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Antimicrobial and anti-biofilm activity of titanium dioxide nanoparticles alone and in combination with erythromycin against methicillin-resistant Staphylococcus aureus

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Keywords:
Ibiofilm Formation
Erythromycin
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ABSTRACT

Background: *S. aureus* is a major pathogen and the predominant bacteria that causes hospital-acquired infections. TiO2NPs have unique characteristics and expanding use for different applications in nanomedicine and have attracted enormous interest in the various rising nanoproducts. **Objective:** To evaluate the anti-bacterial and anti-biofilm activities of TiO2NPs alone and in combination with the macrolide class of drugs (Erythromycin) against *S. aureus* isolated from different clinical specimens. **Methods:** Kirby-Bauer's disk diffusion technique was applied for antimicrobial susceptibility testing against *S. aureus* isolates. The Minimum inhibitory concentrations of TiO2NPs and erythromycin were performed with the broth microdilution method, while biofilm formation was investigated by the Calgary technique. **Results:** At a total of eighty-five strains of S. aureus clinical isolates from patients who are in hospitals in the medical city of Baghdad, 34 (40%) of S. aureus were sensitive to penicillin class (Methicillin) while 51 (60%) were resistant to methicillin, with statistical significance between both groups (P <0.05). In urine samples, the majority of *S. aureus* isolates were 21 (24.7%), followed by sputum with 14 (16.5%) samples. Patients infected with *S. aureus* were significant among age groups of 45–54 years old and patients with Ages between 15 and 35 indicate a lower susceptibility to S. aureus infection.mThe results showed that the prevalence of infection with *S. aureus* was significantly higher among female patients 30 (35.3%) rather than male patients 21 (24.7%), and the male/female ratio was 0.46/1. Vancomycin and imipenem were the most active antibiotics against MSSA and MRSA, with sensitivity of 85 (100%) and 82 (96.5%) for vancomycin and imipenem, respectively, whereas MRSA and MSSA exhibited marked resistance to ciprofloxacin and azithromycin, with sensitivity of 64 (75.3%) and 38 (44.7%), respectively.mTiO2NPs showed excellent biofilm inhibitory activity against MRSA and MSSA isolates, and results showed that TiO2 NPs alone with ½MIC can inhibit biofilm formation by about 40% of MRSA and about 60% of MSSA. Moreover, the combination of TiO2 NPs with erythromycin inhibits biofilm formation by approximately 80–90% for MRSA and MSSA, respectively. **Conclusions:** The TiO2NPs alone and in combination with a macrolide antibiotic (Erythromycin) showed high bactericidal and biofilm-eradicating efficacy against clinical *S. aureus* isolates.

نشاط مضاد للميكروبات ومضاد للبيوفيلم لجسيمات ثاني أكسيد التيتانيوم النانوية بمفرده وبالاقتران مع الإريثروميسين ضد المكورات العنقودية الذهبية المقاومة للميثيسيلين

عصام جمعة ناصر

قسم تقنيات المختبرات الطبية ، كلية الصحة والتقنيات الطبية ، الجامعة التقنية الوسطى ، العراق

الكلمات المفتاحية:
تكوين الأغشية الحيوية
الإريثروميسين
المكورات العنقودية الذهبية
الجسيمات النانوية لثاني أكسيد
التيتانيوم

الملخص

الخلفية: المكورات العنقودية الذهبية هي أحد مسببات الأمراض الرئيسية والبكتيريا السائدة التي تسبب العدوى المكتسبة من المستشفيات. TiO2NPs لها خصائص فريدة واستخدام موسع لتطبيقات مختلفة في الطب النانوي وقد اجتذبت اهتمامًا هائلاً بمختلف المنتجات النانوية الصاعدة. الهدف: تقييم الأنشطة المضادة للبكتيريا والمضادة للأغشية الحيوية من TiO2NPs بمفردها وبالاقتران مع فئة أدوية الماكروليد (Erythromycin) ضد S. aureus المعزولة من عينات سريرية مختلفة. الطريقة: تم تطبيق تقنية Kirby-Bauer للانتشار القرصي لاختبار الحساسية لمضادات الميكروبات ضد عزلات S. aureus. تم إجراء التراكيز المثبطة الدنيا لـ TiO2NPs

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والإريثروميسين باستخدام طريقة التخفيف الدقيق للمرق، بينما تم فحص تكوين الأغشية الحيوية باستخدام تقنية كالجاري. نتائج: في المجموع خمسة وثمانين سلالة من المكورات العنقودية الذهبية المعزولة من المرضى الموجودين في مستشفيات مدينة بغداد الطبية، كانت 34 (40٪) من المكورات العنقودية الذهبية حساسة لفئة البنسلين (الميثيسيلين) بينما 51 (60٪) كانت مقاومة للميثيسيلين، مع دلالة إحصائية بين المجموعتين (P <0.05). في عينات البول، كانت غالبية عزلات بكتريا S. aureus 21 (24.7٪)، تليها عزلات البلغم 14 (16.5٪) عينة. كان المرضى المصابون بالمكورات العنقودية الذهبية معنويا بين الفئات العمرية من 45-54 سنة والمرضى الذين تتراوح أعمارهم بين 15 و35 يشيرون إلى قابلية أقل للإصابة بالبكتريا العنقودية الذهبية. أظهرت النتائج أن انتشار الإصابة بالمكورات العنقودية الذهبية كان أعلى معنويا بين المرضى الإناث 30 (35.3٪) مقارنة مع المرضى الذكور 21 (24.7٪)، وكانت نسبة الذكور / الإناث 0.46 / 1. كان فانكومايسين وإيميبينيم أكثر المضادات الحيوية نشاطًا ضد MSSA وMRSA، بحساسية 85 (100٪) و82 (96.5٪) للفانكومايسين والإيميبينيم، على التوالي، بينما أظهرت MRSA وMSSA مقاومة ملحوظة للسيبروفلوكساسين والأزيثروميسين، مع حساسية 64 (75.3٪) و 38 (44.7٪) على التوالي. أظهر TiO2NPs نشاطًا مثبطًا ممتازًا للبيوفيلم ضد عزلات MRSA وMSSA، وأظهرت النتائج أن TiO2 NPs وحده مع MIC½ يمكن أن يمنع تكوين الأغشية الحيوية بحوالي 40٪ من MRSA وحوالي 60٪ من MSSA. علاوة على ذلك، فإن توليفة TiO2 NPs مع الإريثروميسين يثبط تكوين الأغشية الحيوية بنسبة 80-90٪ تقريبًا لـ MRSA وMSSA، على التوالي. الاستنتاجات: أظهرت TiO2NPs وحدها وبالاقتران مع مضاد حيوي ماكرولايد (Erythromycin) فعالية عالية في القضاء على الجراثيم والغشاء الحيوي ضد عزلات S. aureus السريرية.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a strain of *Staphylococcus aureus* that has developed resistance to -lactam antibiotics such as penicillin and cephalosporin. Since its description in 1961, MRSA strains have been recognized as important nosocomial pathogens that often cause postsurgical wound infections that originate nearly exclusively in healthcare facilities [1].

Staphylococcus aureus is notorious for its resistance to standard antimicrobials. Drug resistance may arise via a number of different processes, including antibiotic inactivation by enzymes, lower affinity for the antibiotics due to target modification, efflux pumps, and antibiotic trapping [2]. Multidrug-resistant bacteria are a potential risk for fatal infections. Clindamycin, macrolides, quinolones, tetracyclines, and trimethoprim-sulfamethoxazole all have high in vitro susceptibilities against MRSA strains, especially those from community-acquired infections. Strains of MRSA, previously seen mostly in healthcare settings, are spreading into the general population [3].

The production of biofilms by bacteria is the primary mechanism that contributes to antibiotic resistance in bacteria. In order to acquire resistance, *Staphylococcus aureus* produces an extracellular polymeric material matrix. This matrix is made up of polysaccharides, nucleic acids, proteins, and lipids. Because they are protected inside this extracellular polymeric material matrix, sessile bacteria are able to tolerate the immunological reactions of the host and thus become less vulnerable to antibiotics, which, in turn, are unable to penetrate into the biofilm [4].

Titanium dioxide is one of the photocatalytic nanomaterials that has been researched and is utilized widely in a wide variety of applications and for a variety of different reasons. TiO2NPs find widespread applications in medicine. TiO2 is often non-toxic, very durable, has a high refractive index, a high absorption of light, and a manufacturing cost that is lower than that of other antibacterial agents [5].

The purpose of this research was to evaluate the anti-bacterial and anti-biofilm activities of TiO2 NPs alone and in combination with drugs from the Macrolide class of drugs (Erythromycin) against MSSA and MRSA isolated from different clinical specimens.

Materials and methods

1- Sample collection and bacterial diagnosis

This study was conducted in the educational laboratories of Baghdad Teaching Hospital for the period between October 2021 and March 2022. The identification of *S. aureus* isolates from 85 different clinical specimens, including urine, sputum, wound, abscess, catheter tip, nasal swab, eye, ear, blood, and semen, was confirmed by Gram stain and cultural characteristics on blood agar (Beta hemolysis). Brain-heart infusion broth was employed for the isolation of bacteria from blood samples, and all isolates of S. aureus were further cultured into a selective and differential medium (Mannitol Salt Agar). Biochemical assays validate the final analytical confirmation of S. aureus by biochemical tests using the API 20 E kit "BioMérieux, Marcy L'Etoile, France".

2- Antibiotic susceptibility testing

The Kirby-Bauer's disk diffusion technique was applied for antimicrobial susceptibility testing (AST) of S. aureus isolates according to Clinical Laboratory Standards Institute (CLSI) guidelines. Two to three colonies of S. aureus were mixed with normal saline to get a final density of 1.5 x 10^8 CFU/ml ("approximately 0.5 McFarland standard turbidity") and then the S. aureus suspension was spread on the plate of Müller-Hinton agar. Different antibiotic discs are distributed on the agar plates and incubated at 37 °C for 24 hours. The zone of inhibition size around antibiotic discs was measured and the final results were documented as sensitive (S) or resistant (R) as per the recommendation of (CLSI) [6].

3- Antibiotics and TiO2 nanoparticles

All antibiotics used in this study were purchased from Bioanalyse (Turkey), which are listed in Table (1) along with the concentration. Hongwu International Group Ltd. (China) supplied hydrophilic titanium dioxide rutile nanoparticles with the following specifications: Appearance (White powder), Purity 99.9%, Size 30-50nm (Rutile) Fig.1.

4- Determination of minimum inhibitory concentrations (MIC) of TiO2NPs and antibiotics

The minimum inhibitory concentrations of TiO2 NPs or antibiotic were performed in accordance with the recommendations of the CLSI (Clinical and Laboratory Standards Institute) guidelines. *S. aureus* isolates were grown in Müller-Hinton broth media and an inoculum

turbidity equal to 0.5 McFarland standard was prepared by a spectrophotometer (APEL CO., LTD, Japan) to obtain a final concentration of approximately 1 $\times 10^6$. Then, 0.1 ml of a two-fold serial dilution of TiO2 NPs or antibiotic was dispensed into a 96-well microtiter plate and the plate was incubated at 37 °C for 24 h. The MIC values were determined by reading absorbance at 600 nm with the microtiter reader "Huma Reader-HS, Human GmbH, Germany."A positive control was S. aureus in Müller-Hinton broth, while a negative control was sterile water with media [7].

5- Anti-biofilm activity of TiO2 NPs and antibiotic

Anti-biofilm activity was measured using a tissue culture plate assay. The TiO2NPs were tested for their ability to inhibit biofilm development using a modified version of the Calgary biofilm technique, either alone or in conjunction with specific antibiotics (Erythromycin). This method relied on measuring the hue of crystal violet in terms of colorimetric quantities. A final concentration of 1 x 10^6 CFU/ml was achieved by diluting the overnight culture of *S. aureus* at 37°C with lysogeny broth. After incubating the suspension at 37 degrees Celsius for 24 hours, 180 microliters of the solution are added to each well of a 96-well microtiter flat-bottom polystyrene plate. Different concentrations of TiO2NPs and erythromycin were applied to each well, and the plates were incubated for 4 hours at room temperature. After incubation for one hour, biofilms were fixed at the bottom of wells by washing the microtiter plates twice or three times with 200 l of phosphate buffered saline. To crystal violet stain biofilms, we added 200 µl of 1% crystal violet to each well, incubated at room temperature for 45 minutes, and then destained with 95% ethanol for 40 minutes at 37°C. The "Huma Reader-HS, Human GmbH, Germany" microtiter plate reader was used to measure the absorbance at 595 nm of each well. According to the formula "[1(A 595 of cells treated with TiO2 NPs/A 595 of non-treated control cells] 100". The absorbance values were used as the primary metric in three separate experiments (A 595 0.4 = +++; 0.3 = ++; 0.2 = +; 0.1 = +/-) [8].

Statistical Analyses

All tests were performed at least three times to ensure accuracy. The Mean SD "Standard deviation" was the statistical distribution used to display the data. Statistical analysis was performed using GraphPad PRISM® 9.3.1 by "GraphPad Software, Inc., La Jolla, CA, USA." The P-values were analyzed using Student's t-test. Assume a level of significance of ($P< 0.05$).

RESULTS

During the period of this study, a total of eighty-five strains of *Staphylococcus aureus* clinical isolates from patients who are in hospitals in the medical city of Baghdad were 34 (40%) sensitive to the penicillin class (Methicillin) compared to the 51 (60%) resistant to methicillin with a statistically significant difference between the two groups ($P< 0.05$) as described in the Fig.2.

The majority of *S. aureus* isolates were found in urine samples, with 21 (24.7%), followed by sputum samples with 14 (16.5%), wound12 (14.2%), abscess10 (11.8%), catheter tip 9 (10.5%), nasal swab and eye representing 5. (5.9%) for each specimen, and ear, blood, and semen represented 3 (3.5%) for each sample, as shown in Table (2).

On the other hand, the results of the statistical analysis demonstrated a significant association between the categories of samples with MSSA and MRSA isolates ($P< 0.05$). Fig. 3.

The results of the current study indicate that the prevalence of patients infected with *S. aureus* was significant among age groups of 45–54 years old and patients aged between 15 and 35 years old, indicating that lower susceptibility to infection with *S. aureus* strains with significantly different between age groups ($P< 0.05$) as indicated in Fig. 4.

Furthermore, the results showed that the prevalence of infection with *S. aureus* (MSSA and MRSA) was significantly higher among female patients 30(35.3%) rather than male patients 21(24.7%) and the male/female ratio was 0.46/1 as presented in Table (3).

Using a modified version of the Calgary approach, each *S. aureus* strain was evaluated for its potential to produce biofilms.

According to the results of this testing, the vast majority of MRSA strains showed significant biofilm development 43 (50.5%) compared to MSSA, which showed the lowest biofilm production 9 (10.5%), as shown in Table (4).

In this investigation, *S. aureus* isolates were tested for antimicrobial susceptibility using a modified Kirby-Bauer disk technique with a different antimicrobial agent class.

Tales 5. shows that the antimicrobial susceptibility pattern of MSSA and MRSA isolates, glycopeptide antibiotic (Vancomycin) and carbapenem antibiotic (Imipenem) were the most efficient antibiotics against MSSA and MRSA isolates, with a total sensitivity of 85 (100%) and 82 (96.5%) for vancomycin and imipenem, respectively. On the other hand, the majority of MSSA and MRSA isolates displayed significant resistance to the quinolone class of antibiotics (Ciprofloxacin) and monobactam antibiotics (Azithromycin), with a total resistance of 64 (75.3%) and 38 (44.7%) for ciprofloxacin and azithromycin, respectively.

The results regarding the biofilm propensity score of *S. aureus* isolates prove that higher levels of antibiotic resistant bacteria have the greatest ability to produce biofilms. The MRSA strain had a high level of biofilm production (+++), in contrast to the MSSA strain, which demonstrated a moderate to low level of biofilm formation (+ to +/-), and there were statistically significant differences between the two strains ($P< 0.05$) as described in the table and Fig.5.

The TiO2 NPs demonstrated outstanding biofilm inhibiting ability against MSSA and MRSS, and the findings suggest that TiO2 NPs with a half-minimum inhibitory concentration (MIC) may prevent biofilm formation in around forty percent of MRSS and approximately sixty percent of MSSA Fig.6. Furthermore, the combination of TiO2 NPs and the macrolide antibiotic (Erythromycin) inhibited biofilm formation by roughly 80–90% for MSSA and MRSS, as shown in Fig.7.

Discussion

Staphylococcus aureus is a significant pathogen that causes a wide variety of illnesses, including skin and soft tissue infections, deep organ abscesses, toxin-mediated disorders, bacterial pneumonia, urinary tract infections, surgical wound infections, septicemia, and meningitis [9]. In Iraq, MRSA and other multidrug-resistant pathogens have become a serious public health problem because of irresponsible and excessive antibiotic usage [10].

All clinically diagnosed *S. aureus* specimens may be MRSA reservoirs that spread infection in a population. From what we've seen, it's clear that *S. aureus* is a substantial contributor to pyogenic infections.

Out of 113 clinical samples containing *S. aureus*, Sapkota *et al.* [11], isolated *S. aureus* from blood (11.28%) and central venous catheter tip (2.26%) compared to sputum (3.76%) and urine (2.26%). The findings of this study indicated the reverse, with urine samples comprising (24.7%) the majority, followed by sputum samples (16.5%). Similar to what was shown by Naimi *et al.*, [12], where vancomycin and imipenem were the most effective antibiotics against *S. aureus*, we found the same thing in the present investigation.

Biofilms induce infectious illnesses and may improve bacteria's drug tolerance. Finding a medication that can eliminate biofilms might help fight infections. Biofilm-associated disorders demand special care due to their drug resistance [13]. Despite the fact that macrolides were among the first-chosen antibiotics in the treatment of biofilm-associated illnesses, many of the microorganisms responsible for biofilm development have developed resistance to them [14].

This study provided strong evidence that *S. aureus* biofilm growth was significantly suppressed when TiO2 NPs were combined with the macrolide antibiotic erythromycin. This synergy might be attributed to the distinct modes of action of erythromycin and TiO2 NPs, since erythromycin inhibits protein synthesis [15], while TiO2 NPs are known to kill germs by a process wherein their positive charge ions are released into the reaction media and bind with the negatively charged thiol groups (-SH) of proteins in the cytoplasmic membrane. Capturing the cell wall and making it more permeable are

both results of this process, as are the changes in the structure of DNA, ribosomes, and enzymes in the cell [16].

CONCLUSIONS

The TiO2NPs alone and in combination with a macrolide antibiotic (Erythromycin) showed high bactericidal and biofilm-eradicating efficacy against clinical MSSA and MRSA isolates. TiO2NPs may be used as an antibacterial and antibiofilm agent in conjunction with antibiotics.

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Table 1: Antibiotics are used against *Staphylococcus aureus* isolates.

Class	Antibiotics	Symbol	Concentration µg/ml
Aminoglycoside	Gentamicin	CN	10
Quinolone	Ciprofloxacin	CIP	10
Tetracycline	Tetracycline	TE	25
Aminoglycoside	Tobramycin	TOB	10
Glycopeptide	Vancomycin	VA	10
Carbapenem	Imipenem	IPM	10
Penicillin	Methicillin	ME	10
Chloramphenicol	Chloramphenicol	C	10
Macrolide	Erythromycin	E	10
Monobactams	Azithromycin	AZM	15
Lincosamide	Clindamycin	DA	10

Table 2: The distribution of *S. aureus* isolates according to the type of specimen.

	Specimen type	No.	%
1.	Urine	21	24.7
2.	Sputum	14	16.5
3.	Wound	12	14.2
4.	Abscess	10	11.8
5.	Catheter tip	9	10.5
6.	Nasal Swab	5	5.9
7.	Eye	5	5.9
8.	Ear	3	3.5
9.	Blood	3	3.5
10.	Semen	3	3.5
Total		**85**	**100 %**

Table 3: The distribution of MSSA and MRSA according to the gender of patients

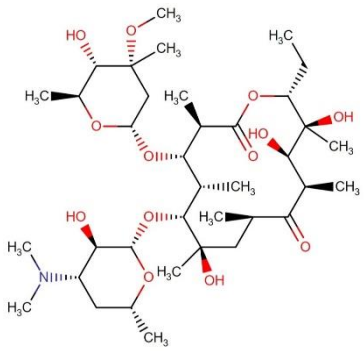
Sex	*Staphylococcus aureus*		Total n (%)
	MRSS n (%)	**MSSA n (%)**	
Males	21(24.7)	6 (7.0)	27
Females	30(35.3)	28(33)	58
Total	51(60)	34(40)	85 (100)

Table 4: Distribution of MSSA and MRSA according to biofilm formation.

Biofilm	*Staphylococcus aureus*		Total n (%)
	MRSS n (%)	**MSSA n (%)**	
Positive	43 (50.5)	9(10.5)	52(61.2)
Negative	8(9.5)	25(29.5)	33(38.8)
Total	51(60)	34 (40)	85(100)

Table 5: Antimicrobial susceptibility patterns and biofilm formation propensity score of Staphylococcus aureus isolates

Antibiotics	MSSA (n = 34)		MRSA (n = 51)		Total sensitive	Total resistant	Biofilm Propensity score
	S [n (%)]	R [n (%)]	S [n (%)]	R [n (%)]	N (%)	N (%)	
Gentamycin	26 (76.5)	8(23.5)	36(70.6)	15 (29.4)	62(72.9)	23(27.1)	+
Ciprofloxacin	12 (35.3)	22(64.7)	9(17.6)	42(82.4)	21(24.7)	64(75.3)	+++
Tetracycline	27(79.5)	7(20.5)	43(84.3)	8(15.7)	70(82.4)	15(17.6)	+
Tobramycin	25(73.5)	9(26.5)	29(56.9)	22(43.1)	54(63.5)	31(36.5)	+
Vancomycin	34 (100.0)	0(0.0)	51 (100.0)	0 (0.0)	85 (100.0)	0 (0.0)	+/-
Imipenem	34 (100.0)	0(0.0)	48(94.1)	3(5.9)	82(96.5)	3 (3.5)	+/-
Azithromycin	29(85.3)	5(14.7)	18(35.3)	33(64.7)	47(55.3)	38(44.7)	++
Chloramphenicol	21(61.8)	13(38.2)	39(76.5)	12(23.5)	60(70.6)	25(29.4)	+
Erythromycin	28(82.4)	6(17.6)	33(67.7)	18(32.3)	61(71.8)	24(28.2)	+
Clindamycin	27(79.4)	7(20.6)	40(78.4)	12(78.4)	67(78.8)	19(21.2)	+



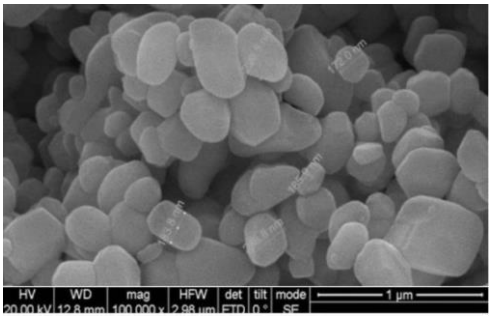
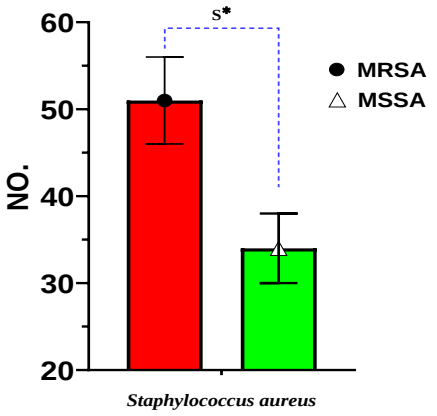
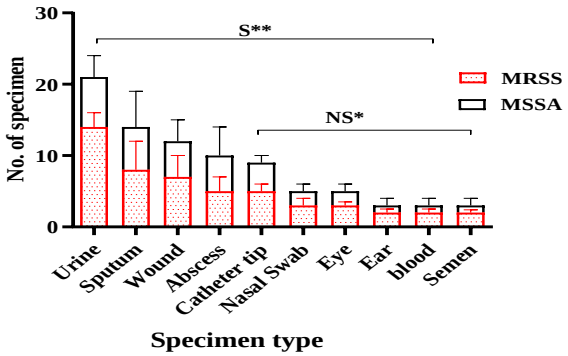


Fig. 1: A- Structures of erythromycin B- A-Morphology of TiO2NPs Hongwu International Group Ltd.



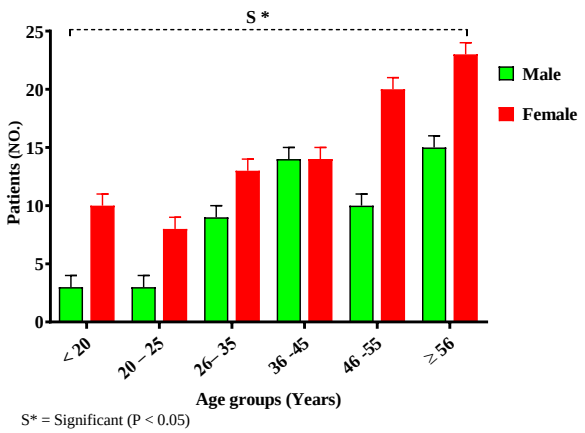
S* = Significant (P < 0.05)

Fig. 2: The number of MSSA and MRSA isolates



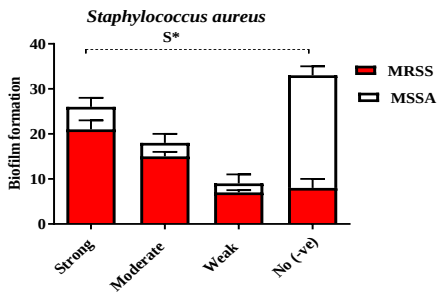
S* = Significant (P < 0.05)
NS* = Non-significant (P > 0.05)

Fig. 3: Correlation between types of samples and MSSA or MRSA isolates.



S* = Significant (P < 0.05)

Fig. 4: Distribution of patients according to gender and age.



S* = Significant (P < 0.05)

Fig. 5: Biofilm propensity score of MSSA and MRSA isolates.

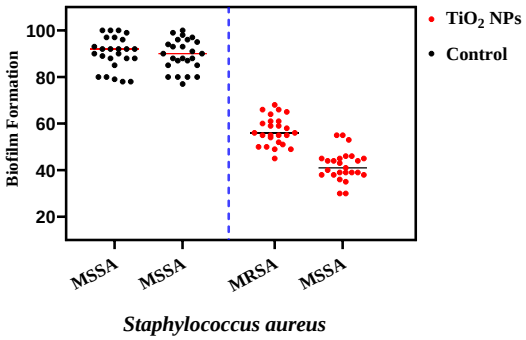


Fig. 6: Biofilm inhibitory activity of TiO2 NPs against MSSA and MRSA isolates.

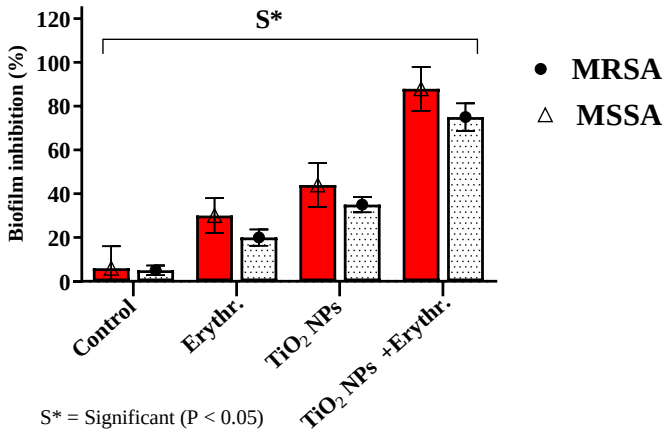


Fig. 7: Anti-biofilm inhibitory activity of TiO2 NPs alone and in combination with erythromycin against MSSA and MRSA isolates.