



The Biological Activity of Mycosynthesized Silver Nanoparticles Against some Pathogenic Bacteria

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Abstract

This study was carried out for the mycosynthesis of silver nanoparticles by *Candida albicans* supernatant. All the isolates used in this study were taken from the patients who existed at Al-Elweya children's teaching hospital in Baghdad, Iraq. Mycosynthesized silver nanoparticles were characterized by color visualization, ultraviolet-visible (UV) spectroscopy, and Fourier transform infrared spectroscopy (FTIR). The UV-Vis spectroscopy examination has shown the highest absorbance (λ_{max}) at the wavelength of 429 nanometers, which indicated the creation of silver nanoparticles. Furthermore, the results of the antibacterial potential of AgNO₃ and AgNPs against *Klebsiella pneumoniae* and *Staphylococcus haemolyticus* bacteria showed the highest effect of AgNO₃ against *Staphylococcus haemolyticus* when the diameter of the inhibition zone reached (14.00 mm). In contrast, the lowest effect of the AgNO₃ was with the diameter of the inhibition zone that reached (11.66 mm). The highest effect of the AgNO₃ against *Klebsiella pneumoniae* by the diameter of the inhibition zone was reached (12.66mm), while the lowest effect was (9.00mm). The highest effect of the AgNPs against *Klebsiella pneumoniae* by the diameter of the inhibition zone reached (16.00mm), while the lowest effect was (13.00mm). The highest effect of the AgNPs against *Staphylococcus haemolyticus* by the diameter of the inhibition zone was reached (17.33mm). Where the lowest effect by the diameter of the inhibition zone was reached (14.00mm). Interestingly, this revealed that *Staphylococcus haemolyticus* was more susceptible to silver nitrate (AgNO₃) and silver nanoparticles (AgNPs) than *Klebsiella pneumoniae*.

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1. Introduction

The curing of infectious diseases sticks on an essential and challenging medical problem due to many factors, like new infections emerging, an increasing population at risk, and a growing number of multi-drug resistant pathogens, thus, there is an urgent need to search and explore therapeutic options through new antimicrobial agents, probably those acting by mechanisms various from current therapeutics. Investigations search for substances possessing direct antimicrobial activity and/or combine (synergy) with classic pharmacological

agents. In addition, recent directions have also looked for products that can restrict the expression of attributes in microbial virulence or activate host immune defence mechanisms [1, 2].

Nanotechnology is a relatively recent branch of science concerned with the synthesis and application of nanoparticles (NPs) with sizes ranging from 1 to 100 nanometers. Many reasons behind this interest such as the unique physicochemical features, including antibacterial properties, catalytic activity, optical properties, electronic properties, and magnetic properties, NPs have been widely researched [3].

Among the different kinds of metallic nanoparticles, silver nanoparticles can be highlighted for their broad-spectrum antimicrobial potency. These nanoparticles adhere to the cell walls and membranes of microorganisms to reach the interior of the cell. They broke the cellular structures, induce reactive oxygen species (ROS) production, and changed signal transduction mechanisms. Several studies reported applications in which good results have been obtained using silver nanoparticles (AgNPs) for the control of pathogenic microorganisms in the fields of health and agriculture [4-6]. Various metals such as Ag, Au, Zn, and Cu have been subjected to nanoformulation for many beneficial applications by adopting different physical, chemical, and biological methods. Biosynthesis of nanoparticles (NPs) can be performed using organisms like bacteria, fungi, and plants or their products, which act as reducing and stabilizing agents. Biosynthesis is relatively simple, clean, sustainable, and economical, and provides excellent biocompatibility in nanoparticles [7, 8]. The fungi, primarily yeast, represent a suitable option for large-scale green nano production. The mechanism of nanoparticle production using fungi is different because fungi excrete large amounts of enzymes used to reduce silver ions to metal nanoparticles [9]. The mechanism of biosynthesis of NPs by using fungi may be intracellular or extracellular. Several studies demonstrated the biosynthesis of extracellular silver nanoparticles utilizing many fungal species. The second method is most widely used because the releasing of NPs from the cells is simpler and easier than the intracellular mechanism. Also, extracellular biosynthesis not required complex procedures for separation and purification steps.

Nonetheless, the NPs dispersion must be purified to eliminate fungal residues and impurities, which can be achieved using simple filtration, membrane filtration, gel filtration, dialysis, and ultracentrifugation [10, 11]. There are many studies on the use of filamentous fungi to manufacture silver nanoparticles, but the use of yeasts, especially *C. albicans*, is limited. Although *Candida* spp. live as normal flora in the human body, in people with a low immune system. They can work as pathogenic microbes and cause disease (candidiasis). The most common *Candida* spp. isolated from patients with candidiasis is *C. albicans*. So the importance of this work lies in the possibility of synthesizing silver nanoparticles using this yeast because of its pathogenicity, and most nanoparticles preparation methods utilize molds. Most studies aim to use nanoparticles as an antifungal for *Candida* spp. because of their high pathogenicity. The main object of this study is to use *C. albicans* for extracellular mycosynthesis of silver nanoparticles (AgNPs) and their bacterial growth inhibitor activity against *K. pneumoniae* and *S. haemolyticus*.

2. Experimental Work

2.1. Chemicals and Reagents

Chemicals and reagents were obtained from private scientific offices in Baghdad, Iraq. Sabouraud dextrose agar medium (Liogilchem-Italy), Potato dextrose broth medium (Condalab-Spain), Blood agar medium (Difco-USA), MacConkey agar medium (Difco-USA), Mannitol salt agar medium (Biolife-Italy), Mueller-Hinton agar medium (Mast-England), Normal saline (PiONEER-Iraq), and AgNO₃ 99.5% (AFCO-China).

2.2. Collection and Identification of Microbial Isolates

Fifty isolates of *C. albicans*, and 15-20 isolates of each of *S. haemolyticus* and *K. pneumoniae* were collected from the patients that existed at Al-Elweya children's teaching hospital in Iraq. The specimens were cultured on specific selective media. After that, the samples were diagnosed morphologically, microscopically, and then diagnosed with the VITEK 2 system. After that the suitable strain of *C. albicans* was employed to the biosynthesis of silver nanoparticles.

2.3. Preparation of the Cultures Media

All the cultures medium used in the study (Sabouraud dextrose agar, Potato dextrose broth, Blood agar, MacConkey agar, Mannitol salt agar, and Mueller-Hinton agar) were prepared according to the preparation methods that mentioned on a bottle of culture medium by the manufacturing company. First, they were sterilized in an autoclave under 121°C and 15 pounds per square inch for 15 minutes. After that, the culture mediums were cast in Petri dishes under sterile conditions inside the biosafety cabinet and saved at 4°C till they were being used [3].

2.4. Preparation of *C. Albicans* Supernatant

The fresh characterized colonies of *C. albicans* were cultured in 50 ml of Potato Dextrose Broth (PDB) medium placed in a falcon tube. 250 ml can ensure aerated circumference for *C. albicans* growth through a clean and sterilized loop inside the hood. Then the culture is incubated for 48 hours in a shaker incubator at 37°C and 150 rpm. After the incubation period, 4000 rpm centrifuge centrifuged the growth medium for 30 minutes, the sediment was removed, and the supernatant was filtrate by a syringe filter with a diameter of 0.22 µm, the supernatant was stored at 4°C until it was used, this preparation method as previously described with some modification [12].

2.5. Preparation of Silver Nitrate(AgNO₃) Solution

In order to prepare 100 ml of 2mM of silver nitrate (AgNO₃) solution, 0.0339 grams of (AgNO₃) was dissolved in 100 ml of deionized water (Di), after preparing the solution was kept in an opaque condition, away from light, to prevent oxidation, and it was used later [13].

2.6. Extracellular Mycosynthesis of Silver Nanoparticles(AgNPs) by the Supernatant of *C. Albicans*

10ml of *C. albicans* supernatant was mixed with 90ml of 2mM silver nitrate solution in a falcon tube and incubated in a shaking incubator at 37°C and 150 rpm for 24 hours in dark conditions. After 24 hours, the color alteration of the medium should be noticed to detect the formation of AgNPs, which is the first indication of the synthesis of the silver nanoparticles, this mycosynthesis method, as previously described with some modification [14].

2.7. Purification of Silver Nanoparticles (AgNPs)

The reaction mixture that contained silver nanoparticles was put in a centrifuge at a speed of 4000 rpm for 30 minutes three times with Milli-Q water to reject the medium residues and other impurities. This procedure was recurred at least three times to assure a better separation of AgNPs, this purification method, as previously described with some modification [12].

2.8. The Characterization of Silver Nanoparticles (AgNPs)

After 24 hours of incubation at a shaking incubator at 37°C and 150 rpm, the color change in the reaction mixture was monitored visually to detect the formation of AgNPs. Next Silver nanoparticles were characterized using a UV-Vis spectrophotometer (UV – 1650 PC SHIMADZU, Chrom Tech, USA) in the Department of Applied Sciences at the University of Technology in Iraq. Before the UV-Vis spectrophotometer examination, the silver nanoparticles (AgNPs) were sonicated for twenty minutes, the existence of the greatest absorbance (λ_{max}) and the production of AgNPs were determined using UV-Vis spectra of silver nanoparticles in the region of 200 to 800 nanometers, and deionized water used as a baseline. Finally, the mycosynthesized AgNPs were examined by FTIR spectrophotometer (Fourier Transform Infra-Red spectrophotometer FT-IR, SHIMADZU, UK) in the Department of Applied Sciences at the University of Technology in Iraq within the range of (400 - 4000) cm⁻¹ to recognize the probable interactions between silver and the molecules, proteins and functional groups that involved in the reduction of silver ions into silver nanoparticles as capping agent [15].

2.9. The Antibacterial Activity of Silver Nitrate (AgNO₃) and Silver Nanoparticles (AgNPs)

The test was done by the well diffusion method, carried out using the Mueller-Hinton agar medium plates. The suspension of each *K. pneumoniae* bacteria and *S. haemolyticus* bacteria was prepared separately by transferring a colony its lifetime is 18-24 hours of each *K. pneumoniae* bacteria and *S. haemolyticus* bacteria to plane tubes containing 3 mL of normal saline and shaking well through a clean and sterile loop. First the optical density of the bacterial suspension was compared with the McFarland standard to obtain a density of 0.5. After that 0.1 ml

of the suspension of the bacteria was transferred and spread over the surface of the Mueller-Hinton agar culture medium evenly using clean and sterile cotton swabs and allow the dishes to dry, the plates were drilled through a clean and sterile well cutter at a rate of 5 wells and then 100 μ l of varying dilutes of 2mM AgNO₃ solution. Silver nanoparticles (AgNPs) (100%, 50%, 25%, and 12.5%) were aseptically filled in the wells, the negative control well (C) was filled with deionized water. Then the plates were incubated in the incubator at 37 °C for 24 hours. After 24 hours, the inhibition zone diameter around the well was measured in (mm). This test was done as previously described with some modification [16].

2.10. Statistical Analysis

The data were analyzed according to (Analysis of Variance) ANOVA in two directions, using the prepared statistical program (SPSS.24).

3. Results and Discussion

3.1. The Extracellular Mycosynthesis of Silver Nanoparticles

After 24 hours of incubation in a shaking incubator within dark conditions at 37°C and 150 rpm, the color change in the reaction mixture from pale yellow to brownish color as shown in (Figure 1). Also, the color alteration was the first indicator of the formation of AgNPs because of the reduction of Ag ions and Plasmon resonance.

These findings are consistent with those of a prior studies conducted by Magdi *et al.* [17], in another previous study by Bahat *et al.* [12], it has been reported that the reduction of metal ions into silver nanoparticles can be when the color of the reaction solution changes into brownish color after 24 hours of incubation, due to the reduction of metal ions.

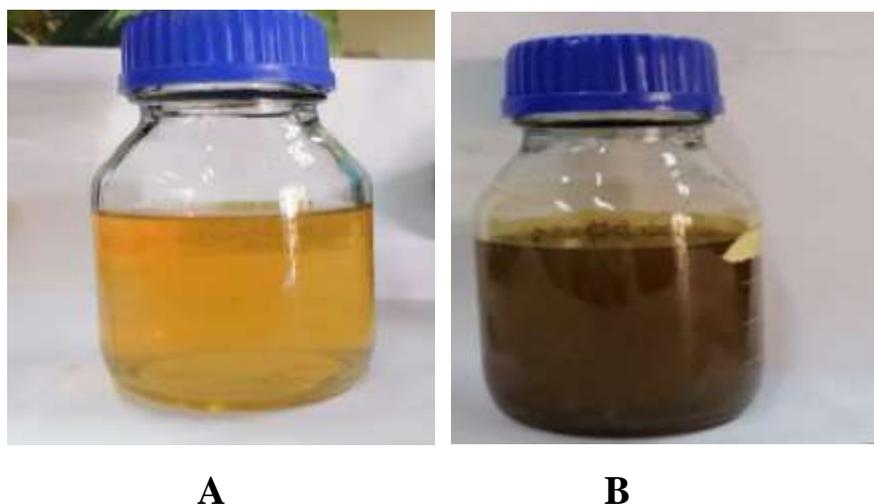


Figure 1: Extracellular mycosynthesis of AgNPs using the supernatant of *C. albicans*. (A) refers to supernatant of *C. albicans* and AgNO₃ solution before mycosynthesis, (B) refers to the mycosynthesis of AgNPs by *C. albicans* after 24 h at 37°C in dark conditions.

3.1. The Characterization of Silver Nanoparticles (AgNPs)

3.1.1. UV-Vis Spectroscopy

The formed AgNPs by *C. albicans* were further specified by the UV-visible spectroscopy, the appearance of the highest absorbance (λ_{max}) at the wavelength of 429 nanometers indicated the creation of silver nanoparticles as shown in (Figure 2). The appearance of a peak at 429 nanometers is because of the phenomenon of the surface Plasmon resonance, which occurs due to the excitation of the surface Plasmon present on the outer surface of the AgNPs which gets excited due to the applied electromagnetic field. These results are so close to a previous study done by Bhat *et al.* [12] which reported, the biosynthesized AgNPs by *C. albicans* showed an absorption peak at 430 nanometers.

Also, the results are in line with another previous study, and the researchers reported that the absorption peak of the biosynthesized AgNPs was 420 nm. [12, 18].

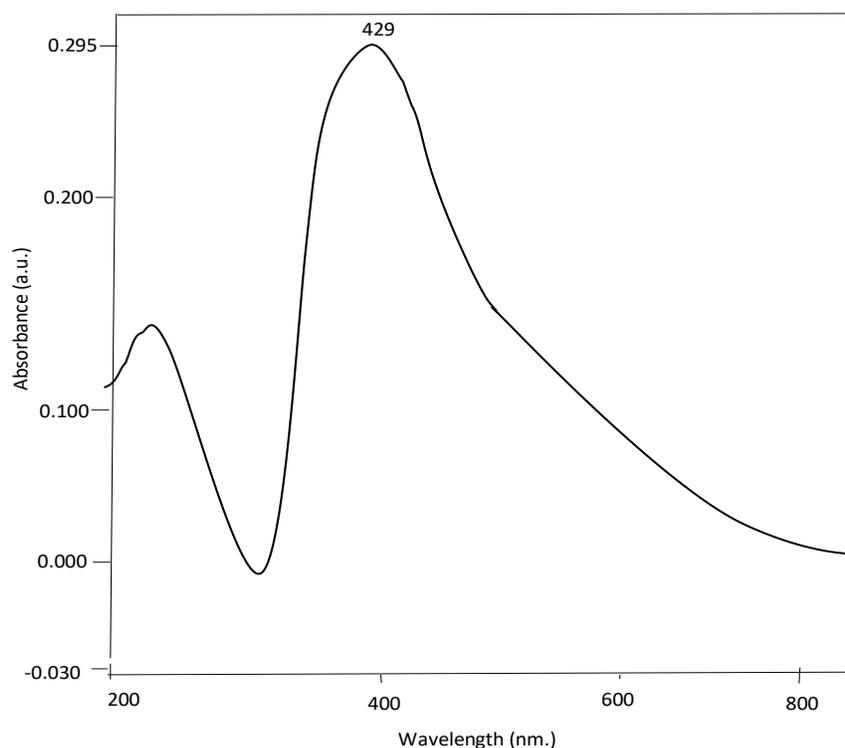


Figure 2: UV-Vis Analysis of Biosynthesized AgNPs.

3.1.2. Fourier Transmission Infrared Spectroscopy (FTIR)

The FTIR test was implemented to recognize the potential biomolecules and functional groups that are responsible for the reduction of Ag ions into AgNPs as a capping agent. The FTIR spectra of synthesized Ag nanoparticles peaks at 3,421; 2,891; 1,647; and 1,363 cm^{-1} as shown in the (Figure 3, Table 1). The formation of bands due to (O–H alcohol) (around 3,421 cm^{-1}), aldehydic C–H stretching (2,891 cm^{-1}), C=O amides (1,647 cm^{-1}), C– N and C– C stretching (1,363 cm^{-1}). The peaks indicate the presence of C=O elongating vibrations in the formation of Ag nanoparticles aggregates, and these results are in agreement with previous studies [12,19].

Table 1. The functional groups present in the FTIR analysis for AgNPs.

Wave number (cm^{-1})	Functional group	Bond type
3.421	Alcohol	O–H
2.891	Aldehyde	C–H
1.647	Amides	C=O
1.363	Amine	C– N and C– C

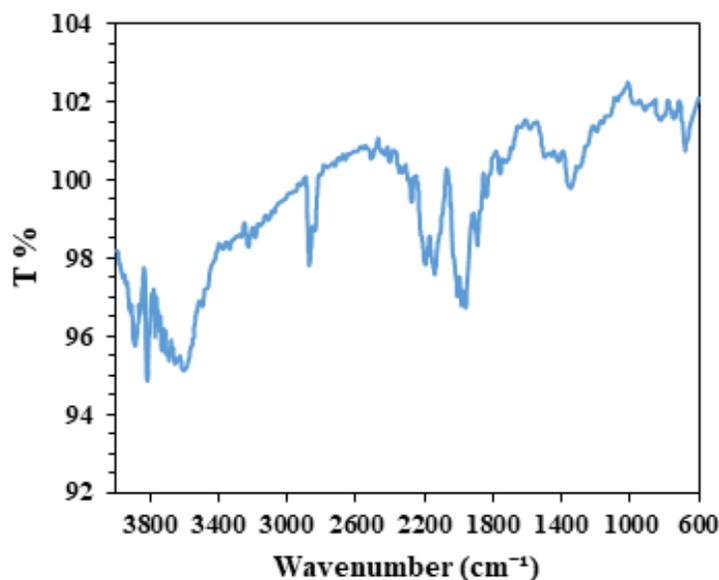


Figure 3: The FTIR analysis of biosynthesized AgNPs.

3.2. Antibacterial Activity of Silver Nitrate and Silver Nanoparticles

3.3.1. Antibacterial Activity of Silver Nitrate (AgNO_3):

Antibacterial activity of 2mM of AgNO_3 against human pathogenic microorganisms, i.e., *K. pneumoniae* bacteria and *S. haemolyticus* bacteria, was evaluated by agar well diffusion methods. An inhibition zones diameters of different dilutions of 2mM AgNO_3 against tested bacteria were measured in (mm) as shown in (Table 2, Figure 4). It was observed that the highest effect of AgNO_3 against *S. haemolyticus* was S (100%) which the diameter of the inhibition zone was 14.00 ± 1.00 mm followed by D3 (50%) which the diameter of the inhibition zone was 13.66 ± 0.58 mm, followed by D2 (25%) which the diameter of the inhibition zone was 12.66 ± 0.58 mm. In comparison, the lowest effect of AgNO_3 was D1 (12.5%) which the diameter of the inhibition zone was 11.66 ± 0.58 mm. The highest effect of AgNO_3 against *K. pneumoniae* was S (100%) which the diameter of the inhibition zone was 12.66 ± 0.58 , followed by D3 (50%) which the diameter of the inhibition zone was 12.66 ± 0.58 mm, followed by D2 (25%) which the diameter of the inhibition zone was 11.66 ± 0.58 mm. While the lowest effect of AgNO_3 was D1 (12.5%) which the diameter of the inhibition zone was 9.00 ± 0.00 mm. The inhibition zone diameter showed that silver nitrate was more efficient against *S. haemolyticus* in comparison to *K. pneumoniae* bacteria as shown in (Figure 4). These results are in line with the finding of a previous study that reported AgNO_3 was more effective against Gram-positive bacteria than Gram-negative bacteria [20]. No antimicrobial activity has been observed by the *C. albicans* supernatant, as shown in (Figure 5). These results are in line with the finding of Hany *et al.* [17], which they reported in their study that the fungal extract did not show any activity against MRSA. Also, Mohammad *et al.* [21] reported that *C. glabrata* supernatant had observed no antimicrobial activity against tested bacteria and *Candida* spp. [17, 20, 21].

Table 2. Antibacterial activity of AgNPs and biosynthesized AgNPs against two types of pathogenic bacteria.

M.O	Inhibition zone in diameter of different concentration			
	Silver nitrate (AgNO_3)			
	D1	D2	D3	S
<i>S. haemolyticus</i>	11.66 ± 0.58	12.66 ± 0.58	13.66 ± 0.58	14.00 ± 1.00
	Silver nanoparticles (AgNPs)			
	14.00 ± 1.00	14.66 ± 1.15	17.00 ± 1.00	17.33 ± 0.58
<i>k. pneumoniae</i>	Silver nitrate (AgNO_3)			
	9.00 ± 0.00	11.66 ± 0.58	12.66 ± 0.58	12.66 ± 0.58
	Silver nanoparticles (AgNPs)			
	13.00 ± 1.00	13.66 ± 0.58	15.33 ± 0.58	16.0 ± 1.00

Note: The symbol (S) refers to 100%, D3 refers to 50%, D2 refers to 25%, D1 refers to 12.5%.

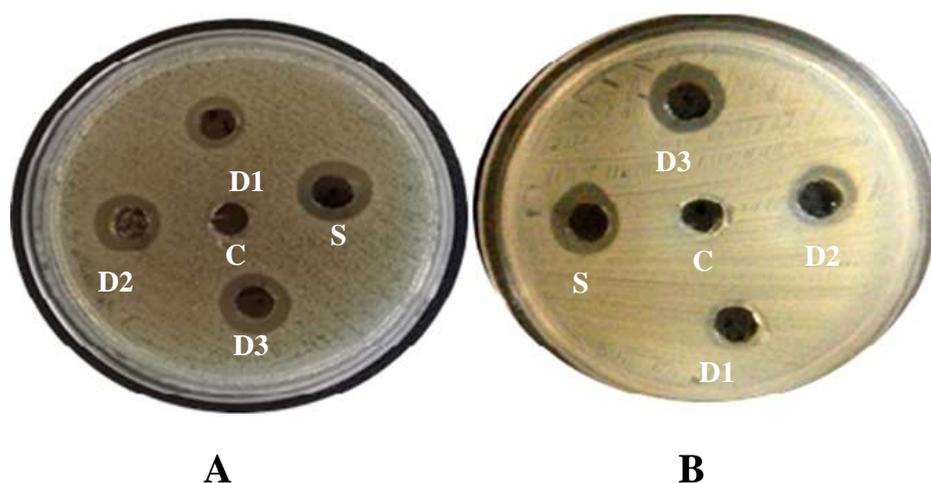


Figure 4: The antibacterial activity of silver nitrate against (A) *S. haemolyticus*, (B) *K. pneumoniae* bacteria.

Note: (S) refers to 100%, (D3) refers to 50%, (D2) refers to 25%, (D1) refers to 12.5%, (C) refers to negative control.

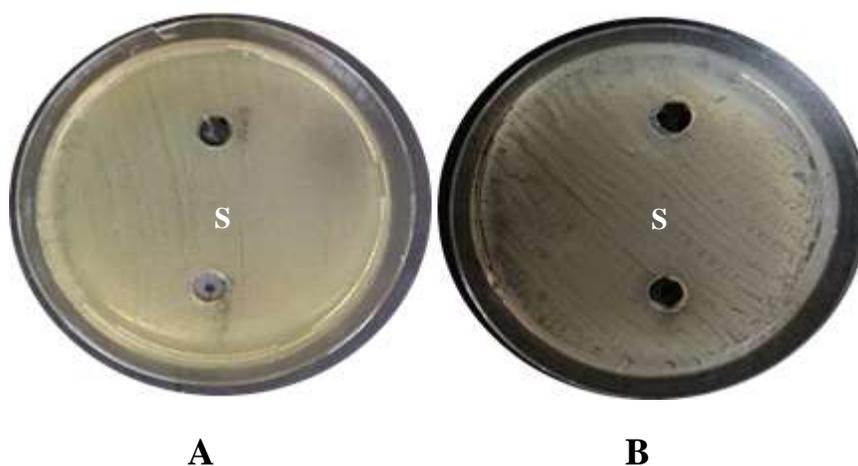


Figure 5: The antibacterial activity of *C. albicans* supernatant against (A) *S. haemolyticus*, (B) *K. pneumoniae* bacteria.

Note: (C) refers to negative control, (S) refers to *C. albicans* supernatant.

3.3.2. Antibacterial Activity of Mycosynthesized Silver Nanoparticles (AgNPs)

The antibacterial activity of silver nanoparticles against two human pathogenic bacteria, i.e., *K. pneumoniae* bacteria, and *S. haemolyticus* bacteria, was evaluated by agar well diffusion methods. An inhibition zone diameter of silver nanoparticles against tested bacteria at different dilutions were measured in (mm) as shown in (Table 2, Figure 6). The highest effect of the AgNPs against *K. pneumoniae* was S (100%) which the diameter of the inhibition zone was 16.00 ± 1.00 mm followed by D3 (50%) which the diameter of the inhibition zone was 15.33 ± 0.58 mm, followed by D2 (25%) which the diameter of the inhibition zone was 13.66 ± 0.58 mm. On the other hand, the lowest effect of the AgNPs was D1 (12.5%) which the diameter of the inhibition zone was 13.00 ± 1.00 mm. Furthermore, the highest effect of the AgNPs against *S. haemolyticus* was the S (100%) which the diameter of the inhibition zone was 17.33 ± 0.58 mm, followed by D3 (50%) which the diameter of the inhibition zone was 17.00 ± 1.00 mm, followed by D2 (25%) which the diameter of the inhibition zone was 14.66 ± 1.15 mm. While, the lowest effect of the AgNPs was D1 (12.5%), which the diameter of the inhibition

zone was 14.00 ± 1.00 mm, as shown in (Figure 6). It was very clear that silver nanoparticles were more efficient against *S. haemolyticus* as compared to *K. pneumoniae* bacteria. These results are in agreement with previous studies that reported AgNPs have more antibacterial activity against Gram-positive bacteria than Gram-negative bacteria [20, 22]. Other research has found that AgNPs have greater antibacterial activity against Gram-negative bacteria than Gram-positive bacteria [20, 22-24]. These differences in AgNPs effectiveness within inhibiting the growth of the two types of bacteria were because of the variations in the cell wall structure of each of *S. haemolyticus* and *K. pneumoniae* bacteria. The cell wall of Gram-positive bacteria contains a thick peptidoglycan layer, which consists of linear polysaccharide chains bound by short peptides, thus forming a more rigid structure leading to difficult penetration of the AgNPs compared to the Gram-negative bacteria where the cell wall possesses a thinner peptidoglycan layer [24, 25]. From the AgNO₃ and AgNPs antibacterial activity results, it is clear that AgNPs were more effective as growth inhibitors for *S. haemolyticus* bacteria and *K. pneumoniae* bacteria than AgNO₃. This is in agreement with previous studies [26-28].

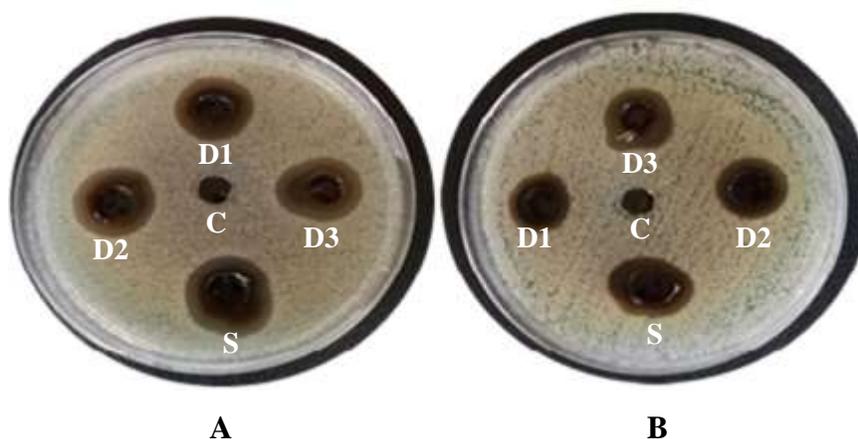


Figure 6: The antibacterial activity of AgNPs against (A) *S. haemolyticus*, (B) *K. pneumoniae* bacteria.

Note: (S) refers to 100%, (D3) refers to 50%, (D2) refers to 25% of AgNPs, (D1) refers to 12.5%, (C) refers to negative control.

4. Conclusions

This study aimed to use the pathogenic *C. albicans* for extracellular mycosynthesis of AgNPs by a simple, cheap, and eco-friendly method. The supernatant of *C. albicans* contains reducing agents that reduces AgNO₃. UV-visible and FTIR spectra were used to characterize the mycosynthesized AgNPs. Extracellular mycosynthesis of AgNPs by yeast, i.e., *Candida albicans*, offers advantages over mycelium fungi in that particle separation is more straightforward, and it takes considerably less time with little complexity compared with mold fungi that require a long time for growing and specific growth conditions. The study finds that AgNPs, and AgNO₃ showed more effectiveness against *S. haemolyticus* in comparison to *K. pneumoniae* bacteria taking the other studies in to account which reported AgNPs and AgNO₃ had more effectiveness against Gram-negative bacteria than Gram-positive bacteria. These variations in bacterial static of AgNO₃ and AgNPs are due to the differences in the cell wall structure of *S. haemolyticus* (Gram-positive) and *K. pneumoniae* (Gram-negative) bacteria. The supernatant of *C. albicans* showed no antibacterial activity against *S. haemolyticus* and *K. pneumoniae* bacteria. So, these mycosynthesized silver nanoparticles in the future could work as an alternative to antibiotics and may lead to the expansion and evolution of adequate pharmaceuticals and represent an alternative remedy for the treatment of bacterial infections.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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