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CONCENTRATION DEPENDENT ANTIBIOFILM ACTIVITY OF TEA TREE OIL AGAINST CLINICAL *ENTEROBACTER CLOACAE* ISOLATED FROM URINARY TRACT INFECTIONS

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ABSTRACT

Background: Biofilm-associated urinary tract infections (UTIs) caused by Enterobacter cloacae represents a significant clinical challenge due to antimicrobial resistance. Natural plant-derived products such as Tea Tree Oil (TTO) gained attention as an antibiofilm agent. **Methods**: A total of 23 clinical E. cloacae isolates sampled from Iraqi patients with UTIs, identified using morphological characteristics, biochemical tests and Vitek-2 compact system. Biofilms forming ability was determined using 96-wells polystyrene flat plate assay. Minimum bactericidal concentrations (MBCs), minimum inhibitory concentrations (MICs) and sub-MICs (SMICs) of TTO determined using resazurin-based assay. Antibiofilm activity was determined via crystal violet assay, at different TTO concentrations by measuring optical densities. The presence of csgA detected via PCR technique and expression levels of csgA quantified via real-time PCR, normalized to the housekeeping gene and analyzed using $2^{-\Delta\Delta Ct}$. **Results**: All E. cloacae isolates were capable of forming biofilms with optical densities ranged from 0.150 to 0.700. Tea tree oil exhibited antibiofilm activity in a concentration-dependent manner. MBCs level significantly reduced biofilm ($p < 0.0001$), with reductions exceeding 50% in several strong and moderate biofilm-formers. MIC levels showed moderate inhibition, whereas SMICs demonstrated limited but detectable inhibition. Molecular analysis revealed that, exposure to TTO resulted in significant downregulation of the csgA compared to control. **Conclusion**: TTO at the level of MBCs, MICs and SMICs demonstrates potential anti-biofilm activity against E. cloacae and may be considered as potential natural In vitro anti-biofilm agent, however, further In vivo studies are required to evaluate its possibility to control biofilm associated-UTIs and molecular levels highlighted these findings.

Keywords: UTIs; Tea Tree Oil; *E. cloacae*; Biofilm; Anti-biofilm

INTRODUCTION

Biofilms are closed structured communities of cells embedded within extracellular polymeric substances (EPS), responsible for providing protection against any stress, antibiotics and host responses and thus lead to persistence of infection (Silva *et al*., 2023). Biofilms presented in many chronic wounds, medical equipment and indwelling devices, where bacterial cells can transition from planktonic cells to biofilm forming cells, which possess phenotypic and genotypic profiles that enhances survival under any environmental stress (Liu *et al*., 2024). Biofilm formation is considered a major virulence factor that many pathogens rely on it for pathogenicity, including *Escherichia coli*, extended-spectrum β-lactamases producers (ESBL), *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and other members of Enterobacteriaceae family such as *Enterobacter cloacae* (Misra *et al*., 2022; Oliveira *et al*., 2025). *E. cloacae* capable of colonizing a wide range of environments, including surgical sites and urinary Foley catheters, where they can form biofilms and enhance their survival and resistance against host defense mechanisms (Cieślik *et al*., 2025). Curli fibers are amyloid structures that play an essential role in adhesion and biofilm formation of bacterial cells. The major structural subunit of curli is encoded by *csg*A, which has been characterized in *E. coli* and *Salmonella enterica*. Adhesion mediated by curli facilitates attachment to surfaces and contributes to the integrity and stability of biofilm. Although most studies have focused on *E. coli*, *csg*A as a curli production gene reported in other members of Enterobacteriaceae, which suggested that biofilm formation in *Enterobacter cloacae* may be driven by the same mechanism. Therefore, evaluating *csg*A expression provides insight into the molecular mechanisms underlying biofilm inhibition by tea tree oil (Barnhart and Chapman, 2006; Evans and Chapman, 2014; Tursi and Tükel, 2018). Embedded bacterial cells of biofilms are significantly higher tolerance to antimicrobial agents compared to planktonic cells. This enhanced tolerance is attributed to protective mechanisms, such as restricted antibiotic diffusion through the EPS, reduced metabolic activity of cells, and elevated horizontal gene transfer within the cellular community of biofilm. These characteristics reported in *Enterobacter cloacae*, where the formation of biofilm contributes to persistence of bacterial cells and antimicrobial resistance in clinical infections (Misra *et al*., 2022; Thöming and Häussler, 2022; Liu *et al*., 2024;). Antimicrobial resistance is related to biofilm infections, when traditional therapeutic agents fail to reduce pathogens, which lead to untreatable chronic recurrent hard to treat infection, so an urgent need in alternative strategies must be taken as interest to prevent biofilms in medical cases (Silva *et al*., 2023). TTO, an essential oil derived from *Melaleuca alternifolia*, is considered a broad-spectrum antibacterial agent and has potential activity to inhibit biofilms of variety of pathogens (Iskandar *et al*., 2025). Terpinen-4-ol, the major component of TTO, a monoterpene responsible for membrane disruption leading to leakage and loss of cellular content and thus lead to impair the growth of planktonic cells in early biofilm steps (Chadha *et al*., 2022). Beyond TTO membrane integrity effect, it interferes with signaling systems of biofilms. Quorum sensing system (QS), a cell-cell communication pathway, that regulate expression of motility, adhesion, EPS matrix production and a variety of virulence factors genes, which are all essential for biofilm formation (Maggio *et al*., 2025).

The present study aims to determine the efficacy of TTO at the level of MBCs, MICs and SMICs anti-biofilm activity against clinical *E. cloacae* isolates isolated from Iraqi patients with UTIs, hypothesizing that TTO may be able to inhibit biofilm formation of the clinically isolated *E. cloacae* and may modulate the biofilm related *csgA* gene expression.

MATERIALS AND METHODS

E. cloacae isolates and Ethical approval

The current study included 23-suspected isolates of *E. cloacae* collected from female UTIs patients from different healthcare facilities in Baghdad governorate between January 2024 and July 2024. All isolates were primary identified using morphological examination via MacConkey agar and a set of routine biochemical tests (oxidase, catalase, indole, methyl red, Voges-Proskauer, citrate utilization and ornithine decarboxylase) (Tille, 2021). Vitek®-2 compact system (BioMérieux, France) utilized for identification and susceptibility of isolates using GN-ID and

AST cards. Ethical approval granted by Iraqi Ministry of Health's Ethical Review Board (No. 43305, 14/11/2023).

Extraction and Dilution of TTO

Tea tree leaves (*M. alternifolia*) purchased from local markets. After collection, all plant parts washed using tap water, dried at 25ºC before being ground into powder. Extraction was carried out using Clevenger, 125g of dried plant material was subjected with 600mL of distilled water and boiled for 2.5 hours. The separated oil from aqueous phase kept at 4º for further use in dark glass bottles to prevent evaporation. Diluted TTO prepared by adding 0.4mL of concentrated TTO mixed with 9.6mL of Dimethyl Sulfoxide (DMSO) to prepare diluted oil concentration of 4% v/v. Serial dilutions were prepared as (1:1) to yield concentrations (2, 1, 0.5, 0.25, 0.125, 0.0625. 0.0312, 0.0156, 0.0078, 0.0039 and 0.0019) % using Mueller-Hinton Broth and each concentration tested using prescribed above method.

Chemical Composition of Tea Tree Oil

The chemical composition of TTO standardized according to the International Organization for Standardization (ISO 4730) (ISO, 2025). The acceptable ranges of each components defined as presented in table (1).

Table (1) Chemical composition of Tea Tree Oil

Compound	Percentage (%)
terpinen-4-ol	30-48
γ-terpinene	10-28
α-terpinene	5-13
1,8-cineole	<15
α-terpineol	1.5-8

Biofilm Quantification Assay

The use of crystal violet consider as golden stander of biofilm quantification assay. As proposed by (Liu *et al*., 2022). The assay carried out with minor modifications. Briefly, triplicates of wells of 96-well polystyrene sterilize microtiter plate inoculated with aliquot of 200 µL of each *E. cloacae* suspensions adjusted to 0.5 McFarland (CFU/mL). Triplicates of TSB consider as negative control. The plates incubated for 24 hours at 37ºc and after incubation period, the content of each well-emptied and triplicate washing steps were applied to each well using 200 µL of phosphate buffer saline and dried at 25 ºc for 35 minutes within sterilized conditions. Eventually, after removing all unattached cells, the attached cells were stained using 0.1% of crystal violet for 15 minutes and then plates emptied again followed by adding 200 µL of Ethanol (95%) to each well. The optical densities determined using ELISA reader (BioRad) at 620nm wavelength in Biotechnology Research Center/Al-Nahrain University. Biofilm formation ability determine using the following criteria: non-former ($OD \leq ODc$), weak former ($ODc < OD \leq 2\times ODc$), moderate former ($2\times ODc < OD \leq 4\times ODc$) and strong former ($OD > 4\times ODc$). The crystal violet assay utilized to quantify biofilm biomass which include cells and extracellular matrix, not distinguish between living and non-living cells.

Determination of MICs and MBCs

The MIC of TTO determined as proposed by (Zandona *et al*., 2025). Sterile flat-bottom polystyrene 96-wells plates utilized. TTO used in range of (0.0019-2)% v/v. Triplicates of 100µL of all *E. cloacae* fresh isolates subjected to 0.5 McFarland standard. An Aliquot of 100µL of each isolate added to each well, followed by adding 100µL of each concentration previously prepared (0.0019-2)% v/v. Negative control consisted of diluted 2%DMSO alone. Positive control represented by each *E. cloacae* isolates without TTO and DMSO. As solvent control, DMSO concentration applied as the same final concentration used to dissolve TTO and included in all wells to exclude any possible effect of the solvent. Plates incubated at 37º for 24 hours and followed by adding 20µL of 0.015% resazurin to each well and plates incubated again at 37º for 1.5 hours. Bacterial biomass was assessed by observing color change observation for each well. To determine MBCs, aliquot of 15µl from wells showing no visible growth were subcultured Muller-Hinton agar plates (Himedia, India) and all plates incubated for overnight at 37ºc. The MBCs defined as lower concentration of TTO resulted no visible growth of colonies on agar surface.

Anti-Biofilm activity of TTO

Modified assay of (Al Rugaie *et al*., 2025), used to estimate the efficiency of TTO on biofilm formation, TTO diluted in Mueller Hinton broth (MHB). Using 96-wells polystyrene plates, Triplicates of 100µL of each *E. cloacae* fresh isolates (0.5 McFarland standard) added to each well, followed by adding 100µL of TTO at the level of MBCs, MICs and SMICs values for each isolate. Plates incubated for 24 hours at 37ºc, and then wells emptied, washed using two folds of 200µl of Phosphate Buffer Saline and stained for 10 minutes with 200µl of Crystal violet followed by adding 200µl of Ethanol 95%. Optical densities measured using ELISA reader (BioRad) at 620 nm. The antibiofilm activity was determined via following formula:

$$\text{Biofilm inhibition (\%)} = [(\text{OD control} - \text{OD test}) / \text{OD control}] \times 100.$$

Detection and Calculation of *csg*A expression

The total DNA of *E. cloacae* strong biofilm formers extracted using extraction kit of DNA provided by (ABIOpureTM, USA). Briefly, five colonies of each *E. cloacae* selected isolates incubated for overnight at 37º in Brain Heart Infusion Broth. After incubation, a 1500 µL tubes filled with aliquot of 1400 µL of each isolates centrifuged at 15000 rpm for 3 minutes, this step followed by adding 20 and 200 µL of proteinase K, Buffer CL and Buffer BL to the pellet and vortexed for 35 minutes at 56º in water bath to ensure lysis of cells. Then, 200 µL of 99.9% Ethanol added and vortexed for few seconds. The whole mixture transferred to latex Mini columns tubes and centrifuged for at 15000 rpm for 5 minutes. Two washing steps applied via BW and TW, followed by centrifugation at 12000 and 13000 rpm for 3 minutes. DNA that binds to filters, transported to new tubes and AE buffer at aliquot of 100 µL added and centrifuged for 5 minutes at 5000 rpm. The tubes conserved within stored at -20º for investigating of *csg*A using PCR. Primers provided by (Macrogen, Korea) and PCR run program for *csg*A prescribed in (Al-Saadi *et al*., 2024). The amplified PCR products were electrophoresed on 2% agarose gel (Promega, USA) and visualized by Red safe (Promega, USA) under ultraviolet trans illuminator (Major Science, Taiwan).
Using GoTag® 1-step RT-qPCR system (Promega, USA) and via using TRIzoleTM Reagent (Thermo Scientific, USA) utilized to extract total RNA and RNA concentration determined using . Briefly, *E. cloacae* selected isolates prepared as broth cultures. Using Muller-Hinton Broth, each selected isolates inoculated in the broth with SMIC level of tea tree oil and incubated for overnight at 37º. Negative control consisted of diluted 2%DMSO. Positive control represented by each *E. cloacae* without tea tree oil and DMSO. Total volume of 1400 µL of each isolates precipitated in Eppendorf tubes and 500 µLof TRIzole (Thermo Scientific, USA) added to the pellet, mixed well and left for 40 minute at room temperature in dark place. Then 200µL of Chloroform added, mixed well for 5 minutes followed by centrifugation at 13000 rpm for 10 minutes. The aqueous phase, which representing RNA was aspirated and transported to new tubes, followed by adding 500 µL of Isopropanol and centrifuged at 13000 rpm for 15 minutes. Isopropanol aspirated and precipitated RNA treated with 70% Ethanol and centrifuged at 12000 rpm for 10 minutes. Real time PCR program of House Keeping gene (16s rRNA) prescribed by (Mohammed and Hussain, 2022). The $2^{-\Delta\Delta CT}$ formula to calculate gene expression suggest by Livak and Schmittgen, used for analysis (Livak and Schmittgen, 2001).

Statistical Analysis

All experiments performed in three technical replicates (n = 3 wells per condition per isolate). The data statistically analyzed in a data sheet and represented as mean and standard deviation (Mean ± SD, n=3). Graphs and significant differences tested using Graphpad Prism 8.0. Comparisons between control to TTO-treated isolates at the level of MBCs, MICs and SMICs) performed using statistical tests depending on data type. Categorical data presented as (growth compared to no growth in MBC/MIC), Fishers exact tested utilized using three replicates per concentration of TTO per isolate of *E. cloacae*. Biofilm biomass quantification comparisons include comparing of control to MBCs, MICs and SMICs. Sub comparisons include comparing MBCs to MICs, SMICs and MICs to SMICs. The Pearson's correlation coefficient utilized to determine the correlation between different parameters, p-values ($p \leq 0.05$) considered statistically significant and values. All statistical analyses performed under consistent experimental conditions to ensure reproducibility.

RESULTS

Identification of *E. cloacae*

All tested isolates gave small lactose fermenter pink dry-mucoid colonies with variable sizes on MacConkey agar. The primary identification via biochemical tests as presented in table (2) revealed that, only 11 isolates (EC3, EC5, EC7, EC8, EC10, EC13, EC15, EC18, EC19, EC22 and EC23) were *E. cloacae*. The primary identification confirmed via Vitek®-2 compact system with accuracy of (96-99)%.

Table 2 Primary identification of *E. cloacae* isolates via Biochemical tests

Isolate	Oxidase	Catalase	Indole	Methyl Red	Vogeus-Proskauer	Citrate Utilization	Ornithine Decarboxylase
EC1	-	+	+	+	-	-	-
EC2	-	+	+	+	-	-	-
EC3	-	+	-	-	+	+	+
EC4	-	+	+	+	-	-	-
EC5	-	+	-	-	+	+	+
EC6	-	+	-	-	+	+	-
EC7	-	+	-	-	+	+	+
EC8	-	+	-	-	+	+	+
EC9	-	+	+	+	-	-	-
EC10	-	+	-	-	+	+	+
EC11	-	+	-	-	+	+	-
EC12	-	+	+	+	-	-	-
EC13	-	+	-	-	+	+	+
EC14	-	+	-	-	+	+	-
EC15	-	+	-	-	+	+	+
EC16	-	+	+	+	-	-	-
EC17	-	+	+	+	-	-	-
EC18	-	+	-	-	+	+	+
EC19	-	+	-	-	+	+	+
EC20	-	+	+	+	-	-	-
EC21	-	+	-	-	+	+	-
EC22	-	+	-	-	+	+	+
EC23	-	+	-	-	+	+	+

Biofilm Quantification Assay

As presented in figure (1), *E. cloacae* isolates were variable in their ability to form biofilms. All of the tested isolates were able to form biofilm and their ability ranged from weak to strong. As baseline, the negative control group showed optical densities of 0.054 and after compared with each tested isolate, a significant increase was observed of optical densities. *E. cloacae* optical density values ranged from 0.150 to 0.700. These isolates classified as 3 (27.27)% as weak formers, 4 (36.36)% as moderate formers and 4 (36.37)% as stronger formers as presented in figure (1) and these values highlighted ability of *E. cloacae* to form biofilms at different ranges.

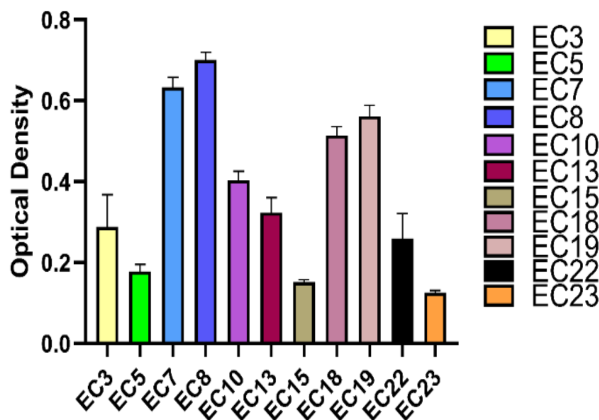

Figure 1 Biofilm formation of *E. cloacae* utilized via crystal violet assay. Each data bar represent triplicates mean of optical density for single isolate. Category of biofilm determined as non-former (OD ≤ ODc), weak former (ODc < OD ≤ 2×ODc), moderate former (2×ODc < OD ≤ 4×ODc), strong former (OD > 4×ODc). All data represented by (Mean ± SD).

Determination of MBCs, MICs and SMICs

As presented in table (3), MBCs, MICs and SMICs of TTO against *E. cloacae* isolates. Concentrations varied according to isolate source. These results demonstrated TTO inhibitory effect against *E. cloacae*.

Anti-Biofilm activity of TTO

The anti-biofilm activity of concentrated TTO (4%v/v) was evaluated against all *E. cloacae* isolates using 96-wells polystyrene plate. The results summarized in figure (2), demonstrated that concentrated TTO showed a significant inhibitory activity against biofilm biomass. After treating strong formers of biofilms with concentrated TTO, a significant ($p < 0.0001$) inhibition of biofilm biomass recorded for isolates (EC7, EC8, EC18 and EC19). EC8 recorded the highest inhibition rate 57.63% (0.268±0.035), followed by EC18, which its rate reach 41.28% (0.302±0.054). Other strong biofilm formers (EC7 and EC19), also significantly their biofilm were inhibited ($p < 0.0001$). Moderate biofilm formers (EC3, EC10, EC13 and EC22) appear to be more vary than strong formers, but still significantly inhibited via TTO. EC3 showed the greatest reduction in biofilm biomass and the most susceptible isolate to TTO. The inhibition rate of EC3 was 54.62% (0.130±0.026), followed by EC13 and EC10 as the inhibition rate reach (46.54 and 37.79)% (0.173±0.065 and 0.250±0.062), respectively and both were highly significant ($p < 0.0001$), while EC22 inhibition was not significant 22.17%(0.202±0.036, ns). The weak biofilm formers (EC5, EC15 and EC23) appear to have different response to TTO not like strong or moderate formers. The biofilm biomass of these isolates appear to be enhance rather than reduced just like other isolates and all of them were not significant (ns).The impact of diluted oil utilized as the same previous design, demonstrated variable but still potent inhibitory effect as anti-biofilm. The diluted TTO tested against each *E. cloacae* isolates at the level of MICs and SMICs. After exposure to TTO at level of MICs inhibition of biofilm, remained consistent, but less than concentrated concentration. Variable differences noticed among isolates. As presented in figure 3, high significant inhibition ($p < 0.0001$) notified after comparing all MBCs level of TTO with control in all isolates of *E. cloacae* except EC23 was not significant (ns). Many significant differences also recoded after using MICs levels of TTO. These differences varied according to isolate source. EC7, EC8, EC18 and EC19 were significantly inhibited ($p = 0.0001$, 0.040, 0.0296 and 0.0008) respectively, while other isolates show non-significant differences (ns) compared to control. Additionally, the levels of TTO-SMICs tested and compared to control. Non-significant inhibition recorded in all isolates except EC7 ($p = 0.025$).

To make comprehensive comparison and for further characterization of TTO anti-biofilm activity, optical densities of isolates treated with TTO at the levels of MBCs compared with those at the levels of MICs and SMICs. Significant reduction in biofilm biomass recorded ($p < 0.0001$) between MBCs and MICs levels within all isolates, which indicated that although bacterial biofilm inhibition. MBCs group demonstrated the lowest optical densities values within all isolates with values nearly to baseline (0.049±0.013), while MICs group exhibited high optical densities (0.331±0.43). These differences highlighted the distinction between TTO bactericidal activities and growth inhibition via MBCs, which achieved nearly complete reduce of biofilm. The same pronounced inhibition recorded when comparing MBCs with SMICs levels, as SMICs optical densities were relatively high (0.358±0.034). These findings highlighted efficacy of SMICs in reducing biofilm establishing. Statistical analysis confirmed that, TTO at the level of MBCs was significantly lower than MICs and SMICs ($p < 0.0001$), which indicated that TTO activity dependent on concentration. At the level of MBCs, a sharp decline of optical densities recorded, which suggests that higher concentration of TTO higher damage to bacterial cells and thus leading to disrupt biofilm integrity. In contrast, lower concentrations of TTO appear to have limited antibiofilm activity, compared to higher concentrations. Overall, these results indicated that TTO required at high concentration to achieve high anti-biofilm activity. A direct comparison between TTO MICs and SMICs levels to achieve their impact on biofilm formation. The statistical analysis revealed non-significant (ns) differences between them. Although both groups displayed variability among isolates, the overall trend consistently favored lower optical densities scored for MICs levels. These results suggested that TTO at the level of MICs represented threshold concentration at which biofilm suppressed, in contrast TTO at the level of SMICs resulted relatively unsuppressed biofilm compared to MICs levels, which reflect that biofilm at the these levels are still persistence.

Table 3 MBCs, MICs and SMICs of TTO against *E. cloacae* isolates determined via resazurin based assay

Isolate	MBC %v/v	MIC %v/v	SMIC %v/v	Growth response	Statistical significance (Fisher's exact test)
EC3	1	0.5	0.25	0.5 (+), 1-2 (-)	**** $p < 0.0001$
EC5	0.25	0.125	0.0625	0.125 (+), 0.25-2 (-)	**** $p < 0.0001$
EC7	2	1	0.5	1 (+), 2 (-)	**** $p < 0.0001$
EC8	2	1	0.5	1 (+), 2 (-)	**** $p < 0.0001$
EC10	1	0.5	0.25	0.5 (+), 1-2 (-)	**** $p < 0.0001$
EC13	0.5	0.25	0.125	0.25 (+), 0.5-2 (-)	**** $p < 0.0001$
EC15	0.25	0.125	0.0625	0.125 (+), 0.25-2 (-)	**** $p < 0.0001$
EC18	2	1	0.5	1 (+), 2 (-)	**** $p < 0.0001$
EC19	1	0.5	0.25	0.5 (+), 1-2 (-)	**** $p < 0.0001$
EC22	1	0.5	0.25	0.5 (+), 1-2 (-)	**** $p < 0.0001$
EC23	0.25	0.125	0.0625	0.125 (+), 0.25-2 (-)	**** $p < 0.0001$

(+): Pink color well (growth) , (-): Blue color well (no growth). Statistical analysis carried out using Fisher's exact test (comparing the concentration MBCs vs. MICs and SMICs, three well (n=3) for each concentration, triplicates for each isolate). P value (****) < 0.0001 indicated significant inhibition in MBCs compared to MICs and SMICs.

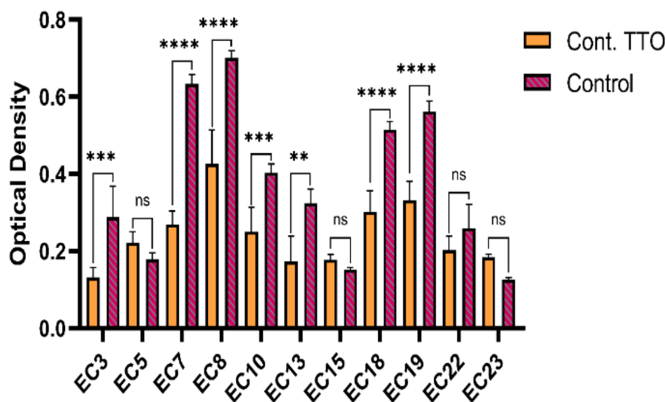

Figure 2 Anti-biofilm activity of Concentrated Tea Tree Oil (TTO) against *E. cloacae* isolated from UTIs patients. All data are represented by (Mean ± SD, n=3). Data expressed as comparison between optical densities of isolates under normal conditions (control groups) vs. optical densities of the same isolates after exposure to TTO. Optical densities measured via ELISA reader at 620nm. The annotation (ns) indicate non-significant differences. Annotation (**), (***) indicate significant differences (p < 0.05) and (****) indicate significant differences (p < 0.0001). Concentrated (Cont.) TTO showed inhibition depended on isolate. Highest inhibition rates observed in EC3, EC7, EC13 and EC18.

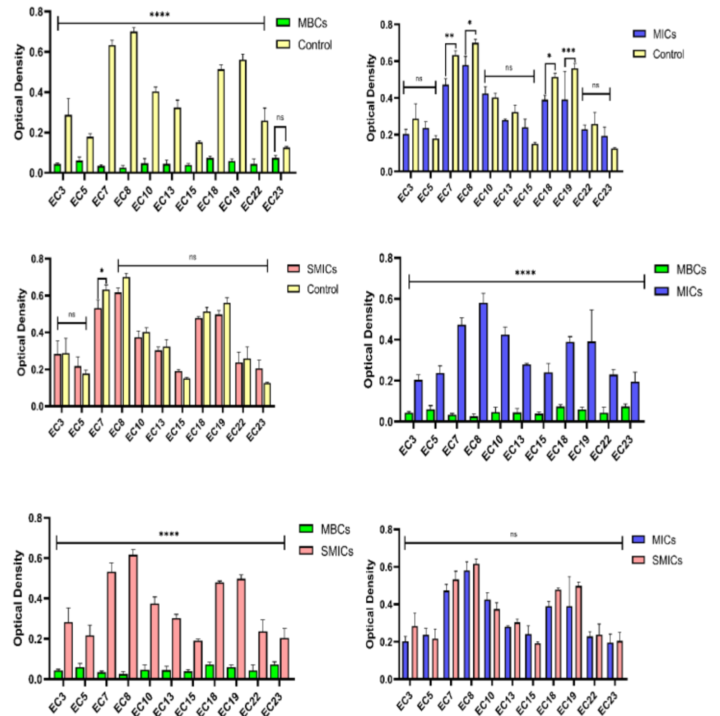

Figure 3 Anti-biofilm activity of Diluted Tea Tree Oil (TTO) against *E. cloacae* isolated from UTIs patients. All data are represented by (Mean ± SD, n=3). Data expressed as comparison between optical densities of isolates under normal conditions (control groups) vs. optical densities of the same isolates after exposure to TTO at the levels of MBCs, MICs and SMICs. Optical densities measured via ELISA reader at 620nm. The annotation (*ns*) indicate non-significant differences. Annotation (**), (***) indicate significant differences ($p < 0.05$) and (****) indicate significant differences ($p < 0.0001$). Concentrated (Cont.) TTO showed inhibition depended on isolate.

*csg*A Detection and Expression Calculation

PCR utilized to detect which of the selected *E. cloacae* harbored *csg*A, which is associated to curli fibers production and biofilm formation. Results revealed that, all selected *E. cloacae* isolates harbored *csg*A gene, after confirmation via agarose gel electrophoresis under UV illumination as shown in figure (4). These results indicated that all strong biofilm formers harbored *csg*A, which means that the genetic traits related to biofilm formation are conserved across isolates. Quantitative Real time-PCR (qPCR) utilized subsequently to evaluate expression of *csg*A, after exposure to SMICs of tea tree oil. Using 16s rRNA, a housekeeping gene, the expression normalized and relative fold change measured using $2^{-\Delta\Delta Ct}$. The selected *E. cloacae* isolates tested under SMIC concentration. As shown in figure (5), all *E. cloacae* isolates expressed downregulation of *csg*A expression after exposure to SMIC. The *csg*A expression was significantly downregulated (p < 0.0001) in all isolates. This significantly downregulation in *csg*A expression in E. cloacae after exposure to SMICs of TTO compared to the untreated control group and the reduction in gene expression came in line with the decrease in biofilm biomass observed by crystal violate assay.

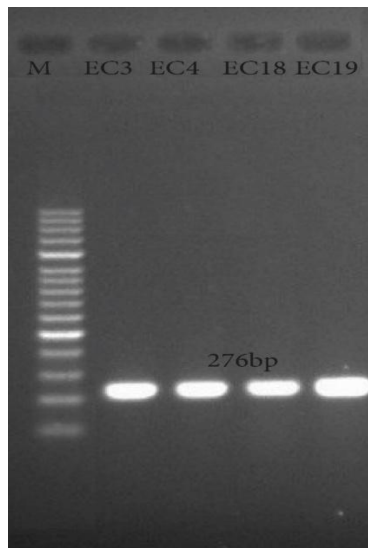

Figure 4 Electrophoresis of *csg*A on 2% agarose gel at 100 volt for 80 minutes. Lane M represent DNA ladder, Lane (EC3, EC4, EC18, EC19) represent amplification of PCR product of *csg*A gene of E. cloacae

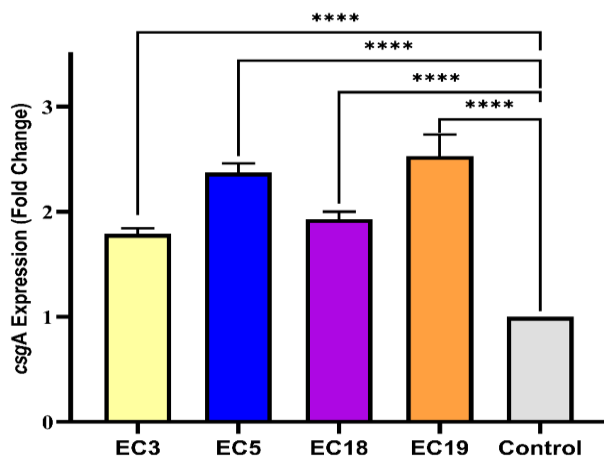

Figure 5 Expression of *csg*A after exposure to SMIC of tea tree oil. Real time-PCR utilized for *csg*A expression after exposure to SMIC level of tea tree oil. Expression levels normalized via 16s rRNA and relative *csg*A fold change calculated via Litvak's formula. Highly significant differences ($p < 0.0001$) recorded in all isolates.

DISCUSSION

Globally, *E. cloacae* consider a threat and increasingly recognized as an important opportunistic pathogen associated with UTIs and other hospitals and healthcare associated infections, particularly in immunocompromised patients. As a member of ESKAPE group, E. cloacae posses a significant global healthcare concern, duo to emerging high levels of antimicrobial resistance, especially in hospitals environment (Oliveira *et al*., 2022; Elbehiry *et al*., 2024). The ability of *E. cloacae* to form biofilms which further complicate treatment, due to these biofilms enhance and provide protection against antibiotics, host defenses and thus lead to chronic and recurrent untreatable infections, especially in catheters associated urinary tract infections (CAUTIs) (Vestby *et al*., 2020; Mishra *et al*., 2020). Biofilms are highly organized bacterial communities embedded within a self-produced EPS matrix. This matrix provide protection and act as a physical and chemical barrier responsible for limiting penetration of antibiotic and facilitates persistence of bacterial cells, leading to increased tolerance and resistant biofilm cells compared to free floating planktonic cells (Carson *et al*., 2006; Cáceres *et al*., 2020). The present study revealed that *E. cloacae* exhibited variable ability to form biofilms (weak-strong), thus highlighting the heterogeneity within clinical isolates, which may influence their susceptibility to antibiotics. In this study, TTO extracted from *M. alternifolia*, demonstrated significant ability to inhibit biofilms activity against clinically isolated *E. cloacae* isolates in a concentration-dependent status. TTO is a well-known essential oil produced as a secondary metabolite with broad-spectrum activity against both Gram-positive and Gram-negative bacteria. This activity primarily attributed to its major bioactive component, terpinen-4-ol (Nazzaro *et al*., 2013; Misra *et al*., 2022). The present work finding showed that TTO significantly reduced biofilm biomass at the level of SMICs, MICs and MBCs level, with the highest inhibitory effect observed at MBC concentrations, where biofilm biomass reduced nearly to the levels of the negative control. These results came in line with previous studies reporting that essential oils exhibit enhanced antibacterial and antibiofilm activity at higher concentrations and this ability attributed to disruption of bacterial plasma membranes and loss of cellular integrity (Hammer *et al*., 2006; Evans and Chapman, 2014). The activity of TTO may be attributed by its lipophilic nature, which enables it to interact with and disrupt bacterial cell membranes. Previously reported that two major TTO components are terpinen-4-ol and α-terpineol, which are responsible for membrane disruption and these components increase cell membrane permeability, leading to cellular contents efflux outside and eventually lead to cell death (Karygianni *et al*., 2016). Additionally, these components have been reported to have more activity against planktonic cells but less activity on biofilms embedded cells, due to the protective nature of the biofilm matrix, which limits penetration of hydrophobic compounds (Fathy *et al*., 2025). Tea tree oil was more effective at MBCs level, compared to MICs and SMICs levels. High concentrations of tea tree oil reduced biofilms of *E. cloacae* and make them nearly too optical density of control, while low concentrations showed limited effect. These findings came in line with nature of essential oils pharmacodynamics, which are likely to be active at high concentrations and can easily penetrate biofilm matrix and lead to sessile cells (planktonic cells) disruption (Touati *et al*., 2025). Among biofilm formers (strong, moderate and weak), different responses of tea tree oil recorded, and strong biofilms produced required high concentrations to achieve the same level of inhibition. These findings suggest that biofilm architecture, density of cells susceptibility profile and thickness of EPS matrix may influence isolates susceptibility to tea tree oil and interfere with diffusion of tea tree oil hydrophobic compounds (Touati *et al*., 2025). Recent evidences, highlighted tea tree oil activity against biofilm formation and its activity beyond the antibacterial activity but also involve in disruption of biofilm at molecular and cellular levels. Essential oils derived from plants interfere and disrupt many biofilm formations steps, including adhesion, EPS integrity and communication system of cells (Quorum sensing system), which are critical for biofilm establishing and maintained (Cordeiro *et al*., 2020). Tea tree oil as antibiofilm agent have significant inhibition of biofilm formation at SMICs level, which suggest its activity in early biofilm stages and quorum sensing system regulated virulence factors. Terpinen-4-ol, major bioactive component of tea tree oil is implicated in cell membranes disruption, increase membrane permeability and compromising metabolism of energy leading to reduce adhesion of bacterial cells and biofilm availability even at SMICs doses (Hoja *et al*., 2025). Monoterpenes of tea tree oil, hydrophobic components can easily penetrate matrix of biofilms, which destabilizing biofilms and EPS and thus lead to detachment of cells before maturation (Evans and Chapman, 2014). Additionally, in another studies, tea tree oil with its components could interfere with quorum sensing signaling pathways leading to downregulation of biofilm essential genes and virulence factors of gram positive and gram negative bacteria (Cordeiro *et al*., 2020). For instance, tea tree oil tested and reduce biofilm formation of *S. mutans* and *S. sobrins* by inhibiting biofilm adhesion step, a crucial step in biofilm formation (Hoja *et al*., 2025). Combinations of tea tree oil with other essential oils to perform synergistic activity to enhance biofilm reduction, is a promising strategy against MDR-bacterial infections (Xie *et al*., 2025). This may explain why higher concentrations of TTO required achieving significant inhibition in strong biofilm-forming isolates in the present study. Additionally, cells within biofilms exhibit reduced metabolic activity and thus altered gene expression, which further contributing to antimicrobial tolerance. Collectively, these findings indicate that beyond tea tree oil antibiofilm activity, more likely to interfere with adhesion, EPS matrix and quorum sensing system, which are relevant and crucial mechanism for *E. cloacae* (Cordeiro *et al*., 2020). qPCR analysis revealed significant downregulation of *csg*A expression after exposure to SMICs levels of TTO. This finding indicates that TTO not only disrupts biofilms of *E. cloacae* at the structural level but also interferes with the genetic regulation of biofilm formation genes. The present study demonstrated significantly downregulation of *csg*A. Since the *csg*A gene encode for the major subunit of curli, the reduction of gene expression may explain the decrease of biofilm biomass. Similar findings by recent studies reported that essential oils may modulate the expression of biofilm-related genes and virulence factors in Gram-negative bacteria, therefore downregulation ability of TTO, which affect biofilm-associated genes, may be attributed to that TTO interference with QS, which responsible for regulation of biofilm formation, however, the molecular mechanism by which TTO affect expression of *csg*A gene directly, not investigated in the present work and remains hypothetical. The essential oils such as TTO, showed to inhibit QS signaling pathways, which lead to reducing the expression of many genes involved in bacterial adhesion, EPS production, and biofilm maturation and stability (Noumi *et al*., 2018; Wang *et al*., 2019; Guillín *et al*., 2021; Barak *et al*., 2025). Medically, the anti-biofilm activity of tea tree oil against *E. cloacae* demonstrates may suggest its potential use in the prevention and treatment of biofilm-associated infections, particularly catheter-associated urinary tract infections (UTIs) that can be acquired in hospitals. Tea tree oil may be incorporated into coatings for medical devices, such as urinary catheters, to reduce or prevent bacterial colonization and inhibit biofilm formation. Furthermore, it can be used as an adjunct to conventional antibiotics that fail to treat UTIs, as it may reduce the development of antibiotic resistance (Haines *et al*., 2022; Navarro *et al*., 2022; Oder *et al*., 2024). One limitation of the present work, is that the specific molecular mechanism of TTO

action on biofilm formation of *E. cloacae* was not directly investigated and the proposed machinery disused based on previously studies to explain the observed TTo antibiofilm activity. Further molecular studies required to confirm these mechanisms.

CONCLUSIONS

Tea tree oil exhibited antibiofilm activity through mechanisms, including bacterial cells membranes disruption, inhibition of quorum sensing system, reduction of adhesion and interfere with biofilm maturation. Tea tree oil consider a promising candidate to control biofilms especially in UTIs caused by *E. cloacae*.

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