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Assessment the Modulation effect of using Green synthesis ZnO NPs against Multidrug Resistant *Klebsiella pneumoniae* isolated from respiratory tract infection

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Abstract

Klebsiella pneumoniae is one of common hospital-acquired bacteria causing nosocomial pneumonia, urinary tract infections, and intra-abdominal infections. The aim of this study is investigating the Modulation effect of Zinc Oxide nanoparticles (ZnO NPs) against multidrug resistant *K. pneumonia* which was isolated from Respiratory Tract Infections (RTIs). The results of isolation and characterization of bacterial specimens showed that 20.81% of RTIs were *Klebsiella pneumoniae*. The strongest isolate showed resistant for most usable antibiotics selected. Simultaneously, ZnO NPs were produced by an aqueous extract of Green Tea leaves as a reducing and stabilizer agent. The Ultraviolet-Visible (UV-Vis) spectrum was indicated a successful production of ZnO NPs at 383 nm. X-Ray Diffractometry (XRD) pattern showed peaks at 2θ positions matching to standards and indicating a formation of hexagonal (wurtzite) shape of ZnO NPs, with an average size of 22nm. Fourier Transform- Infrared (FT-IR) spectra of ZnO NPs revealed the participating of Green Tea biomolecules in the synthesis process. The minimum inhibitory concentration (MIC) of ZnO NPs against *K. pneumoniae* was 3.2 mg/mL. The results of using nanoparticles showed a morphological changing in *K. pneumoniae* colonies, and a modulation effect occurred against some antibiotic resistance of *Klebsiella pneumoniae* such as Gentamycin and Levofloxacin.

Keywords: Zinc Oxide, Nanoparticle, Green tea, *Klebsiella pneumoniae*, Antibiotic resistance.

تقييم التأثير التحويري لدقائق اوكسيد الزنك النانوية المصنعة بطريقة خضراء على بكتريا الكلبسيلا الرئوية المتعددة المقاومة والمعزولة من اخماج الجهاز التنفسي

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الخلاصة

تعد بكتيريا الكلبسيلا الرئوية واحدة من البكترية المسببة للأمراض الشائعة التي تنقل في المستشفيات ، أذ تسبب التهابات المسالك البولية ، والالتهاب الرئوي المتنقل عن طريق المستشفيات ، والالتهابات داخل البطن. ان الهدف من هذه الدراسة هو الكشف عن تأثير دقائق اوكسيد الزنك النانوية في تغيير مقاومة بكتريا الكلبسيلا الرئوية المتعددة المقاومة للمضادات الحيوية والمعزولة من اخماج الجهاز التنفسي. وقد بينت نتائج عملية العزل والتشخيص للعينات البكتيرية ان الكلبسيلا الرئوية تشكل نسبة 20,81% من عينات اخماج الجهاز التنفسي. وقد تم اختيار العزلة الاكثر مقاومة للمضادات الحيوية الشائعة الاستعمال. في نفس الوقت ، تم انتاج

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دقائق اوكسيد الزنك النانوية باستعمال المستخلص المائي لاوراق الشاي الاخضر و الذي يمثل كعامل مختزل ومثبت للدقائق النانوية. ومن خلال فحص جهاز المطياف الضوئي ، تم التأكد من نجاح انتاج دقائق اوكسيد الزنك النانوية وعلى طول موجي 383 نانومتر. وان جهاز مقياس انكسار اشعة اكس اظهر قمم موجية لمواقع ثيتا قورنت بمواد قياسية تؤكد تكون دقائق اوكسيد الزنك النانوية بشكل عشاري الاضلاع ، وان معدل حجم الدقائق هو 22 نانومتر. فيما بينت نتائج جهاز فوريه تحويل طيف الاشعة تحت الحمراء اشتراك الجزيئات الحيوية لمستخلص الشاي الاخضر في عملية التخليق.وكان التركيز الادنى لدقائق اوكسيد الزنك النانوية القادر على تثبيط عزلات الكلبسيلا الرئوية 3.2 ملغم/ مل. أظهرت نتائج استعمال الدقائق النانوية حدوث تغييرفي شكل المستعمرات البكتيرية ، اضافة الى تغيير في اختبار فحص حساسية لبعض المضادات الحيوية ، مثل الجنتامايسين والليفوفلوكساسين.

Introduction

Klebsiella pneumoniae is a common hospital-acquired bacteria, causing nosocomial pneumonia, urinary tract infections, and intra-abdominal infections. *K. pneumoniae* is also a community-acquired bacteria [1]. Historically, it has been recognized as pulmonary bacteria since discovery for more than 100 years ago and was described as the agent of Friedlander's pneumonia, a severe form of lobar pneumonia with high mortality [2]. The classic clinical presentation is dramatic: toxic presentation with sudden onset, high fever, hemoptysis (currant jelly sputum), and can produce extensive hemorrhagic in the lung [3, 4]. On the other hand, *K. pneumoniae* is a multidrug-resistant (MDR) which can cause high morbidity and mortality due to limited treatment options [5]. *K. pneumoniae* can be found in the respiratory tracts and feces of approximately 5% of normal individuals [3]. The field of nanotechnology is one of the most active areas of research in modern material sciences, and has an impact all affairs of human life and creates a growing horizons in life sciences, especially in biomedical and biotechnological branches [6]. The Nanoparticles show new properties based on specific characteristics in the material such as morphology and distribution and possess a special size property with a high ratio of the surface area to volume, decreased size of the particles lead to greater surface: volume ratio [7]. ZnO NPs have received noticed attention because of their antimicrobial, UV blocking, and high catalytic and photochemical activities [8]. The researchers have investigated the antimicrobial effect of ZnO NPs against a wide range of bacterial infections. Reference [9] has reported significant antimicrobial activity of ZnO NPs. NPs have conventionally produced by chemical and physical methods, such as sol-gel process, microwave, hydrothermal method, chemical vapour deposition, and chemical precipitation [10, 11], and these methods involve using of hazardous reagents in synthesis of nanoparticles. Because of environmental issues, there is an urgent need to find and develop an ecofriendly methods of synthesis of nanomaterials; therefore, there are growing development environmentally-friend processes for nanoparticle synthesis without using toxic chemicals [12]. As a result, biological methods for nanoparticle synthesis have been suggested as possible ecofriendly alternatives to chemical and physical methods by using microorganisms, enzymes, and plants or plant extracts. Biological methods of nanoparticle synthesis have paved the way for the "green production" of nanoparticles which have shown better control over crystal growth and their stabilization [13]. Green tea leaves are extremely rich in the flavonol group of polyphenols that are known as "catechins", which had higher antioxidant activity. The antioxidants are very good reducing agents for metal ions; therefore, they are favored for use in green synthesis of nanoparticles. Furthermore, Green tea has high contents of amino acids, lipids, and proteins that help in stabilizing the formation of nanoparticles and inhibit particle agglomeration [14]. Because of the green synthesis of ZnO NPs and experiments on their antimicrobial activities are still limited, this research worked on the green tea associated-synthesis of ZnO NPs and to test their antimicrobial activity and modulation of antibiotic resistance in *K. pneumoniae*. The aim of this study was investigating the Modulation effect of Zinc Oxide nanoparticles (ZnO NPs) on antibiotic resistance of multidrug resistant *K. pneumonia* which was isolated from Respiratory Tract Infections (RTIs).

Materials and methods

Bacterial strains

Respiratory tract infection clinical samples (269 samples) were collected from out and inpatients of Baghdad Teaching Hospital and National Center of Educational Laboratories (Medical City /Baghdad /Iraq). The specimens included Sputum and bronchial wash. The selected colony were chosen by

culturing on selective media (MacConkey agar media/ Himedia), then identified by VITEK2 system and chromogenic media (CHROMagar/France).

Antimicrobial susceptibility test

Clinical *K. pneumoniae* isolates were tested of their susceptibility to ten antibiotics related to respiratory tract infection (RTI) treatment (Amikacin, Azithromycin, Cefotaxime, Chloramphenicol, Ciprofloxacin, Doxycycline, Methicillin, Meropenem, Ticarcillin-clavulanic acid and Tigecycline) by Kirby–Bauer disk diffusion method [15], Clinical and Laboratory Standards Institute (CLSI, 2017) [16] relied for result interpretation. The isolate that showed most resistance for most usable antimicrobial disks was selected for further experiments.

ZnO NPs preparation

Zinc acetate dihydrate (Zn $(CH_3COO)_2$ $.2H_2O$)/Aldrich solution (0.02 M) was prepared by dissolving 0.239 gm in 100 mL of Deionized water completely. Amount of 20 gm of dried green tea leaves/local market, was soaked in 100 mL of Deionized water and boiled for 10 minutes. After cooling to room temperature, green tea extract was filtered with gauze and then Whatman No. 1 filter paper. The different volume of green tea extracts 0.5,1 and 2 mL were mixed separately and homogeneously with zinc acetate solution (100 mL) and adjusting the pH at 12 by adding NaOH solution (1M) with stirring for 2 hours. The reacted precipitant was washed three times with deionized water and Ethanol 95%, and dried at 60 °C to produce pale-white powder. The ZnO NPs powder was preserved in dark and air-tight vials. This method was modified from Senthilkumar and Sivakumar procedure [7].

Characterization of ZnO NPs

UV-Vis spectrophotometry was employed to confirm the synthesis of NPs in the solution. Furthermore, synthesized nanoparticles were characterized using Atomic Force Microscopy (AFM) and X-ray diffractometer (Shimadzu/Japan) with Cu Kα radiation (λ= 0.15406 nm), ZnO NPs powder was prepared for X-ray diffraction (XRD) pattern, then the results were compared with ZnO NPs X-ray diffraction standards, Debye-Scherrer equation was used to calculate the average crystalline size [17]:

$$\boldsymbol{D_{hkl} = \frac{k \times \lambda}{\beta_{hkl} \times \cos\theta_{hkl}}}$$

Where, D_{hkl} represent the perpendicular of crystalline size to the normal line of (*hkl*) plane, *k* is a constant = 0.9, λ is the X-ray wavelength, β_{hkl} is the full width at half maximum of the (*hkl*) diffraction peak and θ_{hkl} is the Bragg angle of (*hkl*) peak. FT-IR spectra were recorded for ZnO NPs to identify the biomolecules that participated in the process of synthesis.

MIC assay of ZnO NPs

A sterile ZnO NPs stock solution with (12.8 mg/mL) concentration was diluted by using double fold serial dilution, 5 mL of the sterile ZnO NPs (stock solution) was diluted with 5 mL of sterile Mueller-Hinton broth to obtain (6.4 mg/mL) concentration. The above step was repeated several times to obtain other dilutions (3.2, 1.6, 0.8, 0.4 mg/mL), leaving one as a positive control (inoculated media without ZnO NPs) and another one as a negative control (flask included nanoparticles and media without inoculation). 0.1 mL of the bacterial inoculum (1.5 X 10^8 cell/mL) was added, and the flasks were incubated in a shaker incubator at 120 rpm at 37 ℃ for 24 h. The lowest concentration of the ZnO NPs that inhibited the growth of the bacterial isolate in the broth, was identified as the minimum inhibitory concentration (MIC) [18].

Antibiotic susceptibility test (AST)

This test was performed *in vitro* for both virulent and ZnO NPs-exposed isolates to monitor the morphological alteration. The antibiotic susceptibility test detects the MICs of some antibiotics against selected *K. pneumoniae* isolate. This test was processed by VITEK2 system (Biomerieux/France) with using two samples, one of them was represented as control (virulent isolate), and another one was cultured in Brain Heart Infusion broth with the half value of ZnO NPs MIC. Before VITEK2 test, samples were taken from the two cultured media and re-cultured on MacConkey agar to detect the morphological alteration in *K. pneumoniae* colonies.

Result and Discussion

Bacterial identification

Several tests of colony morphology and biochemical tests were performed to identify bacterial isolates. The characteristic parameters showed that out of 269 of respiratory tract specimens, 56 (20.81%) were *Klebsiella pneumoniae*, through a Gram-negative, non-motile, small straight rods and arranged in single cells or in pairs under light microscope, in addition to a mucoid texture, large size, round regular colonies and lactose fermenter on MacConkey agar. The positive result of *Klebsiella pneumonia* was further confirmed by VITEK2 test (Figure-1) and chromogenic media (Figure-2).

Identification Information	**Analysis Time:**	3.85 hours	**Status:** Final
Selected Organism	99% Probability **Bionumber:**	**Klebsiella pneumoniae ssp pneumoniae** 6607734753565010	
ID Analysis Messages			

Biochemical Details																	
2	APPA	-	3	ADO	+	4	PyrA	+	5	IARL	-	7	dCEL	+	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	+
17	BGLU	+	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	+	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	+	29	TyrA	+	31	URE	+	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	+	37	MNT	+	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	+
46	GlyA	+	47	ODC	-	48	LDC	+	53	IHISa	-	56	CMT	-	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Figure 1- Identification test by VITEK2 system (*K. pneumoniae* with probability 99%).

Figure 2- *K. pneumoniae* culture on CHROM agar media

Antimicrobial susceptibility test

A broad spectrum antibiotics were selected to study the susceptibility pattern of clinical strains of *K. pneumoniae*, as shown in Table-1. The results revealed increases in the percentage of resistant against several antimicrobial agents recently used in Iraq (during the recent decade) such as Azithromycin, Methicillin and Ticarcillin-clavulanic acid. The most resistant isolate is KP18 which resisted Amikacin, Azithromycin, Cefotaxime, Ciprofloxacin, Doxycycline, Methicillin, Meropenem, Ticarcillin-clavulanic acid and Tigecycline, with intermediate resistant to Chloramphenicol.

Table 1- The number and percentage of *K. pneumoniae* isolates in antimicrobial sensitivity test

No.	Antibiotic	Symbol	Resistant No. (%)	Intermediate No. (%)	Sensitive No. (%)
1	**Amikacin**	**AK**	13 (**23.21%**)	4 (**7.14%**)	39 (**69.64%**)
2	**Azithromycin**	**ATH**	34 (**60.71%**)	2 (**3.57%**)	20 (**35.71%**)
3	**Cefotaxime**	**CTX**	13 (**23.21%**)	8 (**14.29%**)	35 (**62.50%**)
4	**Chloramphenicol**	**C**	3 (**5.36%**)	3 (**5.36%**)	50 (**89.29%**)
5	**Ciprofloxacin**	**CIP**	21 (**37.50%**)	0 (**0%**)	35 (**62.50%**)
6	**Doxycycline**	**DXT**	15 (**26.79%**)	5 (**8.93%**)	36 (**64.71%**)
7	**Methicillin**	**MEC**	36 (**64.29%**)	13 (**23.21%**)	7 (**12.50%**)
8	**Meropenem**	**MEM**	10 (**17.86%**)	2 (**3.57%**)	44 (**78.57%**)
9	**Ticarcillin-clavulanic acid**	**TIM**	28 (**50%**)	13 (**23.21%**)	15 (**26.79%**)
10	**Tigecycline**	**TGC**	1 (**1.79%**)	2 (**3.57%**)	53 (**94.64%**)

Green synthesis of ZnO NPs

The result of the green synthesis of ZnO NPs showed a gross conversion of color from light yellow to brownish pale-white after two hours of reaction, due to the formation of ZnO NPs in the solution. The yielded ZnO NPs was pure pale-white color after the washing procedure and removing the impurities Figure-3.

A- Before reaction **B- After reaction**

Figure 3-Formation of ZnO NPs after 2 h

UV-Vis spectra

UV-Vis spectrum of ZnO NPs Figure-4, confirmed synthesizing ZnO NPs by detecting the maximum absorption with highly blue-shifting occurred around 385 nm approximately. The production with 0.5, 1, and 2 mL of Green tea showed maximum absorption at 355, 367, and 366 respectively; the result revealed that 1 mL of green tea extract was the best volume to produce ZnO NPs, this result agreed with Senthilkumar and Sivakumar [7].

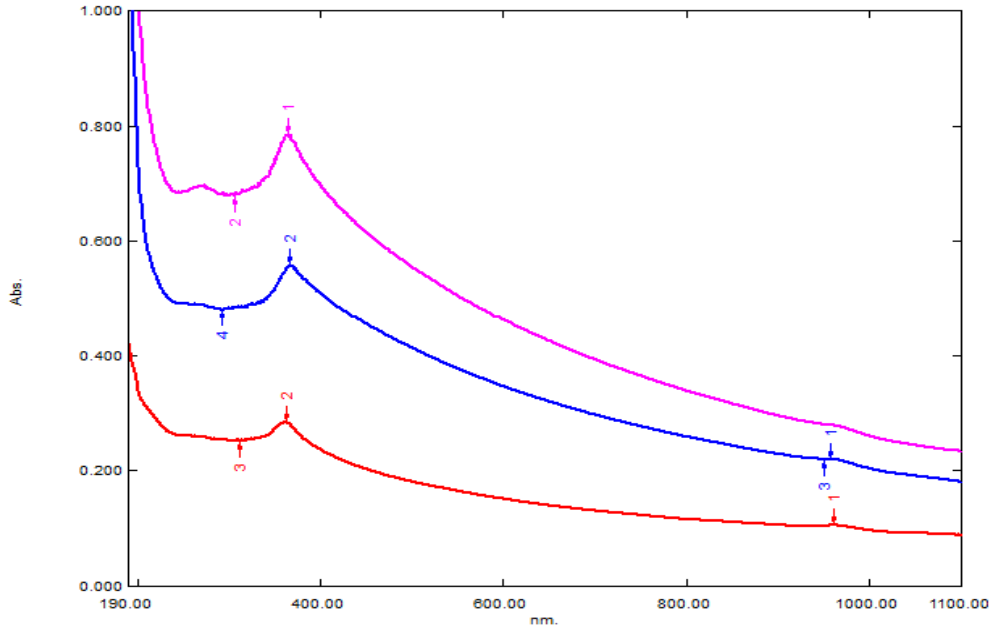


Figure 4- UV-Vis spectra of green-synthesized ZnO NPs (Upper: production with 2 mL of green tea, medal: with 1 mL, lower: with 0.5 mL).

X-Ray Diffraction study

The XRD spectra of ZnO nanoparticle powder as shown in (Figure-5), confirmed formation of hexagonal (wurtzite) structure of the ZnO NPs by showing prominent peaks corresponded to the diffraction planes (100), (002), (101), (102), (110), (103) and (112) which agreed with International Centre for Diffraction Data (Standard Card No. 36-1451) (Figure-6). The average size of green-synthesized ZnO NPs was calculated by Scherrer formula was 22 nm. Whereas, Jiang, and Cai [19] exhibited the same crystallite growth rate at (38–50 nm)

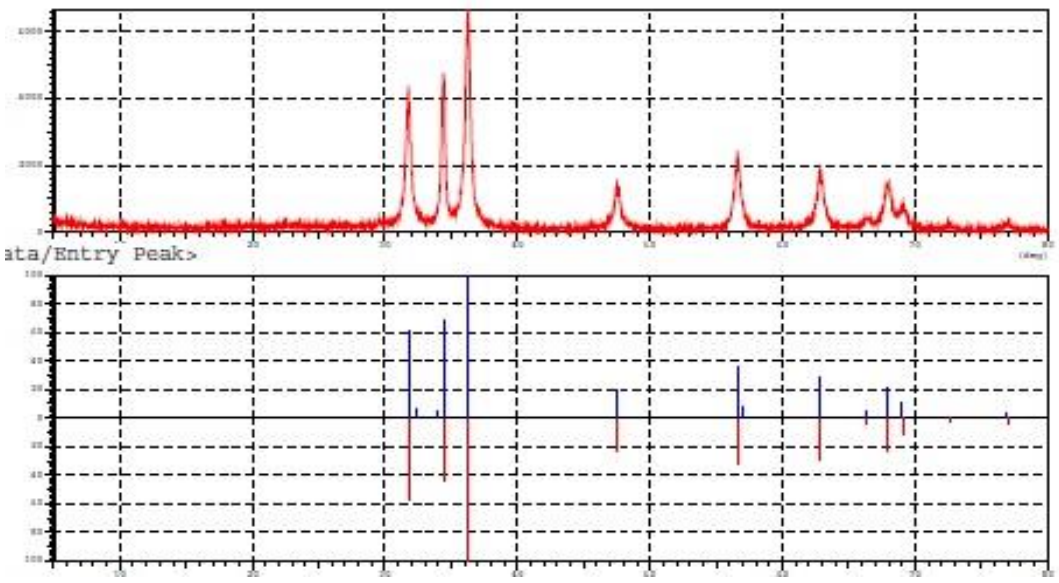


Figure 5- XRD spectra of green-prepared ZnO NPs.

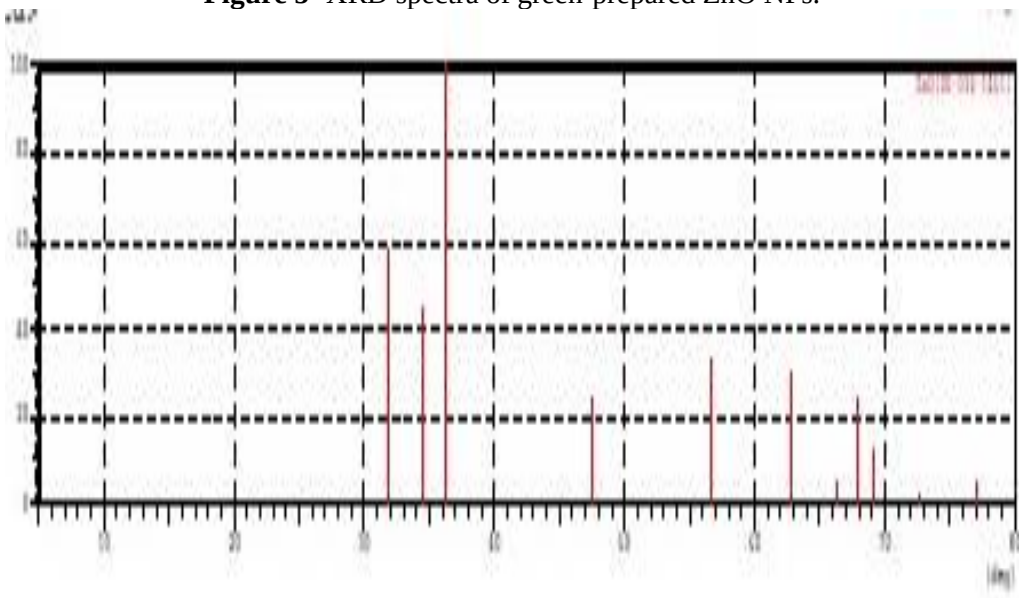


Figure 6-Standard Card No. 36-1451 spectra of ZnO NPs with wurtzite structure.

FT-IR analysis

The interpretation of FT-IR spectrum involves the corresponding of the absorption bands with specific chemical compounds in the tested sample. In this way, the compounds exist in Green tea which was responsible for the reduction and stabilization processes in the green production of ZnO NPs can be identified. The FT-IR spectrum presented in (Figure-7) showed bands at 493 and 433 cm^{-1} in the IR spectrum, which was the characteristic peaks of ZnO molecules. The result of C–H out of plane bending indicated at 892 cm^{-1}, and C–O stretching in amino acids showed a band at 1026 cm^{-1}. The C–N stretch of amide-I in proteins showed a band at 1413 cm^{-1}. Whereas the bands at 1595 cm^{-1} was pointed the presence of C=C stretch in aromatic ring and C=O stretch of polyphenols. C–H stretch in alkanes and O–H stretch in carboxylic acid bands appeared at 2929 and 2860 cm^{-1} respectively. Finally, the bands at 3477, 3425 and 3404 cm^{-1} were appeared because of the stretching vibrations of O–H groups which present in water, phenols and alcohol and N–H stretching in amines.. Thus, from these results, it can be observed that polyphenols, polysaccharides, carboxylic acid and proteins of green tea participated in the reduction and stabilization (capping) actions of ZnO NPs synthesis. In addition, it can be concluded that the existing of the high percentage of phenolic groups were responsible for the reduction process, and the presence of amino acids and amide linkages in proteins were responsible for the stabilizing of ZnO NPs as Senthilkumar, and Sivakumar [7] mentioned.

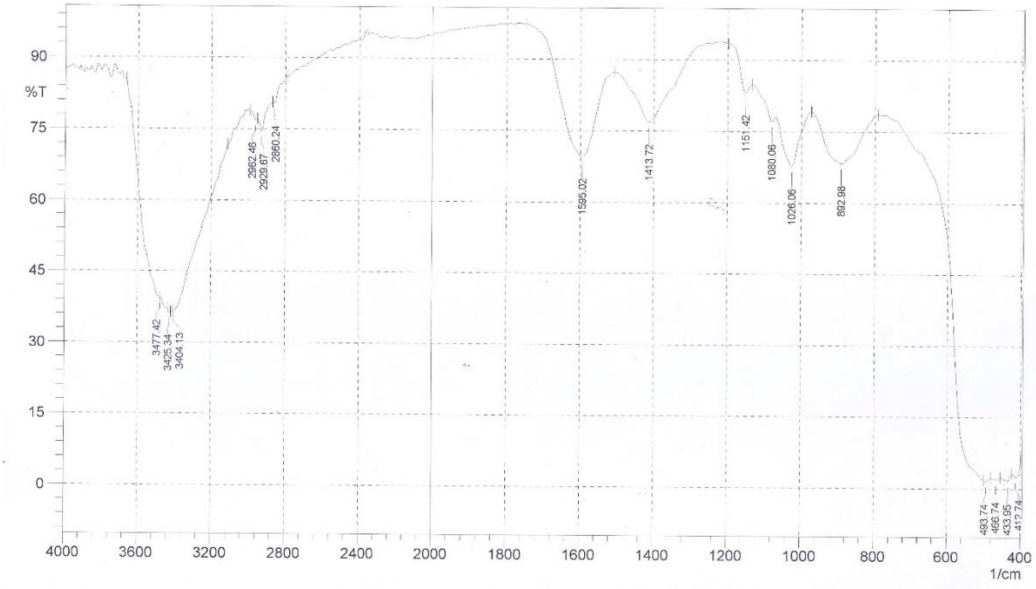


Figure 7-FT-IR spectra green tea-prepared ZnO NPs.

Antimicrobial studies

Determination of ZnO NPs MIC

The result of minimal inhibitory concentration (MIC) of ZnO NPs toward *K. pneumoniae* was 3.2 mg/mL. This result was disagreeing with Sultan and coworkers [20] who indicated that, the MIC of chemical prepared ZnO NPs was 1 mg/ml. The antimicrobial effect may due to significant characteristics of nanoparticles especially the higher surface volume ratio which gives greater contact with the microbial surface and provides enhanced activity. Additionally, the nanometer size of nanoparticles facilitates their entry into the bacterial cell membrane to enable inhibition mechanisms inside the cell. ZnO NPs generate hydrogen peroxides which chemically interact with cell membrane [7]. Also the antimicrobial activity of nanoparticles may involve a production of reactive oxygen species (ROS) and the accumulation of nanoparticles in the bacterial cytoplasm. ROS can cause cell membrane dysfunction [21] and cell death by oxidizing lipids of cell membrane [22]. Nanoparticles can also insert the mitochondria of cells through various pathways and can produce oxidative stress which leads to cell death by apoptosis [23].

Antimicrobial Sensitivity test (AST)

K. pneumoniae treated with half amount of ZnO NPs MIC was further cultivated on MacConkey agar media. The results showed a morphological changing in the colony shape and structure (smaller, less brightness with granular shape, decreased mucus production compared with control isolate) (Figure-8). This test was used to detect a conversion in susceptibility against antimicrobial agent when compared with control isolate. The results of VITAK2 showed that the MIC of Gentamycin and Levofloxacin was decreased from 8 to 4 µg/mL and the degree of resistance was changed from intermediate to sensitive and from resistant to intermediate respectively Figures-(9 and 10). This conversion may due to the effect of ZnO NPs on the efflux pump as [24] or due to a DNA mutation because of the excessive production of ROS [25]. A modulation in this experiment was improved throughout synergistic effect of ZnO NPs with antimicrobial agent against virulent treated isolate compared with the control.

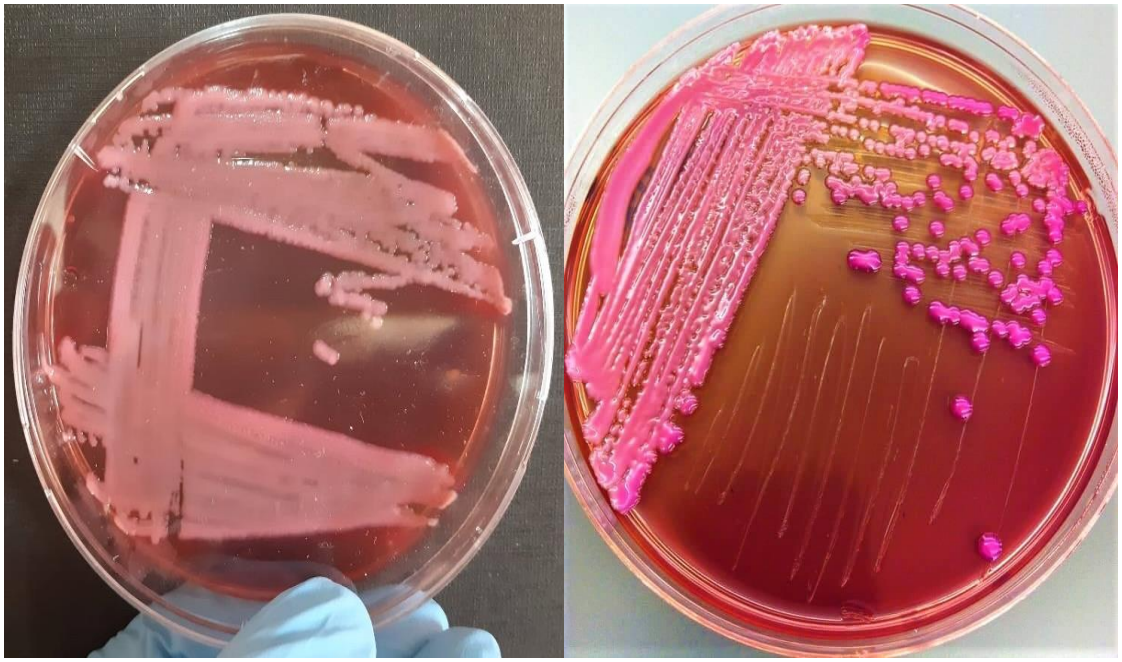
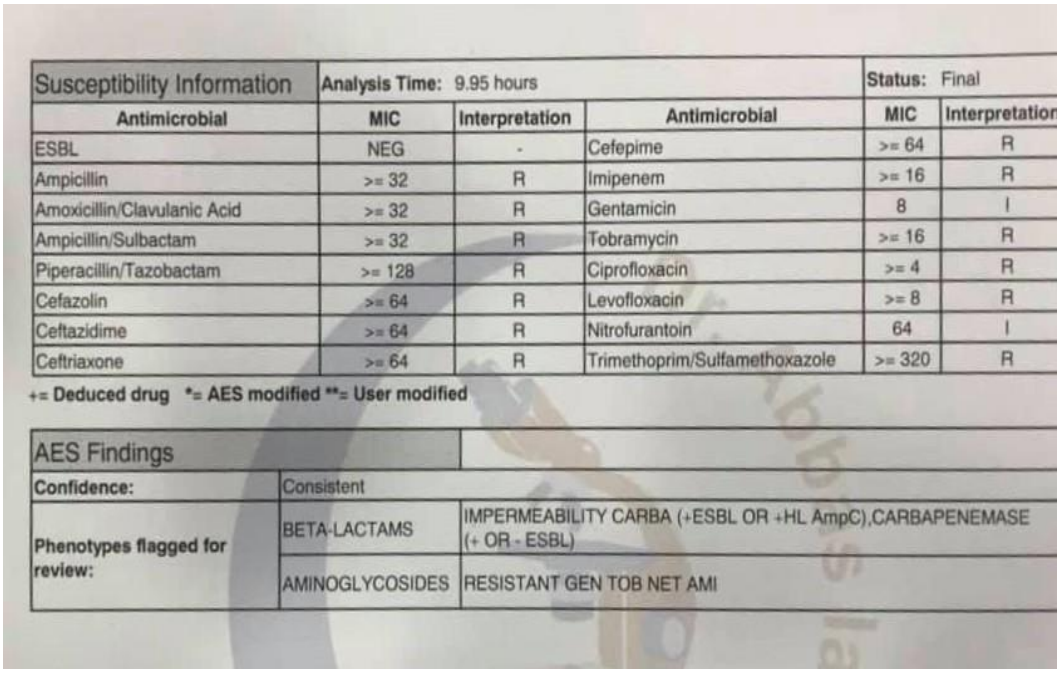


Figure 8- Right plate: Bright, mucoid and virulent *K. pneumoniae*, Left plate: Morphological changing in *K. pneumoniae* colonies (Flat, less brightness with granular shape, less mucoid compared with control isolate) cultured on ZnO NPs-containing media.

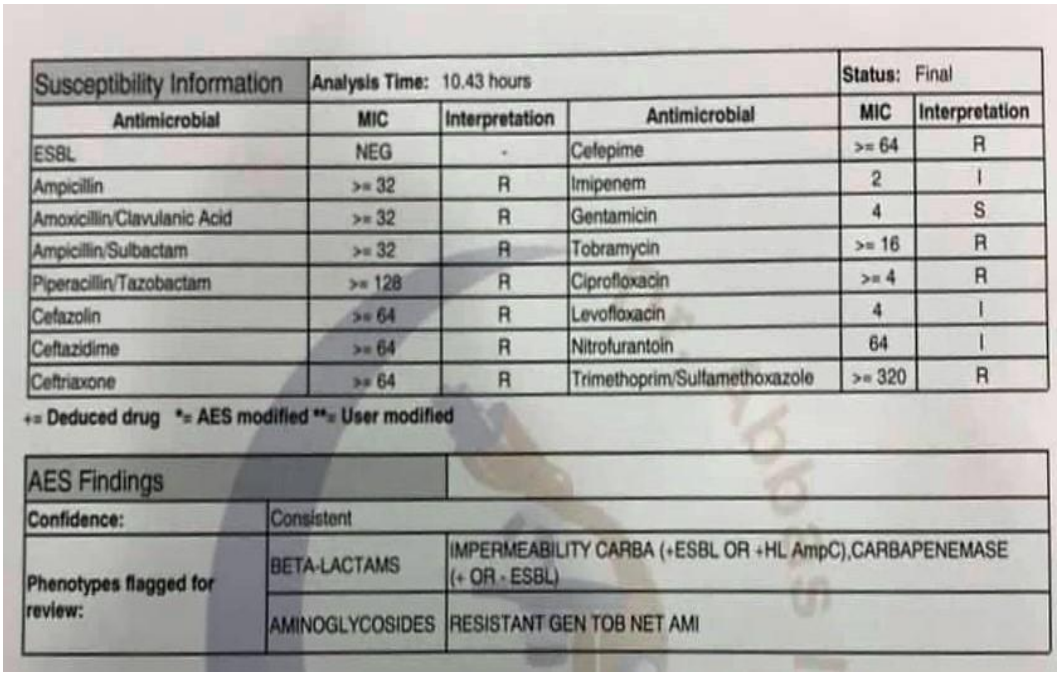


Susceptibility Information	Analysis Time: 9.95 hours			Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
ESBL	NEG	-	Cefepime	>= 64	R
Ampicillin	>= 32	R	Imipenem	>= 16	R
Amoxicillin/Clavulanic Acid	>= 32	R	Gentamicin	8	I
Ampicillin/Sulbactam	>= 32	R	Tobramycin	>= 16	R
Piperacillin/Tazobactam	>= 128	R	Ciprofloxacin	>= 4	R
Cefazolin	>= 64	R	Levofloxacin	>= 8	R
Ceftazidime	>= 64	R	Nitrofurantoin	64	I
Ceftriaxone	>= 64	R	Trimethoprim/Sulfamethoxazole	>= 320	R

+= Deduced drug *= AES modified **= User modified

AES Findings		
Confidence:	Consistent	
Phenotypes flagged for review:	BETA-LACTAMS	IMPERMEABILITY CARBA (+ESBL OR +HL AmpC),CARBAPENEMASE (+ OR - ESBL)
	AMINOGLYCOSIDES	RESISTANT GEN TOB NET AMI

Figure 9- AST test of virulent isolate of *K. pneumoniae* by VITEK2 system



Susceptibility Information	Analysis Time: 10.43 hours			Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
ESBL	NEG	-	Cefepime	>= 64	R
Ampicillin	>= 32	R	Imipenem	2	I
Amoxicillin/Clavulanic Acid	>= 32	R	Gentamicin	4	S
Ampicillin/Sulbactam	>= 32	R	Tobramycin	>= 16	R
Piperacillin/Tazobactam	>= 128	R	Ciprofloxacin	>= 4	R
Cefazolin	>= 64	R	Levofloxacin	4	I
Ceftazidime	>= 64	R	Nitrofurantoin	64	I
Ceftriaxone	>= 64	R	Trimethoprim/Sulfamethoxazole	>= 320	R

+= Deduced drug *= AES modified **= User modified

AES Findings		
Confidence:	Consistent	
Phenotypes flagged for review:	BETA-LACTAMS	IMPERMEABILITY CARBA (+ESBL OR +HL AmpC),CARBAPENEMASE (+ OR - ESBL)
	AMINOGLYCOSIDES	RESISTANT GEN TOB NET AMI

Figure 10- AST test of *K. pneumoniae* isolate by VITEK2 system after exposing to ZnO NPs (decreasing in MIC of Gentamycin and Levofloxacin).

Conclusion

This study demonstrates the ability of ZnO NPs to play a role as an antimicrobial agent, in addition to its modulation activity for some antibiotic used against *K. pneumoniae*. Our result suggests a possibility of using some antibiotics with incorporation of ZnO NPs in a synergistic effect, to prevent multidrug resistant bacteria that cause dangerous respiratory tract infections. Further studies could be helpful to formulate conjugated nano-drug as an antimicrobial agent on a large scale level through standardized regulatory conditions.

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