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Antimicrobial Activity of Zinc Oxide, titanium Dioxide and Silver Nanoparticles Against Mithicillin-Resistant *Staphylococcus aureus* Isolates

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Abstract
The different investigation has been carried out on the biological activities of silver, zinc oxide and titanium dioxide nanoparticles. But no study till now has identified the activity of particles' size or type, nor a study has proved their best synergism activity with antibiotics in one experiment against the same isolates.
In this study different sizes of the three types of nanoparticles (NPs) was used to detect the antibacterial activity against methicillin resistant *Staphylococcus aureus*, which was isolated from patients (with burns and wound) at Erbil hospitals. Different antibiotics also, was evaluated against the same isolates. The Mithicillin-Resistant *S. aureus* (MRSA) isolates showed multidrug-resistant to most using antibiotics like; ampicillin, amoxicillin, gentamicin, tetracycline clindamycin and erythromycin. The minimum inhibitory concentration (MIC) of these antibiotics at then was conjugated with the nanoparticles' MIC to show the synergism effect by well diffusion method.
The results showed that the phenotypic inhibition zones had been increased minimum 2 mm to maximum 18 mm, that confirmed the synergism activity when the antibiotics conjugated with NPs. These results signify that these nanoparticle potentate the antimicrobial action of (beta lactums, aminoglycosides, tetracycline, macrolides and lincosamides) a possible utilization of nano compound in combination effect against MRSA .

Keywords: Nanoparticles, MRSA, Synergistic Effect.

Introduction:
Staphylococcus aureus is one of the most significant human pathogens that cause both nosocomial and community-acquired infections. The *S. aureus* strains that resist methicillin and oxacillin have spread worldwide. Infections with Mithicillin-Resistant *S. aureus* (MRSA) strains, which resist a wide range of antibiotics (multidrug-resistant), are associated with considerable injury and mortality [1].
The emergence of bacterial resistance to antibiotics and its spreading are major health difficulties, leading to treatment problems for a large number of drugs [2]. Thus there has been increasing interest in the use of inhibitors of antibiotic resistance for combination therapy [3].
Metallic nanoparticles are attracting a great deal of attention because of their potential of succeeding specific processes and selectivity, especially in biological and pharmaceutical applications [4]. The effective antimicrobial properties of these materials are mainly due to their nanoparticles size, which providing them unique chemical and physical properties as increased surface to volume ratio and high reactivity [5]. They act as Nano-antibiotics and their potential of controlling infectious diseases have been explored and demonstrated by various researchers.
Small particles exhibited higher antimicrobial activity than big particles. That result can be due to high particle penetration when these particles have smaller sizes [6]. Silver, zinc and titanium have been used mostly for the synthesis of stable diffusions of nanoparticles [7,8].
Nowadays NPs are called “a wonder of modern medicine”. The antibiotics can kill perhaps a half dozen different disease-causing organisms, while NPs can kill some 650 cells [9]. The aim of this study focus on using Nano-biotechnology for antimicrobial chemo-dynamic therapy.

Material and Methods
Bacterial strains: *S. aureus* isolates investigated in this study originated from the strain collection of patient's burns and wounds at Erbil hospitals. There were about 53 isolates of *S. aureus* collected from 150 patients.
Antimicrobial susceptibility testing: Fifty three of *S. aureus* isolates were tested by standard disc diffusion method for the most used antibiotics: ampicillin, amoxicillin, gentamicin, tetracycline, clindamycin and erythromycin; according to NCCLS (2006) [10]. Then, it had been ensured by Vitek 2 compact system [11].
Tested Nanoparticles: The nanoparticles that used was silver (Ag NPs) with size 20 nanometer, zinc oxide (ZnO NPs) with size 20,30,50-150 nanometer and finally titanium oxide (TiO_2NPs) with size 10,50,100 nanometer prepared by M K Impex Corp., CANADA company. These NPs were set by the company in a plastic freight with a stowage 100 gm. as powder. The NPs stock solution were prepared by adding 100 mg of NPs powder into 10 ml of deionized water with high shaking to separate the NPs accumulation for 5 min. to obtain a homogenous solution which then, was sterilized by autoclaving and left at room temperature. The final concentration was 10 mg\ml.
The Measurement of minimum inhibitory concentration of NPs and antibiotics:

Minimum inhibitory concentration (MIC) is the lowest concentration that inhibits the visible growth of bacteria. The dilution by micro-titer plate method was applied by using Muller Hinton broth to detect the MIC of NPs depending on NCCLs in micro-titer plate wells. By which the results that got from dilution were in these concentrations : 2600, 1300, 650, 325, 162.5, 81.25, 40.6, 20.3, 10.15, 5.07 microgram\ml. The microtiter plates were placed in incubator overnight at 37^{5}C and the growth was noticed after 18 hr in the wells. The lowest concentration that inhibited the visible growth of bacteria was determined as MIC. The antibiotics' MIC were obtained by the same way [12].

The measurement of conjugated NPs with antibiotic

A 0.1 ml of overnight bacterial suspension was transported and spread onto Muller Hinton agar plates after comparing it with McFarland No. 0.5 turbidity standard, this solution has aspecific optical density to provide a turbidity comparable to that of bacterial suspension containing 1.5×10^{8} CFU/ml. Four wells were made on the plate with 5 mm diameter by sterile boring cork. The MIC of NPs, which was determined in the mentioned above steps was added to the well No.1 and No.2 respectively. A 100 microliter (include 50 microliter of the antibiotic MIC that determined by Micro Scan apparatus plus 50 microliter of NPs together) were added into well No. 3, this well gave the result of using conjugated NPs with antibiotic. The distal water was added to the well No.4 to make it as a negative control. The plates were incubated overnight at 37ºC . The diameter of inhibition zones around the wells was measured in mm. The test was repeated to take the average of the effect [13].

Results and Discussion

Antimicrobial agents' effect

The results revealed that from all 53 isolates, there were 44 isolates (83%) showed resistant to the methicillin; while 7 isolates (13.2%) were sensitive to the methicillin and only 2 isolates (3.8%) were intermediate resistance to the methicillin. These results agreed with the results of a local study by Alhasani (2011), who showed that the rate of Methicillin resistance *S. aureus* (MRSA) was 81% [14].

The isolates showed a high virulence for the used antibiotics. All isolates was resistant to amoxicillin antibiotic and there were 51 isolates (96,2%) resistant to ampicillin antibiotic, while only two isolates (3,8%) were sensitive to it. The gentamicin resistance result's was 88.6% which confirms Samir's study (2007), who found that the rate of resistant to Gentamicin in *S. aureus* was 80% [15]. The results also showed decreasing in tetracycline susceptibility. The rate of resistance was 94.3%, whereas one isolate 1.9% was intermediate. The resistance to other antibiotics like clindamycin and erythromycin also investigated in this study and the results showed high resistant of *S. aureus* isolates to both antibiotics in the rate of resistance 40% and 51% respectively. The antimicrobial agents results are cleared in the Figure:1 .

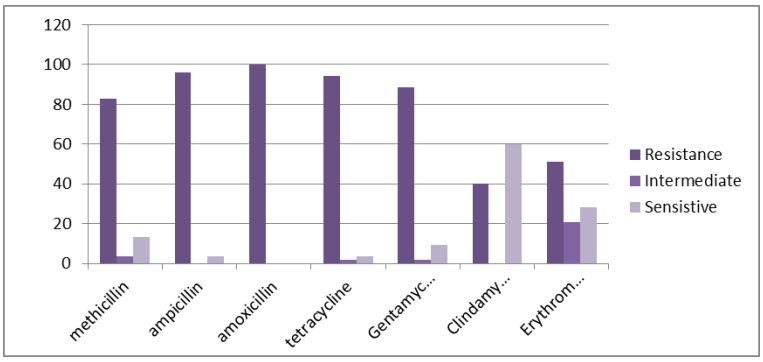

Figure (1-1) antimicrobial agents susceptibility.

A. Antimicrobial Effect of the Nanoparticles

Twelve of MRSA isolates that exhibited resistance to the most common antibiotics like: ampicillin, amoxicillin gentamycin, tetracycline, clindamycin and erythromycin were in exposure of nanoparticles to show their antimicrobial effect.

The Antimicrobial Effect of the Silver Nanoparticles

The silver 20 nm showed a phenotypic inhibition on agar plate 9 mm. That was obtained after measuring its MIC by series of dilution method. The MIC average was approximately 166 µg\ml. This results agreed with Nilda Vanesa *et al* (2009), who noticed an antimicrobial activity against MRSA by using four different sizes of Ag NPs [16].

Morones *et al* (2005) explained the antibacterial activity of silver nanoparticles on Gram positive bacteria, such as *S. aureus*, suggested that silver nanoparticles attach to the surface of the cell membrane and interrupt its function, penetrate bacteria, and release silver ions [17,18].

Silver's mode of action also, supposed to be dependent on Ag^{+} ions, which strongly inhibit bacterial growth through suppression of enzymes, electron transport system components and through interference with DNA functions [19].

The Antimicrobial Effect of the Zinc Oxide Nanoparticles

Zinc oxide 20 nm by the virtue of its MIC average (256 µg\ml) in muller hinton broth, the inhibition zones average was 11 mm. while ZnO 30 nm. showed phenotypic inhibition zone average 7 mm on agar plate at MIC average about 341 µg\ml. finally the isolates showed sensitivity to ZnO 50-100 nm in MIC 160 µg\ml. The inhibition zone was 9 mm.

These results agreed with Iram *et al* (2015), who observed the noticeable antibacterial activity of ZnO NPs against *S. aureus* [20].

There are several mechanisms that have been suggested to explain the antibacterial activity of ZnO nanoparticles. The production of hydrogen peroxide from the surface of ZnO is considered as an effective mean for the inhibition of bacterial growth [21].

Another possible mechanism for ZnO antibacterial activity is the release of Zn^{2+} ions that most likely involve the disruption of the cell membrane lipids and proteins that resulted in the leakage of intracellular contents and eventually the death of cells [22].

The Antimicrobial Effect of the Titanium Dioxide Nanoparticles

Depending to the isolates sensitivity to TiO_2 10 nm, the MIC was (112 µg\ml) and the inhibition zones average was 8 mm. while the isolates showed phenotypic inhibition zone average 7 mm on agar plate at MIC average about 128 µg\ml for TiO_2 30 nm. Finally the isolates showed sensitivity to TiO_2 50 nm in MIC 128 µg\ml and the inhibition zone was 6 mm. These results confirm Roy *et al* (2010), who detected the obvious antibacterial activity of the TiO that tested on *S. aureus* [23].

The antibacterial activity of TiO2 that found perhaps due to a reaction of the TiO2 surface with water. On exposure to ultraviolet (UV) irradiation, TiO2 releases free radicals such as OH, O^-_2, HO^-_2, and H_2O_2, Which is regarded a potent oxidizing power that usually results in circumstance of bacteria and other organic substances [24]. The MIC of these different types and sizes of nanoparticles are in table:1**.**

Table (1) The MIC & Inhibition zone of NPs.

–	**Ag 20 nm**	**ZnO 20 nm**	**ZnO 30 nm**	**ZnO 100 nm**	**TiO 10 nm**	**TiO 30 nm**	**TiO 50 nm**
MIC	166 µg\ml	256 µg\ml	341 µg\ml	160 µg\ml	112 µg\ml	128 µg\ml	128 µg\ml
I Z	9 mm	11mm	7 mm	9 mm	8 mm	7 mm	6 mm

IZ = inhibition zone

B. The Study of the Synergistic Effects of the Nanoparticles with Antibiotics

The MIC of the antibiotics that exhibited inactivity against the isolates was conjugated with the MIC of NPs to study their effects together by well diffusion method. The relationship between these antibiotics & nanoparticles is described in the below table (Table2).

Table (2) The effect of conjugated NPs with Ab.

Ab.	**IZ**	**The effect of conjugated NPs with Ab. (mm)**						
----	**Ab. E.**	**Ag 20 nm**	**ZnO 20 nm**	**ZnO 30 nm**	**ZnO 50 nm**	**TiO 10 nm**	**TiO 30 nm**	**TiO 100 nm**
Am	0	11	15	12	16	18	13	16
AX	0	9	12	11	11	10	11	9
GM	6	19	20	18	14	11	11	10
TE	2	6	9	6	7	10	7	6
CA	8	17	18	20	19	15	12	12
E	2	19	18	20	21	13	11	14

The results showed that the average of inhibition diameter increased when the antibiotic MIC compacted with the different sizes of the NPs MIC. This supported Fayaz *et al* (2010), who noticed that the NPs mini size enable it to penetrate the microbe membrane cell [25]. Robert *et al* (2013), also confirmed that the activity of the Ampicillin and amoxyclav will increase if the Silver (Ag^+) NPs concentration increases. That's led to inhibit the two type of bacteria (Gram positive and negative). Other study mentioned that the activity of Gentamicin increases against S. aureus when it compacted with (Ag^+) NPs [26].

The results of the synergism effect of Ag NPs with Tetracycline and Erythromycin also exhibited an increasing in their inhibition zone. The increasing in diameter length was approximately about 4 mm and 17 mm respectively, while Zarina *et al* (2014) found that the diameter of the inhibition areas for the same antibiotics increased about 2mm and 3mm respectively[27]. Moreover, the antibacterial activities of clindamycin were increased in the presence of Ag-NPs against the isolates. The result showed that the inhibition zone enlarged about 9 mm. This is support Ahmad's *et al* (2007), who found increasing of the NPs activity after it compacted with clindamycin [28].

The results also showed a noticible synergistic effect when some of the mentioned antibiotics compacted with the different sized of Zinc Oxide (ZnO) Nanoparticles, as explained in (table 2).

Bhande *et al* (2013) showed that the effects of the antibiotics rise if they will conjugate with the Zinc Oxide (ZnO) Nanoparticles [29]. It was easily to notice their activity against the bacteria that resist wide spectrum ampicillin, which could cause urinary tract infections. This study also suggested that the conjugation of Zinc Oxide (ZnO) Nanoparticles with the antibiotics increase the permeability of the bacterial plasma membrane, that enable the plasma protein flowing out of the cell throw the interrupted cell membrane.

Similarly, erythromycin conjugated ZnO particles gave the same smart results of enlarging in inhibition area. This is also, support Iram *et al* (2015), who found that erythromycin conjugated ZnO NPs (≤50nm) lowered the MIC of his resistant strains of S. aureus [20].

The observous increasing of inhibition zone When the gentamycin conjugates with ZnO supports Voicu *et al* (2013), who detect by his bacteriological tests the a synergistic activity of ZnO-gentamicin nanoparticles [30].

The two clindamycin and Tetracycline also exhibited an inhibtional activity against S. aureus. Clindamycin showed a high inhibition zone When it comjugated with ZnO 30 nm.

TiO2 effect alone was somehow low, while it exibted an obsorvious defference when conjugated with the antibiotics. The results showed that the effect of TiO2 NPs was very close to Roy et al (2010) [23]. He found that the increasing in effect after it conjugated with the antibiotics as follows: with ampicillin and gentamycin 9 mm, with amoxicillin 7 mm, with erythromycin and clindamycin 6 mm and finally with tetracycline 5 mm.

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فعالية دقائق الفضة ، أوكسيد الزنك و ثنائي أوكسيد التيتانيوم النانوية ضد عزلات المكورات العنقودية الذهبية المقاومة للميثيسيلين

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الملخص

أن بحوث عديدة حملت على عاتقها دراسة التأثير النانوي لدقائق الفضة وثنائي أوكسيد التيتانيوم وأوكسيد الزنك على البكتريا, لكن أيا منها لم يحدد الفعالية الأكفأ لحجم ما أو نوع ما من أنواع هذه الدقائق دونما غيره, كما لم يثبت أكفأ فعالياته التآزرية مع المضادات الحيوية في تجربة واحدة وعلى العزلات ذاتها.

في هذه الدراسة أختبرت الأنواع الثلاثة لهذه الدقائق وبأحجام مختلفة لتحديد النوع الاكثر فعالية وتركيزه المؤثر على بكتريا المكورات العنقودية الذهبية المقاومة للمثيسيلين والمعزولة من حروق وجروح المرضى الراقدين في مستشفيات أربيل . وتم اختبار فحص الحساسية ايضا لهذه العزلات لغرض تحديد المضادات التي تقاومها البكتريا, فوجد انها مقاومة لأكثر المضادات شيوعا في الاستخدام كمضادات الامبسلين, الاموكسيسيلين, الجنتاميسين, التيتراسايكلين, الكليندامايسين و الايرثروميسين.

كما تم تحديد التركيز المثبط الادنى للدقائق والمضادات التي قاومتها البكتريا من خلال سلسلة من التخافيف, ومن ثم تم دمج هذين التركيزين لملاحظة التأثير التآزري للمضاد مع الدقائق النانوية من خلال انتشارها من حفر وسط النمو well diffusion method , وأظهرت مناطق تثبيط ظاهرية بأقطار تفوق اقطار تثبيط المضاد وحده أو الدقائق النانوية وحدها بزيادة ملحوظة يتراوح مقدارها 2 – 18 مليمتر مما يثبت التأثير التآزري لهذه الانواع من الدقائق مع مضادات مجاميع : beta lactums , tetracycline , aminoglycosides , macrolids واخيرا مجموعة lincosamides .

الكلمات المفتاحية: الدقائق النانوية , المكورات العنقودية الذهبية المقاومة للميثيسيلين , الفعالية التآزرية