ISSN: 0067-2904

In vitro and Molecular Study of Synergistic Impact of Eugenol and Fosfomycin against Clinically-isolated, Fosfomycin-Resistant *Escherichia coli*

Ali Attaallah Ibrahim [1,2 *], Rana Kadhim Mohammed [1]
[1] *Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq*
[2] *Abi Ghraib General Hospital, Baghdad Al-Karkh Health Directorate, Iraqi Ministry of Health, Baghdad, Iraq*

Received: 12/11/2023 Accepted: 1/2/2024 Published: 28/2/2025

Abstract

Antibiotic resistance is a significant problem for which new treatments are required. There is growing concern about Fosfomycin resistance in *Escherichia coli*; understanding this resistance will help develop effective treatment strategies and preserve the efficacy of antibiotics. This study aimed to investigate the synergistic effects of Eugenol and Fosfomycin against Uropathogenic *Escherichia coli* (UPEC) and their possible potential as co-treatment. This study identified and isolated UPEC isolates from urine samples, with 63.6% being identified as UPEC. Antibiotic susceptibility tests showed that 99.3% of the UPEC isolates were resistant to multiple types of antibiotics [multidrug-resistant (MDR)]. The study examined the antibacterial properties of Fosfomycin and Eugenol using the parameters of agar well diffusion, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), fractional inhibitory concentration index (FICI), and time-kill curves. The MIC for Fosfomycin was 512-1024 µg/mL and for Eugenol was 1.25-5 µg/mL, while the MBC for Eugenol was 5-10 µg/mL and for Fosfomycin was 2048 µg/mL. Synergistic effects were considerable, where the addition of Eugenol at 1/4 MIC concentration resulted in 1/8 MIC of Fosfomycin. . The highest bactericidal activity for most UPEC isolates was recorded at 4-8 hours using Eugenol, 8-12 hours using Fosfomycin, and 4-8 hours using co-treatment. The study also used molecular assays to identify the expression levels of Fosfomycin resistance genes (*murA*, *glpT*, and *cyaA*) under different treatments, which revealed positive espression in all the isolates. The results showed variable gene expression changes in response to the different treatments. In conclusion, this study shows that Eugenol and Fosfomycin co-treatment is effective against UPEC.

Keywords: Fosfomycin-resistant UPEC; Eugenol; *murA*, *glpT*, and *cyaA* genes; synergistic.

دراسة مختبرية وجزيئية للتأثير التآزري لليوجينول والفوسفوميسين ضد الاشيريكيا القولونية المقاومة للفوسفوميسين المعزولة سريريًا

علي عطا الله ابراهيم[1، 2*]، رنا كاظم محمد[1]
[1] قسم التقنيات الاحيائية، كلية العلوم، جامعة بغداد، بغداد، العراق.

* Email: ali.ata@sc.uobaghdad.edu.iq

[2] مستشفى ابي غريب العام، دائرة صحة بغداد الكرخ، وزارة الصحة، بغداد، العراق.

الخلاصة

مقاومة المضادات الحيوية هي مشكلة رئيسية وتتطلب علاجات جديدة. هناك قلق متزايد بشأن مقاومة الفوسفوميسين في الإشريكية القولونية. فهم هذه المقاومة سيساعد في تطوير استراتيجيات علاجية فعالة والحفاظ على فعالية المضادات الحيوية. هدفت هذه الدراسة إلى التحقيق في التأثير التآزري لليوجينول والفوسفوميسين ضد الإشريكية القولونية المسببة لأمراض المسالك البولية (UPEC) وعلاجهم المشترك المحتمل. حددت هذه الدراسة وعزلت عزلات UPEC من عينات البول، وكانت 63.6٪ منها UPEC. أظهرت اختبارات حساسية المضادات الحيوية أن 99.3٪ من عزلات UPEC كانت مقاومة لأنواع متعددة من المضادات الحيوية [متعددة المقاومة للأدوية (MDR)]. فحصت هذه الدراسة الخواص المضادة للبكتيريا للفوسفوميسين واليوجينول باستخدام انتشار بئر الأجار وتحديد الحد الأدنى للتركيز المثبط للنمو (MIC) والحد الأدنى للتركيز القاتل للبكتيريا (MBC). التأثيرات التآزرية لمؤشر التركيز المثبط الكسري (FICI). كشفت منحنيات وقت القتل عن التأثير التآزري القاتل للبكتيريا للفوسفوميسين واليوجينول. كانت MIC للفوسفوميسين 512–1024 ميكروغرام / مل، واليوجينول كان 1.25–5 ميكروغرام / مل، وكان MBC لليوجينول 5–10 ميكروغرام / مل والفوسفوميسين كان 2048 ميكروغرام / مل. كانت التأثيرات التآزرية كبيرة، حيث أدى 4/1 MIC من اليوجينول إلى تقليل ل 8/1 MIC من الفوسفوميسين. قتل اليوجينول معظم عزلات UPEC خلال 4–8 ساعات، والفوسفوميسين خلال 8–12 ساعة، والعلاج المشترك خلال 4–8 ساعات. استخدمت الدراسة أيضًا التحاليل الجزيئية لتحديد المعلمات المتعلقة بجينات مقاومة الفوسفوميسين الجينومية murA و glpT و cyaA ، ومستويات التعبير عنها تحت معالجات مختلفة. كانت الجينات murA و glpT و cyaA موجبة في جميع العزلات. أظهرت النتائج تغيرات متغيرة في تعبير الجين استجابةً للمعالجات المختلفة. في الختام، تُظهر هذه الدراسة أن العلاج المشترك باليوجينول والفوسفوميسين فعال ضد UPEC.

Introduction

E. coli, particularly UPEC, is the predominant culprit behind bacterial urinary tract infections (UTIs), demonstrating a significant role in community-acquired UTIs and a notable proportion of hospital-acquired infections. UPEC, the most common extraintestinal pathogenic *E. coli* (ExPEC), accounts for approximately 80% of community-acquired and 20% of hospital-acquired UTIs [1].

UPEC possesses distinct genetic characteristics and virulence factors that distinguish it from its nonpathogenic counterparts. Pathogenicity islands (PAIs) are genetic elements closely linked to the virulence and pathogenicity of ExPEC [2].

Fosfomycin, a bactericidal antibiotic, is primarily used to treat uncomplicated UTIs (approved for lower UTIs in the US and certain European countries) [3]. It demonstrates efficacy against a spectrum of bacteria, including enteric Gram-negative species like *E. coli*, *K. pneumoniae*, and *E. cloacae*, as well as Gram-positive cocci such as *S. aureus*, *S. pneumoniae*, and *E. faecalis*. Fosfomycin has also been explored as a treatment option for infections caused by multidrug-resistant (MDR) bacteria [4].
Fosfomycin, a phosphonic acid derivative, exerts its distinct mode of action by irreversibly inhibiting bacterial cell wall synthesis through disruption of UDP-N-acetyl glucose amine enol pyruvyl transferase (MurA), a pivotal enzyme in peptidoglycan biosynthesis [5]. Resistance to fosfomycin can occur through various mechanisms including reduced antibiotic permeability, target enzyme impairment, and enzymatic inactivation [6].

In *E. coli*, fosfomycin uptake primarily relies on two nutrient transport systems: the glycerol-3-phosphate transporter (GlpT) and glucose-6-phosphate transporter (UhpT), which

require the presence of cAMP-CRP. Mutations in the genes associated with these pathways can diminish antibiotic uptake and confer varying levels of fosfomycin resistance. Adequate cAMP levels are essential for the full expression of the fosfomycin transporters GlpT and UhpT in Enterobacteria, with cAMP production depending on adenyl cyclase CyaA activity, further modulated by phosphotransferase PtsI. Mutations in cyaA or ptsI reduce intracellular cAMP levels, hampering the synthesis of both fosfomycin transporters and diminishing antibiotic uptake [6].

MurA, an essential enzyme targeted by fosfomycin, is inactivated by irreversible binding of the antibiotic to the protein. Mutations affecting the fosfomycin-binding site in MurA, particularly Cys115, result in resistance to this antibiotic [7].
Traditional medicine has long harnessed the therapeutic potential of plant extracts, which are valuable resources for pharmaceutical applications. Medicinal plants contain many compounds with essential properties and minimal side effects, making them attractive to the pharmaceutical industry. In contrast to conventional treatments, which are associated with significant side effects and high costs, medicinal plant extracts offer a more natural and holistic approach to healthcare. This growing interest has prompted pharmaceutical companies to incorporate these extracts as raw materials in their drug development processes [8].

One such plant is clove (*Syzygium aromaticum*), which is renowned for its medicinal use. Clove plants are rich in beneficial compounds including essential oils, flavonoids, saponins, and tannins. Eugenol is the primary component of clove essential oil, accompanied by compounds such as β-caryophyllene, which contribute to the distinctive aroma and medicinal properties of clove [9]. Clove extracts have diverse medicinal applications, with their antimicrobial effects attributed to their ability to disrupt the cell walls and membranes of microorganisms, positioning them as potential natural alternatives to conventional antibiotics [10].

Eugenol, the chief constituent of clove oil, has been extensively studied for its wide-ranging properties including antioxidant, analgesic, antimutagenic, anti-inflammatory, and antimicrobial activities. It has been shown to be effective against numerous bacteria, including *S. aureus* and *E. coli*, by damaging the cell membranes and causing intracellular component leakage [11]. A study on eugenol showed that this compound has synergistic activity with various antibiotics, such as vancomycin, penicillin, ampicillin, and erythromycin, and the combination of these compounds allowed a reduction in MIC values by 5–1000 times compared to the MIC values of individual compounds used alone [12].
The aims of this study is to investigate the synergistic effects of Eugenol and Fosfomycin against UPEC and their possible potential as effective co-treatment.

Materials and methods

Samples Collection and Diagnosis

Urine samples were collected from patients suspected of having UTIs and transported to the laboratory under sterile conditions. Urine samples were inoculated onto Brain Heart Infusion (BHI) agar and Blood agar, then incubated at 37°C overnight. *E. coli* was identified by performing standard laboratory techniques, including Gram staining, biochemical tests, API system (API 20E), VITEK 2 compact system, and genotypic detection. The Ethics Committee at the Department of Biotechnology/ College of Science/ University of Baghdad, approved the study protocol (Reference: CSEC/0222/0044) on February 10, 2022.

Susceptibility test

The antimicrobial susceptibility of *E. coli* was determined using the Kirby-Bauer disk diffusion method, according to the Clinical and Laboratory Standards Institute's (CLSI, 2022) guidelines [13]. The antibiotics Ampicillin, Amoxicillin-clavulanate, Fosfomycin, Nitrofurantoin, Ciprofloxacin, Levofloxacin, Nalidixic acid, Norfloxacin, Cefepime, Cefazolin, Trimethoprim, and Trimethoprim-sulfamethoxazole were used. The zone of inhibition around each disk was measured and interpreted as indication of sensitive, intermediate, or resistant bacteria, according to the CLSI (2022) guidelines.

Eugenol Extraction and Purification

Eugenol from clove (*S. aromaticum*) was extracted using steam distillation by Clevenger [14]. The clove was commercially obtained from a local market in Baghdad, Iraq. It was purified by steam distillation and extracted using dichloromethane (CH_2Cl_2) as the organic solvent.

Characterization of Eugenol by High-performance liquid chromatography (HPLC)

The extracted clove oil (Eugenol) was injected (20 µL) into the HPLC according to the optimum conditions. HPLC analysis was performed using a Sykam S 2100 Quaternary Gradient HPLC Pump (Germany). The specifications of the column of examination were 25*4.6 mm 5-micron C18, flow rate 0.8 ml/min, wavelength λ 210 nm, mobile phase 0.1% H_3PO_4 (1N) and D.W, temperature 25ºC, and volume of injection 20 µl [15].

Calculation of concentration of the sample

$$\text{Sample concentration}=\frac{\text{Area of sample}}{\text{Area of standrd}}\times \text{concentration of standard} \times \text{dilution factor} \quad \ldots\ldots\ldots\ldots 1$$

Antibacterial activity in vitro

Agar well diffusion method

The agar well diffusion method is widely used to evaluate the antibacterial activity of antibiotics and plant extracts [16, 17]. This study tested the effectiveness of Fosfomycin and Eugenol against *E. coli*. Bacterial suspensions were prepared from fresh colonies, and the concentration was adjusted to 1.5×10^8 colony-forming units (CFU)/ml (McFarland turbidity). Each strain was inoculated by streaking the bacteria onto a Mueller-Hinton agar (MHA) plate. Then, a hole with a diameter of 6 mm was punched aseptically with a sterile cork borer, and 50 µL of the Fosfomycin antibiotic (1024 µg/ ml) and/or Eugenol extract solution (80 µg/ ml) was introduced into the well. The antibacterial agent (antibacterial activity) diffuses into the agar medium and inhibits the growth of the microbial strain, which was tested after overnight incubation at 37°C.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC):

The MIC values of Eugenol and Fosfomycin were determined by the agar dilution method. The serial dilutions for Fosfomycin ranged from 0.5 to 4096 µg/ml, whereas those for Eugenol ranged from 1.25 to 160 µg/ml. The bacterial culture was diluted to a concentration adjusted to 0.5 McFarland turbidity, equal to 1.5×10^8 CFU/mL. Eugenol and Fosfomycin concentrations were added to MHA in a petri dish, solidified, and incubated at 37°C overnight. The MIC was determined as the lowest concentration of Eugenol and Fosfomycin that inhibited the visible growth of *E. coli*. MBC was determined when no bacterial growth was observed at the minimum concentration [18, 19].

Synergism (checkerboard) assay

The synergy between Fosfomycin and Eugenol, *E. coli* was studied using the checkerboard assay [20, 21]. Different concentrations of Fosfomycin and Eugenol were used. The FIC index was determined using the following formulae: FIC index = $FIC_{Eugenol}+ FIC_{Fosfomycin}$, $FIC_{Eugenol}= MIC_{Eugenol\ (in\ combination)} /MIC_{Eugenol\ (alone)}$, and $FIC_{\ Fosfomycin}= MIC_{Fosfomycin\ (in\ combination)}$

/$MIC_{Fosfomycin\ (alone)}$. An FIC index of ≤ 0.5 indicates the synergetic effect of the combination; values between 0.5 and 1 indicate that the combination is additive; values between 1 and 4 indicate indifference; and an FIC index > 4 indicates that the combination is antagonistic [22].

Time Killing (TK)

The time-kill assay was performed as previously described [23]. The antibacterial activities of Fosfomycin and Eugenol against Fosfomycin-resistant UPEC isolates were assessed by the time-kill assays. The reduction in CFU/mL after 18 hours was also measured. Fosfomycin and Eugenol (corresponding to the MIC) were incubated with the UPEC isolates. As a control, MHB was added instead of either Fosfomycin or Eugenol. All the samples were incubated at 37°C. CFU was counted by spreading 5µl of a 10-fold diluted sample on the surface of MHA in 0 hrs and after 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 hrs of incubation.

DNA Extraction

From a 1ml culture, chromosomal DNA was isolated using a PrestoTM Mini gDNA Bacteria Kit (Geneaid, Thailand). Chromosomal DNA, after purification, was kept at -20°C. A nanodrop device was used to measure DNA concentration and purity. One µl of each DNA sample was used to measure optical density (O.D) at 260 nm and 280 nm wavelengths.

Thermal Cycler; Polymerase Chain Reaction (PCR) Technique

PCR was performed to amplify specific DNA fragments, using the primers (Table 1). The reaction mixture contained template DNA (chromosomal) (3 µl), primers [1 µl from each of forward and reverse primers (10 poml)], and GoTaq®Green Master Mix (10 µl). The PCR mixture was completed to 20 µl by adding deionised nuclease-free water. PCR was performed under the conditions listed in Table 2 for uniplex PCR of each gene. The temperature and time of the PCR program were optimized using a gradient PCR. The PCR products were detected using a gel image analysis device and a UV light source, following electrophoresis of the samples on 1.5% (w/v) agarose (Promega, USA) in 1X TBE buffer stained with a safe stain.

Table 1: Primer sequences used in this study.

Gene	Primer	Nucleotide sequence (5′ to 3′)	PCR product bp	Reference
16S rRNA	Forward	TGTCGTCAGCTCGTGTTGTG	130	[24]
	Reverse	ATCCCCACCTTCCTCCAGTT		
murA	Forward	CGGTATCGACGATTTCCGGT	190	Designed in this study
	Reverse	TTAGGCGCGACCATCAAACT		
glpT	Forward	GAAGTCCACGGTGTAGCCAA	160	Designed in this study
	Reverse	CGGCTTCCTGATCTACGGTC		
cyaA	Forward	TTTGCCAGCGAAGGGATCAT	166	Designed in this study
	Reverse	GCGATGACGAGTAGAAGCGA		

Table 2: Program conditions for uniplex PCR amplification of each gene in this study.

Gene	Initial denaturation (ºC/min)	No. of cycle	Denaturation (ºC/min)	Annealing (ºC/min)	Extension (ºC/min)	Final extension (ºC/min)
16S rRNA	95/5	35	94/1	55/1	72/1	72/10
murA	95/5	35	94/1	56/1	72/1	72/10
glpT	95/5	35	94/1	56/1	72/1	72/10
cyaA	95/5	35	94/1	56/1	72/1	72/10

Gene Expression

Real Time-PCR was used to determine gene expression levels to identify the behaviors of some factors contributing to the resistance of UPEC isolates after being under stress with MIC concentrations of Fosfomycin, Eugenol, and co-treatment (combination of Fosfomycin with Eugenol).

The present study investigated gene expression in ten pathogenic *E. coli* isolates (resistant to Fosfomycin). The purified RNA isolated from each isolate was used to determine the expression of the target genes (*murA*, *glpT*, and *cyaA*), which are considered crucial chromosomal resistance factors for Fosfomycin in UPEC. The expression level of each gene was calibrated and normalized to that of the housekeeping gene *E. coli 16S rRNA* using real-time PCR [25]. RNA was extracted from bacterial cultures using a commercial RNA extraction kit. RNA was reverse-transcribed into cDNA, which was amplified using specific primers (Table 1).

The components of the reaction mixture of one-step RT-PCR, template (3 µl), primers [1 µl from each of forward and reverse primers (10 poml)], qPCR Master Mix (10 µl), and Deionized Nuclease–Free water were added to the PCR mixture to obtain a final volume of 20 µL. The reaction conditions were set using a two-step method (Table 3). All templates were run in triplicates.

Table 3: Program conditions of qRT-PCR.

Gene	RT. Enzyme Activation (ºC/min)	Initial denaturation (ºC/min)	No. of cycle	Denaturation (ºC/min)	Annealing (ºC/min)	Extension (ºC/min)
16S rRNA	37/ 5	95/ 5	40	95/ 0.5	55/0.5	72/0.5
murA	37/ 5	95/ 5	40	95/ 0.5	56/0.5	72/0.5
glpT	37/ 5	95/ 5	40	95/ 0.5	56/0.5	72/0.5
cyaA	37/ 5	95/ 5	40	95/ 0.5	56/0.5	72/0.5

Statistical Analysis

Statistical analysis of the mean ± standard deviation was performed using One-way ANOVA tests, and statistical analysis of qualitative data was performed using the Chi-square ($\chi 2$) test by means of SPSS 25 and GraphPad Prism 8. Statistical significance was set at a *p-value* of $P \leq 0.05$ [26].

Results and Discussion

Isolation of samples and identification of E. coli

All collected urine samples, totaling hundred and fifty, underwent an initial culturing process on BHI agar and blood agar. Of 250 urine samples, 220 (88 %) showed bacterial growth on both BHI and Blood agar. Upon microscopic examination, the results showed that 53 isolates (24.1%) were categorized as Gram-positive while 167 isolates (75.9%) were classified as Gram-negative bacteria. The distribution of the clinical samples based on sex and age is shown in Table 4.

Table 4: Distribution of clinical samples according to sex and age.

Group		Frequency	Percentage %	*p-value*
Age (year)	15-30	80	32	<0.0001*
	31-45	96	38.4	
	46-60	48	19.2	
	>60	26	10.4	
Sex	Male	88	35.2	<0.0001*
	Female	162	64.8	

Data are presented as the chi-square ($\chi 2$) goodness of fit. * Statistically significant at $p \leq 0.05$.

The current study showed that the incidence of urinary tract infections in women surpassed that in men by more than two-fold. The biological and social disparities between males and females contribute significantly to the increased vulnerability of women to microbial infections compared to men [27]. When considering UTIs, it has been observed that women are disproportionately affected compared with men. This can be attributed to various factors such as the anatomical proximity of the female urethra to the anus. Additionally, decreased levels of estrogen hormones during menopause contribute to increased susceptibility to urinary tract infections, primarily due to the absence of protective vaginal flora [28]. The primary factors contributing to the increasing prevalence of UTIs in older males are the growth of the prostate gland and the presence of neurogenic bladder [29]. Other researchers have also documented this connection, since their investigations have demonstrated a correlation between prostate illness in males and elevated UTIs. The rising prevalence of UTIs in young girls can be attributed to several variables, including heightened sexual activity, recent use of a diaphragm combined with spermicide, and past medical records of recurrent UTIs [30].

E. coli was identified depending on the morphological features of culture media, biochemical tests, API system (API 20E), and VITEK 2 compact system. Genotypic identification using PCR to detect the *16S rRNA* gene among all isolates was also performed in the current study. The results showed that 100% of the isolates were *E. coli*, using *16S rRNA*, with an amplified size of 130bp. Figure 1 illustrates the bands of the positive results compared to the DNA ladder (100pb). Out of 220 isolates, *E. coli* was found in 140 isolates (63.6%); it was more distributed in patients than other bacterial isolates. Molecular diagnosis used the *16S rRNA* gene, which was confirmed by [31, 32], as the final diagnosis of bacteria.

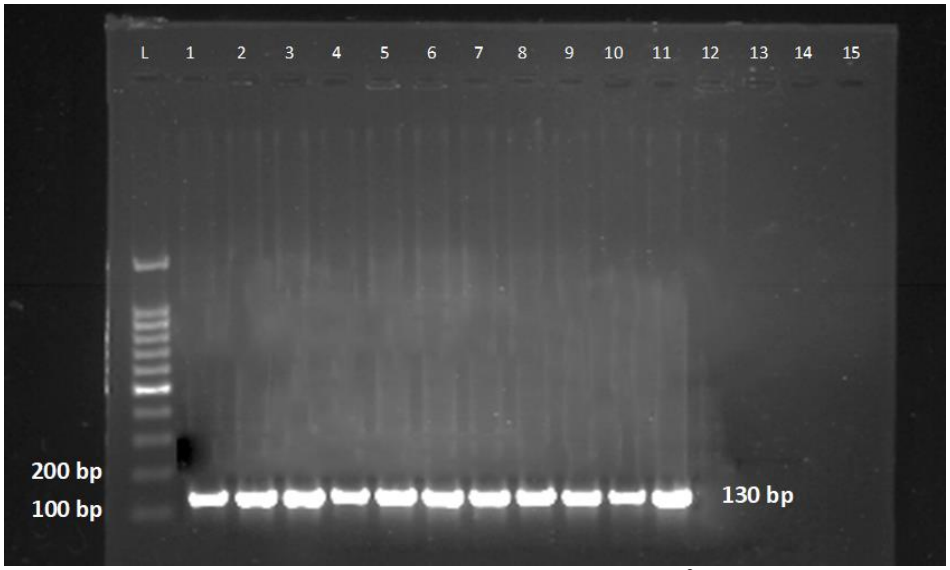

Figure 1: Agarose gel electrophoresis (1.5% agarose, 7 V/cm^2 for 80min) of amplified *16S rRNA* gene (130 bp) from UPEC using conventional PCR. Lane L: 100bp DNA ladder. Lanes 1-11: Amplicons *16S rRNA* gene for UPEC. Lanes 12-14: Control: DNA extracted from different bacterial isolates (*K. pneumoniae, P. aeruginosa, and S. aureus*). Lane 15: negative control (replacement of DNA template with water in the PCR mixture).

Numerous pathogens can cause UTIs due to variations in the susceptibility of the host to pathogens. These variations are influenced by biological and environmental factors that promote diversity in hosts, pathogens, vectors, and social factors, including the disease control efforts of individuals [33]. Enterobacteriaceae possesses many variables that are

accountable for their adherence to the uroepithelium. Gram-negative aerobic bacteria can colonize the urogenital mucosa using adhesin, pili, fimbriae, and P1-blood group phenotypic receptors. Numerous virulence factors, both secreted and surface-associated, contribute to the ability of *E. coli* isolates to cause UTIs, which explains their high prevalence [34]. In this study, we isolated 140 *E. coli* isolates (63.6%), suggesting that *E. coli* is the primary cause of UTIs. When *E. coli* was isolated from UTIs in Iraq, two previous studies [35, 36] reported that it was the most prevalent agent, with percentages of 56% and 42%, respectively.

Antibiotic susceptibility testing

The susceptibility test of one hundred and forty UPEC showed varied levels of resistance to antibiotics (Table 5). Most isolates were resistant to ampicillin (99.3 %), cefazolin (99.3 %), nalidixic acid (96.4 %), trimethoprim (96.4 %), trimethoprim-sulfamethoxazole (95.7 %), nitrofurantoin (76.4 %), cefepime (73.6 %), ciprofloxacin (62.9 %), norfloxacin (60.7 %), and levofloxacin (60 %). The lowest rates of resistance were to fosfomycin (7.1 %) and amoxicillin-clavulanate (21.4 %). A Statistically significant difference ($p \leq 0.05$) was found between resistance to different types of antibiotics and between intermediate resistance and sensitivity to the same antibiotic.

Table 5: Percentage results of antibiotic susceptibility tests against *E. coli* isolates.

Antimicrobial class	Antibiotic	Resistance		Intermediate		Sensitive		*p-value*
		No.	%	No.	%	No.	%	
Penicillins	Ampicillin	139	99.3	1	0.7	0	0	<0.0001*
β-lactam combination agents	Amoxicillin-clavulanate	30	21.4	54	38.6	56	40	0.011*
Fosfomycins	Fosfomycin	10	7.1	11	7.9	119	85	<0.0001*
Nitrofurans	Nitrofurantoin	107	76.4	5	3.6	28	20	<0.0001*
Quinolones and fluoroquinolones	Ciprofloxacin	88	62.9	6	4.3	46	32.8	<0.0001*
	Levofloxacin	84	60	0	0	56	40	<0.0001*
	Nalidixic acid	135	96.4	1	0.7	4	2.9	<0.0001*
	Norfloxacin	85	60.7	2	1.4	53	37.9	<0.0001*
Cephems	Cefepime	103	73.6	12	8.6	25	17.9	<0.0001*
	Cefazolin	139	99.3	0	0	1	0.7	<0.0001*
Folate pathway antagonists	Trimethoprim	135	96.4	0	0	5	3.6	<0.0001*
	Trimethoprim-sulfamethoxazole	134	95.7	1	0.7	5	3.6	<0.0001*
p-value		<0.0001*		<0.0001*		<0.0001*		

Data are presented as the chi-square ($\chi 2$) goodness of fit. * Statistically significant at $p \leq 0.05$.

This study examined the development of antibiotic resistance in UPEC isolates obtained from UTI patients. Development of resistance to β-lactam antibiotics is associated with the synthesis of several classes of β-lactamases. Plasmids frequently contain genes encoding several forms of β-lactamases, commonly called *bla* genes [37]. In a previous study, specific genetic mutations that confer resistance to nitrofurantoin were discovered. Mutations in *nsfA* and *nfsB*, which encode oxygen-insensitive nitroreductases, have been identified as the underlying causes of nitrofurantoin resistance [38]. Resistance to UPEC has grown owing to the widespread use of quinolones and fluoroquinolones for treating UTIs worldwide [39]. Mutations in DNA supercoiling catalyzing gyrA and gyrB commonly cause quinolone resistance in *E. coli*. A quinolone resistance-determining region (QRDR) was identified in the N-terminal sequence of gyrA (amino acids Ala-67–Gln-106). This sequence is correlated with

phenotypic resistance to quinolones and fluoroquinolones [40]. Additional mechanisms by which *E. coli* develops resistance to quinolones and fluoroquinolones include efflux pumps and reduction of antibiotic absorption, resulting from alterations in the outer membrane porin proteins [41]. Dihydrofolate reductase (DHFR), an enzyme whose promoter mutation targets frequent overproduction, is the leading cause of bacterial resistance to trimethoprim [42]. The resistance of *E. coli* to trimethoprim-sulfamethoxazole due to chromosomal mutations (often single point mutations) in *dhfr* or *dhps* genes is commonly the cause of resistance to these antibiotics [43].

Multiple drug resistance (MDR)

The MDR results shown in Table 6 indicated very high multiple resistance to various antimicrobial classes; 139/140 (99.3%) isolates were MDR. The obtained results showed that three isolates (2.1%) had resistance to all the seven antimicrobial classes, while five isolates (3.6%) showed multiple resistance to three classes. The highest percentage was observed in the group which resisted five antimicrobial class groups; 85 isolates (60.7%).

Table 6: Multiple drug resistance of *E. coli*.

No. of antimicrobial class	No.	%	*p-value*
One	1	0.7	<0.0001*
Three	5	3.6	
Four	21	15	
Five	85	60.7	
Six	25	17.9	
Seven	3	2.1	
Total	140	100	

Data are presented as the chi-square ($\chi 2$) goodness of fit. * Statistically significant at $p \leq 0.05$.

Multidrug resistance analysis revealed that 139/140 (99.3%) isolates were MDR. The prevalence of drug-resistant *E. coli* has increased in recent years. Several contributing factors include mutations, horizontal gene transfer, and careless antibiotic usage [44, 45]. The selection of optimal antibiotics for treatment is vital to reduce the spread of antibiotic resistance from bacteria to other bacteria worldwide, and the resulting threats to economic loss and human health. The results of antibiotic susceptibility tests conducted on microbiological isolates are crucial for determining which antibiotic will be most effective in curing an infection. Selecting an appropriate antibiotic and establishing its worth and dose are crucial steps in preventing the development of resistance and maximizing therapeutic efficacy [39].

Characterization of Eugenol by HPLC

The results of the HPLC analysis are shown in Figure 2A for the Eugenol standard and Figure 2B for the Eugenol sample. Upon evaluating the graphs, the peak corresponding to Eugenol surfaced at retention times of 13.78 and 13.91 minutes for the Eugenol standard and Eugenol sample, respectively. A comparative analysis of the peak areas of the Eugenol standard and Eugenol sample showed that the percentage of Eugenol present in the sample was relatively high (67%).

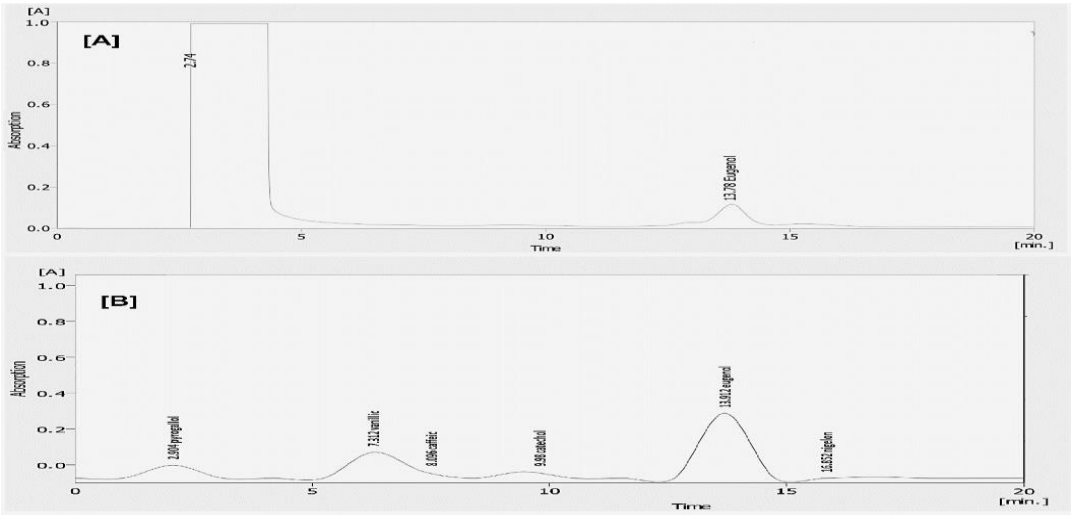

Figure 2: HPLC chromatogram of Eugenol. A: Eugenol standard, B: Eugenol sample.

Antibacterial activity in vitro (Agar well diffusion, MIC, MBC, and synergistic)

Eugenol showed antibacterial effects at 80 µg/mL against UPEC (10 to 18 mm), and Fosfomycin showed antibacterial effects at 1024 µg/mL against UPEC (10 to 30 mm). The zone of inhibition showed little effect when combined with Fosfomycin (10–35 mm).

The MIC values of 10 isolates (A23, A24, A25, A26, A28, A33, A85, A88, A90, and A101), which were resistant to most antibiotics used in this study, are shown in Table 7. The MIC of Fosfomycin was observed between 512-1024 µg/mL, while the MBC was 2048 µg/mL. The MIC of Eugenol was observed between 1.25-5 µg/mL and the MBC was between 5-10 µg/mL.

The synergy between Fosfomycin and Eugenol is shown in Table 7.

Table 7: MBC, MIC and FICI of Eugenol and Fosfomycin against UPEC isolates.

Isolates	Agents(µg/mL)	MBC	MIC		FICI	Interpretation
			Alone	Combination		
A 23	Eugenol Fosfomycin	5 2048	1.25 512	0.312 256	0.75	Additive
A 24	Eugenol Fosfomycin	5 2048	5 512	1.25 128	0.5	Synergy
A 25	Eugenol Fosfomycin	5 2048	2.5 512	0.625 256	0.5	Synergy
A 26	Eugenol Fosfomycin	5 2048	5 512	1.25 256	0.75	Additive
A 28	Eugenol Fosfomycin	5 2048	5 512	1.25 128	0.5	Synergy
A 33	Eugenol Fosfomycin	10 2048	2.5 1024	0.625 256	0.5	Synergy
A 85	Eugenol Fosfomycin	10 2048	2.5 1024	0.625 256	0.5	Synergy
A 88	Eugenol Fosfomycin	10 2048	5 1024	1.25 128	0.375	Synergy
A 90	Eugenol Fosfomycin	10 2048	5 1024	1.25 256	0.5	Synergy
A 111	Eugenol Fosfomycin	10 2048	2.5 1024	0.625 256	0.5	Synergy

For the A 88 isolate, the combination of Eugenol and Fosfomycin exerted a robust synergistic effect, where the addition of Eugenol at 1/4 MIC resulted in 1/8 MIC of Fosfomycin (FICI =

0.375). For A 28, A 33, A 85, A 90, and A 111 isolates, 1/4 MIC of Fosfomycin was observed after being combined with 1/4 MIC of Eugenol (FICI = 0.5). For A 25 isolate, 1/2 MIC of Fosfomycin was observed after being combined with 1/4 MIC of Eugenol (FICI = 0.5). For A23 and A 26 isolates, adding 1/4 MIC Eugenol could be additive with Fosfomycin, resulting in 1/2 MIC (FICI= 0.75).

Time-kill curves

Figure 3 shows a time-kill curve describing viability following treatment with Fosfomycin and/or Eugenol, based on findings from an MIC experiment. Within 4–8 hrs, Eugenol exhibited a bactericidal activity to *E. coli*, while that for Fosfomycin was within 8-12 hrs. Also, the co-treatment of Fosfomycin with Eugenol showed bactericidal activity within 4-8 hrs.

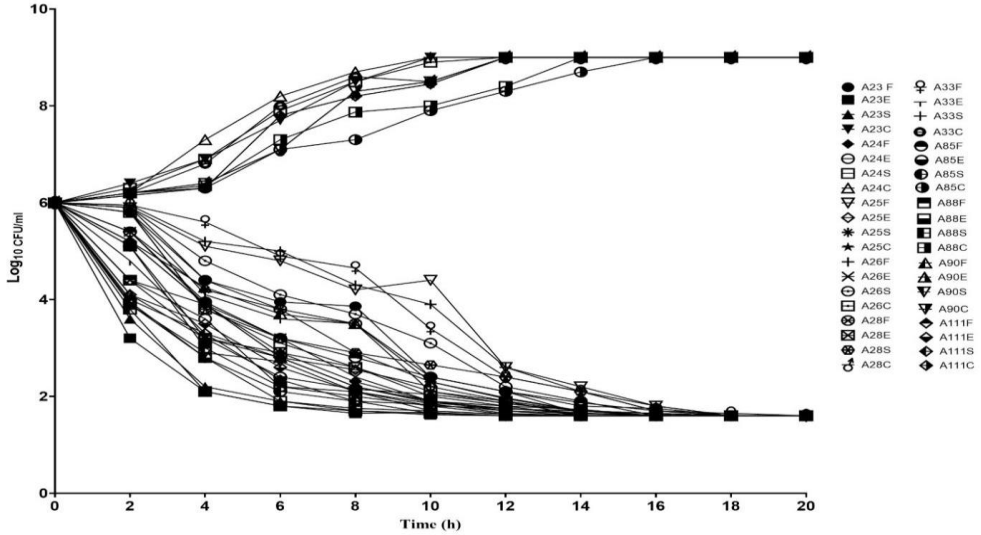

Figure 3: Time–kill curves of *E. coli* isolates. Cell viability (log_{10} CFU/mL) is plotted for cultures grown at concentrations of Fosfomycin and Eugenol relative to isolates' specific MICs and synergistic. F: Fosfomycin (1024 µg/ml), E: Eugenol (5 µl/ml), S: synergistic [co-treatment of Fosfomycin (512 µg/ml), with Eugenol (2.5 µl/ml)], C: control (untreated).

Eugenol has been observed to induce changes in membrane permeability, releasing intracellular contents and subsequent cellular harm. The impact of this perturbation on the cytoplasmic membrane is assessed by quantifying intracellular ATP levels. The presence of divalent cations increases the MIC value of Eugenol, indicating an interaction with the membrane. Eugenol also induces physiological and morphological changes in *E. coli*, as observed through TEM and SEM tests [46]. Eugenol has free hydroxyl groups in its structure that may be responsible for the antimicrobial activity verified in this study, as its free hydroxyl groups confer the antimicrobial activity of nitric oxide (NO). It was deduced that the hydroxyl group in eugenol is linked to proteins, thereby preventing enzymatic action. The cell membrane is ruptured in the presence of the essential oil because it is rich in lipophilic compounds. This damage directly affects the maintenance of cellular pH and the balance of inorganic ions. The main factors responsible for this damage are monoterpenes and sesquiterpenes, which have varying effects on microorganisms [47]. These findings suggest that eugenol and its derivatives can be used as antimicrobial agents.

Detection of Fosfomycin resistance-related genes

One hundred and forty UPEC isolates were tested for chromosomal Fosfomycin resistance *murA*, *glpT* and *cyaA* genes using the Uniplex PCR molecular detection method.

I- *murA* gene (UDP-N-acetylglucosamine-3-O-enolpyruvyl transferase)

The amplified desired fragment of the *murA* gene (190bp) used genomic DNA extracted as a template of UPEC isolates. This detection observed a positive result for all 140/140 (100%) isolates by using genomic DNA as a template, with the molecular size of amplified products as displayed in Figure 4.

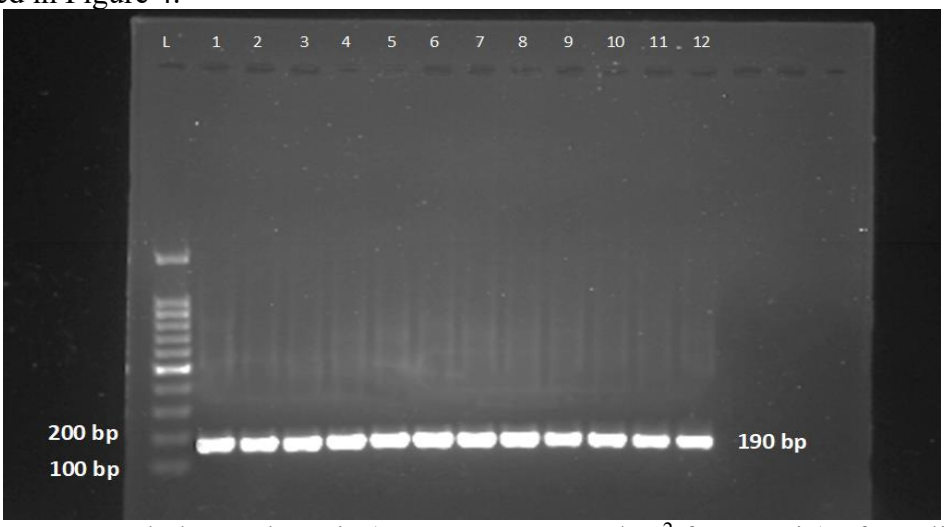

Figure 4: Agarose gel electrophoresis (1.5% agarose, 7 V/cm^2 for 80 min) of amplified *murA* (190 bp) from UPEC using conventional PCR. Lane L: 100bp DNA ladder. Lanes 1-12: Amplicons *murA* gene for UPEC, all lanes represent positive results.

II- *glpT* gene (α-glycerol-3-phosphate transporter)

The amplified desired fragment of the *glpT* gene (160bp) used genomic DNA extracted as a template of UPEC isolates. This detection observed a positive result for all 140/140 (100%) isolates using genomic DNA as a template, with the molecular size of amplified products as displayed in Figure 5.

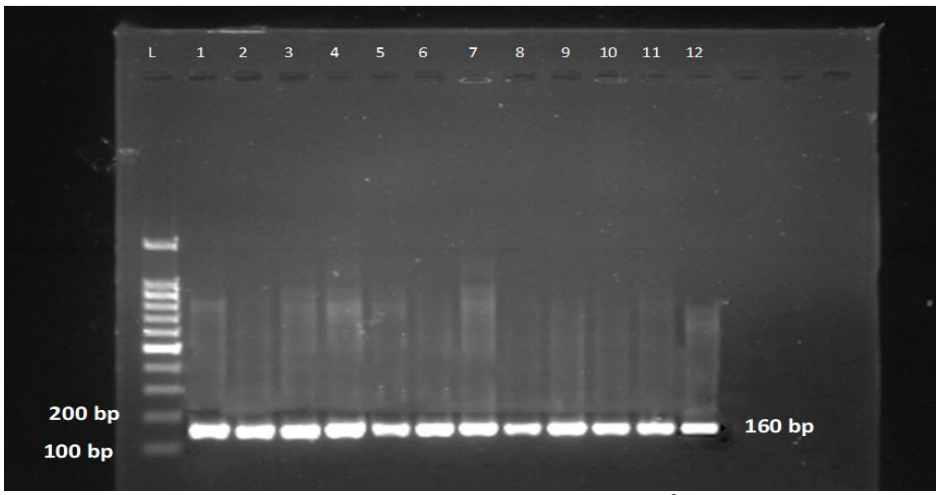

Figure 5: Agarose gel electrophoresis (1.5% agarose, 7 V/cm^2 for 80 min) of amplified *glpT* (160 bp) from UPEC using conventional PCR. Lane L: 100 bp DNA ladder. Lanes 1-12: Amplicons *glpT* gene for UPEC, all lanes represent positive results.

III- *cyaA* gene (adenyl cyclase)

The amplified desired fragment of the *cyaA* gene (166bp) used genomic DNA extracted as a template of UPEC isolates. This detection observed a positive result for all 140/140 (100%) isolates by using genomic DNA as a template, with the molecular size of amplified products as displayed in Figure 6.

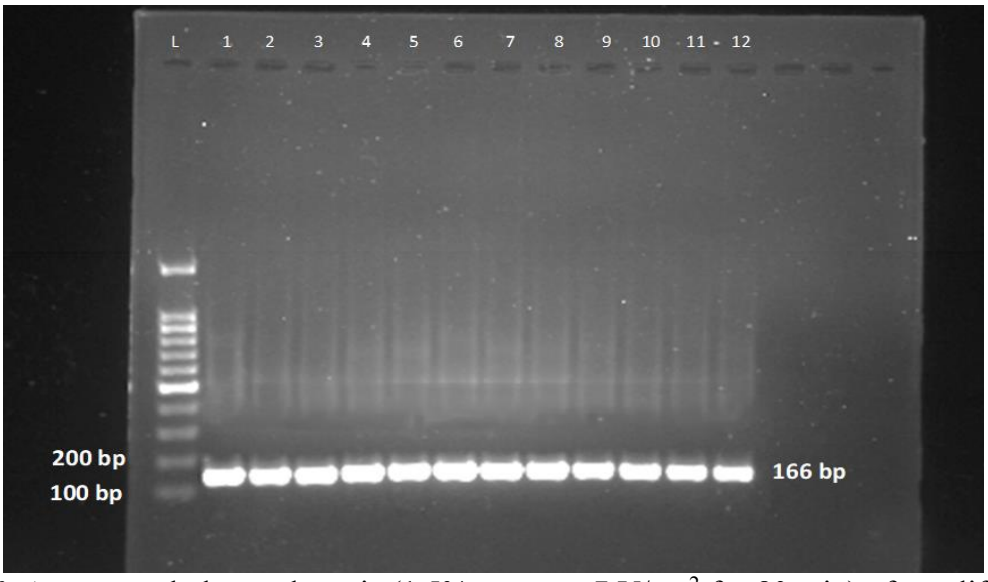

Figure 6: Agarose gel electrophoresis (1.5% agarose, 7 V/cm^2 for 80 min) of amplified *cyaA* (166 bp) from UPEC using conventional PCR. Lane L: 100 bp DNA ladder. Lanes 1-12: Amplicons *cyaA* gene for UPEC; all lanes represent positive results.

Determination Gene Expression Level by RT-PCR

In the present study, the expression of the targeted chromosomal Fosfomycin resistance *murA, glpT* and *cyaA* genes was estimated for ten isolates that were subjected to stress with MIC concentrations of Fosfomycin, Eugenol, and synergistic (Fosfomycin with Eugenol) and compared with the control (Untreated).

The *16S rRNA* gene served as the housekeeping gene for all other genes. This practice is being questioned as it becomes increasingly clear that some housekeeping genes may vary considerably in specific biological samples. The result of *16S rRNA* for this study showed Ct values ranging from 19.12 to 20.18.

The effects of Fosfomycin and Eugenol on the expression of *murA, glpT* and *cyaA* genes in UPEC isolates are as follows:

I- *murA* gene (UDP-N-acetylglucosamine-3-O-enolpyruvyl transferase)

The results effects of Fosfomycin and Eugenol on the expression of *murA* gene in UPEC isolates are shown in Table 8 and Figure 7.

The analysis of *murA* gene expression, in comparison to the control (untreated) group, revealed a notable reduction in gene folding expression across all isolates, regardless of treatment status. The Ct values corresponding to the *murA* gene throughout all treatment conditions play a crucial role in providing insights into the initial mRNA content inside the samples. The data indicate that the concentration of Fosfomycin and/or Eugenol was influenced by and correlated with the more excellent value of Ct in all treatment conditions, which suggests a decrease in gene folding expression.

Table 8: The mean gene folding expression for *murA* gene in different statuses.

Status	Mean	± Std. Deviation	F- value	*p-value*
Untreated	1	0	20.1	<0.0001*
Eugenol	0.76	0.1		
Fosfomycin	0.81	0.09		
Eugenol and Fosfomycin	0.7	0.1		

Data presented as One-way ANOVA. * Statistically significant at p-value ≤ 0.05.

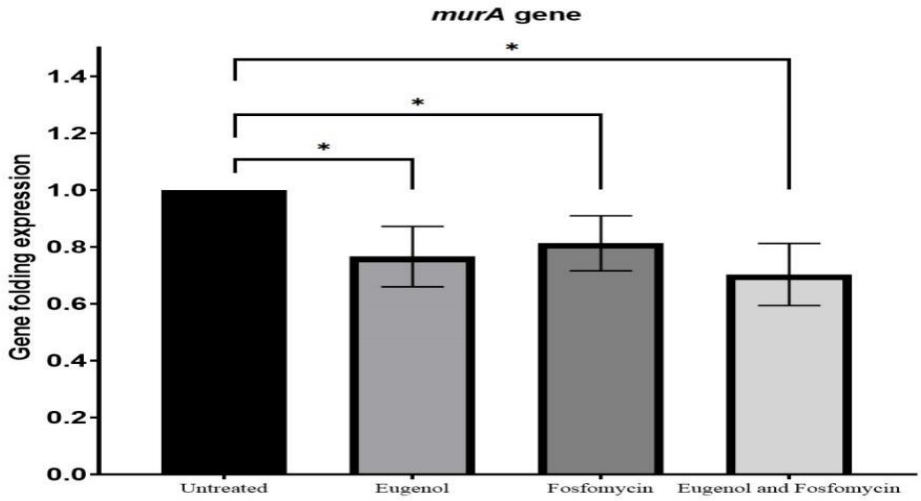

Figure 7: The mean gene folding expression for *murA* gene in different statuses.

II- *glpT* gene (α-glycerol-3-phosphate transporter)

The results of the effects of Fosfomycin and Eugenol on the expression of *glpT* gene in UPEC isolates are shown in Table 9 and Figure 8.

All treatment statuses revealed increased gene expression for the *glpT* gene compared to the control (untreated) group. However, the co-treatment of Fosfomycin with Eugenol exhibited a significant impact. Ct levels are associated with the *glpT* gene across all treatment conditions. The results of this study show that a rise in its expression corresponds to a reduction in Ct and a corresponding drop in Fosfomycin concentration in the co-treatment of Fosfomycin with Eugenol. The study observed that the co-treatment resulted in a significant upregulation of *glpT* expression, with a fold increase of 6.02.

Table 9: The mean gene folding expression for the *glpT* gene in different statuses.

Status	Mean	± Std. Deviation	F- value	*p-value*
Untreated	1	0	25.94	<0.0001*
Eugenol	1.28	0.12		
Fosfomycin	1.40	0.28		
Eugenol and Fosfomycin	6.02	2.96		

Data presented as One-way ANOVA. * Statistically significant at *p*-value ≤ 0.05.

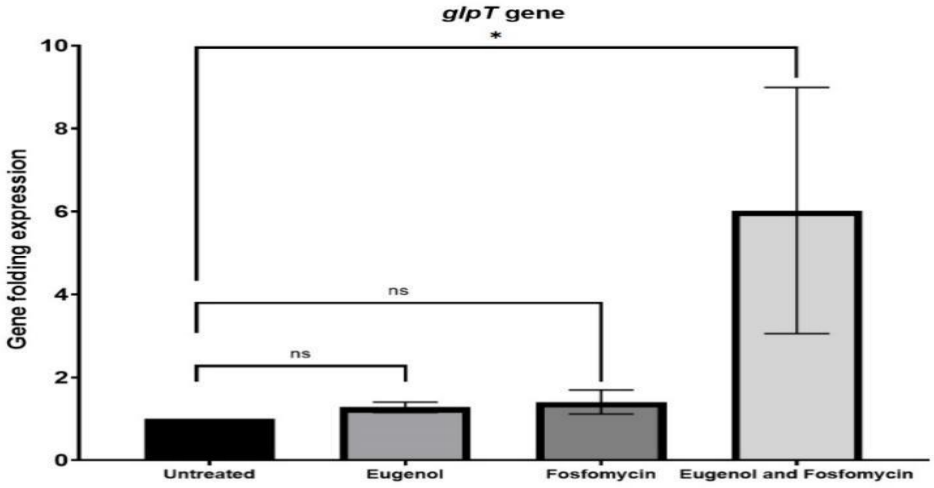

Figure 8: The mean gene folding expression for the *glpT* gene in different statuses.

III- *cyaA* gene (adenyl cyclase)

The results of the effects of Fosfomycin and Eugenol on the expression of *cyaA* gene in UPEC isolates are shown in Table 10 and Figure 9.

The findings about gene folding expression of the *cyaA* gene across all treatment conditions indicated a notable increase in gene expression relative to the control (untreated) group. However, the co-treatment of Fosfomycin with Eugenol exhibited a significant impact. The Ct values of the *cyaA* gene across different treatment statuses reveal a notable correlation between the reduction in Ct and the decline in Fosfomycin concentration upon the co-treatment with Fosfomycin and Eugenol. This correlation suggests a concurrent elevation in the expression of the *cyaA* gene. The study observed that the co-treatment of Fosfomycin with Eugenol resulted in a significant upregulation of *cyaA* expression, with a fold increase of 2.72.

Table 10: The mean gene folding expression for *cyaA* gene in different statuses.

Status	Mean	± Std. Deviation	F- value	*p-value*
Untreated	1	0		
Eugenol	1.52	0.42		
Fosfomycin	1.69	0.45	11.38	<0.0001*
Eugenol and Fosfomycin	2.72	1.19		

Data presented as One-way ANOVA. * Statistically significant at p-value ≤ 0.05.

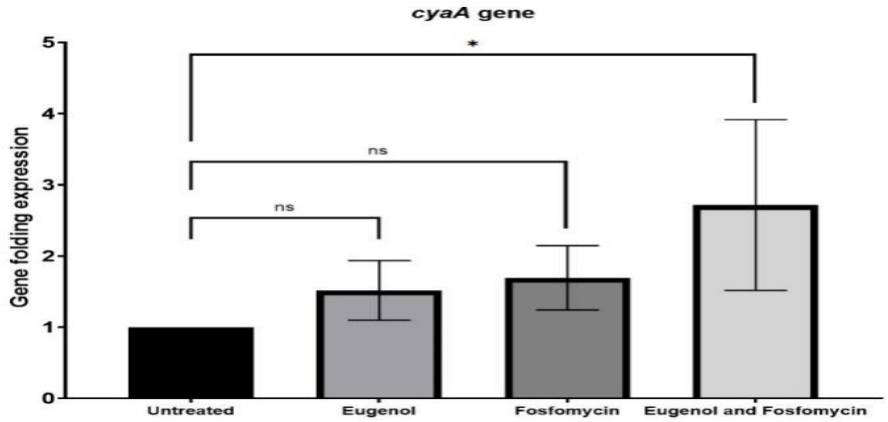

Figure 9: The mean gene folding expression for the *cyaA* gene in different statuses.

Analysis of RNA expression using techniques like real-time PCR has traditionally used reference or housekeeping genes to control for errors between samples [48]. However, the development of resistance to Fosfomycin is mainly attributed to mutations in the *murA* gene, as well as genetic mutations in the glycerol-3-phosphate transporter (glpT) gene and upregulation of transportation systems (*cyaA*) gene [7, 49, 50]. Modifying the antibiotic MurA (UDP-N-acetylglucosamine enolpyruvyl transferase) target is a prevalent mechanism leading to Fosfomycin resistance. This method involves the irreversible binding of the antibiotic to the protein, hence rendering the enzyme inactive. Resistance to the antibiotic Fosfomycin can be shown in *E. coli* when a mutation occurs in the Fosfomycin-binding region of MurA, specifically at the amino acid residue Cys115 [51].

The crystal structures of some significant facilitator superfamily (MFS) transporters have been characterized. The first structures were of the glycerol 3-phosphate/phosphate exchanger *glpT* [52]. MFS family members play an essential role in drug transport or drug resistance. Resistance to the Fosfomycin antibiotic frequently occurs due to mutations in the MFS genes [53].
Any mutations within the genes encoding *cyaA* result in a notable reduction in intracellular cyclic adenosine monophosphate (cAMP) levels and, consequently, a subsequent decrease in the synthesis of Fosfomycin transporters [54].

Conclusions

In conclusion, the findings of this study underscore the gravity of antibiotic resistance, as a staggering percentage of UPEC isolates displayed resistance to multiple antibiotic classes, leaving limited treatment options. However, Fosfomycin and amoxicillin-clavulanate emerged as promising, with notably lower resistance rates. The experimental approaches employed in this study, including agar well diffusion, MIC, MBC, FICI, and time-kill curves, collectively demonstrated the substantial synergistic bactericidal actions of Fosfomycin and Eugenol. These results suggest that co-treatment with these compounds can effectively combat UPEC, potentially offering a viable therapeutic strategy. Furthermore, the molecular assays examining genomic Fosfomycin resistance genes (*murA*, *glpT*, and *cyaA*) and their expression levels under different treatments shed light on the genetic aspects of resistance and its modulation. The presence of these resistance genes in all isolates, combined with variable gene expression changes in response to treatments, adds depth to our understanding of resistance mechanisms.

Conflict of interest statement

The authors declare that they have no conflict of interest in the publication.

References

[1] G. M. De Souza, E. R. D. S. Neto, A. M. da Silva, M. V. M. D. S. Iacia, M. V. P. Rodrigues, V. Cataneli Pereira, and L. K. Winkelstroter, "Comparative study of genetic diversity, virulence genotype, biofilm formation and antimicrobial resistance of uropathogenic Escherichia coli (UPEC) isolated from nosocomial and community acquired urinary tract infections," *Infect Drug Resist*, pp. 3595–3606, 2019.

[2] J. Geurtsen, M. de Been, E. Weerdenburg, A. Zomer, A. McNally, and J. Poolman, "Genomics and pathotypes of the many faces of Escherichia coli," *FEMS Microbiol Rev*, vol. 46, no. 6, p. fuac031, 2022.

[3] A. Lalezadeh, P. Ghotaslou, and R. Ghotaslou, "The Detection of Fosfomycin-Modifying Enzymes (fos) in Uropathogenic Enterobacterale, Azerbaijan, Iran," *Canadian Journal of Infectious Diseases and Medical Microbiology*, vol. 2023, 2023.

[4] A. Goer L. S. Blanchard, A. Van Belkum, K. J. Loftus, T. P. Armstrong, S. G. Gatermann, D. Shortridge, B. J. Olson, J. K. Meece, T.R. Fritsche, and M. Pompilio, "Multicenter Evaluation of the Novel ETEST Fosfomycin for Antimicrobial Susceptibility Testing of Enterobacterales, Enterococcus faecalis, and Staphylococcus Species," *J Clin Microbiol*, vol. 60, no. 7, pp. e00021-22, 2022.

[5] L. Shan, Q. Wenling, P. Mauro, and B. Stefano, "Antibacterial agents targeting the bacterial cell wall," *Curr Med Chem*, vol. 27, no. 17, pp. 2902–2926, 2020.

[6] D. Zheng, P. J. Bergen, C. B. Landersdorfer, and E. B. Hirsch, "Differences in Fosfomycin Resistance Mechanisms between Pseudomonas aeruginosa and Enterobacterales," *Antimicrob Agents Chemother,* vol. 66, no. 2, pp. e01446-21, 2022.

[7] C. El-Khoury, E. Mansour, Y. Yuliandra, F. Lai, B. A. Hawkins, J.J. Du, E. J. Sundberg, N. Sluis-Cremer, D. E. Hibbs, and P. W. Groundwater, "The role of adjuvants in overcoming antibacterial resistance due to enzymatic drug modification," *RSC Med Chem*, vol. 13, no. 11, pp. 1276–1299, 2022.

[8] S. Sreekumar and N. C. Nisha, "Plants are renewable source of medicine and nanoparticles: A review," *Current Aspects in Pharmaceutical Research and Development*, vol. 8, pp. 44–56, 2022.

[9] A. M. Teles, J. V. Silva-Silva, J. M. P. Fernandes, A. L. Abreu-Silva, K. D. S. Calabrese, Mendes N. E. Filho, and F. Almeida-Souza, "GC-MS characterization of antibacterial, antioxidant, and antitrypanosomal activity of Syzygium aromaticum essential oil and eugenol," *Evidence-Based Complementary and Alternative Medicine*, vol. 2021, pp. 1–12, 2021.

[10] A. B. Eldin, M. Ezzat, M. Afifi, O. Sabry, and G. Caprioli, "Herbal medicine: the magic way crouching microbial resistance," *Nat Prod Res*, pp. 1–10, 2023.

[11] M. Ulanowska and B. Olas, "Biological Properties and prospects for the application of eugenol—A review," *Int J Mol Sci*, vol. 22, no. 7, p. 3671, 2021.

[12] S. Hemaiswarya and M. Doble, "Synergistic interaction of eugenol with antibiotics against Gram negative bacteria," *Phytomedicine*, vol. 16, no. 11, pp. 997–1005, 2009.

[13] CLSI, Performance standards for antimicrobial susceptibility testing, CLSI supplement M100. Clinical and Laboratory Standards Institute Wayne, PA, 2022.

[14] J. V. DeFrancesco, "Extraction and Analysis of Eugenol from Cloves," *The Journal of Forensic Science Education*, vol. 3, no. 1, 2021.

[15] I. F. A. Karm, "Investigation of active compound in clove (Syzygium aromaticum) extrac and compared with inhibitors of growth of some types of bacteria causing food poisoning," *Iraqi Journal of Agricultural Sciences*, vol. 50, no. 6, 2019.

[16] C. Valgas, S. M. de Souza, E. F. A. Smânia, and A. Smânia Jr, "Screening methods to determine antibacterial activity of natural products," *Brazilian journal of microbiology*, vol. 38, pp. 369–380, 2007.

[17] Q. S. Atwan and N. H. Hayder, "Eco-friendly synthesis of Silver nanoparticles by using green method: Improved interaction and application in vitro and in vivo," T*he Iraqi Journal of Agricultural Science*, vol. 51, pp. 201–216, 2020.

[18] B. L. Deatherage, J. C. Lara, T. Bergsbaken, S. L. R. Barrett, S. Lara, and B. T. Cookson, "Biogenesis of bacterial membrane vesicles," *Mol Microbiol*, vol. 72, no. 6, pp. 1395–1407, 2009.

[19] A. A. Majeed and M. A. Al-Aubydi, "Assessment the Modulation effect of using Green synthesis ZnO NPs against Multidrug Resistant Klebsiella pneumoniae isolated from respiratory tract infection," *Iraqi Journal of Science*, pp. 1221–1231, 2019.

[20] C. Duque, A. C. A. Souza, K. L. Aida, J. A. Pereira, K. S. Caiaffa, V. R. D. Santos, and A. Prakki, "Synergistic antimicrobial potential of EGCG and fosfomycin against biofilms associated with endodontic infections," *Journal of Applied Oral Science*, vol. 31, p. e20220282, 2023.

[21] S. A. Fayyad, M. R. Majeed, and S. S. Mahmoud, "Evaluation of synergistic effect of nicotinic acid with imipenem as antibiofilm for clinical Pseudomonas Aeruginosa isolates," *Iraqi Journal of Science*, pp. 50–56, 2019.

[22] H. H. H. Al-hamedawy and S. S. Mahmoud, "Synergistic Effect of Linezolid, Tigecycline, and Vancomycin on Staphylococcus Aureus Isolated From Iraqi Patients with Diabetic Foot Ulcers," *Iraqi Journal of Science*, pp. 36–42, 2019.

[23] J. J. Gaudereto, L. V. P. Neto, G. C. Leite, E. P. S. Espinoza, R. C. R. Martins, G. Villas Boa Prado, and S. F. Costa, "Comparison of methods for the detection of in vitro synergy in multidrug-resistant gram-negative bacteria," *BMC Microbiol*, vol. 20, pp. 1–7, 2020.

[24] Y. Wang, L. Kong, J. Liu, and H. Ma, "Synergistic effect of eugenol with Colistin against clinical isolated Colistin-resistant Escherichia coli strains," *Antimicrob Resist Infect Control*, vol. 7, no. 1, pp. 1–9, 2018.

[25] O. Z. Al-Hayali, M. F. A. L. Marjani, and A. Maleki, "Evaluation of Rosmarinus officinalis Leaves Essential Oils Activity Against Vancomycin Intermediate Staphylococcus aureus (VISA) Isolated from Baghdad Hospital Patients," *Iraqi Journal of Science*, pp. 2153–2167, 2023.

[26] M. Bland, *An introduction to medical statistics*. *Oxford university press*, 2015.

[27] L. Gay, C. Melenotte, I. Lakbar, S. Mezouar, C. Devaux, D. Raoult, and J. L. Mège, "Sexual dimorphism and gender in infectious diseases," *Front Immunol*, vol. 12, p. 698121, 2021.

[28] G. E. Ashuntantang, V. D. Garovic, I. P. Heilberg, and L. Lightstone, "Kidneys and women's health: key challenges and considerations," *Nat Rev Nephrol*, vol. 14, no. 3, pp. 203–210, 2018.

[29] M. Mantzorou and C. Giaginis, "Cranberry consumption against urinary tract infections: clinical state-of-the-art and future perspectives," *Curr Pharm Biotechnol*, vol. 19, no. 13, pp. 1049–1063, 2018.

[30] H. Yazdi, C. Restrepo, C. Foltz, M. Hammad, P. H. Chung, L. G. Gomella, and J. Parvizi, "Symptomatic benign prostatic hyperplasia: a risk factor for periprosthetic joint infection in male patients," *JBJS*, vol. 102, no. 7, pp. 543–549, 2020.

[31] N. N. Omar and R. K. Mohammed, "A Molecular Study of Toxic Shock Syndrome Toxin gene (tsst-1) in β-lactam Resistant Staphylococcus aureus Clinical Isolates," *Iraqi Journal of Science*, pp. 825–837, 2021.

[32] R. K. Mohammed and A. A. Ibrahim, "Analysis of Nucleotide Sequences Similarity and Protein Prediction of Some Resistance Genes in Escherichia coli Isolated from Iraqi Patients with Urinary Tract Infections," *Arch Razi Inst*, vol. 77, no. 3, 2022.

[33] J. Josephs-Spaulding, T. J. Krogh, H. C. Rettig, M. Lyng, M. Chkonia, S. Waschina, and C. Kaleta, "Recurrent urinary tract infections: unraveling the complicated environment of uncomplicated rUTIs," *Front Cell Infect Microbiol*, vol. 11, p. 562525, 2021.

[34] R. K. Mohammed and A. A. Ibrahim, "Distribution of dfrA1 and cat1 antibiotic resistance genes in uropathogenic Escherichia coli isolated from teens pregnant women in Iraq," *Iraqi Journal of Science*, pp. 3340–3353, 2022.

[35] A. A. Muhaimeed and A. M. Ghareeb, 'Prevalence of Multi-Antibiotic Resistance marAand Quorum Sensing luxS Genes and Evaluation of Biofilm Formation in Uropathogenic Escherichia coli', *Iraqi Journal of Biotechnology*, vol. 22, no. 1, pp. 252–261, 2023.

[36] H. A. Salman, A. kamil Alhameedawi, S. M. S. G. Muhamad, and Z. Taha, "Prevalence of Multi-Antibiotic Resistant Bacteria Isolated from Children with Urinary Tract Infection from Baghdad, Iraq," *Microbiol Biotechnol*, vol. Lett 2022, no. 50, pp. 147–156, 2022.

[37] W. Adamus-Białek, A. Baraniak, M. S. Wawszczak, Głuszek, B. Gad, K. Wróbel, and P. Parniewski, "The genetic background of antibiotic resistance among clinical uropathogenic Escherichia coli strains," *Mol Biol Rep*, vol. 45, pp. 1055–1065, 2018.

[38] Y. Wan, E. Mills, R. C. Leung, A. Vieira, X. Zhi, N. J. Croucher, and S. Sriskandan, "Alterations in chromosomal genes nfsA, nfsB, and ribE are associated with nitrofurantoin resistance in Escherichia coli from the United Kingdom," *Microb Genom*, vol. 7, no. 12, 2021.

[39] R. Urban-Chmiel, A. Marek, D. Stępień-Pyśniak, K. Wieczorek, M. Dec, A. Nowaczek, and J. Osek, "Antibiotic resistance in bacteria—A review," *Antibiotics*, vol. 11, no. 8, p. 1079, 2022.

[40] C. Chen, Y. Wang, H. Nakaminami, E. S. Kim, G. A. Jacoby, and D. C. Hooper, "Escherichia coli GyrA Tower Domain Interacts with QnrB1 Loop B and Plays an Important Role in QnrB1 Protection from Quinolone Inhibition," *Antimicrob Agents Chemother*, vol. 65, no. 7, pp. 10–1128, 2021.

[41] M. R. A. Karam, M. Habibi, and S. Bouzari, "Urinary tract infection: Pathogenicity, antibiotic resistance and development of effective vaccines against Uropathogenic Escherichia coli," *Mol Immunol*, vol. 108, pp. 56–67, 2019.

[42] A. Wróbel, K. Arciszewska, D. Maliszewski, and D. Drozdowska, "Trimethoprim and other nonclassical antifolates an excellent template for searching modifications of dihydrofolate reductase enzyme inhibitors," *J Antibiot (Tokyo)*, vol. 73, no. 1, pp. 5–27, 2020.

[43] M. Sánchez-Osuna, P. Cortés, M. Llagostera, J. Barbé, and I. Erill, "Exploration into the origins and mobilization of di-hydrofolate reductase genes and the emergence of clinical resistance to trimethoprim," *Microb Genom*, vol. 6, no. 11, 2020.

[44] S. A. Ali and H. O. M. Al-Dahmoshi, "Detection of Efflux Pumps Gene and Relation with Antibiotics Resistance in Uropathogenic Escherichia Coli (UPEC) Isolated from Patients with Cystitis," *Iraqi Journal of Science*, pp. 2388–2397, 2022.

[45] H. H. Al-Hasnawy, M. R. Judi, and H. J. Hamza, "The dissemination of multidrug resistance (MDR) and extensively drug resistant (XDR) among uropathogenic E. coli (UPEC) isolates from urinary tract infection patients in Babylon Province, Iraq," *Baghdad Science Journal*, vol. 16, no. 4 Supplement, pp. 922–986, 2019.

[46] G. E. Jeyakumar and R. Lawrence, "Mechanisms of bactericidal action of Eugenol against Escherichia coli," *J Herb Med*, vol. 26, p. 100406, 2021.

[47] S. F. Hamed, Z. Sadek, and A. Edris, "Antioxidant and antimicrobial activities of clove bud essential oil and eugenol nanoparticles in alcohol-free microemulsion," *J Oleo Sci*, vol. 61, no. 11, pp. 641–648, 2012.

[48] M. R. Yourick, B. A. Sandkam, W. J. Gammerdinger, D. Escobar-Camacho, S. P. Nandamuri, F. E. Clark, and K. L. Carleton, "Diurnal variation in opsin expression and common housekeeping genes necessitates comprehensive normalization methods for quantitative real-time PCR analyses," *Mol Ecol Resour*, vol. 19, no. 6, pp. 1447–1460, 2019.

[49] K. Zurfluh, A. Treier, K. Schmitt, and R. Stephan, "Mobile fosfomycin resistance genes in Enterobacteriaceae—an increasing threat," *Microbiologyopen*, vol. 9, no. 12, p. e1135, 2020.

[50] I. Portillo-Calderón, M. Ortiz-Padilla, J. M. Rodríguez-Martínez, B. de Gregorio-Iaria, J. Blázquez, J. Rodríguez-Baño, and F. Docobo-Pérez, "Contribution of hypermutation to fosfomycin heteroresistance in Escherichia coli," *Journal of Antimicrobial Chemotherapy*, vol. 75, no. 8, pp. 2066–2075, 2020.

[51] C. Dos Santos, L. S. Dos Santos, and O. L. Franco, "Fosfomycin and nitrofurantoin: classic antibiotics and perspectives," *J Antibiot (Tokyo)*, vol. 74, no. 9, pp. 547–558, 2021.

[52] S. Sauve, J. Williamson, A. Polasa, and M. Moradi, "Ins and Outs of Rocker Switch Mechanism in Major Facilitator Superfamily of Transporters," *Membranes (Basel)*, vol. 13, no. 5, p. 462, 2023.

[53] S. Sharma, V. Kaushik, M. Kulshrestha, and V. Tiwari, "Different Efflux Pump Systems in Acinetobacter baumannii and Their Role in Multidrug Resistance," *Advances in Microbiology, Infectious Diseases and Public Health*, vol. 17, pp. 155–168, 2023.

[54] G. C. Leite, L. V Perdigão-Neto, R. C. R. Martins, C. Rizek, A. S. Levin, and S. F. Costa, "Genetic factors involved in fosfomycin resistance of multidrug-resistant Acinetobacter baumannii," *Infection, Genetics and Evolution*, vol. 93, p. 104943, 2021.