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Synergistic Effect of Biosynthesized Silver Nanoparticles Combined with Antibiotics against *Pseudomonas aeruginosa* and *Proteus mirabilis* Isolated from Acute Otitis Externa

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Abstract

Background: the acute external ear infections is an infectious disease recognize to be polymeric in most of the cases, usually associated with superadded by either fungus, bacteria or both of destroyed skin and it is underlying structure.

Objective: To evaluate the synergistic effect of bio prepared silver nanoparticles(Ag-NPs) with antibiotics *for Pseudomonas aeruginosa and Proteus mirabilis***.**

Methods: In this study, we use the Proteus mirabilis ATCC 16404 & the standard strains **of** *Pseudomonas aeruginosa* ATCC 27853 to test the inhibitory effect of Ag-NPs alone or by using diffusion technique with antibiotics. The biosynthesis of Ag-NPs was done by using the basidiomycete, mushroom (Agaricus *Bosporus).* The synthesized Ag-NPs was characterized by UV/Vis spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR) and Scanning electron microscopy (SEM). Antibacterial activity was determined by agar well diffusion by zones of growth inhibition and this activity was evaluated by calculating the increase in the folded area of inhibition.

Results: The synthesized Ag-NPs were (4.5-35 nm) as confirmed by SEM. Spectrum detection of analysis showed peaks between 500-4000 cm-1. Biological formation of Ag-NPs was shown by changing the color of the intermixture of AgNPs (fungal cell filtrate with1mmol/ litter of silver nitrate) from clear yellow color to brown color at variable volumes and different concentration (20, 30, 40 and 50 µl) was evaluated in versus to bacterial isolates. The way it has been found that Ag-NPs was the most efficient solution in the inhibition of bacterial growth with the concentration of 50 µl. Estimation of the synergistic result was tested via method of disc diffusion opposite *Pseudomonas aeruginosa* and *protues mirabilis.*

The results showed that Ag-NPs was the most efficient in the inhibition of bacterial growth with the concentration of (50 µl) and a significant combined influnece were revealed for all measured antibiotics combined with AgNPs at extremely little amount of both AgNPs and antibiotics.

Conclusion: This study concluded that the nanoparticles synthesized from *Agaricus bisporus* have great potential as antimicrobial compound against tested pathogenic microorganisms. However, synthesis of nanoparticles can potentially eliminate the problem of chemical agents, which may have adverse effects on its application

Keywords: synergistic effects; Ag-NPs; antibiotics*; Pseudomonas aeruginosa; Proteus mirabilis; Acute otitis externa*

INTRODUCTION

Otitis externa or external ear infection refers to a set of inflammatory diseases on the integumentary infection of the ear external canal, usually associated with superadded by either fungus, bacteria or both of destroyed skin and it is an underlying structure [1]. There is a multiple factors act, changing the skin layers mainly the superficial layers, making the way for infection to occur, resulting in bacterial otitis externa, the main reason of infection in the external ear [2]. The systematic disorders like chronic anemia, endocrine disorders - especially diabetes mellitus, low body concentration of vitamin and different type of skin lesion like seborrhea, any type of eczema and psoriasis resulting in decrease the protective mechanism against infectious assaults in the external canal of the ear, resulting in otitis externa [3].

Silver nanoparticles assume noteworthy part of the field for science and pharmaceutical. The silver nanoparticles antibacterial effect (AgNPs) is a very well-known and applied for technological and medical purposes [4]. The usual pathogens responsible for AOE are *Pseudomonas aeruginosa, P. mirabilis, Staphylococcus* spp. and various gram bacilli and a culture of the canal will usually demonstrate a mixed growth of theses organism [5*]. P. aeruginosa* leading to a nosocomial infection, it is a Gram-negative opportunistic pathogen in human. In particular, it may lead to a recurrent or chronic lung infection in a patient with cystic fibrosis, also causing an increase in death rate by the formation of high virulence factors and the incorrect or poor response of the host [6]. *P. mirabilis* causes 90% of *Proteus* infections. *P. mirabilis* causes 90% of *Proteus* infections and is believed to be the most common cause of infection-related otitis media [7].

The strong toxicity of silver against a wide range of microorganisms is well known and silver nanoparticles showed to be promising antimicrobial materials. So, the present study aimed to evaluate the synergistic effect of bio prepared silver nanoparticles using edible mushroom as bioreactance with antibiotics *for Pseudomonas aeruginosa and Proteus mirabilis* isolated from acute otitis media.

MATERIALS AND METHODS

Collection, isolation, and susceptibility of Clinical Samples

Goldenberg's inclusion criteria used to select eighty patients with acute otitis externa for this study, these criteria include, no previous any treatment and tympanic membrane intact for diagnosis of otitis externa. All patients selected in this study had material gained from the infected ear via a swab through a skilled professional and it was elated in Stuart's culture medium. Samples were taken from patients of ages ranged between (20 -65) years old who were attending the clinical outpatient of Otorhinolaryngology of Ad-Diwaniyah teaching hospital and private clinics at Ad-Diwaniyah city during the period from November 2016 to April 2017. We take cultures and sent for investigation and the disc spread agar were used for susceptibility tests, and the outcome studied according to following laboratory result and clinical examination [8-9].

Preparation of Crude Extract of *Agaricus bisporus*

Ag-NPs which were used synthesized by fresh edible mushroom *Agaricus bisporus* from commercial sources. About 20 gm of mushroom was weighted out and rinsed well with two times distilled water, then crushed plus transferred to a beaker of 100ml of sterile distilled water. This mixture is stirred for about 2 hours, filtered using Whatt man No.1 filter paper. The extract of mushroom can be preserved for further experiments by storing it at 40° C. [13]. Samples of different concentrations of the mushroom extract and AgNO3 was prepared to derive the most efficient preparatory method for efficient and faster synthesis of silver nanoparticles. Sample 1 was prepared using 50ml of extracted mushroom put with 50 ml of 1mM AgNO3 solution. A control sample was prepared by mixing 40 ml of 1mM AgNO3 (approximately 8.5 mg) directly to 10 ml of Distilled water [10].

Characterization of Ag-NPs

1. Visual detection and UV-Visible Spectroscopy

Synthesis of Ag-NPs using *Agaricus bisporus* extract was observed by the transformation of the color yellow color to dark brown color within 12 hours. additionally, its recognized by UV-Visible Spectroscopy (UltraViolet-1600 PC Shimadzu). The mushroom extract & AgNO3 response process was monitored by Ultraviolet-Visible spectroscopy by a setting of 2.0 nm, amongst the wavelength two hundred to seven hundred nm. **[**11].

2. Scanning Electron Microscopy (SEM)

The characterization of the size of AgNps was done by scanning electron microscope, the AgNps synthesized using mushroom extract was allowed to dry completely and grounded well to a powder. SEM- specimen required to be completely dry since the specimen is in high vacuum. The morphology of AgNps is apparently spherical, were observed that are in AgNps size of 30 ± 15nm and poly-dispersed [11].

3- FTIR spectroscopy measurements

The residual of silver nanaoparticles via *Agaricus bisporus* extract was centrifuged at 1000 rpm for 15 min to remove the unwanted impurities then supernatant again was repeated. Pellets obtained were washed with deionized water to get the pure AgNps. The sample completely air dried at room temperature; collected powdered AgNPs were taken (FTIR analysis) in the range of 250 to 4250 cm-1(14) [11].

Antibacterial activity and Ag-NPs

Antibacterial activities of antibiotics were determined by disc diffusion method according to [9]. The Combination of Ag-NPs and antibiotics against bacterial isolates were done by disc diffusion method. The antibiotics used in our study belong to several classes and have various cellular targets, modes of action and bacterial resistance mechanisms penicillin G, ampicillin, cefotaxime, gentamycin, and rifampicin. To regulate the synergistic result of Ag-NPs the discs were saturated with newly set Ag-NPs and these discs were used for antibacterial action tests. Antibacterial action was quantified by the equation $(B^2 - A^2)/A^2$, where A and B are the zone of inhibition of antibiotic and antibiotic with Ag-NPs, respectively [12].

Combination of Antibiotics and Ag-NPs

Antibacterial activities of antibiotics were determined by disc diffusion method (Kirby-Bauer method) according to [8]. The mixture of Ag-NPs and antibiotics opposite bacterial isolates were done by disc diffusion method. To regulate the synergistic result of Ag-NPs the discs were saturated with newly set Ag-NPs and then these discs were used for antibacterial action tests [13].

RESULTS AND DISCUSSION

Visual detection

Ag-NPs were visually detected by changing color from yellow to dark brown figure (1). The reduction of silver ions to Ag-NPs (Ag+ to Ag0) lead to changing color from transparent or light yellow to brown [13]. The control did not show any change in its initial color when incubated under the same conditions [14].

Fig.1: Colloid of mushroom and AgNO3

UV/ Visible Spectrophotometry

Figure (2) showed the UV-Vis spectrophotometry (1600) has also been used to detect the production of silver nanoparticles. The results containing the biosynthesized silver nanoparticles observed a peak in the range of 430 nm which is the defined range of the Ag-NPs, taken after every 24 hours for 3 days. The production of Ag-NPs from *Agaricus bisporus* which agreement with the work of [14].

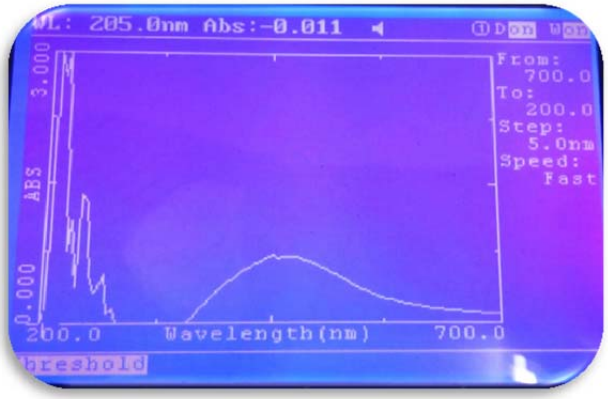


Fig.2: Peak of silver nanoparticles synthesized by *Agaricus bisporus* UV/Vis spectroscopy

Scanning Electron Microscopy (SEM)

Characterization by SEM of AgNPs was observed. this study revealed a uniform arrangement of particles having a size in the range of 5-35nm and spherical in shape figure (3), Whereas [15] recorded synthesized silver nanoparticles by Pleurotus sajorcaju of size range 5-50. While [20] obtained that silver nanoparticle synthesis by *Ganoderma lucidium*. They also reported the polydisperse nature of their nanoparticles, however, their size ranged between 10 to 70 nm.

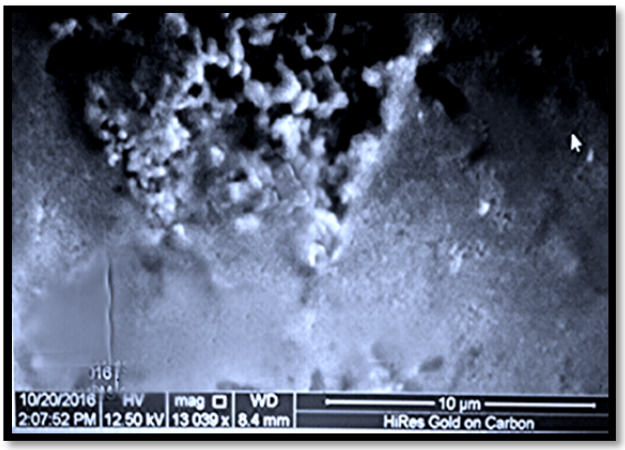


Fig.3: Electron Microscopy of silver nanoparticles synthesized by *Agaricus bisporus*

FTIR

The interface between silver nanoparticles and proteins was investigated via FT-IR, recognized the particles found in mushroom abstracts supposed to be in charge of the decrease of silver ions to AgNPs and established the covering substances for the constancy of this bio reduced nonmetal figure (4). FT-IR assessment revealed the ranges between 250 to 4250 cm-1 of AgNPs which exhibited the absorption and positioned at 2250 – 2800, 1500 – 1700 and 1000 of these 2250-2100 signifies C=C Alkynes (stretch), 1500 – 1700 for C=C amide (stretch) and 1000 for C-O Alcohols, Ethers, Esters and Carboxylic acid (stretch) [5]. Around 0.02 mg of sample was combined with 100 mg of the scanning range of 250-4250 cm−1 were gained with the resolution of $2cm^{-1.}$ F KBr.

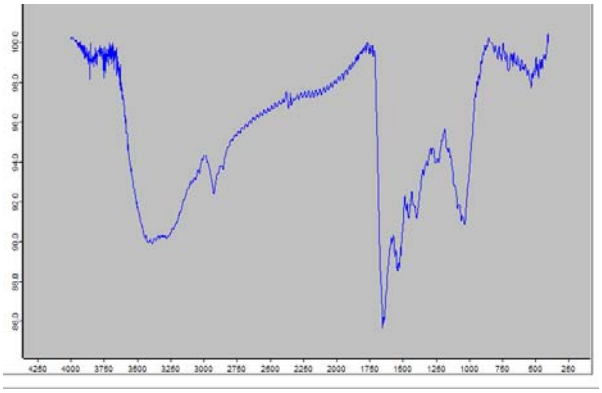


Fig.4: FT-IR assessment revealed the scales amongst 250 to 4250 cm-1 of silver nanoparticles.

Combination of AgNPs and Antibiotics

The results showed that all bacterial isolates of *Ps. aeruginosa* (table 1) and P.*mirabilis* (table 2) showed high resistant to all tested antibiotics . The maximum activity of inhibition (25 mm) was observed when combined the AgNPs with rifampicin against *Ps. aeruginosa* and was 20mm against *P. mirabilis* to nitrofurantoin and rifampicin while the lower inhibitory effect was 15 mm to ampicillin against each *Proteus mirabilis* and Ps. *aeruginosa*. That combination of some antibiotics including (Ampicillin, Ceftazidime) and Ag-NPs against *Ps. aeruginosa and* showed strong synergistic effects at the extremely little amount of equally Ag-NPs and antibiotics. The present study was differed with [21] who found that zone inhibition (17.5 mm) to amoxicillin against *Ps. aeruginosa* and [22] who revealed that inhibitory activity (19 mm). This inhibitory effect is due to that nanoparticles have the capability to terminate the constancy of lipopolysaccharides permitting a rise in penetrability of the external membrane and the peptidoglycan structure and is distinguished and arrested by antibiotics immediately [23].

This study examined the effects of Ag-NPs with edible mushroom in combination with several antibiotics. *Ps. aeruginosa* isolates revealed the highest response were 60% for both Ampicillin and Cefotaxime. rifampicin and gentamycin gave anti-biofilm rate of 50% and 40% respectively.

Combination of nanoparticles with antibiotics inhibited effectively the growth of bacteria than antibiotics alone. There was the relatively synergistic effectiveness of some a wide-ranging of antibiotics in combination with Ag-NPs against Ps. *aeruginosa and P. mirabilis* (Fig.5). The antibacterial action of the established antibiotics augmented noticeably when collective with AgNps as was demonstrated by the markedly reduced of the Ag-NPs

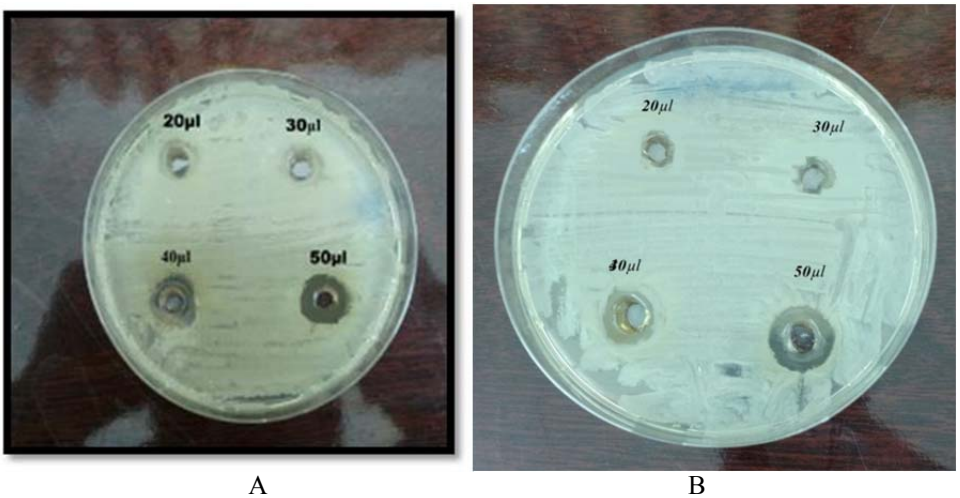
amount against the established bacteria. The combined effect of antibiotics and Ag-NPs was demonstrated at extremely low amount of Ag-NPs of (volume of 50 ml of mushroom extract with 50ml AgNO3) is the most effective against the growth of bacteria, on the other hand the results concluded that the inhibition zone diameters were increased by using 50 µl Ag-NPs by edible mushroom *Agaricus bisporus*. Species of mushroom exhibited a positive for the synthesis of Ag-NPs are protein rich and a pharmaceutically significant type of fungi. The precise mode of action behind the change of AgNO3 to Ag-NPs by mushroom abstract still not clear. Nevertheless, the enzymes outside the cells are may be in charge of the procedure of nanoparticles biosynthesis [22]. The spectrum by giving higher inhibition zones against isolates ranges, as the highest inhibition zone obtained in bacterial isolates *Ps. aeruginosa* (15mm) and less inhibition zone it was *P.mirabilis* (13mm) because of the maximum resistant capacity of the bacterial isolates. Though advantageous as antimicrobial agents, silver nanoparticles have adverse effects on cells, for example, the generation of free radical which are harmful to both eukaryotic cell and bacteria [23-24].

(Table 1) Zone of growth inhibition (mm) of *P. aeruginosa* against different antibiotics combined with or without Ag-NPs at content of 30 µl per disc)

	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

(Table 2) Zone of inhibition (mm) of different antibiotics against *P.mirabilis*, (in absence and in presence of (Ag-NPs) at content of 30 µl per disc)

	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



A B

Fig.5: Inhibitory effect of different concentration and volumes of Ag-NPs against (*A*)*P. aeruginosa* and (B) *P.mirabilis.*

CONCLUSION

The current study concluded that mushroom (Agaricus *bisporus)* synthesized by Ag-NPs revealed significant inhibitory action and has great potential as antimicrobial compound against tested pathogens because of the tested resistant bacteria to an antibiotic, established their vulnerability to antibiotics combined with Ag-NPs. Though, production of nanoparticles may strongly terminate the chemical agent's problem, which has possible side effects against its application.

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