RESEARCH ARTICLE

Evaluation of Antibacterial Effect of Zinc Oxide Nanoparticles Synthesized by Wet-chemically Method on Multidrug-resistant Uropathogenic Bacteria

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ABSTRACT

Zinc oxide (ZnO) nanoparticles have been attractive for their antibacterial effects along with their safety, suitable physicochemical properties, and cost effectivity. The aim of this study was to synthesize ZnO nanoparticles and investigate their antibacterial effects on multidrug resistant (MDR) uropathogenic bacteria. ZnO nanoparticles were constructed by wet chemical method with inhibitor. Structural and optical evaluation of zinc oxide nanoparticles was performed by Ultraviolet absorption spectroscopy, X-ray diffraction (XRD), and Scanning Tunneling microscopy. Uropathogenic bacteria were isolated from the patient pediatric urine and identified through biochemical testing. Antibiotic resistance pattern of the isolates was determined by disk diffusion method and the antibacterial activity of ZnO nanoparticles was detected by pour plate method at the concentrations of 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, and 0.8 g/l. The produced ZnO nanoparticles had a hexagonal shape with the crystal size of 15 nm and an optical size of 3 nm. The minimum effective concentration of these nanoparticles that inhibited the bacterial growth was 0.05 g/l. *E.coli* is generally found to be the most frequent cause of UTI in children in Isfahan, in the center of iran, with the mean prevalence of 64%. Most cases of resistance were related to Ampicillin, Amoxicillin, Tetracycline and the highest sensitivity was related to Chloramphenicol, Gentamicin, and Trimethoprim/Sulfamethoxazole. The results of the present study, in accordance to the previously approved safe characteristics of ZnO nanoparticles, concentration of 0.05 g/l is proposed to be evaluated for *in vivo* usage for treatment of urinary tract infections.

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INTRODUCTION

Urinary tract infection (UTI) means the presence of a threshold number of pathogenic bacteria (10^5 CFU / mL) in the urine and is the most common acquired bacterial infection among children and women [1,2]. More than half of women have experienced a urinary tract infection at least once in their lifetime [3]. UTI has a high mortality rates in many countries and its treatment is very expensive [4]. Various Gram-negative pathogenic bacteria such as *Escherichia coli*, *Proteus mirabilis*, and *Klebsiella pneumoniae* are involved in urinary tract infections [5]. According to the World Health

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Organization (WHO), excessive prevalence of urinary tract infections is due to *Escherichia coli* because of the resistant of its strains to a wide range of antibiotics [6]. *Escherichia coli* is a Gram-negative bacterium commonly found in the intestinal microbiome of mammals, birds, and fish, and is a major human pathogen that causes urinary tract infections, enterocolitis, and sepsis in humans. So far, more than 10 different pathotypes of *E.coli* have been identified that can cause similar diseases through various mechanisms [7, 8]. In recent years, increased drug resistance transmission among pathogens is a major problem that is the main cause of the emergence of new resistant strains, especially multidrug-resistant strains (MDR) ones. Transmission of resistance factors to susceptible strains elevates treatment cost and disease treatment failure (DTF) probability, leading to an increased disability and mortality [9, 10]. Therefore, finding an alternative treatment to overcome increasing bacterial drug resistance is essential [11]. Antibacterial agents are very important in various industries such as food, cosmetics, chemicals, textiles, and medicine [12]. Increasing studies are conducted on the use of non-organic antibacterial agents such as metal oxides, preferably in the form of nanoparticles, because these non-organic compounds show strong antibacterial activity at low concentrations. They are also much more stable in severe conditions and are considered as non-toxic compounds. Some of them even contain essential mineral elements for the human body [13, 14]. Controlling the growth of bacterial biofilms using nanoparticles has attracted great notice for medicinal purposes [4]. Metal nanoparticles damage DNA and kill bacteria through various mechanisms such as oxidation of membrane lipids, altering the permeability of the bacterial cell wall and releasing the cell contents [15]. Nanotechnology refers to the design, characterization, production, and use of structures and tools with the shape and the size of nanometer scale (1-100 nm) [13]. The use of nanoparticles is a promising therapeutic strategy to overcome the growing emergence of multidrug-resistant bacteria [11]. Nanoparticles are of great importance due to their catalytic, optical, magnetic, and electrical properties [16]. One of the most important nanoparticles is zinc oxide (ZnO), which is used industrially in most countries and is recognized as a safe compound by the US Department of Food and Drug Administration (FDA) [17]. ZnO nanoparticles are relatively inexpensive and UV resistant structures that have antimicrobial activity at normal pH [4]. These nanoparticles have attracted a great deal of attention in fundamental studies and potential applications in various research fields from physical chemistry to medical sciences [18]. They are known for their use in the treatment of urinary tract infections as one of the most effective non-organic nanoparticles [19]. The most important feature of ZnO nanoparticles is their small size and large surface area, which increases their chemical and biological properties and antimicrobial activity in usage for elimination of Gram-negative and Gram-positive bacteria [20]. Among metal oxide nanoparticles, zinc oxide nanoparticles have been reported as antimicrobial agent against pathogenic microorganisms that have the most toxic effects on *Escherichia coli* [21, 22]. The mechanism of antimicrobial activity of ZnO nanoparticles is similar to other nanoparticles, and it acts mainly through the destruction of the bacterial cell wall [17]. Some procedures have been employed to synthesize of nanoparticles, including co-precipitation, combustion, sol-gel, microwave, sol-gel auto-combustion, hydrothermal and wet chemical method [23]. Among the nanoparticle synthesis methods, the wet chemical method has advantages such as synthesis at room temperature, production of high purity products, relatively high production efficiency and high chemical reactivity [24]. To make nanoparticles by chemical or controlled sedimentation methods from metal salts such as Zn, and Cd, as well as alkali metal sulfides like sodium sulfide, sodium hydroxide, or potassium hydroxide in the presence of a chemical solution such as 2-mercaptoethanol, thiophenyl or polyethylene glycol is used and the particle size is controlled by adjusting the used materials concentrations and pH values. After the reaction is complete, the stabilized nanoparticles in the product are precipitated and extracted from the solution [25]. The aim of this study was to investigate the antimicrobial effect of zinc oxide nanoparticles synthesized by wet chemical method on multidrug-resistant europathogenic *Escherichia coli*, *Proteus mirabilis* and *Klebsiella pneumoniae*.

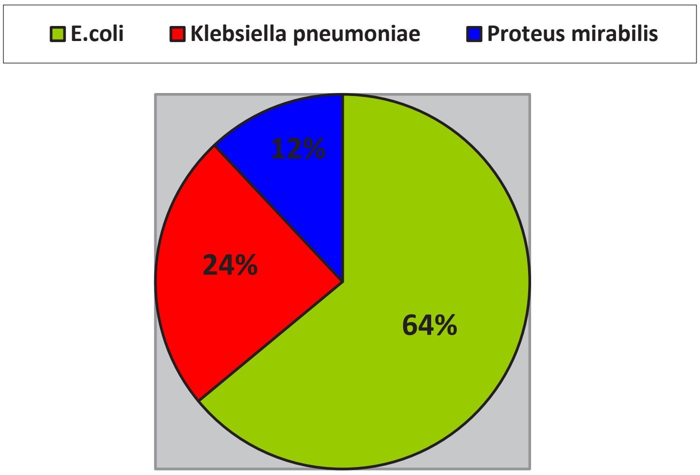

Fig. 1. Causative microorganisms for UTI in children.

MATERIALS AND METHODS

Synthesis of ZnO nanoparticles

Wet chemical method with inhibitor was used in order to synthesize zinc oxide nanoparticles [26]. The basis of the inhibition method is to prevent the growth and accumulation of particles during the reaction by creating a coating layer around them. This is accomplished by inserting an inhibitor into the reaction as well as setting steps. All chemicals used in this study were prepared by the German Merck company. In the synthesis pathway, 2.61 g of zinc nitrate (N_2O_6Zn, $4H_2O$), 400 µl of organic inhibitor 2-Mercaptoethanol ($HSCH_2CH_2OH$), and 0.8 g of sodium hydroxide were dissolved in 100 ml of deionized distilled water, respectively. Then, the zinc nitrate solution was poured into the reaction container (which was a single-aperture balloon) and while homogenized by a magnetic stirrer, the inhibitor (2-Mercaptoethanol) entered the balloon drop by drop through the soxhlet. At the end of this step, the sodium hydroxide solution was slowly added to the contents of the container to complete the reaction. In this experiment, the flow was adjusted so that one drop entered the balloon every 4 seconds. Based on the experience, at this rate, the particle size distribution was narrower. At this stage, after a few minutes, the effects of nanoparticle formation in the balloon could be seen. Solution entry rate, magnet rotation speed, and other parameters remained unchanged until the end of the experiment, so that all nanoparticles were produced under the same conditions. As a result of the reaction, ZnO nanoparticles began to precipitate inside the container. The obtained product was washed 3 times with distilled water and placed in an oven at 50 °C for 24 hours to dry.

Characterization of zinc oxide nanoparticles

X-ray diffraction (XRD)

The X-ray region in the electromagnetic spectrum is between the γ-ray and the ultraviolet ray. Using this spectral region, information can be obtained about the structure, material type, and values of the elements. X-ray diffraction is a powerful method to examine crystalline materials [27]. To investigate the crystal structure of the synthesized nanoparticles, the obtained powder was subjected to X-ray diffraction (XRD) test (Bruker D8 ADVANCE λ = 0.154 nm Cu Kα radiation) and the data was analyzed by Xpert High Score software.

UV-visible absorption spectrometer

UV-visible was used to study the optical properties and particle size of the synthesized materials. The UV-visible absorption spectrometer measures and plots the absorption coefficient of matter in response to visible and ultraviolet wavelength radiation (from 200 to 600 nm). The adsorption spectra were taken from the nanoparticles produced in the colloidal state where the device was calibrated with distilled water, to ensure that the resulting absorption spectrum was only for semiconductor nanoparticles. What was given as input to the device was a very dilute solution that was poured into a quartz container that was not absorbed in the above spectral region. The

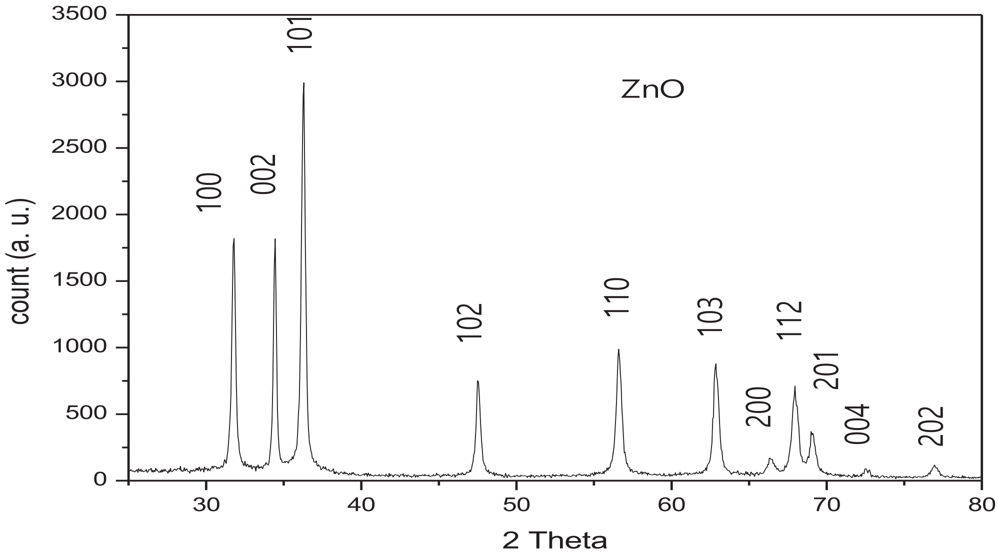

Fig. 2. XRD diagram of zinc oxide nanoparticles.

device used was UNICO UV-2100 spectroscopy. As shown in Fig. 2, the wavelength of the sample adsorption shoulder was reduced relative to the wavelength of the bulk ZnO adsorption shoulder (367 nm).

Scanning Tunneling Microscope (STM)

Scanning tunneling microscope (NATSICO, Iran) was used to study the particle size distribution.

Bacterial identification and antibiotic susceptibility testing

Positive urine culture samples of children with urinary tract infections were collected from several medical diagnostic laboratories in Isfahan. A total of 100 urine cultures were examined, that twenty-five samples were Multi-drug resistant bacteria that caused urinary tract infections in children. Identification was done based on culture characteristics, Gram stain and routine standard biochemical tests and differential culture media such as Sulfide Indole Motility (SIM), Triple Sugar Iron Agar (TSI), Simmons Citrate Agar, Urease, Methyl Red and Voges-Proskauer. Urine samples were collected suprapubicly or by transurethral catheterization from patients younger than 2 years old and using the Midstream Specimen of urine in patients with 2 years of age in sterile containers.

Samples were cultured on blood agar and EMB agar by using a standard calibrated loop (0.01mL) and the plates were incubated at 37 °C for 24hours. The result of equal or more than 10^5 CFU/mL and the cytology of the urine through microscopic detection of bacteriuria and PMNs (≥10 leukocytes/mm^3) were considered as positive UTI and a less than 10^2 CFU/ml was interpreted as negative UTI. The result of 10^2-10^4 CFU/ml was repeated [28, 29].

The isolated strains were probably E. coli, Proteus mirabilis and Klebsiella pneumoniae. After identifying the bacteria, the antibiotic resistance pattern of the studied strains using 14 antibiotic discs (Padtan Teb, Iran) including; Ceftazidime (CAZ), 30 µg; Ampicillin (AM), 10 µg; Amikacin (AN), 30 µg; Chloramphenicol (C), 30 µg; Nalidixic acid (NA), 30 µg; Cefepime (FEP), 30 µg; Nitrofurantoin (FM), 300 µg; Amoxicillin (AMX), 25 µg; Ciprofloxacin (CP), 5 µg; Gentamicin (GM), 10 µg; Imipenem (IPM), 10 µg; Ceftriaxone (CRO), 30 µg; Tetracycline (TE), 30 µg; And Trimethoprim/Sulfamethoxazole (SXT), 1.25 /23.75 µg was performed by disk diffusion method (Kirby-Bauer) on Mueller-Hinton agar (MHA) after preparing the microbial suspension equivalent to McFarland standards (1.5 × 10^8 CFU/ml). The mean diameters of growth inhibition zones (mm) were reported after 3 repetitions and the results were reviewed in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2020) [30]. Finally, the bacterial isolates that showed maximum resistance were isolated.

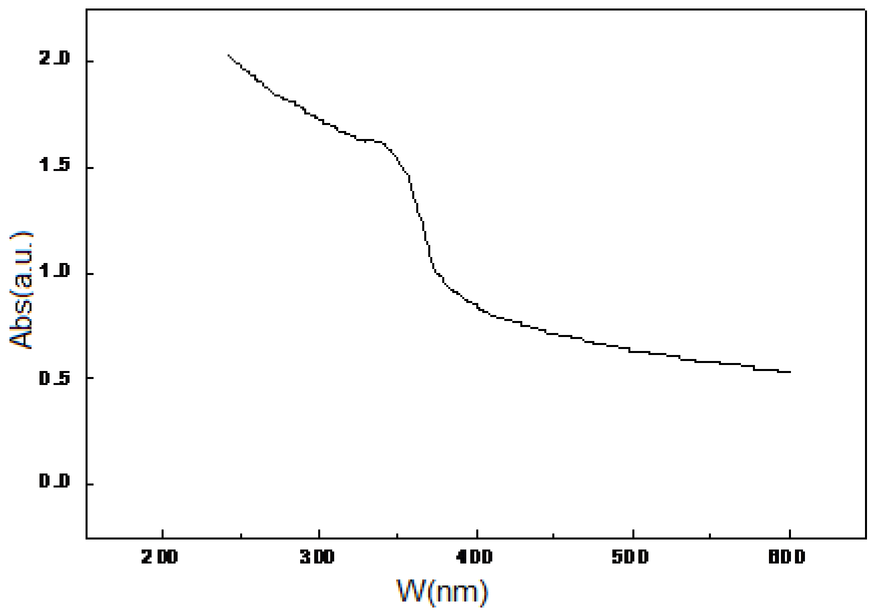

Fig. 3. Absorbance spectrum of zinc oxide nanoparticles.

Investigation of antibacterial activity of nanoparticles

To evaluate the antibacterial effect of nanoparticles on MDR strains of *Escherichia coli*, *Proteus mirabilis*, and *Klebsiella pneumonia*, pour plate method was used [31]. Prior to the test, all tools and materials used in the test were sterilized by autoclave and all steps were performed under the biological cabinet. First, 7 different concentrations of nanoparticles (0.0125 g/l, 0.025 g/l, 0.05 g/l, 0.1 g/l, 0.2 g/l, 0.4 g/l, and 0.8 g/l) were prepared in 1 ml distilled water; then vortexed and autoclaved. After re-vortex, each solution was poured into a sterile petri dish (10 cm diameter), and 19 ml of sterile melted MHA culture medium which was reached to the temperature of about 45 °C was added to each of them and rotated in order to culture medium and nanoparticle suspension were thoroughly mixed. Samples lacked nanoparticles were considered as controls. The media were left until solidification. Then, a loop full of the each previously cultured bacterial suspension was transferred to a physiological saline falcon tube. The falcons were vortexed and then the optical absorbance of the bacterial solution was adjusted to 0.8-0.13 (1.5×10^8 CFU/mL). Then the bacteria were cultured by spreading on MHA containing nanoparticles and control sample and finally incubated at the temperature of 37 °C for 24 hours.

Statistical analysis

The data were analyzed using One-way ANOVA and Tukey's post hoc tests using Graphpad prism software 8.

RESULTS AND DISCUSSION

Antibiotic resistance is now considered as a global problem. Due to the increasing use of antibiotics, and subsequent increase in antibiotic resistance, treatment of bacterial infections has faced many difficulties. Antibiotic resistant UTI is becoming more common globally, increases morbidity and doubles healthcare costs. Therefore, finding an alternative treatment to overcome increasing bacterial drug resistance is essential. The use of ZnO nanoparticles is a promising therapeutic strategy to overcome the growing emergence of multidrug-resistant bacteria.

During the study period a total of 25 bacterial isolates from urinary tract infections was collected from children. Similar to the results of Poladfar et al in Shiraz [32], *E. coli* was the most isolated organism accounting for which accounted for 16 (64%) followed by *Klebsiella pneumonia* for 6 (24%), and *Proteus mirabilis* for 3 (12%) (Fig. 1). Based on the findings, most cases of resistance

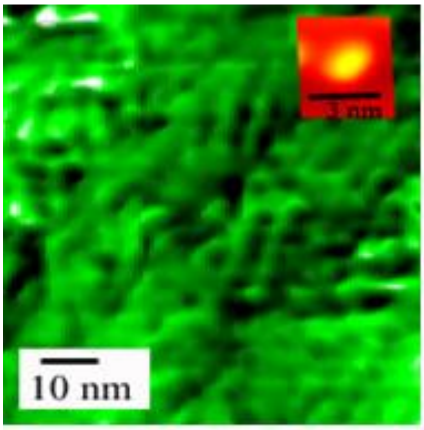

Fig. 4. STM image Two-dimensional of the generated nanoparticle.

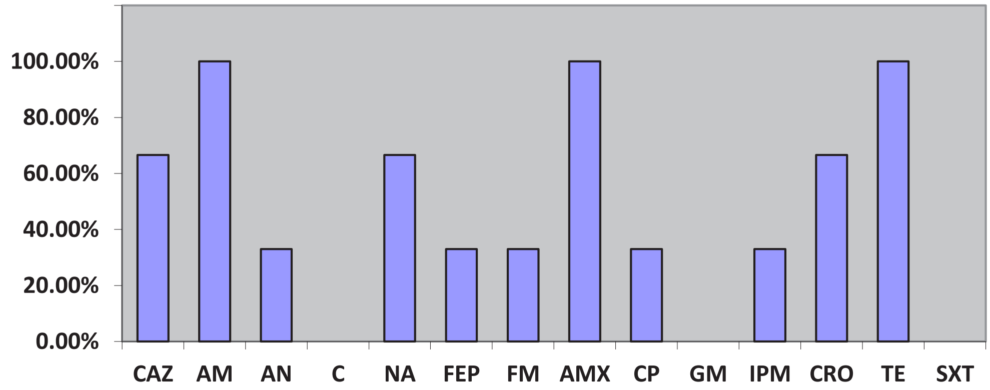

Fig. 5. Antibiotic resistance patterns of isolated uropathogens.

were related to Ampicillin (100%), Amoxicillin (100%), Tetracycline (100%) and the highest sensitivity were related to Chloramphenicol (100%), Gentamicin (100%), Trimethoprim/Sulfamethoxazol (100%) (Fig. 5). Our results were almost similar to a another study in Fasa on *E. coli* isolates recovered from urine cultures exhibited the high sensitivity to Gentamicin (65.6%) and the highest resistance to Ampicillin (100%), and Co-trimoxazole (68.7%) [33]. In Sanandaj, a study showed E. coli as the most common cause of UTI, with the highest resistance to ampicillin (43.87%) and the lowest resistance to nitrofurantoin (3.62%) [34].

Zinc oxide is an essential ingredient of many enzymes, sun screens, and ointments for pain and itch relief. They are more effective against microorganisms such as *Escherichia coli*, *Proteus mirabilis* and *Klebsiella pneumonia*. Mechanism of action has been ascribed to the activation of zinc oxide nanoparticles by light, which penetrate the bacterial cell wall via diffusion. It has been confirmed of the bacterial cells that zinc oxide nanoparticles disintegrate the cell membrane and accumulate in the cytoplasm where they interact with biomolecules causing cell apoptosis leading to cell death [35].

ZnO nanoparticles were prepared by wet chemical method and mercaptoethanol was used as an inhibitor, similar to the synthesis method used by Dowlatababdi et al [26]. The advantage of the wet chemical synthesis method is based on inhibiting the prevention of the particles growth and agglomeration during the reaction through creating an envelope around them.

The structural and optical properties of nanoparticles synthesized by wet chemical method were investigated by XRD and UV-visible absorption spectrometer (Figs. 2 and 3). The data obtained from the XRD spectrum in Fig. 2 shows

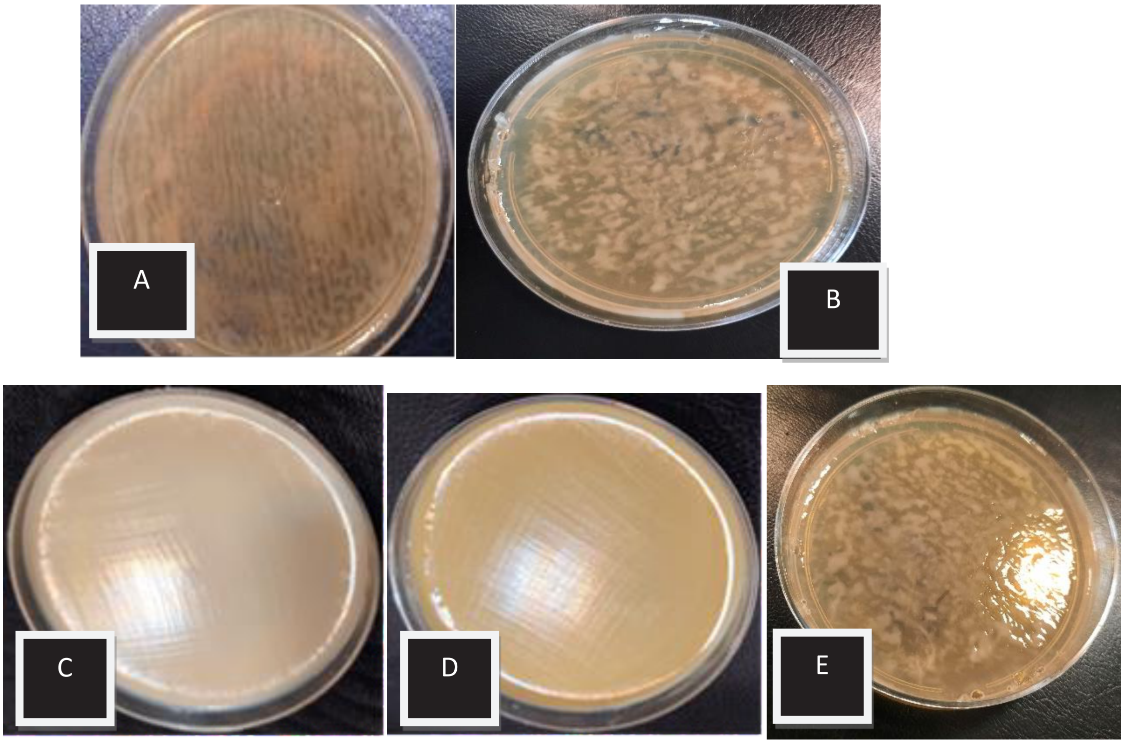

Fig. 6. Antibacterial test for different concentrations of ZnO nanoparticles on *E. coli*. A: Control. B: Concentration of 0.0125 g/l. C: Concentration of 0.025 g/l. D: Concentration of 0.05 g/l. E: Concentration of 0.1 g/l.

that the diffraction pattern is related to zinc oxide and the maxima resulting from the pages (100), (002), (101), (102), (110), (103), (200), (112), (201), (004), and (202) are clearly seen in it. Examination of the diffraction spectrum of the produced sample revealed that the phase is related to pure hexagonal zinc oxide nanoparticles (wurtzite). The crystal size for the most intense maximum was calculated, the maximum (101) which resulted is 15 nm. As can be seen in Fig. 3, the absorption shoulder wavelength of the sample is about 350 nm, which is reduced compared to the massive ZnO absorption shoulder wavelength (367 nm), and the optical size was estimated using the Brass ratio method of 3 nm. Fig. 4 shows images of the produced zinc oxide nanoparticles prepared by tunneling microscope and particles with a size of 3 to 7 nanometers are visible in it. Antibacterial mechanisms of ZnO nanoparticles, including oxidation of membrane lipids, alteration of bacterial cell wall permeability, and release of cell contents are effective in the killing of bacteria [15]. According to the results of antibiotic resistant test in the present study, (Table 1), the studied bacteria were resistant to several drugs. The antibacterial properties of zinc oxide nanoparticles synthesized against Gram-negative bacteria including *Escherichia coli*, *Proteus mirabilis*, and *Klebsiella pneumonia* were evaluated by pour plate method, and the results of which can be seen in Table 1 and Fig. 6. As it is clear, the number of colonies on the control sample is very high in contrast to test samples, so this nanoparticle had high antibacterial properties. The minimum concentration of nanoparticles that showed inhibitory effect on the bacterial growth was 0.05 g/l.

CONCLUSIONS

The results of our study showed that *E.coli* was generally found to be the most frequent cause of UTI in children in Isfahan, in the center of Iran, with the mean prevalence of 64%. According to the results of this study and the comparison between previous studies, Chloramphenicol, Gentamicin and Trimethoprim/ Sulfamethoxazole are still effective drugs for the empirical treatment of urinary tract infections.

Zinc oxide nanoparticles had strong antibacterial properties and were effective on urinary tract infecting bacteria including *Escherichia*

Table 1. Mean resistance and susceptibility of the isolated uropathogenic bacteria to antibiotics.

	SE= 1.145			SE= 1.575			SE= 1.544		
	E. coli			*Proteus mirabilis*			*Klebsiella pneumonia*		
Antibiotic	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
Ceftazidime (CAZ) 30 µg			*	(27mm)*					*
Ampicillin (AM) 10 µg			*			*			*
Amikacin (AN) 30 µg			*	(20mm)*			(19mm) *		
Chloramphenicol (C) 30 µg	(20mm) *			(20mm)*			(22mm)*		
Nalidixic acid (NA) 30 µg			*			*		(15mm) *	
Cefepime (FEP) 30 µg	(30mm) *			(33mm) *					*
Nitrofurantoin (FM) 300 µg	(23mm) *					*	(21mm) *		
Amoxicillin (AMX) 25 µg			*			*			*
Ciprofloxacin (CP) 5 µg			*		(23mm)*		(28mm)*		
Gentamicin (GM) 10 µg	(18mm)*			(18mm) *			(19mm) *		
Imipenem (IPM) 10 µg			*	(25mm) *				(21mm)*	
Ceftriaxone (CRO) 30 µg			*	(32mm) *					*
Tetracycline (TE) 30 µg			*			*			*
Trimethoprim/ Sulfamethoxazole (SXT) 1.25 /23.75 µg	(20mm) *			* (27mm)			(17mm)*		

coli, *Proteus mirabilis*, and *Klebsiella pneumoniae* that were resistant to several antibiotics. Due to the growing resistance of bacteria to antibiotics, it is essential to find alternative treatments to overcome increasing drug resistance. Zinc oxide nanoparticles show strong antibacterial activity in low concentrations. They are stable in various sever conditions and are considered as a non-toxic compound. Therefore, they can be a good alternative for infection treatment after checking for non-toxicity. In this study, a wet chemical method with inhibitor was used to synthesize zinc oxide nanoparticles. The produced nanoparticles had a crystal size of 15 nm and an optical size of 3 nm. To evaluate the antibacterial properties, pour plate method was also used. The results show that ZnO nanoparticles with a concentration of at least 0.05 g/l have a high inhibitory effect on the growth of multidrug resistant bacteria.

ETHICAL CONSIDERATIONS

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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CONFLICT OF INTEREST STATEMENT

All authors declare that no conflicts of interest exist for the publication of this manuscript.

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