EVALUATION OF ANTIBACTERIAL ACTIVITY OF NICKEL OXIDE NANOPARTICLES AGAINST *ESCHERICHIA COLI*

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ABSTRACT

The study's goals are to use pyocyanin pigment produced by the clinical isolate *Pseudomonas aeruginosa* as a reducing and stabilizing agent for nickel oxide nanoparticles (NiO NPs) and their use it antibacterial agents against biofilm-producing, multidrug-resistant Escherichia coli isolated from various clinical sources. The antibiotic susceptibility test of *E. Coli* isolates was shown to be resistant to ceftriaxone, cotrimoxazole, piperacillin-triazole, tetracycline, and ticarcillin, while sensitive to Amikacin, Amoxicillin, Nitrofurantoin, and Imipenem. NiO NPs are synthesized by using nickel sulfate $NiSO_4$ (10g) with a concentration of pyocyanin (10mg/10ml). The NiO NPs synthesized were characterized by various techniques such as AFM, UV-VIS, and FTIR. The result showed that the wavelength of NiO was 211nm and the average diameter of NiO was 63.59 nm, and the concentration of NiO NPs was 0.5 mg/ml, showing that the maximum inhibition zones of *E.coli* were 22 mm. Biosynthesis of NiO NPs using pyocyanin was shown to have promising activity as an antibacterial against the biofilm-producing *E. coli*.

Key Words: biofilm, antimicrobial activity, pyocyanin, *E.coli*, Nickel oxide nanoparticles (NiO NPs).

الفعالية ضد البكتيرية لدقائق اوكسيد النيكل النانوي ضد بكتريا *E.coli* المعزولة من مصادر سريرية تقييم تأثير دقائق النيكل النانوية المصنعة بالطريقة البايولوجية

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المستخلص

ان الهدف من هذة الدراسة هو استخدام صبغة البايوسيانين المنتجه من بكتريا *Psedoumonas aeruginosa* المعزولة من مصادر سريرية كعامل مختزل ومثبت لاوكسيد النيكل النانوي (NiO NPS) واستخدامه كمضاد بكتيري ضد بكتريا *E.coli* المنتجه للغشاء الحيوي والمقاومه للمضادات الحيوية المعزولة من مصادر سريرية. ان نتيجة فحص اختبار الحساسية لبكتريا *E.coli*اضهر مقاومتها للمضادات الحيوية الاتية (Piperacillin– ,Amoxicillin,Cotrimoxazole ,Ceftriaxone Tazole and Tetracyclin) بينما اضهرت حساسيتها تجاه (Amikacin ,Ticarcillin,Nitrofurantoin and Imipenem)تم تصنيع NiO NPs باستخدام كبريتات النيكل $NiSO_4$ (10 جم) مع تركيز من البيوسيانين (10 مجم/ 10مل) وتم توصيفة بعدة طرق مختلفة مثل (AFM , UV–VIS, FTIR) اضهرت نتائج الطول الموجي 211nm وحجم النيكل النانوي هو63.59nm والتركيز المثبط هو 0.5mg/ml والذي اضهر اعلى مستوى تثبيط ضد بكتريا *E.coli* وهو 22mm. التصنيع الحيوي للاوكسيد النيكل النانوي عن طريق البايوسيانين اضهر فعالية قاتلة للبكتريا المنتجة للغشاء الحيوي.

الكلمات المفتاحية: الغشاء الحيوي، الفعاليه المضادة للبكتريا، البايوسيانين، *E.coli*، اوكسيد النيكل النانوي (NiO NPs)

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NTRODUCTION

Nanotechnology is the study of extremely small structures mostly with sizes ranging from (1 to 100 nm), which also provide them with exceptional characteristics when compared to microscopic-sized particles (3). NiO nanoparticles (NiO NPs) have gained popularity due to their wide range of characteristics such as transfer of electron capability, super-capacitance, electrocatalysis, and excellent chemical stability (9). mostly, as a result, numerous physical and chemical approaches have also been effectively also used to produce NiO NPs (16). High surface area NiO nanoparticle films have also been created using processes such as laser liquid ablation, electrodeposition, spin coating process, sol-gel, chemical bath deposition, as well as spray pyrolysis (23). The easer method for creative nanoparticles is the green synthesis method: which is an environmentally friendly system due to the use of plant extracts (leaves, flowers, seeds, and peels), bacteria, fungi, and also enzymes for the synthesis of nickel oxide nanoparticles, rather than large quantities of chemicals (1). Synthesis of NiO by bacteria and fungi extract 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 pyocyanin pigment was found to be formed by gram-negative *Pseudomonas aeruginosa* (8). *Pseudomonas aeruginosa*'s capacity to create the blue-green pigment pyocyanin is one of the distinguishing features. Pyocyanin is a member of the whole phenazine family, which seem to be heterocyclic chemicals generated naturally, but with side chains replaced at different places around their rings mostly by different types of bacteria. (4). Pyocyanin is abundantly available in the sputum of cystic fibrosis patients infected with *P. aeruginosa* as well as plays an important role even in the pathogen's pathogenicity (20,24). *Escherichia coli* commonly colonizes human infants' gastrointestinal tracts within a few hours after birth. *E. coli* and its human host typically cohabit in excellent health as well as a mutual benefit instead for decades. (10) Certain *E. coli* strains are also primarily responsible for disease and mortality in infections of medical device-associated such as with urethral and otherwise intravascular catheters, prosthetic joints as well as shunts, and prosthetic grafts. (7) Biofilms, by encapsulating organisms in an extracellular biochemical matrix, cause them to avoid the host immunological response, become more virulent, and contribute most to the evolution of antibacterial treatment resistance. (11) According to a National Health Institutes research, biofilm is present in more than 80% of all bacterial infections. Biofilms have been linked to a variety of medical disorders, including Infections of the upper respiratory tract and urogenital disorders. (12) Infections from oral plaque and indwelling medical equipment These biofilms are also exceedingly difficult to eliminate since antibiotic resistance can still multiply 1,000 times. culture Tissue or microtiter plate (TCP), congo red agar method (CRA) tube method (TM), bioluminescent assays, and fluorescent microscopic examinations are also all methods for detecting biofilm (13). This study was aimed at purifying pyocyanin from Pseudomonas aeruginosa and was used to biosynthesize nickel oxide nanoparticles as a reducing and stabilizing agent. As well as study the potential application of the synthesized nanoparticles in vitro as antibacterial activity against human pathogenic bacteria.

MATERIALS AND METHODS

Identification of bacterial isolates was collected from patients suffering from urinary tract infections, from Hospital (Al-Yarmouk educational hospital, Al-Ramadi Hospital, and Pediatric Teaching Hospital in the medical city) during the period from 1st October 2020 to 1st January 2021. All bacterial species isolates were identified via conventional biochemical assays. Antibacterial activity and otherwise manufacture of NiO NPs were investigated using bacterial strains of multi-drug resistance *E.coli* (17).

Detection of Biofilm (qualitative method

Detection of the Biofilm formation for isolates of *E. coli* by using the Congo red agar this procedure includes:

The Congo red agar method was used to

evaluate E. coli's capsule ability to generate biofilm mostly as a presumptive test instead for the formation of biofilm (2). The dye of Congo red agar was employed as a pH indicator in this experiment. Plates that contain Congo red agar media were already seeded and incubated for 24 hours at 37^{o}C in an aerobic environment. Following this time, colonies that have been shown as dark red or blackish and had a dry or quality crystalline were designated biofilm producers. Biofilm nonproducers were showing red colonies with a smooth look.

Detection of Biofilm (quantitative method)

Each isolate was inoculated in a tube containing 5ml of Trypticase soya broth (TSB) and incubated at 37^{o}C for 18-24 hours (stationary phase), after which 200l (180 ml of media+20 ml of bacteria) was pipetted into the bottom of polystyrene microtiter plates. Overnight incubation was performed at 37^{o}C. Cultures were then washed 3 times in the wells using saline containing phosphate buffer. The plate was then air-dried at room temperature for 15 min, and then stained with 200 ml of 0.1% crystal violet for about 15 min. After the stain was fully removed, 200 ml of 95% ethanol was added to each of the stained wells and left for 10 to 15 minutes at room temperature. The optical density of the wells of the plate was measured by using a micro ELISA auto reader at 630 nm. Then they used Sterile TSB as a negative control of the test. To compensate for background absorbance, the mean of (OD) is optical density and the reading value of control mean (C) was taken from the test (T) values (6). Table (1) measures the intensity of biofilm:

Table 1. Intensity of biofilm

Adherence of Biofilm Formation	Interpretation
ODs ≤ ODc	Non –adherent
ODc < ODs ≤ 2 * ODc	Weakly adherent
2 * ODc < ODs ≤ 4 * ODc	Moderately adherent
4 * ODc < ODs	Strongly adherent

Pyocyanin pigment production: Each Sample of *P. aeruginosa* isolate was collected inoculated on LB broth media (15) and incubated at 37˚C for 72 hrs.

Extraction of pyocyanin

Extraction of pyocyanin by autoclaving the broth solution for 15min at 120˚C then centrifuging at 5000 rpm for 10 min, take up the supinated that contains pyocyanin (14).

Synthesis of nickel oxide nanoparticles

This is the first study to use pyocyanin for the Biosynthesis of NiO nanoparticles, the Process of the synthesis of nickel oxide nanoparticles was done by utilizing nickel sulfate ($NiSO_4$, 99%). The method of synthesis is done by two solutions: Solution (A) is prepared as follows: 10 gm of Nickel Sulfate $NiSO_4$ in 50 ml deionized distilled water DDW dispersed by ultrasonication bath for 30 minutes. Also, solution (B) was prepared by dissolving 10 gm/ml from pyocyanin and dispersed by ultra-sonication bath for 60 minutes then the mix of solution (A) and (B) in the flask then put the flask in the shaker in the darkroom overnight. Then centrifugated for 10 min at 5000 rpm. The precipitate of a solution containing the whole nickel oxide nanoparticles was washed twice with deionized distilled water to get rid of the remnants of the pyocyanin pigment. The resulting nanoparticles precipitation was dried in the oven at 60°C for 30 minutes. Finally, the black powder was stored in a dark container for further characterization and use, and the procedure of producing NiO nanoparticles was carried out following (3).

Antibacterial activity of NiO Nanoparticles

The agar well diffusion method was used to assess bacterial susceptibility to NiO NPs. The Mueller Hinton medium was used in this test. *E.coli* cultivated overnight at 37°C. After an incubation period, the standard inoculum for each bacterial isolate at a concentration of 1.5 X 10^8 CFU / mL was formed according to the standard solution of 0.5 McFarland. A sterile small swab has been dipped inside the tube containing 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 (0.5, 0.25, 0.12, 0.06, 0.03) mg/ mL of NiO NPs were dispensed into separate wells followed by overnight incubation at 37°C. After incubation, the

bacterial susceptibility diameters were measured and reported for the inhibition zones. The well containing sterile distilled water alone was takeup as a negative control (18).

RESULTS AND DISCUSSION

Bacterial isolation in culture media: 200 samples of bacteria grown on MacConkey agar (Figure1), 140 lactose fermenting *E.coli* isolates (70%) appear as dry circular form, dark pink color, and are always surrounded by a dark pink region of precipitated bile salts. While 40 isolate of Klebsiella pneumonia (20%) appears as large mucus and dark pink colony on MacConkey agar and 20 isolates (10%) of pink or red growing colonies of *Enterobacter spp*. it could be said that *E.coli* comprises the major cause of UTIs in patients In Iraq similar results are mentioned when they isolated *E.coli* from the same source in a survey conducted, and Mohamed *et al.*, 2017 reported that *E.coli* was the most common agent whose percentage (50.4%, 37.9%, 49%, 27.5%, 64.4%, 46.7%, 43.9%, and 47.4%).

Figure 1. *E. coli* on MacConkey agar

Antibiotic Susceptibility Test of UPEC

All the isolates went through the susceptibility test, for ten different antibiotics by the disc diffusion method recommended by the clinical and laboratory standards institute (CLSI, 2017) guidelines that result in (Table2). showed varying levels of resistance,

Table2. Antimicrobial resistance of biofilm-producing and nonproducing *E. coli*

Antibiotic type	Biofilm producer E. coli (n = 107)		Biofilm nonproducer E. coli (n = 33)	
	Frequency	%	Frequency	%
Amoxicillin- clavulanic acid	103	96.2%	30	90.9%
Amikacin	18	16.8%	5	15.1%
Nitrofurantoin	13	12.1%	2	6.0%
Ciprofloxacin	81	75.7%	17	51.5%
Ceftriaxone	75	70%	17	51.5%
Cotrimoxazole	73	68.2%	19	57.5%
Imipenem	10	9.3%	3	9.0%
Piperacillin-tazobactam	83	77.5%	29	87.8%
Ticarcillin	87	81.3%	27	81.8%
Tetracycline	76	71.0%	26	78.7%

In this study, *E. coli* showed the highest percent of resistance to amoxicillin followed by Ticarcillin and Tetracycline in both biofilm and non-biofilm *E. coli.*

Pyocyanin pigment extraction

The green pigment appeared on the surface of the broth and after shaking the flask the green pigment was distributed in the broth medium (Figure2).

UV–VIS spectral analysis pyocyanin

The pyocyanin developed by *pseudomonas aeruginosa* is characterized by scanning a UV-visible spectrophotometer (Shimadzu, Japan) in (Figure3) to detect the maximum absorption(3), the result showed the absorbance of pyocyanin pigment at 316nm. the result showed the absorbance of pyocyanin pigment at 316nm and agrees with (13).

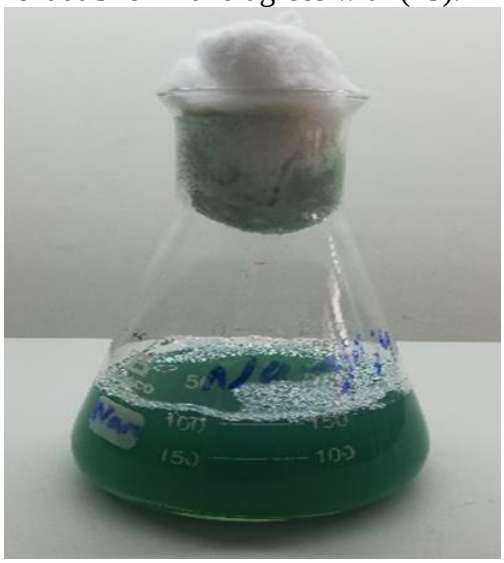


Figure 2. Pyocyanin pigment

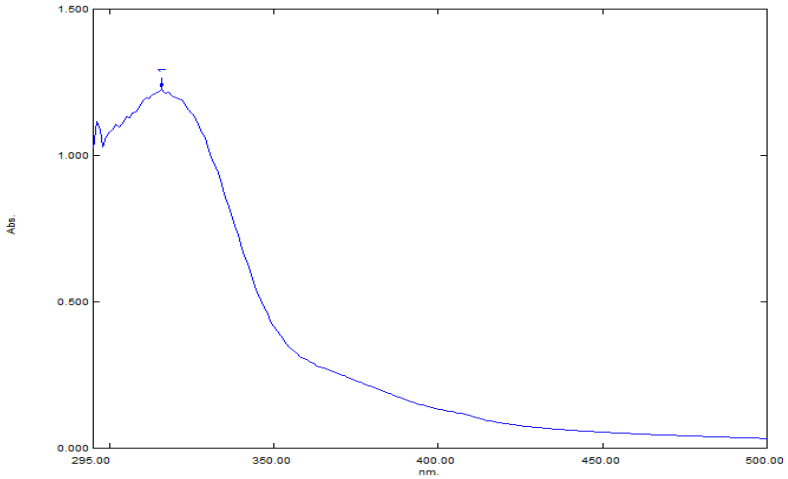


Figure 3. The UV-VIS of pyocyanin

Detection of Biofilm (qualitative method)

The results of the congo red test proved a remarkably high capability of UPEC isolates to form a biofilm since 107 (76%) of isolates were biofilm phenotype positive. (Figure4)

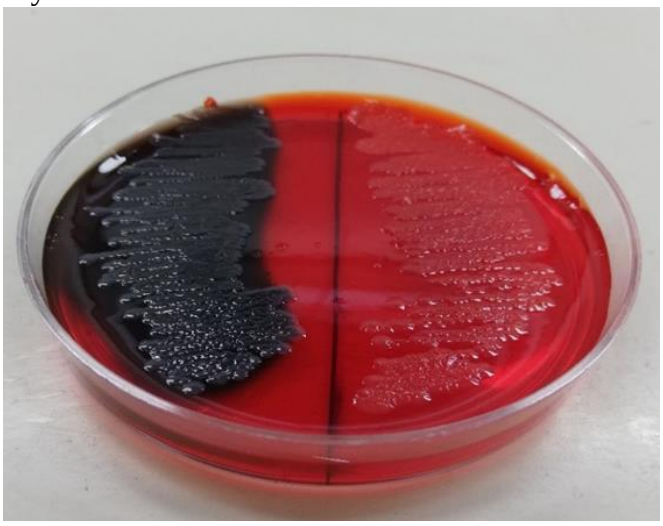


Figure4. The black colony of *E.coli* producing biofilm and the red colony of *E. coli* non-producing biofilm

Detection of biofilm production by microtiter plate assay: All the UPEC isolates showed biofilm formation ability with different potential capacities under the same conditions of experimentation (Figure5) the results of isolates were confined between three groups, strong biofilm producers in four isolates (5%), moderate biofilm producers in ten isolates (11%), and weakly biofilm producers seventy 76 isolates (84%). perhaps, there are 84% of isolates give weak producers of biofilm because most of the isolates take up from children aged from (1-15years).

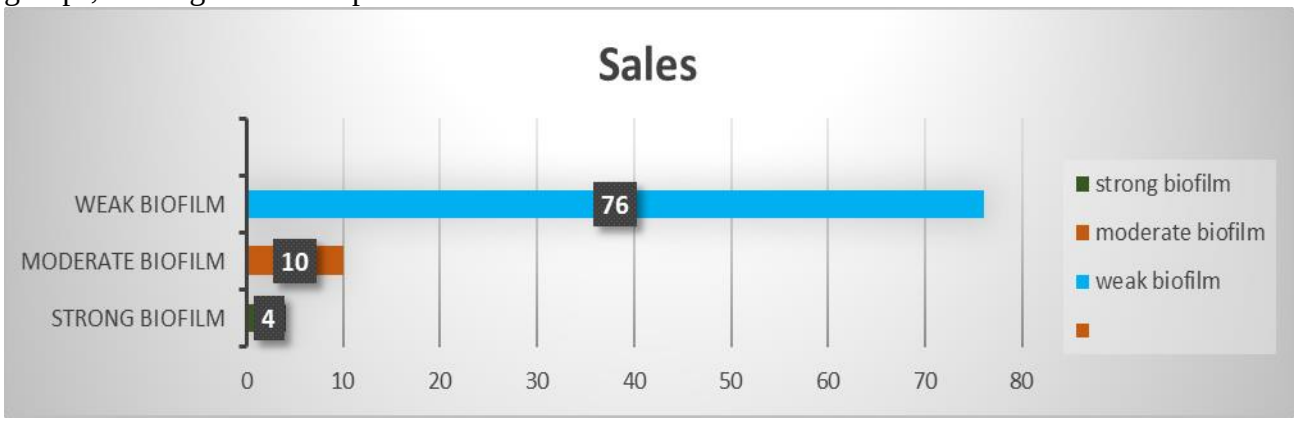


Figure5. Percentage of the application of *E.coli* producing biofilm

Characterization of biological synthesis NiO NPs: UV–VIS spectral analysis N Io

The biosynthesis of NiO NPs is characterized by scanning a UV-visible spectrophotometer (Shimadzu, Japan) (3) in (Figure6) to detect the maximum absorption. Absorbance is measured at 211 nm

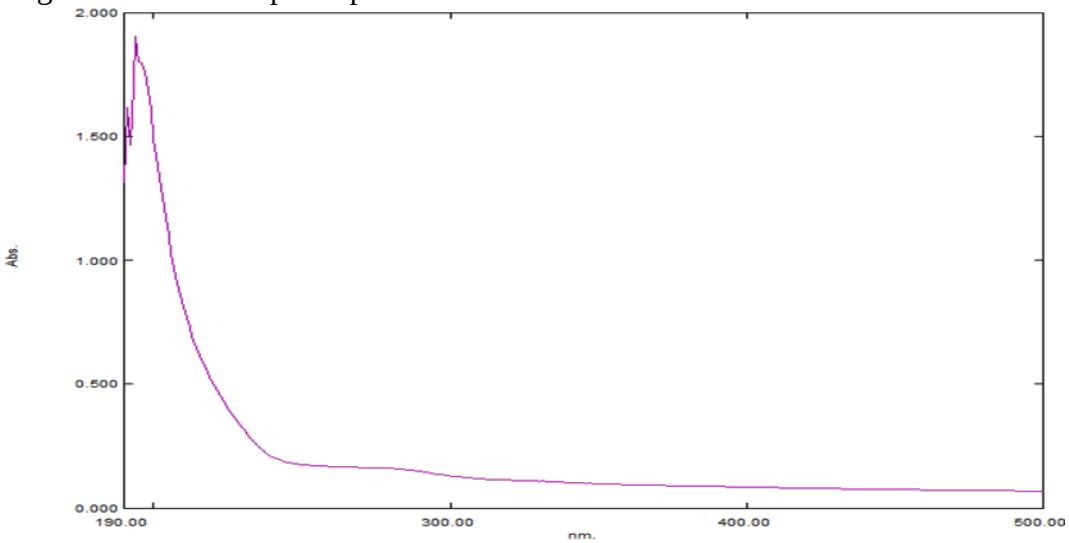


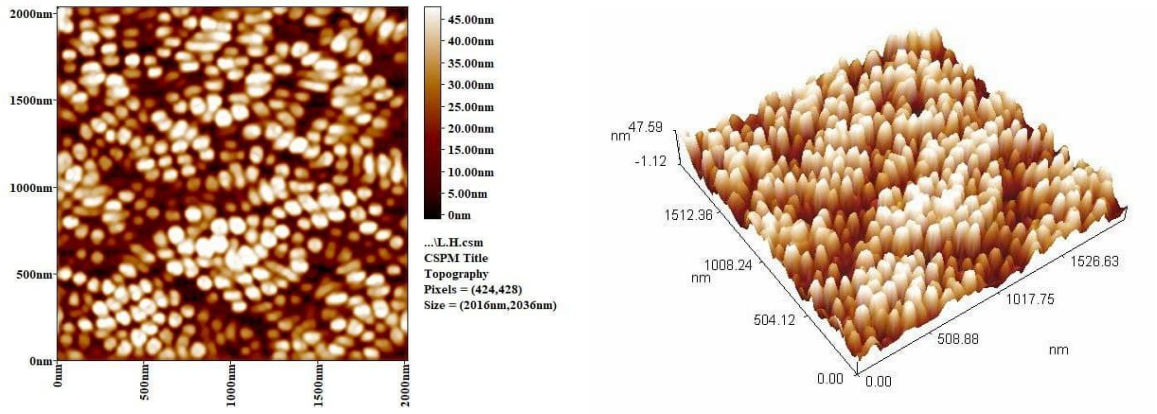
Figure6. The UV-VIS of NiO

Atomic force microscopy (AFM):

The surface shape formation of the NiO NPs was studied by atomic force microscopy to show that NiO NPs 2D and 3D(22). (Figure7). AFM images show that the biosynthesized NiO NPs are spherical. The size of an average diameter of 63.59 nm, (Table3) was also measured by AFM (Figure8). This result agrees with (20).

Table3. The average diameter of NiO nanoparticle 2D 3D

Avg. Diameter: 63.59 nm	**<=10% Diameter: 45.00 nm**
<=50% Diameter: 60.00 nm	**<=90% Diameter: 75.00 nm**



2D **3D**

Figure7. Atomic force microscopy (AFM) of NiO NPs synthesized using pyocyanin illustrates 2D and 3D

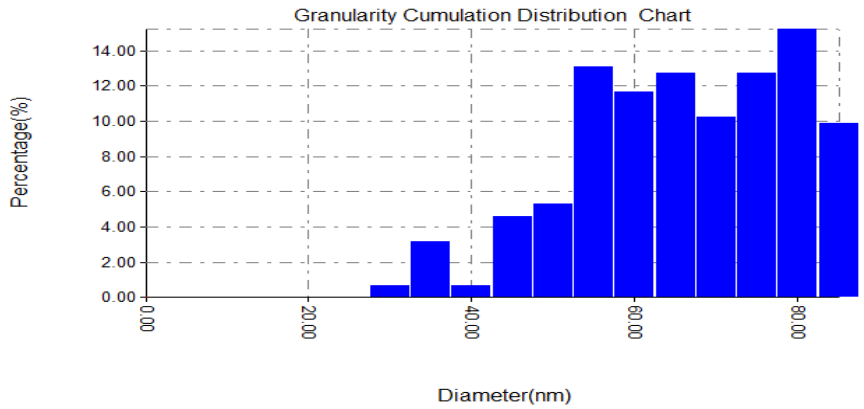


Figure 8. The average size of nickel nanoparticles

Fourier transform infrared (FTIR)

FTIR spectrum has determined the functional groups of nanoparticles. (Figure6) Represents the absorption spectrum of Biologically synthesized nanoparticles in FTIR (Table4) showing the bands are observed around 3429.2-3402.2 cm^{-1}, corresponding to the stretch movement of the hydroxyl group OH Alcohols and H bonds Phenols. The last is around at 1633.59cm^{-1} related to N-H bond Amines and the visible at 3481.27-3234.4cm^{-1} O=bond is due to the Oxide of metals it is due to amines compounds(21), the study agrees with (21)

Table 4. FTIR of NiO NPs

	Frequency of Absorption (cm^{-1})	Bonds	Compound class of Functional Groups
Pyocyanin	3429.2-3402.2	O–H stretch N–H stretch	Alcohol, phenols, Amine
	3282.62-3259.47	H– bonded N-H stretch	Amine, Alkene
	1633.59	N–H bend	Amine, Alkene
	1143.71	C=C stretch	Aliphatic Ether
	1101.28	C–O stretch C–O stretch	Aliphatic Ether
Pyocyanin+$NiOSO_4$	3338.55-3245.97	C=C stretch	Alkene
	1635.52	C=C stretch	Alkene
	1143.71	C–N stretch	Amine
	1095.49	C–O stretch	Alcohol
	630.68	Metal Oxygen	NiO
NiO NPs	3481.27-3234.4	O–H stretch N–H stretch	Alcohol, phenols, Amine
	1652.88	H– bonded	Alkene
	1093.56	C=C stretch	Alcohol
	617.18	C–O stretch Metal Oxygen	NiO

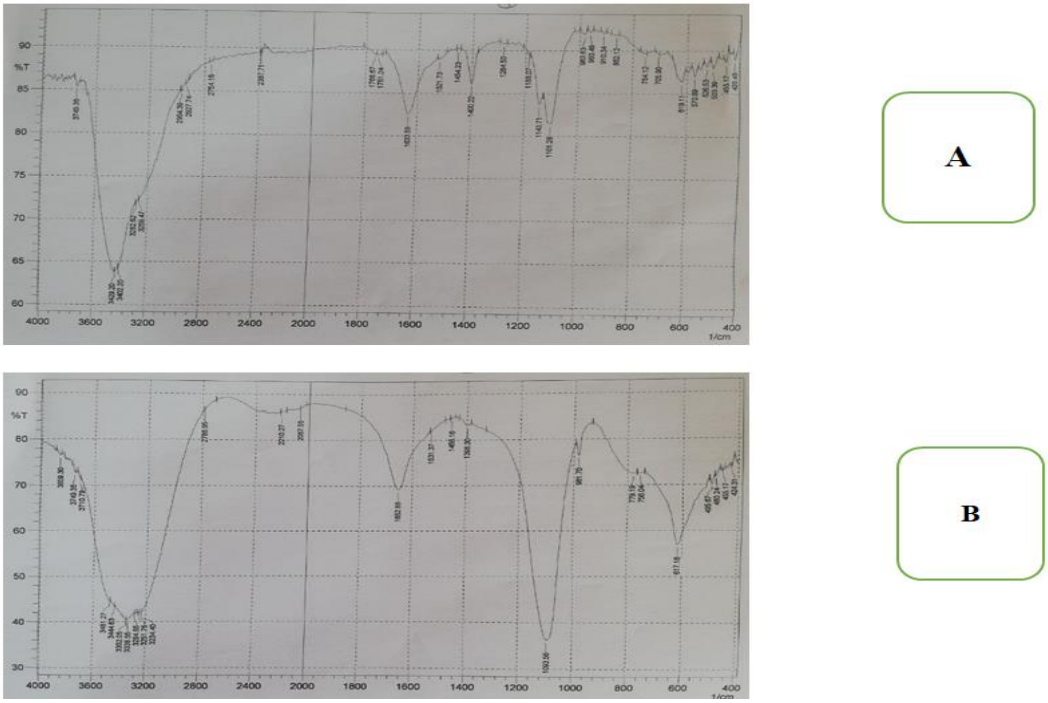


Figure 9. FTIR images of NiO NPs synthesized using pyocyanin pigment, A: pyocyanin pigment. B: NiO NPs

Antibacterial susceptibility test

This is the first study to use NiO nanoparticles as an antibacterial for MDR- *E. coli* that have strong biofilm production. The results of using NiO NPs as antibacterial agents were found to be directly dependent upon the NiO NPs concentration. (Table5) shows that the maximum inhibition zones of *E. coli* were 22 mm at a concentration of 0.5 mg/ml of NiO NPs, Whereas the minimum inhibition zones were located at 0.006 mg/ml of NiO NPs concentrations, the inhibition zone depended on the concentration of NiO NPs. The main mechanism of NiO NPs toxicity is potentially associated with metal oxides carrying a positive charge even though the microorganisms (19). Negative charges; these results in electromagnetic interaction between microbes and metal oxides leading to oxidation and finally death of microbes (25). The bactericidal action of NiO nanoparticles on bacteria is of extreme importance due to the ability of pathogenic bacteria to join the body (5). The antimicrobial effect of NiO against fungi and bacteria has been demonstrated and communicated in modern research, see (Figure10).

Table5.The inhibition zone of the Antibacterial effect of NiO NPs on *E.coli*

No	NiO con. (mg/ml)	Zone of diameter (mm)
1	0.5	22
2	0.25	18
3	0.12	16
4	0.06	14
5	0.03	12

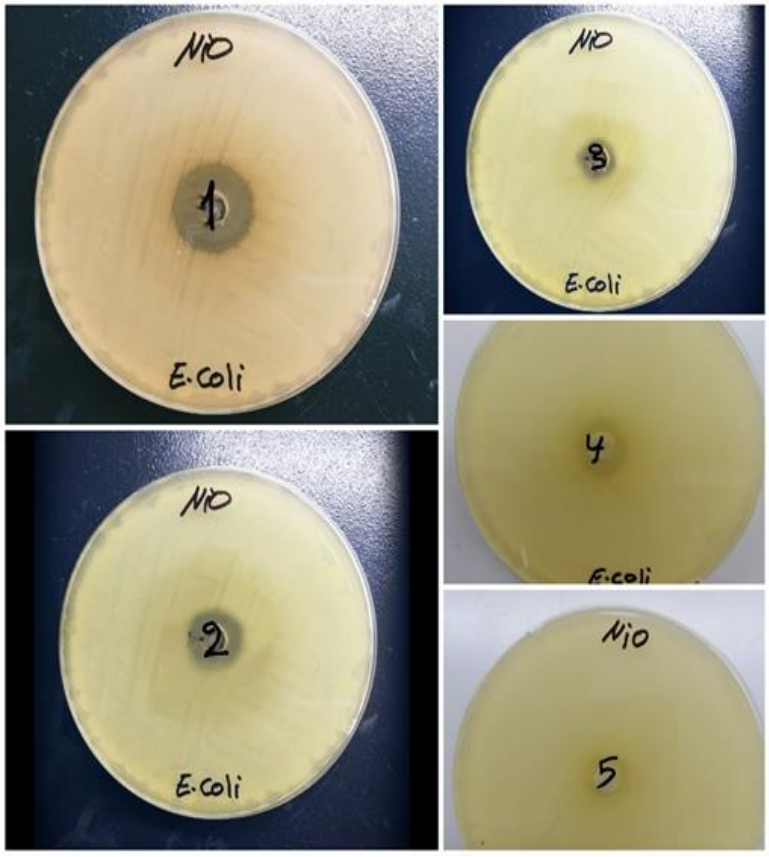


Figure10. antibacterial activity of NiO NPs on *E. coli*

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