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The Effect of *Citrus reticulata* and *Rheum rhabarbarum* on Gene Expression of *fimB* and *fimE* Genes in *Escherichia coli* Isolates Taken from Iraqi Prostate Patients

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Abstract: *Citrus reticulata essential oil Rheum rhabarbarum* (rhubarb) extract as herbal alternatives to conventional antibiotics in combating multidrug-resistant (*MDR*) *Escherichia coli* isolated from patients with benign prostatic hyperplasia (BPH) suffering from urinary tract infections (UTIs). A total of 120 urine samples were collected from male patients (aged 45–87), from which 29 *E. coli* strains were identified. Among them, six isolates (E1, E3, E9, E14, E16, and E18) showed strong antibiotic resistance and high biofilm-forming ability.*C. reticulata* oil exhibited strong antibacterial activity, fully inhibiting all six MDR *E. coli* isolates at 10,000 and 20,000 ppm, while rhubarb extract showed no inhibition.Biofilm production by the most virulent isolates significantly decreased when treated with *C. reticulata* oil ($P \leq 0.01$).Gene expression analysis revealed that citrus oil downregulated fimE (a gene that suppresses fimbriae expression) and upregulated fimB (a gene that activates fimbriae), suggesting a dual role in modulating bacterial adhesion mechanisms.Gas chromatography–mass spectrometry (GC-MS) analysis showed that the main active compound in citrus oil was D-Limonene (76.17%), which is known for its antimicrobial properties. *C. reticulata* essential oil demonstrates significant potential as a natural antimicrobial and antibiofilm agent against MDR *E. coli*, offering a promising approach for treating UTIs in patients with BPH, especially in the face of increasing antibiotic resistance.

Keywords: Citrus oil, rhubarb extract, *E. coli*, biofilm formation.

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1. Introduction

Prostate enlargement is also known as benign prostatic hyperplasia (BPH), when tissue around the prostate gland grows larger, it squashes the prostate gland and makes it harder to urinate, This enlargement may also slow down urine flowing out from the bladder, trapping some urine in it, or can cause bladder or kidney damage [1].

Benign prostatic hyperplasia (BPH) can cause urinary retention, increasing the risk of urinary tract infections (UTIs) in men. Both conditions often coexist in older adults, with each potentially worsening the other. Understanding their interaction is key for effective diagnosis and treatment [2].

A urinary tract infection (UTI) is an infection that affects any part of the urinary tract, including the kidneys, ureters, bladder, and urethra. [3]. UTIs rank as the second most frequent type of infectious disease, with an estimated 150 million cases diagnosed annually

worldwide. Despite antibiotic use in UTI management, the socioeconomic burden associated with recurrent UTIs (rUTIs) is considerable [4].

Uropathogenic *Escherichia coli* (UPEC)-induced UTIs begin at the urethra, where the bacteria adhere to the epithelial cells and colonize the bladder after ascending through the ureters to reach the kidneys [5].

E. coli is a facultative anaerobe and can grow in a wide range of temperatures with an optimum temperature of 37°C. *E. coli* is the most numerous facultative anaerobe presenting in the lower intestinal tract of birds and mammals. *E. coli* is genetically diverse and includes both commensal strains and pathogenic strains. [6].

According to the increasing of bacterial resistance against antibiotics, essential oils are the positive healthy options to control diseases caused by these resistant microbes. Citrus reticulata, popularly known as mandarin, is a subtropical fruit of the Rutaceae family [7]. Chemical composition plays a key role in flavor and aroma, making it an integral part of food and other products. It is important to study the chemical composition to understand the utility of *C. reticulata* in various applications [8].

Another active medicinal plant is *Rheum rhabarbarum,* commonly known as rhubarb, is a fascinating plant species, *Rheum rhabarbarum* contains various bioactive compounds contributing to its functional properties. Phytochemicals found in *R. rhabarbarum* include flavonoids, anthraquinones, organic acids, tannins, catechins, and other compounds. These phytochemicals account for the functional properties of rhubarb [9]. Some flavonoids such as quercetin, kaempferol, and their glycosides have antioxidant, anti-inflammatory, antibacterial, antiviral, and hepato-protective properties [10]. The aim of this study is to investigate herbal alternatives to antibiotics from the Citrus reticulata essential oil and *Rheum rhabarbarum* extract in order to control *E. coli* isolated from UTI. Control type 1 fimbriae expression by *fimB* and *fimE* are site-specific recombinases in *E. coli* that which are essential for adhesion and virulence [11]. They regulate the inversion of the *fimS* promoter, switching fimbrial expression ON or OFF in response to factors like temperature and host signals [12]. Generally fimB promotes the ON phase, while *fimE* promotes the OFF phase [13]. These genes also interact with broader regulatory networks beyond the *fim* operon [14].Their activity is crucial for *E. coli* pathogenesis in urinary tract and extraintestinal infections [15].

2. Materials and method

2.1 Bacterial collection and isolation

Samples were collected from five hospitals in Baghdad during November–December 2022 and July–September 2023, with ethical approval.A total of 120 bacterial isolates were obtained from the urine of patients have prostates and UTI were age between (45 – 87) years. with benign prostatic hyperplasia (BPH) under sterile, cooled conditions.The isolates were cultured on various agar media and incubated for 18–24 hours at 37 °C under aerobic conditions.All isolates underwent identification tests, including morphological examination and VITEK 2 system [16].

2.2Antibiotic Sensitivity Test by vitik2

Antibiotic susceptibility was tested using the VITEK 2 Compact system from bioMérieux. Pure colonies grown on blood or MacConkey agar were suspended in sterile saline to match a

0.5 McFarland standard. The suspension was loaded into AST cards for Gram-positive or Gram-negative bacteria.Cards were automatically sealed, incubated, and read by the device. Results were reported as Susceptible, Intermediate, or Resistant according to CLSI uidelines.

2.3 Collection and extraction of plants

The plant specimens used in this study consisted of the above-ground portions of peel of *Citrus reticulata* (Iraqi) and *Rheum rhabarbarum* roots, which were obtained from a local market in Baghdad. Following collection, the plants underwent a thorough cleaning process, including rinsing with tap water, and were subsequently air-dried at room temperature. They were then stored in pristine conditions until they were ready for use.

In a round-bottomed flask, 100 g of dried sample of C. reticulata with 1 L of D.W was placed, then subordinated to hydro distillation for 2 hours by heating to 60 ºC by using the Clevenger apparatus, the aqueous stage was divided into two layers: the under layer included water was neglected, while the toper layer involved the oils gathered, after that stored in a refrigerator in a glass vials were locked and marked until used [17]. The alcoholic extract of rhubarb is prepared using soxhlet apparatus. The root was collected from a local market in Baghdad,50 gm. of Rheum root were extracted with ethanol 250 ml (70%) for seven-hour. The extract was dried and kept [18].

Subsequently, the extracts was stored at a temperature of 4º C until it was ready for utilization. To create the stock solution, the concentrated oil and alcoholic extracts were combined with Dimethyl sulfoxide (DMSO) and diluted. Subsequently, various concentrations (20000 ppm, 10000 ppm and 5000 ppm) were prepared by mixing specific volumes of the stock solution with specific volumes of DMSO.

2.3.1. Determination of minimal inhibitory concentration of plant extracts (MIC)

The agar dilution method was used to determine the MIC of four plant-based substances against E. coli, following CLSI guidelines.Essential oils and plant extract were dissolved in DMSO, with stock solutions prepared at 500,000 ppm.Dilutions ranging from 20,000 to 5,000 ppm were incorporated into Müller-Hinton agar.Plates were inoculated with *E. coli* and incubated at 37°C for 24 hours. MIC was defined as the lowest concentration showing no visible bacterial growth [19].

2.3.2. Preparation of 1/2 MIC Concentration

To evaluate the sub-inhibitory effects of *Citrus reticulata* essential oils, and *Rheum rhabarbarum* extract, a 1/2 MIC concentration was prepared following the agar dilution technique as described by CLSI guidelines (CLSI, M07-A9). After determining the MIC value, the 1/2 MIC was calculated and obtained through serial dilution of a 500,000 ppm stock solution (prepared by dissolving each oil or extract in DMSO). The diluted concentration was incorporated into molten Müller-Hinton agar, poured into sterile Petri dishes, and used in subsequent experiments to assess sub-inhibitory effects.

2.3.3. Investigate the phytochemical compounds of the active extract using GC-MS technique

The Chemical Analysis Center in Iraq scientific laboratories conducted Gas Chromatography-Mass (GC-MS) analysis on the active extract using a Shimadzu gas chromatograph. The

procedure for this analysis was performed in the same manner.

2.4 Biofilm formation

Detection of biofilm formation phenotypically was done by using the microtiter plate method according to the method described by Hassan et al [21]. Biofilm formation by (29) *E. coli* isolates was evaluated on 96-well polystyrene plates using LB broth with 1% glucose.Plates were incubated at 37°C for 24 hours, then washed with PBS and fixed with methanol.Wells were stained with 0.1% crystal violet, washed, dried, and destained using 33% glacial acetic acid.Optical density at 600 nm was measured to quantify biofilm, and results were classified based on a cut-off value.Isolates showing strong biofilm formation were chosen for further testing.

2.5 Molecular study

2.5.1 . Extraction of bacterial DNA

The genomic DNA of the twenty *E. coli* isolates that strong biofilm producing was extracted using Kit, as indicated in Table (2-1). The kit's manufacturer protocol was followed (Geneaid™ DNA Extraction Kit, Geneaid Biotech Ltd / Taiwan), wherein DNA was extracted from fresh bacterial cultures.

Table (2-1): The primers used in this study

Genes	Oligonucleotide Primer	Sequence (5'→3')
fimE	**Forward**	**GCAACGGGAGCCAGAGATTA**
	Reverse	**CAGTTAGCACGTTCCTGGGT**
fimB	**Forward**	**TCCGGGCGAGTGAAATTTGT**
	Reverse	**GGTGCGTTGTTGAAAAGCCT**
GAPDH/HKG	**Forward**	**ACTTACGAGCAGATCAAAGC**
	Reverse	**AGTTTCACGAAGTTGTCGTT**

2.5.2. Conventional PCR study

A. Primer Preparation

The primers used in this study were designed manually using sequence data obtained from NCBI GenBank and analyzed with appropriate bioinformatics tools.The primers listed in Table 3-4 were received lyophilized and dissolved in nuclease-free water at 100 pmol/µl. A working solution was prepared by diluting 10 µl of primer with 90 µl of nuclease-free water to obtain 10 pmol/µl. Both stock and working solutions were stored in a freezer until PCR amplification.

B. PCR amplification

PCR reactions were prepared in 20 µl volumes by mixing PCR premix, 2 µl of each primer, and 3 µl of DNA template.Nuclease-free water was added to complete the volume, and a negative control lacking DNA was included.Reactions were run in a thermocycler using the conditions shown in Table (2-2) for amplifying fimB and fimE genes.

Table (2-2):Program used to amplify *fimB* and *fimE* genes.

Stage	Temperature	Time	Cycle
Initial denaturation	94	5 min	1
Denaturation	94	30 second	35
Annealing	58	30 second	
Extension	72	30 second	
Final extension	72	5 min	1

C. Agarose gel electrophoresis

Agarose gel electrophoresis was used to confirm gene presence and PCR specificity following Green and Sambrook [22]. A 1% agarose gel was prepared in TBE buffer, stained with ethidium bromide, and poured into a casting tray.PCR products mixed with loading dye were loaded alongside a

DNA ladder, electrophoresed, and visualized under UV light.

2.5.3 One step quantitative real time PCR assay (qRT-PCR)

A. Reaction setup and thermal cycling protocol

The components of the RT-PCR mixtures are listed in the table below (2-3). The housekeeping gene GAPDH was used as an internal control.

Table (2-3): The components of the RT-PCR mixtures

Component	Volume (µl)	Final concentration
Luna Universal One-Step Reaction Mix (2X)	10	1X
Luna Warm Start ® RT Enzyme Mix (20X)	1	1X
Forward primer (10 pmol/µl)	0.8	0.4 µM
Reverse primer (10 pmol/µl)	0.8	0.4 µM
Template RNA	variable	20 ng/ul
Nuclease-free water	to 20	

B. Quantitative real time PCR (qRT PCR)

Quantitative real-time PCR was performed as follows:

1. All reaction components were thawed at room temperature and then placed on ice. Once fully thawed, each component was briefly mixed by inversion, pipetting, or gentle vortexing.
2. All components were added to and combined in a sterile PCR tube, then thoroughly but gently mixed by pipetting or vortexing.
3. The RNA template was added to the qPCR tubes, which were then sealed with flat, optically transparent caps.
4. The tubes were briefly spun to remove bubbles (1 minute at 2,500–3,000 x g) and then placed in the thermocycler.
5. The thermocycler was programmed and run according to the protocol outlined below table (2-4).

Table (2-4): (qRT-PCR) program used in the study

CYCLE STEP	TEMPERATURE (°C)	TIME	CYCLES
Reverse Transcription	55	10 minutes	1
Initial Denaturation	95	1 minute	1
Denaturation	95	10 seconds	40-45
Extension	60	30 seconds	
Melt Curve	60-95	various	1

A melting curve was generated with temperatures ranging from 95°C to 60°C, increasing by 0.5°C every 15 seconds.

C. Data analysis of qRT-PCR

Normalization of the data was achieved by referencing the GAPDH housekeeping gene. Subsequently, gene expression levels were quantified as fold changes using the ΔΔCT method described by Livak and Schmittgen [23]. Gene expression levels were calculated using a series of equations based on this methodology. For each sample, including the calibrator and unknown samples, the difference in threshold cycle values (ΔCT) between the target gene and the reference gene was determined.

ΔCT = CT target gene – CT reference gene… (1)

The ΔΔCT value was calculated by determining the difference between the ΔCT value of the unknown sample and the ΔCT value of the calibrator sample.

ΔΔCT = ΔCT sample – ΔCT Calibrator … (2)

The normalized amount of the target gene in each sample was adjusted using the formula $2^{-\Delta\Delta CT}$. This adjusted

value was then used to compare and contrast the expression levels across the various samples.

Fold change = 2-ΔΔCT … (3)

Changes in the relative expression of mRNA levels were assessed using the comparative threshold cycle (CT) value method. This approach involved computing the differences in gene expression levels between the isolates using the 2-ΔΔCT formula.

3. Results and Discussion

3.1 Isolation and identification of bacteria

One hundred and twenty of bacterial isolates from the beginning of November- December 2022 to July, August-September 2023. Bacterial isolates were collected from fife hospitals in Baghdad, in patients have prostates and UTI were age between (45 – 87) years. For primary identification of isolates was depended on morphological colony (color, shape, size, and texture) appeared on different agar culture media incubated at 37 °C for 24 hr., bacterial isolates were identified initially by conventional bacterial culturing procedures (morphological check on selective and differential cultures media) as ABC streaking method on culture medium, Vitek 2 system. Common bacterial isolates were *E. coli* (29 isolate).

Generally, the results of studies were somewhat similar to other studies of Atala [24] that reported respectively *S . aureus* bacteria was isolated from patients with UTI in Iraqian hospitals with diffrerent resistant for antibiotics, and the results appeared use the same identification and Phylogenetic tree between local isolates as reported by Majeed *et al* [25].

3.2 Antibiotic susceptibility test of resistance bacteria

The antimicrobial susceptibility of all isolates was evaluated using VITIK, as detailed by the Clinical and Laboratory Standards Institute [26], for the antibiotic susceptibility test. Each bacterial isolate that was collected participated in the antibiotic susceptibility assay. Presently, identifying effective treatments for antibiotic-resistant bacteria presents a considerable challenge. As shown in the results below figure (3-1) and table (3 - 1), the *E. coli* isolates that exhibit the highest levels of multidrug resistance are 1, 3, 9, 14, 16, and 18.

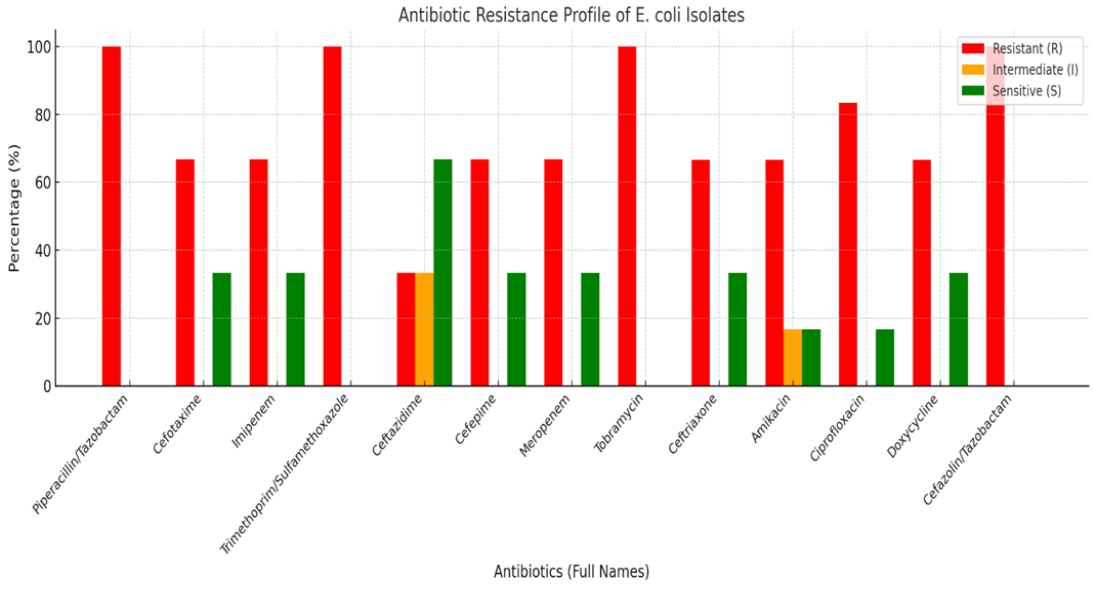

Figure (3-1): Antibiotic resistance profile of *E. coli* isolates by vitik2

Table (3-1): Inhibition effect of different antibiotics against *E coli* resistant isolates.

E.coli No.		TZO	CTX	IPM	SXT	CAZ	FEP	MEM	TOB	CRO	AMK	CIP	DOX	CZT
1	R	+	+		+			+	+		+	+	+	+
	I					+								
	S			+			+							
3	R	+		+	+	+	+	+	+	+		+		+
	I										+			
	S		+							+			+	
9	R	+	+	+	+		+		+	+	+	+		+
	I							+						
	S					+							+	
14	R	+	+	+	+	+	+	+		+	+	+	+	
	I													
	S								+					+
16	R	+	+		+			+	+	+	+	+	+	+
	I					+								
	S			+			+							
18	R	+		+	+		+		+			+		+
	I										+			
	S		+			+		+		+			+	
No	R	6	4	4	6	2	4	4	5	4	4	6	3	5
	I	0	0	0	0	2	0	0	0	0	1	0	0	1
	S	0	2	2	0	2	2	2	1	2	1	0	3	0
%	R	100	66.70	66.70	100	33.33	66.70	66.70	83.30	66.0	66.67	100	50.00	83.33
	I	0	33.3	33.3	0	33.33	33.3	33.3	16.67	33.3	16.67	0	0	16.67
	S	0	0	0	0	33.33	0	0	0	0	16.67	0	50.00	0
P-value		0.01 **	0.01 **	0.01 **	0.01 **	1.0 NS	0.01 **	0.01 **	0.01 **	0.01 **	0.01 **	0.01 **	0.01 **	0.01 **

R= Resistant, I= Intermediate, S= Susceptible

Antibiotic resistance greatly affects public health. Most strains of *E. coli* are innocuous or beneficial. Nevertheless, some strains of pathogenic *E. coli* can lead to food and water-borne diseases in humans and animals. The table (4 -2) and figure (3-1), the *E. coli* isolates that exhibit the highest levels of multidrug resistance are 1, 3, 9, 14, 16, and 18and many pathogenic *E. coli* strains are multi-drug-resistant against conventional antibiotics, the P-value was ** (P≤0.01),to combat pathogenic drug-resistant bacteria, new bioactive agents need to be developed. Multidrug-resistant *E. coli* isolates showed complete resistance to ciprofloxacin, TZO, and TMP-SMX, similar to the findings reported by Hassan et al. [27] and Al-Tawfiq et al. [28], indicating widespread ESBL and plasmid-mediated resistance genes. Resistance to carbapenems (66.7–83.3%) was statistically significant (*P = 0.01*), consistent with Wu et al. [29], suggesting carbapenemase-producing strains such as NDM or KPC. High aminoglycoside resistance (66.7%) aligns with the study by Ramirez and Tolmasky [30], which links this to

aac(6')-Ib enzymes carried on mobile genetic elements. Biofilm-related resistance mechanisms, as discussed by Guo et al. [31], were evident, with biofilm-embedded cells being 10–1000× more resistant, in agreement with Singh et al. [32].The prioritization of MDR E. coli as a critical threat pathogen by WHO aligns with Aslam et al. [33], who emphasized the urgent need for new antimicrobial strategies.

3.3 Biofilm formation capacity of bacterial isolates.

The results revealed that were strongest biofilm producers of *E. coli* isolates; and the *P value* was significant ($P \leq 0.01$) in table (3-2).

Table (3-2): Biofilm forming capacity of bacterial isolates.

Isolate no.	Mean OD 600	Biofilm strength	Isolate code	Mean OD 600	Biofilm strength
E1	0.303	Strong	E16	0.404	Strong
E2	0.277	Moderate	E17	0.247	Moderate
E3	0.412	Strong	E18	0.259	Moderate
E4	0.277	Moderate	E19	0.362	Strong
E5	0.260	Moderate	E20	0.257	Moderate
E6	0.244	Moderate	E21	0.372	Strong
E7	0.260	Moderate	E22	0.251	Moderate
E8	0.266	Moderate	E23	0.246	Moderate
E9	0.435	Strong	E24	0.285	Moderate
E10	0.254	Moderate	E25	0.243	Moderate
E11	0.311	Strong	E26	0.296	Moderate
E12	0.332	Strong	E27	0.384	Strong
E13	0.258	Moderate	E28	0.281	Moderate
E14	0.425	Strong	E29	0.251	Moderate
E15	0.264	Moderate	C	0.065	

*** E1-E29: *E coli* isolates 1-29, C: Negative control, Cut off value: 0.0745.**

Among the 29 *E. coli* isolates, 31% were strong biofilm producers, while 69% showed moderate levels, with no weak formers—consistent with Hassan et al. [27**].** The high OD values in isolates E9, E14, and E3 suggest robust biofilm production, which correlates with increased antibiotic resistance, as reported by Guo et al. [35**].** These findings align with Singh et al. [32**],** who highlighted biofilm's role in chronic infections and treatment failure. The absence of weak producers may reflect clinical selection pressure favoring biofilm-forming phenotypes, such trends underscore the urgency for biofilm-targeted therapies, including agents that disrupt biofilm architecture., similar to Aslam et al. [34].

3.4 Minimum inhibition concentration (MIC) of essential oil and alcoholic extract against resistant bacteria

MIC test should be carrying out to determine the susceptibility of isolates. The most antibiotic resistant *E. coli* isolates were experienced to determine the minimum inhibitory concentration of both extracts, by utilizing the agar dilution process with serial concentrations of both of them (20000 ppm , 10000 ppm, 5000 ppm and control), Table (3 - 3).

Table (3-3): MIC results of of *C. reticulata* oil and *R. rhabarbarum* extract against the resistant *E. coli* isolates.

Treatments	Conc. (ppm)	*E.coli* resistant isolates					
		1	3	9	14	16	18
Citrus oil	20000	-	-	-	-	-	-
	10000	-	-	-	-	-	-
	5000	+	+	+	+	+	+
Rheum extract	20000	+	+	+	+	+	+
	10000	+	+	+	+	+	+
	5000	+	+	+	+	+	+
Control	20000	+	+	+	+	+	+
	10000	+	+	+	+	+	+
	5000	+	+	+	+	+	+
+ve: No. -ve No.		7 5	7 5	7 5	7 5	7 5	7 5
+ve: % -ve: %		58.33 41.67	58.33 41.67	58.33 41.67	58.33 41.67	58.33 41.67	58.33 41.67
P-value		0.049 *	0.049 *	0.049 *	0.049 *	0.049 *	0.049 *
* ($P \leq 0.05$).							

The results show that citrus oil at concentrations of 10,000 and 20,000 ppm completely inhibited all six multidrug-resistant *E. coli* isolates (1, 3, 9, 14, 16, and 18), while at 5,000 ppm it lost efficacy (100% survival). This suggests a clear dose-dependent antibacterial effect, which is consistent with findings by Bakkali et al. (33), who demonstrated strong antimicrobial action of citrus essential oils due to compounds like limonene and citral. In contrast, Rheum extract showed no inhibitory activity at any tested concentration; all isolates remained viable, similar to the untreated control group. This lack of effect contrasts with earlier reports on Rheum's antimicrobial properties, possibly due to strain-specific resistance or low bioactive compound concentration [22]. The statistically significant P-value (0.049) across all treatments indicates a meaningful difference in antibacterial activity between citrus oil and Rheum extract. These results support previous work by Nazzaro et al. [36], who emphasized that plant extracts vary widely in their efficacy depending on concentration, phytochemical composition, and the resistance profile of bacterial strains. Moreover, the complete inhibition by higher citrus concentrations aligns with Tao et al. [37] who noted essential oils could penetrate biofilms and alter membrane permeability in Gram-negative bacteria such as E. coli. The 41.67% inhibition across multiple treatments further emphasizes the potential of essential oils as alternative or adjunct therapies against MDR pathogens [23].

3.5 Measuring the ability of isolates to form biofilms that treatment with oil essential

The essential oils were extracted, determined the active compounds, then the MIC of oils, the synergistic effect of two oils mixture were determined and its effect on the formation of biofilms in the most strongly biofilm-forming isolates (3, 9, 14 and 16) according to result table 3-4.

Table (3-4): Measuring the ability of isolates to form biofilms that treatment with mandarin oil.

Treatment	Isolate 3	Isolate 9	Isolate 14	Isolate 16
Citrus oil	0.0851 ±0.003	0.1648 ±0.042	0.1053 ±0.012	0.1317 ±0.025
Control	0.2726 ±0.005	0.2713 ±0.001	0.2639 ±0.006	0.2844 ±0.004
LSD	0.0217 **	0.0861 **	0.0299 **	0.0513 **
P-value	0.0001	0.0011	0.0002	0.0001
** ($P \leq 0.01$).				

The results showed that Citrus oil exhibited antibiofilm effect against resistant *E. coli*, regardless of the temperature. This observation has potential applications in food safety, as the combination can be used to eliminate foodborne pathogens without the need for heat treatment. Combining essential oils could be a strategy to overcome bacterial resistance according to Scotti et al.,[38]. To date, using combinations of essential oils to maintain the antimicrobial efficacy of active ingredients against resistant *E. coli* has not been reported as study Iseppi et al., [39]. With the increasing usage of antibiotics, bacteria have developed resistance mechanisms, and foodborne pathogens have been found to be resistant to antibiotics. Simple heat treatment is often ineffective in eliminating resistant pathogens from foods according to Shi et al., [40].

3.6 Ruslt of molecular study

3.6.1 Detection of fim gene in E. coli isolates

A set of primers was used for fim gene detection in isolates of *E. coli* by using the monoplex PCR technique. The result showed that of the (1 , 3, 9 ,14 ,16 ,18) *E. coli* isolates (100 %) in Figure (3 - 2).

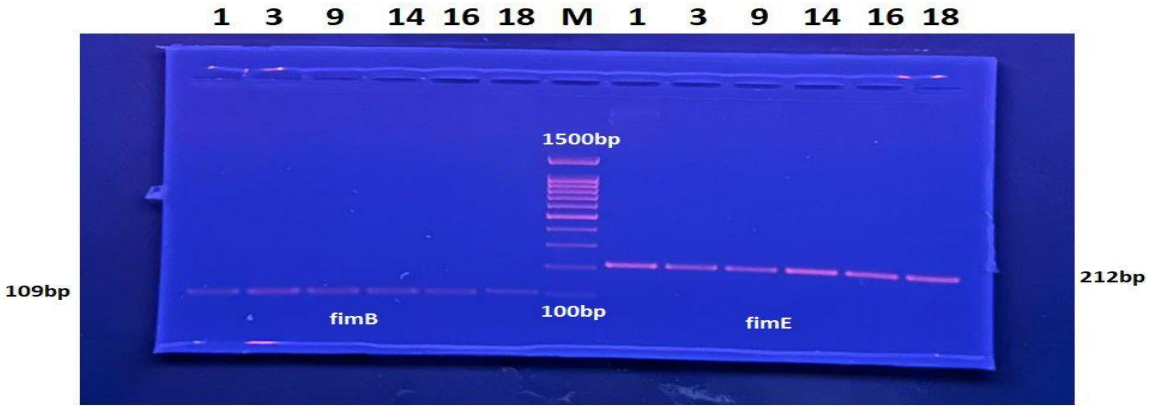

Figure (3-2): Visualization of *E. coli fim* gene by 1.5% agarose gel analysis stained with EtBr. The shown bands are representative of PCR products amplified from *E. coli* isolates, and lane M represents the DNA ladder.

This finding indicates that these isolates are indeed *E. coli*, since the fim gene is typically present in *E. coli* strains. Therefore, detecting this gene in any isolate strongly supports its identification as *E. coli* agree to Abdulla and Abdulrahman [41].

3.6.2. Effect of Citrus oil on *fim E* gene expression.

Table (3-5) show the study effect of stringent response initiated by control on the expression of *fim E* genes, the RNA was extracted from the established biofilm under normal and oil effect to measure the expression of the genes using qRT-PCR.

Table (3-5): Fold change of *fimE* gene expression/ Citrus oil

Isolate no.	Control			Citrus oil			ΔΔCt	Fold change	Result
	Hkg	*fimE*	ΔCt	Hkg	*fimE*	ΔCt			
3	16.77	18.92	2.15	13.32	15.61	2.29	0.14	0.90751916	Decrease
9	16.21	16.07	-0.14	14.02	17.6	3.58	3.72	0.07588718	Decrease
14	16.14	16.57	0.43	14.82	18.59	3.77	3.34	0.09875516	Decrease
16	16.05	17.95	1.9	15.18	17.3	2.12	0.22	0.85856544	Decrease
LSD (P-value)	--	--	--	--	--	--	--	0.702 * (0.0392)	--
* (P≤0.05).									

Table (3-6) showed the fold change of *fim E* gene expression Citrus oil, extracted from Citrus reculata has an antibacterial effect on *E. coli* and changes its expression of *fimE* gene. However, the mechanism of this gene regulation is still unclear the oil decrease expression of *fimE* gene .*E. coli* is a normal flora of the human body and a common pathogenic bacterium that causes urinary tract infections (UTIs). UTI is mainly caused by the stimulation of urethra and bladder by the bacterial adhesion and invasion in the mucosa and then the fimbriae on the surface of the bacterial cell bind with the glycoprotein receptor in the host material. *fim-E*, which is one of the fimbriae, plays a major role [24].

Table (3-6) : Fold change of *fim-B* gene expression/ citrus oil

Isolate no.	Control			Citrus oil			ΔΔCt	Fold change	Result
	Hkg	*fimB*	ΔCt	Hkg	*fimB*	ΔCt			
3	16.77	23.14	6.37	13.32	15.65	2.33	-4.04	16.449821	Increase
9	16.21	26.01	9.8	14.02	16.34	2.32	-7.48	178.52719	Increase
14	16.14	19.98	3.84	14.82	17.05	2.23	-1.61	3.05225184	Increase
16	16.05	29.14	13.09	15.18	18.56	3.38	-9.71	837.53171	Increase
LSD (P-value)	--	--	--	--	--	--	--	12.942 ** (0.0001)	--
**** (P≤0.01).**									

The table (3- 6) show ability of Citrus oil increase gene expression of *fimB* gene and the *P value* was significant ** (*P≤0.01*) in most isolate contrary to the results of the table (3-6) Where the gene's function is inhibited, this explains why it works on one *fimE* gene and not another. According to our knowledge alone as Nascimento et al., 2023 and Kairey et al.,2023; qburnene and the mixture were the first-time compounds interestingly worth the study. This suggests that the ability of essential oils to repress the most critical genes of UPEC and EAEC was a perception. At all time points, the expression of *fimB* increased after exposure to Citrus oil. Our report provides important information on better understanding how *E. coli* is influenced by Citrus oil exposure and at which time point the expression of fimB was significant. This can be applied to developing other novel therapeutic strategies. Further studies are needed to investigate the potential therapeutic application. In conclusion, in the *E. coli* experimental model, 2% tea tree oil induced the increased expression of the type 1 fimbriae *fimB* gene in a time-dependent manner. Our research suggests that the increased expression

of the *fimB* gene in response to tea tree oil may lead to the associated enhanced attachment ability of type 1 fimbriae on epithelial sites of urinary tract-like cells [25] ; [26].

3.7 GC-MS results

The effect of Citrus oil related to the antibacterial effect of its active compounds that analysed using GC-MS analysis. A study of the essential oil compounds of mandarin showed that the oil contains a spectrum of active compounds distributed in the form of 5 peaks in the analysis report, as shown in table (3-7), fig (3-3) below.

Table (3-7): Compounds present in the oil extract of Citrus using GC-MS analysis.

No.	Compound name	Retention time	Percentage %
1	.alpha.-Pinene	6.863	5.03
2	beta.-Myrcene	8.396	7.90
3	D-Limonene	9.675	76.17
4	gamma.-Terpinene	10.458	10.23
5	Cyclohexane, 1-ethenyl-1-methyl-2	18.079	0.67

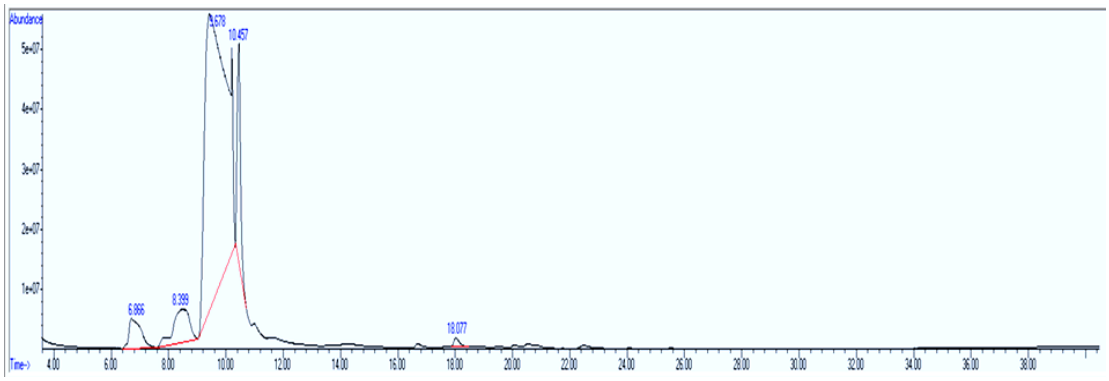

Figure (3-3): GC-MS profile of Citrus oil extract.

As the above results, Citrus oil consist of 5 active compounds, the higher percentage is 76.17% for D-Limonene while the lower percentage is 0.67% for Cyclohexane. A result by Boughendjioua, *et al.,* [42] identified 28 compounds representing a total of 99.41%. The essential oil is constituted mainly of: D-Limonene (85.10%), Sabinene (2.49%), Linalyl acetate (2.00%), Copaene (1.80%) and α-Pinene (1.75%) totalling approximately 93.14%.

In another previous result, the chemical composition of hydrodistilled oils obtained from the leaves of six Citrus reticulata Blanco (mandarin) cultivars grown in Nigeria were examined by GC and GC/MS, the result of their chemical composition was further submitted to cluster analysis. Fifty-seven constituents were characterized accounting for 88.2-96.7% of the total oils. Sabinene, g-terpinene, p-cymene, d-3-carene and (E)- b-ocimene were observed in great variability in all the oils. Other constituents include linalool, myrcene, terpinen-4-ol and cissabinenehydrate. In addition, limonene, terpinolene, β-pinene, and α-pinene were detected in appreciable concentrations. β-sinensal and α-sinensal were isolated by preparative GC and characterized by one- and two-dimensional NMR techniques [27].

Conclusion

Between 120 bacterial isolates from UTI, 29 isolates are *E. coli* isolates. According to the results of AST, six isolates showed the highest bacterial multidrug resistance. The result showed that the Citrus oil had a high efficiency as antibiofilm effect against resistant *E.*

coli isolates with MIC equal to 10000 ppm, while showed no effect to rhubarb extract. The fim gene segment was located in all isolates identified as *E. coli* using convention testing. Biofilm formation with plant extract decreased significantly when compared to biofilm developed under normal conditions across all isolates. High prevalence of key genes associated with biofilm formation in *E. coli* isolates. Specifically, the *fim E* and *fim B* genes. According to the results of GC-MS analysis, the phytochemical screening showed that mandarin oil composed of 5 active compounds. The higher percentage is 76.17% for D-Limonene while the lower percentage is 0.67% for Cyclohexane.

Funding Statement

This study suggestd that the antibacterial and antibiofilm effect was related to D-Limonene active compound.

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