Synergistic Effects of Biosynthesized Nanoparticles Combined with Antibiotics against *Pseudomonas aeruginosa and Proteus mirabilis*

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Abstract

Objective: This project designed to determined the effect of edible mushroom-Ag-Nps in combined with conventional antibiotics against selected multidrug biofilm forming pathogens.

Methods: biofilm producer bacteria were isolated and identified using routine cultural and biochemical tests from clinical specimen, in addition to that bacterial standard strains were used. Silver nanoparticle was prepared by using edible mashroom as bio-reductant. The biosynthesis of Ag-Nps was characterized by changing mushroom extract color from clear yellow to brownand uv/visual spectrophotometer , electronic microscope .then ,the Ag-Nps was tested against bacterial strains and biofilm producer bacteria using diffusion method as antibacterial agents in combination with antibiotics.

Results: Biosynthesized Ag-Nps characterized by visual spectroscopy, electronic microscope (SEM) were ranged (5-50 nm) in size and Fourier Transform Infrared Spectroscopy (FTIR) in peak 430 cm-1 refers to proteins formation .Different volumes and concentration of AgNps (20, 30, and 50 µl)were tested against selected multi drug biofilm pathogens showed the Ag-NPs at concentration 50 µl was the most efficient in the inhibition of bacterial growth . On the other hand, Those assessment of the consolidated impact were examined utilizing circle dissemination strategy against Methicillin Resists *Staph aureus*(*MRSA*),*Eschershia coli, Pseudomonas aeruginosa and Protues mirabilis.* Results recorded a synergetic effect of Ag-Nps in association with resistant antibiotics.

Conclusion: greater potential as antimicrobial compounds against pathogenic micro-organisms, and that they Combination of nanoparticles with antibiotics inhibited effectively ability to form biofilm than antibiotics alone, from this we can conclude that Ag-NPs have may be used in the remediation of infectious diseases ,so Ag-NPs with antibiotics show maximum antibacterial activity, and so his may lead to developed of new pharmaceuticals therapeutic needs**.**

KEYWORDS; Ag-NPs, MDR, *Mushroom (Agaricus bisporus)* , Antibiotics.

Introduction

Nanotechnology may be a standout amongst the majority quickly developing ranges for science (1). Nanotechnology may be the capacity will worth of effort at those atomic, atomic degree around 1–100 nm clinched alongside span (2). Silver nanoparticles (Ag-NPs) assume a paramount part in the field about science and medication (3). Ag-NPs could be connected securely to help when those successful focuses against different sorts for organic entities bring been decided. Recently, we showed that Ag-Nps show antibacterial exercises, including death cells demise (4).

Mushrooms have been part of the normal human diet for thousands of years and in recent times, the amounts consumed have risen greatly, involving a large number of species. Mushroom was infections (5). Mushrooms have been part of the normal human diet for thousands of years and in recent times, the amounts consumed have risen greatly, involving a large number of species. Mushroom was infections(6).

Methodology

Bacterial isolates of *Methicillin Resistance Staph aureus* ATCC 43300, *Ecoli* ATCC **28739** , *Pseu* . ATCC 27853, and *Proteus mirabilis* ATCC 16404 were obtained from the central health laboratory Baghdad City (Iraq).While the Clinical Isolates of Multi drug resistant bacteria were attended the general teaching hospitals in AL-Diwaniyah city. These isolates mainly isolated from various clinical samples including medical devices, urinary catheter tips, urine sample, blood and pus.

Preparation of Crude Extract of Edible Mushroom (*Agaricus bisporus)*

Ag-NPs used were synthesized from edible mushroom fresh mushrooms *Agaricus bisporus* (white button mushrooms) were procured from commercial sources. About 20 gm. of the mushroom was weighted out and washed thoroughly with double distilled water. Then crushed and transferred to a beaker containing 100ml of sterile distilled water. This mixture is stirred for about 2 hours and then filtered using Whatt man No.1 filter paper. Mechanically reduction of Ag+ to Ag^0. The extract of mushroom can be preserved for further experiments by storing it at 40° C (8).

Characterization of Ag-NPs

1. Visual detection and UV-Visible Spectroscopy

Synthesis of Ag-NPs using *Agaricus bisporus* extract was observed by the color change from yellow to dark brown within 24 hours. Further, it has been characterized by UV-Visible Spectroscopy (UV-1600- PC Shimadzu). The process of reaction between $AgNO_3$ and mushroom extract was monitored by UV-Visible spectra with resolution of 2.0 nm, between the wavelength 200 to 700 nm (9, 10).

2. Scanning Electron Microscopy (SEM)

Characterization the shape and size of biosynthesized(Ag-NPs) were done by analyzing with Scanning electron microscope at Al-kuffa university college of science / center of scientific research. The dark brown colored silver nanoparticles were obtained washed and centrifuged for 20 min at 10.000 g (8).

3. Fourier transmission infrared (FTIR) spectroscopy measurements

The residual solution of Ag-NPs by *Agaricus bisporus* extract after reaction was separated at 10000 rpm for 15 min to remove the unwanted impurities and then supernatant is again centrifuged 10 time for 15 min the resulting solution was repeated. Pellets obtained were washed with deionized water to get the pure Ag-NPs. The sample was completely air dried at room temperature; the collected powdered Ag-NPs were taken to F.T.I.R analysis in the range of 250 - 4250 cm-1

Antibacterial Activity of Ag-NPs

Antibacterial activity of Ag-NPs using *Agaricus bisporus* extract were determined by agar well diffusion method(8). volumes of Ag-NPs and several concentration was investigated by agar well diffusion method to determine the better volume and concentration. Ag-NPs were added to agar wells which were loaded with (20µL, 30µL,and 50µL) Ag-NPs suspension .The plates were incubated at 37°C for 24 hours. After incubation, the plates were analyzed for the zones of inhibition. The activity was evaluated by calculating the increase in folded area.

Antibacterial activity and Ag-NPs

Antibacterial activities of antibiotics were determined by disc diffusion method according to CLSI (2016) (12).The Combination between Ag-NPs and antibiotics against bacterial isolates were done by disc diffusion method. Bacterial resistance action penicillin G ,ampicillin ,cefotaxime ,gentamycin and rifampicin.To determine the synergistic effect of Ag-NPs the discs were impregnated with freshly prepared Ag-NPs and then these discs were used for antibacterial activity assays (13). Antibacterial activity was quantified by the equation $(B^2 - A^2)/A^2$, where A and B are the zone of inhibition for antibiotic and antibiotic with Ag-NPs, respectively(14) .

Results

***Visual detection of AgNps**

Ag-NPs were visually detected by changing color of the suspension(figure 1), containing cell free filtrate and silver nitrate .The reduction SNPs to Ag-Nps (Ag^+ to Ag^0) lead to change of color from transparent or light yellow to brown, which indicated the formation of Ag-NPs in the reaction mixture.

Figure (1): Colloid of mushroom and AgNo3 .

UV/ Visible Spectrophotometer

Figure (2) shows the UV-Vis spectrophotometry (1600)has also been used to detect the synthesis of Ag-Nps . The results containing the synthesized Ag-NPs observed in a peak of 430 nm . Ag-NPs taken every 24 hours for 3 days.

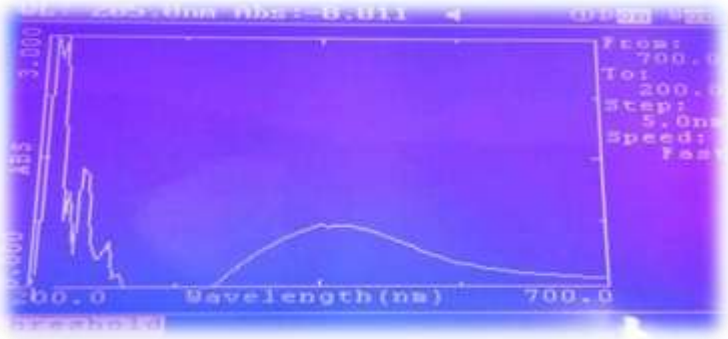


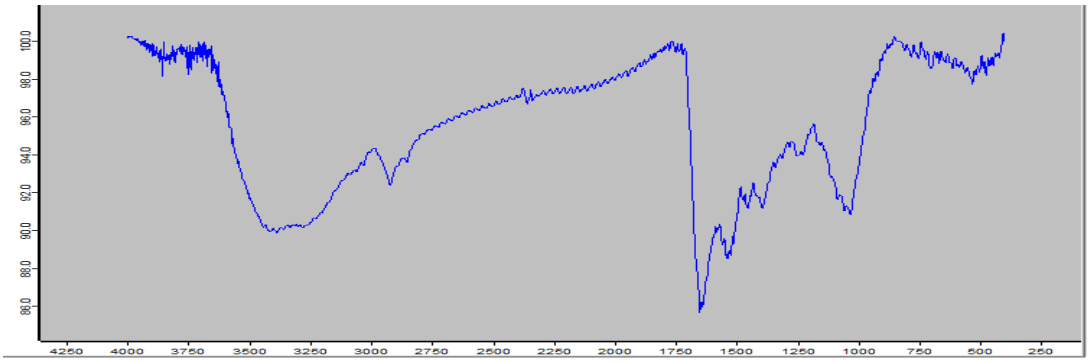
Figure (2):Peak of Ag-NPs **by *Agaricus bisporus* UV/Vis spectroscopy**

Scanning Electron Microscopy (SEM)

Characterization of Ag-NPs were observing SEM. revealed a uniform arrangement of particles having size in the range of 5-35nm and spherical in shape (figure 3).

***Fourier Transmission Infrared Spectroscopy (FTIR)**

The interaction between Ag-NPs and proteins was analyzed by FTIR **.** characterization identified the molecules present in mushroom extracts thought to be responsible for the reduction of silver ions to silver nanoparticles and confirmed the capping agents for the stability of this bio reduced nanometer (figure 4) . FT-IR measurements showed the spectra between 250 to 4250 cm-1 of Ag-NPs which showed the absorption and centered at 2250 – 2800, 1500 – 1700 and 1000 of these 2250-2100 represents C=C Alkyne (stretch).



Figure(4):**FTIR spectra of** Ag-NPs **by *Agaricus bisporus* in Iraq**

Effect of Optimum Volumes and Concentrations of AgNps

The effect of Ag-NPs against standardization bacteria were examined(table1; figure5). it found that the Ag-NPs concentration of 50 µl of mushroom extract was the most effective against the growth against standardization bacteria(table 1), on the other hand the results concluded that the inhibition zone in diameters were increased by using 50 µl Ag-NPs by edible mushroom *Agaricus bisporus* figure (6).

The spectrum by giving a higher inhibition zones against isolates ranges, as the highest inhibition zone obtained in bacterial isolates *E.coli* ≥20 mm and less inhibition zone it was *MRSA* ≥14 mm because of the maximum resistant capacity of the bacterial isolates.

Table(1)Zone of Growth Inhibition (mm) of Standard bacterial tested with Different Concentration of Ag-Nps Against

Tested isolated With AgNps and AgNo3	***Proteus mirabilis* 16404 ATCC**	***Ps. aeruginosa* 27853 ATCC**
AgNO3 (control)	**12**	**12**
Nanoparticles (Ag-NPs) test	**15**	**18**

Figure(5)Inhibition Zone of growth tested isolates.

Determination the Effect of Increasing Fold Area with Antibiotics and Ag-NPs

According to the antibiotic resistant test, Gram negative bacteria isolates showed high resistance to antibiotics than gram positive bacteria. The results showed that all bacterial isolates showed perfect resistant to all antibiotics used. The antibacterial activities of Ampicillin, Cefotaxime, rifampicin, Oxacillin and penicillin G increased in the presence of Ag-NPs edible mushroom *A. bisporus* against bacterial isolates. The diameters of inhibition zones (mm) around different antibiotics with and without Ag-NPs against test strains ,however the combination of

Ag-NPs with antibiotics noteworthy enhanced the antibacterial activities of all the antibiotics as revealed by fold increase in the antibacterial activities of the antibiotics are shown in (Table 1).

The antibacterial activities of antibiotics were observed maximum fold area in combination with Ag-NPs against *E. coli* (5.2) .

Rank 2- Increased fold area in finde of AgNps for *P.aeruginsa*

	No. *P. aeruginosa* isolates																													
	6			9			10			11			16			18			19			20			22			23		
Antibiotic	Ab	Ab - Ag	I.F	Ab	Ab- Ag	I.F	Ab	Ab- Ag	I.F	Ab	Ab - Ag	I.F	Ab	Ab - Ag	I.F	Ab	Ab - Ag	I.F	Ab	Ab - Ag	I.F	Ab	Ab - Ag	I.F	Ab	Ab - Ag	I.F	Ab	Ab - Ag	I.F
Ampicillin	-	14	4.4	-	10	0.22	-	14	4.4	-	10	0.22	-	12	3	-	10	0.22	-	12	3	-	14	4.4	-	14	4.4	-	10	0.22
Cefotaxime	7	13	2.5	-	10	0.22	7	13	2.5	7	13	2.5	7	13	2.5	7	13	2.5	-	10	0.22	7	13	2.5	-	10	0.22	7	13	2.5
Gentamicin	-	10	0.22	7	14	3	-	10	0.22	11	16	1.11	7	14	3	7	14	3	7	14	3	7	14	3	7	14	3	-	10	0.22
Rifampicin	-	10	0.22	-	10	0.22	-	10	0.22	-	10	0.22	-	10	0.22	9	16	2.1	9	16	2.1	9	16	2.1	9	16	2.1	9	16	2.1

Rank 3- Increased fold area in finde of AgNps for *P.mirabilis*

	No. *P.mirabilis* isolates																													
	1			2			5			6			7			13			14			15			17			19		
Antibiotic	Ab	Ab - Ag	I.F	Ab	Ab- Ag	I.F	Ab	Ab- Ag	I.F	Ab	Ab - Ag	I.F	Ab	Ab - Ag	I.F	Ab	Ab - Ag	I.F	Ab	Ab - Ag	I.F	Ab	Ab - Ag	I.F	Ab	Ab - Ag	I.F	Ab	Ab - Ag	I.F
Ampicillin	7	15	3.5	7	15	3.5	-	12	3	-	12	3	-	12	3	-	12	3	10	15	1.2	10	15	1.2	7	15	3.5	10	15	1.2
Cefotaxime	-	10	1.7	-	10	1.7	-	10	1.7	-	10	0.22	-	10	1.7	-	10	1.7	-	10	0.22	-	10	1.7	-	10	0.22	-	10	1.7
Gentamicin	-	10	1.7	8	16	3	-	10	1.7	8	16	3	7	13	2.5	7	13	2.5	8	16	3	8	16	3	8	16	3	-	10	1.7
Rifampicin	-	10	1.7	10	15	1.2	-	10	1.7	10	15	1.2	-	10	1.7	10	15	1.2	10	15	1.2	-	10	0.22	10	15	1.2	-	10	0.22

In the present study bacterial ability to produce biofilm were applied on 73 isolates ,**35 resist to all antibiotics** which used while other 38 isolates gave resistant to **4-5** antibiotics . These isolates selected according to the multi-drug resistance pattern , the number of strong

biofilm producers were 33, moderate 18 and weak or non-biofilm producers 22 by TM . while the results detected by TCP 38 as strong, 23 as moderate and 12 as weak/non biofilm producers

This study examined the effects of Ag-NPs with edible mushroom in combination with several antibiotics. These isolates selected according to MDR, TM and TCP as the most isolates obtained highest OD value, The isolates showed different response to the antibiotics after they were treated. Figure (3) .The highest response to Cefotaxime and Gentamycin were 70% by *E. coli*, while Ampicillin and Rifampicin were 40%,50% respectively. *Ps. aeruginosa* isolates the highest response were 60% for both Ampicillin and. Cefotaxime. Rifampicin, and Gentamycin gave anti biofilm rate of 50% and 40% respectively.

The highest level of inhibition of *P.mirabilis* showed in figure (5) 60% for ampicillin and Cefotaxime while 50% for Gentamycin and Rifampicin .Additionally the result showed that the OD values acquired by Ag-NPs with antibiotics were less than the OD values obtained by antibiotics

The response of *MRSA* for Penicillin G and Ampicillin were 40% for isolates inhibited the biofilm and 60% for each Cefotaxime and Gentamycin while 20% for Rifampicin figure 5

(A) (B)

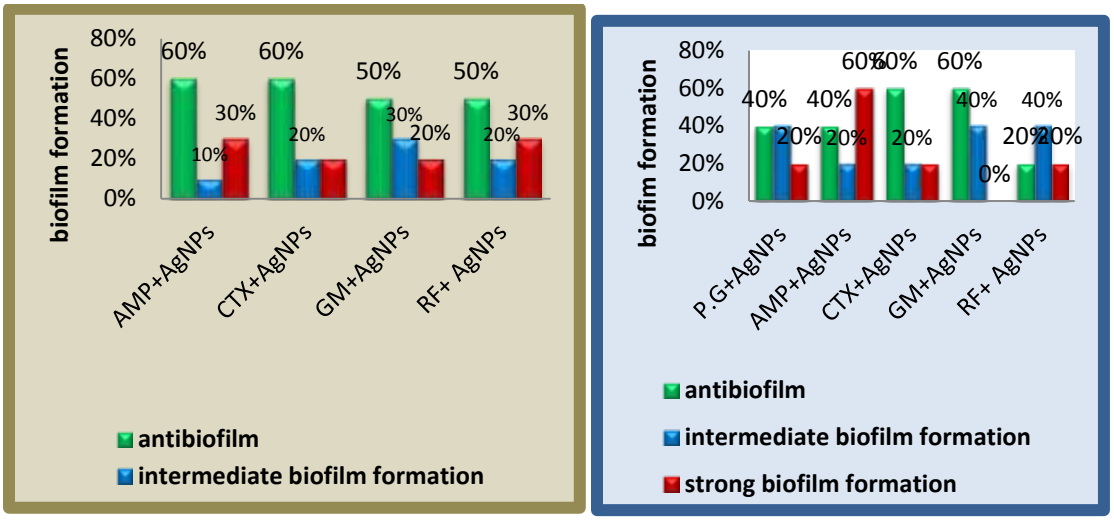


Figure 5. percentage of Inhibition in biofilm formation determined by using the TCP method, showing the effect of addition of nano-Ags in combination with various conventional antibiotics. (a) *P. aeruginosa*, (b) *P.mirabilis*,

Discussion:

Ag-NPs were visually detected by changing color of the suspension(figure 1), containing cell free filtrate and silver nitrate .The reduction of silver ions to Ag-NPs (Ag^+ to Ag^0) lead to change of color from transparent or light yellow to brown, which indicated the formation of Ag-NPs in the reaction mixture .The results containing the synthesized Ag-NPs observed in a peak

of 430 nm . Ag-NPs taken every 24 hours for 3 days. The production of Ag-NPs from *Agaricus bisporus* which agreement with the work of (19). Similar result ranging of 420-430 done by (20).

Characterization of Ag-NPs were observing SEM. revealed a uniform arrangement of particles Whereas Nithya & Ragunathan (20) recorded synthesized silver nanoparticles by *pleurotus sajorcaju* of size range 5-50. (10) Obtained that Ag-NPs by *Ganoderma lucidium* They also reported the polydisperse nature of their nanoparticles, 10 to 70 nm.

NPs using *pleurotus sajor caju(Mushroom)* was tested against the *Ps. aeruginosa* and *p.mirabilis* produced zone of inhibition of 12mm, 14mm; respectively. Nithya & Ragunathan (20) suggested that the Ag-NPs from *Agaricus bisporus* explored medicinally and nutritionally important species of dried mushrooms , the fungi produce many proteins and enzymes involved in synthesis of Ag-NPs and are simpler to grow both in laboratory and industrial level and also the yield is high (24).

Thus, Birla *et al.*(14) were indicated the synergistic activity observed was better in *E. coli* and *Ps. aeruginosa* than *Staph. aureus*. Paná˘cek (25) revealed that Ag-NPs can be combined with antibiotics more effective combination against various pathogenic microbes.

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