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Combination effect of edible mushroom – sliver nanoparticles and antibiotics against selected multidrug biofilm pathogens

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Objective This project was designed to determine the effect of edible mushroom-Ag-Nps in combination with conventional antibiotics against selected multidrug biofilm-forming pathogens.
Methods Biofilm-producing bacteria were isolated and identified using routine cultural and biochemical tests from clinical specimen. In addition to that, standard bacterial strains were used. Silver nanoparticles were prepared using edible mushroom as bio-reductant. The biosynthesis of Ag–Nps was characterized by changing mushroom extract color from clear yellow to brown and UV/Visual spectrophotometer and electronic microscope. Then, the Ag–Nps were tested against bacterial strains and biofilm-producing bacteria using diffusion method as antibacterial agents in combination with antibiotics.
Results Biosynthesized Ag–Nps characterized by visual spectroscopy, scanning electronic microscope (SEM) were ranged (5–50 nm) in size, and Fourier Transform Infrared Spectroscopy (FTIR) in peak 430 cm-1 refers to protein formation. Different volumes and concentration of Ag-Nps (20, 30, and 50 µl) tested against selected multi drug biofilm-producing pathogens showed Ag–NPs at concentration 50 µl. They were more efficient in the inhibition of bacterial growth. On the other hand, assessment of the consolidated impact was examined utilizing circle dissemination strategy against methicillin-resistants, such as *Staphylococcus aureus* (*MRSA*), *Escherichia coli, Pseudomonas aeruginosa and Proteus mirabilis*. Results recorded a synergetic effect of Ag–Nps in association with resistant antibiotics.
Conclusions Compounds with greater potential as antimicrobial against pathogenic micro-organisms in combination with nanoparticles inhibited effectively to form biofilm than antibiotics alone. From this, we can conclude that Ag–NPs may be used in the remediation of infectious diseases. So Ag–NPs with antibiotics show maximum antibacterial activity. This may lead to develop new drugs for therapeutic needs.
Keywords Ag–NPs, MDR, *mushroom* (*agaricus bisporus*), antibiotics

Introduction

Nanotechnology may be a standout amongst the majority of quickly developing fields of science.[1] It has worthy effect at those atomic, atomic and supramolecular levels around size (1–100 nm) clinched alongside span.[2] Silver nanoparticles (Ag–NPs) assume a paramount part in the field of science and medication.[3] We showed that Ag–Nps show antibacterial exercises, and demise overall cell.[4]

Edible mushrooms were normal human diet for thousands of years and in recent times, the amounts consumed have risen greatly, involving a large number of species.[5] Fungi constitute a very favorable object of nano biotechnological studies.[6]

Recently, there has been significant interest toward the issues posed eventually Tom's perusing those biofilm mode of bacterial furthermore contagious Growth. As stated by open publication from national organization about health, more than 60% of known microbial spoiling is initiated. Eventually, Tom's perusing biofilm-shaping pathogens are MRSA, *E.coli*, *Pseudomonas aeruginosa*, what's more *Proteus mirabilis*. 30% for unending wounds, for example, foot, leg and weight ulcers being especially defenseless with biofilm infections.[7] So, this work is designed to determine the effect of edible mushroom-Ag–Nps in combination with conventional antibiotics against selected multidrug biofilm-forming pathogens.

Methods

Bacterial Isolates

Bacterial isolates of *Methicillin Resistance S. aureus* ATCC 43300, *E. coli* number **28739**, *P. aeruginosa* No. 27853, and *P. mirabilis* No. 16404 were obtained from the Central Health Laboratory Baghdad City (Iraq). The clinical isolates of multi-drug-resistant bacteria were obtained from the general teaching hospitals in AL-Diwaniyah city. The samples were collected from various sources of body.

Preparation of Crude Extract of Edible Mushroom (Agaricus Bisporus)

Ag–NPs were synthesized from edible fresh mushroom *Agaricus bisporus* (white button mushrooms). They were procured from commercial sources. About 20 gm. of the mushroom was weighted out and cleaned with ionized distilled water. Then crushed and transferred to a beaker containing 100 ml of sterile distilled water. This mixture was stirred for about 2 h and then filtered using Whatt man No.1 filter paper. Mechanically, Ag^{+} was reduced to Ag^{0}. The extract of mushroom was preserved under 40°C for further experiments.[8]

Preparation of Biosynthesized Silver Nanoparticles by Using Edible Mushroom

Four concentrated sample mushroom extracts and $AgNO_3$ were prepared to derive the most efficient preparatory method. Sample no. 1 was prepared by using 50 cm^3 mushroom colloid which was added to 50 cm^3 of 1 mM $AgNO_3$ aqueous solution. Sample no. 2 was prepared using 10 ml mushroom extract added to 40 ml of distilled water into which 1 mM of $AgNO_3$ (approximately 8.5 mg) was added. Sample no. 3 was prepared using 450 ml of distilled water taken in a conical flask into which 50 ml of mushroom extract was added. The above mixture was stirred well and about 1mM of $AgNO_3$ (approximately

86.5 mg) was added. Control sample was prepared by mixing 40 ml of 1 mM $AgNO_3$ (approximately 8.5 mg) directly to 10 ml of distal water (D.W) extract).[8]

Characterization of Ag-NPs

Visual Detection and UV-Visible Spectroscopy

Synthesis of Ag–NPs using *A. bisporus* extract was observed by changing color from yellow to dark brown within 12–24 h. Further, it has been characterized by UV-visible spectroscopy (UV-1600-PC Shimadzu). The process of reaction between $AgNO_3$ and mushroom colloid was monitored by UV-visible spectra with resolution of 2.0 nm, between the wavelength 200 and 700 nm.[9,10]

Scanning Electron Microscopy (SEM)

Characterization of Ag–Nps were done through analyzing with SEM; SEM. Ag–NPs synthesized using mushroom extract was allowed to dry completely and grounded well to a powder than specimen is normally required to be completely dry since the specimen is at high vacuum. Morphology of Ag–NPs is apparently spherical, it is observed that Ag–NPs formed in size range of (5–50 nm) and poly-dispersed.[8]

FTIR

The colloid of Ag–NPs by *A. bisporus* extract was separated at 10 000 rpm for 15 min to remove the unwanted impurities. Then supernatant was again centrifuged 10 times for 15 min. The resulting solution was repeated. Pellets obtained were washed with deionized water to get pure Ag–NPs. The sample was completely air-dried at room temperature; then collected powdered Ag–NPs were taken or FTIR analysis in wave length of 250 to 4500 cm^{-1}.[11]

Antimicrobial Stir of Ag–Nps

Antibacterial activity of Ag–Nps using *A. bisporus* extract was determined by agar well diffusion method.[8] Volumes of Ag–NPs and several concentration were investigated by agar well diffusion method to determine the better volume and concentration. Ag–NPs were added to agar wells, which were loaded with 20 µl, 30 µl, and 50 µl, Ag–NPs suspension. The activity was evaluated by calculating the increase in folded area.

Antibacterial Activity and Ag–Nps

Antibacterial activities of antibiotics were identified with reference to CLSI (2016).[12] Antibacterial activities of the combination of Ag–NPs and antibiotics against bacterial isolates were done by disc diffusion method. Penicillin G, ampicillin, cefotaxime, gentamycin and rifampicin. To mush red the synergistic effect of Ag-NPs. Discs were impregnated with freshly prepared Ag–NPs, and then these discs were used for antibacterial activity assays.[13] Antibacterial activity was calculated according to the formula $(B2-A2)/A2$, where A and B are the zone of inhibition for antibiotic and antibiotic with Ag–NPs, respectively.[14]

Bacterial Ability to Produce Biofilm by Tissue Culture Plate and Tube Methods

Tube Method

Tube method was performed according to Pramodhini et al.[15]

Biofilm Activity of Ag–NPs by TCP Method

Tissue culture plate assay was performed as given in the literature.[4,17] To investigate the biofilm activity of Ag–NPs alone and in combination with antibiotics with minor modifications, this method was widely used and considered as standard test for detection of biofilm formation. This method was applied on bacterial isolates. These isolates were selected according to the previous tests (Tube Method). Also, the media was used to evaluate biofilm formation; tryptone soya broth (TSB), individual wells of sterile, polystyrene, 96-well TCPs were filled with 170 µl of the single populations of the bacterial species equivalent to the McFarland No. 0.5 at 10^5 CFU.

Results

Visual Detection of AgNps

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

UV/ Visible Spectrophotometer

Figure 2 shows the UV-Vis spectrophotometry (1600) used to detect the synthesis of Ag–Nps. The result containing the synthesized Ag–Nps was observed in a peak of 430 nm. Ag–NPs were taken every 24 h for 3 days.

Fig. 1 **Colloid of mushroom and $AgNO_3$.**

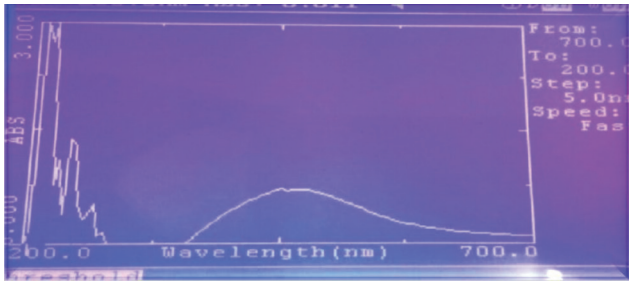

Fig. 2 **Peak of Ag-NPs by *Agaricus bisporus* UV/Vis spectroscopy.**

Scanning Electron Microscopy (SEM)

Characterization of Ag–NPs was observed using SEM. It revealed a uniform arrangement of particles having size in the range of 5–35 nm and spherical in shape (Fig. 3).

Fourier Transmission-Infrared Spectroscopy (FTIR)

The interaction between Ag–NPs and proteins was analyzed by FTIR. Characterization revealed the molecules present in mushroom extracts though reduction of silver ions to silver nanoparticles and confirmed the agents (Fig. 4). FT–IR measurements showed the spectra between 250 and 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).

Effect of Optimum Volumes and Concentrations of Ag–Nps

The effect of Ag–NPs against standardized bacteria were examined (Table 1, Fig. 5). It found that Ag–NPs concentration of 50 µl of mushroom extract was the most effective (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 *A. bisporus* (Fig. 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 was *MRSA* ≥ 14 mm because of the maximum resistant capacity of the bacterial isolates.

(Note) A = 50 ml / 50 ml (mushroom and $AgNO_3$), B = 10 ml/40 ml(water with I molar $AgNO_3$) and C = 50 ml/450 ml water with 1 molar $AgNO_3$; 1 molar = 8.5 mg of $AgNO_3$

Determination of 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

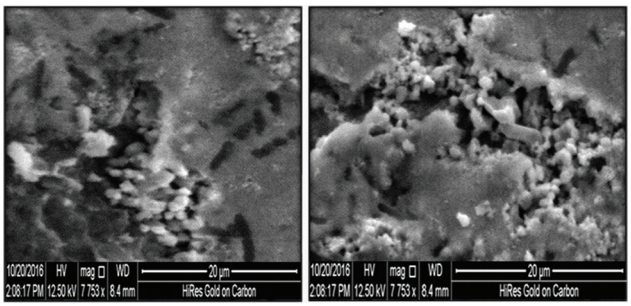

Fig. 3 **Scanning Electron Microscopy of Ag–NPs by *Agaricus bisporus* in Iraq.**

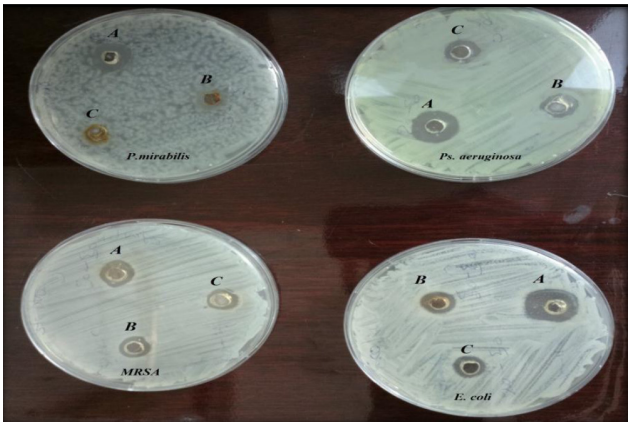

Fig. 5 **Inhibition zone of growth tested isolates.**

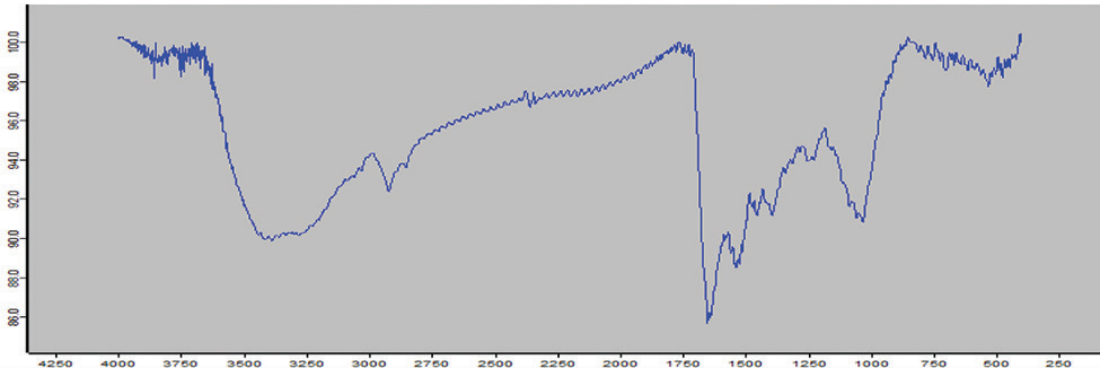

Fig. 4 **FTIR spectra of Ag-NPs by *Agaricus bisporus* in Iraq.**

Table 1. **Rack (1) area of growth inhibition (mm) of standard bacterial tested against with different concentration of Ag–Nps .**

Standard Isolates / Tested isolated with AgNps and $AgNO_3$	Zone in growth inhibition (mm)			
	MRSA 43300 ATCC	*E. coli* 28739 ATCC	*Proteus mirabilis* 16404 ATCC	*Ps. aeruginosa* 27853 ATCC
$AgNO_3$ (control)	10	16	12	12
Nanoparticles (Ag-NPs) test	14	20	15	18

Gram-positive bacteria. The results showed that all bacterial isolates showed perfect resistant to all antibiotics used, ampicillin, cefotaxime, gentamycin, rifampicin, oxacillin. Penicillin G increased in the presence of Ag–Nps edible mushroom *A. bisporus* against bacterial isolates. However, the combined Ag–Nps and antibiotics enhanced the antibacterial activities as revealed (Rack 1).

The antibacterial activities of antibiotics were observed maximum fold area in combination with Ag–NPs against *E. coli* (Fig. 5).

In the present study, bacterial ability to produce biofilm was 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. 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 (Fig. 3). The highest response to cefotaxime and gentamycin were 70% by *E. coli*, while ampicillin and rifampicin were 40% and 50%, respectively. *P. aeruginosa* isolates showed high response, that is, 60% for both ampicillin and cefotaxime. Rifampicin and gentamycin gave anti biofilm rate of 50% and 40%, respectively.

P. mirabilis showed the highest level of inhibition (Fig. 5), that is, 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 that inhibited the biofilm and 60% for each cefotaxime and gentamycin while 20% for rifampicin (Fig. 5).

Discussion

Ag–NPs were visually detected by changing color of the suspension (Fig. 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. The results containing the synthesis of Ag–NPs observed in a peak of 430 nm. Ag–NPs were taken every 24 hours for 3 days. The production of Ag–NPs from *A. bisporus,* which agreed with the work of Haq et al.[19] Similar result ranging 420–430 was achieved as done by Nithya and Ragunathan.[20]

Characterization of Ag–NPs was observed in SEM. It revealed a uniform arrangement of particles whereas Nithya and 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, 10–70 nm.

Mushroom species found positive for the production of Ag–NPs are rich in protein and are medicinally important group of fungi. The exact mechanism behind the conversion of $AgNO_3$ to Ag–NPs by mushroom extract was not known. Silver have a high affinity towards phosphorus and sulphur compounds. A study of on the antibacterial activity of Ag–NPs against Gram-negative bacteria 22 revealed that Ag–Nps obstruct the cell function by concern to the surface of the cell membrane.[21,22] Ag–NPs are the effective killing agent of broad spectrum of Gram-negative bacteria such as *E. coli* and *P. aeruginosa* and Gram-positive bacteria such as MRSA; also suggested that silver ions (Ag^+) released from silver nanoparticles can interact with phosphorus moieties in DNA, resulting in inactivation of DNA replication.

NPs using *Pleurotus sajor-caju* (Mushroom) was tested against the *P. Aeruginosa*. *P. mirabilis* produced zone of inhibition of 12 mm, 14 mm; respectively. Nithya and 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 the synthesis of Ag–NPs and also the yield is high.[24]

Thus, Birla et al.[14] indicated increased area of *E. coli, P. aeruginosa* than *Staphaureus*. Panáček[25] revealed that Ag–NPs can be combined with antibiotics for more effective combination against various pathogenic microbes.

The present study agreed Bhosale et al.[26] Synthesis of nanoparticles is harsh against Gram-negative bacteria. Devika et al.[27] revealed that activity was observed in Ag–Nps with antibiotics, and maximum activity was against *E. coli*, and minimum activity was against *S. aureus*. Bacteria have great ability

Table 2A. **Zone of inhibition (mm) of different antibiotics against *S. aureus* (in absence and in presence of Ag–NPs at content 30 µl per disc.**

	No. *MRSA* isolates														
	12			2			3			7			10		
Antibioties	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
Penicillin S 29-R 28	8	10	0.59	8	10	0.59	12	16	0.93	8	10	0.59	10	18	2.24
Ampicillin	-	10	0.22	7	13	2.5	7	13	2.5	-	10	0.22	7	18	2.5
Cefotaxime	-	10	0.22	10	14	0.96	7	10	1.5	-	10	0.22	9	13	1.77
Gentamican 15 -R12	-	10	0.22	7	13	2.5	-	10	0.22	-	10	0.22	7	15	2.5
Rifampicin 20-R16	-	12	1.5	8	10	0.59	8	10	0.59	9	15	1.77	9	13	1.77
Oxacillin	-	12	1.5	-	10	0.22	-	12	1.5	-	12	1.5	-	10	0.22

Note- 1. Ab; Antibiotic, AgNps; Silver nanoparteclis, IF; Increased fold area 2. The discs' diameter (6 mm) were used to musser the increase area in columns.

Table 2B. **(Rack 2 b). Zone of inhibition (mm) of different antibiotics against *E. coli* at 50 µl per disc**

		No. *E.coli* isolates																													
		1			4			8			6			12			14			18			22			23			25		
Antibioties	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	-	15	5.2	11	13	0.39	9	15	1.7	-	10	0.22	-	11	2.3	7	15	3.5	-	10	0.22	7	15	3.5	7	15	3.5	-	15	5.2
Cefotaxime	7	12	1.3	11	13	0.39	11	13	0.39	-	10	0.22	11	14	0.5	11	14	0.5	9	14	1.4	8	16	3	9	14	1.4	8	16	3
Gentamican	8	15	2.5	8	18	4	8	15	2.5	6	10	1.7	8	18	4	10	18	2.2	10	18	2.2	12	15	0.5	12	15	0.5	7	15	3.5
Rifampicin	12	15	0.5	11	13	0.3	11	14	0.3	10	14	0.4	8	15	2.5	11	15	0.5	10	15	1.2	8	15	2.5	10	15	1.2	10	15	1.2

Table 2C. **Area of inhibition (mm) of *P. aeruginosa* (in absence and in presence of Ag–Nps at 50 µl per disc)**

		No. *P. aeruginosa* isolates																													
		6			9			10			11			16			18			19			20			22			23		
Antibioties	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
Gentamican	-	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	-	16	0.22	9	16	2.1	9	16	2.1	9	16	2.1	9	16	2.1	9	16	2.1

Table 2D. **Area of inhibition (mm) of *P. mirabilis* (in absence and in presence of (Ag–NPs) at 50 µl per disc)**

		No. *P.mirabilis* isolates																													
		1			2			5			6			7			13			14			15			17			19		
Antibioties	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	12	7	15	3.5	10	15	12
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
Gentamican	-	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	10	1.2	-	10	0.22	10	15	1.2	-	10	0.22

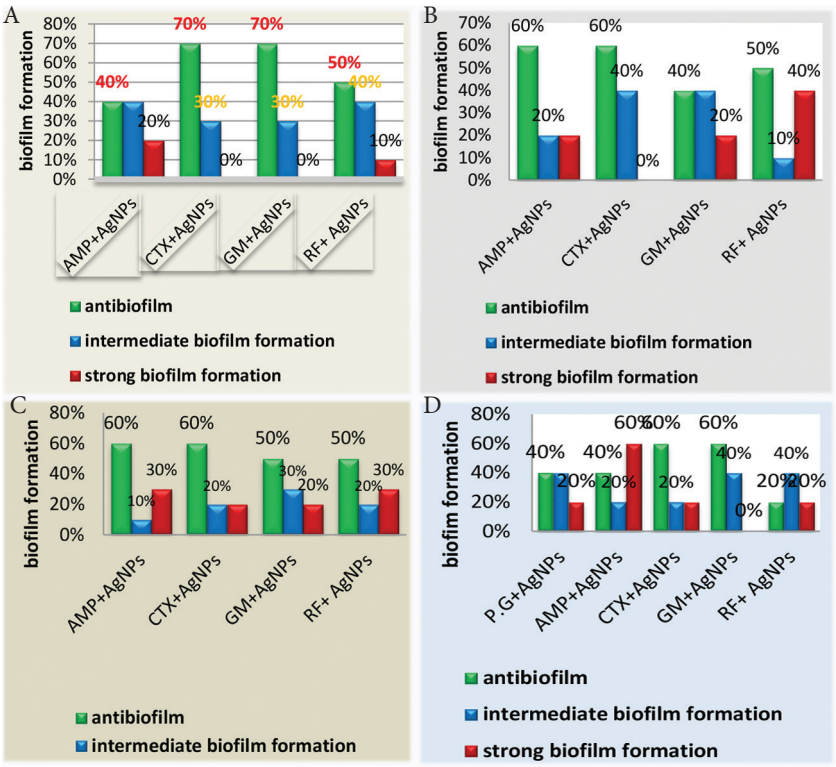

Fig. 5 **Percentage of inhibition in biofilm formation determined by using the TCP method, showing the effect of addition of nano-Ags in combination with various conventional antibiotics. (a) *E. coli*, (b) *P. aeruginosa*, (c) *P.mirabilis*, (d) MRSA.**

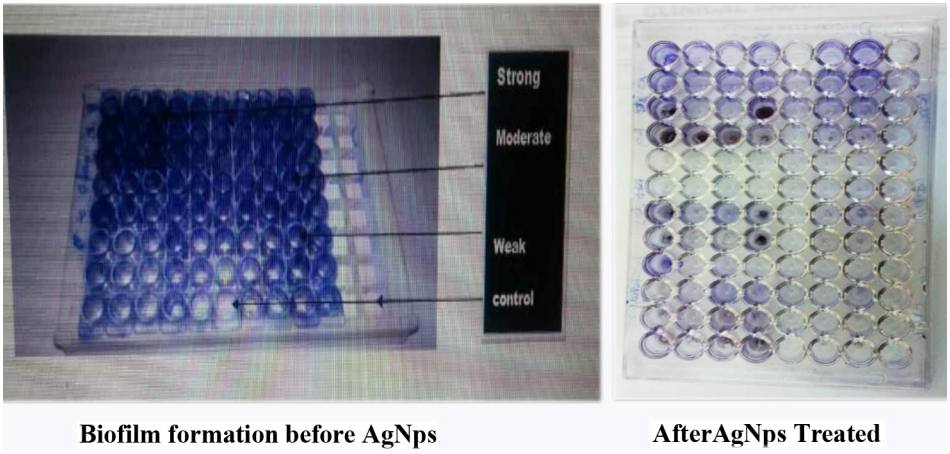

Fig. 6 **TCP method of biofilm for *E.coli* shows the effect (before and After) of treated with Ag–Nps result from left to right.**

of developing resistance against antibiotics. Yamanaka *et al.*[28] confirmed in permeability of the outer membrane and the peptidoglycan structure and is recognized and captured by antibiotics immediately.[29]

Ahmed demonstrated that out of 118 Gram-positive bacteria, the percentage of biofilm producers by TCP method was 52.0%, which is higher than in TM (48.0%).[30] biofilm producer as (moderate and strong). In another study, the incidence of biofilm producers among 81 isolates of G+ve and G-ve were 51 (63%) strong bacteria biofilm and 30 (37%).[31] Moderate bacteria biofilm, in which Gram-positive bacteria generally higher than Gram-negative bacteria in producing biofilm. Hwang *et al*reported different results between inhibition of biofilm formation by TCP method.[16] The effect of Ag–NPs was effective than various conventional antibiotics; ampicillin, chloramphenicol, kanamycin. against *P. aeruginosa.* While this result agreed with their results, they revealed that combinations of Ag–NPs and the three conventional antibiotics (Ampicillin, Kanamycin and chloramphenicol) also appeared to actively inhibit biofilm formation to varying degrees. It was observed that Ag–NPs inhibit the formation of biofilm.[32]

The response of MRSA for penicillin G and ampicillin was 40% for isolates inhibited the biofilm, and 60% for each cefotaxime and gentamycin while 20% for rifampicin (Fig. 5). This result agreed with Khalid *et al.*[33] revealed that Ag–NPs had an inhibitory activity on biofilm formation greater than 55%. Combinations of Ag–NPs and antibiotics showed a greater inhibitory activity than Ag–Nps alone, and anti-biofilm activity was due to Ag–Nps.

Ag–NPs are also active against bacterial biofilm,[34] Inhibited of biofilm formation with ability of Ag–Nps to prevent the adhesion to various surfaces.[35] Sangiliyandi *et al*. indicated that Ag–Nps alone inhibited biofilm activity by approximately 20%.[36] While combinations of Ag–Nps and ampicillin inhibited biofilm activity in Gram-negative and Gram-positive bacteria by 70% and 55%, respectively.[34]

The greater potential as antimicrobial compounds against pathogenic micro-organisms, and that the combination of nanoparticles with antibiotics inhibited effectively the ability to form biofilm by TCP method than antibiotics alone. From this, we can conclude that Ag–NPs with antibiotics show maximum antibacterial activity, and this may lead to develop new pharmaceuticals for therapeutic needs. It is proven that the Ag–Nps synthesized from *Agaricus bisporus*have synergistic effect of antibiotics at very low concentrations.

Conflict of Interest

None.

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