

Tobramycin-Silver Nanocomposite: A New Trend of Antimicrobials against Resistant Strains

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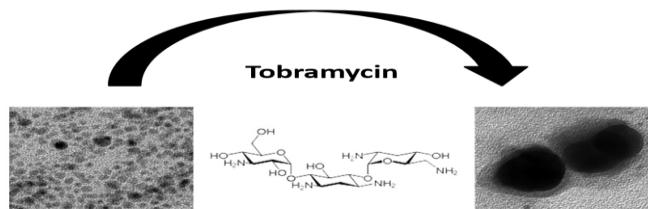
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Abstract: Effectiveness of many antibiotics has been remarkably decreased due to bacterial resistance. the present work aims to explore a novel antimicrobial nanocomposite with greater efficacy against resistant bacteria. Tobramycin-silver nanocomposite was synthesized and characterized by Transmission Electron Microscopy, UV-VIS Spectroscopy, Zetasizer and Fourier Transform Infrared spectroscopy. Antimicrobial activity of tobramycin, silver nanoparticles and Tobramycin-silver nanocomposite was tested against *Staphylococcus aureus*, *pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Proteus mirabilis* and *acinetobacter baumannii*, the results revealed that Tobramycin-silver nanocomposite had enhanced antimicrobial efficacy against the selected resistant strains in range between 250% and 980% than Tobramycin alone.

Key words: Bacterial resistance, antimicrobial agent, Tobramycin-silver nanocomposite, silver nanoparticles.

Graphical abstract



I. Introduction

Tobramycin (TOB) is an aminoglycoside produced by the bacterium *Streptomyces tenebrarius*. Its mode of action through targeting the decoding aminoacyl site on the 16S ribosomal RNA, induces misreading during translation step leading to cell death (Davies and Davis, 1968); (Davis, 1987). For several years Tobramycin had been the antibiotic of choice in the treatment of clinical infections due to its bactericidal activity against many bacterial strains such as *Pseudomonas aeruginosa*. It is particularly prescribed for the treatment of septicemia, lower respiratory infections, complicated and recurrent urinary tract infections, soft tissue and serious skin infections including burns and peritonitis, ophthalmic and central nervous system infections caused by organisms resistant to other antibiotics, including other aminoglycosides (Pignatello et al., 2011).

Later on, The emergence of resistance to multiple antimicrobial agents in pathogenic bacteria has become a significant global public health threat, causing considerable patient mortality and morbidity (Payne, 2008). This resistance was due to high selective pressure of antimicrobials commonly used in hospitalized patients, particularly in extended-spectrum cephalosporins, aminoglycosides, carbapenems and fluoroquinolones led to evolution (Loeffler et al., 2003). Bacterial resistance has been occurred to aminoglycosides specially tobramycin, because of the reduced penetration of the antibiotic inside cells and its low affinity for the bacterial ribosome or drug deactivation by microbial cell enzymes (Brunton, 1991). This crucial problem forced scientists to discover new antibiotics and chemically modifying existing antimicrobial drugs (Boucher et al., 2009).

Recently, Nanotechnology was applied to the therapeutic fields, the roles of nanometals like silver, platinum and gold, in drug delivery and therapy such as the development of materials with antimicrobial properties (Ali et al., 2014; Ali, 2013; Eldin et al., 2014; Meng et al., 2010). One of the recent strategies in overcoming resistance lie on exploring novel antimicrobial nanomaterials and nanocomposite. Various nanocomposite has shown a great efficacy against pathogenic microbes leading to control bacterial infections. (Sibanda and Okoh, 2007). The antimicrobial nanomaterials also improve pharmacokinetics and accumulation, while reducing the adverse effects of antibiotics (Rai et al., 2009). The conjugation between silver and antibiotics such as, amoxicillin, Penicillin G, erythromycin and vancomycin, leaded to enhancing and synergistic antimicrobial effects against Gram-positive and Gram-negative bacteria (e.g., *E. coli* and *S. aureus*) (Ali et al., 2014; Dar et al., 2013; Fayaz et al., 2010). The main advantages of the preparation of antimicrobial nanomaterials that it could be cost effective compared to antibiotics synthesis, and also they are quite stable for long-term storage with a prolonged shelf-life. (Weir et al., 2008).

This work focus on increasing Tobramycin activity against different widely spread resistant microorganisms such as *Staphylococcus aureus*, *pseudomonas aurignosa*, *Klebsiella pneumonia*, *Proteus mirabilis* and *Acinetobacter bauminaii* by conjugation between Tobramycin with silver nanoparticles (AgNPs) forming (TOB-AgNPs) nanocomposite as a novel antimicrobial nanomaterials.

II. Materials and Methods

A. Isolation and identification of clinical isolates

Forty eight isolates were obtained from pus, wound, sputum, urine and blood samples from patients admitted in different wards of Egyptian hospitals. The clinical isolates were identified by standard microbiological procedures including cultivation on Mannitol salt agar, blood agar, Cetrimide agar, MacConkey agar. Then a lot of tests were performed including gram's staining, colony morphology, sugar fermentation tests and biochemical tests such as oxidase test, Catalase test, Coagulase test and IMViC according to (Kateete et al., 2010) for *Staphylococcus aureus*, (Anbuselvi, 2013) for *Pseudomonas* species, (NG et al., 2010) for *Enterobacteriaceae* and (Filimon and Tara, 2004) for *Acinetobacter bauminaii* and finally the identification of the strains was confirmed by using API STAPH, 20NE and 20E.

B. Antibiotic susceptibility of isolates by disk diffusion method

Antimicrobial susceptibility tests were performed by the Kirby-Bauer disk diffusion method against a panel of antimicrobial standards as follow; Piperacillin-Tazobactam (TZP 110); Cefepime (FEP 30); Imipenem (IPM 10); Meropenem (MEM 10); Gentamycin (CN 10); Tobramycin (TOB 10); Levofloxacin (LEV 5). Results were recorded and compared with the standard levels to know whether isolates were sensitive, intermediate, or resistant to the concerned antibiotics. The interpretation of inhibition zones of test culture was done according to (Cockerill et al., 2012).

C. Synthesis of Silver nanoparticles (AgNPs)

The silver nanoparticles (AgNPs) were prepared by chemical reduction method according to (Ratyakshi and Chauhan 2009) with slight modification. In brief, for sterilization, glassware were cleaned with aqua regia, and rinsed copiously with deionized water (**Milli-Q, Millipore, purified**) and autoclaved at 120 °C. 1 mM AgNO₃ (Sigma) stock solution was prepared (0.17 g AgNO₃ in 100 ml H₂O) in 100 ml volumetric flask. 1mM trisodium citrate (**Sigma**) solution was prepared (0.3 g sodium citrate + 100 mL H₂O) in 100 mL volumetric flask. 10 mL of AgNO₃ stock solution were taken and diluted to 50 ml in beaker and heated up with stirring at 400 rpm till boiling. Once boiling obtained 1mL of Trisodium Citrate solution was added, the solution was left on the heater till the clear golden yellow color of AgNPs is obtained.

D. Preparation of Tobramycin-Silver nanocomposite (TOB-AgNPs).

Tobramycin-Silver Nanocomposite (TOB-AgNPs) was prepared according to (Raja and Singh, 2013) and (Kora and Rastogi, 2013) with little modification as following; A stock solution (1.024 mg/mL) of tobramycin (**99 %**) (**Epico, Egypt**) was prepared and stored at -20 °C. For the conjugation of silver nanoparticles with tobramycin, 1 ml of the tobramycin stock solution was added to 20 ml of AgNPs solution under stirring and left in dark for 18 h incubation at room temperature. The golden yellow color of silver nanoparticles disappears indicating the formation of tobramycin capping layer over the surface of silver nanoparticles forming (TOB-AgNPs) nanocomposite, then stored at 4 °C and used for all further experiments. Both of AgNPs and (TOB-AgNPs) Nanocomposite were characterized by High Resolution Transmission Electron Microscope (TEM) (Tecnai, FEI Netherland), UV-Vis-NIR spectrophotometer (Varian, Cary 5000), Zeta sizer nano ZS (Malvern, UK), Fourier Transform Infrared Spectroscopy(FTIR) (Jasco, Japan). The preparation and characterization processes were done in the Nanotechnology & Advanced Materials Central Lab, Agriculture Research Center, Egypt.

E. Determination of minimum inhibitory concentration (MIC)

The antimicrobial activity of AgNPs, Tobramycin and TOB-AgNPs nanocomposite were assessed using the standard microdilution method according to (Wikler et al., 2007) against different strains, The MIC was read after 24 h of incubation at 37 °C visually and by using the ELISA reader.

F. Statistical Analysis

Statistical presentation and analysis of the present study was conducted, using the mean, standard Deviation, [ANOVA] test using the Statistical Package for the Social Sciences (SPSS) software (SPSS 17, Chicago, IL, USA).

III. Results & discussion

A. Isolation and identification

Isolation and identification of 48 isolates by biochemical tests and API showed that (16.6%) 8 isolates were *Staphylococcus aureus*, (29.2%) 14 isolates were *Pseudomonas aeruginosa*, (31.2%) 15 isolates were *Klebsiella pneumonia*, (4.2%) 2 isolates were *Proteus mirabilis* and (18.8%) 9 isolates were *Acinetobacter baumannii*. The isolates were obtained from burns (20), wound (15), urine (5), sputum (4) and blood (4) as described in table (1).

<u>Source</u>	<u>Acinetobacter baumannii</u>	<u>Proteus mirabilis</u>	<u>Klebsiella pneumonia</u>	<u>Pseudomonas aeruginosa</u>	<u>Staphylococcus aureus</u>	<u>Total No.</u>
<u>Blood</u>	1	0	1	1	1	4
<u>Wound</u>	4	0	4	5	2	15
<u>Burn</u>	2	2	5	8	3	20
<u>Sputum</u>	1	0	1	0	2	4
<u>Urine</u>	1	0	4	0	0	5
<u>Total no.</u>	9	2	15	14	8	48
<u>%</u>	18.8	4.2	31.2	29.2	16.6	

Table (1): Distribution of resistant bacteria among clinical specimens:

B. Antibiotic susceptibility of isolates by disk diffusion method

The Majority of the isolates showed multidrug resistance profiles where it has been found that 91.6% of isolates were resistant to tobramycin while 6.3% only of them were sensitive to it and 2.1% were intermediate. Results also showed that 85.5% of the isolates were resistant to levofloxacin and 83.4% were resistant to gentamycin, cefepime and imipenem but in case of meropenem and tazobactam/piperacillin resistant was 81.2% as illustrated in figure (1). These results showed that the most predominant antibiotic which the majority of isolates resist was Tobramycin. That was the mean reason for choosing Tobramycin to be under study and the core goal of this work was to reactivate the Tobramycin efficacy and reduce its microbial resistance through conjugation with silver nanoparticles.

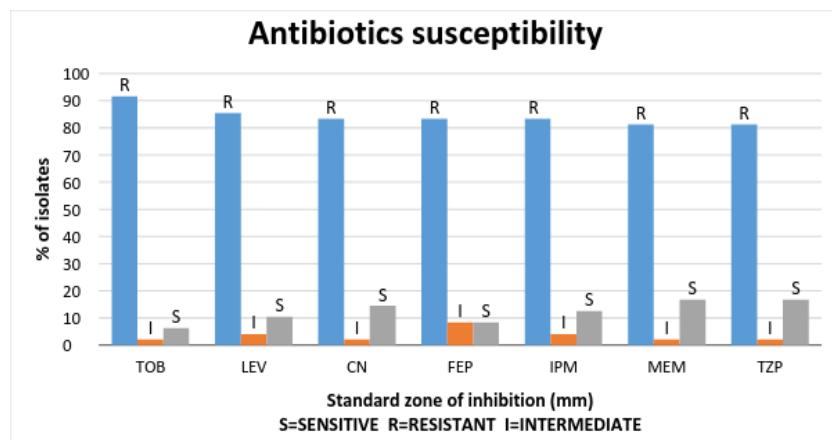


Figure (1): Antibiotics susceptibility against resistant bacteria **TOB**: Tobramycin; **LEV**: Levofloxacin; **MEM**: Meropenem; **CN**: Gentamycin; **FEP**: Cefepime; **IPM**: Imipenem; **TZP**: Piperacillin-Tazobactam

C. Characterization of AgNPs and TOB-AgNPs

Spectrophotometer measurements of silver nanoparticles (AgNPs) showed sharp characteristic absorption peak at 415 nm which corresponding to the plasmonic absorption band of spherical shaped silver nanoparticles with accordance with (Behera et al., 2013; RATYAKSHI and CHAUHAN, 2009). While both of Tobramycin and Tobramycin- Silver Nanocomposite (TOB-AgNPs) showed no absorption in the range from 300 nm to 800 nm as shown in Figure (2).The disappearance of the characteristic AgNPs peak may be explained in term of forming cape of tobramycin around the silver nanoparticles that masking the silver plasmonic absorption band and forming new conjugate of (TOB-AgNPs) Nanocomposite.

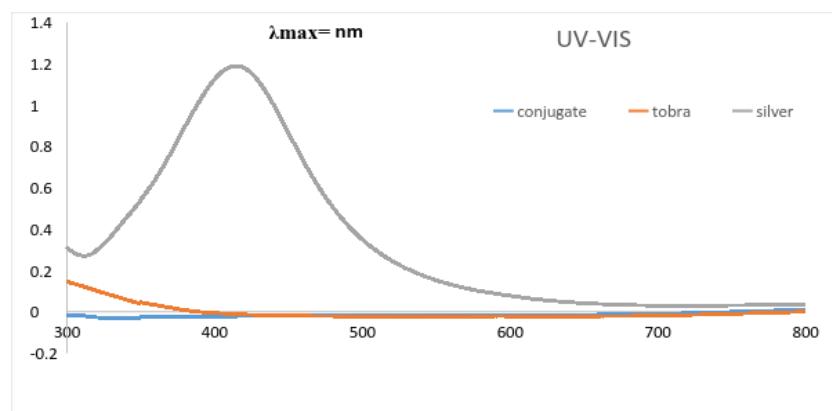


Figure (2): UV-Vis spectrophotometry of silver nanoparticles, Tobramycin and (TOB-AgNPs) Nanocomposite representing absorbance characteristics of each material.

This assumption was clearly illustrated by High Resolution Electron Microscopic (HRTEM) imaging, figure (3a), Silver nanoparticles are well distributed spherical shaped with average size 5 ± 3 nm. After conjugation process, Tobramycin formed a cap over the surface of silver nanoparticles forming silver/tobramycin core shell Nanocomposite as shown in figure (3b). The type of conjugation was not evaluated under this study, so it may be through chemical bond formation or physical Van der Wall interaction.

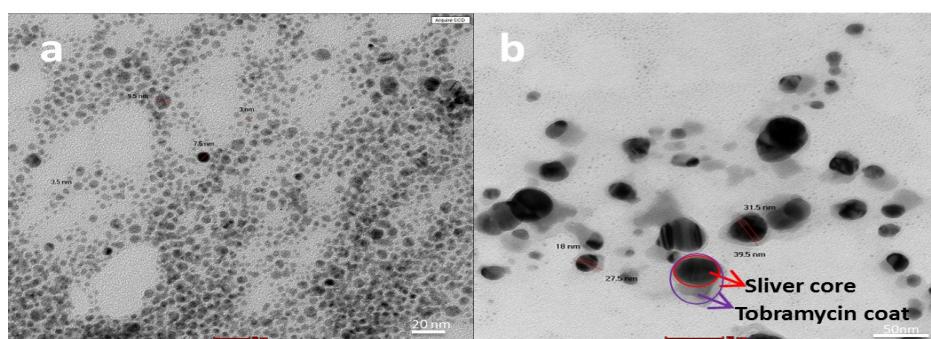


Figure (3): (a) HRTEM micrograph of silver nanoparticles with average size 5 ± 3 nm. (b) HRTEM micrograph of (TOB-AgNPs) Nanocomposite indicating particle size growth and formation of Tobramycin coat around silver core.

TOB-AgNPs nanocomposite TEM image showed the formation of well-formed cap of tobramycin around the spherical silver nanoparticles. Another findings in accordance with the measurements of particle size analyzer based on dynamic light scattering technique. Figure (4a) shows the particle size distribution of silver nanoparticles with average size **5 nm**. While that of TOB-AgNPs Nanocomposite, the size became **45.3 nm** (**figure 4b**) which indicate particle size growth that support the assumption of formation of tobramycin cap around the silver nanoparticles that was clearly observed by HRTEM images.

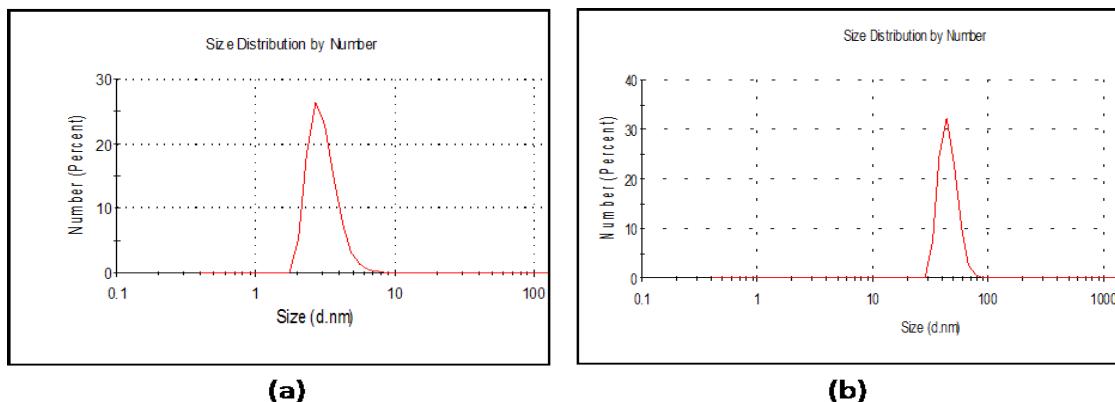


Figure (4): (a) particles size analysis of AgNPs. (b) Particle size analysis of TOB-AgNPs nanocomposite.

Also an important findings were zeta potential measurements, where for AgNPs the surface net charge -38.4 mV, Fig 5(a). On the other hand, zeta potential for TOB-AgNPs nanocomposite showed a noticeable change in the surface net charge which turned to the positive side +14.7 mV Fig 5(b), which also confirmed the formation of TOB-AgNPs nanocomposite.

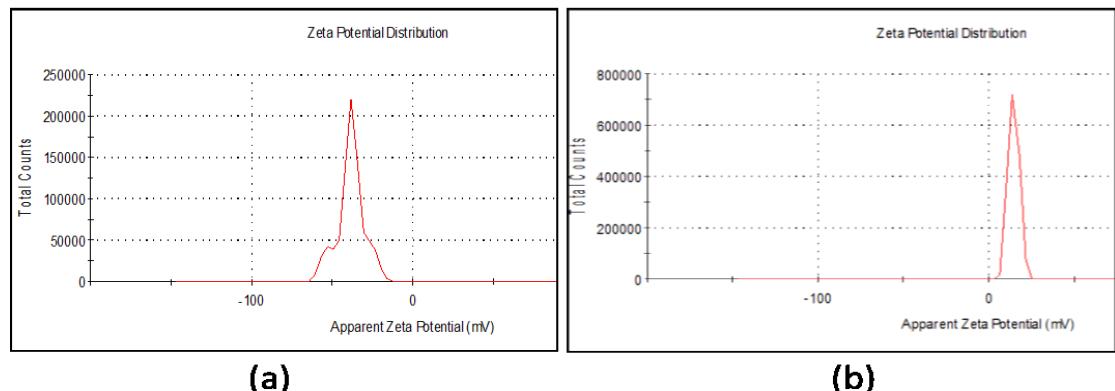


Figure (5): (a) Negative Net charge on AgNPs (Citrate capping) -38.4 mV .(b) Negative Net charge on TOB-AgNPs nanocomposite +14.7 mV.

A more specific characterization tool that confirm the conjugation formation was FTIR spectrum. FTIR analysis of AgNPs showed a broad absorption bands appearing at 3425 cm^{-1} is assigned for O-H stretching vibration (Das et al., 2013). Presence of the sharp peak at 2922 cm^{-1} was assigned to C-H stretching vibration. Also, The sharp and strong absorption band at 1636 cm^{-1} and 1429 cm^{-1} assigned to the stretching vibration of N-H and C=O group respectively where 1058 cm^{-1} assigned to C-N stretching (Marimuthu et al., 2011).The FTIR analysis of pure TOB gave strong absorption bands around 3363 cm^{-1} , 2921 cm^{-1} attributed to the stretching of amine and hydroxyl groups, and the stretching of aliphatic CH respectively. Other peaks at 1602 , 1456 , 1380 cm^{-1} respectively attributed to the bending of NH group, CH_2 scissoring, OH bending and finally 1023 cm^{-1} corresponding to C-O & C-N which totally agree with (Pignatello et al., 2011). FTIR of TOB-AgNPs nanocomposite showed also peaks at 3454 cm^{-1} & 2924 cm^{-1} corresponding to hydroxyl group and stretching of aliphatic CH respectively. Disappearance of Tobramycin original peaks at 1456 cm^{-1} of CH_2 scissoring , 1380cm^{-1} of OH bending and 1023cm^{-1} of C-O & C-N .Also Appearance of new peak at 2072 cm^{-1} confirmed that a new conjugate might be formed.

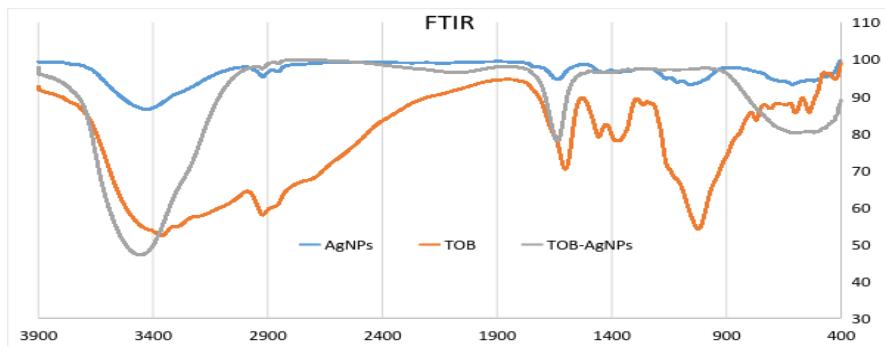


Figure (6): FTIR of silver nanoparticles, Tobramycin and Tobramycin-silver nanoparticles nanocomposite representing effect of conjugation process on chemical bonds formation.

D. Determination of minimum inhibitory concentration (MIC)

The antibacterial activity of the AgNPs, Tobramycin and TOB-AgNPs were tested against the Gram-positive *Staphylococcus aureus* & the Gram-negative bacteria *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Proteus mirabilis* and *Acinetobacter baumannii*. Results is presented in Table (2) in $\mu\text{g/ml}$.

Each experiment was repeated three times, and the resulting bacterial growths corresponding to a particular sample were reported as the mean \pm standard deviation ($n = 3$).

Strain	Silver	Tobramycin	Silver-tobramycin	ANOVA		Tukey's test			% efficacy
	Mean \pm SD	Mean \pm SD	Mean \pm SD	f	P-value	S&T	S&C	T&C	
<i>Acinetobacter baumannii</i>	56.50 \pm 19.57	2218.3 \pm 591.2	226.2 \pm 78.40	20.83	0.0020	**	ns	**	980%
<i>Klebsiella pneumonia</i>	226.2 \pm 78.40	341.3 \pm 147.8	135.7 \pm 0.0	3.415	0.1023	ns	ns	ns	250%
<i>Pseudomonas aeruginosa</i>	181.0 \pm 78.40	341.3 \pm 147.8	135.7 \pm 0.0	3.754	0.0876	ns	ns	ns	250%
<i>Proteus mirabilis</i>	271.5 \pm 0.0	682.7 \pm 295.6	181.0 \pm 78.40	6.880	0.0280	ns	ns	*	375%
<i>Staphylococcus aureus</i>	271.5 \pm 0.0	853.3 \pm 295.6	113.1 \pm 39.20	15.38	0.0043	*	ns	**	750%

Table (2): Statistical analysis for MIC for AgNPs, Tobramycin and TOB-AgNPs nanocomposite.

S=Silver

T=Tobramycin

C= TOB-AgNPs

Nanocomposites

***	Extremely significant	< 0.001
**	Very significant	0.001 to 0.01
*	Significant	0.01 to 0.05
ns	Not significant	>0.05

Antimicrobial susceptibility was performed using microdilution method. Results showed that the efficacy of TOB-AgNPs nanocomposite increased by 980% than Tobramycin alone against *Acinetobacter baumannii*, the difference in MIC was very significant between (AgNPs and Tobramycin) and (TOB-AgNPs & tobramycin) where MICs of both AgNPs and TOB-AgNPs nanocomposite were lower than Tobramycin by 40 and 10 times respectively. In case of *Klebsiella pneumonia* and *Pseudomonas aeruginosa* the difference in MIC was non-significant between AgNPs, tobramycin and TOB-AgNPs nanocomposite and the efficacy of nanocomposite increased only by 250% than Tobramycin alone. The difference in MIC between TOB-AgNPs nanocomposite and tobramycin was significant against *Proteus mirabilis* where the efficacy of TOB-AgNPs nanocomposite increased by 375% than Tobramycin alone. Finally, in case of gram positive bacteria (*Staphylococcus aureus*) the difference in MIC between TOB-AgNPs nanocomposite and tobramycin was very significant and the efficacy of TOB-AgNPs nanocomposite increased by 750% than Tobramycin, also the difference in MIC between AgNPs and tobramycin was significant where MICs of AgNPs and TOB-AgNPs nanocomposite were lower than tobramycin by 3 and 10 times respectively.

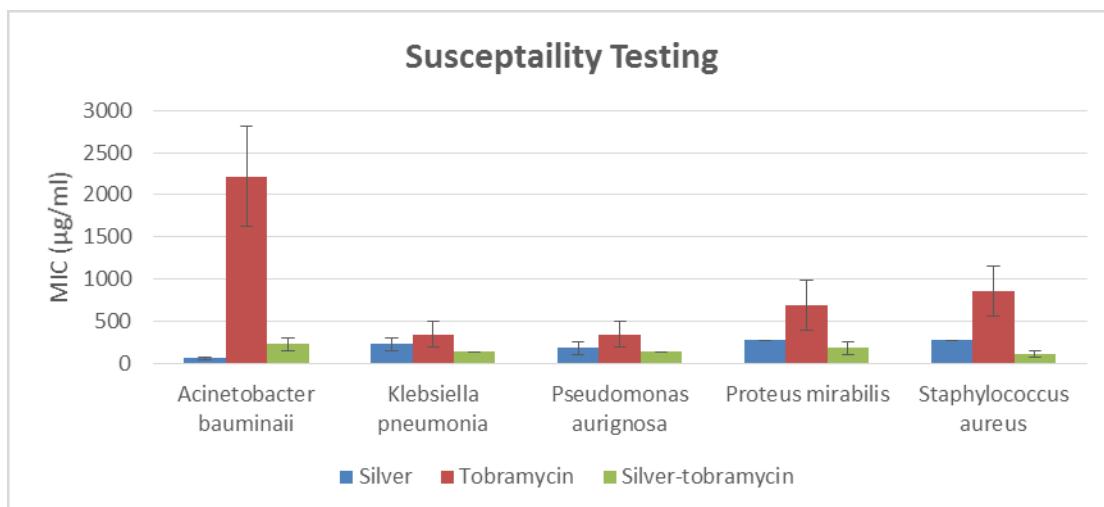


Figure (7): Statistical analysis of MIC ($\mu\text{g}/\text{ml}$) represents antimicrobial activity of Tobramycin, Silver Nanoparticles and TOB-AgNPs nanocomposite.

The obtained results indicate that the conjugation between AgNPs and tobramycin can enhance the activity of tobramycin and reduce the microbial resistance of different species under study, that may attributed to; First, the small size and large surface area of AgNPs of the composites which facilitates the penetration of the conjugate through the bacterial cell wall and can easily reach the nuclear content of bacteria (Lok et al., 2006), that reinforces the idea that the greatest the surface area the greatest the antibacterial activity (Jeong et al., 2005). Second, the formation of TOB-AgNPs nanocomposite may resulted in a new antibiotic formulation which totally differ from pharmacokinetic point of view than that of Tobramycin alone and cannot be recognized by bacterial strains that previously known to be resistant to Tobramycin. Third, the conjugation of Tobramycin with AgNPs forming well-arranged Tobramycin molecules over the surface which can be easily targeted to the sites of action on the bacterial cell wall.

IV. Conclusion

In this study, Tobramycin conjugated with silver nanoparticles in order to increase its antimicrobial activity. Clinical isolates were collected from different Egyptian hospitals, identified by standard microbial procedures and confirmed by API. The isolates were *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Proteus mirabilis* and *Acinetobacter bauminaii*. Antibiotic susceptibility were also performed against different classes of antibiotics. The results revealed that 91.6% of isolates were resistant to tobramycin. Minimum inhibitory concentrations (MIC) of AgNPs, Tobramycin and TOB-AgNPs were determined using microdilution method. A comparative study were established between Tobramycin and TOB-AgNPs, results showed that the activity of TOB-AgNPs nanocomposite increased by 980%, 250%, 250%, 375% and 750% than Tobramycin alone against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Proteus mirabilis* and *Acinetobacter bauminaii* respectively. The obtained results indicate that the conjugation between AgNPs and Tobramycin can enhance the activity of Tobramycin and reduce the microbial resistance against gram positive and gram negative bacteria.

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