Antimicrobial Activity Zinc Oxide ZnO Nanoparticles Against Biofilm Formation of Uropathogenic E. coli

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***Abstract*— UroPathogenic Escherichia coli (UPEC) are the main reason of urinary tract infections (UTIs) that consider as among the most prevalent pathological states in both community and hospital settings. Twenty isolates of E. coli were isolated from 50 urine samples obtained from different Hospitals at Baghdad, Iraq from patients with UTIs at (40%) isolation percentage. These isolates were diagnosed by biochemical tests, and confirmed by Analytic Profile Index (API 20E) as E. coli isolates. They were detected for the ability they had to biofilm formation by microtiter plate method. Five of them were considered as a biofilm former. These isolates were tested for the effect of ZnO on their biofilm formation pre-mature and mature biofilm by using 4 concentrations (500, 250, 125 and 62.5µg/ml). The results showed that all concentrations of ZnONPs showd antibiofilm activity against pre mature and post mature biofilm. The best effect was recorded against pre mature biofilm.**

***Index Terms*— E. coli, UTI, Uropathogenic E. coli, Biofilm, nanoparticles, ZnO.**

I. INTRODUCTION

This In 1885 Escherichia coli were at first isolated from infants' feces by German pediatrician Theodor Escherich (1857–1911) (1,2). E. coli belong to family Enterobacteriaceae (3). It is a Gram-negative bacterium, non-spore-forming and usually motile by peritrichous flagella (3,4). The genus Escherichia commonly resides in the lower gut of warm-blooded animals, environment, and foods (5,6). It is oxidase-negative, grows using simple carbon sources, citrate-negative, methyl red-positive, Voges–Proskauer-negative and colonies are display a green sheen selective media as eosin methylene blue (EMB) (2).

Urinary tract infections (UTIs) are one of main causes that lead to morbidity affecting 150 million people each year worldwide (7). E. coli is the most frequent cause of acute urinary tract infections, neonatal meningitis, , abscesses in a various organ systems, urinary tract sepsis, acute enteritis in humans and animals, it is a general cause of haemorrhagic colitis often referred to as 'bloody diarrhoea' and commonly linked to a dysentery-like disease affecting humans known as 'traveller's diarrhoea' (8,9). Also it is one of the most frequent hospital-acquired pathogens which can cause abdominal infections and bloodstream infections (BSI) (10). Uropathogenic E. coli (UPEC) is responsible for 80-90% of community- acquired and 30-50% of hospital acquired UTIs. It is the primary cause of UTIs (11,12). A growing threat to global health is the emergence and rapid spread of carbapenem and extended-spectrum cephalosporin resistance in Enterobacteriaceae. Furthermore, the prevalence of antibiotics resistance E. coli is rising, posing a serious threat to human health worldwide (13,14).

Besides being the primary causative agent for recurrent UTIs, the infectivity associated with indwelling medical device-related is also caused by E. coli biofilm (15). It is biofilm made up of a bacterial colony embedded in a matrix of extracellular polymeric substances (EPS). It shields the microorganism from environmental conditions which could otherwise lead to infection and this diversity biofilm's structural components are exacerbated by the emergence of antibiotics resistance, making it more difficult to eradicate (16,17). Biofilms offer a setting that resistance to antibiotic penetration and facilitate the horizontal transfer of virulence genes, thereby promoting the development of Multidrug-resistant organisms (MDRO) (18,19). The capacity of UPEC to form biofilms makes use of various virulence factors determine its ability to invade, grow, ascend and persist in the uroepithelium (20,21).

The need for high doses of antibiotics, which frequently result in intolerable toxicity,is increased due to the increased of resistance to antimicrobial agents. As a result, researchers looked for different approach for treating bacterial infections and developed nanostructures as novel antimicrobial agents (22,23,24). The prices mechanism of actions of nanoparticles are still unclear, but they may be depends on variety of factors including bacterial species, concentrations, surface modifications, compositions, and intrinsic compositions of the particles. Of all of the metal oxide nanoparticles, Zinc oxide nanoparticle (ZnO-NP) were found as the most toxic against E. coli. Its produce reactive oxygen species that kill the bacteria by disrupts membrane integrity (25,26).

The range of cellular target of conventionally antimicrobial agents is limited and their effectiveness against biofilm is also limited, this highlights the necessity for investigated alternative therapies such as nanomaterial for efficient drug delivery to prevent the growth of biofilm (27,28). This study aimed to: isolation and diagnosis of E. coli from patients suffering from UTI, detection the isolates ability to form biofilm by microtiter plate method and detection the ability of ZnO-NP to effect on mature and premature UPEC biofilm.

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II. MATERIALS AND METHODS

A. Specimens Collections and Isolates Diagnosis

Fifteen urine specimens were collected in sterilized containers from three hospitals in Baghdad, Al-Imam Ali hospital, Ibn Al-baladi hospital, and Fatema Al-Zahraa hospital. The samples first have been directly transferred to the lab for examination and diagnosis. All the specimens were cultured onto the MacConkey and EMB agar by streak plate method to observe the colony morphology. The organisms exhibiting the distinctive colonial morphology was of E. coli was repeatedly subculture onto EMB agar, incubated for 24 hours at 37°C in order to obtained a pure culture with homogenous colonies (29). Then, the suspected colonies were transferred to culture on blood agar for hemolysis activity, Triple Sugar iron agar (TSI) and MR-VP for sugar fermentation, Urea agar for urease production, Peptone water for indole test and Simone citrate for citrate utilization test (Himedia/ India) tubes for 24h incubated at 37C(30).

B. Detection of Biofilm Formation

The biofilm formation was bacterial isolates was detecting by microtitter plate method (31). All the obtained bacterial isolatesfrom the previous stage were cultured in Brain Heart Infusion broth (BHI) containing 1% glucose, 96-well polystyrene tissue culture plates was used and then incubated at a temperature of 37C for. Washed 3 times by the DW, and the adhering cells in the wells have been fixed with 200µl of the absolute methanol for 20min; the plates have been emptied and then left overnight to dry. Adhering cells have been stained of with 0.1% crystal violet in a 200µl volume for 15 min, and the excess stain has been descanted and left for drying at room temperature overnight. 200µl of 96% of the ethanol in each one of the wells were used for dye fixation. Plate has been read at 490nm by spectrophotometer. Each isolate was carried out in triplicates and the results have been compared with absorbance of the wells that contain sterile BHI broth as the control.

C. Preparation of ZnO NPs Suspension

The preparation of nanoparticles was done in accordance to (32); After adding 100 ml of the ZnO to 10ml of the sterile DW, thoroughly shake the mixture. The suspension solution was subjected for 30 min of ultra-sound (40 kHz), followed by autoclaved at 121C for 20min and cooling period to room temperature.

D. Detecting Anti-biofilm Activities of ZnO Against Bacterial Isolates

The previously mentioned procedure in biofilm formation was followed but with various concentration levels of the ZnO (500, 250, 125 and 62.5µg/ml). NPs which have been added with the bacterial suspension to the wells, as triplicate for every value of the concentration, and then the plate was incubated for 24hr at 37C, after the period of incubation, and have been washed, stained, and read OD at 490nm.

E. Statistical Analysis

The program SAS (2018) (33) was utilize to ascertain the impact of variant factors in the study parameter. In this study a significant comparison between means was made using the Least significant difference –LSD test (Analysis of Variation-ANOVA).

III. RESULTS

A. Bacterial Isolates

Twenty E. coli isolates from 50 urine specimens were identified by using culture media and biochemical test as shown in the table (1) in (40%) isolation percentage. E.coli colonies on MacConkey agar showed up a tiny, pink colonies (Lactose fermentor), but colonies on EMB agar showed up a green metallic sheen. Colonies that tested positive for Lactose- and indole and negative for citrate utilization test were presumptively identified as E.coli.

Table (1): Results of biochemical test of E. coli isolates

No.	Biochemical test and media	Result
1	MacConkey agar	Small, smooth pink colonies
2	Eosin Methylene Blue (EMB)	Green metallic Sheen
3	Triple sugar iron agar (TSI)	A/A +(gas) –(no H_2S)
4	Urease test	-ve
5	Indole test	+ve (red ring)
6	Methyle Red Voges Proskauer test (MRVP)	+ for MR, -ve for VP
7	Simmone citrate Utilization test	-ve (green color)

B. Biofilm Assay

The results showed that all isolates were biofilm producers. Fourteen isolates were produced moderate biofilm in percentage (70%), while other six isolates were moderate biofilm producers in percentage (25%). As Demonstrated in table (2) and figure (1).

Table (2): The OD value and biofilm production result of E coli isolates.

Isolate no.	OD1	OD2	OD3	Average	ODC	2*ODC	4*ODC	Type of biofilm
E 66	0.218	0.228	0.209	0.218	0.061	0.122	0.244	Moderate
E 96	0.243	0.266	0.25	0.253	0.061	0.122	0.244	Strong
E 75	0.248	0.218	0.221	0.229	0.061	0.122	0.244	moderate
E 89	0.237	0.159	0.168	0.188	0.061	0.122	0.244	moderate
E 51	0.233	0.179	0.17	0.194	0.061	0.122	0.244	moderate
E 90	0.233	0.195	0.212	0.213	0.061	0.122	0.244	moderate
E 103	0.245	0.221	0.16	0.208	0.061	0.122	0.244	moderate
E 55	0.231	0.169	0.187	0.195	0.061	0.122	0.244	moderate
E 54	0.252	0.217	0.245	0.238	0.061	0.122	0.244	moderate
E 1	0.335	0.206	0.236	0.259	0.061	0.122	0.244	Strong
E 72	0.206	0.166	0.191	0.187	0.061	0.122	0.244	Moderate
E 79	0.233	0.262	0.241	0.245	0.061	0.122	0.244	strong
E 91	0.18	0.167	0.161	0.169	0.061	0.122	0.244	Moderate
E 52	0.223	0.226	0.18	0.213	0.061	0.122	0.244	Moderate
E 2	0.194	0.244	0.21	0.216	0.061	0.122	0.244	Moderate
E 92	0.215	0.261	0.303	0.259	0.061	0.122	0.244	Strong
E 80	0.283	0.291	0.29	0.288	0.061	0.122	0.244	Strong
E 65	0.228	0.27	0.281	0.259	0.061	0.122	0.244	Strong
E 57	0.249	0.217	0.227	0.231	0.061	0.122	0.244	Moderate
E 71	0.207	0.223	0.271	0.233	0.061	0.122	0.244	Moderate

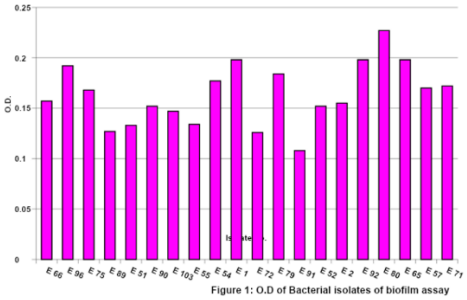

Figure 1: O.D of Bacterial isolates of biofilm assay

C. Effect of ZnO NPs Against Biofilm

Five E. coli isolates that gave highest OD value of biofilm production were selected from the 20 E. coli isolates (E92, E79, E80, E65 and E1) to show the effect of ZnONPs against pre-

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mature and mature biofilm as listed in table (3), figure (2) and table (4), figure (3) respectively. The results showed that all concentrations of ZnONPs gave antibiofilm activity as premature biofilm against E.coli with different degrees incompare with control, table (3).

Table (3): The effect of Zno NPs against pre-mature biofilm

Bacterial isolates	ZnO NPs concentrations (µg/ml)					LSD (P-value)
	Control	500	250	125	62.5	
E 92	0.259	0.061	0.055	0.054	0.056	0.044 * (0.046)
E 79	0.245	0.057	0.059	0.069	0.067	0.051 (0.039)
E 80	0.288	0.060	0.057	0.057	0.083	0.059 * (0.033)
E 65	0.259	0.066	0.058	0.055	0.062	0.051 * (0.038)
E 1	0.259	0.061	0.056	0.058	0.058	0.048 * (0.042)
LSD (P-value)	0.137 NS (0.874)	0.029 NS (0.891)	0.0252 NS (0.877)	0.0294 NS (0.763)	0.0257 NS (0.726)	---
* (P ≤0.05), NS: Non- Significant.						

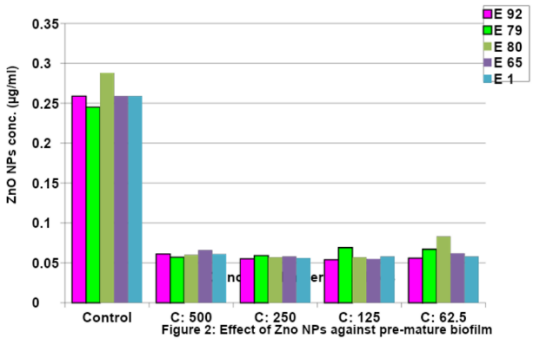

Figure 2: Effect of Zno NPs against pre-mature biofilm

On the other hand, the current findings revealed that ZnO NPs were affected on mature biofilm of E.coli. The higher effect was achieved against bacterial isolates E79 and E65 by all concentrations, while there was no effect against bacterial isolates E92 and E1, table (4) and figure (3).

Table (4): The effect of ZnO NPs against mature biofilm

Bacterial isolates	ZnO NPs concentrations (µg/ml)					LSD (P-value)
	Control	500	250	125	62.5	
E 92	0.0684	0.108	0.26	0.40	0.117	0.317 * (0.049)
E 79	1.75	0.145	0.380	0.356	0.11	0.521 ** (0.0057)
E 80	0.288	0.088	0.301	0.191	0.101	0.283 NS (0.295)
E 65	1.121	0.147	0.296	0.104	0.096	0.569 ** (0.0006)
E 1	0.259	0.320	0.222	0.117	0.112	0.291 NS (0.602)
LSD (P-value)	0.549 ** (0.0078)	0.307 NS (0.118)	0.211 NS (0.703)	0.359 NS (0.065	0.087 NS (0.794)	---
* (P ≤0.05), ** (P ≤0.01), NS: Non- Significant.						

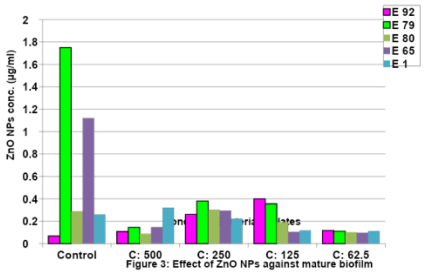

Figure 3: Effect of ZnO NPs against mature biofilm

IV. DISCUSSION

UPEC are a causative agent in the great majority of UTIs, pyelonephritis, cystitis as well as infectious complications that can cause acute renal failure in both renal transplant patients and healthy individuals (34). UPEC expresses a wide range of virulence factors to overcome the mucosal barrier's inertia (35). When the UPEC breaches normally sterile urinary tract, the host's inflammatory responses are set off, which result in the production of cytokine, the infiltration of neutrophils and the exfoliation of infected bladder epithelial cells (36).

When compare to other lactose fermenter bacteria, E. coli grow more readily on EMB agar which enhanced its proliferation. Large amount of lactose caused E. coli to produce extremely dark, metallic shiny colonies devoid of Klebsiella and Enterobacter (37). This could be because pink colonies didn't form as much acid accumulated (38,39). Recurrent and complicated UTIs are frequently caused by bacteria that form biofilm (40,41). Twenty off fifty urine specimen (or 40% from the total) were diagnosis as E. coli. Because it is the primary causative pathogen of approximately 80% of recurrent UTI in women, E. coli responsible for these infections (42). Ninety percent of UTIs caused by UPEC, which is colonize the faeces or perineal region and travel up the urinary tract to the bladder (43). Adhesins or fimbriae, siderophore systems, and toxins are among the distinct virulence factors that distinguish UPEC strains and are closely related with the colonization and persistence of bacteria in the urinary tract (44).

In all 407 urine samples that were taken from patients at the University of Benin Teaching Hospital (UBTH), Benin, Edo State, Nigeria, who were either inpatient or outpatient and had suspected UTI. Male and female yield 162 (39.8%) and 245 (60.2%) E. coli isolates respectively (45). A total of 76 urine specimens were taken from of a patients who were experienced recurrent UTIs. After specimens underwent bacteriologically analysis, E. coli a counted for fifty (65.8%) of isolated bacterial strains. Using the microtiter plate assay, 100% of isolated UPEC strains showed a biofilm positive phenotype under optimal condition, 29 (58%) of these isolates categorized as weakly positive biofilm producers, and 21 (42%) categorized as highly positive biofilm producers (21).

Antibacterial activity ZnO-NP against E. coli O157:H7was discovered. As the ZnO-NP concentration rises the inhibitory effects also rise. The finding suggests that ZnO-NP may cause bacterial cell wall distortion and damage and this could lead to intracellular contents leaking out and ultimately the death of the bacterial (46). Depending on the nanoparticles concentrations, ZnO-NP exhibiting inhibitory effects on biofilm formation in UPEC isolates; these effects were more pronounced at MIC concentration than sub MIC concentrations dramatically reduced flu gene in UPEC isolates with strong biofilm but it is unable to prevent biofilm formation (11).

According to the researches by Applerot et al (47) and Musarrat et al (48), ZnO-NP can dramatically reduce the amount of biofilm that form by E. coli strains. In their investigation, ZnO-NP had a stronger inhibitory effect than in one carried out by (11). This discrepancy results from the various approaches taken in the assessment of biofilm formation. Furthermore, size of nanoparticles and bacterial type are significant variables that could influence the nanoparticle's inhibitory effect. Smaller

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particles have a stronger antibacterial effect because they have a larger surface area to volume ratio. Additionally, ZnO-NP inhibits P. aeruginosa from form biofilm and this may be useful in treatment of biofilm (49). The most likely mechanism for the bacterial cell ability to fight biofilms is that the nanoparticles treatment altered the permeability of their cell membranes, allowing nucleic acids to leak out of the cells (50).

CONCLUSION

From the previous results it was concluded that ZnO-NPs showed high activity against E.coli biofilm in both pre and post mature stage but the best effect was achieved against pre-mature one.

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