Mitigation of Virulence in Resistant *Escherichia coli* by Diclofenac Sodium, Phenotypic and Genotypic Study

Sara M. Eltabey[1,*], Ali H. Ibrahim [2], Mahmoud M. Zaky [2], Moustafa M. Saleh [3,*]

[1] *Microbiology program, Botany Department, Faculty of Science, Port Said University, Egypt.*
[2] *Botany Department, Faculty of Science, Port Said University, Port Said, Egypt.*
[3] *Microbiology and Immunology Department, Faculty of Pharmacy, Port Said University, Port Said, Egypt.*

Correspondence:* Sara M. Eltabey; Microbiology program, Botany Department, Faculty of Science, Port Said University, Port Said, Egypt, Email: saraheltabay@sci.psu.edu.eg.
***Moustafa M. Saleh, Microbiology and Immunology Department, Faculty of Pharmacy, Port Said University, Port Said, Egypt, Email: Mostafa.mohamed@pharm.psu.edu.eg

ABSTRACT

Escherichia coli is one of the most ubiquitous pathogens causing several life-threatening diseases. Currently, antibiotics are failing fast while rates of drug-resistant bacteria are increasing worldwide with almost no new drugs coming to market and even last-resort antibiotics are becoming ineffective. Anti-virulence is a new approach targeting bacterial virulence and unlike classic antibiotics is aimed at disarming pathogens rather than killing them or inhibiting their growth. Inactivation of the bacterial arsenal of virulence factors will attenuate pathogens and render them susceptible to natural host defenses. The effect of the FDA-approved drug diclofenac sodium at a sub-inhibitory concentration (¼ MIC) on some *Escherichia coli* virulence factors was evaluated phenotypically and genotypically. Phenotypically, biofilm formation ability, proteases production and motility behavior were found to be significantly decreased under the effect of diclofenac sodium. Genotypically, qRT-PCR was used to evaluate the relative expression levels *of papC, fimH, ompT_m, stcE* and *flic* genes regulating virulence factors production. Importantly, the relative expression levels of all the tested genes showed significant downregulation under the effect of diclofenac sodium. The current research revealed that diclofenac sodium has a promising anti-virulence effect against resistant *Escherichia coli.* As a result, it is suggested that, in addition to or as an adjuvant to traditional antimicrobials, diclofenac sodium could be a possible solution to treat infections caused by resistant *Escherichia coli.*

Keywords: Diclofenac sodium; E. coli; Virulence inhibitors; Anti-virulence therapy.

1.   Introduction

Escherichia coli (*E. coli*) is a Gram-negative bacterium that lives in the human gut and belongs to the *Enterobacteriaceae* family and can spread through faeces or water routes into the environment to cause intestinal or extraintestinal infections (Lorenz *et al.* 2020). Pathogenic *E. coli* is a common bacterium that causes serious diseases such as urinary tract infections (UTIs), bacteremia, pneumonia, and neonatal meningitis (NM) in both the community and in hospitals (Metlay, *et al.* 2019; Nicolle *et al.* 2019). *E. coli* is the main cause of 50% of hospital-acquired UTIs and 85% of community-acquired UTIs. Each year, over 150 million people in the world suffer from UTIs, which has significant economic costs (Terlizzi *et al.* 2017). Complications of UTIs affect a variety of human organs including the bladder, ureters, and urethra, rendering it one of the most severe and contagious diseases affecting people globally (Rahman *et al.* 2019). In addition, *E. coli* is one of the major causes

of bacteremia in hospitalized patients. *E. coli* bacteremia is a significant problem, particularly for the elderly after the age of 55 to 60. If no preventive actions are taken, the number of *E. coli* bacteremia cases can be anticipated to rise in line with the world's elderly population, which is expected to grow from 617 million in 2015 to 1 billion by 2030 and to 1.6 billion by 2050. UTIs are the primary factors in more than 50% of cases of *E. coli* bacteremia (Bonten *et al.* 2021).

Also, *E. coli* can cause pneumonia which most frequently affects elderly people with medical conditions that weaken host defenses and cause high morbidity. *E. coli* pneumonia is frequently obtained in hospital settings as the organism may enter the respiratory tract through dispersion from a primary site in the gastrointestinal tract or the genitourinary tract or through aspiration of oropharyngeal secretions (John *et al*. 2022). Moreover, in developed countries, *E. coli* is the main cause of NM and nearly 30% of all NM cases are caused by this pathogen. Large cohort studies have shown that NM correlated to *E. coli* remains to be an important factor in sepsis-related morbidity and mortality in infants (Liu *et al.* 2021). A variety of virulence factors enables *E. coli* to survive in challenging conditions and play a significant role in the ability of the bacteria to produce infections (Vaish *et al.* 2016).

The virulence factors of *E. coli* are either associated with the surface of the bacterial cell or secreted and exported to their site of action. The most important virulence factors produced by *E. coli* include the polysaccharide capsule, biofilm formation ability, adhesins, proteases, toxins, motility behavior, and iron acquisition factors (Sarowska *et al.* 2019). Biofilms are highly organized communities of microorganisms that are embedded in a self-produced extracellular polymeric substance (EPS) and attached to biological or non-living surfaces. These biofilm communities possess unique properties that are not present in freely-living cells, such as the ability to protect themselves from external stress that enhancing antimicrobial resistance (AMR) and the transfer of antibiotic resistance genes between bacterial species. According to the National Institutes of Health, a staggering 80% of all human body infections are associated with biofilms. Microorganisms that form biofilms have the capacity to colonize various medical devices, including urinary catheters, thereby increasing mortality and morbidity rates, and transforming existing infections into chronic diseases. Additionally, biofilms play a significant role in the majority of chronic infections, such as UTIs and chronic wound infections (Ballén *et al*. 2022).

Virulence factors production in *E. coli* is controlled and regulated by several genes including *papC, fimH, ompT_m*, *stcE,* and *flic*. It was found that the *papC* is considered the biofilm-associated gene encoding the outer membrane assembly platform, and is responsible for the production of P fimbriae that play a crucial role in the pathogenesis of UTIs and biofilm formation (Schiebel, 2017). In addition, the *fimH* gene is encoding for proteinaceous filamentous adhesins known as type I fimbriae of *E. coli* that facilitate the initial attachment during biofilm formation, and are essential for *E. coli* capacity to form biofilms on abiotic surfaces (Schiebel, 2017). *OmpT* protease is regarded as a pivotal virulence factor in strains causing cystitis, pyelonephritis, and urosepsis as opposed to asymptomatic strains (Desloges *et al*. 2019). Importantly, the urogenital tract is protected from uropathogenic *E. coli* colonization by producing antimicrobial peptides (AMPs) as one of the innate defenses. It was found that the *ompT_m* gene regulates the production of *ompT* protease in uropathogenic *E. coli*, which enables the pathogen to resist AMP killing and cause a variety of UTIs (Desloges *et al.* 2019). On the other hand, the *stcE* gene is encoding for the production of some other proteases that contribute to the cleavage of some of the host mucosal glycoproteins on the host cell surface leading to facilitating adherence of *E. coli* to host cells (Grys *et al.* 2006). On the contrary, the bacterial flagellum is comprised of a polymer of flagellin subunits that are encoded by the *fliC* gene. More specifically, the *fliC* genetic sequence is accountable for the production of a subunit protein that get into polymerization, and as a result, yields the curved and elongated filaments of the flagella from the basal body of *E. coli* (Hidalgo *et al.* 2011).

The spread of multidrug-resistant (MDR) *E. coli* is a major global concern. The term MDR bacteria means bacteria that have acquired resistance to a minimum of three distinct classes of antimicrobial drugs, and it is commonly observed within healthcare facilities (Wang *et al.* 2019). The misuse and the overuse of antibiotics, according to the World Health Organization (WHO), accelerate the development of MDR *E. coli*, which becomes more difficult to treat as the antibiotics used to treat them become less effective (Totsika, 2016; Elfaky *et al.* 2022). The inability of current therapeutic techniques to successfully treat resistant *E. coli* infections emphasizes the requirement for novel therapeutic approaches (Foroogh *et al*. 2021). Significantly, a "disarm - don't kill" strategy aims to produce agents that can replace failing antibiotics while also eliminating two of their essential drawbacks; drug resistance and killing of commensal bacteria (Totsika, 2016). Therapeutic switching is a potential strategy for identifying off-label uses for existing approved drugs that are unrelated to their regular therapeutic uses. This drug repositioning method has the advantage of saving time, and money and the lowest

possible failure risk (Saleh *et al.* 2022).

The FDA-approved drug diclofenac sodium is a non-steroidal anti-inflammatory drug (NSAID) and is commonly used to cure various inflammatory diseases in addition to its analgesic activity (Xie *et al.* 2023). In previous research, it was proved that diclofenac can be used as a virulence inhibitor to limit bacterial colonization and reduce biofilm formation by interfering with quorum sensing-controlling virulence in *Pseudomonas aeruginosa* (Abbas, 2015). In addition, another study showed how diclofenac sodium reduced the development of biofilm in strong strains of *Klebsiella pneumoniae* and *E. coli* that are associated with UTIs (Baldiris *et al.* 2016).

In the present study, we aimed to investigate the potential inhibitory effects of the FDA-approved drug diclofenac sodium on the production of *E. coli* virulence factors by phenotypic and genotypic methods. Our results could ameliorate trials trying to find a different solution to combat *E. coli* infections and downgrade the spread of AMR.

2.   Material and Methods

2.1.   *Culture media and chemicals used in the current study*

The media used in this study include Tryptone Soya Broth (TSB), Luria-Bertani (LB) broth, and Mueller Hinton agar (MHA) which were purchased from Merk, Germany. Diclofenac sodium was kindly gifted from IDI Pharmaceuticals, Port Said, Egypt.

2.2.   *Bacterial isolates*

Six *E. coli* clinical isolates were used in the current investigation which were provided from the stock culture collection of the Microbiology and Immunology Department, Faculty of Medicine, Suez Canal University. These isolates were previously isolated from UTIs patients admitted at the Suez Canal University Specialized Hospital.

2.3.   *Antibiotic susceptibility testing of the isolates*

The antibiotic susceptibility testing of the tested isolates was performed on MHA using the disc diffusion method against ^ antimicrobials of different groups   including levofloxacin (LEV), meropenem (MRP), cefotaxime (CTX), trimethoprim/sulphamethoxazole (SXT), amikacin (AK), piperacillin (PRL), doxycycline (DO), and amoxicillin/clavulanic acid (AMC) that were purchased from (Oxoid, UK). Briefly, overnight cultures of the investigated isolates in TSB were diluted to reach the turbidity of 0.5 MacFarland. An aliquot of 10 µl of each isolate was inoculated on MHA plates. To achieve uniform distribution, the plates were swabbed three times perpendicularly. By sterile forceps, the antimicrobial discs were placed on the surface of the media. After overnight incubation, the diameter of each zone of inhibition around discs was measured in mm and the results were interpreted according to CLSI 2021.

2.4.   *Minimum inhibitory concentration (MIC) of the diclofenac sodium*

The MIC of diclofenac sodium was determined via the broth microdilution method (Abbas *et al.*2017). In brief, the overnight cultures of the tested isolates were diluted with TSB to obtain a cell density of 10^6 CFU/ml. A 2-fold serial dilution solution in LB broth was made using the tested drug with varying concentrations (0.25, 0.5, 1, 2, 4, 8, 16, 32, and 64 mg/ml) in addition to control wells without the drug. In microtiter plates, 100 µl of bacterial suspensions were added to 100 µl of the prepared diluted tested drug. After overnight incubation at 37°C, the lowest concentration of the tested drug inhibited the visible growth of bacteria and was determined to be the MIC value.

2.5.   *Evaluation of the effect of sub-MIC of diclofenac sodium on the bacterial growth*

Overnight cultures of the tested isolates were added to LB broth in the presence and absence of sub-MIC (¼ MIC) of diclofenac sodium and was adjusted to a turbidity of 0.5 MacFarland and incubated overnight at 37°C. The turbidities of treated and untreated isolates were measured at OD600 nm spectrophotometrically and the results were compared (Saleh *et al.* 2022).

2.6.   Phenotypic assessment of virulence factors inhibition in E. coli by diclofenac sodium

2.6.1. Biofilm inhibition assay

The assay of inhibition of biofilm formation in the tested isolates was measured quantitatively by the tube method as follows (Avdić *et al.* 2017). Overnight cultures were used to prepare suspensions of the tested isolates in LB broth that were adjusted to contain 10^6 CFU/ml in falcon tubes with and without sub-MIC (1/4 MIC) of diclofenac sodium. Following overnight incubation, the falcon tubes contents were emptied and washed three times by sterile distilled water to get rid of any planktonic bacteria. Consequently, the tubes were dyed for 15 minutes with 5 ml of 1% crystal violet solution and washed with water. After the tubes were dried in an inverted position, 5 ml of 96% ethanol was added to each tube and incubated for an additional 15 minutes. The absorbance of 1 ml of the ethanol/crystal violet solution was measured at 595 nm. The tubes inoculated with untreated isolates were used as a control.

2.6.2. Total proteases inhibition assay

Total proteases assay was carried out using the modified skimmed milk method (Fekry *et al*. 2022). The bacterial isolates' overnight cultures were inoculated in LB broth to reach the turbidity of the 0.5 McFarland standard (equal to OD_{625} of 0.08-1) in the presence and absence of sub-MIC (1/4 MIC) of diclofenac sodium. An aliquot of 200 µl of culture supernatants, obtained by centrifugation of the bacterial cultures at 10000 rpm for 10 min, is incubated with 1 ml skimmed milk (1.25% in sterile distilled water) at 37°C for 30 min. Tubes that had not been inoculated were utilized as a negative control. The optical density of skimmed milk for treated and untreated isolates was measured at 600 nm using a spectrophotometer to determine the proteolytic activity.

2.6.3. Swimming motility inhibition assay

The present investigation was carried out to evaluate the assay for inhibiting swimming motility, in accordance with procedures previously described by (AL-Ghonaiem, Ibrahim & Al-Salamah, 2010). To summarize, subsequent to an overnight growth in LB broth, 5 µl of both treated and untreated isolates of cultures were introduced into the center of a 0.4% soft LB agar plate, which were then incubated at 28°C. The progression of bacterial colonies was measured by observing their forward migration after 24 hours.

2.7.   RNA extraction and relative gene expression determination using qRT PCR

E. coli isolate (E1) was chosen as a representative isolate to estimate the relative expression levels of the genes regulating the production of virulence factors in *E. coli* in treated and untreated isolate to illustrate how the virulence factors in *E. coli* could be inhibited by diclofenac sodium at the molecular level. Briefly, the tested isolate (E1) was cultured overnight at 37 °C in TSB with and without ¼ MIC of diclofenac sodium until the bacteria reached the middle log phase (OD_{600} of 0.5–0.6). The pellets of the tested isolate were obtained by centrifuging at 6000 g for 10 min; following the manufacturer's instructions and RNA was extracted using the TRIzols Reagent (15596026, Life Technologies, USA). Reverse transcription followed by qRT-PCR of virulence factors regulating genes *papC, fimH, ompT_m, stcE* and *flic* was carried out according to the QuantiTects Reverse Transcription Kit's (Qiagen, Germany) procedures. The Rotor-Gene Q (Qiagen, USA) was used to conduct qRT-PCR analysis using the primers listed in Table (1). The thermal cycling protocol consisted of an initial denaturation step at 95 °C for duration of 10 minutes, followed by a series of 45 cycles. Each cycle involved a denaturation step at 95 °C for duration of 10 seconds, an annealing step at 60 °C for 15 seconds, and an extension step at 72 °C for 15 seconds. The relative expression values of each gene were standardized using the housekeeping gene 16S rRNA. The relative gene expression in the treated and untreated isolates was compared using the $2^{-\Delta\Delta CT}$ method (Livak 2001).

Table 1 Primers used in qRT-PCR.

Gene	Primer sequence	Annealing temperature	References
16s rRNA	F/ AGT TTG ATC MTG GCT CAG R/ GGA CTA CHA GGG TAT CTA AT	55°C	(Magray *et al.* 2011)
PapC	F/ TGA TAT CAC GCA GTC AGT AGC R/ CCG GCC ATA TTC ACA TAAC	65°C	(Schiebel 2017)
FimH	F/ TGC AGA ACG GAT AAG CCG TGG R/ GCA GTC ACC TGC CCT CCG GTA	57°C	(Schiebel 2017)
OmpT_m	F/ TTT GAT GCC CCA GAT ATC TAT CGG R/ GGC TTT CCT GAT ATC CGG CCA TG	56°C	(Desloges *et al.* 2019)
StcE	F/ AAG GGC CCC TCT GAG GTG TCT GTTAAA CCC GTG G R/AAA AA TGG CCA CGA AGT GGCCGC ACC GTC TCA GG	60°C	(Grys *et al.* 2006)
fliC	F/ ACA GCC TCT CGC TGA TCA CTC AAA R/ GCG CTG TTA ATA CGC AAG CCA GAA	59°C	(Hidalgo *et al.* 2011)

F = Forward, *R* = Reverse

2.8. Statistical analysis

The software package utilized for data analysis was GraphPad Prism 8.0.2. The calculations were based on the average values ± standard errors that were obtained from three biological experiments, wherein each experiment consisted of three technical replicates. The impact of the drug that was examined on *E. coli* virulence factors was evaluated by One Way ANOVA, in accordance with Tukey's Multiple Comparison test, at $P < 0.05$ for significance.

3. Results

3.1. Antibiotic susceptibility and resistance profile of the tested isolates

The tested isolates revealed a high resistance pattern against the tested antibiotics. Regarding the tested six isolates, 100% resistance was found against amoxicillin/clavulanic acid, piperacillin, cefotaxime, and trimethoprim/sulphamethoxazole. Furthermore, four isolates showed a resistance rate of 67% against levofloxacin while two isolates showed a resistance rate of 33% against doxycycline. On the other hand, no resistance was found against both amikacin and meropenem as illustrated in Table (2).

Table 2 Antibiotic susceptibility pattern of the tested isolates.

Antibiotic / Isolates	LEV	MRP	CTX	SXT	AK	PRL	DO	AMC
E 1	R*	S	R	R	S	R	R	R
E 2	R	S	R	R	S	R	S	R
E 3	S	S	R	R	S	R	S	R
E 4	S	S	R	R	S	R	R	R
E 5	R	S	R	R	S	R	S	R
E 6	R	S	R	R	S	R	S	R

* R=Resistant, S=Sensitive

3.2. Determination of MIC of diclofenac sodium

The broth microdilution method was used to assess the MIC of diclofenac sodium. It was found that diclofenac sodium inhibited the growth of the tested *E. coli* isolates at 16 mg/ml.

3.3.   The effect of sub-MIC of the tested drug on E. coli growth

The inhibitory activities against *E. coli* virulence factors were tested at a concentration equivalent to ¼ MIC of the sub-MIC on bacterial viable growth. Notably, no discernible change in growth was seen between the treated and untreated isolates, suggesting that using sub-MIC of diclofenac sodium has no significant effect on bacterial growth as in Fig. 1.
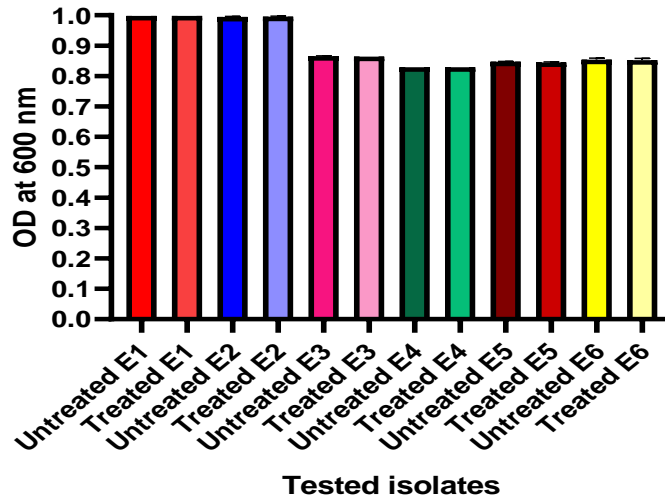

Fig. 1. The impact of ¼ MIC of diclofenac sodium on bacterial growth after 24 hours of incubation. The experiment was done in triplicate. *, Significant $P < 0.05$ was considered significant.

3.4.   The influence of diclofenac sodium on the production of E. coli virulence factors

3.4.1.   Biofilm inhibition assay

Diclofenac sodium exhibited a significant reduction in the biofilm formation ability of all the tested isolates from 100% in control untreated isolates to percentages ranging from 15% to 89 % in treated isolates (Fig. 2).
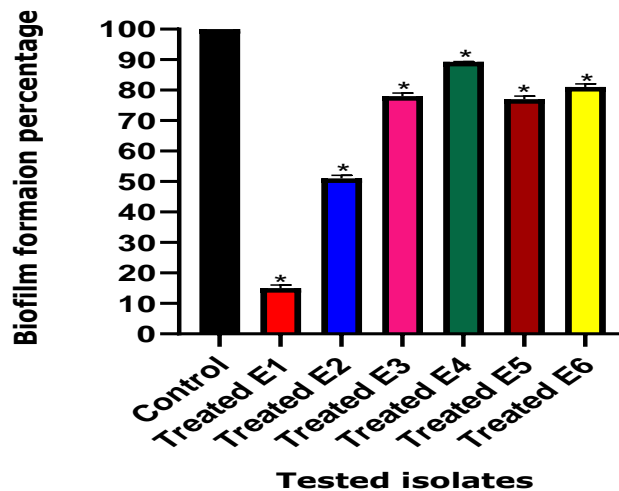

Fig. 2. The effect of ¼ MIC of diclofenac sodium on biofilm formation in *E. coli*. The results represented the means ± standard errors of three experiments. * Significance, the P value was < 0.05 for significance.

3.4.2. Total proteases inhibition assay

Diclofenac sodium showed a significant decrease in proteolytic activity from 100% in control untreated isolates to percentages ranging from 9% to 63% in treated isolates. Non-significant reduction was observed by the isolate (E3) from 100% in control untreated isolates to 98% in treated isolate, as shown in Fig. 3.
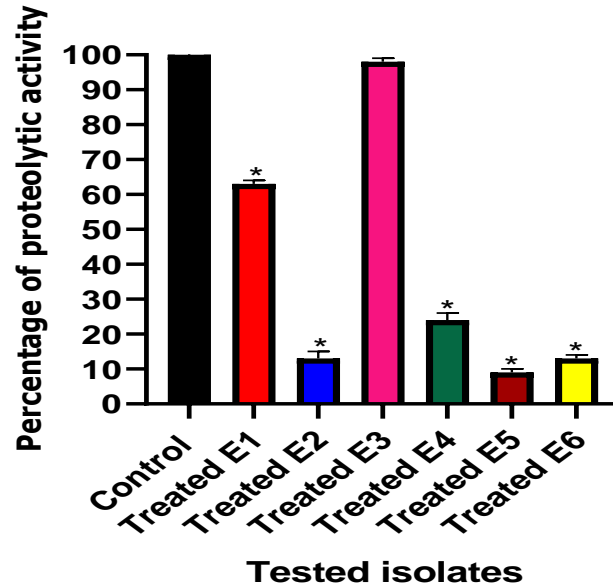

Fig. 3. The effect of ¼ MIC of diclofenac sodium on proteases activity in *E. coli*. The results represented the means ± standard errors of three experiments. * Significance, the P value was < 0.05 for significance.

3.4.3. Assessment of motility inhibition

Diclofenac sodium has demonstrated a significant decrease in motility behavior, from 100% in control untreated isolates to percentages ranging from 15% to 56% in treated isolates, as shown in Fig. 4.
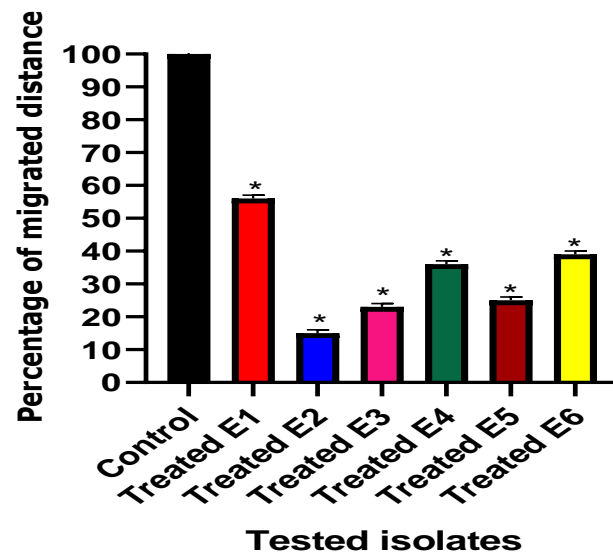

Fig. 4. The inhibitory effect of sub-MIC of diclofenac sodium on swimming motility of *E. coli* isolates. The results represented the means ± standard errors of three experiments. * Significance, the P value was < 0.05 for

significance.

3.5.   *Estimation of relative gene expression of virulence factors encoding genes in E. coli by qRT-PCR*

At the molecular level, isolate E1 was selected as a representative example to confirm the inhibitory ability of diclofenac sodium on virulence factors regulating genes in treated and untreated *E. coli* isolate using qRT-PCR and was analyzed using the $2^{-\Delta\Delta Ct}$ method. Remarkably, the relative expression levels of *papC, fimH, ompT_m, stcE* and *flic* regulating genes were significantly reduced in the diclofenac sodium-treated isolate in comparison to the control untreated isolate. Importantly, diclofenac sodium decreased *papC* relative expression level by 40%, while *fimH* gene relative expression was suppressed with a percentage of 50% in diclofenac sodium-treated isolate. Moreover, diclofenac sodium significantly lowered *ompT_m* relative expression levels by 50%, while *stcE* and *flic* relative expression level was reduced by a percentage of 60%, and 40%, respectively, in the diclofenac treated isolate Fig. 5.
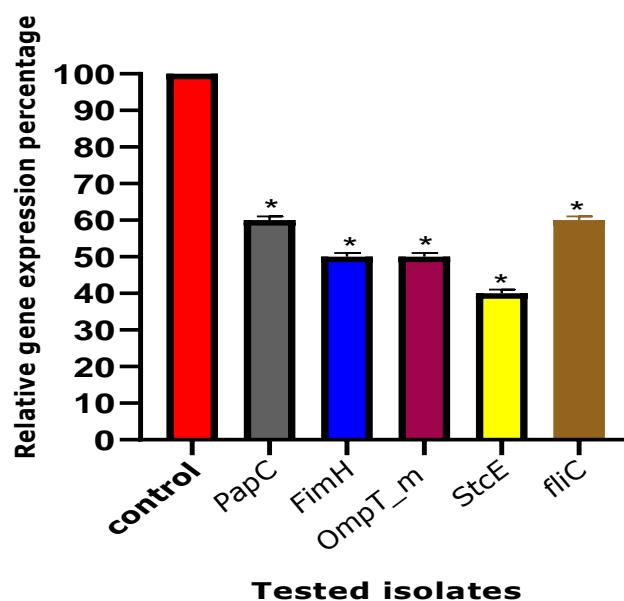

Fig. 5. The impact of diclofenac sodium at ¼ MIC on virulence factors regulating genes (*papC, fimH, ompT_m, stcE* and *flic*) in *E. coli* treated isolate. The results represented the means ± standard errors. * Significance, the P value was < 0.05 for significance.

4.   Discussion

Antibiotic resistance is a global public health crisis that appears unavoidable. In the modern era, MDR strains of *E. coli* have appeared remarkably, and many antimicrobial drugs are becoming less effective as a result (Am *et al*. 2016). Along these lines, MDR *E. coli* has become a significant public health issue in many countries leading to treatment failure, high rate of mortality, and serious stress on the health system (Ibrahim *et al*. 2012). The ability of *E. coli* to develop a variety of virulence factors contributes to its pathogenicity and its capacity to cause serious diseases such as NM, UTIs, and bacteremia (Vaish *et al*. 2016). The anti-virulence strategy is a new approach that is expected to render antimicrobial treatments superior to traditional antibiotics (Totsika, 2016). The purpose of using anti-virulence drugs is to repurpose medications that disarm the pathogenic bacteria instead of killing them (Calvert *et al*. 2018).

Based on previous studies (Abbas, 2015; Jangra *et al*. 2022), that proved the effectiveness of diclofenac sodium as an anti-virulence agent in other bacterial species, we tested the effect of diclofenac sodium as a possible anti-virulence agent in *E. coli*.

In the current research, the tested isolates revealed 100% resistance against AMC, PRL, CTX, and SXT. Also, the tested isolates showed a resistance rate with percentages of 67% and 33% against LEV and DO, respectively. On

the other hand, no resistance was found against AK and MRP. Similarly, in previous research, MRP and AK demonstrated the greatest sensitivity against the tested *E. coli* isolates, with percentages of 72.4%, and 96.5%, respectively (Essawy *et al.* 2018), while AMC and CTX displayed the highest rate of resistance, with percentages of 68.4% and 42.1% respectively (Algammal *et al*. 2022). Also, a previous report indicated that *E. coli* isolates demonstrated notable resistance to LEV and DO, with resistance rates of 18% and 37% respectively (Aly, Essam & Amin 2012). Moreover, in previous study, the *E. coli* isolates showed 85.7% resistance against PRL and 100% resistance against SXT (Ali *et al.* 2022). Based on the current findings, it is possible to attribute the high susceptibility rates of AK and MRP to the accurate selection of these antibiotics for therapeutic purposes, as this approach ensures the preservation of the antibiotics' efficacy. Conversely, the increasing resistance rate of the remaining antibiotics can potentially be linked to their widespread empirical utilization and inadequate implementation of infection control measures. These associations have been previously reported in the literature (Al-Baz *et al*. 2022; El-Mahdy, 2022).

In the current study, diclofenac sodium inhibited the growth of the tested isolates at MIC of 16 mg/ml. The effect of diclofenac sodium might be due to DNA synthesis disruption (Abbas, 2015). Further phenotypic and genotypic tests were carried out at a concentration of ¼ MIC.

E. coli starts infection by adhering to the host cell to cause the disease. The main virulence factors involved in this adhesion mechanism are adhesins such as pili, which enhance attachment and help bacteria colonize their host's system and biofilm formation (Ghazaei, 2021). Nevertheless, the biofilm is formed when EPS form a matrix to shield the bacteria from environmental challenges and also reduce the penetration of antimicrobial drugs. Additionally, the EPS matrix hinders the inner zones of the biofilm from absorbing oxygen and nutrients, producing some cells, known as persisters, to enter a vegetative state making them inaccessible by antimicrobial drugs (Leão *et al.* 2020). Collectively, with typical antibiotic dosages, it is nearly impossible to eradicate a biofilm after it has been developed on an abiotic or tissue surface. Despite the limitations, in light of one possible solution to this issue is the use of antibiotics in combination with substances that have anti-biofilm activity (Liu *et al.* 2021).

In the present research regarding the phenotypic tests, the biofilm formation ability of *E. coli* was evaluated under the effect of diclofenac sodium. Importantly, an obvious inhibitory effect of the tested drug on the production of biofilm was observed which is found to be in line with previous research revealed that diclofenac sodium limited the biofilm formation ability in *E. coli* by a percentage of between (11.4-52.2%) (Reśliński *et al.* 2015). Also, in an earlier study, diclofenac sodium reduced the biofilm formation ability by 50.1% in MDR *E. coli* which is following our results (Baldiris *et al*. 2016). Likewise, in earlier research similar to our results, diclofenac sodium exhibited an anti-biofilm activity in *S. aureus* and reduced its pathogenicity (Alves *et al.* 2021). Furthermore, the previous report (Abbas *et al.* 2020) revealed that diclofenac sodium significantly reduced the biofilm formation in *S. aureus* with a percentage ranging between 22.67 to 70% which is close to some extent to our results. Moreover, in a previous report, diclofenac sodium reduced the biofilm formation ability by a percentage of (90%) in *Proteus mirabilis* (Hegazy, 2016). Additionally, when combined with fluconazole or voriconazole, it was found that diclofenac sodium reduced the development of biofilm in *Candida tropicalis* (Brilhante *et al.* 2020).

The mechanism by which diclofenac sodium disrupts the biofilm development in bacteria might be attributed to bacterial adhesion interference, EPS interference, or bacterial cell surface properties alternation (Abbas, 2015).

On the other hand, proteases in *E. coli* are playing a pivotal role in regulating the levels of some proteins and in degrading defective or abnormal proteins. Thus, bacterial proteases are pivotal for cell survival and stress response, which increases the toxicity of resistant bacteria (Luo *et al.* 2008). Fortunately, using anti-virulence drugs targeting the proteases is an attractive target that can substantially minimize the resistance development by preventing colonization and penetration of the host (Kaya *et al.* 2022). Similar to our data showed that proteases production was reduced by diclofenac sodium in *E. coli* by about 64 %, the previous research done by (Abbas, 2015) reported that proteases have been significantly decreased by diclofenac sodium with a percentage of 52.58% in *P. aeruginosa*. In another study, treatment of *P. aeruginosa* with the NSAID, tenoxicam, lowered proteases production by a percentage of (14-46%) which is to some extent similar to our findings (Askoura *et al.* 2020). Furthermore, in accordance with our results, the earlier research (El-Mowafy *et al.* 2014) revealed that the NSAID, aspirin, has a proteases inhibitory efficacy of about 58% in *P. aeruginosa*. An important note needs to be highlighted that E3 isolate was not inhibited by diclofenac sodium in testing its proteolytic activity. That may be due to intrinsic differences in their virulence factor production compared to the other tested strains. It is possible that this strain has evolved alternative mechanisms that are not affected by the tested drug.

The role of flagella-mediated motility is associated with pathogenicity. Specifically, it performs a pivotal role in

increasing attachment, expediting the development of biofilms, and regulating the immune system (Erhardt, 2016). The swimming motility assessment was conducted both with and without sub-MICs of the tested drugs. Results indicated that the tested drugs significantly reduced the swimming motility of the examined isolates, with untreated isolates exhibiting 100% motility. Similar to our results, in a previous investigation, it was observed that the provision of diclofenac to *P. aeruginosa* led to a significant reduction in motility by 84% (Ulusoy *et al.* 2013).

The molecular understanding of *E. coli* pathogenesis is critical in the fight against this important human pathogen since it may aid in the development of new therapeutic techniques. Considering the phenotypic results suggesting that diclofenac sodium lowered the production of the virulence factors (biofilm and proteases) in *E. coli*, it was crucial to evaluate the influence of diclofenac sodium on bacterial virulence regulating genes such as biofilm-associated genes (*papC, and fimH*) and protease-associated genes (*ompT_m and stcE*) using qRT-PCR.

Significantly, the levels of the relative gene expression of all the tested genes were decreased by a percentage ranging between 40%-50% under the effect of diclofenac sodium in our research. In the current study, the *fimH* gene relative expression level was suppressed with a percentage of 50% in diclofenac sodium-treated isolate. In a previous study, the natural substances curcumin, thymol, and eugenol downregulated the relative expression of the *fimH* gene in multi-virulent *E. coli* which is in accordance with the current results (Elfaky *et al.* 2022). In another report, it was reported that diclofenac sodium showed significant downregulation of the biofilm-regulating genes in *S. aureus* by a percentage ranging between 38.55% and 61.05% (Abbas *et al.* 2020). Also, in previous research, it was discovered that *Medicago sativa* (Alfalfa) extract prevents *E. coli* from producing biofilms by reducing the expression levels of *papC* which is in accordance with our data (Chamachar *et al.* 2022). In another report, the biofilm-associated genes and protease-associated genes in *P. aeruginosa* were downregulated under the effect of the NSAID, aspirin, by a percentage between (69-72%) and (38-72 %), respectively (El-Mowafy *et al.* 2014). Furthermore, it was reported that the NSAID, tenoxicam, showed a significant downregulation of proteases regulating genes by a percentage ranging between (6-27%) in *P. aeruginosa* which is to some extent in accordance with our results (Askoura *et al.* 2020).

5. Conclusion

The establishment of alternative therapies has become necessary due to the challenges of dealing with antibiotic resistance. In this regard, the use of non-antibiotic medications such as diclofenac sodium as an anti-virulence agent, whether alone or as an adjuvant to traditional antibiotics, is a promising strategy.

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