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RESEARCH PAPER

Real-Time PCR Analysis of Virulence Genes Inhibition in Uro-pathogenic *E. Coli* by Metal Nanoparticles, Phytol, and Cefixime

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ABSTRACT

This study was aimed to find out the inhibitory effect of phytol, metal nanoparticles (Zirconium-NPs, Zinc-NPs, and Cerium-NPs) and cefixime on the virulence genes of most important causative agent of urinary tract infections (UTI) bacteria, Uro-pathogenic *Escherichia coli* (UPEC). The Real Time PCR was then used to analyze the virulence genes *fimH, sfa, cnf1, bolA, csgD, and pgaC*. These genes were overall down regulated by all treatments, particularly when treating with Phytol + Cefixime. Here, we employed this combinatorial approach and found the most down regulated gene as the *fimH* gene, an essential gene for UPEC adhesion to host tissues, to have a fold value of 11.11. Maximal down regulation of *cnf1 gene* involved in tissue damage was noted in Phytol+ Cefixime (fold value 333.33) illustrating less cytotoxic drug. Similarly, ZrNPs and ZnNPs had strong inhibition of *fimH* fold value = 5.95 (ZrNPs), *sfa* and *pgaC* genes. Differential effect in down regulating the *bolA* gene (biofilm formation gene) was observed most of the treatments except treatments of Phytol+ CeNPs and Cefixime+ ZnNPs. Moreover, Curli production and biofilm integrity were significantly reduced in CsgD, critical for curli and biofilm formation, by a fold value of 2.19 when it was subjected to Cefixime + ZnNPs treatment. For multi drug resistant infections, these medicines combined may be useful in improving UTI treatment, all surfaces characterized by using advanced techniques such as XRD and FESEM.

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INTRODUCTION

Urinary tract infections (UTIs) are a serious health problem, but they are a frequent bacterial infection. The infections are caused by species of *E. coli, K. pneumoniae, P. mirabilis, E. faecalis and S. saprophyticus. UPEC (Uropathogenic E. coli)* is the most common cause of both the complicated and uncomplicated UTI. UPEC has adapted to intracellular life within the urothelium and to colonization by modification to the basis of colonization, adhesion, invasion and intracellular replication [1]. Mandatory expression of some of these UPEC strains with severe or recurrent UTI such as Type1 fimbriae (*fimH), S fimbriae (sfa),* cytotoxic necrotizing factor (*cnf1), bolA, pgaC and csgD* are some of these virulence genes encoded.

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To colonize, or evade, circumvent or even invade and initiate an unpleasant inflammatory response, they assist the organism in establishing a residency on the host's surface or overcome a defensive response of the host [2,3].

The development of therapeutic alternatives for biofilm-associated UTIs will be aided by knowledge of the biofilm formation and antimicrobial resistance determinants of Uro-pathogenic *E. coli* strains, Fimbria adhesions, curli, and non-fimbria surface adhesions are expressed on the cell surface to aid in colonization and persistence through UPEC and host cell interactions, facilitating intra-cellular invasion as well as cell aggregation and biofilm formation. The expression of these surface proteins is regulated by phase variation [4]. The development of biofilms is really closely linked to heightened resistance to antibiotic treatment. In fact, infections caused by biofilms present a serious problem in clinical settings. Thus, Improving the effectiveness of therapies may be accomplished by creating sophisticated drug delivery systems that can efficiently target and penetrate biofilms [5]. One of the most important issues facing world health in the twenty-first century is antibacterial resistance. It happens when bacteria, change and become resistant to medications meant to eradicate them, making cures useless and diseases enduring. Known as "superbugs," this phenomenon is rapidly spreading and poses a serious risk to modern medicine, human life, and the security of the global health system [6]. Phytol (3,7,11,15-tetramethylhexadec-2-en-1-ol) is diterpene, which comes from long-chain unsaturated acyclic alcohols, has a variety of biological effects, such as antibacterial and anti-inflammatory properties. phytol is found in essential oils and has lipophilic capability to cross cell membrane, it is hypothesized to possess antibacterial activity and drug-enhancing potential [7,8]. Organic nanoparticles (NPs) are made of organic substances like lipids or polymers and have shown remarkable promise in medication delivery. They are therefore more suited for biological applications because of their composition, and their significance in the pharmaceutical and nanomedicine industries is growing daily [9]. The goals of nanotechnological advancements are to: (a) enhance the drug's pharmacokinetic and pharmacodynamic characteristics generally without altering its molecular structure; (b) deliver the drug precisely to the target structure; (c) ensure that biological barriers can be avoided; and (d) make production simple and straightforward [10,11]. The development and evaluation of innovative therapeutic approaches, such as phytol, and multi-nanoparticles such as Zirconium Oxide (ZrO), Zinc oxide (ZnO), and Cerium oxide (CeO_2) are required to address the resistance mechanisms of biofilm formation and virulence genes in Uro-pathogenic *E. coli* isolates from patients with UTIs. The present study aimed to assess experimental of novel strategies that depend on using phytol and multi-nanoparticles (ZrO, ZnO, CeO) alone and combined between them and with cefixime antibiotic, as typically abundant that used in UTI treatment. Assessment of quantified changes in virulence gene expression of UPEC by Real-time PCR.

MATERIALS AND METHODS

Study design and bacterial identification

This study was approved by the Research Ethics Committee (204-1223-2024) at the University of Anbar, College of Medicine/Microbiology department in Anbar governorate-Ramadi city, Iraq. One hundred twenty-five samples collected during the period from 1st November 2024 to15th January 2025 from pathogenic bacteria isolates were obtained from urine samples collected from patients who were referred to Ramadi Teaching Hospital. Inclusion criteria included all age group patients (above one month) from both genders who were already not antibiotic treatment and those who were diagnosed medically with UTI, and the doctors sent them to do urine culture in the laboratory. The patients currently taking antibiotics were not included in the study. Then, the 48 isolates of *E. coli* were identified using standard bacteriological and biochemical tests, then confirmed diagnosis by Vitek 2 System. The pure colonies were preserved, and the sub-culture of these isolates was performed according to the guidelines of microorganism care and the Research Committee of the Ministry of Health (Ethics committee approval code: 2021/2). One colony of the isolated bacteria at 37 C° was combined with a plate of nutrient agar (HIMEDIA/India) in order to preserve the bacteria long enough to keep their genetic characteristics. Five milliliters of BHI broth were then added to this combination, and a single colony supplemented with 15% sterile glycerol was added. Finally, for 3-6 months, the slants were kept at -20 C° [12].

Antimicrobial Susceptibility Testing

Using fifteen antibiotic disks from (HIMEDIA Limited, India) included Nitrofurantoin, Gemifloxacin, Amikacin, Levofloxacin, Gatifloxacin, Cefazolin, Ceftriaxone, Trimethoprim, Ciprofloxacin, Gentamicin, Minocycline, Fosfomycin, Nalidixic acid, Meropenem and Cefixime. Method according to the guidelines of Clinical and Laboratory Standards Institute 35th Edition (CLSI), 2025 [13]. Those isolates were considered as Multi-Drug Resistant (MDR) if they were resistant to at least one agent of ≥ 3 classes of antibiotics [14].

Nanoparticles, Phytol, and cefixime solution

All nanoparticles (ZrO_2, ZnO, CeO_2) are imported from *Nanoshel Company*, USA, as a powder (Purity: 99.9%, APS: 20nm, Purity: 99.5%, APS: 10-30nm, Purity: 99.99%, APS: 30nm) respectively. Nanoparticles were confirmed by SEM examination and XRD Fig. 1. Phytol (Natural Products), 97% (GC), CAS Number: 7541-49-3 MF: $C_{20}H_{40}O$ with MW: 296.5, solution imported from AK Scientific Inc, Union City, California, USA. Cefixime is used as a powder from Pharma International, Jordan.

Bacterial Culture and Treatment

Antimicrobial test and minimum inhibitory concentration (MIC) determination

At this stage, 96-well plates were created with 100 µL serial concentrations (3.9, 7.8, 15.625, 31.25, 62.5, 125, 250, and 500 mg/mL) of phytol, ZrO_2, ZnO, and CeO_2 at concentrations of (3.9, 7.8, 15.625, 31.25, 62.5, 125, 250, and 500 µg/mL) and Cefixime (8, 16, 32, 64, 128, 256, 512,1024) µg/mL). 100 µL of brain heart infusion broth with the same concentration of the investigated substances was added to each well. Then, using the following estimates, calculate the predicted bacterial cell density (number of CFU of *E. coli*): McFarland (0.5) = 1.5×10^8 were incubated at 37 °C. Following incubation, 30 µL of resazurin 0.15 mg/ml was added to each well. The wells were then re-incubated for 24 hours before the findings were reported. Blank or negative controls were the same liquid medium that contained the same quantity of the analyzed substances but was free of microorganisms. Bacterial cultures that were not subjected to any treatments served as the positive control. A shift in color from blue to pink signified

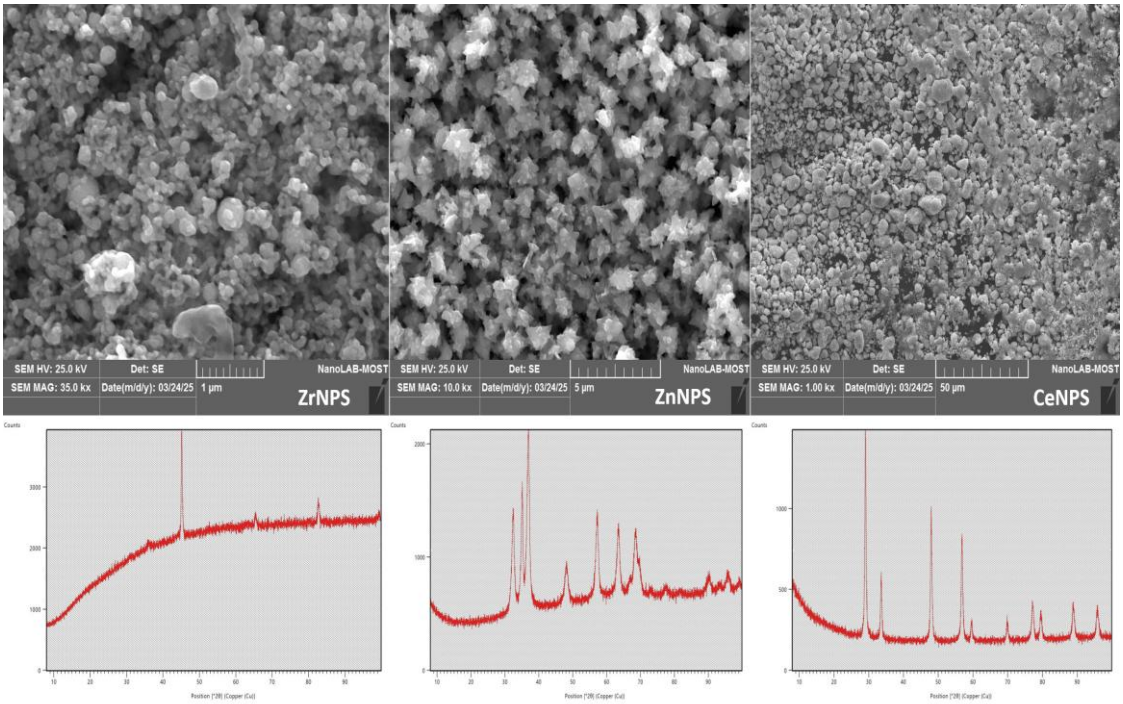

Fig. 1. SEM and XRD images of the nanoparticles. A- ZrNPs, B- ZnNPs, C- CeNPs

Table 1. UPEC treated with sub-MIC.

No.	Treatments	No.	Treatments
1	Phytol-treated	9	Phytol + CeNPs
2	Cefixime-treated	10	Cefixime + ZrNPs
3	ZrNPs-treated	11	Cefixime + ZnNPs
4	ZnNPs-treated	12	Cefixime + CeNPs
5	CeNPs-treated	13	ZrNPs + ZnNPs
6	Phytol + cefixime	14	ZrNPs + CeNPs
7	Phytol + ZrNPs	15	ZnNPs + CeNPs
8	Phytol + ZnNPs	16	Control (untreated)

Table 2. Primer sequence for RT-PCR analysis.

Virulence factor	Genes		Oligonucleotide sequence (5′-3′)	Amplicon size (bp)	Accession No.
Housekeeping	*16srRNA*	F	AGTCTGCAACTCGACTCCATG	122	MT649856.1
		R	CTTTTGCAACCCACTCCCATG		
Type 1 fimbriae D-mannose specific adhesin	*fimH*	F	ATCCATTTCCTACCACCAGCG	143	CP055259.1
		R	AAGCACGGCAATTAATGAGCC		
Biofilm adhesin polysaccharide	*pgaC*	F	TTGAGGAAACCATACACGCCG	125	CP054224.1
		R	ATGGGGAATTTGTGCAGCCAT		
Synthesis of curly fimbriae and cellulose	*csgD*	F	TTGCCAGCTACCTGATTACGC	108	CP054556.1
		R	CGCCGATACGCAGCTTATTCA		
Membrane permeability, motility, cell morphology and biofilm development	*Bola*	F	TATCGTCACAATGTCCCAGCC	188	CP054232.1
		R	TGCAACCCTTCCCACTCCTTA		
Cytotoxic necrotizing factor 1	*cnf1*	F	CTGCTATACCTGGTTTGGCGA	107	CP054236.1
		R	AACGCTGCTAAGTACCTCCTG		
S fimbrial adhesins	*Sfa*	F	CGCCGAGCTGAACAAGAATAC	83	CP054236.1
		R	AACTTTCAAGCCCGGTCATTTT		

a decrease in resazurin and, thus, bacterial proliferation. Every experiment was carried out three times [15]. As previously reported [16,17], the microdilution chequerboard plates were made in triplicate but with certain modifications to the *E. coli* strains and by using the interaction between the two treatments.

Use of resazurin in the microdilution checkerboard assays

In the microdilution chequerboard experiments, resazurin was applied to each bacterial microtiter plate following a 24-hour incubation period. When color change was then observed 48 hours later. Based on the best outcomes from the MIC testing, this dye contact time was chosen. The chequerboard the formula below was used to determine the fractional inhibitory concentration index or FICI: FICI is equal to (MICBcombi/MICBalone) + (MICAcombi/MICAalone). A FICI of less than 0.5 indicated synergistic effect, a FICI of greater than 0.5 but less than 4.0 indicated no interaction, and a FICI of greater than 4.0 indicated antagonism [18]. Determine sub-inhibitory concentrations (sub-MIC) of each treatment to avoid bactericidal effects. Treatments were divided into sixteen experimental groups in Table 1.

RNA Extraction & cDNA Synthesis

Harvest bacterial cells after 12 hrs. from treatment, then extract total RNA using TRIzol reagent. Quantify RNA concentration and purity by using Thermo-Scientific Nano-Drop 8000 Microvolume UV-Vis Spectrophotometer. Reverse

transcriptase RNA into cDNA using *easy script* one step gDNA removal and cDNA synthesis super mix kit (Transgene-China).

Real-Time PCR

Design and validate primers for target genes (*fimH*, *pgaC*, *csgD*, *bolA*, *cnf1*, *sfa*) and *16srRNA* as a housekeeping gene (Reference gene) Table 2.

Perform Real-Time PCR using SYBR Green and analyze gene expression by using the Livak equation $2^{\wedge(-\Delta\Delta Ct)}$ method [19].

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 10.5.0 and Microsoft Excel v 2016.

RESULTS AND DISCUSSION

Collection and identification

Among 125 patients, 73.6% was female (N=92) while male was 26.4% (N=33). There was a statistically significant difference ($p < 0.05$) favored female, the results showed 48 isolates were found to be Uro-pathogenic *Escherichia coli* (UPEC). The microscopical features are Gram-negative, straight, bacilli-shaped, non-spore-forming, cultural features slightly convex, moist, lactose ferment on MacConkey agar that showed pink colonies, while on Eosin methylene blue (EMB) greenish metallic sheen colonies and HiCrome™ UTI Agar colonies appear Purple to magenta color Fig. 2. Biochemical characterization Catalase, indole, methyl red, lactose, and glucose ferment are positive, but oxidase, Voges-Proskauer, and citrate utilization are negative. Triple sugar iron (TSI) Yellow/Yellow (A/A), Gas +ve, H_2S – ve [20,21].

The Antibiotic Susceptibility Testing

In this study, antibiotics were used to establish a resistant pattern of *E. coli* to several broad-spectrum antibiotics that are associated with a high risk of UPEC infection. The result showed in Table 3 all isolate's resistance to Cefixime was around (100%), and approximately (86%), (82%), (and 82%) of them were highly resistant to Cefazoline, Ceftriaxone, and Nalidixic acid, respectively. Furthermore, around (78%) of isolated *E. coli* were resistant to Trimethoprim and (74 %) to Ciprofloxacin and Gemifloxacin. In addition, around (70%), (62%), (60%), (44%), (26%) of this *E. coli* were resistant to Levofloxacin, Gatifloxacin, Minocycline, Gentamicin, and Amikacin respectively. In contrast, over (80%) of *E. coli* were highly susceptible to Meropenem and (68%) to Fosfomycin and Nitrofurantoin.

Antimicrobial effect of Nanoparticles, Phytol and Cefixime

The results showed minimum inhibitory concentration MICs of (CrNPs, ZnNPs, CeNPs, and cefixime) at 125, 62.5, 125, and 512 µg/mL could inhibit the growth of the clinical isolates of *E. coli* respectively. However, a MIC of Phytol 250 mg/mL was necessary to kill the clinical isolates, whereases checkerboard results reveal in (Table 4) the MIC of cefixime decreased to 32 when mixed with ZrNPs or ZnNPs and 64 when mixed with CeNPS or Phytol while the MIC of cefixime 512 µg/mL used alone. This study showed the best synergistic action between ZnNPs and cefixime by calculating the value of Fractional inhibitory concentration FIC index equal to 0.187 Where it appeared that the value of FIC is less than 0.5

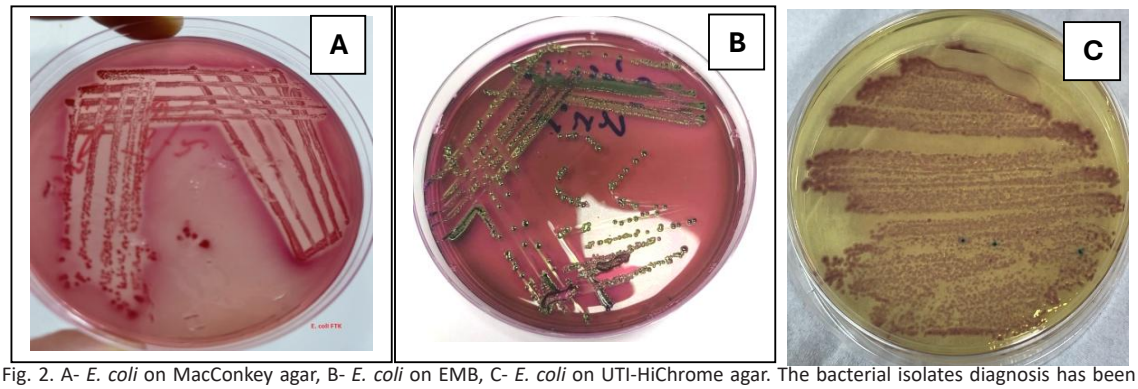

Fig. 2. A- *E. coli* on MacConkey agar, B- *E. coli* on EMB, C- *E. coli* on UTI-HiChrome agar. The bacterial isolates diagnosis has been confirmed using the VITEK2 system.

(0.187 and 0.375) for *E. coli* and this indicates the synergistic action between these treatments, as shown in (Table 4).

Effect of Treatments on Virulence Gene Expression
Extraction of RNA

Ribosomal Nucleic Acid (RNA) was extracted from MDR-UPEC isolates before and after treatment with Sub-MIC of different treatments (Table 1), alone and in combination. All samples of RNA were extracted with a TRIzol™ Reagent purification kit. The concentration of RNA ranged between (122 – 135) ng/µl.

Real Time PCR (qPCR)

A Two-step RT-PCR process was used to detect the effect of sub – MIC of ZrNPs, ZnNPs, CeNPs, Phytol and cefixime alone and combined with on gene expression levels (fold changes) *of fimH, sfa, cnf1, bolA, csgD* and *pgaC* compared to housekeeping gene *(16srRNA*) at the levels of nps, nmol graphene, nmol graphene oxide and nmol semiconductor NPs, the amounts by which they influence gene expression were quantitated by SYBR green (Fig. 3). The results of this investigation showed down regulation of the gene expression levels when treated with all treatments or in

Table 3. Antimicrobial susceptibility pattern of isolated UPEC based on the Clinical and Laboratory Standards Institute (CLSI) guidelines (2025).

Antibiotic (Disk Concentration)	R %	I %	S %
	Aminoglycosides		
Amikacin (AK;10 µg)	26	4	70
Gentamicin (GEN;10 µg)	44	28	28
	Cephalosporin		
Cefazolin (CFZ;30 µg)	86	2	12
Ceftriaxone (CTR;30 µg)	82	8	10
Cefixime (CFX;15 µg)	100	0	0
	Fluoroquinolones		
Ciprofloxacin (CIP;5µg)	74	2	24
Levofloxacin (LEV; 5µg)	70	4	26
Gatifloxacin (GAT; 5µg)	62	18	20
Gemifloxacin (GEM; 5µg)	74	8	18
	Quinolones		
Nalidixic acid (NA; 30µg)	82	6	12
	Tetracyclines		
Minocycline (MI; 30µg)	60	12	28
	Phosphonic acid derivatives		
Fosfomycin (FO; 200µg)	4	28	68
	Carbapenems		
Meropenem (MEM; 10µg)	12	8	80
	Nitrofurans		
Nitrofurantoin (NIT; 100µg)	16	16	68
	Antifolate		
Trimethoprim (TR; 5µg)	78	8	14

all combinations as compared with untreated bacteria (control), usually those expression equal to one. Type 1 fimbriae are encoded by the *fimH* gene and are important virulence factors in uro-pathogenic E. coli strains, as they play an important role in UPEC adhesion to host tissues. We observed all treatments down regulated this gene, with the greatest result in Phytol+ Cefixime (fold value = 11.11). Dealing with the UPEC is dealt with and these treatments may inhibit the cells' ability to adhere to the urothelial cells, so that the problem is less severe. ZrNPs and Cefixime+ ZnNPs also caused considerable effects on fimH down regulation with fold values of 5.95 and 1.28 respectively, demonstrating an involvement in the blocking of UPEC attachment mechanisms. Thus,S- fimbrial adhesins encoded by the sfa gene are essential for UPEC adhesion to host cells. Inhibition of the cells' ability to adhere to the urinary tract lining is suggested by all treatments (including combinations such as Phytol + Cefixime (fold value = 12.35) and ZnNPs (fold value = 1.46) and ZrNPs (fold value = 12.82)) resulting in down regulation. Different to Cefixime and Phytol alone, Cefixime + ZnNPs (fold value = 3.10) and Phytol + ZnNPs (fold value = 7.04) show moderate effects on UPEC adhesion and virulence suggesting that some combinations may exert better effects in this regard. The cytotoxic necrotizing factor 1 encoded by the cnf1 increases virulence and damages host tissues. As shown in Fig. 3, this gene was all down regulated but significantly down regulated, except for Phytol+ Cefixime combination which had 333.33-fold value. Therefore, phytol-based treatments and antibiotics teamed together could possibly improve UPEC cytotoxicity, possibly by reducing the local damage in case of an infection. Moreover, Cefixime + ZnNPs (fold value = 111.11) and Cefixime + ZrNPs (fold value = 62.5) other combinations had significant effect on *cnf1* down regulation suggesting a role of nanoparticles in reducing virulence factor. It also genes a part to play in stress responses and biofilm formation. Diverse treatments, except for Phytol+ CeNPs and Cefixime+ ZnNPs, displayed down regulation in genes expression, and the Phytol+ CeNPs showed up regulation (fold value = 1.375). Under certain conditions, phytol-based treatments in combination with antibiotics may produce a differential biofilm formation effect.

The biofilm formation shows strong down regulation for Phytol (fold value = 27.03), Cefixime (fold value = 12.66), ZrNPs (fold value = 11.36) Fig. 3 and hence these treatments can reduce the expression of biofilm forming genes in UPEC. Curli fibers, which are involved in the formation of biofilm and UPEC virulence, are synthesized by the *csgD*. These gene were down regulated most of treatment and the strongest effects on down regulation of this gene was in Phytol (fold value = 1.74) and Cefixime+ ZnNPs (fold value = 2.19). Both down regulation of *csgD* through all of the treatments indicates that these treatments have the ability to disrupt curli production thus diminishing UPEC ability to form biofilms and overall virulence. Cefixime along with ZnNPs could also result in significant down regulation of biofilm formation and adhesion and thus may be working synergistically to reduce biofilm formation and the adhesion. *pgaC* gene encodes for polysaccharide

Table 4. synergism between difference treatment against *E. coli*

MIC alone (A)		MIC-Combination (A)		MIC alone (B)		MIC-Combination (B)		FIC (A)	FIC (B)	FICI	Conclusion
Phytol	250	Phytol	62.5	CFX	512	CFX	64	0.25	0.125	0.375	Synergism
Phytol	250	Phytol	15.625	ZrNPs	125	ZrNPs	31.25	0.0625	0.25	0.3125	Synergism
Phytol	250	Phytol	15.625	ZnNPs	62.5	ZnNPs	15.625	0.0625	0.25	0.3125	Synergism
Phytol	250	Phytol	125	CeNPs	125	CeNPs	31.25	0.5	0.25	0.75	Additive
ZrNPs	125	ZrNPs	31.25	CFX	512	CFX	32	0.25	0.0625	0.3125	Synergism
ZnNPs	62.5	ZnNPs	7.812	CFX	512	CFX	32	0.12499	0.0625	0.187492	Synergism
CeNPs	125	CeNPs	62.5	CFX	512	CFX	64	0.5	0.125	0.625	Additive
ZrNPs	125	ZrNPs	31.25	ZnNPs	62.5	ZnNPs	7.812	0.25	0.125	0.375	Synergism
ZrNPs	125	ZrNPs	15.625	CeNPs	125	CeNPs	62.5	0.125	0.5	0.625	Additive
ZnNPs	62.5	ZnNPs	7.812	CeNPs	125	CeNPs	62.5	0.12499	0.5	0.624992	Additive

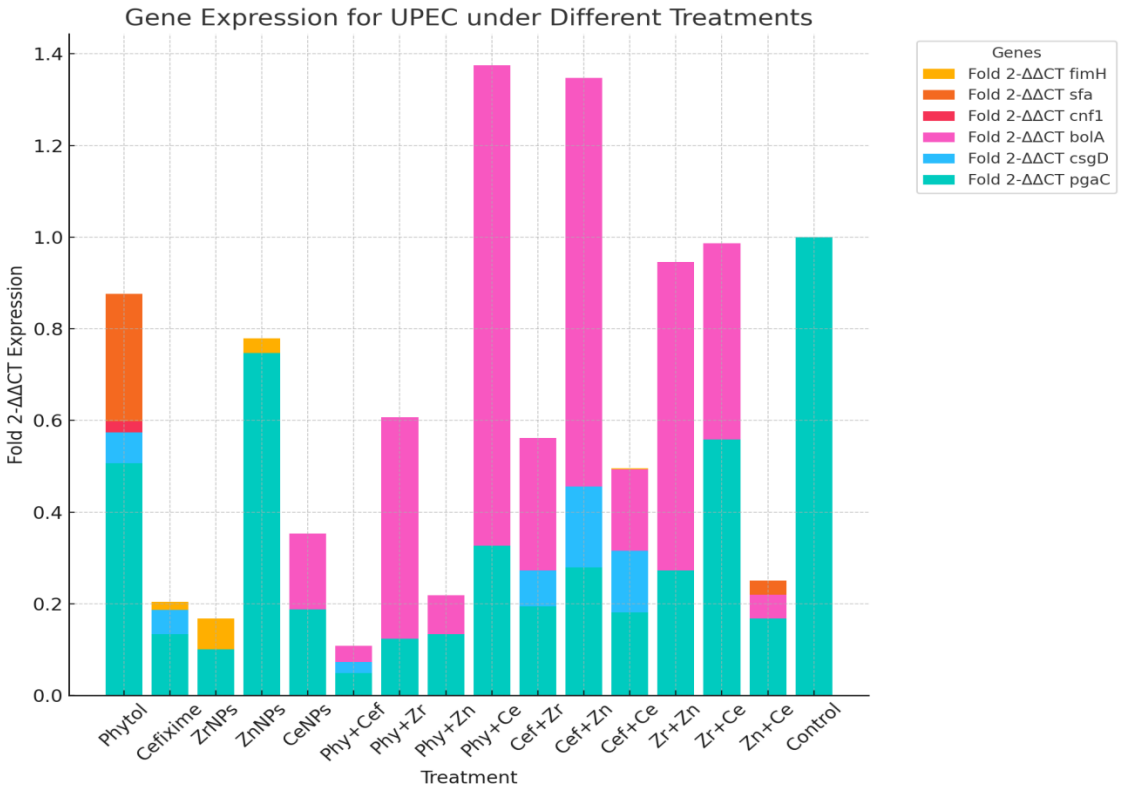

Fig. 3. A gene expression (fold $2^{-\Delta\Delta CT}$) represented for Uro-pathogenic *E. coli* (UPEC) under different treatments and for the expression levels of *fimH*, *sfa*, *cnf1*, *bolA*, *csgD*, and *pgaC* genes. It is apparent from the chart that most treatments had down-regulation on the gene expression.

synthesis of biofilm matrix. Phytol (fold value = 1.98), Cefixime (fold value = 7.52) and ZrNPs (fold value = 10) treatments all down regulated the biofilm formation as evidenced by decreased film matrix and thus advance the use of antibiotics for treating the biofilm associated UTI. The strongest down regulation for the combination of Phytol+ Cefixime (fold value = 20.83) indicates that the phytol and cefixime combined are highly effective in disrupting biofilm integrity when used together.

The increase in MDR strains of Uro-pathogenic *Escherichia coli* (UPEC) pose more difficulties in treating urinary tract infections (UTIs). In order to examine the effects of phytol, cefixime, as well as metal nanoparticles (CrNPs, ZnNPs, and CeNPs) on the expression of UPEC virulence genes, this study was conducted. They found that the synergy it promoted, and the downregulation of some important virulence components. We summarize these findings below in more detail with clinical consequences combined with insight from the molecule. The *fimH* gene encodes type 1 fimbriae, a very important adhesin which allows UPEC to adhere to the lining of the host's urinary tract. This study findings demonstrated that fimH was down regulated by all treatments, Phytol + Cefixime and its fold value was most (11.11). This finding is consistent with a well-accepted theory in the literature that *fimH* contributes to UPEC bihost capacity to attach to urothelial cells, binding to urothelial mannosylated receptors [22]. Probably, down-regulation of this gene will be able to affect the bacteria's capability of sticking, which is the very first stage for infection process. This fit with recent research that indicates that the hook as a potent phytochemical can inhibit bacterial adhesion and prevent the formation of biofilms [23]. It has been suggested that phytol may disrupt *fim* operon through interference with the regulatron of the transcription factor fimB [24]. Similar effect on down regulation of *fimH* was observed for ZnNPs and ZrNPs including moderate impact at fold value of 1.28 for ZnNPs + Cefixime. This agrees with the idea that nanoparticles may

increase the antimicrobial efficacy of antibiotics through increased delivery into bacterial membranes and biofilms [25]. The *sfa* gene encodes S fimbrial adhesins, another essential element in bacterial adhesion. The treatment of proteins utilizing ZrNPs and Phytol + Cefixime had their effect on all proteins tested, with the most apparent manifested in ZrNPs (fold value = 12.82) and Phytol + Cefixime (fold value = 12.35). Inhibiting S fimbriae can considerably reduce the pathogenicity of UPEC, as they are required for UPEC to adhere to the host's epithelial cells [26]. Cefixime + ZnNPs and Phytol + ZnNPs down regulated moderately, however, with *sfa*, which is curious, since that would imply that specific antibiotic and nanoparticle combinations have stronger impacts on UPEC adherence. These results provide support for the notion that combining nanoparticles with conventional antibiotics may lead to synergistic effects with better treatment outcomes. [27]. The *cnf1* gene encodes cytotoxic necrotizing factor 1, a major toxin responsible for UPEC ability to cause tissue damage and promote inflammation, and *cnf1* induces host cell Rho GTPase activation, leading to inflammation and tissue damage [28]. Our study revealed that Phytol + Cefixime had the most dramatic effect on *cnf1* down-regulation (fold value = 333.33). In contrast, other treatments, such as Cefixime + ZnNPs (fold value = 111.11) and Cefixime + ZrNPs (fold value = 62.5), also demonstrated significant inhibition. This is particularly important since *cnf1* is a key virulence factor implicated in the pathogenesis of pyelonephritis and other severe UTI complications [29]. Phytol may inhibit the *cnf1* promoter or destabilize its mRNA, while nanoparticles could degrade the toxin via ROS-mediated cleavage. The ability of Phytol combined with Cefixime to drastically reduce *cnf1* expression suggests that phytol-based treatments could help mitigate UPEC cytotoxic effects, potentially improving clinical outcomes by reducing tissue damage and inflammation. This idea is supported by the growing body of literature on plant-derived compounds, which are known to possess anti-inflammatory and cytotoxicity-reducing properties [29,30]. *bolA* gene is associated with bacterial stress responses and biofilm formation. More noteworthily, the fold value of *bolA* was up regulated in Phytol + CeNPs and Cefixime + ZnNPs (fold value = phytol 27.03, cefixime 12.66)) while other treatments like Phytol (fold value = phytol 27.03) and Cefixime (fold value = cefixime 12.66) did got down regulation. Certain combinations may up regulate due to different effects on biofilm formation and this may imply that Phytol and nanoparticles in combination may promote or inhibit biofilm formation under some conditions. The persistence of UPEC in the urinary tract, especially in chronic infections, is mostly dependent on its ability to form biofilm. This indicates that most of these agents might have the ability to downregulate UPEC's production of stable biofilms, a primary driver of long-term infection and antibiotic resistance [31]. Curli fibers, important for the structural integrity of biofilms, are produced by the synthesis that is controlled by the *csgD* gene. Down regulation of *csgD*, most of treatments, such as Phytol (fold value = 1.74) and Cefixime + ZnNPs (fold value = 2.19) suggested that these treatments might disrupt curli production and impaired biofilm integrity. Finally, the inhibition of curli fibers is essential for the stability of biofilms, and may have a huge impact against UPEC's ability to persist in the host [32]. The results show that Phytol and nanoparticles could prevent biofilm formation by damaging *csgD*, which is a major curli regulator. It can help disrupt the efficacy of antibiotics, which is especially helpful against biofilm associated infections, a type that is often resistant to treatment [33], and similarly all treatments down regulated the pgaC gene involved in the biosynthesis of biofilm matrix polysaccharides, but most significantly by the Phytol + Cefixime combination (fold value = 20.83). It is important that the *pgaC* gene is necessary for the structural integrity of biofilms and the inhibition of the *pgaC* gene further supports the idea that Phytol and Cefixime may increase penetration of antibiotics to biofilms, infecting UPEC and degrade poly-β-1,6-N acetylglucosamine (PNAG), one of the major biofilm binding molecules [34]. Findings of this study are most compelling one where synergistic action between nanoparticles (ZnNPs) and Cefixime utilized. This is coupled with a huge decrease in the MIC of Cefixime as assessed in an in vivo setting. FIC index values (0.187 for ZnNPs + Cefixime) indicate antibiotic combination is enhancing antimicrobial efficacy against the biofilm associated UPEC. Thus, significantly, a biofilm formation is a major UPEC resistance factor against treatment. It is known that nanoparticles improve targeted drug delivery vehicle, increase antibiotic penetration and decrease bacterial

resistance to conventional antibiotics [35]. With ZnNPs (FICI=0.187), a 16-fold reduction of cefixime MIC (of 512 to 32 µg/mL) matched with current nano-antibiotic synergy frontier. Disruption of the OM-IM periplasmic bridge by zinc oxide nanoparticles is found to occur when they bind to Braun's lipoprotein (LPP) leading to catastrophic membrane invagination [36]. Recent breakthroughs elucidate phytol multi target actions: Quorum Sensing Interference: Binds *SdiA* (*LuxR* homolog) and therefore suppresses *lsrACDB* (autoinducer 2 uptake genes) [37]. Metabolic Reprogramming: Inhibits acetate kinase (*ackA*), starving UPEC of acetyl-CoA for virulence factor synthesis [38].

CONCLUSION

This study presents compelling evidence that phytol, metal nanoparticles, and cefixime can markedly down-regulate essential virulence genes in UPEC, thereby interfering with bacterial adhesion, biofilm formation, and cytotoxicity. The identified synergistic effects between nanoparticles and antibiotics highlight their promise as complementary treatments for biofilm-associated urinary tract infections. The results indicate a new strategy for addressing antibiotic resistance in UPEC, particularly concerning multidrug-resistant strains. Additional in vivo studies and clinical investigations are essential to confirm the efficacy of these treatments in human infections and refine therapeutic strategies.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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