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, vitecek 2, and confirmed by PCR technique, targeting the 16S rRNA and blaOXA-51 genes. Antimicrobial susceptibility tests were performed using Vitecek 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/sulfonamide, 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 \( \geq 0.1 \) mg/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

**Abstract**

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

Dalia Mohammed Ahmed1, Laith Ahmad Yaaqoob2, Sehand kamaludeen Arif3

1,3 Department of Biology, College of Science, University of Sulaimani, Sulaymaniya, Iraq
2Department of Biotechnology, College of Science, Baghdad University, Baghdad, Iraq

*Corresponding author : Sehand.arif@univsul.edu.iq*

DOI: https://doi.org/10.37940/AVJS2020.13.2.13

Received: 3/8/2020 Accepted: 26/11/2020

This article is licensed under a CC BY (Creative Commons Attribution 4.0)
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, photocatalysts, electrical electrodes, and gas sensors (3).

Various synthesis methods of TiO2 nanostructures are available, such as pyysical 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 discovered 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 nanoparticle 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, blaOXA 51 and RNA β 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’-TACCTTGTTACGACTTCACCCCA-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 blaOXA-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’-TGGATTGCACCTTCATCTG-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 soy 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 37°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 = OD_C= Non biofilm producer.
2. OD_C < OD_T ≤ 2OD_C = Weak biofilm producer.
3. 2OD_C < OD_T ≤ 4OD_C =Moderate biofilm producer.
4. 4OD_C < 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
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* (10⁸) 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 ultrasonic 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 TiCl4 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 determined by using agar well diffusion method. Bacterial isolates were cultivated overnight at 37 °C on Mueller Hinton medium. After incubation, standard inoculum for each bacterial isolate at a concentration of 1.5 X 10⁸ CFU / mL was formed and compared with the standard solution of 0.5 McFarland. A sterile swab has 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 (Table 1). 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\textsubscript{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/ sulphamamide, while it showed an intermediate resistance to levoflaxacin (Table 2). 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\textsubscript{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\textsubscript{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\textsubscript{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.*
*baumannii*, it was suggested that the identification of this species may be based solely on the detection of an *blaOXA*-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 TiO₂ NPs**

**Atomic force microscopy (AFM)**
AFM images show that the synthesized TiO₂ 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 TiO₂ NPs was studied by atomic force microscopy, the 2D and 3D of TiO₂ 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 nanoparticles 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-Scherrer, and XRD patterns all titanium oxide nanoparticles were in form of crystalline as shown in the equation below (41).

\[ D = \frac{K \lambda}{\beta \cos \theta} \]

Where:
- D: is the average crystallite size (Å)
- K: is the shape factor (0.9)
- \( \lambda \): is the wavelength of X-ray (1.5406 Å) Cu Kα radiation
- \( \theta \): is the Bragg angle
- \( \beta \): 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 37°C. 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 proper wavelength during the photocatalytic 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 referred 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**

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

A: Amplified fragments of \( \text{bla}_{\text{OXA}-51} \) gene in clinical MDR \( A. \text{baumannii} \) (1), Negative control (2), \( A. \text{baumannii} \) ATCC 19606 (3), Lane M: DNA ladder 100bp.

B: PCR amplification of 16S RNA gene fragment, clinical MDR \( A. \text{baumannii} \) (1 and 2), \( A. \text{baumannii} \) ATCC19606 (3), Lane M: DNA ladder 100bp.

Fig 4. Absorption pattern of purified pigment, isolated from \( Serratia \text{ marcescens} \)
Table 3. Estimation size of TiO$_2$ NPs and Average size of titanium nanoparticles.

| Diameter (nm)< | Volume e(%) | Cumulative on(%) | Diameter (nm)< | Volume e(%) | Cumulative on(%) | Diameter (nm)< | Volume e(%) | Cumulative on(%) |
|----------------|-------------|------------------|----------------|-------------|------------------|----------------|-------------|------------------|
| 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           | 90.00          | 19.83       | 99.14            |
| 65.00          | 14.66       | 34.48            | 80.00          | 19.83       | 99.14           |                |             |                  |

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 baumannii | 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. baumannii.

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.

References
1. Dos Santos Ramos MA, Da Silva PB, Spósito L, De Toledo LG, Bonifácio BV, Rodero CF, Dos Santos KC, Chorilli M, Bauab TM. Nanotechnology-based drug delivery systems for control of microbial biofilms: a review. *Int J Nanomedicine*, 2018;(13)1179-1213.
2. Raghunath A. Perumal E. Metal oxide nanoparticles as antimicrobial agents: a promise for the future. *Int. J. Antimicrob. Agents*, 2017; 49(2):137-152.

3. Masihuzz Zaman, Ejaz Ahmad, Atiyatul Qadeer, Gulam Rabbani, Rizwan Hasan Khan. Nanoparticles in relation to peptide and protein aggregation *Int J Nanomedicine*, 2014; 9(9)899–912.

4. Mishra V, Sharma R, Dut Jasuja N and Gupta D K. A review on green synthesis of nanoparticles and evaluation of antimicrobial activity, *International Journal of Green and Herbal Chemistry*, 2014; 3(1): 081.

5. Mohanpuria, P., Rana, N.K. & Yadav, S.K. Biosynthesis of nanoparticles: technological concepts and future applications. *J Nanopart Res*, 2008; 10, 507–517.

6. Kanafari A. et al. Review of Prodigiosin, Pigmentation in *Serratia marcescens*. *Online journal of biological science*, 2006;(6)1,1-13.

7. Harris AKP, Williamson NR, Slater H et al. The *Serratia* gene cluster encoding biosynthesis of the red antibiotic, prodigiosin, shows species- and strain-dependent genome context variation. *Microbiol*. 2004, (150)3547–60.

8. Iguchi A, Nagaya Y, Pradel E, Ooka T, Ogura Y, Katsura K, Kurokawa K, Oshima K, Hattori M, Parkhill J, Sebaihia M, Coulthurst S, Gotoh N, Thomson NR, Ewbank JI, Hayashi T. Genome evolution and plasticity of *Serratia marcescens*, an important multidrug resistant nosocomial pathogen. *Genome Biol Evol*.2014; (6)2096–2110.

9. Danevčič T, Borić Vezjak M, Zorec M, Stopar D. Prodigiosin - A Multifaceted Escherichia coli Antimicrobial Agent. *Plos one*. 2016;11(9): e0162412.

10. Dozie-Nwachukwu, S.O., Obayemi, J.D., Danyuo, Y.T. *et al*. Biosynthesis of Gold Nanoparticles and Gold/Prodigiosin Nanoparticles with *Serratia marcescens* Bacteria. *Waste Biomass Valor*, 2017;(8)6, 2045-2059.

11. Akl B., Nader, M., El-Saadony, M. Biosynthesis of Silver Nanoparticles by *Serratia marcescens* ssp sakuensis and its Antibacterial Application against some Pathogenic Bacteria. *Journal of Agricultural Chemistry and Biotechnology*, 2020; 11(1), 1-8.

12. Seifert, H. and Dijkshoorn, L. Overview of the medical characteristics, taxonomy, and epidemiology of *Acinetobacter*. In: Bergogne-Berezin, E., Friedman H., and Bendinelli M: *Acinetobacter biology and pathogenesis*. Springer, 2008; p.19-47.

13. Organization WHO, WHO Publishes List of Bacteria for Which New Antibiotics Are Urgently Needed, WHO, Geneva, Switzerland, 2017. http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/.

14. Yaw Adjei Anane,Teke Apalata ,Sandep Vasaikar ,Grace Emily Okuthe,and Sandle Songea. Molecular Detection of Carbapenemase-Encoding Genes in Multidrug-Resistant *Acinetobacter baumannii* Clinical Isolates in South Africa. *International Journal of Microbiology*.2020. (1). pp.1-10.

15. Karah, N., Haldorsen, B., Hegstad, K., Simonsen, G. S., Sundsfjord, A., Samuelsen, Ø., & Norwegian Study Group of *Acinetobacter*. Species identification and molecular characterization of *Acinetobacter* spp. blood culture isolates from Norway. *The Journal of antimicrobial chemotherapy*, 2011, 66(4), pp.738–744.

16. Lee MJ, Jang SJ, Li XM, Park G, Kook JK, Kim MJ, Chang YH, Shin JH, Kim SH, Kim DM, Kang SH, Moon DS. Comparison of
rpoB gene sequencing, 16S rRNA gene sequencing, gyrB multiplex PCR, and the VITEK2 system for identification of Acinetobacter clinical isolates. Diagn Microbiol Infect Dis, 2014;78, pp.29–34.

17. Ghaima K. K., Saadedin S., M., Jassim K., A. Isolation, molecular identification and antimicrobial susceptibility of Acinetobacter baumannii isolated from Baghdad hospitals. International Journal of Scientific and Research Publications, 2016;6(5).

18. Abdullah Z. H., Merza N. S. Phenotypic and Molecular detection of Acinetobacter baumannii isolated from patients in Duhok City-Iraq. Science journal of university of Zakho, 2019; 7(4), pp. 132-137.

19. Freeman J, Falkiner FR, Keane CT. New method for detecting slime production by coagulase negative staphylococci. J Clin Pathol, 1989; 42(872)-4.

20. Hassan A, Usman J, Kaleem F, Omair M, Khalid A, Iqbal M. Evaluation of different detection methods of biofilm formation in the clinical isolates. Braz J Infect Dis. 2011;15(4).

21. Nabajit Deka. Comparison of Tissue Culture plate method, Tube Method and Congo Red Agar Method for the detection of biofilm formation by Coagulase Negative Staphylococcus isolated from Non-Clinical Isolates. International journal of current Microbiology and Applied Science, 2014;3(10), 810-815.

22. Higgins PG, H. Wisplinghoff H, Stefanik D, Seifert H. Selection of topoisomerase mutations and overexpression of adeB mRNA transcripts during an outbreak of Acinetobacter baumannii. J Antimicrob Chemother. 2004; (54),821–823.

23. Woodford N, Ellington MJ, Coelho JM, Turton JF, Ward ME, Brown S, et al. Multiplex PCR for genes encoding prevalent OXA carbapenemases in Acinetobacter spp. Int. J. Antimicrob. Agents. 2006; (27),351–353.

24. Christensen G D, Simpson W A, Younger J J, Baddour L M, Barrett F F, Melton D M, Beachey E H. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. J Clin Microbiol. 1985; 22(6),996–1006.

25. Jesline, A., John, N.P., Narayanan, P.M. et al. Antimicrobial activity of zinc and titanium dioxide nanoparticles against biofilm-producing meticillin-resistant Staphylococcus aureus. Appl Nanosci, 2015;(5),157–162.

26. Chen, W.C., W.J.Yu, C.C.Chang, J.S.Chang, S.H. Huang and C.H. Chang. Enhancing production of prodigiosin from Serratia marcescens C3 by statistical experimental design and porous carrier addition strategy. Biochemical Engineering Journal, 2013; (78),93-100.

27. KG Rao, CH Ashok, KV Rao, CS Chakra, P Tambur. Green Synthesis of TiO2 Nanoparticles Using Aloe Vera Extract. International Journal of Advanced Research in Physical Science, 2015;2(1A),28-34.

28. Krithika K, Sruthi C.V. and Greetharamani D. Production of silver nanoparticles from Serratia marcescens and its application as antibacterial agent. Scrutiny International Research Journal of Agriculture, Plant Biotechnology and Bio Products, 2014;1(5).

29. Providencia Joyanes, Maria del Carmen Conejo, Luis Martinez-Martinez, Evelio J. Perea. Evaluation of the VITEK 2 System for the Identification and Susceptibility Testing of Three Species of Nonfermenting Gram-Negative Rods Frequently Isolated from Clinical Samples. J Clin Microbiol. 2001; 39(9), 3247–3253.

30. Funke, G., D. Monnet, C. deBernardis, A. von Graevenitz, and J. Freney. 1998. Evaluation of the VITEK 2 system for rapid identification of medically relevant gram-
negative rods. J. Clin. Microbiol. 1998; (36), 1948–1952.

31. Ling T. K., Tam P. C., Liu Z. K., Augustine, Cheng F.B. Evaluation of VITEK 2 Rapid Identification and Susceptibility Testing System against Gram-Negative Clinical Isolates. Journal of Clinical Microbiology, 2001; 39 (8), 2964-2966.

32. Menglan Zhou, Yao Wang, Chang Liu, Timothy Kudinha, Xiaolin Liu, Yanping Luo, Qiwen Yang, Hongli Sun, Jihong Hu, Ying-Chun Xu. Comparison of five commonly used automated susceptibility testing methods for accuracy in the China Antimicrobial Resistance Surveillance System (CARSS) hospitals. Infect Drug Resist. 2018; (11), 1347–1358.

33. Misbah S, AbuBakar S, Hassan H, et al. Antibiotic susceptibility and REPPCR fingerprints of Acinetobacter spp. isolated from a hospital at ten years. J Hosp Infect. 2004; (58): pp.254-261.

34. Dortet L, Legrand P, Soussy CJ, Cattoir V. Bacterial identification, clinical significance, and antimicrobial susceptibilities of Acinetobacter ursingii and Acinetobacter schindleri, two frequently misidentified opportunistic pathogens. J Clin Microbiol. 2006; (44): 4471–8.

35. Biglari S, Alfizah H, Ramliza R, Rahman MM. Molecular characterization of carbapenemase and cephalosporinase genes among clinical isolates of Acinetobacter baumannii in a tertiary medical centre in Malaysia. Journal of Medical Microbiology, 2015; (64), 53–58.

36. Al-Doori H, Rasheed M, Rasheed H, Musleh M. Molecular Detection of blaOXA Genes in Acinetobacter baumannii Collected from Patients with Various Infections. 2019.

37. Puyuan Li, Wenkai Niu, Huan Li, Hong Lei, Wei Liu, Xiangna Zhao, Leijing Guo, Dayang Zou, Xin Yuan, Huizing Liu, Jing Yuan, Changqing Bai. Rapid detection of Acinetobacter baumannii and molecular epidemiology of carbapenem-resistant A. baumannii in two comprehensive hospitals of Beijing, China. Front Microbiol. 2015;(6): 997.

38. Evans B. A., Hamouda A., K. J. Towner K.J. and Amyes S.G.B. OXA-51-like b-lactamases and their association with particular epidemic lineages of Acinetobacter baumannii. European Society of Clinical Microbiology and Infectious Diseases, 2008;(14)3, pp. 268-275.

39. Khanafari A., M.M.Assadi and F.A.Fakhr. Review of prodigiosin, pigmentation in Serratia marcescens Qods Sqr., Tajrish Sqr. Tehran, Iran Department of Forest Sciences, Faculty of Forestry, The University of British Columbia, 2006; (6):1–13.

40. Yang, H. Atomic force microscopy (AFM): principles, modes of operation and limitations. 1st ed., Nova Science Publishers, Incorporated, 2014.

41. Pusit Pookmanee and Sukon Phanichphant. Titanium dioxide powder prepared by a solgel method, Journal of Ceramic Processing Research, 2009; 10(2): 167.

42. Vijayalakshmi, R. and V. Rajendran. Synthesis and characterization of nano TiO2 via different methods. Arch.Appl.Sci.Res. 2012;4(2):1183–1190.

43. Ruzicka F, Hola V, Votava M et al. Biofilm detection and clinical significance of Staphylococcus epidermidis isolates. Folia Microbiol (Praha) 2004; 49(5):596-600.

44. Baqai R, Aziz M, Rasool G. Urinary tract infection in diabetic patients and biofilm formation of uropathogens. Infect Dis J Pakistan. 2008 17(1):7–9.

45. Knobloch JK et al. Evaluation of different detection methods of biofilm formation in Staphylococcus aureus. Med Microbial Immunol, 2002; 191(2):101-6.

46. Masoumi S, Shakibaie M R, Gholamrezazadeh M. and Monirzadeh F. Evaluation Synergistic Effect of TiO2,
ZnO Nanoparticles and Amphiphilic Peptides (Mastoparan-B, Indolicidin) Against Drug-Resistant *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Acinetobacter baumannii*. Arch Pediatr Infect Dis. 2018; 6(3): e57920.

47. Rana Mujahid Abdullah. A Study the Effect of TiO2 Nanoparticles Combination with Antibiotics and Plant Extracts Against Some Gram Negative Bacteria. Baghdad Science Journal,2016;13(3):0425.

48. Kareem P.A., Alsammak E.G.H., Abdullah Y.J., Bdaïwi Q.M. Estimation of antibacterial activity of zinc oxide, titanium dioxide, and silver nanoparticles against multidrug-resistant bacteria isolated from clinical cases in Amara City, Iraq. *Drug Invention Today*, 2019;11(11):2887-2890.

49. Jesline, A., John, N.P., Narayanan, P.M. et al. Antimicrobial activity of zinc and titanium dioxide nanoparticles against biofilm-producing methicillin-resistant *Staphylococcus aureus*. *Appl Nanosci*.2015; (5):157–162.

50. Albaïaty, Lubna. Titanium dioxide nanoparticles as antibacterial agents against some pathogenic bacteria. *Drug Invention Today*.2019;(12) 5.

51. Zhang, H., Chen, G. Potent antibacterial activities of Ag/TiO2 nanocomposite powders synthesized by one-pot sol-gel method. *Environmental Science and Technology*.2009;34(8),2905-2910.

52. Ravishankar, R.V., Jamuna, B.A. Nanoparticles and their potential applications as antimicrobials. In: Mendez-Vilas, A.(Ed.), *Science Against Microbial Pathogens: Communicating Current Research and Technological Advances*. Formatex, Badajoz,2011, pp.197-209.

53. Holt, K.B., Bard, A. J. Interaction of silver (1) ions with the respiratory chain of *Escherichia coli*: an electrochemical and scanning electrochemical microscopy of micromolar Ag. *Journal of biochemical Industry*, 2003; 44(39),13214-13223.

54. Chen W-J, Tsai P-J, Chen Y-C. Functional Fe3O4/TiO2 core/shell magnetic nanoparticles as photokilling agents for pathogenic bacteria. *Small*. 2008;(4):485-491.

55. López de Dicastillo C, Patiño C, Galotto MJ, Vásquez-Martínez Y, Torrent C, Alburquerque D, et al. Novel hollow titanium dioxide nanospheres with antimicrobial activity against resistant bacteria. *Beilstein Journal of Nanotechnology*. 2019 ;(10):1716-1725.

56. Carol López de Dicastillo, Matias Guerrero Correa, Fernanda B. Martínez, Camilo Streit and Maria José Galotto. Antimicrobial Effect of Titanium Dioxide Nanoparticles, DOI: http://dx.doi.org/10.5772/intechopen.90891.

57. Bali R, Razak N, Lumb A, Harris AT. The synthesis of metallic nanoparticles inside live plants. In: Proc. 2006 *Int. Conf. Nanosci. Nanotechnology, ICONE*. 2006. pp. 224-227.

58. Moghaddam AB, Moniri M, Azizi S, Rahim RA, Bin AA, Saad WZ, et al. Biosynthesis of ZnO nanoparticles by a new *Pichia kudriavzevii* yeast strain and evaluation of their antimicrobial and antioxidant activities. *Molecules*. 2017;22(6):872.