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Antibacterial Effect of Nanoparticles Against Antibiotic Resistant Gram Positive and Gram-Negative Bacteria

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Abstract

The increasing emergence of bacterial resistance occurred mainly due to continuous persistent exposure to antibiotics causing high morbidity and mortality so studies in controlling the infections caused by these strains are required. Nanoparticles have been reported as non-antibiotic therapeutic agents, that have antibacterial effect against many pathogens including bacteria and fungi. Fifty bacterial isolates were divided into two groups 25 methicillin-resistant Staphylococcus aureus (MRSA) and 25 carbapenem-resistant gram-negative isolates collected from different infection sites from patients admitted to AL-Zahraa university hospital, The isolates were identified by routine culture and sensitivity using disc diffusion susceptibility test and by the Vitek 2 automated system. The synthetic nanoparticles AgNPs, ZnONPs and chitosan NPs were characterized by TEM, EDX and FTIR. The antibacterial effect of NPs was screened using agar well diffusion method. The cytotoxicity of NPs towards human lung fibroblasts was determined using cell culture assays. The minimum inhibitory concentration (MIC) of NPS and antibacterial effects alone and in combination with antibiotic ciprofloxacin were determined against twenty isolates, ten MRSA and ten carbapenem-resistant E. coli and klebsiella isolates using the microdilution method. The biocompatible concentration of AgNPs (6 µg/mL), ZnONPs (500 µg/mL) and chitosan (100 µg/mL) were non-cytotoxic but also showed no antibacterial effects However, when combined with antibiotic ciprofloxacin, the biocompatible concentration of NPs resulted in significant inhibition of bacterial growth for multiple bacterial species. This study presents a promising strategy with further testing in vivo, to develop novel antimicrobial agents and strategies to confront emerging antimicrobial resistance.

Keywords: Nanoparticles, Antimicrobial, Resistance, Synergistic.

1. Introduction

Antibiotics are powerful medications that fight certain infections and can save lives when used properly. However, the massive prescription of antibiotics and their non-regulated and extensive usage has resulted in the development of extensive antibiotic resistance in microorganisms and the emergence of super bacteria; this has been of great clinical significance and is considered a major crisis The problem of antibiotic resistance, which has limited the use of cheap and old antibiotics, has necessitated the need for a continued search for new antimicrobial compounds.

Nanotechnology is rapidly advancing and is used for a wide range of applications in medicine. The potential of nanoparticles as antimicrobial agents is considered as an alternative approach to overcome the challenge posed by multidrug resistance in bacteria. Nanoparticles are a wide class of materials that include particulate substances, which have one dimension less than 100 nm at least. The unique physiochemical properties of the nanoparticles combined with the growth inhibitory capacity against microbes has led to the upsurge in the research on nanoparticles and their potential application as antimicrobials. Many nanoparticles such as silver, copper, chitosan, and metal oxide nanoparticles like titanium oxide or zinc oxide have been reported to have antibacterial property

Nanoparticles have a multi-level mode of action influencing many bacterial structures and metabolic processes including inactivating bacterial enzymes, disrupting cell wall, metabolic processes and increasing cell permeability.

However, it is imperative to carry out extensive studies on these nanomaterials to determine their impact on normal tissues prior to wide scale applications and to evaluate the impact on humans and the environment.

2. Patients and Methods

Fifty clinical bacterial isolates were randomly collected from AL-Zahraa university hospital. twenty-five MRSA isolates and twenty-five carbapenem-resistant gram-negative isolates out of 82 MRSA isolates and 48 carbapenem-resistant gram-negative isolates that were isolated from clinically significant infections sites during the period of October 2019 to October 2020. Identification was done by the traditional microbiological methods and was confirmed by Vitek 2 automated system.

2.1 Antibiotics susceptibility test

Antibiotics susceptibility test was determined by the standard disc diffusion method according to CLSI document M02-A12 (CLSI 2018) [1] and by the Vitek 2 automated system.

2.2 Nanoparticles synthesis

Silver nanoparticles (AgNPs), Zinc oxide nanoparticles (ZnO NPs) and chitosan NPs were synthesized and prepared in the Naqaa Foundation for scientific research and Nanotechnology- Egypt according to (Zhou and Wang,2012) [2], (Suntako,2015) [3] and (Tang et al., 2006) [4].

2.3 Characterization of nanoparticles

Characterization of silver, zinc oxide and Chitosan nanoparticles size and shape by Transmission Electron Microscope (TEM) was done using JOEL GEM 1010 transmission electron microscope at 70 KV at The Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University.

Characterization of silver nanoparticles and zinc oxide using Energy Dispersive X-ray microanalysis (EDX) for elemental analysis and Characterization of chitosan NPs using Fourier-transform infrared spectroscopy (FTIR) for functional groups was done at (RCMB), Al-Azhar University.

2.4 Antibacterial effect of nanoparticles

Qualitative screening was accomplished by the agar well diffusion method to detect their antibacterial effect ***(Valgas et al., 2007)*** [5]. They were confirmed by the determination of (MICs) by broth

microdilution according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2018) [1].

2.5 Cytotoxic effect of nanoparticle

Human lung fibroblast normal cells were used to evaluate the cytotoxic effect of nanoparticles and the study was done at the tissue culture unit in The Regional Center for Mycology and Biotechnology.

2.6 Synergistic effect of nanoparticles with antibiotics

The MIC by broth microdilution for ciprofloxacin alone and with each of the three nanoparticles (Ag-NPs 6 µg/ml, ZnO-NPs 500 µg/ml and Chitosan- NPs: 100 µg/ml) were detected for randomly selected 10 MRSA and 10 carbapenem-resistant E. coli and klebsiella isolates according to (CLSI 2018) and (El-Azizi, 2016) [6].

3. Results

Figures 1 and 2 demonstrate the distribution of MRSA isolates and carbapenem-resistant isolates according to the type of clinical samples. The Carbapenem-resistant gram-negative members and their percentages are demonstrated in Table 1. The Antibiotic susceptibility test results of MRSA and Carbapenem-resistant gram-negative isolates are demonstrated in Tables 2 and 3.

3.1 Characterization of nanoparticles

3.1.1 Characterization of silver, zinc oxide and Chitosan nanoparticles size and shape by Transmission Electron Microscope (TEM)

The TEM Micrograph revealed the size, shape and general morphology of the nanoparticles and showed spherical-shaped silver nanoparticles with an average size of 10.16 nm, spherical-shaped zinc oxide nanoparticles with an average size of 9.064 nm and spherical-shaped Chitosan nanoparticles with an average size 10.625 nm (Figure 3).

3.1.2 Characterization of silver and zinc oxide nanoparticles using energy-dispersive X-ray microanalysis

The EDX analysis of silver nanoparticles showed percentage relative composition of elements such as sulfur (S) 3%, Silicon (Si) 2%, Copper (cu) 5%, Zinc (Zn) 8% and Silver (Ag) 71.5% while EDX analysis of zinc oxide nanoparticles yielded 97.9% of zinc (Figure 4).

3.1.3 Characterization of Chitosan nanoparticles using Fourier-transform infrared spectroscopy

The peak of the OH group (the main functional group of chitosan) was seen at 3448 cm-. The band 1635cm-1 represents C=O stretching in the amide group and the peak of the PO4 group of tripolyphosphate (TPP) was at 702cm-1 **(Figure 5)**

3.2 Assessment of Antibacterial Activities of Ag-NPs, ZnO-NPs and Chitosan Nanoparticles:

3.2.1 Screening of the antibacterial effect of nanoparticle

Table 4 demonstrates that both MRSA and carbapenem-resistant isolates were sensitive to Ag-NPs, and chitosan nanoparticles and non-responsive to ZnO-NPs.

By comparing the antibacterial effect of nanoparticles between the two groups studied, we found that the antibacterial effect was greater on carbapenem-resistant and highly significant with Ag-NPs (400 µg/ml) and significant with chitosan nanoparticles Table .5, Figure .6.

3.2.2 The minimum inhibitory concentration of nanoparticles was confirmed using broth microdilution method

Table 6 demonstrates the MIC results of Ag, chitosan and ZnO nanoparticles on ten MRSA and ten carbapenem-resistant isolates. By comparing the MIC results between the two groups studied, we found that the antibacterial effect was greater on

carbapenem-resistant than MRSA isolates (Table 7).

3.2.3 Comparison between Minimum Inhibitory Concentration of different Nps on carbapenem-resistant *E. coli* and *Klebsiella pneumonia*

E. coli was more sensitive than Klebsiella pneumonia with no difference between the two strains regarding the effect of ZnO-NPs**.** Table 8

3.3 Cytotoxicity assay

3.3.1 ZnO nanoparticles

Inhibitory activity against human lung fibroblast normal cells was detected with $CC_{50=}$ 650 ±5.4 µg/ml.

3.3.2 Chitosan nanoparticles

Weak inhibitory activity against human lung fibroblast normal cells was detected with CC_{50} = > 2000 µg/ml.

3.3.3 Ag nanoparticles

Inhibitory activity against human lung fibroblast normal cells was detected with $CC_{50=}$ 5.87 ± 0.17 µg/ml (Table 9)

3.3.4 Evaluation of the synergistic effect of nanoparticles with antibiotics

Tables 10 and 11 demonstrate a significant synergistic increase in the antibacterial effect of ciprofloxacin when added to the three nanoparticles in both MRSA and carbapenem-resistant isolates.

Our results showed that chitosan nanoparticles were safe while Ag nanoparticles were most effective but showed cytotoxic effects and were dose dependent.

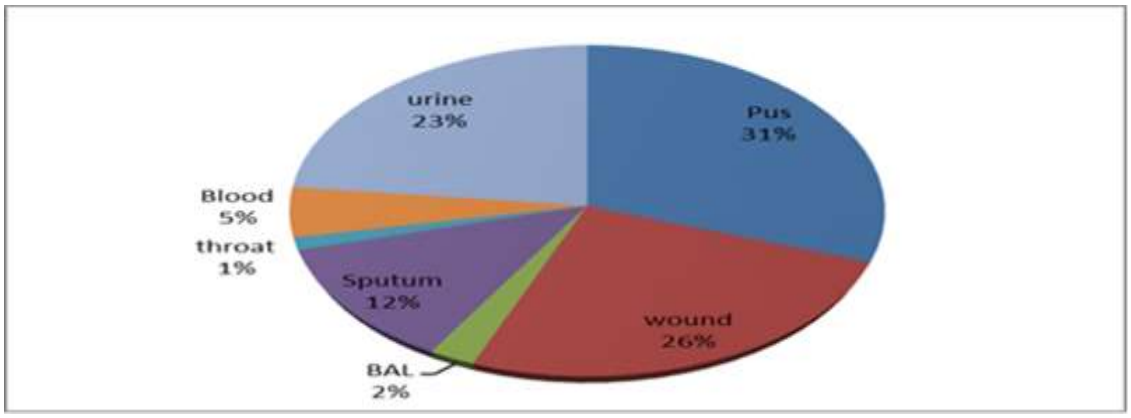

Figure (1): Distribution of MRSA isolates according to the type of clinical specimen.

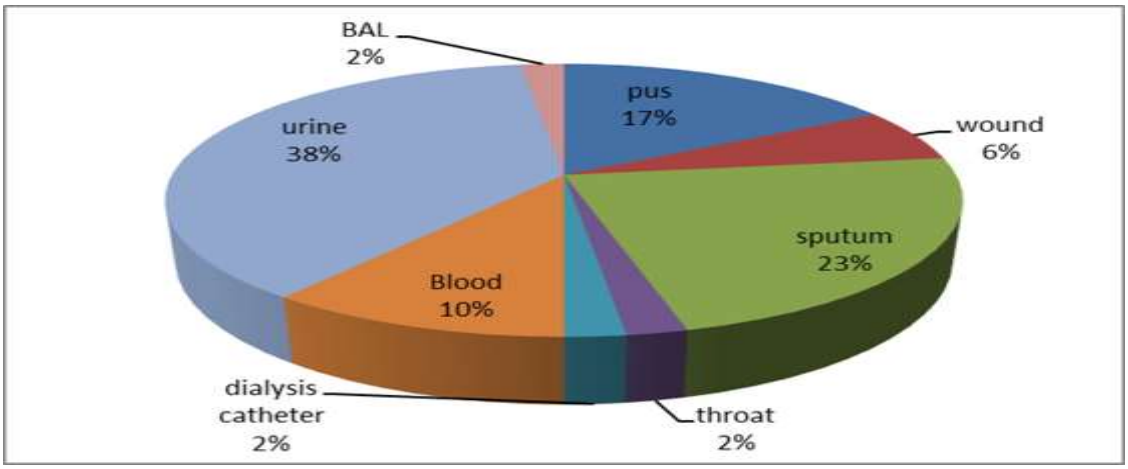

Figure (2): Distribution of carbapenem-resistant gram-negative isolates according to the type of clinical specimen

Table (1): Identification of carbapenem-resistant gram-negative isolates.

Total carbapenem-resistant gram-negative isolates (total NO. 48)	Number of isolates and percentage
K. pneumoniae	18(37.5%)
pseudomonas aeroginsa	14 (29.1%)
Escherichia coli	13 (27%)
Entrobacter cloaca	2(4.1%)
Serratia fonticola	1(2 %)

Table (2): Antibiotic susceptibility of MRSA.

	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
P	0	0.0%	0	0.0%	25	100.0%
OX	0	0.0%	0	0.0%	25	100.0%
GM	11	44.0%	3	12.0%	11	44.0%
CIP	11	44.0%	2	8.0%	12	48.0%
LEV	13	52.0%	0	0.0%	12	48.0%
MXF	13	52.0%	0	0.0%	12	48.0%
E	12	48.0%	1	4.0%	12	48.0%
DA	13	52.0%	3	12.0%	9	36.0%
QD	25	100.0%	0	0.0%	0	0.0%
LZD	25	100.0%	0	0.0%	0	0.0%
VA	25	100.0%	0	0.0%	0	0.0%
TE	12	48.0%	0	0.0%	13	52.0%
TGC	25	100.0%	0	0.0%	0	0.0%
RD	25	100.0%	0	0.0%	0	0.0%
SXT	20	80.0%	0	0.0%	5	20.0%

Table (3): Antibiotic susceptibility of carbapenem-resistant gram-negative strains.

	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
AMP	0	0.0%	0	0.0%	24	100.0%
SAM	0	0.0%	0	0.0%	24	100.0%
TZP	0	0.0%	0	0.0%	24	100.0%
CZ	0	0.0%	0	0.0%	24	100.0%
FOX	0	0.0%	0	0.0%	24	100.0%
CAZ	0	0.0%	0	0.0%	24	100.0%
CRO	0	0.0%	0	0.0%	24	100.0%
FEP	0	0.0%	0	0.0%	24	100.0%
MEM	0	0.0%	0	0.0%	24	100.0%
AK	11	45.8%	0	0.0%	13	54.2%
GM	9	37.5%	6	25.0%	9	37.5%
TOB	4	16.7%	1	4.2%	19	79.2%
CIP	0	0.0%	1	4.2%	23	95.8%
LEV	0	0.0%	0	0.0%	24	100.0%
SXT	3	12.5%	0	0.0%	21	87.5%

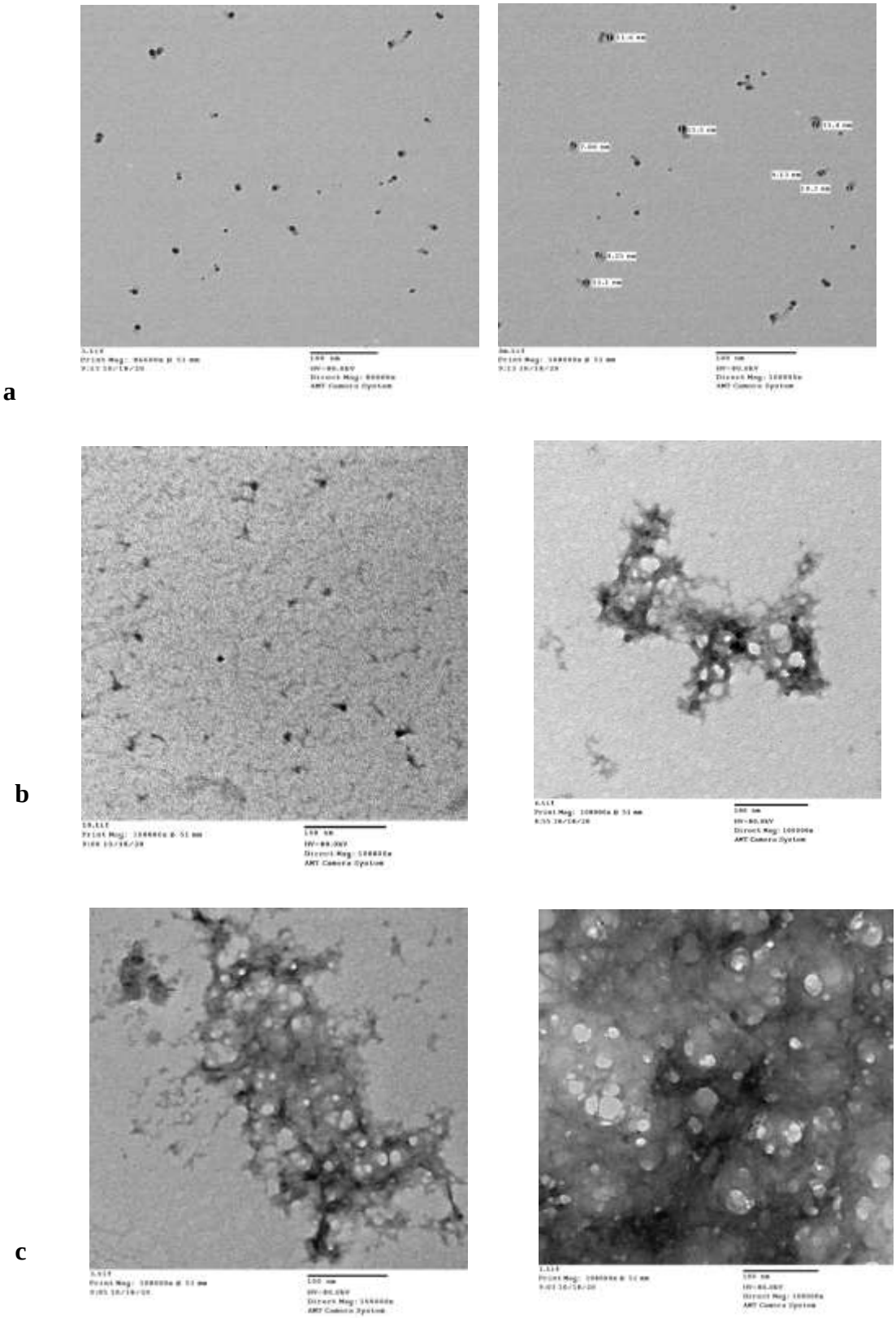

Figure (3): Images of silver(a), zinc oxide(b) and Chitosan nanoparticles(c) under Transmission Electron Microscope.

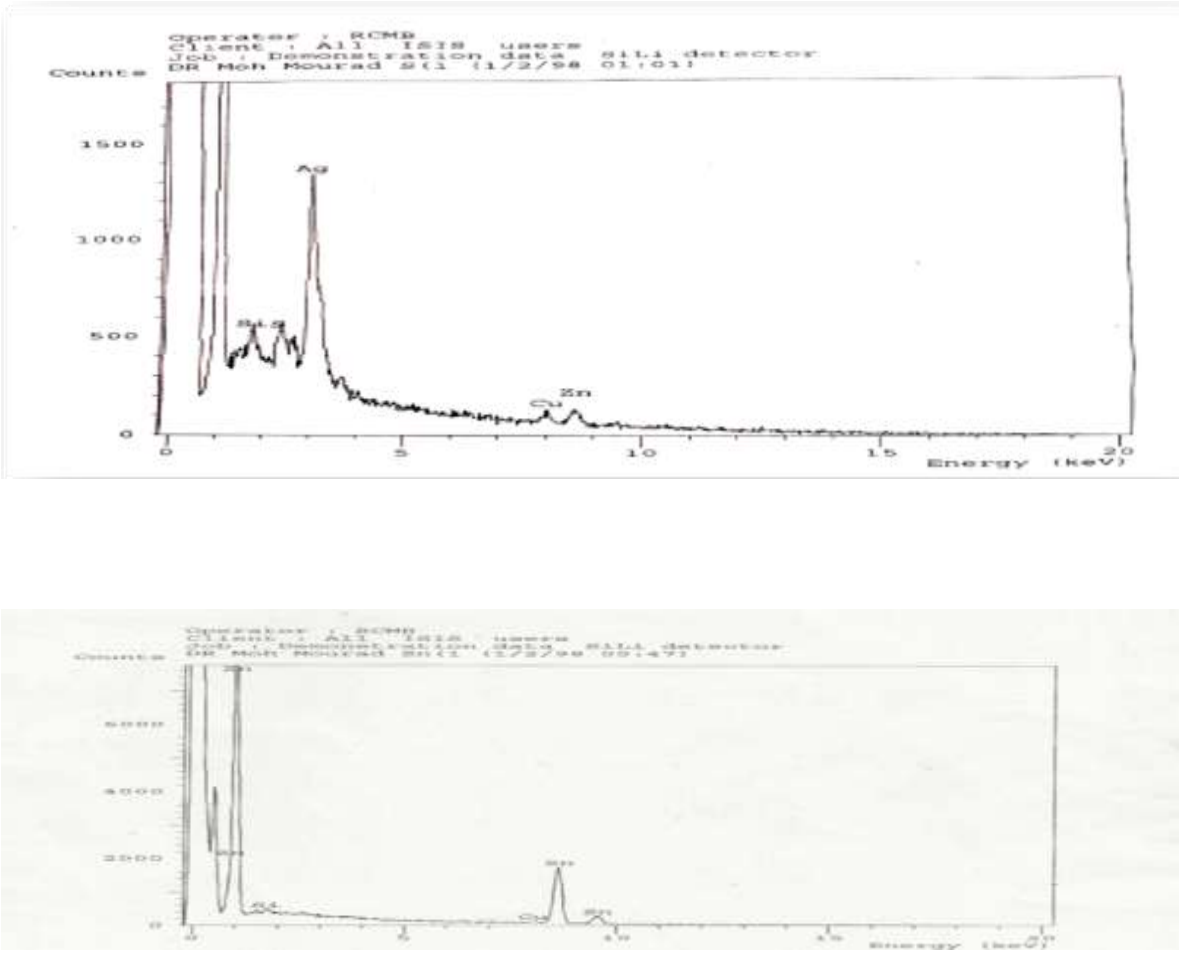

Figure (4): Energy Dispersive X-ray microanalysis of silver (a) and Zinc oxide nanoparticles (The Regional Center for Mycology and Biotechnology)

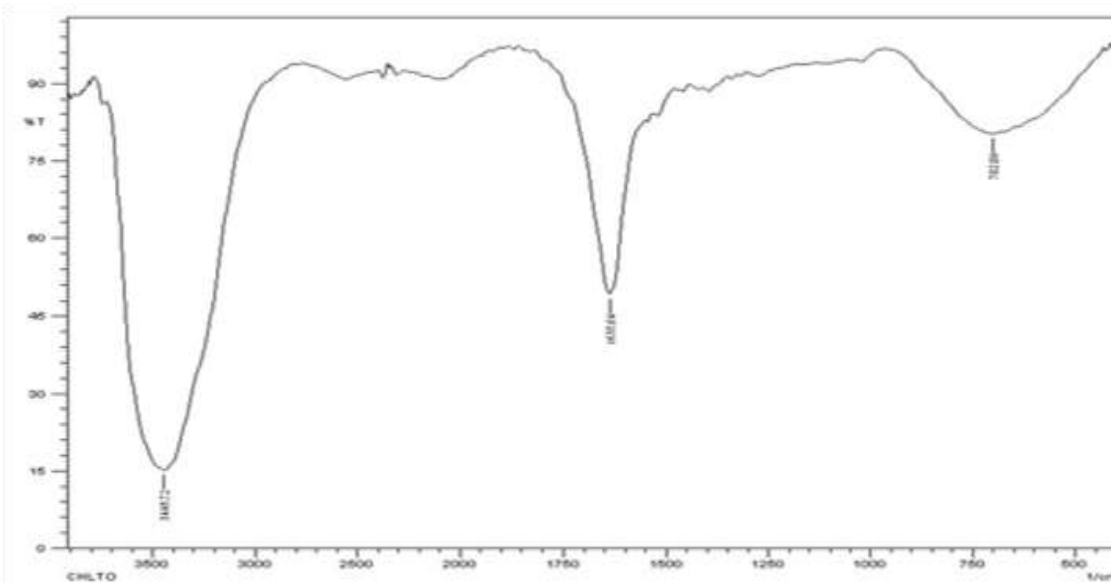

Figure (5): FTIR Analysis of Chitosan Nanoparticles

a

b

Figure (6): Antibacterial activities of Nanoparticles by Agar well diffusion method in MRSA(a) and carbapenem-resistant isolates (1 for ZnO-NPs, 2 for Chitosan and 3 for Ag-NPs)

Table (4): Results of antibacterial effect of nanoparticles on 25 methicillin-resistant staphylococcus aureus and 25 carbapenem-resistant gram-negative isolates by good diffusion through measuring the zone of growth inhibition in mm.

Nanoparticle	Response	MRSA		CARBA	
		No.	%	No.	%
Zn oxide (2000 micro gm/ml)	Non-responsive	25	100.0%	24	95.8%
	Responsive	0	0.0%	1	4.2%
Chitosan (2000 micro gm/ml)	Non-responsive	16	64.0%	18	75.0%
	Responsive	9	36.0%	6	25.0%
Ag (400 micro gm/ml)	Non-responsive	3	12.0%	9	37.5%
	Responsive	22	88.0%	16	62.5%

Table (5): Comparison between antibacterial activities of different Nps on carbapenem-resistant and MRSA isolates by agar well diffusion through measuring the zone of growth inhibition in mm

		Group 1 MRSA No. = 25	Group 2 CARBA No. = 25	Test value	P-value	Sig.
Chitosan (2000 micro gm/ml)	Median (IQR)	12 (11 – 12)	13 (12 – 14	2.512•	0.027	S
	Mean ± SD	11.8 ± 0.84	13.0 ± 0.87			
	Range	11 – 13	12 – 14			
Ag (400 micro gm/ml)	Median (IQR)	14 (11 – 16)	16.5 (16 – 20	3.192•	0.003	HS
	Mean ± SD	14.53 ± 3	17.86 ± 3.19			
	Range	11 – 20	15 – 29			

Table (6): Range and Interquartile Range of a minimal inhibitory concentration of nanoparticles against MRSA and carbapenem-resistant gram-negative strains.

	MRSA		CARBA	
	Range	**Median (IQR)**	**Range**	**Median (IQR)**
Zn Oxide (micro gm/ml)	250 – 500	500 (250 – 500)	500 – 500	500 (500 – 500)
Chitosan (micro gm/ml)	31.25 – 125	125 (31.25 – 125)	15.6 – 125	23.43 (15.6 – 31.25)
Ag (micro gm/ml)	12.5 – 100	25 (18.75 – 62.5)	6.25 – 50	12.5 (9.38 – 18.75)

Table (7): Comparison of Minimum Inhibitory Concentration of different Nps on carbapenem-resistant and MRSA isolates.

		Group 1	**Group 2**	**Test value**	**P-value**	**Sig.**
		MRSA	**CR**			
Zn Oxide (micro gm/ml)	Median (IQR)	500 (250 – 500)	500 (500 – 500)	-1.069•	0.285	NS
	Mean ± SD	416.67 ± 129.1	500.0 ± 0.0			
	Range	250 – 500	500 – 500			
Chitosan (micro gm/ml)	Median (IQR)	125 (31.25 – 125)	23.43 (15.6 – 31.25)	-2.101•	0.036	S
	Mean ± SD	89.29 ± 45.75	39.05 ± 42.80			
	Range	31.25 – 125	15.6 – 125			
Ag (micro gm/ml)	Median (IQR)	25 (18.75 – 62.5)	12.5 (9.38 – 18.75)	-1.975•	0.048	S
	Mean ± SD	40.63 ± 37.05	17.19 ± 14.47			
	Range	12.5 – 100	6.25 – 50			

Table (8): Comparison between Minimum Inhibitory Concentration of different Nps on carbapenem-resistant E. coli and Klebsiella pneumonia

		Group 2A	**Group 2B**
		E. coli	**Klebsiella pneumoniae**
Chitosan (micro gm/ml)	Median (IQR)	15.6 (15.6 – 15.6)	31.25 (31.25 – 125)
	Mean ± SD	15.6 ± 0.0	62.5 ± 54.13
	Range	15.6 – 15.6	31.25 - 125
Ag (micro gm/ml)	Median (IQR)	9.38 (6.25 – 18.75)	12.5 (12.5 – 31.25)
	Mean ± SD	12.5 ± 8.84	21.88 ± 18.75
	Range	6.25 – 25.0	12.5 – 50
Zn Oxide (micro gm/ml)	Median (IQR)	500 (500 – 500)	500 (500 – 500)
	Mean ± SD	500.0 ± 0.0	500.0 ± 0.0
	Range	500 – 500	500 – 500

Table (9): Comparison between cytotoxicity effects of different silver nanoparticles against WI-38 cells (human lung fibroblast normal cells)

Different NPs	**Nps conc. (µg/ml)**	**Viability %**
ZnO NPs	650	61.79%
Chitosan NPs	=2000	87.91
Ag NPs	5.87	65.21%

Table (10): Results of Combination of ciprofloxacin with nanoparticles against Methicillin-resistant staphylococcus aureus by broth microdilution method

	Range	**Mean ± SD**	**Median (IQR)**	**Test value**	**P-value**	**Sig.**
Cip alone	15.62 – 500	173.44 ± 186.11	125 (31.25 – 250)	-	-	-
Cip+Ag NPs (6 microgram/ml)	3.9 – 500	141.01 ± 202.73	31.25 (7.8 – 250)	-2.207	0.027	S
Cip+Zno NPs (500 microgram/ml)	3.9 – 500	135.54 ± 205.8	23.43 (7.8 – 250)	-2.032	0.042	S
Cip+Chit. NPs (100 microgram/ml)	0.97 – 500	131.14 ± 208.52	15.6 (3.9 – 250)	-2.371	0.018	S

Cip: ciprofloxacin, Ag NPs: silver nanoparticles, Zno NPs: zinc oxide nanoparticles, Chit. NPs: Chitosan nanoparticles, IQR: Interquartile Range, P-value: probability values: significant, Mann-Whitney test, P>0.05: Non-significant (NS); P <0.05: Significant; P <0.01: Highly significant.

Table 11: Results of the Combination of ciprofloxacin with nanoparticles against Carbapenem-resistant gram-negative isolates by broth microdilution method

	Range	Mean ± SD	Median (IQR)	Test value	P-value	Sig.
Cip alone	15.6 – 250	135.94 ± 88.41	125 (62.5 – 250)	-	-	-
Cip+Ag (6 microgram/ml)	1.9 – 250	90.03 ± 95.08	46.88 (15.6 – 125)	-2.366	0.018	S
Cip+Zno (500 microgram/ml)	15.6 – 500	226.56 ± 170.09	250 (62.5 – 250)	-1.997	0.046	S
Cip+Chit (100 microgram/ml	1.9 – 250	67.38 ± 72.74	46.88 (31.25 – 62.5)	-2.371	0.018	S

Cip: ciprofloxacin, Ag NPs: silver nanoparticles, Zno NPs: zinc oxide nanoparticles, Chit. NPs: Chitosan nanoparticles, IQR: Interquartile Range, P-value: probability values: significant, Mann-Whitney test, P>0.05: Non-significant (NS); P <0.05: Significant; P <0.01: Highly significant.

4. Discussion

The emergence of gram-positive and gram-negative antibiotic-resistant pathogens including MRSA and carbapenem-resistant strains are a major threat and burden for healthcare systems worldwide. World health leaders have described antibiotic-resistant microorganisms as “nightmare bacteria” that “pose a catastrophic threat” to people in every country in the world **(Luepke et al., 2017; Alvarez et al., 2019) [7, 8].**

Staphylococcus aureus is a bacterial species capable of colonizing and causing infections in a wide range of hosts. It is the cause of serious infections in humans and the number one of hospital-associated infections.

In the present study, the highest MRSA isolates were recovered from pus (31%) followed by wound (26%) followed by urine samples (23 %) This higher frequency of MRSA in pus specimen has been previously reported especially in diabetic foot infections, surgical wounds, and burn patients **(Garoy et al., 2019) [9].** Also, in a study conducted by **Ibrahim et al. (2020) [10]** MRSA isolates were mostly found in pus (57.1%) followed by urine samples (33.3%), the high MRSA isolates recovered from the investigated urine clinical samples indicating increasing prevalence of Staphylococcus aureus in urinary tract infection. In Egypt, it was found that more than half of hospitals had at least one carbapenem-resistant isolate and half (47.9%) of Enterobacteriaceae isolates were, carbapenem-resistant which is higher than estimates reported from other Arab, African, or Asian countries **(Kotb et al., 2020) [11].**

In our study, the most common carbapenem-resistant isolates were K. pneumoniae (37.5%) followed by pseudomonas aeroginsa (29.1%) and E. coli (27%) Similar results were reported in other studies **(Kotb et al., 2020 and Amalia et al., 2019) [11,12].**

Antimicrobial efficacy of NPs was evaluated by many researchers against a broad range of microbes, including many strains of bacteria, fungi, and viruses. Nano-sized particles are well-established as a promising alternate to antibiotic therapy because they possess unbelievable potential for solving the problem associated with the development of multidrug resistance in pathogenic microorganisms, hence also regarded as next-generation antibiotics **(Rai et al., 2014) [13]**.

In the current study, AgNPs was generated by chemical reduction of silver nitrate while ZnO nanoparticles were synthesized by the precipitation method and chitosan nanoparticles were synthesized by dissolving chitosan in acetic acid which all are established methods for synthesis of nanoparticles. Several studies stated that NPs can be widely synthesized in numerous ways, such as physical, chemical, photochemical, irradiation, laser, green synthesis and biological methods **(Iravani, 2014) [14]. Chou et al. (2005) [15] and Lim et al. (2005) [16]** have reported the chemical procedure for the production of silver nanoparticles**.**

ZnO nanoparticles have been synthesized by **Mazhdi and Khani (2012) [17]** and **Moharram et al. (2014) [18]** via the precipitation method, also **Banerjee et al. (2002) [19]** synthesized chitosan nanoparticles chemically by acetic acid and tripolyphosphate.

In the current study, TEM analysis showed spherical-shaped silver nanoparticles with average size 10.16 nm, spherical-shaped zinc oxide nanoparticles with average size 9.064 nm and spherical-shaped Chitosan nanoparticles with average size 10.625 all of which agreed with the reported literature **(Pal et al., 2007; Srinivasan et al., 2013; Patra and Baek, 2017; Alsammarraie et al., 2018) [20, 21, 22, 23].**

The EDX analysis of silver nanoparticles showed a strong signal at 3 keV in the silver region and thus confirmed the presence of silver nanoparticles in the prepared sample indicating the reduction of Ag+ ions. **Magudapathy et al. (2001) [24]** reported that metallic silver nanoparticles generally show an optical absorption peak approximately at 3 keV. **Gopinath et al. (2010) [25]; Kusumaningrum et al. (2018) [26]** and many others emphasized the same findings.

The EDX analysis of ZnO NPs yielded 97.9% of metallic zinc, **Bhuyan et al. (2015) [27]** and **Suresh et al. (2015) [28]** and many others emphasized the same findings.

According to our results of FTIR analysis, the peak of OH group (the main functional group of chitosan) was seen at 3448 cm-. The band 1635cm-1 represents C=O stretching in amide group and the peak of PO4 group of tripolyphosphate was at 702cm-1 Similar results were observed by **Lam et al. (2012) [29]** and **Mohammadpour et al. (2010) [30].**

The antibacterial activities of different NPs were assayed against common bacterial species namely carbapenem-resistant and MRSA. Agar diffusion tests were performed as a qualitative test to observe and predict the nanoparticles antibacterial behaviour. These methods have many advantages over other methods, such as simplicity, low cost, the ability to test a high number of microorganisms and antimicrobial agents. However, it is not able to determine the MIC, as it is impossible to determine the diffusion of the antimicrobial agent in the agar **(Balouiri et al., 2016) [31].**

In the present study, NPs exhibited a significant inhibitory effect against the tested isolates. The sensitivity of bacterial pathogens towards nanoparticles was found to vary depending on the strains used.

Gram-negative bacteria were more susceptible to the antibacterial effect of NPs than Gram-positive bacteria. This discrepancy could be due to differences in the membrane structure and the composition of the cell wall **(Kim et al., 2007; Shahverdi et al., 2007) [32, 33].**

Our results are similar to results reported by **Deljou and Goudarzi, (2016) [34],** who found a higher antibacterial activity against E. coli (22 mm) and Salmonella typhi (19mm) than S. aureus (15 mm) and S. epidermis (16 mm)**.** Also, **Majeed et al. (2016) [35]** showed that the highest zone of inhibition was observed with E. coli of followed by S. aureus. using AgNPs

Another study by **Prakash et al. (2013) [36]** who demonstrated the antibacterial activity of silver nanoparticles against MDR pathogens namely Klebsiella pneumoniae, Micrococcus luteus and Staphylococcus aureus. The maximum antibacterial efficacy was observed against multidrug-resistant Klebsiella pneumoniae (18mm), and the moderate activity was against multidrug resistant staphylococcus aureus (10 mm) and Micrococcus luteus(11mm). Similar results were demonstrated by **Ninganagouda et al. (2013) [37] and Singh et al. (2013) [38]** who reported that biosynthesized AgNPs have better growth inhibition against Gram-negative than Gram-positive bacteria. They found that the major mechanism through which AgNPs manifest antibacterial properties was either by anchoring or penetrating the bacterial cell

wall. But these results disagreed with **Anima and Saravanan (2009) [39]** and **Fayaz et al. (2010) [40]** who observed that antibacterial activity was maximum in the case of MRSA, intermediate in MRSE, whereas the antibacterial activity seen against S. typhi and K. pneumoniae were moderate. And Yasir **et al. (2018) [41]** and **Jalal et al. (2019) [42]** recorded the antibacterial effects of silver nanoparticles on Staph. aureus (20 mm) and E. coli (18 mm).

Also, **Wisam et al. (2018) [43]** showed the diameter of the inhibition zones of biosynthesized AgNPs against Bacillus subtilis (31 mm) and Escherichia coli (30 mm).

In the present study the MIC activity of NPs on MRSA and Gram-negative carbapenem-resistant E coli and Klebsiella showed that carbapenem-resistant strains are more sensitive than MRSA to Chitosan NPs and Ag-NPs effect with lower MIC values ranging in MRSA (31.25 – 125) µg/ml for Chitosan nanoparticles and (12.5 – 100) µg/ml for Ag-NPs and in carbapenem-resistant E coli and Klebsiella (15.6 – 125) µg/ml for Chitosan nanoparticles and (6.25 – 50) µg/ml for Ag-NPs with non-significant effect of ZnO-NPs on both carbapenem-resistant and MRSA

Other studies demonstrated that the antimicrobial activity of AgNPs on Gram-negative bacteria was dependent on the concentration of AgNPs and was closely associated with the formation of pits in the cell wall of bacteria. Accumulation of the AgNPs in the pits results in the permeability of the cell membrane, causing cell death **(Sondi and Salopek-Sondi, 2004) [44].**

Considering ZnO-NPs our results are in accordance with results of **Lakshmi et al. (2012) [45]** who observed no significant variation amongst gram-positive and gram-negative bacteria

Considering chitosan NPs our results are in accordance with results of **Joshi et al. (2009) [46]** and who observed that antibacterial activity was more evident against gram-negative bacteria than gram-positive bacteria.

In the present study, cell viability and metabolic activity studies were conducted using by exposing the normal human fibroblast cell line (WI-38) cells to NPs at different concentrations for 24 hours. The results of the assay showed a dose-dependent decrease in the percent viability of the cells. In the present study, cytotoxicity data were fitted to a sigmoidal curve to calculate the CC50 of NPs that caused a 50% inhibition in comparison to untreated controls.

Inhibitory cytotoxic activity of AgNps against normal human lung fibroblast cells was detected under these experimental conditions with CC50 5.87 µg/ml.

Moteriya and Chanda (2020) [47] found that the cytotoxic effect of AgNPs on HeLa cell line was dose-dependent, and the cell death increased with increasing concentration of AgNPs and cell viability was 100% at concentration 50 ug/ml.

The synthesized ZnO NPs showed inhibitory cytotoxic activity against normal human lung fibroblast cells with CC50 650µg/ml. Similar dose-dependent cytotoxic effect of ZnO NPs on human liver carcinoma cells and sliver nanoparticles on HeLa cancer cells is reported by **Chung et al. (2015) [48]** and **Moteriya and Chanda (2016) [47]. Park et al. (2011) [49]; Gurunathan et al. (2013) [50]** suggested that nanoparticles induce ROS generation, DNA fragmentation, and membrane leakage which resulted in the cell death. **Prashanth et al. (2015) [51]** reported the green synthesized ZnO nano powder showed a better cytotoxic effect as compared to commercial ZnO Nano powder against breast cancer cell line. However, the action of nanoparticles depends on size, shape, type of cells and also dose and time dependence. The precise concentration at which NPs may be cytotoxic is still unresolved in the literature, primarily due to the wide range of differing methodologies used to produce

the nanoparticles and the subsequent in vitro testing systems utilized (Gaiser et al., 2013; Niska et al., 2016; Nakkala et al., 2017; Kshirsagar et al., 2018; Shi et al., 2018)[52,53,54,55,56] .

Our results are similar to results reported by Kean et al. (2005) [57] and Zhang et al. (2008) [58] who studied In vitro cytotoxicity of chitosan NPs on MCF7(breast cancer cells) with CC50 >10 mg/mL.

Also, Mao et al. (2005) [59] reported that the cytotoxicity of chitosan NPs on L929 (normal fibroblast cell line from subcutaneous connective tissue of mouth) was >1000 μg/m.

By using broth microdilution, the efficacy of ciprofloxacin was synergistically increased with silver, ZNO and chitosan NPs with (P-value 0.027), (P-value 0.042) and (P-value 0.018) respectively.

Nanoparticles used at concentrations shown to be biocompatible can synergistically increase the antibacterial effectiveness of antibiotics against Gram-negative carbapenem-resistant isolates and MRSA.

5. Conclusion

1-Silver, Zinc Oxide and chitosan nanoparticles have a strong antimicrobial effect against multi-drug resistant Gram-positive and Gram-negative hospital isolates.

2- Nanoparticles used at concentrations shown to be biocompatible can synergistically increase the antibacterial effectiveness of antibiotics against Gram-negative carbapenem-resistant isolates and MRSA.

3- Chitosan nanoparticles were safe while Ag nanoparticles were most effective but showed cytotoxic effects and were dose-dependent.

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