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BIOSYNTHESIS AND CHARACTERIZATION OF ZNO NANOPARTICLES FROM AQUEOUS EXTRACT OF *CAMELLIA SINENSIS* AND DETERMINE ITS ANTIBACTERIAL ACTIVITY AGAINST MULTIDRUG RESISTANCE BACTERIA

Ali Hassan Mahmoud and Zainab Muhammed Nsaif
College of Science, Diyala University, Iraq.
*Email: zalzubaidii@yahoo.com

ABSTRACT

The current study involved collecting 225 samples of different age groups and from different clinical sources (burns and wounds). Selective and differential media, Microscopic Examination, Biochemical test, IMVIC tests, and Vitek 2 system were used to identify the bacterial species. The results showed that the bacterial isolates were distributed on *Staphylococcus aureus, Pseudomonas aeruginosa, Staphylococcus epidermidis, Klebsiella pneumoniae, Escherichia coli* and *Proteus mirabilis.* All isolates were multidrug-resistant to 12 antibiotics from different classes and according to the susceptibility test, isolate distinction in 2 groups (75 %) of MDR isolates were resistant to (5-9) antibiotics, while (25 %) were susceptible. Zinc Oxide nanoparticles synthesized was done by a green method with Zinc acetate dehydrate as a precursor and aqueous extract of *Camellia sinensis*as a reducing agent, color-changing to pale-white was an indication of the formation of ZnONPs. The average size and shape of the nanoparticles were detected by using Atomic Force Microscopy (AFM) which was 40 nm with a spherical shape. Scanning Electron Microscopy (SEM) showed the ZnO NPs have spherical, radial, and cylindrical structures. The wavelength range was measured by Ultraviolet-visible spectroscopy (UV-Vis) for monitoring the formation of the nanoparticles, which showed a sharp peak at 325 nm. The average crystallite size of ZnONPs was estimated using Debye Scherrer's formula were 20-40nm by using X-ray Diffraction (XRD). Fourier-transform infrared spectroscopy (FT-IR) spectra have been used for ZnONPs to detect the functional groups found in the synthesis process via green tea extract.

***Keyword*:** ZnO NPs. Biosynthesis, *Camellia sinensis,* Green tea, Antibacterial activity
Running title: Biosynthesis and characterization of ZnO Nanoparticles

Introduction

The production or modification of new antimicrobial compounds available to enhance the antibacterial activity for treatment, disinfection, or antisepsis is a highly important field of study. In this endeavor, Nanotechnology is emerging as a multidisciplinary field of science in which a wide range of metal nano nanoparticles (NPs) have been synthesized. The produced NPs have a unique size with more surface area to volume ratio that promoted their reactivity with the surrounding molecules (Gunalana *et al*., 2012). Therefore conventionally both physical and chemical methods are used to synthesize them, however, these methods have various demerits including expensive, toxic by-products, critical conditions of temperature and pressure, and long-time reactions (Geraldes *et al*., 2016). Whereas green synthesis of NPs; involve non-toxic, cheap, and generally available plant sources that are environment friendly (Salem *et al*., 2016). Those medicinal plants were rather used in the synthesis of NPs that already recognized for biomedical properties and having a huge range of natural products (Agarwal, *et al*., 2017). These bioactive phytochemicals are reacted to reduce metals into metal oxide and showing good stability in the formation of NPs (Mishra & Sharma, 2015). However, reports were available on inorganic metals such as Ti, Mg, Fe, Zn, S, and Al. Among these metals; ZnO has got exceptional position due to its wide applications in various fields of science (Dhanemozhi *et al*., 2017).

ZnO NPs were reported to have improved UV protection and enhanced opacity used for UV protections (Sundrarajan *et al*., 2015), as well as these nanoparticles showed raised catalytic, photochemical, and antimicrobial properties (Awwad *et al*., 2014), and were documented to rupture the lipid bilayers of the bacterium and fungal cell wall and revealed significant antibacterial and antifungal activity (Senthilkumar & Sivakumar, 2014). Production of ZnO NPs is a huge scope of this study to synthesize and evaluate its antimicrobial activity. zinc oxide nanoparticles have anti-microbial activity, as they have a wide surface area, which provides good connectivity to bind to their membrane cells and then penetrate into the cell, that the passage of this metal particle across the plasma membrane, bacteria lose control over the membrane permeability of incoming materials of bacteria, which leads to their death (Hendiani *et al*., 2015).

Green tea leaves were used to synthesize ZnO NPs. It is known as "Camellia sinensis" well-known for its Phenolic contents (Saravanak kumar *et al*., 2016), it is rich in bioactive phytochemicals that refer to as an anti-septic, anticancer, and antimicrobial agent (Rani *et al*., 2014). Consequently, these properties are enhanced in the production of ZnO NPs.

Throughout the current study, ZnO NPs were synthesized by using the leave extract of Camellia sinensis, and its antibacterial activity was assessed. Additional produced ZnO NPs were characterized by UV–visible, FTIR, XRD, and SEM.

Material and Methods

Isolation and identification of pathogenic bacteria

Two hundred twenty-five swabs of different age groups of both genders and different clinical sources burns and wounds were collected from patients in Baquba General Hospital and Medical City in Baghdad; after transplantation in the selective and differential cultures media; the bacterial isolates were diagnosed firstly, as well as Microscopic Examination, Biochemical tests, IMVIC tests and VITEC compact 2 system was done for confirmation (Pincus, 2011).

Antibiotic susceptibility test for pathogenic bacterial isolates

All isolates of bacteria were tested for antimicrobial sensitivity test according to the CLSI (2020), have been subjected to 12 types of the antibiotics for each bacteria to estimate the possible resistance or sensitivity to the antibiotics from different groups.

Preparation of *Camellia sinensis* (Green tea)

The Green tea leaves were washed well to clean from impurities and then dried in sunlight for 2 days and crushed with anElectric grinder to obtain a fine powder, which was kept in a sterile and closed glass vial at 4°C until further investigations.

Biosynthesis of ZnO Nanoparticles from aqueous extract *Camellia sinensis*

Zinc acetate dihydrate with 98% purity was checked from sigma. 2 gm of zinc acetate dihydrate was dissolved in a flask containing 70 ml of Deionized distilled water (ddH_2O) and then mixed for five minutes. 10 gm of Green tea leaf add to flask containing 100 ml of ddH_2O with magnetic stirrer bar medium size to mix the extract completely and then place the flask on the magnetic stirrer hot plate for 2 hours at 90°C with 1200 rpm /second. Then the extract is cooled at room temperature and separated via filter paper (Whatman No. 40). Then 30ml from green tea extracts mixed with 70 ml of Zinc acetate dihydrate, the solution was dried at 70 °C in a vacuum oven for 24h to produce pale-white ZnO nanoparticles. In the end, the solution calcination at 100°C for 1h and kept for further studies (Senthilkumar *et al*., 2014).

Characterizations of ZnO Nanoparticles

The characterization of ZnO NPs was performed using Fourier Transform Infra-Red Spectroscopy (FT-IR) Shimadzu (Germany) to show specific functional groups. ZnO NPs was confirmed by measuring the wavelength in the UV-VIS spectrum. Structural characterization was analyzed to obtain information about Crystallite Size and Crystal Structures by using an X-ray Diffractometer (XRD). The size surface topography and granularity volume of the ZnO nanoparticles was performed by using Atomic force microscopy (AFM). The morphology analysis, shape, and particle size of samples were performed by Scanning electron microscope (SEM).

Antibacterial Activity of the ZnO Nanoparticles

The synthesized ZnO NPs were evaluated for antimicrobial activity; well diffusion method (Hasan *et al*., 2009). Antibacterial activity was determined against Gram-positive and Gram-negative pathogenic bacteria (multiple drugs resist). For the antibacterial assay, all isolates were spread uniformly on Muller Hinton agar plates, five wells (5 mm) were made by sterilized cork borer in the plate. Four different concentrations (12.5, 25, 50,100 mg /ml) were prepared from the stock solution of ZnO NPs, the fifth well has been taken as control by adding 100 µl of ddH2O. Three repeats made for each microbe and then incubated for 24 h at 37 °C. The effectiveness of each nanoparticles concentration was determined by measuring the diameter of the inhibition zone around each hole and then compared with the negative control (Obedat *et al*., 2012).

Results and Discussion

The results showed in the table (1) that from the total 225 wound swab two species of bacteria belong to the Gram-positive, namely; *Staphylococcus aureus* and *Staphylococcus epidermidis* which isolated from wounds at a ratio of 37(58.7%) and 23 (61%) while they were isolated from burns at a ratio of 26(41.3%) and 15(39%). Also, this results showed that four species belong to the Gram-negative, namely *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli,* and *Proteus mirabilis* which isolated from wounds at a ratio of 28(59.5%), 18(60%), 14(63.6%) and 11(100%) respectively while they were isolated from burns at a ratio of 19(40.5%), 12(40%), 8(36.4%) and 0 respectively. The results appeared that there were 15 specimens did not have growth as the smears were taken from the patients; this results may be due to many reasons, the techniques that used was improper like the smears were not typically sufficient represented to had the contaminants and the using of sterilizers or the antibiotics.

Table 1 : The number of the isolates for each bacterium.

The Bacteria	No. (%) of Wounds isolates	No. (%) of Burns isolates	Total of isolates
Staphylococcus aureus	37 (58.7%)	26 (41.2%)	63
Pseudomonas aeruginosa	28 (59.5%)	19 (40.4%)	47
Staphylococcus epidermidis	23 (61%)	15 (39%)	38
Klebsiella pneumoniae	18 (60%)	12 (40%)	30
Escherichia coli	14 (63.6%)	8 (36.4%)	22
Proteus mirabilis	11 (100%)	0	11

Antimicrobial susceptibility test

Antimicrobial susceptibility testing was performed for six of the aforementioned pathogenic bacteria to identify the isolates of bacteria which have multiple drug resistance (MDR).The results showed in the table (2) shown that *S. aureus* were resist to Tetracycline, Azithromycin, Ciprofloxacin, Levofloxacin, Gentamicin, Ofloxacin, Clarithromycin, Streptomycin, Vancomycin and sensitive to Chloramphenicol, Clindamycin and Doxycycline while *S. epidermidis* were resist to Vancomycin, Ciprofloxacin, Gentamicin, Ofloxacin, Doxycycline, Clarithromycin, Azithromycin, Tetracycline, Levofloxacin and sensitive to Chloramphenicol Streptomycin and Clindamycin.

P. aeruginosa wereresist to Amikacin, Aztreonam, Tobramycin, Piperacillin, Gentamicin, Imipenem, Ceftazidime, Cefepime and Meropenem while sensitive for Levofloxacin, Ofloxacin and Ciprofloxacin while *E. coli* showed resistance toCefpodoxime, Cefoxitin, Ticarcillin-clavulanate, Imipenem, Trimethoprim, Azithromycin, Aztreonam, Ampicillin,Cefuroxime and sensitive to Levofloxacin, Doxycycline Tetracycline as shown in table (3). *K. pneumonia* exhibited resistance to Gentamicin, Piperacillin, Trimethoprim, Streptomycin, Ciprofloxacin, Cefuroxime, Nitrofurantoin, Cefotaxime, Ofloxacin,and sensitive to Imipenem, Amikacin and Chloramphenicol while the isolates of *P. mirabilis* showed toresistance to Chloramphenicol, Gentamicin, Nitrofurantoin, Amikacin, Trimethoprim, Cefuroxime, Streptomycin, Cefotaxime, and sensitive to Imipenem, Ofloxacin, Ciprofloxacin Piperacillinas shown in table (4).

Table 1 : Antimicrobial sensitivity test of *S. aureus* and *S.epidermidis* isolates.

Antibiotics	***S. aureus***	***S. epidermidis***
Tetracycline	R	R
Azithromycin	R	R
Ciprofloxacin	R	R
Levofloxacin	R	R
Gentamicin	R	R
Ofloxacin	R	R
Clarithromycin	R	R
Streptomycin	R	S
Vancomycin	R	R
Chloramphenicol	S	S
Clindamycin	S	S
Doxycycline	S	R

Table 3 : Antimicrobial sensitivity test of *P. aeruginosa* and *E. coli* isolates.

Antibiotics	***P.aeruginosa***	**Antibiotics**	***E.coli***
Amikacin	R	Cefpodoxime	R
Aztreonam	R	Cefoxitin	R
Tobramycin	R	Ticarcillin-clavulanate	R
Piperacillin	R	Imipenem	R
Gentamicin	R	Trimethoprim	R
Imipenem	R	Azithromycin	R
Ceftazidime	R	Aztreonam	R
Cefepime	R	Ampicillin	R
Meropenem	R	Cefuroxime	R
Levofloxacin	S	Levofloxacin	S
Ofloxacin	S	Doxycycline	S
Ciprofloxacin	S	Tetracycline	S

Table 4 : Antimicrobial sensitivity test of *K. Pneumonia and P. mirabilis*.

Antibiotics	***K. pneumoni a***	***P. mirabilis***
Gentamicin	R	R
Piperacillin	R	S
Trimethoprim	R	R
Streptomycin	R	R
Ciprofloxacin	R	S
Cefuroxime	R	R
Nitrofurantoin	R	R
Cefotaxime	R	R
Ofloxacin	R	S
Imipenem	S	S
Chloramphenicol	S	R
Gentamicin	S	R

Biosynthesis of ZnO Nanoparticles

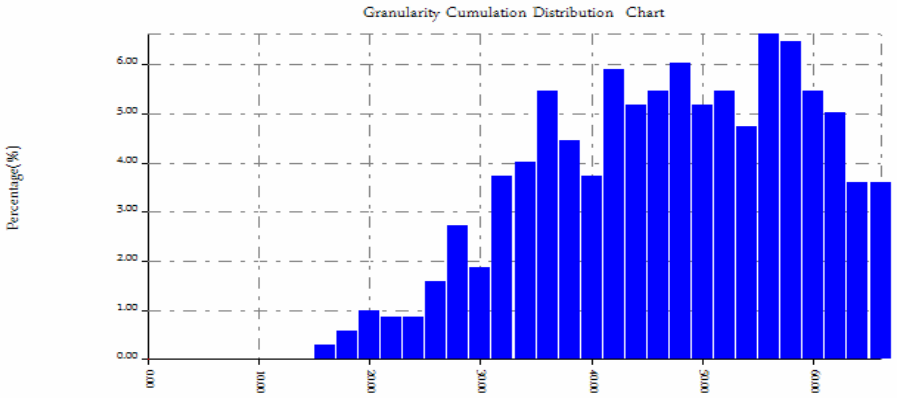
The green synthetic method of ZnO NPs is a recent approach that is the explanation of cheap and ecofriendly methods. Therapeutically important plants have such phytochemicals that act to steady and reduce metal oxides for the synthesis of nanoparticles with precise shape and size (Rani *et al*., 2014). Further such famous phytochemicals are involved in the bio controlling mechanism of growth of pathogenic microorganisms (Zhang *et al*., 2008). In the current study, for the biosynthesis of ZnO NPs; dried ground leaves of *C. sinensis* were used to prepare the extract, and a solution of Zinc acetate dihydrate was poured in 100 mL of fresh leave extract. Pale yellow ZnO nanoparticles instantly appeared that grew larger within seconds and finally precipitate down leaving a supernatant layer as shown in Figure 1. The green tea extracts contain flavonols and catechins that serve as reduction agents to ZnO nanoparticles for the zinc acetate. The phenolic composite exhibits great antioxidants agent and this process was very good to reduce metal particles, thus promoting the green nanoparticles synthesis. Change in color is the primary test for nanoparticles synthesis shown in Figure (3), color-changing to pale-white is an indication of the formation of ZnO Nanoparticles (Iravani *et al*., 2011). One of the most important uses of biosynthesis of zinc oxide nanoparticles is that it is cheap, environmentally safe, riskless, easy to operate, and low in toxicity (Heer *et al*., 2017).

Fig. 1 : The biosynthesis of ZnO NPs from *Camellia sinensis.*

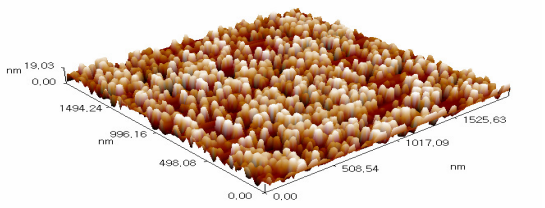
Characterization of Zinc oxide Nanoparticles

Atomic Force Microscopy (AFM)

Atomic force microscopy (AFM) was used to validate the surface shape of the biosynthetic zinc oxide nanoparticles by using *Camellia sinensis* (green tea) aqueous extract and then take a 2D and 3D image with the AFM. The results showed the variation in the phenotypic characteristics of the zinc oxide nanoparticles. Figure (2) indicated that ZnONPs formed by Green tea in a nano size 40 nm. The current study succeeded in achieving good results in reaching the size of nanoparticle for zinc oxide that were less than what found in previous study Al-Ogaidi, (2017) which obtained 88nm in size.



A



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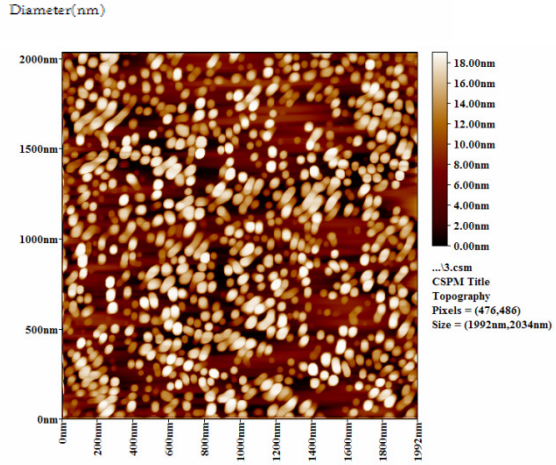

B

Fig (2):A- The range of size of biosynthesized ZnO nanoparticles. B-Topography of two-dimensional zinc oxide nanoparticles.C-Topography of three-dimensional zinc oxide nanoparticles.

Scanning Electron Microscopy (SEM) analysis

The surface phenotype of synthesized zinc oxide nanoparticles was explored Green tea by scanning electron microscopy (SEM). Figure (3) showed that these particles were under different enlargement forces, irregular and polymorphic. These particles have between rectangular, spherical, radial, and cylindrical structures. Medium size ranges between 29-55 nanometers. The current study succeeded in achieving good results in reaching a narrow range of nanoparticle sizes for zinc oxide which this result agreement with Dhanemoz *et al*. (2017).

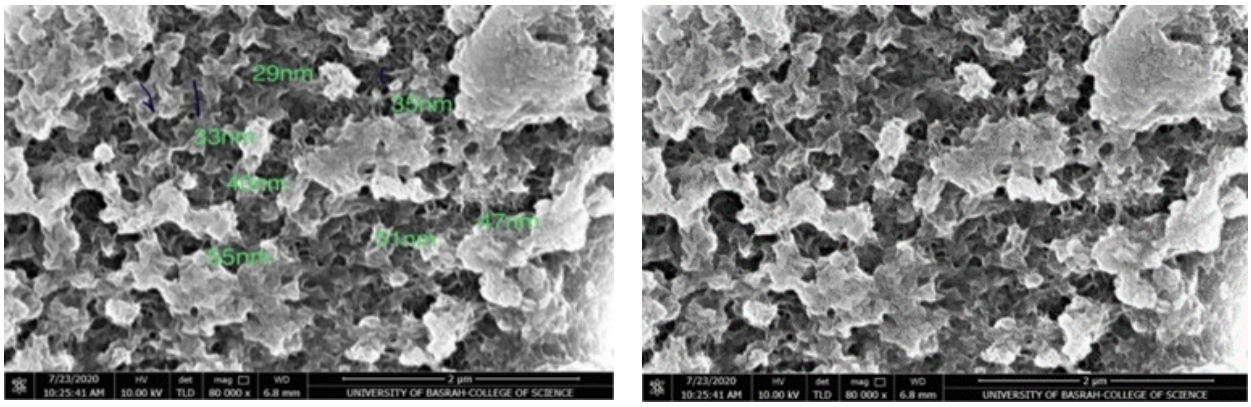

Fig. 3: Biosynthesized ZnONPs by green tea under SEM with diverse shapes and diameter ranging (29-55 nm)

UV-Vis spectrum

Adopts Visible-UV Spectroscopy enables the transmission of visible light and/or transmission of ray's ultraviolet during the sample to determine the presence of a substance that absorbs light inside the sample. The absorbance peak was reported at 325 nm in Figure (4) this result was similar to Irshad *et al*. (2018) noting the ZnO absorption peak was (330 nm). The reduction of the zinc acetic dehydrates to ZnO nanoparticles in the green tea leaf extract were developed as a pale white powder. Through synthesis, the adding of the tea extracts was accompanied by an immediate color change which signaled the beginning of ZnO NP formation. The reaction color ranged from colorless to white the change in color indicates the formation of ZnO nanoparticles as indicated by the UV visual spectra. The highly blue-shifted maximum absorption occurring about 325 nm confirms the formation of the nanoscale ZnO component since the maximum absorption for the bulk ZnO occurs at around 385 nm (Senthilkumar *et al*., 2014).

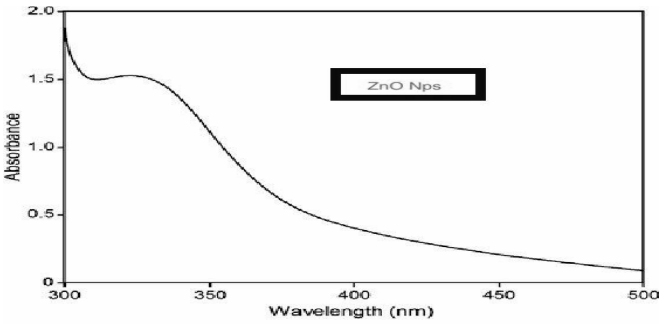

Fig. 4 : UV-Vis spectrum of synthesized ZnO NPs by green tea.

Fourier-transform infrared spectroscopy (FTIR)

FTIR was performed to show specific functional groups in ZnO NPs**.** Figure (5) show the ZnONPs formed by green tea IR set, the band is around 3000 cm–1 due to extending O – H group vibrations in water, alcohol, and phenols and N – H extending in amines. In alkanes, the C – H stretch and in carboxylic corrosive O – H stretch show up separately at 2937 and 2890 cm–1. The 1624 cm–1 band was ascribed in the fragrant ring to the C = C stretch and polyphenols to the C = O stretch. The amide-I in protein stretch C – N gives the band 1415 cm–1. A band at 1026 and 1049 cm–1 is caused by the C – O expansion in amino corrosive. The additional three peaks which appear in the ZnO NPs IR spectrum at 943 and 677 cm–1 are the characteristic peaks of ZnO molecules (Subhan *et al*., 2014). Last but not least, the powerless band at 617 cm–1 is C – H's after effect out of the plane bend. This result was near Al-Ogaidi, (2017). Thus it can be observed from the IR collection that green tea was rich in polyphenols, carboxylic corrosive, polysaccharide, amino corrosive, and protein. The presence of phenol may act as a good reduction agent, an amide in the protein group was responsible for stabilizing nanoparticles in ZnO.

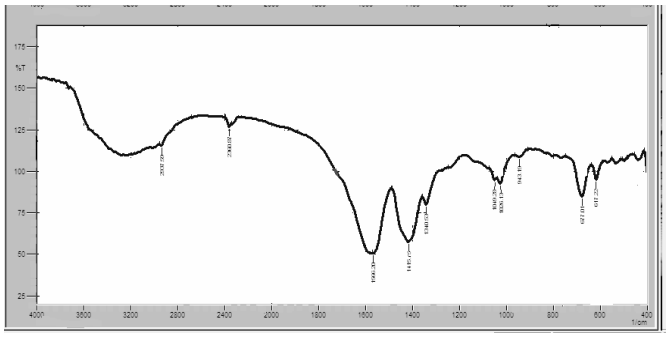

Fig. 5 : FT-IR spectra of ZnONPs synthesis by green tea extract

X-ray Diffraction (XRD)

X-ray diffraction technology is widely used for crystal structure and crystal size of solids materials. The XRD of ZnO NPs prepared and calcination at 100°C Extract the water and achieve a higher crystallization. Figure (6) shows prominent peaks corresponding to the levels of diffraction found at 2θ interval values 31.7° (100), 34.4° (002), 47.5° (102), 56.5° (110), and 62.8° (103) were in good agreement with the Joint Committee on Powder Diffraction Standards (JCPDS) Card No. 36 confirms the hexagonal structure of

ZnO NP. The average particle size (D) was determined using the Debye Scherrer formula (Bokuniaeva *et al*., 2019)

$$D = 0.9 \lambda / \beta \cos \theta$$

Where λ is the wavelength of X-ray source (CuKα line – 0.1541 nm), β is the full width at half maximum (FWHM) in radians and θ is Bragg's diffraction angle. It was found that the measured value of D is 20-40 nm this result was close to Irshad *et al*. (2018) who mention the average size was 30-40 nm.

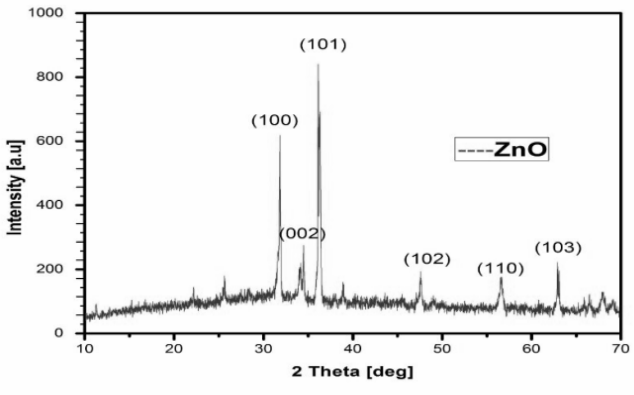

Fig. 6 : XRD spectra of BiosynthizedZnO NPs.

Antibacterial activity of ZnONps against pathogenic bacteria

ZnO NPs showed a remarkably antibacterial effect against both Gram-positive and Gram-negative bacteria (multidrug-resistance) as shown in Table (5) and Figure (7), The ZnO NPs showed the highest areas of inhibition zones against Gram-positive *S. aureus* and *S. epidermidis* at concentration 100 mg/ml were 33mm and 31mm respectively while recorded at concentration 12.5 mg/ml lowest areas of inhibition zones against the same isolates reaching 15mm and 14mm respectively, otherwise ZnONPs shown at concentration 25mg/ml the inhibition zones were 19mm and 18mm respectively against the same isolates and at concentration 50mg/ml the inhibition zones were 27mm and 25mm respectively.

Oppositely the effect of Zinc oxide against Gram-negative *P. aeruginoas, K. pneumoniae, E. coli* and *P. mirabilis* which show at concentration 100 mg/ml highest areas of inhibition zones reaching (28, 28, 27, 26) mm respectively while at concenteration12.5 mg/ml showed lowest areas of inhibition zones were (13, 13, 13, 12) mm respectively against the same isolates, The inhibitions zones of ZnONPs for other concentrations 25mg/ml and 50 mg/ml were diverse amongst the tested (Gram-negative) isolates. These results were very close to Irshad *et al*., (2018) and Al-Ogaidi,(2017).

Table 5 : The Effect of ZnO NPs on bacterial growth.

Type of isolate	12.5 mg/ml	25 mg/ml	50 mg/ml	100 mg/ml
Staphylococcus aureus	15	19	27	33
Staphylococcus epidermidis	14	18	25	31
Pseudomonas aeruginosa	13	17	23	28
Klebsiella pneumoniae	13	17	24	28
Escherichia coli	13	16	23	27
Proteus Mirabilis	12	15	21	26

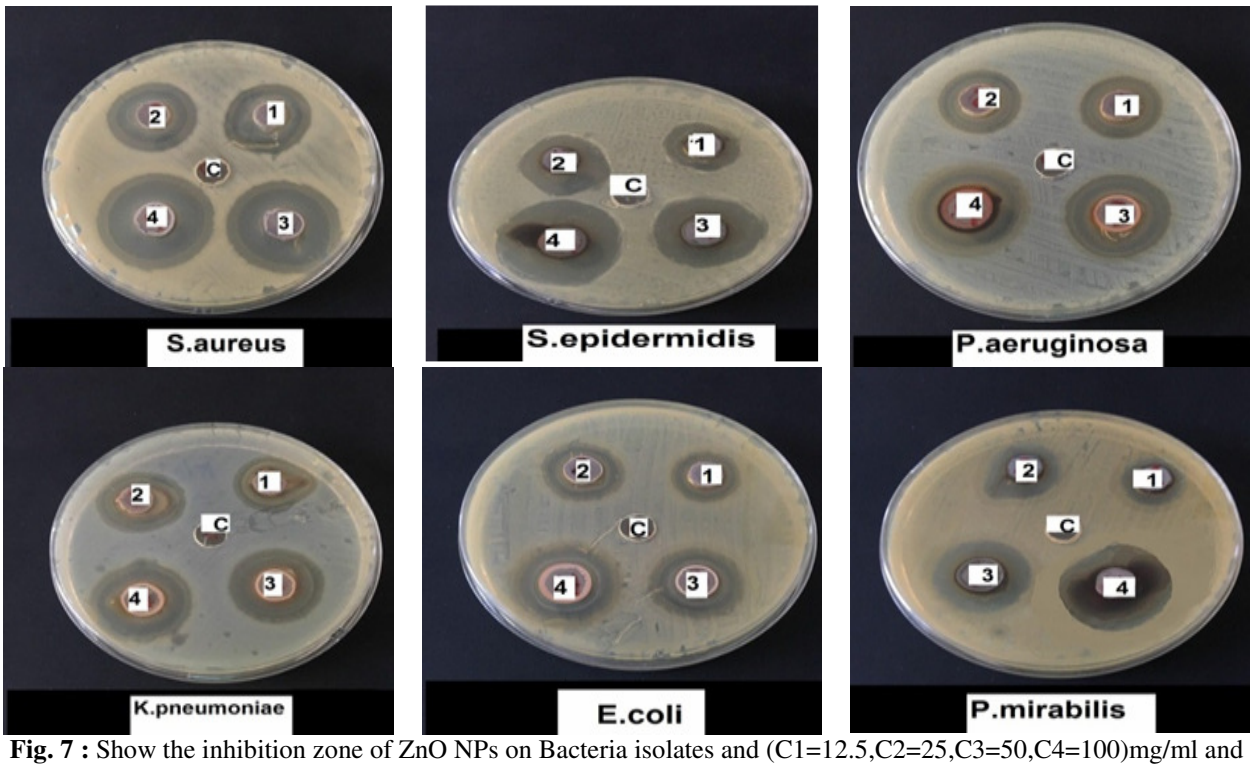

Fig. 7 : Show the inhibition zone of ZnO NPs on Bacteria isolates and (C1=12.5,C2=25,C3=50,C4=100)mg/ml and C = Negative Control (ddH_2O)

The current study showed that the green biosynthesis of zinc oxide particles from green tea aqueous extract has great efficacy in getting rid of pathological bacteria with multiple resistance isolated from burn and wound samples. Based on the differences in the bacterial structure the activity of ZnO on the gram-positive bacteria was more than its activity on gram-negative bacteria because the interaction between nanoparticles and cell surfaces would differ that lead to an effect on the penetrability of membranes since the entrance of nanoparticles inside bacterial cell induces oxidative stress consequently leading to inhibit cell growth and ultimately cell death (Prasad *et al*., 2014). 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 the enhanced activity. ZnO NPs generate hydrogen peroxides that chemically interact with the cell membrane (Senthilkumar *et al*., 2014). Also, the antimicrobial activity of nanoparticles may involve the production of reactive oxygen species (ROS) can cause cell membrane dysfunction and cell death by oxidizing lipids of the cell membrane (Dutta *et al*., 2012).

Conclusions

Staphylococcus aureus was the most commonly isolated from burns and wounds infections and then *Pseudomonas aeruginosa*. All bacteria isolated from burns and wounds had multiple antibiotics resistance (resistance to more than one antibiotic). The biosynthesis of ZnONPs by aqueous extract *Camellia sinensis* has antibacterial activity against Gram-positive and Gram-negative bacteria. The activity of ZnO on the gram-positive bacteria was more than its activity on gram-negative bacteria. AFM, SEM, XRD, UV-vis, and FTIR were used to investigate ZnONPs. The antibacterial activity of ZnONPs has more sufficient from antibiotics in inhibition pathogenic bacteria isolated from wounds and burns. The biosynthesis of zinc oxide nanoparticles from plant extracts was a simple method, environmentally friendly, economical cheap.

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