EVALUATION THE EFFECT OF GREEN SYNTHESIS TITANIUM DIOXIDE NANOPARTICLES ON *ACINETOBACTER BAUMANNII* ISOLATES

M. R. Hamza **L. A. Yaaqoob**
Researcher **Asist. Prof.**
Dept. Biot., Coll. Scie., University of Baghdad, Iraq
Raheemmaryam285@gmail.com laith.yaaqoob@sc.uobaghdad.edu.iq

ABSTRACT

Acinetobacter baumannii **is gram negative opportunistic coccobacilli,the most important agent in nosocomial infections with high mortality rate. This bacteria is characterized by (biochemical tests, polymerase chain reaction (PCR) and vitek-2 system). The antibiotic susceptibility result of *A. baumannii* isolates were shown resistant to (Methicillin, Nitrofurantion), while sensitive to (colistin, Ciprofloxacin).This study was aimed to biosynthesis of titanium dioxide nanoparticles by using prodigiosin pigment produced from clinical isolate *Serratia marcescens* as reducing and stabilizing agent for Titanium dioxide nanoparticles (TiO_2 NPs) and used as antibacterial agent for *Acinetobacter baumannii* isolated from different clinical sources. TiO_2 NPs synthesized by using titanium chloride $TiCl_4$ (5 ml/50 ml) in deionized water with concentration of prodigiosin (10 mg/ml)and adjusted pH at 7 and the temperature at 50 ˚C. TiO_2NPs synthesized was Characterized by various technique, such as (AFM , UV-VIS, FTIR , XRD and FE-SEM). The result showed that wavelength(366)nm was optimum for TiO_2NPs that have crystalline shape at average volume (47.52 nm). Green synthesized TiO_2 NPs have shown several applications such as biomedical, anticancer, bio sensing, catalysis etc.**

Key Words: optimization, antimicrobial activity, prodigiosin

تقييم تأثير دقائق التيتانيوم النانوية المصنعة بالطريقة الخضراء على بكتريا *Acinetobacter baumannii*

مريم رحيم حمزة ليث احمد يعقوب
باحث استاذ مساعد
قسم التقنيات الأحيائية، كليه العلوم، جامعة بغداد، بغداد، العراق.

المستخلص:

تعد بكتريا *Acinetobacter baumannii* العصوية السالبة لصبغة كرام من العوامل الاكثر اهمية في التهابات المستشفيات ذات معدل الوفيات المرتفع. تم تشخيص البكتريا بواسطة (الاختبارات الكيميائية و تفاعل البلمرة المتسلسل PCRو vitek–2) . اظهرت نتائج اختبار الحساسية لعزلات بكتريا *A.baumannii* المقاومة لكل من (الميثيسيلين و النايتروفيورانتن) بينما كانت حساسة لكل من (الكوليستين و السبروفلوكساسين). هدفت الدراسة الحالية الى تخليق دقائق التيتانيوم النانوية باستخدام صبغة البرودجيوسين المنتجة من العزلة المرضية *Serratia marcescens* كعامل مختزل ومثبت لدقائق التيتانيوم النانوية واستخدامها كعوامل مضادة لبكتريا *Acinetobacter baumannii* المعزولة من مصادر سريرية مختلفة .صنعت جزيئات التيتانيوم النانوية باستخدام $TiCl_4$ بحجم)5 مل/ 50 مل من الماء منزوع الايونات (وصبغة البرودجيوسين بتركيز (10ملم/مل) ، حيث تم تضبيط دالة الحموضة على 7 ودرجة الحرارة عند 50 مئوية. تم توصيف الدقائق النانوية المصنعة باستخدام عدة تقنيات منها. (SEM–AFM, UV–VIS, FTIR, XRD and FE) بينت النتائج ان الطول الموجي 366 نانوميترهو الامثل لدقائق التيتانيوم النانوية والتي تمتلك شكلا كرويا بمعدل حجم (47.52) نانوميتر. تمتلك الدقائق النانوية المصنعة بالطريقة الخضراء عده استعمالات طبية حياتية مثل مضادة للخلايا السرطانية، التحسس الحيوي، محفزات وغيرها.

الكلمات المفتاحية: تحسين، فعالية مضادة للبكتريا، البرودجيوسين.

***Received:12/11/2019, Accepted:16/3/2020**

INTRODUCTION

Due to its broad variety of applications in almost all forms of industries, from textiles to medicine and electronics, everybody is based on nanotechnology. Titanium dioxide (TiO_2) is solid inorganic material, which is a metal oxide in white colour , non-flammable, less soluble, thermally stable and not structured as hazardous according to the UN Globally Harmonized System (GHS) for Chemicals Classification and Labeling (1) . TiO_2 is produced by the elements atomic number 22 titanium from the IV B group and atomic number 8 oxygen from the VI A group . At different temperatures it can exhibit three various phases in nano range, such as Anatase, Rutile and Brookite . Anatase has been shown to have excellent chemical and physical charcteristics for environmental remediation in these processes (18) . It also has charecterstics of high quality, such as non-wettability, hydrophobicity and wide band gap. It is therefore used in different industrial applications: self-cleaning, charging systems, photo catalysis, color sensitized solar cells, microelectronics, textiles, chemical sensors, antibacterial products and electrochemicals (22) . There are different types of methods for the synthesis of titanium dioxide nano-particles including: solution combustion (29) , sol-gel (12), hydrothermal (24), solvothermal (8), microwave assisted (10), co-precipitation (13), chemical vapour deposition (14) and green synthesis. The green synthesis method is an environmentally friendly system due to the use of plant extracts (leaves, flowers, seeds and peels), bacteria, fungi and enzymes for the synthesis of titanium dioxide nanoparticles, rather than large quantities of chemicals (25). Green synthesis supplies more advantages than chemical methods and physical methods, since it is simple to process, very cost-effective and scalable for large-scale production. This process did not require high pressure, expensive machines, high temperatures and toxic chemicals. The secondary metabolite prodigiosin was found to be formed by gram negative *Serratia marcescens*, *Serratia rubidaea Rugamonas rubra*, *Pseudomonas magneslorubra*, *Alteromonas rubra*, *Vibrio gazogenes* and Gram positive *actinomycetes*, such as *Streptomyces longisporus ruber* and *Streptoverticillium rubrireticuli* forms prodigiosin and / or by- product of this molecule (15). Prodigiosin have a wide range of applications including; Antibacterial, Antifungal, Antimalarial, Antitrypanosomal, Anti algae, Anticancer, Insecticidal, Immunosuppressive activities (27). Acknowledging the value of developing environmentally friendly methods for synthesizing biologically active nanoparticles. Prodigiosin are now mediating new developments in the field of nanotechnology. The genus *Acinetobacter* consists of several species but *Acinetobacter baumannii* is the most widespread member linked directly with hospital-acquired infections (16). *A. baumannii* is a gram-negative, strictly aerobic, glucose non-fermenting coccobacillus (3). This bacterium had been considered a low-level pathogen, in spite of its capacity to cause vast and severe infections including: skin, bloodstream, secondary meningitis, urinary tract and soft tissue infections(17). This study was aimed to the purified a prodigiosin from *Serratia marcescens* and used to biosynthesize of titanium dioxide nanoparticles as reducing and stabilizing agent . As well as study the potential application of the synthesized nanoparticles in vitro as antibacterial activity against human pathogenic bacteria (*A. baumannii*).

MATERIALS AND METHODS

Bacterial isolation and culture media

The bacterial isolates (*A. baumannii*) used in the present study was collected from four hospitals in Baghdad/Medical city including: the Martyr Ghazi Al-Hareery Hospital for Surgical Specialties, Baghdad Teaching Hospital, Burns Specialty Hospital, and Child Care Hospital at (12/ 2018 till 4/2019) include 195 clinical specimens comprising; wounds, urine, burns and sputum .Then all specimens were cultured by streaking on MacConkey agar and blood agar thereafter incubated at 37 ˚C for 24 hours,This media was prepared according to the instruction of manufacturer company (Himedia). The bacterial isolate (*S. marcescens*) used in the present study for prodigiosin production was isolated from the Martyr Ghazi Al-Hareery Hospital for Surgical Specialties and Baghdad

Teaching Hospital from (urine and sputum) of infected patients. Then all specimens were cultured by streaking on nutrient agar thereafter incubated at 30 ˚C for 24 hours. The biochemical tests, morphological characteristics and identification using manual and/or automated methods (Vitek II, bioMe ´rieux, Marcy l'Etoile, France) for both bacteria(*A. baumannii* and *S. marcescens*) and polymerase chain reaction(PCR) by 16 SrRNA and blaoxa-51 only for *A. baumannii* were performed .

Prodigiosin production:Fermentation media Preparation was based on Chen and coworkers with modification (9) . In briefly Medium was prepared by mixing the components by (g/l) included Peptone 5 as nitrogen source ,sucrose 10 as carbon source, $MgSO_4.7H_2O$ 0.61, $MnSO_4.4H_2O_2$ 2, $CaCl_2.2H_2O$ 8.82 and $FeSO_4.4H_2O$ 0.33. The pH was set to 7.0 and then sterilized at 121° C for 15 minutes by autoclaving. After sterilization, the medium left to cool and inoculated with 2% of (1×10^8) selected bacteria isolates (*S. marcescens*) and incubated in shaker incubator at 30 ° C for 72 hours at 200 rpm.

Extraction and purification of prodigiosin

The crude Prodigiosin was extracted from *S. marcescens* cell-free broth culture obtained after 30 hours of incubation. The culture medium was centrifuged at 8000 rpm for 15 minutes. Then the supernatant was discarded and 250 ml of methanol was added to the harvested cell , thoroughly mixed at room temperature for 3 hours. The resulting mixture was then centrifuged for 20 min at 8000 rpm, collecting and filtering the supernatant through a filter paper (0.2 µm, milipore filter) . Rotary evaporator was used to concentrate the methanol filtrate at 70˚C and twice amount of chloroform was then added to extract the red pigment. The two solvents were mixed vigorously in a reparatory funnel . Chloroform phase (organic phase) was collected and dried at 45 ˚C . The resulting pigment was then dissolved in a small amount of methanol and stored in a dark bottle in a refrigerator for further tests . Prodigiosin was purified according to (9).

Synthesis of titanium dioxide nanoparticles

Titanium chloride ($TiCl_4$, 99%) (ACROS ORGANICS/FRANCE) was used for preparation of titanium nanoparticles. Method of synthesis is done by two solutions:

Solution (A) is prepared as follows: 5 ml of $TiCl_4$ in 50 ml deionized water (DI) dispersed by ultra-sonication bath for 30 minutes. In addition, solution (B) prepared by dissolving of 10mg\ ml from prodigiosin and dispersed by ultra-sonication bath for 60 minutes. The two solutions (A and B) are mixed by magnetic stirrer at pH 7 about 30 min.and then left over night in the dark room. The solution contains titanium nanoparticles , separated and concentrated for 30 minutes by centrifugation at 6000 rpm then washed twice by DI water and also precipitated for 30 minutes by centrifugation at 6000 rpm. There after dried in the oven at 60 ˚C for 30 minutes to obtain a white powder , and kept in dark vial for further characterization and applications.

Antibacterial test(in vitro): Antibacterial activity of TiO_2 NPs was investigated using gram-negative bacteria *Acinetobacter baumannii* . The minimal inhibition concentration (MIC) of TiO_2 NPs was estimated by using of agar well diffusion technique (20). The synthetic TiO_2 from (Hongwu, China) used as negative control in a same concentration of green TiO_2 NPs that used in all experiment . Almost 25 ml of the Müller Hinton agar sterilized medium was poured into sterilized petri dishes and permitted to solidify at room temperature. The overnight growth of tested bacteria was transported and spread over the agar medium by separately using a sterile cotton swab, wells were made. After that, different concentrations of green TiO_2 NPs (3.9, 7.81, 15.62, 31.25, 62.5, 125,250.500 µg/ml) , were prepared and added with negative control (synthetic TiO_2) at same the concentration to the wells. The plates were incubated for 24 hours at 37 ° C. The inhibition zone around the well had been measured after incubation (23) .

RESULTS AND DISCUSSION

Bacterial isolation in culture media: *A.baumannii* isolates, on MacConkey agar were non-lactose fermenting colonies ;while on blood agar the colonies appeared as non-hemolytic opaque creamy colonies. *S.marcescens* appear as red colony on nutrient agar.

Vitek- 2 compact system for *A.baumannii* and *S.marcescens*

In the current study the selected bacterial isolate was identified by using vitek- 2 compact system. Both *A.baumannii and S. Marcescens* had 99% in similarity with selected isolates.

Identification of *A.baumannii* by PCR

All bacterial isolates were positive to 16SrRNA and blaOXA-51 genes table 1.

Table 1. Sequence of 16SrRNA and blaOXA51 primers

Gene name	Primer Name	Sequence	Product size
16SrRNA	F	5'-CAGCTCGTGTCGTGAGATGT-3'	150 bp
	R	5'-CGTAAGGGCCATGATGACTT-3'	
blaOXA51	F	5'-TAATGCTTTGATCGGCCTTG-3'	353 bp
	R	5'-TGGATTGCACTTCATCTTGG-3'	

Production of prodigiosin

Serratia A3 showed maximum production of prodigiosin. The prodigiosin concentration provided by this isolate was (10.89 mg / l) after three days of incubation. The maximum production of prodigiosin had been observed at 48 h and that production had been completed by 72 h (9). The prodigiosin produced by *Serratia marcescens* is characterized by UV-visible spectrophotometer (Shimadzu, Japan) in order to detect the maximum absorption (ÿmax). Absorbance in measured 530 nm (15).As showen in figure (1) :

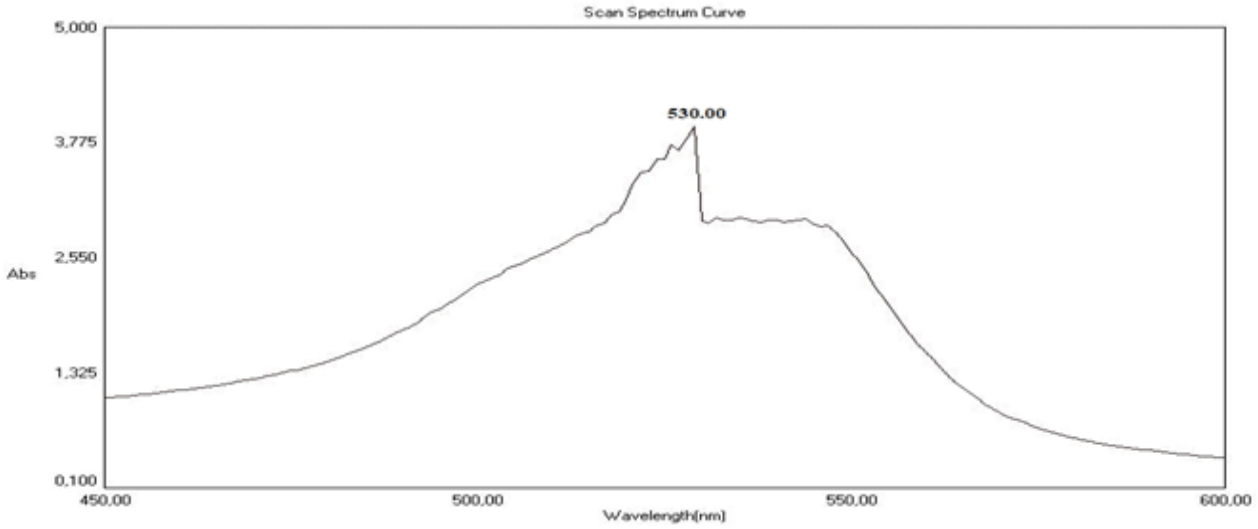
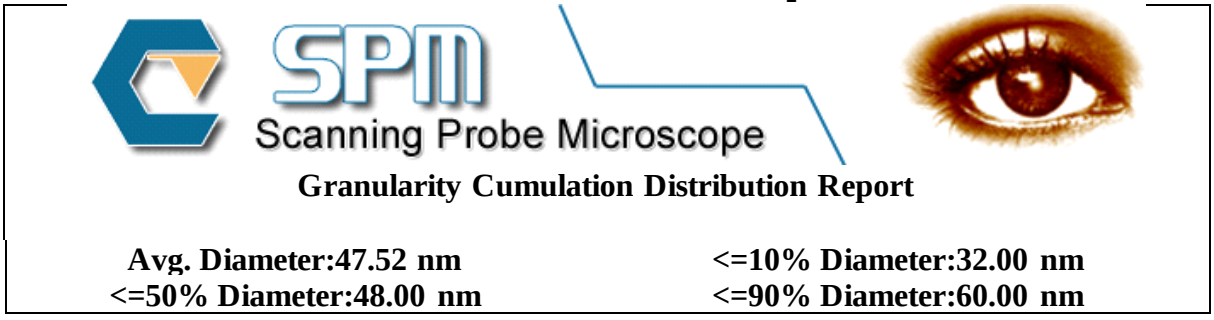


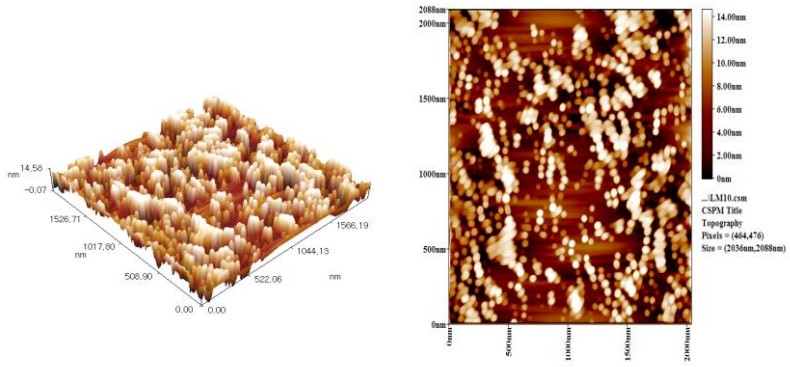
Figure1. Absorption pattern of purified pigment, isolated from *Serratia* sp.

Characterization of green synthesis TiO_2 NPs

Atomic force microscopy (AFM): 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 (28) (Fig 2). AFM images show that the synthesized TiO_2 NPs are in spherical shape and the size of an average diameter was 47.52 nm(Fig3).

Table 2. Estimation size of TiO_2NPs





(3D) (2D)

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

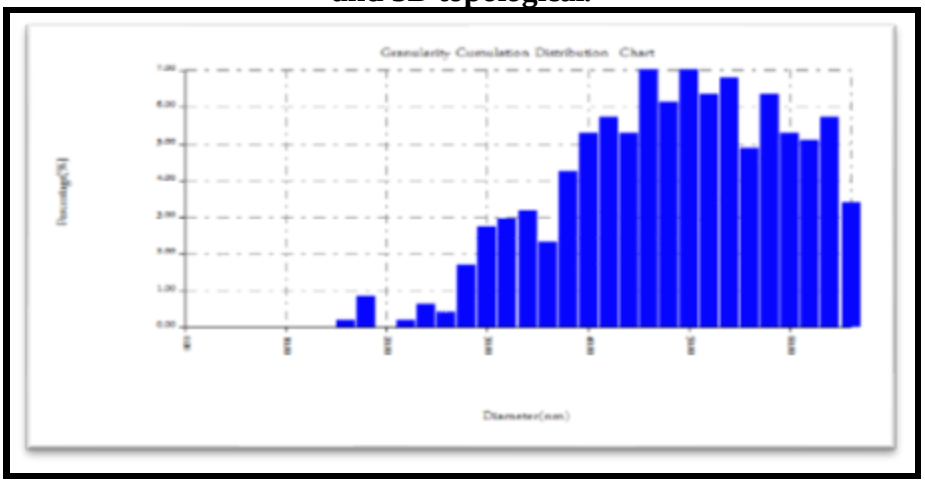


Figure 3. Average size of titanium nanoparticles

X-ray diffractometer

The XRD pattern of TiO_2nanoparticles was obtained from green synthesis as show in Fig .4. XRD patterns show that all TiO_2 NPs in anatase phase , and these findings were in good agreement with the JCPDS number of the card 21-1272. Peaks were absorbed at 25°, 38°, 48°, 53°, 55°, 62° and 75° along with miller indices values (1 0 1), (0 0 4), (2 0 0), (1 0 5), (2 1 1), (2 0 4) and (2 1 5) respectively.

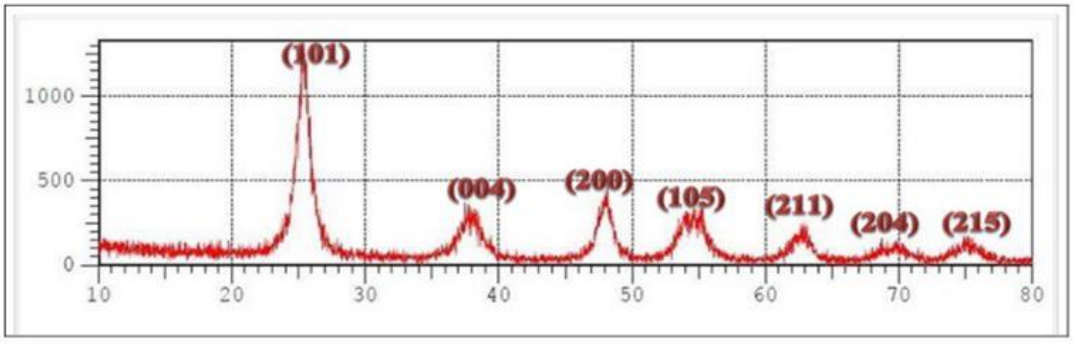


Figure 4. XRD pattern of TiO_2 nanoparticles

As the width of the peak increases the particle size decreases, which is similar to that of nano material, that obtained the lattice parameters a = b=0.3785 nm and c=0.9513 nm. The average size of crystallite was determined by the equation of Debye-Schereer is(23nm), as stated below .

$$D = [K\lambda / \beta\cos\theta] \text{ Å} \quad (21)$$

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.

Fourier transform infrared (FTIR) spectroscopy analysis

FTIR spectrum has determined the functional groups of nanoparticles. (Fig. 5) represents the absorption spectrum of green synthesized nanoparticles. An intense peak at 3398.34 cm-1 was visible due to OH stretching mode. The occurrence of the peak properties at 1629.74 cm-1 suggested the presence of crystallographic H_2O molecules, i.e. O-H bend. The wide peak at 455.17 cm-1 and 572.82 cm-1 respectively represented the Ti – O band and Ti – O – Ti skeletal frequency (19).

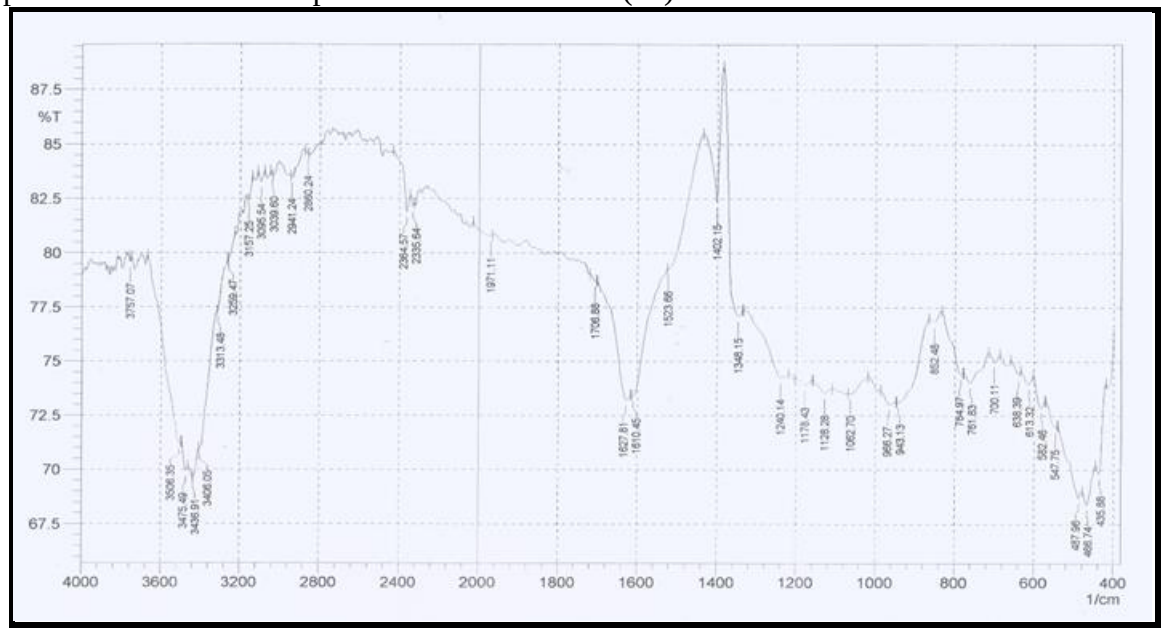


Figure 5. FTIR images of TiO_2 NPs synthesized using prodigiosin

UV–VIS spectral analysis

Milky white colloidal solution was developed to signify the conversion of titanium chloride ($TiCl_4$) into nano-sized TiO_2 particles. In addition, they investigated their physical characteristics using UV-Visible spectroscopy . Therefore, the synthesis of nano-sized TiO_2 particles were dedicated to the absorption spectra in (366) nm (Fig. 6). This result similar with the absorption spectra 362 nm by(5) .The UV-vis peaks showed the direct recombination of the electrons in the conduction band and the valence band holes (11).

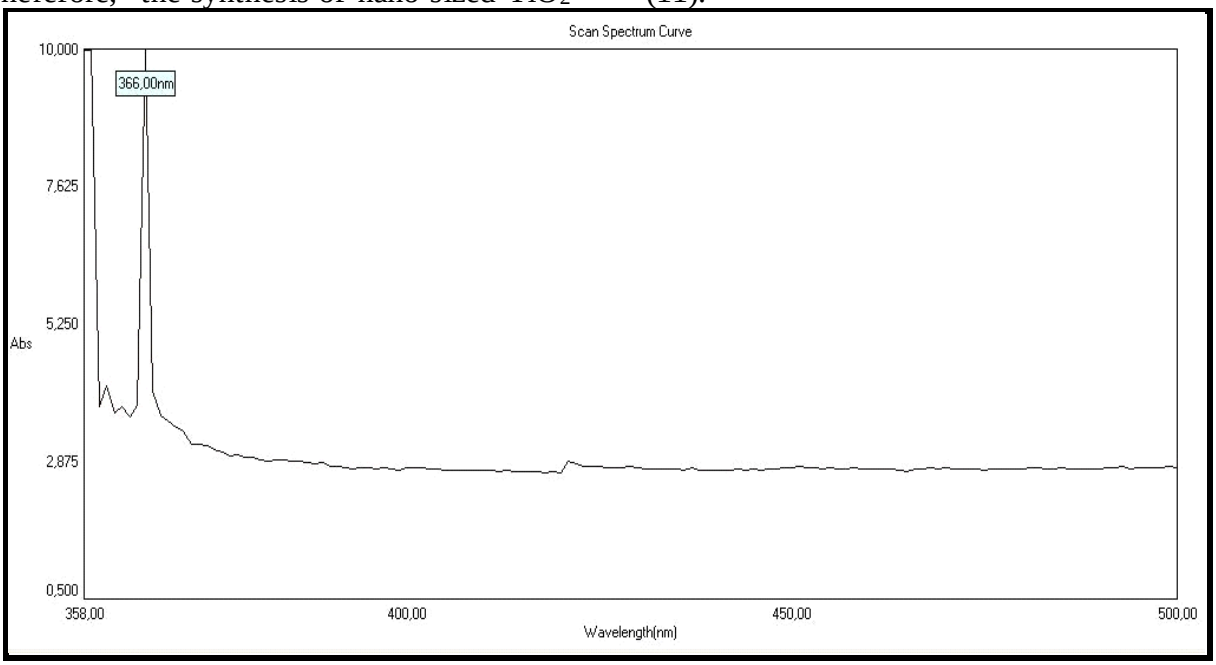


Figure 6. UV- VIS images of TiO_2 NPs synthesized using prodigiosin

Field emission scanning electron microscopy

Through applying the FESEM, images were taken of the sample at a magnification of 50 kx. Based on (fig. 7), all the samples exhibit smooth planes and uniform shape in the form of TiO_2 nano clusters. It has been invistigated that the particle size increases with increase in calcination temperature due to the agglomeration of smaller particle at high temperature. Low temperature leads to better boundaries between nanoparticles. As a result, the shape of the NPs changed to sphere (26).

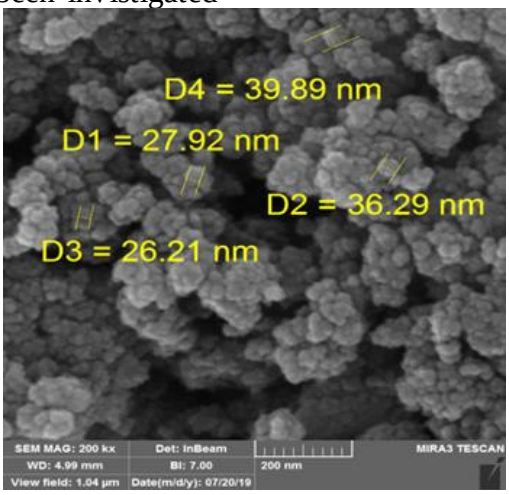


Figure 7. FE-SEM images of TiO_2 NPs synthesized using prodigiosin

Antibacterial susceptibillity test

The results of using TiO_2 NPs as antibacterial agents were demonstrated in (Fig. 8). The antibacterial activity was found to be directly dependent upon the TiO_2 NPs concentration. Table 3 showen that the maximum inhibition zones of *A. baumannii* were 28 mm at concentration 500 µg/ml of TiO_2 NPs, Whereas the minimum inhibition zones were located at 7.81 µg / ml of TiO_2 NPs concentrations,these result were agreement with(2) . The difference in inhibition diameter may be due to different interactions between TiO_2 NPs and the microorganism, and due to the susceptibility of bacteria used in the current study . The main mechanism of TiO_2 NPs toxicity is potentially associated with metal oxides carries the positive charge even though the microorganisms bear negative charges; this results in electromagnetic interaction between microorganisms and metal oxides leading to oxidation and finally death of microorganisms (4,30) . Bactericidal action of TiO_2 nanoparticles on bacteria is of extreme importance due to the ability of pathogenic bacteria to join the food chain of the ecosystem (6,30). The antibacterial activity of TiO_2NPs was due to the capability of TiO_2 particles to cause free hydroxyl radicals (OH^-) (7). The antimicrobial effect of TiO_2 against fungi and bacteria has been demonstrated and communicating in modern research.

Table 3. Antimicrobial activity of TiO_2NPS against *A.baumannii*

No.	TiO_2 NPs concentration µg/ml	Zone Dimeter (mm) *A.baumannii*
1	3.9	Nill
2	7.81	10
3	15.62	13
4	31.25	16
5	62.5	19
6	125	21
7	250	24
8	500	28

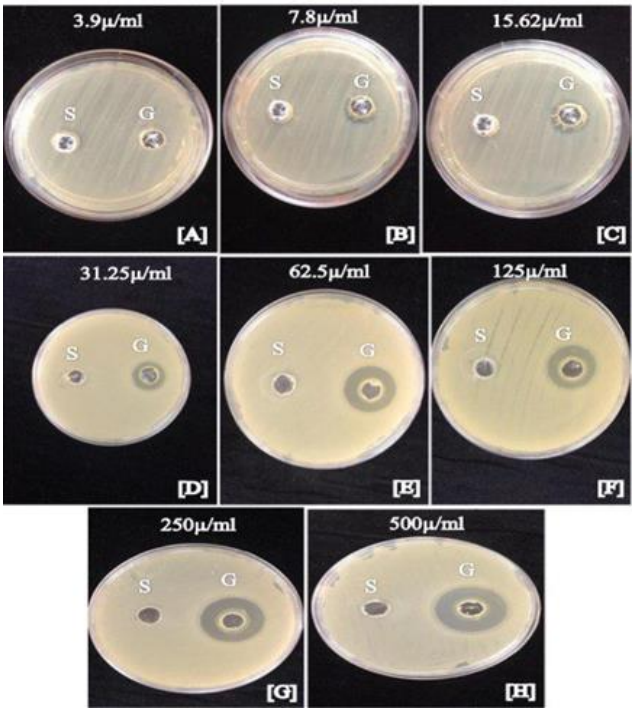


Figure 8. Antimicrobial activity of TiO_2NPS against *A.baumannii* at different concentration : (A) 3.9 µg /ml , (B)7.81 µg /ml , (C) 15.62 µg /ml, (D) 31.25 µg /ml, (E) 62.5 µg /ml (F) 125 µg /ml , (G) 250 µg /ml, (H) 500 µg /ml. G: Green TiO_2NPs synthesized by prodigiosin , S: Synthetic TiO_2 from (Hongwu, China).

Green synthesis of nanoparticles makes use of environmental friendly non-toxic and safe reagent. Purified prodigiosin was characterization by FT-IR and using for synthesis TiO_2NPs by green method. The suitable concentration of reducing agent (prodigiosin) was (10 mg/L). The best size of $Ticl_4$ was 5 ml in 50 ml deionized water, Temperature of reaction was 50℃, pH of reaction was 7 and Time suitable for reaction was 30 minutes. TiO_2NPs by green method was characterization by UV–visible Spectroscopy where a final SPR band at 366nm.The crystallinity determined by X-ray Diffraction (XRD) titanium with a lattice parameter of a = 0.3785 Å which were in good agreement with reference of the (FCC). The size was estimated 47.52 nm and surface morphology of the TiO_2NPs by atomic force microscopy (AFM) and give 3D topological for TiO_2NPs. The FT-IR measurements were recorded to identify the major functional groups for TiO_2NPs. Titanium dioxide nanoparticles application in vitro as antimicrobial activity against human pathogenic bacteria gram negative such as (*A.baumannii*) show good activity and minimum inhibitory concentration also counting were 7.81µg/ml for both bacteria.

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