in it, which allows the exit of cell components to the outside, as well as by interfering with the construction of proteins necessary for cell life as it inhibits its structure as well as by distorting DNA and causing mutations and cellular abnormalities, the antibacterial gentamicin came second as it belongs to the group of aminoglycosides extracted from *Micromonospora purpurea* bacteria.Gentamicin is one of the most powerful antibiotics that treat infections caused by aerobic negative bacteria, and it can be combined with other antibiotics to treat positive bacteria such as staphylococcus. Gentamicin works to kill bacteria by inhibiting formation of bacterial protein, as it binds with the 30s ribosome and thus the bacteria are unable to complete the formation of mRNA, and thus the bacteria are unable to complete their growth and die (Viveiros *et al.*,2010).

### Toxicity test

The result of this test showed that the fungal isolate under test lack ability of the production of aflatoxins, are one of the most dangerous toxins produce by some types of Aspergillus species. The medium used is characterized by facilitating the production of toxin because it provides the necessary nutrients and because it gives abundant filamentous growth and without any organs or reproductive units that deplete energy and food and for this reason the color of growth appears white in the (Fig 7).

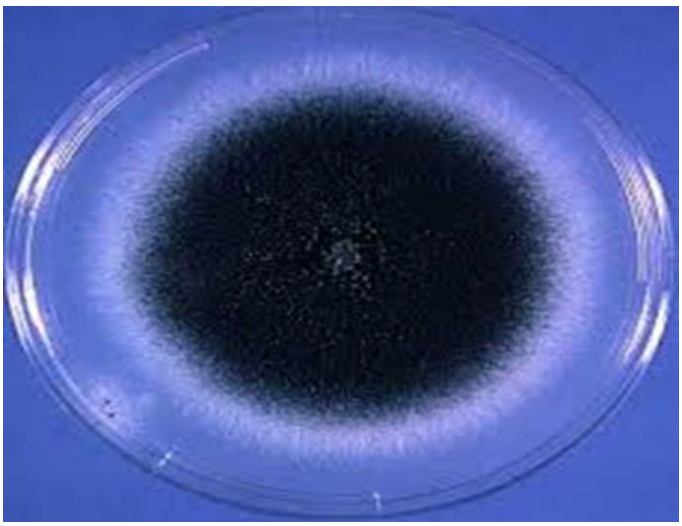

**Fig. 7. The negative result of the production of mycotoxins**

## Conclusion

*A.niger* is very effective in the synthesis of gold nanoparticles in inexpensive media **.**Gold nanoparticles inhibit the growth of pathogenic bacteria under test with results comparable to antibiotics with side effects**.** The produced nanomaterial is non-toxic and has limited side effects.

## Acknowledgements

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### Conflict of interests

The authors declare that there is no competing interest.

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