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Original Article

Thymol Enhances Antibiotic Sensitivity and Inhibits Biofilm Formation in Urinary Tract Infection Isolates of Escherichia coli From Gilan Province, Iran

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Abstract

Background: The present study aimed to investigate the effect of thymol on growth inhibition, biofilm inhibition, and antibiotic sensitivity of *Escherichia coli* isolated from urinary tract infection in Gilan province, Iran.

Methods: The disc diffusion and broth microdilution methods were used to investigate the antimicrobial effect of thymol on 30 urinary tract infection isolates of *E. coli*. Additionally, the time-kill assay was used to investigate the effectiveness of thymol at different time intervals over a period of 24 hours. The anti-biofilm effect of thymol was investigated using the microplate method. Moreover, the effect of thymol on *fim*H gene expression was investigated. To investigate the effect of thymol on the sensitivity of *E. coli* isolates to cefotaxime and ciprofloxacin, the checkerboard assay was used.

Results: ThymolFF exhibits inhibitory effects on the growth of *E. coli* isolates and the diameter of the zone of growth inhibition caused by 5 mg of thymol in the studied isolates varied between 15 and 30 mm and minimum inhibitory concentration (MIC) of thymol against MDR *E. coli* isolates ranged from 1 to 4 mg/mL. Depending on the concentration and the exposure time, bacterial killing was promoted by thymol treatment. The use of thymol decreased the biofilm formation of the isolates by more than 50% compared to the control and also bacterial treatment with thymol led to down-regulation of the *fim*H gene. Furthermore, enhancing the antibacterial effect of cefotaxime and ciprofloxacin by thymol was demonstrated.

Conclusion: The present study found that thymol, a terpenoid of plant origin, exhibited strong killing efficiency, inhibiting the biofilm formation, and enhanced antibiotic sensitivity of *E. coli* isolated from urinary tract infection.

Keywords: *Escherichia coli,* Thymol, Biofilm, Antimicrobial

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Introduction

Urinary tract infections (UTIs) are the most common cause of hospital infections and the second most common infection among humans. More than 150 million cases of UTIs are reported per year worldwide, affecting all age groups. Most UTIs are caused by bacteria that are part of the normal flora, such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus* species. The treatment of UTIs is costly and is commonly treated with antibiotics, which leads to rapid adaptation of microbiota of the gut and vagina and the development of antibiotic-resistant bacteria (1-3). About 80%-90% of non-hospital UTIs are caused by *E. coli* (1). This bacterium is a member of the *Enterobacteriaceae* family which is usually resistant to antibiotics. This resistance is due to multiple intrinsic and acquired mechanisms present in the bacterium. Normally, beta-lactam antibiotics are prescribed to treat *E. coli* infections, but the emergence and variety of beta-lactamase-producing species have limited the effectiveness of these strains (2). In recent years, because of the resistance of pathogens to common antibiotics and increasing interest in using natural antimicrobial substances, research on the effectiveness of antimicrobial plant substances has increased (3). Plants produce a wide range of secondary metabolites with many desirable biological functions which can protect plants against plant pathogens and against abiotic stresses (4,5). Interactions between active plant substances and antibiotics can lead to antagonistic, additive, or synergistic effects. Usually, the use of several antimicrobial substances together can disrupt

several different biochemical processes which destroy microbial agents. Thymol (2-isopropyl-5-methyl phenol) is the most important phenolic monoterpene extracted from plants of the *Lamiaceae* family, including thyme, oregano, basil, and spearmint. Various studies have shown that thymol has strong antiseptic, anti-inflammatory, and antioxidant properties. According to the Food and Drug Administration (FDA), this substance is considered safe and can be used as a food additive (3,6,7). Moreover, the association between thymol and commercial antibiotics has been studied in several works and the results are promising. Thymol enhanced the antibacterial effect of neomycin against *Staphylococcus aureus* and showed synergistic effects on the activity of ceftriaxone against *Enterococcus faecalis* (8). Besides, thymol in combination with streptomycin and kanamycin showed synergic effects against *K. pneumoniae* biofilms (9). In another study, thymol has been shown to be synergistic with streptomycin and gentamicin against *S. aureus* and with streptomycin against *Streptococcus agalactiae.* In addition, thymol showed synergistic activity with chloramphenicol against *Acinetobacter baumannii* (10). Thymol increases the sensitivity of colistin-resistant gram-negative bacteria to colistin and may be a promising treatment option against infections caused by these bacteria (11). However, there is a lack of studies on the synergistic activity of thymol combined with antibiotics which are commonly used in the treatment of UTIs caused by drug-resistant *E. coli* isolates. The purpose of this study was to investigate the effect of thymol on growth inhibition, biofilm inhibition, and antibiotic sensitivity of *E. coli* isolated from UTI using time-kill assay, microtiter plate method, and checkerboard assay in Gilan province, Iran.

Materials and Methods

Test Bacteria

In this study, a total of 30 *E. coli* were isolated from UTIs in Gilan province, Iran, by transferring clean-voided midstream urine samples to blood agar and MacConkey (MC) agar media using a 0.01-ml platinum loop. After incubation at 37 °C for 24 hours, UTI was diagnosed as described previously (2) and isolates were identified by biochemical tests which include catalase, oxidase, motility, indole production, urease production, methyl red, citrate utilization, growth on triple sugar iron agar (TSI) and lysine iron agar (LIA), and oxidative/fermentative degradation of glucose (3).

The resistance pattern of *E. coli* to selected antibiotics was established using an antibiogram test (disk diffusion) following the CLSI 2021 guidelines. Antibiotic discs used in this study included gentamicin (10 µg), ceftazidime (30 µg), cefotaxime (30 µg), ampicillin/sulbactam (30 µg), meropenem (10 µg), piperacillin (100 µg), imipenem (10 µg), piperacillin/tazobactam (36 µg), and ciprofloxacin (5 µg), which were purchased from Himedia (India). *E. coli* ATCC 25922 was included for quality control, and breakpoints were in accordance with CLSI guidelines. Then, the number of strains with multiple drug resistance (MDR) was determined based on CDC definition (12).

Biofilm Forming Ability

Phenotypic assessment of the biofilm-forming ability of test isolates has been done by microplate method. Briefly, standard overnight cultures (1.5×10^8 CFU/mL) of test bacteria have been diluted 100 folds in Tryptic soy broth containing 1% glucose. Subsequently, 200 µL of each dilution was transferred to individual wells of a 96-well plate and incubated for 48 hours at 37 °C. After incubation, the wells were washed with PBS (three times) to remove planktonic bacteria and then fixed with methanol for 20 minutes. Thereafter, each well was stained with 200 µL of 0.02% crystal violet and washed with distilled water for 5 minutes. After drying the plates and adding 200 µL of 33% glacial acetic acid to each well, biofilm was quantitatively analyzed by reading the OD of the wells at 570 nm with an ELISA reader. The level of biofilm formation has been determined according to OD values ($OD \leq ODc$ = no biofilm producer; $ODc < OD \leq 2\times ODc$ = weak biofilm producer; $2\times ODc < OD \leq 4\times ODc$ = moderate biofilm producer; $4\times ODc < OD$ = strong biofilm producer) (13).

Investigation of the Inhibitory Effect of Thymol on Escherichia coli Isolates

The disc diffusion method was used to investigate the antimicrobial effect of thymol on clinical isolates of *E. coli*. For this purpose, 100 µL of standard overnight bacterial suspension (1.5×10^8 CFU/mL) was cultured on Mueller Hinton Agar. Afterwards, the standard discs were coated with 1, 2.5, 5, and 10 mg of thymol and transferred to the culture. Finally, the plates were incubated for 24 hours at 37 °C and the diameter of the zone of inhibition was measured. This experiment was performed in triplicate.

Determination of Minimum Inhibitory Concentration of Thymol

The minimum inhibitory concentration (MIC) of thymol against 10 selected MDR *E. coli* isolates was obtained by broth microdilution method. To perform this experiment, serial dilution of thymol including 0.5-32 mg/mL was prepared in 100 µL of Mueller Hinton broth medium. Then, 100 µL of standard overnight bacterial suspension (1.5×10^8 CFU/mL) was added to each well. Then, the plate was incubated for 24 hours at 37°C. This experiment was done in duplicate (10).

Investigation of the Anti-biofilm Effect of Thymol

The anti-biofilm effect of thymol was investigated using the microplate method. The procedure was the same as the phenotypic evaluation of biofilm-forming ability, with the difference that the trypticase broth medium contained a sub-MIC of thymol for each isolate (ranging from 0.5 to 2 mg/mL). All tests have been performed in triplicate and the percentage of inhibition was calculated as described previously (14).

Time-kill Assay for Thymol

The effectiveness of thymol against clinical isolate of MDR *E. coli* has been determined by time-kill assay at different time intervals over a period of 24 hours. In these assays, overnight bacterial suspension (5×10^5 CFU/mL) was incubated in a 96-well plate containing adjusted Mueller Hinton broth supplemented with thymol in separate wells. For the determination of cell viability, aliquots were removed from each tube after 0, 2, 4, 8, 12, and 24 hours of incubation and subsequently were diluted serially (1:10) with PBS. Each dilution was cultured on Muller-Hinton agar plates. The time-kill experiment was performed in triplicate (5).

Investigation of the Effect of Thymol on Antibiotic Sensitivity of Escherichia coli Isolates

The checkerboard assay was used to investigate the effect of thymol on the sensitivity of *E. coli* isolates to cefotaxime and ciprofloxacin. In brief, in a 96-well microplate, thymol and selected antibiotic were diluted in MHB and 8 gradient concentrations of each, including 1/32×MIC-4×MIC, were prepared. Each horizontal row of wells contained a similar level of antibiotic concentration and each longitudinal column had an equal amount of thymol. Each well had a total volume of 200 µL, including 100 µL of bacterial suspension (5×10^5 CFU/mL), 50 µL of thymol, and 50 µL of cefotaxime/ciprofloxacin. Moreover, several control wells, including drug-free, bacteria-free, and single antibacterial agent, were used. *E. coli* ATCC 25922 was used as sensitivity control strain. After 24 hours of incubation at 37 °C, the MIC values were read. Each test was done three times. Synergy was determined by the calculation of the fractional inhibitory concentrations index (FICI) as described earlier (15).

Investigation of the Effect of Thymol on fimH Gene Expression

Total RNA was extracted from *E. coli* isolate treated with a sub-MIC of thymol (1 mg/mL) using the Cinagen kit based on the manufacturer's protocols and subsequently subjected to DNase treatment. Bacterial culture without the presence of antimicrobial substances was used as a control. Qualitative and quantitative analysis of extracted RNA was done using agarose gel electrophoresis and a NanoDrop spectrophotometer, respectively. Then, 0.2 µg of the extracted RNA was immediately used to synthesize cDNA using the Cinagen RT kit and thermal treatment was performed at 85 °C for 5 seconds, followed by incubation at 42 °C for 60 minutes. Then, the synthesized cDNA was used as a template in real-time PCR. In this study, the *gap*A gene was used as an internal control. Primer sequences for amplification of *fim*H gene were the same as described previously (16). The polymerase chain reaction was done using the Genet bio kit (CAT. NO: Q9210, South Korea). The reaction was performed under the following conditions: initial annealing steps at 95 °C for 1 minute, 35 thermal cycles of 95 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds. Gene expression change was calculated using $2^{-\Delta\Delta CT}$ method (16). A no-template sample was included as a negative control in each run for each gene. This reaction was repeated 3 times.

Statistical Analysis

In this study, *t* test was used to analyze the difference between the control and treatment groups in SPSS. A *P* value of <0.05 was considered statistically significant.

Results

Test Bacteria

Among tested isolates, 25 isolates showed MDR phenotype and all of them showed biofilm-forming ability in phenotypic assay. Among them, 10 isolates which were MDR, strong biofilm former, and resistant to both antibiotics (cefotaxime and ciprofloxacin) were selected for further investigations.

Antibacterial Effects of Thymol against Escherichia coli Isolates

The effect of thymol on inhibiting the growth of *E. coli* isolates was investigated by diffusion method and MIC determination. The diameter of the zone of growth inhibition caused by 5 mg of thymol in the studied isolates varied between 15 and 30 mm (Figure 1). The MIC of thymol against MDR *E. coli* isolates ranged from 1 to 4 mg/mL.

The Effect of Thymol on Antibiotic Sensitivity of Escherichia coli Isolates

The measurement of the FIC index of thymol and cefotaxime in 10 MDR *E. coli* isolates (Table 1) showed that thymol and cefotaxime had additive and synergism effects in 8 and 2 isolates, respectively. The use of thymol in combination with cefotaxime reduced the MIC values of this antibiotic 2 to 8 times, but in most cases, no decrease was observed in the MIC of thymol. Thymol and ciprofloxacin had an additive effect in all the tested isolates (Table 2), and the use of thymol in combination with this antibiotic caused a 2-64-fold decrease in the

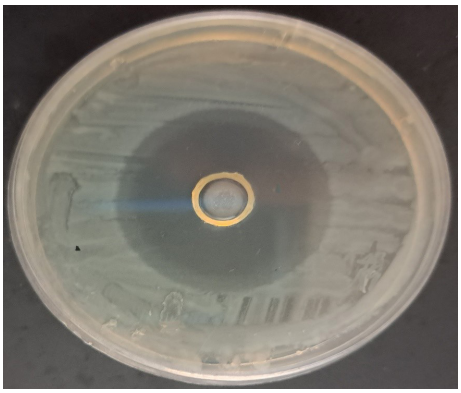

Figure 1. Growth Inhibition of *Escherichia coli* Isolate Caused by 5 mg of Thymol

Table 1. The Effect of the Combination of Cefotaxime and Thymol on MDR *Escherichia coli* Isolates

Bacterial test	1	2	3	4	5	6	7	8	9	10
MIC of cefotaxime	64	32	64	64	1024	512	512	128	32	128
MIC of thymol	4	1	2	2	2	1	4	2	1	2
MIC of cefotaxime in combination with thymol	4	16	4	8	256	256	64	64	16	16
MIC of thymol in combination with cefotaxime	4	1	2	2	1	1	2	2	1	2
FIC index	1.12	1.5	1.06	1.25	0.75	1.5	0. 5	1.5	1.5	1.12

MIC: Minumim inhibitory concentration, FIC: Fractional inhibitory concentration.

Table 2. The Effect of the Combination of Ciprofloxacin and Thymol on Multidrug-Resistant *Escherichia coli* Isolates

Bacterial Test	1	2	3	4	5	6	7	8	9	10
MIC of ciprofloxacin	128	64	16	1024	1024	16	512	64	64	128
MIC of thymol	4	2	4	2	2	1	4	2	1	2
MIC of ciprofloxacin in combination with thymol	16	1	1	256	32	8	16	32	4	8
MIC of thymol in combination with ciprofloxacin	4	2	4	2	2	1	4	2	1	2
FIC index	1.12	1.02	1.06	1.25	1.03	1.5	1. 03	1.5	1.06	1.06

MIC: Minumim inhibitory concentration, FIC: Fractional inhibitory concentration.

MIC of ciprofloxacin. Enhancing the antibacterial effect of cefotaxime and ciprofloxacin by thymol (obtained from the disc diffusion method) is presented in Figures 2 and 3.

Investigation of the Anti-biofilm Effect of Thymol

Thymol inhibited the biofilm of *E. coli* in all studied isolates compared to the control ($P<0.05$). The use of thymol decreased the biofilm formation of the isolates by more than 50% compared to the control. The inhibitory effect of thymol on the biofilm formation of each test isolate is shown in Figure 4.

Time-kill Assay for Thymol

The bactericidal activity of thymol against the selected *E. coli* isolates with regard to the changes in the log10 CFU/mL of viable cells is shown in Figure 5. In the time-kill curve assay (Figure 1), after 24 hours of incubation at 37 °C, the bacterial population reached approximately 12 log10 CFU/mL in the control group. When test bacteria were treated with thymol at a concentration of 0.5 × MIC, a gradual decrease in bacterial counts was observed during incubation, and the bacterial population showed a reduction of 2.8-3.4 log10 after 24 hours of exposure. At MIC of thymol, the viability of test bacteria was sharply decreased and no viable cells were detected after 8-12 hours in tested isolates. A sharp drop in the number of viable *E. coli* cells was detected in treatment with thymol at 2 × MIC and no viable cells were observed within 2-4 hours of incubation. The concentration of thymol and the duration of exposure were also considered to be important factors for promoting bacterial reduction following treatment with thymol.

The Effect of Thymol on the Expression Level of the fimA Gene

The presence of the *fim*H gene in the studied isolates of *E. coli* was detected by the PCR. Examining the expression of

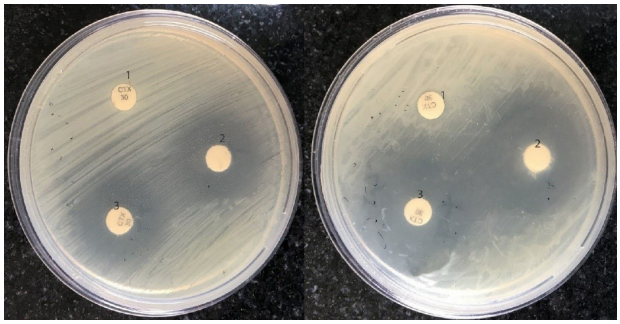

Figure 2. Growth Inhibition of *Escherichia coli* Isolates by Cefotaxime (1), Thymol (2), and the Combination of Thymol and Cefotaxime (3) Using the Disc Diffusion Method

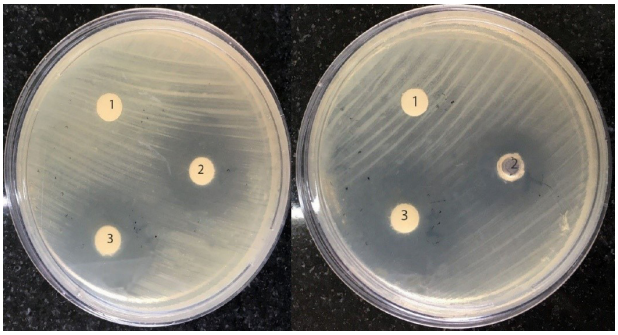

Figure 3. Growth Inhibition of *Escherichia coli* Isolates by Ciprofloxacin (1), Thymol (2), and the Combination of Thymol and Ciprofloxacin (3) Using the Disc Diffusion Method

*fim*H in *E. coli* isolates treated with a sub-MIC of thymol showed that the difference between the treatment and control groups was significant at the probability level of 1% and thymol significantly caused a reduction in the expression of this gene compared to the control group (Figure 6).

Discussion

Urinary tract infections are the most common cause of hospital infections and the second most common

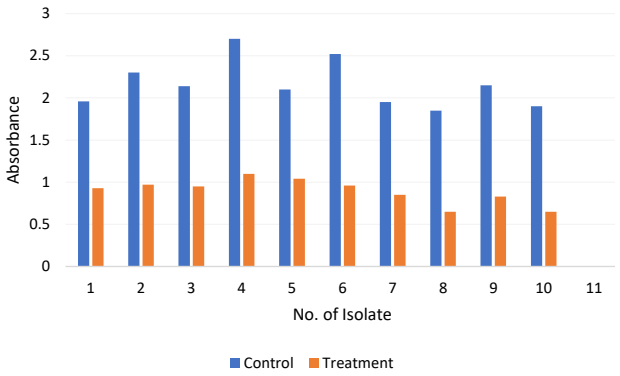

Figure 4. The Anti-biofilm Effect of Thymol on Multidrug-Resistant *Escherichia coli* Strains. The diagram shows the absorbance of *E. coli* isolates in the absence (control) and presence of sub-MIC of thymol (treatment). Thymol decreased the biofilm formation of the isolates by more than 50% compared to the control

infection in humans. About 80%-90% of non-hospital UTIs are caused by *E. coli*. Since the resistance to antibiotics in the treatment of infections has increased in recent years, the need for new resources and solutions to deal with infections and pathogenic bacteria is felt (1,4,17). The secondary metabolism of plants produces many types of molecules with potential antimicrobial activity. Most of these molecules have weaker antibacterial activity compared to antibiotics but they can act as synergism with antimicrobial drugs to enhance their effect. Thymol is the main active ingredient in thyme oil and extract, and their antimicrobial, antifungal, and antibacterial properties have been proven (5). In the present study, the antimicrobial effect of thymol on urinary isolates of *E. coli* and its synergistic effect with cefotaxime and ciprofloxacin, which are widely used drugs in the treatment of UTIs, were studied. Thymol has an inhibitory effect on all MDR isolates studied. The lowest growth inhibitory concentration (MIC) of thymol in the examined isolates ranged from 1 to 4 mg/mL. In the agar diffusion method, using the combination of thymol with cefotaxime and ciprofloxacin caused an increase in the diameter of the zone of growth inhibition of the studied bacteria. In addition, thymol in combination with cefotaxime had additive (83.33%) and synergistic (16.66%) effects and in combination with ciprofloxacin in all cases (100%), there were additive effects on tested *E. coli* isolates. The use of thymol in combination with these antibiotics significantly reduced the MIC of the antibiotic. The use of thymol in combination with cefotaxime caused a 2- to 8-fold decrease in the MIC of cefotaxime, but in most cases, a decrease in the MIC of thymol was not observed. Besides, the use of thymol in combination with ciprofloxacin caused a 2- to 64-fold decrease in the MIC of ciprofloxacin.

The use of herbal antimicrobial compounds in combination with antibiotics can reduce the MIC of antibiotics and prevent the increase in drug resistance (18,19). Since essential oils alone do not have a specific target against bacteria, the risk of resistance is negligible

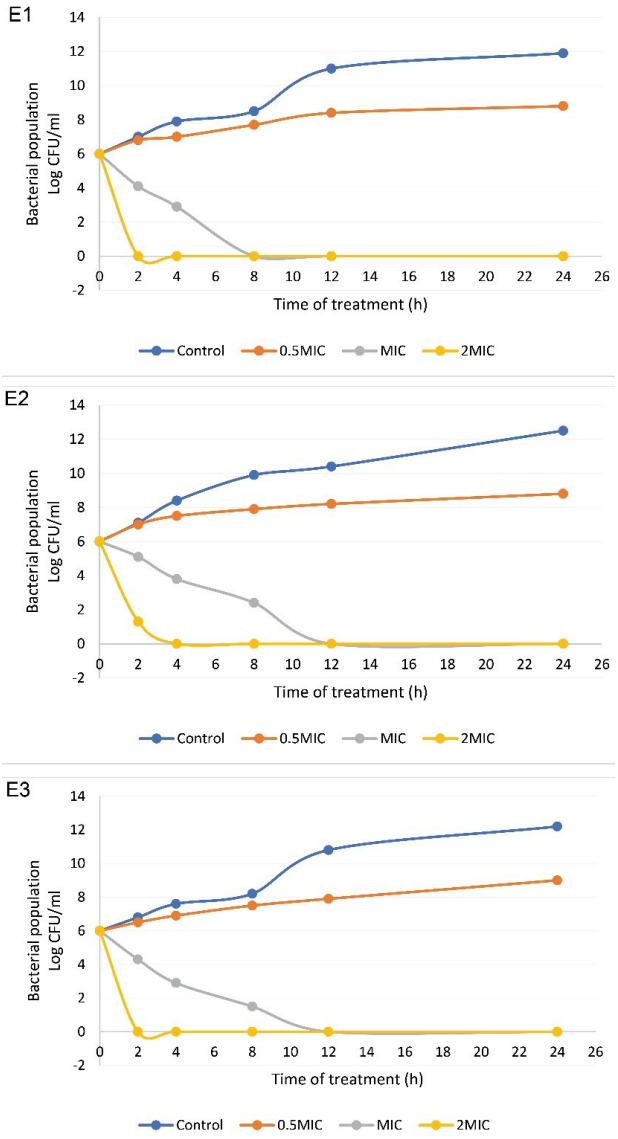

Figure 5. Bacterial Killing Kinetics of Thymol at 0.5 × MIC, MIC, and 2 × MIC Compared with Control against Selected *E. coli* Isolates. Treatment with thymol at 0.5 × MIC led to a reduction of 2.8-3.4 log10 CFU/mL after 24 hours of exposure. No viable cells were detected after 8–12 hours in tested isolates by thymol at MIC and *E. coli* cells decreased sharply by thymol at 2 × MIC and no viable cells were observed within 2-4 hours of treatment

(20) and they can act as antibiotic adjuvants, promising new therapeutic applications (21). Antibiotics may have a different and stronger mechanism of action in the presence of essential oils and thus prevent bacterial resistance (22). Thymol significantly decreased the MIC values of cefotaxime and ciprofloxacin against test bacteria. It even caused the recovery of the sensitivity of the studied bacteria to cefotaxime and ciprofloxacin (according to the MIC). During the last ten years, the effectiveness of herbal compounds in combination with antibiotics, and the use of their combination in reducing the effective concentration of antibiotics, reducing side effects, and preventing the spread of drug resistance have been reported (23,24). Although it is not clearly known how plant compounds can decrease bacterial resistance to antibiotics, several mechanisms are proposed in this context. Due to the hydrophobic nature of plant

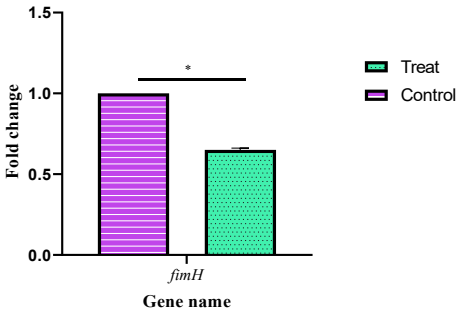

Figure 6. Changes in Expression Level of *fim*H Gene in *E. coli* Isolates Treated with Thymol

essential oils and their components, they interact with lipid-containing bacterial membranes and increase their permeability (25,26). The change in the permeability of the target membranes can increase the absorption of antibiotics by bacterial cells. Another plausible explanation is that the plant molecules inhibit the efflux pump activity in bacterial cells that are responsible for reducing the concentration of antibiotics in bacteria (27). Additionally, active compounds such as plant extracts are known as important sources of destroying biofilms. Herbal medicines and their derivatives not only play an effective role in the treatment of biofilm-based diseases but also have fewer side effects compared to chemical medicines (28). The presence of compounds such as flavonoids including quercetin, kaempferol, naringenin, and apigenin, which have the ability to suppress the chromium sensing system for cell-to-cell communication, can explain the inhibitory effect of thymol on biofilm formation. These compounds suppress the expression of genes involved in bacterial biofilm formation and biofilm formation regulators (29-31). It is noteworthy that our results confirmed that bacterial treatment with thymol leads to down-regulation of *fim*H which is a biofilm-associated gene.

Moreover, to determine the bactericidal power of various concentrations of thymol, a time-kill assay was performed against *E. coli* isolates. In this assay, thymol showed killing activities against tested isolates in a concentration and time-dependent manner. The main reason for the killing activity of thymol is its interaction with microbial plasma membranes which can cause them to collapse (32). Additionally, a previous related study revealed the advanced killing potential of thymol against a time and temperature-optimized attached *Listeria monocytogenes* population in lettuce (33). Moreover, the killing potential of thymol against *C. albicans*, *S. mutans*, and *Enterobacter spp.* has been elucidated (34,35).

The present study has some limitations. It only investigated the effect of thymol on a limited number of *E. coli* isolates; thus, larger samples should be investigated in further studies to confirm the findings. In addition, further research is needed to investigate the effect of thymol and its combination with antibiotics on other bacterial species and in vivo conditions to support the use of thymol in clinical therapy.

Conclusion

The present study found that thymol, a terpenoid of plant origin, exhibited strong killing efficiency, inhibiting the biofilm formation, and enhanced antibiotic sensitivity of *E. coli* isolated from UTI. These findings indicated that thymol can be used as a novel antibacterial agent against MDR and biofilm-associated infections. The results showed that it can be used in combination with antibiotics to enhance their antibacterial potential.

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Authors' Contribution

Conceptualization: Leila Asadpour.
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Methodology: Fatemeh Dizani, Leila Asadpour.
Project administration: Leila Asadpour.
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Supervision: Leila Asadpour.
Validation: Fatemeh Dizani, Leila Asadpour.
Visualization: Leila Asadpour.
Writing–original draft: Fatemeh Dizani.
Writing–review & editing: Leila Asadpour.

Competing Interests

The authors declare that there is no conflict of interests.

Ethical Approval

Not applicable.

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