Research Article

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Biosynthesis of TiO2 nanoparticles using prodigiosin and evaluating its antibacterial activity against biofilm producing MDR- *Acinetobacter baumannii*

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Abstract

A rising number of hospital infections were caused by multi drug resistant *A.baumannii*. This microorganism has become a big global concern for clinicians. This study aimed to evaluate the antimicrobial activity of biosynthesized TiO2 nanoparticles against biofilm producing multi drug resistant *A. baumannii*. Bacteria were isolated from burn wounds. The selected isolate was identified using the routine biochemical assays, viteck 2, and confirmed by PCR technique, targeting the 16S rRNA and *blaOXA-51* genes. Antimicrobial susceptibility tests were performed using Viteck 2 system and the biofilm production was tested by using microtiter plate method. S marcescens was used for production of the prodigiosin which characterized later by UV-visible spectroscopy and then was used for biosynthesis of titanium dioxide nanoparticles (TiO2) NPs. Atomic force microscopy, X-ray diffractometer and field emission scanning electron microscopy were used for characterization of TiO2 NPs. Antimicrobial activity of TiO2 NPs was examined by well diffusion assay using concentration of 0.4- 0.006 mg/ml. The studied isolate was beta-lactamase producer and showed resistance to aminoglycosides, quinolones, furanes and trimethoprim/ sulphonamide, PCR amplification of 16S rRNA and *blaOXA-51* genes was used for detection of *A baumannii*. The selected isolate was a strong biofilm producer with 5.9 times more than the OD values of the control. Atomic force microscopy images showed that the synthesized TiO2 NPs were in spherical shape with an average diameter of 67.49 nm. The TiO2 NPs inhibited the bacterial growth at concentrations of ≥ 0.1mg/ ml and a maximum zone of inhibition recorded was 22 mm at concentration of 0.4 mg/ ml. Biosynthesis of TiO2 NPs using prodigiosin was showed a promising antibacterial activity against strong biofilm producing MDR- *A. baumannii*.

Keywords: *A. baumannii*, Antimicrobial activity, Biofilm, Prodigiosin, TiO2 nanoparticles

التصنيع الحيوي لجسيمات ثاني اكسيد التيتانيوم النانوية باستخدام البروديجيوسين وتقييم نشاطه ضد بكتريا *A.baumannii* المُنتج للغشاء الحيوي والمقاوم للأدوية المتعددة

الخلاصة

تعد بكتريا *A.baumannii* المقاومة للأدوية المتعددة سببا رئيسيا فى ارتفاع معدلات الاصابة فى المستشفيات وباتت تشكل معضلة كبيرة للاطباء.هدفت هذه الدراسة إلى تقييم فعالية جسيمات TiO2 النانوية المصنعة ضد بكتريا *A. baumannii* المقاومة للأدوية المتعدة والمكونة للغشاء الحيوي البكتيري .تم عزل البكتيريا من مسحات الجروح الناتجة عن الحروق ومن ثم شخيصت العزلة باستخدام الفحوصات الكيميائية الحيوية الروتينية ، Viteck 2 ، وتم تأكيد التشخيص بواسطة تقنية ال (PCR) ، باستهداف جينات S 16rRNA و bla_{OXA-51}. تم إجراء اختبار الحساسية للبكتريا للمضادات الحيويه باستخدام نظام Viteck 2 واجري اختبار إنتاج الغشاء الحيوي باستخدام طريقةmicrotiter plate استخدمت بكتريا *S. marcescens* لإنتاج البروديجيوسين الذي تم فيما بعد توصيفه بواسطة UV-visible spectroscopy والذي بدوره تم استخدامه للتصنيع الحيوي لجسيمات TiO2 . استخدمت فحوصات ال Atomic force microscopy وX-ray diffractometer وfield emission scanning electron microscopy لتوصيف جسيمات TiO2 النانوية. تم فحص فاعليه فعالية جسيمات TiO2 النانوية ضد العزلة المذكورة عن طريق Agar well diffusion assay باستخدام تراكيزمن 0.4 - 0.006 مجم / مل .العزلة المدروسة كانت منتجة لانزيم beta-lactamase وأظهرت مقاومة لمجموعة المضادات (للأمينوغليكوزيدات والكينولونات والفيوران وتريميثوبريم / سلفوناميد) .استخدم ال PCR لتضخيم جين 16SrRNA و bla_{OXA-51} لتاكيد تشخيص *A. baumannii* . العزلة المدروسة أظهرت مستوى عالي من انتاج الغشاء الحيوي 5.9, مرات أكثر من قيم OD للسيطرة. أظهرت الصور المجهرية للقوة الذرية أن TiO2 NPs المُصنَّع كان في شكل كروي بمتوسط قطر يبلغ 67.49 نانومتر. ثبط TiO2 NPs نمو البكتيريا بتركيزات ≥ 0.1 مجم / مل وكانت منطقة التثبيط القصوى المسجلة 22 مم بتركيز 0.4 مجم / مل .التصنيع الحيوي للجسيمات TiO2 النانوية باستخدام البروديجيوسين, أظهر نشاطًا واعدًا ضد بكتريا *A. baumannii* المُنتج للغشاء الحيوي والمقاوم للأدوية المتعددة

Introduction

Nanotechnology is a neoteric industry which utilizes macro-molecular nano-scale material (1-100 nanometers) (1). Several types of nanoparticles, varying in size, form, surface area and function, have been developed. Nanoparticles of metal and metal oxide are generally noticeable in combating microbial species because of their specific properties (2)

Recently titanium dioxide nanoparticles have attracted a lot of interest, in addition to approval by the United States Food and Drug Authority. Titanium dioxide nanoparticles are potentially highly effective in biological field, medical field, environmental field, solar energy cells, photo-catalysts, electrical electrodes, and gas sensors(3).

Various synthesis methods of TiO2 nanostructures are available, such as pysical method, chemical method, biological method and hybrid technique (4).

Recently, the biological methods have become the most environmentally sustainable and low cost approach compared to other methods (5).

A red pigment, Prodigiosin, was discovred in *S marcescens,* it is an important secondary metabolite (6). The generation of prodigiosin in different strains, in particular through a quorum sensing mechanism, seems to be induced by cell density at later stages of bacterial growth (7,8). Prodigiosin plays a crucial role in nanoparticles biosynthesis, in addition to its unique anticancer properties, it also found to have antibacterial, antiprotozoal, and anti-inflammatory activity (9). Also, it is used or nanoparticular biosynthesis of gold and silver (10,11).

In recent years *Acinetobacter baumannii* has become primarily a multi-drug resistant bacterium and a major concern (12). WHO reported it among major antibiotic resistant "pathogen priorities" highlighting its serious threats to public health (13,14).

Advanced molecular methods have been employed today to identify *A. baumannii*, such as 16S rRNA, *bla*$_{OXA\ 51}$ and RNA ß subunit (*rpo*) genes amplification and sequencing (15,16,17,18).

According to a National Health Institutes report, over 80 % of all bacterial diseases contain biofilm. Biofilms are associated with various medical conditions which of, upper respiratory tract infections, urogenital infections, dental plaque and indwelling medical devices infections. (19)

These biofilms are extremely difficult to eradicate as the antibiotic resistance can be increase 1,000 times (20). There are different techniques for biofilm detection, like, tissue culture or microtiter plate (TCP), tube method (TM), congo red agar method (CRA), bioluminescent tests, and fluorescent microscopic examinations (21).

The aims of this study are to synthesize, characterize and evaluate the antibacterial activity of biosynthesized TiO2 NPs using prodigiosin pigment against biofilm producing multidrug resistant *A. baumannii* using well diffusion method.

Materials and Methods

Bacterial isolation and Identification

Twenty bacterial isolates were collected from Burn, Reconstructive and plastic Surgery Hospital in Sulaimani -KRG- Iraq during the period of 01/7/2019 to 20/12/2019. All Bacterial isolates were identified on the basis of routine biochemical assays and confirmed by VITEK 2 system (Biomérieux, France) using VITEK® 2 GN ID Card and VITEK® 2 GP ID Card (according to the manufacturer's instructions).

One isolate of multidrug-resistant *A. baumannii* was selected for further experiments. *A. baumannii* ATCC (19606) and *Serratia marcescens* strains supplied from biology department college of science,

University of Sulaimani, KRG-Iraq, and used as a standard strain for molecular detection and also for antibacterial activity and biosynthesis of TiO2 NPs.

Molecular detection of *A. baumannii*

A-Extraction of DNA

Genomic DNA was extracted from an overnight culture in tryptic soy broth using Bacterial Genomic DNA Kit (Geneaid, Taiwan) according to the manufacturer's protocol.

B-Detection of 16S ribosomal RNA gene

Specific 16S rRNA primers were used for identification of the isolate as described by (22). In brief, a 1500-bp fragment of the ribosomal RNA gene was amplified using the following primers F (5′TGGCTCAGATTGAACGCTGGCGGC-3′) and R(5′- TACCTTGTTACGACTT CACCCCA-3′). The final reaction volume was 25 µl, and the cycling conditions were as follows: an initial denaturation at 95°C for 4 minutes, followed by 30 cycles of denaturation at 95°C for 40 seconds, annealing at 64°C for 40 seconds, extension at 72°C for 40 seconds, and a final extension at 72°C for 5 minutes. The PCR-amplified products were stained with ethidium bromide and analyzed by 1% agarose gel electrophoresis. Images were acquired using a Bio-Rad Gel Doc XR^+ imaging system.

C- Detection of *bla*$_{OXA-51}$ gene

To confirm the identity of the isolates at the level of species, OXA-51 gene was amplified with expected amplicon size of 353bp (23, 18) using primers: F (5′-TAATGCTTTGATCGGCCTTG-3′) and R (5′-TGGATTGCACTTCATCTTGG-3′).

The final volume was 25 µl, and the cycling conditions were as follows: an initial denaturation at 94°C for 5 minutes, followed by 30 cycles at 94°C for 25 seconds, 56.5°C for 40 seconds, 72°C for 50 seconds and a final extension at 72°C for 6 minutes. The PCR-amplified products were stained and analyzed as mentioned above.

Detection of Biofilm

A. baumannii was inoculated in a 5ml Trypticase soya broth (TSB) and grown to stationary phase then culture was diluted at 1:100. Subsequent, 200µl of diluted culture was pipetted into wells in 96-well flat bottom polystyrene microtiter plates. Incubation was carried out at 37º C for 24 hours. Cultures were then aspirated and the wells were washed 3 times with phosphate buffer saline (Ph 7.2). The plate was then air dried for 15 min at room temperature then stained by adding 200 µl of 0.1% crystal violet for 15 min. After removing the stain, 200µl of 95% ethanol were added to each stained well and left for 10 to 15min at room temperature. The optical density of the wells was measured at 590 nm using micro ELISA auto reader. Sterile TSB was used as a negative control. To compensate for background absorbance, the optical density (OD) reading value of control (C) was deducted from the test (T) values (24,25).

The intensity of biofilm was measured as below:

1. $OD_T = ODc$ = Non biofilm producer.
2. $ODc < OD_T \leq 2ODc$ = Weak biofilm producer.
3. $2ODc < OD_T \leq 4ODc$ =Moderate biofilm producer.
4. $4ODc < OD_T$ = Strong biofilm producer.

Antibiotic susceptibility test

Susceptibility tests were performed using VITEK 2 system with AST-GN48 TEST KIT susceptibility card for Gram negative bacteria (Biomérieux, France) according to the manufacturer's instructions.

Prodigiosin production

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Production of the prodigiosin was executed by inoculating the *S. marcescens* to the batch fermentation media. The batch fermentation media was prepared according to (26). The fermentation medium was consisting of Peptone 5g/l as nitrogen source, sucrose 10g/l as carbon source, 0.61Mm of MgSO4.7H2O, 2mM of MnSO4.4H2O2, 8.82mM of CaCl2.2H2O and 0.33mM of FeSO4.4H2O. The pH of the medium was adjusted at 7.0. After the process of sterilization for 15 minutes, the amount of 2% of *S. marcescens* (10^8) Mcfarland inoculum was transported to the batch fermentation medium, then incubated at 30 ° C in a shaker incubator for 72 hours at 200 rpm.

Extraction and purification of prodigiosin

The extraction of Prodigiosin the red pigment was carried out after 30 hours of incubation from the cell-free broth culture of *S. marcescens.* Subsequently the amount of 250 ml of methanol was added to the harvested cells after centrifugation of the culture medium for 15 minutes at 8000 rpm and stirred completely for 3 hours at room temperature. Thereafter, a second centrifugation was done for the remaining mixture for 20 minutes at 8000 rpm, the supernatant was collected and filtrated through 0.2 µm, milipore filter (Gema medical, Spain). The process of concentration of methanol filtrate at 70˚C was accomplished by Rotary evaporator. The extraction of the crude prodigiosin was done by adding double volume of chloroform (organic phase), the red elutes was collected and dried at 45 ˚C to get the red powder of the prodigiosin, finally, the obtained red powder was dissolved in methanol for the purpose of storage. The process of extraction of prodigiosin was carried out according to (26).

Synthesis of titanium dioxide nanoparticles

Process of the synthesis of titanium dioxide nanoparticles was done by utilizing tanium chloride (TiCl4, 99%). Ten mg of red powder of prodigiosin was dissolved in 1ml deionized water in a sterilized flask and dispersed by ultra-sonic bath for 60 minutes. On the other hand, 5 ml of TiCl4 was dissolved in 50 ml deionized distilled water and was dispersed for 30 minutes by ultra-sonic bath. After the dispersion time completed, the two separate solutions (the prodigiosin solution and the $TiCl_4$ solution) both were mixed and stirred thoroughly by using magnetic stirrer at pH 7 for 30 minutes and thereafter the mixture was left in a dark room overnight. The solution that include the precipitate of the titanium dioxide nanoparticles, centrifuged at 6000 rpm for 30 minutes, washed two times by DDW, then was centrifuged for the second time at 6000 rpm to concentrate the precipitate. The obtained nanoparticles precipitation was dried at 60 ˚C for 30 minutes in an oven. Finally, the resulting white powder was preserved in a dark bottle for further characterization and applications, the process of synthesizing TiO2 nanoparticle was done according to (27, 28).

Antibacterial Activity of TiO2 Nanoparticles

Bacterial susceptibility to TiO2 NPs was det ermined by using agar well diffusion method. Bacterial isolates were cultivated overnight at37 ° C on Mueller Hinton medium. After incubation, standard inoculum for each bac terial isolate at a concentration of 1.5 X 10^8 CFU / mL was formed and compared with the standar d solution of 0.5 McFarland. A sterile swab ha s been dipped into the suspension and subsequently inoculated on the Muller Hinton agar (MH) plate to evenly cover bacteria on the plate surface.

Wells of 6 mm diameter were made aseptically on MH agar plates and 0.1mL of various
concentrations (0.4mg/mL, 0.2 mg/mL, 0.1 mg /mL,
0.05mg/ mL,0.025mg / mL,0.0125mg /mL, 0.006mg / mL)
of TiO2 NPs were dispensed into separate wells followed by overnight

incubation at 37 °C. After incubation, bacterial susceptibility diameters in the inhibition zones were reported. Wells containing sterile distilled water alone were used as a negative control and well was contained gentamycin as a positive control (25).

Results and Discussion

Bacterial identification

Vitek 2 system was identified the isolate as follows: *A. baumannii* (99% probability) using VITEK® 2 GN ID Card which is able to identify more than 150 fermentative & non-fermentative Gram-negative bacilli (Table1). VITEK2 compact device incorporates many advantages that may be of clinical significance for routine bacterial identification such as easy technique rapid detection and high degree of automation (29).

Funke et al (1998) showed that within 3 h, the VITEK 2 system identified correctly 84.7% of selected species belong to enterobacteriaceae family and 70 different species of non-enteric bacilli, while 0.8% being misidentified strains and 1.2% being non-identified strains **(30)**. In the present study, *A. baumannii* was identified by VITEK 2 system and also was confirmed by molecular technique targeting two specific genes (16S rRNA and bla_{oxa-51} genes) as shown below.

Antibiotic susceptibility assay

Minimum inhibitory concentration of the tested bacteria were obtained by using automated system (Vitek 2) with specific cards representing different classes, In a current study a substantial increase to most antibiotic classes was observed, particularly antibiotics chosen for treating *A. baumannii* infections, it was beta-lactamase positive and showed resistance to aminoglycosides, quinolones, furanes and trimethoprim/ sulphonamide, while it showed an intermediate resistance to levofloxacin (Table2). Many of the antimicrobial agents can be reliably tested through VITEK 2 system. Vitek 2 is a closed system that can avoid cross-contamination or contamination of the environment (31). It can manage number of specimens at the same time automatically, preparation and processing of samples is simple to do, reduce processing and operating times dramatically, increase the performance of standard clinical laboratories (30,31)

In China, a large study which included 851 hospitals for evaluating the performance of five commonly used automated susceptibility testing systems (Vitek 2, Phoenix, Microscan, TDR, and DL) against ESBL-producing *Escherichia coli* and KPC-producing *Klebsiella pneumonia*, except for cefepime and meropenem, Vitek 2 system seemed to have provided a reasonably reliable and conservative evaluation of MIC (32).

Molecular Detection of MDR- *A baumannii*

A. baumannii was identified by targeting two genes, 16S rRNA and bla_{OXA}-51 gene by two separated PCR reaction.

Primers targeted 16S rRNA gene were able to detect *A baumannii* in both clinical isolate and *A. baumannii* ATCC 9606 (Fig. 3). Our results were in agreement with previous studies (17,18,29,33 and 34) which also recorder a16S rRNA as a target for identification of *A. baumannii.*

bla_{OXA}-51 gene was also successfully amplified in both clinical and ATCC (19606) *A. baumannii,,* the current findings were consistent with numerous studies that reported the existence of *bla*OXA-51 gene in all clinical *A. baumannii* isolates but not found in other *Acinetobacter spp* (14, 18, 35, 36 and 37), since, obviously, these genes are unique to *A.*

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baumannii, it was suggested that the identification of this species may be based solely on the detection of an *bla*$_{OXA}$-51 gene (38).

Production of prodigiosin

The spectra of absorption of the prodigiosin pigment which has been extracted from our sample of *Serratia marcescens* (ATCC 15365) was showed maximum production of the red pigment and demonstrated an absorption peak at 526 nm wavelength (39), the characterization of the prodigiosin was done by using UV-visible spectroscopy (Shimadzu, Japan), so as to reveal the ultimate absorption of this red pigment, as shown in fig.4.

Characterization of green synthesis TiO_2 NPs

Atomic force microscopy (AFM)

AFM images show that the synthesized TiO_2 NPs are in spherical shape and the size of an average diameter was 67.49 nm (table 3 and fig.5). The surface morphology of the TiO_2 NPs was studied by atomic force microscopy ,the 2D and 3D of TiO_2 NPs were given topology **(40).**

X-ray diffractometer

Analyzing our green synthesized titanium oxide nanoparticles by using prodigiosin was done by X-ray diffractometer. The XRD pattern showed that the peaks obtained from synthesized TiO2 nanoparticles were matched with the standard diffraction data of TiO2 nanoparticles (JCPDS number of the card 21-1272). Peaks were at =25°, 38°, 48°, 53°, 55°, 62° and 75° and referred to (1 0 1), (0 0 4), (2 0 0), (1 0 5), (2 1 1), (2 0 4) and (2 1 5) as shown in fig.6.

The interlacing parameters of titanium oxide nanoparticels were a = 0.3785Å, these parameters have been compatible with the reference of face-centered cubic (fcc) crystal lattice of metallic titanium. According to the Debye-Schereer, and XRD patterns all titanium oxide nanoparticles were in form of crystalline as shown in the equation below (41).

$D = [K\lambda / \beta\cos\theta]$ Å

Where:

D: is the average crystallite size (Å)

K: is the shape factor (0.9)

Λ: is the wavelength of X-ray (1.5406 Å) Cu Kα radiation

θ : is the Bragg angle

β ; is the corrected line broadening of the nanoparticles.

Field emission scanning electron microscopy

FESEM images measured the topography of the synthesized nanoparticles, the images were magnified at 50kx based on (fig. 7), the whole nanoparticle samples demonstrate smooth planes and orderly organized in a shape of titanium oxide nanoparticles bunches, researches were revealed that increasing in the rate of calcination temperature will have an important effect on the size of the nano particles changing them from small to large particles by means of agglomeration, however, better small size nano particles were formed by low temperatures leading to formation of spherical shape of nano particles (42).

Detection of biofilm production

After incubation for 24 hr, the tested isolate produced biofilm in the TSB medium at 37ºC.The mean OD of biofilm production was (0.674), which was 5.9 times more than the control (Mean OD=0.114), which indicate that *A. baumannii* was a strong biofilm producer (Table 4 and Fig 8).

Previous studies were evaluated three screening methods for biofilm production, tissue culture or 96 wells microtiter plate (TCP), tube method (TM) and congo red agar method (CRA), they were reported that the TCP method as superior to the TM and CRA methods (43,44). Knobloch *et al.* (7) recorded that TCP method was able to detect 57.1% of their isolates as a biofilm producer in contrast to CRA method

which detected only 3.8% of them (45).

Deka (2014) also compared the results of TCP Method with CRA method, they found that TCP method detected 83% as biofilm producer while CRA method detected only 20% as biofilm producer (21).

Antimicrobial activity of TiO2 NPs against Biofilm producing bacteria

A biofilm producing MDR *A. baumannii* was selected for the evaluation of antibacterial activity of green synthesized TiO2 NPs. The mean of three replicates of the diameter of inhibition zones around each well with TiO2 NPs solution is represented in fig 9. It was found that at concentrations of ≥ 0.1 mg/ ml of TiO2 NP was able to inhibit bacterial growth. A maximum zone of inhibition was 22 mm at concentration of 0.4 mg/ ml, while minimum zone of inhibition was 11 mm at concentration of 0.1 mg/ ml. Similar results antibacterial activity of TiO2 NP were recorded by (46, 47, 48, 49). A recent research in Iraq, used commercial TiO2 NPs (35nm), has shown that the antibacterial activities of TiO NPs range from 31.25 µg / ml to 500 µg / ml against many grams positive and negative bacteria including *A. baumannii* . Jesline,2015 and Albaiaty, 2019 also recorded that TiO2 NPs were able to inhibit bacterial growth at 500 µg / ml with maximum zone of inhibition of 14 mm against strong biofilm-producing MRSA isolates (49,50). Abdullah (2016) reported that, TiO2 NP exhibited an antibacterial activity against some biofilm producing gram negative bacteria, but when they were combined with antibiotics the effect was greater (47).

Another work from Iraq (48) was concluded that the particle size may has an effect on the antibacterial activity of the NP, They have used three different particle sizes (10, 50 and 100 nm) of TiO2 against *A. baumannii* , they were found that 50 nm particle size was exhibited the best effect at concentration (25,000 µg/ml), while a higher concentration (50,000 µg/ml) was needed when they were used 10 nm and 100 nm NP to reach the same antibacterial activity.

TiO2 nanoparticles due to their small size and high surface to volume ratio undergo a higher level of interaction with the bacterial cells surface than the larger particles, resulting a high antibacterial activity (51). According to several studies, it was believed that the metal oxides carry the positive charge while the microorganisms carry negative charges; this causes electromagnetic attraction between microorganisms and the metal oxides which leads to oxidization and finally death of microorganisms, they are cause pits or holes of bacterial cell wall could be associated with internalized particles, leading to increase of permeability and cell death (52,53).

One of the principal mechanisms of TiO2 action is the production of reactive oxygen species (ROS), when exposed to light at the pro per wavelength during the photo catalysis process on its surface (54, 55). The search for new antimicrobial substances has been directed on metal oxide nanoparticles. Specifically, titanium dioxide (TiO2) as an attractive antimicrobial compound; because it is non-toxic (widely recognized as safe substance), chemically stable, inexpensive and also due to its photocatalytic nature (56).

The biosynthesis approaches are often ref erred to as "green synthesis," which were based on divers biologically available natural resources, including live plants (57), plant extracts, algae, fungi, yeasts (58) and bacteria for nanoparticles synthesis.

These biological processes were regarded as safe, cost effective, biocompatible, un toxic, stable and environmentally friendly (56).

Table 1: Identification of *A baumannii* by using Vitek 2 system

Identification information	Card: GN	Lot Number: 2410762113	Expires: Dec 28 2019 12:00 CST
	Completed: Sep 4 2019 18:04 CDT	Status: Final	Analysis Time: 6:00 hours
Selected organism	99% Probability Bionumber :02410101035500350 *Acinetobacter baumannii* Confidence : Excellent identification		

Table 2 : Antibiotic susceptibility test of *A. baumannii* using Vitek 2 System

Identification information		Analysis time: 6:00 hours		Status: Final	
Selected organism		99% Probability *Acinetobacter baumannii* Bionumber: 0241010103500350			
Susceptibility Information		Analysis Time: 8:00 hours			Status:Final
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
ESBL			Ertapenem		
Ampicillin	>= 32	R	Meropenem	>=16	R
Ampicillin/ Sulbactam	>= 32	R	Amikacin		
Piperacillin	>= 128	R	Gentamicin	>=16	R
Cefazolin	>= 64	R	Tobramycin	>=16	R
Cefoxitin	>= 64	R	Ciprofloxacin	>= 4	R
Ceftazidime	>= 64	R	Levofloxacin	4	I
Ceftriaxone	>= 64	R	Nitrofurantoin	>=512	R
Cefepime	>= 64	R	Trimethoprim/Sulfamethoxazole	>= 320	R

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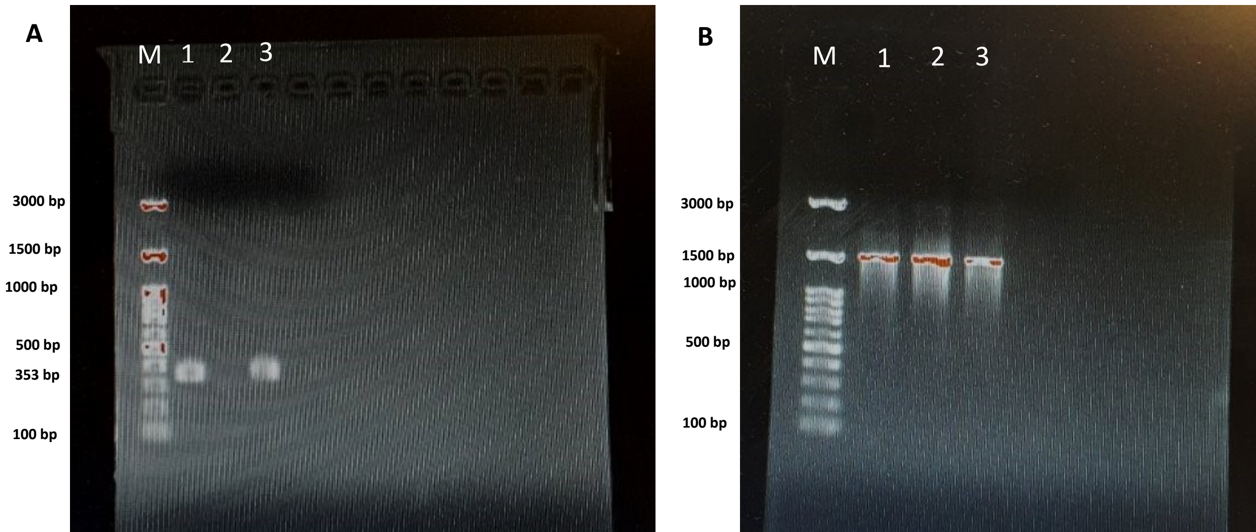


Fig 3.: The gel electrophoresis image of PCR product:

A: Amplified fragments of *bla*$_{OXA}$-51gene in clinical MDR *A. baumannii* (1), Negative control (2), *A. baumannii* ATCC 19606 (3), Lane M: DNA ladder 100bp.
B: PCR amplification of 16S RNA gene fragment, clinical MDR *A. baumannii* (1 and 2) , *A. baumannii* ATCC19606 (3), Lane M: DNA ladder 100bp.

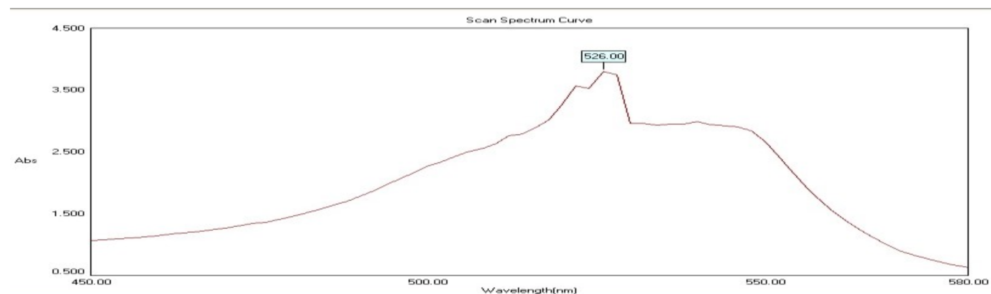
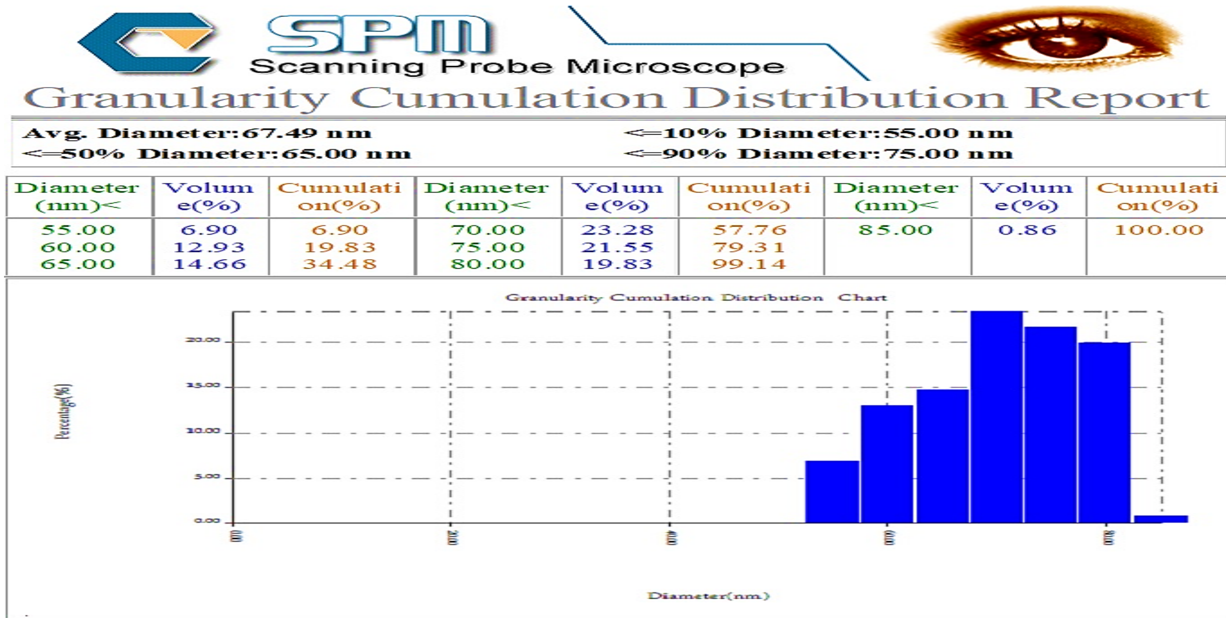


Fig 4. Absorption pattern of purified pigment, isolated from *Serratia marcescens*

Table 3. Estimation size of TiO_2 NPs and Average size of titanium nanoparticles.

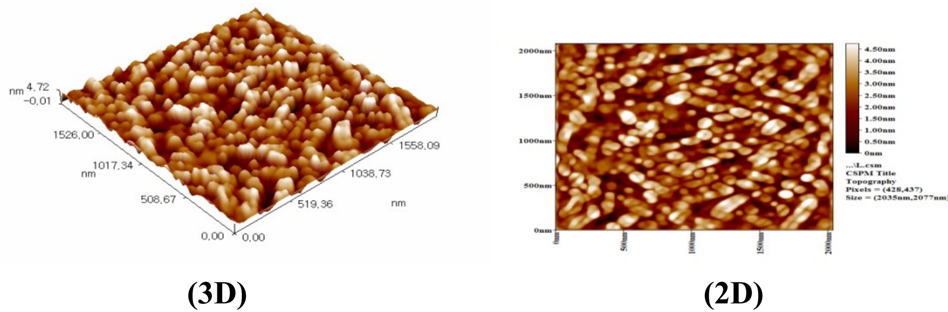


SPM Scanning Probe Microscope

Granularity Cumulation Distribution Report

Avg. Diameter:67.49 nm <=10% Diameter:55.00 nm
<=50% Diameter:65.00 nm <=90% Diameter:75.00 nm

Diameter (nm)<	Volume(%)	Cumulation(%)	Diameter (nm)<	Volume(%)	Cumulation(%)	Diameter (nm)<	Volume(%)	Cumulation(%)
55.00	6.90	6.90	70.00	23.28	57.76	85.00	0.86	100.00
60.00	12.93	19.83	75.00	21.55	79.31			
65.00	14.66	34.48	80.00	19.83	99.14			



(3D) (2D)

Fig 5. Atomic force microscopy of TiO_2 NPs synthesized using prodigiosin illustrate 2D and 3D topological.

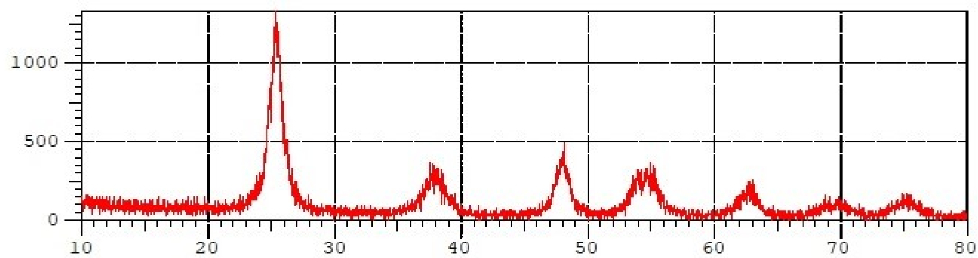


Fig 6. XRD pattern of TiO_2 nanoparticles

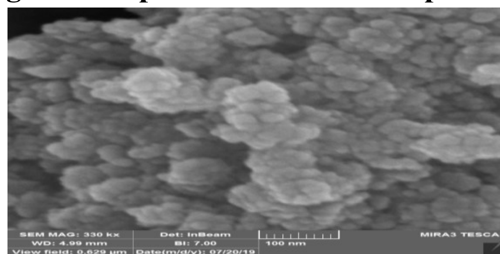


Figure 7. FE-SEM images of TiO_2 NPs synthesized using prodigiosin

Table 4: The mean OD of biofilm production and intensity of different isolates

	A baumanni	Control
Mean OD*	0.674	0.114
Biofilm Intensity**	5.6	No Biofilm
Biofilm production	Strong Biofilm producer	No Biofilm

*: Represent 24 repeated tests; **: number of folds greater than control OD values.

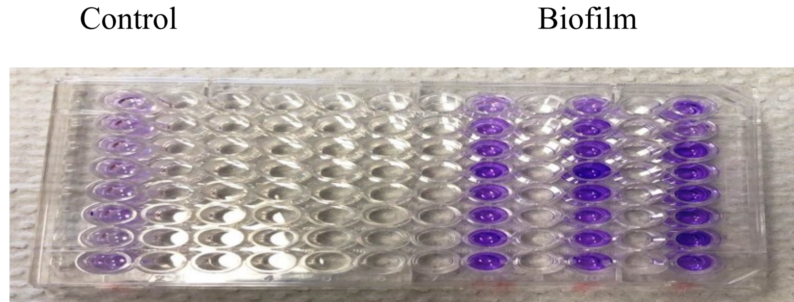


Fig 8: Detection of biofilm by using 96 well microtiter plate assay.

Fig 9: Antimicrobial activity of biosynthesized TiO2 NPs against *A. baumanni*.

A: 0.00625 mg/ml, B: 0.4 mg/ml.

Conclusions

According to our knowledge this is a first study in KRG- IRAQ used Prodigiosin for Biosynthesis of TiO2 nanoparticles which showed a promising antibacterial activity against strong biofilm producing MDR- *A. baumannii.*

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