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Original Article

# Molecular Profiling and Phytochemical Approach to Combat Multidrug-Resistant *Escherichia coli* O157: Insights from $(GTG)_5$-PCR Genotyping and Melissa officinalis Extract Antibacterial Activity

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## Abstract

***Background & Objectives:*** The emergence and global spread of multidrug-resistant (MDR) *Escherichia coli* O157 represents a critical public health challenge, causing severe foodborne outbreaks and life-threatening complications such as hemolytic uremic syndrome (HUS). Rapid adaptation and genetic diversity inof *E. coli* O157 complicate treatment, while limited efficacy of conventional antibiotics underscores the urgent need for alternative strategies. This study aimed to investigate the genetic diversity of *E. coli* O157 isolates and evaluate the antibacterial potential of *Melissa officinalis* extract as a natural therapeutic option for controlling infections caused by this pathogen.

***Materials & Methods:*** Twenty-three *E. coli* O157 isolates from diverse animal sources were genotyped using $(GTG)_5$-PCR. Antibiotic susceptibility of each strain was assessed via Kirby-Bauer disk diffusion against Norfloxacin, Tobramycin, Imipenem, Azithromycin, Doxycycline, and Levofloxacin. Antibacterial activity of aqueous-ethanolic leaf extract of *M. officinalis* leaf extract (100 mg/mL) was tested using agar well diffusion.

***Results:*** $(GTG)_5$-PCR revealed five distinct genotypic clusters, demonstrating high genetic heterogeneity. Antibiotic testing showed variable susceptibility, with Norfloxacin most effective (32-34 mm) and Tobramycin least (10-19 mm). Clusters D and E exhibited multidrug resistance. *M. officinalis* extract displayed strong bactericidal activity against all isolates, with an average inhibition zone of 32 mm, exceeding several conventional antibiotics.

***Conclusion:*** *E. coli* O157 isolates exhibit substantial genetic diversity and variable antibiotic resistance. The potent antibacterial activity of *M. officinalis* extract highlights its potential as a natural alternative or complementary therapy. Integrating molecular genotyping with phytochemical screening offers a promising approach to combat MDR pathogens.

***Keywords:*** *Escherichia coli* O157, Antibiotic resistance, Melissa officinalis, Phytochemical antibacterial, Genotyping, $(GTG)_5$-PCR.

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## Introduction

*Escherichia coli* O157 is recognized as one of most virulent enterohemorrhagic pathogens responsible for severe foodborne outbreaks and hemolytic uremic syndrome (HUS) worldwide (1). The increasing of multidrug-resistant (MDR) strains has posed a major challenge to clinical and public health sectors (2). The spread of antibiotic resistance has led to a significant challenge for both clinical management and public health systems (3).

As a zoonotic bacterium, *E. coli* O157:H7 exhibits a remarkable ability to adapt to diverse environmental niches through genetic variability, contributing to its persistence in animal hosts, food chains, and environmental reservoirs (4). Horizontal gene transfer via plasmids, transposons, and integrons facilitates the rapid acquisition and dissemination of antibiotic-resistance genes (5). Exposure to stressors, including antimicrobial agents, can induce the expression of stress-response and virulence genes, enhancing survival under adverse conditions (6). Collectively, these mechanisms contribute to the emergence of multidrug-resistant clones, highlighting the importance of molecular monitoring, resistance-gene surveillance, and integrated One Health strategies for managing this pathogen (4,5).

Molecular typing approaches, including repetitive extragenic palindromic PCR (REP-PCR) such as $(GTG)_5$-PCR, have become essential tools for differentiating *E. coli* O157 isolates based on genetic diversity. The $(GTG)_5$-PCR fingerprinting method utilizes repetitive trinucleotide sequences to generate strain-specific patterns, facilitating the understanding of epidemiological relationships and tracking the spread of pathogenic clones (4). Consequently, there is a critical need for innovative therapeutic strategies with strong antimicrobial activity.

In recent years, phytochemicals derived from medicinal plantsedicinal herbs have gained considerable attention as potential sources of antimicrobial agents. *Melissa officinalis*, a member of the *Lamiaceae* family, contains biologically active compounds such as rosmarinic acid, caffeic acid, and flavonoids, which exhibit antibacterial and antioxidant properties (7-9). Studies have demonstrated that extracts of *M. officinalis* inhibit both Gram-positive and Gram-negative bacteria, including *E. coli* (10).

In theThe present study aimed to perform the molecular genotyping of the *E. coli* O157 isolates using $(GTG)_5$-PCR, characterize their antibiotic resistance profile and determine the antibacterial potential of the aqueous-alcoholic extract of *M. officinalis* leaves. The integration of molecular and phytochemical approaches maywill offer novel insights into strategies for controlling multidrug-resistant bacterial pathogens.

## Materials and Methods

***A) Bacterial isolates:*** This study was conducted as a descriptive cross-sectional investigation. A total of twenty-three *Escherichia coli* O157 isolates (designated EC1–EC22 and EDL933 as a a reference and control strain controltrol) were collected from animal sources and slaughterhouses located in different geographical regions of Fars Provinc. The isolates were confirmed using through biochemical assays (IMViC tests), serological identification using *E. coli* O157 antisera and molecular verification via PCR targeting the *rfbE* gene, which is specific to the O157 serogroup (1).

***B) Antibiotic susceptibility testing:*** Antibiotic susceptibility of the isolates was determined

using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar (MHA), following the Clinical and Laboratory Standards Institute (CLSI, 2023) guidelines. The antibiotics tested included: Norfloxacin (10 µg), Tobramycin (10 µg), Imipenem (10 µg), Azithromycin (15 µg), Doxycycline (30 µg), Ciprofloxacin (30 µg), Levofloxacin (5 µg). After incubation at 37°C for 24 h, After incubation at 37°C for 24 hours, the diameters of the inhibition zones were measured in millimeters, and the isolates were categorized as Sensitivesusceptible (S), Intermediate (I), or Resistant (R) according to CLSI breakpoints (11). All assays were performed in triplicate to ensure accuracy and reproducibility.

***C) Extraction of Melissa officinalis:*** Fresh leaves of *Melissa officinalis* were collected, washed and shade-dried. The dried leaves were powdered and extracted using an aqueous-ethanolic solvent (70:30 v/v) by maceration for 72 hours. The filtrate was concentrated under reduced pressure at 40°C using a rotary evaporator to obtain a semi-solid extract, which was stored at 4°C until further use (3,12).

***D) Determination of antibacterial activity of the extract:*** The antibacterial activity of the *M. officinalis* extract was determined using the agar well diffusion method on Mueller–Hinton agar against the *E. coli* O157 isolates. Wells of 6 mm diameter were filled with 100 µL of the extract at a concentration of 100 mg/ml, Platesplates were incubated at 37°C for 24 h, and inhibition zones were measured in millimeters (13).

***E) (GTG)$_5$-PCR genotyping:*** Genomic DNA of the isolates was extracted using the boiling method, as previously described (13). Briefly, bacterial cells were suspended in sterile distilled water and heated at 100°C for 10 minutes to lyse the cells and release DNA. The lysate was then centrifuged, and the supernatant containing genomic DNA was collected for PCR analysis. Genetic fingerprinting was performed using the (GTG)$_5$- primer (5′-GTGGTGGTGGTGGTG-3′), as outlined by (14,15). PCR amplification was carried out in a 25 µL reaction mixture containing 2 µL of template DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM primer, and 1 U of Taq polymerase. Thermal cycling conditions consisted of an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, and extension at 72°C for 2 minutes, with a final extension at 72°C for 10 minutes (16).

***F) Gel Electrophoresis and Visualization:*** PCR products were separated on a 1.5% agarose gel containing ethidium bromide (0.5 µg/mL) in 1× TAE buffer. Electrophoresis was carried out at 100 V for 90 minutes. Gels were visualized under a UV transilluminator, and digital images were captured for analysis (14,16).

***G) Data Analysis and Dendrogram Construction:*** The banding patterns generated by (GTG)$_5$-PCR were analyzed using NTsys software (version 2.02). Bands were scored as binary data (present= 1, absent= 0). Genetic similarity among isolates was calculated using the Dice coefficient, and a dendrogram was constructed using the unweighted pair group method with arithmetic mean (UPGMA). Clusters were defined at an 80% similarity threshold to identify major genotypic groups (16).

***H) Statistical Analysis:*** All experimental data were expressed as mean ± standard deviation (SD). Statistical comparison of inhibition zone diameters between the *Melissa officinalis*

extract and standard antibiotics was performed using one-way analysis of variance (ANOVA) followed by post-hoc Tukey's test. A probability value of $p < 0.05$. Statistical analyses were conducted using SPSS software (version 20.0).

## Results

***A) Antibiotic susceptibility profiles:*** Antimicrobial susceptibility testing of the *E. coli* O157 isolates (EC1–EC22) and the reference strain EDL933 revealed considerable heterogeneity in resistance patterns. Inhibition zones across all tested antibiotics ranged from 10 to 34 mm. Norfloxacin (NOR) largest inhibitory effects (32–34 mm) that indicatingate strong activity against most isolates and, Tobramycin (TOB) exhibited the lowest inhibitory effects (18mm), while Imipenem (IMP) displayed intermediate inhibition in several strains. Azithromycin (AZM) and Doxycycline (D30) demonstrated moderate to high sensitivity, with inhibition zones ranging from 17 to 34 mm. Levofloxacin (LP) showed variable inhibitory effects among isolates. Notably, EC4, EC20, EC21and EC22 exhibited intermediate (I) or resistant (R) phenotypes, suggesting the emergence of adaptive resistance mechanisms. Overall, these findings highlight the diverse antibiotic susceptibility profiles of *E. coli* O157 isolates, which may contribute to the persistence and dissemination of multidrug-resistant strains in clinical and environmental settings. A detailed summary of the antibiotic susceptibility data is presented in Table 1.

**Table 1.** Antibiotic susceptibility of *E. coli* O157 isolates (ECoRC1- ECoRC22).

| | ***Melissa officinalis* extraction (mm)** | **CN30 (mm)** | **NOR10 (mm)** | **TOB10 (mm)** | **LP (mm)** | **IMP (mm)** | **AZM (mm)** | **D30 (mm)** |
|---|---|---|---|---|---|---|---|---|
| **EC1** | 32 | 0 | 28 (S) | 18(S) | 20 | 22(I) | 23(S) | 18(S) |
| **EC2** | 32 | 0 | 30 (S) | 14(I) | 25 | 25(S) | 22(S) | 19(S) |
| **EC3** | 33 | 1.3 | 32 (S) | 15 (S) | 25 | 25(S) | 22(S) | 18(S) |
| **EC4** | 33 | 0 | 3 (S) | 12® | 26 | 22(I) | 25(S) | 19(S) |
| **EC5** | 33 | 0 | 32 (S) | 15 (S) | 24 | 28(S) | 25(S) | 21(S) |
| **EC6** | 34 | 0 | 32 (S) | 15(S) | 22 | 25(S) | 25(S) | 18(S) |
| **EC7** | 32 | 0 | 32 (S) | 15(S) | 23 | 27(S) | 23(S) | 19(S) |
| **EC8** | 33 | 0 | 28 (S) | 18(S) | 22 | 24(S) | 28(S) | 21(S) |
| **EC9** | 33 | 0 | 33 (S) | 19(S) | 28 | 22(I) | 23(S) | 17(S) |
| **EC10** | 33 | 0 | 31 (S) | 17(S) | 29 | 30(S) | 27(S) | 19(S) |
| **EC11** | 32 | 0 | 32 (S) | 19(S) | 22 | 22(I) | 25(S) | 17(S) |
| **EC12** | 34 | 0 | 30 (S) | 18(S) | 29 | 24(S) | 24(S) | 15 (S) |
| **EC13** | 32 | 0 | 31 (S) | 18(S) | 25 | 28(S) | 24(S) | 19(S) |
| **EC14** | 33 | 0 | 31 (S) | 18(S) | 28 | 26(S) | 25(S) | 18(S) |
| **EC15** | 34 | 0 | 34 (S) | 18(S) | 21 | 27(S) | 24(S) | 17(S) |
| **EC16** | 32 | 0 | 32 (S) | 17(S) | 26 | 26(S) | 24(S) | 18(S) |
| **EC18** | 32 | 0 | 32 (S) | 17(S) | 25 | 28(S) | 24(S) | 21(S) |
| **EC19** | 33 | 0 | 29 (S) | 16 (S) | 24 | 26(S) | 22(S) | 16(S) |
| **EC20** | 33 | 0 | 30 (S) | 14(I) | 12 | 25(S) | 0® | 15(S) |
| **EC21** | 34 | 0 | 23 (S) | 10® | 11 | 24(S) | 12® | 17(S) |
| **EC22** | 34 | 0 | 25 (S) | 17(S) | 22 | 18® | 22(S) | 17(S) |
| **EDL993** | 34 | 0 | 33 (S) | 15(S) | 29 | 26(S) | 29 (S) | 18 (S) |

CN30: Gentamicin (30 µg), NOR10: Norfloxacin (10 µg), TOB10: Tobramycin (10 µg), LP: Lomefloxacin, IMP: Imipenem, AZM: Azithromycin, D30: Doxycycline (30 µg). Interpretation of inhibition zones: S = Susceptible, I = Intermediate, R = Resistant (according to CLSI guidelines).

***B) Antibacterial activity of Melissa officinalis:*** The aqueous–ethanolic extract of *Melissa officinalis* leaves exhibited a strong inhibitory effect against all tested *E. coli* O157 isolates. The average inhibition zone of 32 mm against *E. coli* O157 isolates, which was significantly higher than that of most antibiotics tested. Such as Tobramycin (TOB, 15–19 mm) and Azithromycin (AZM, 22–28 mm) (Table 1).

***C) Statistical Analysis:*** Statistical comparison (ANOVA, $p < 0.05$) revealed a significant difference between the inhibition zone produced by the plant extract and those of β-lactam antibiotics.

***D) $(GTG)_5$-PCR genotyping:*** The isolates designated EC1 to EC22 are displayed in the dendrogram under the labels ECOR1 to ECOR22The $(GTG)_5$-PCR profiles revealed polymorphic banding patterns among the *E. coli* O157 isolates, with DNA fragments ranging from 250 to 3000 bp and totally 23 band was detected. Dendrogram analysis demonstrated genetic diversity, grouping the isolates into five major clusters (Figure 1).

The $(GTG)_5$-PCR dendrogram of *Escherichia coli* O157 isolates (Figure 1) revealed genetic heterogeneity among the 22 isolates and as well as the reference strain EDL933. At an 80% similarity threshold, the isolates were classified into five principal genotypic clusters (A–E).

Cluster A included isolates ECOR1, ECOR4, ECOR6, ECOR8, ECOR10, ECOR12, ECOR16, and EDL933, which exhibited high genetic relatedness (≥90% similarity). These isolates predominantly displayed antibiotic susceptibility, particularly to norfloxacin, tobramycin, azithromycin, and doxycycline. Cluster B included ECOR2, ECOR3, ECOR5, ECOR7, ECOR9, ECOR11, ECOR13, ECOR14, and ECOR17, which demonstrated moderate variability in antimicrobial susceptibility, with several isolates exhibiting intermediate resistance to imipenem and tobramycin. Cluster C, containing ECOR15 and ECOR19, exhibited moderate genetic similarity (~82%) and reduced susceptibility to levofloxacin and imipenem.

Cluster D grouped ECOR18 and ECOR20, which showed lower inhibition zone diameters and elevated resistance to azithromycin and levofloxacin, indicating potential acquisition of resistance determinants and genetic divergence.

Cluster E, encompassing ECOR21 and ECOR22, represented the most resistant isolates, characterized by markedly small inhibition zones (TOB, 10–12 mm; AZM, 12 mm; IMP, 18 mm). Overall, all isolates remained susceptible to norfloxacin, whereas imipenem and tobramycin occasionally exhibited intermediate resistance.

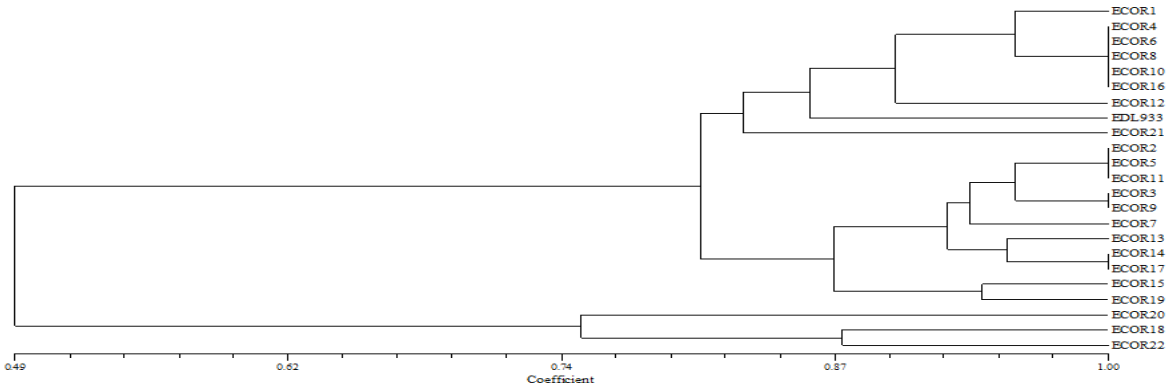


**Fig 1.** $(GTG)_5$-PCR fingerprinting profiles of *E. coli* O157 isolates.

## Discussion

The findings of this study revealed a considerable level of antibiotic resistance among several of *Escherichia coli* O157 isolates, reflecting the global concern regarding the emergence of multidrug-resistant (MDR) strains (17). The detection of intermediate or resistant phenotypes to imipenem (IMP, β-lactam) and azithromycin (AZM, macrolide) highlights the adaptive capacity of *E. coli* O157 to diverse antimicrobial agents, potentially mediated by porin channel mutations, efflux pump overexpression, and horizontal gene transfer mechanisms (18). The $(GTG)_5$-PCR analysis demonstrated high genomic diversity among isolates, indicating that the *E. coli* O157 population is genetically heterogeneous. This heterogeneity plays a vital role in pathogenicity and resistance dissemination. Previous studies have reported similar results using repetitive sequence-based typing methods, confirming the discriminative power of $(GTG)_5$-PCR for epidemiological tracking of *E. coli* O157 (19).

A remarkable finding of in this research study is the potent antibacterial activity of the aqueous-ethanolic extract of *Melissa officinalis* leaves, which produced an average inhibition zone of 32 mm, exceeding the effects of several conventional antibiotics, including tobramycin (TOB, aminoglycoside) and azithromycin (AZM, macrolide). The high efficacy of *Melissa officinalis* extract may be due to the synergistic action of phenolic compounds, including rosmarinic acid, ursolic acid, and caffeic acid, which are known to disrupt bacterial membranes, inhibit quorum sensing, and inhibit biofilm(12). Notably, no bacterial colony growth was detected within the inhibition zones, indicating a clear bactericidal effect rather than a merely bacteriostatic action (12). These observations align with prior researchresearches emphasizing the potential of *M. officinalis* as a natural antimicrobial agent against Gram-negative bacteria (7,20). Moreover, the phytochemicals in lemon balm, another name for *Melissa officinalis,* can target multiple bacterial pathways simultaneously, reducing the likelihood of resistance development compared to single-target antibiotics (13). The clustering analysis revealed a strong correlation between genotypic similarity and antibiotic susceptibility profiles. Isolates within the same genetic cluster tended to exhibit comparable resistance patterns, particularly toward β-lactam and macrolide antibiotics. For example, Cluster A isolates, which were closely related to EDL933, were showed largely susceptibility to β-lactam and macrolide antibioticssusceptible, whereas Clusters D and E contained strains displaying multidrug resistance.

These observations suggest that antibiotic resistance in *E. coli* O157 may have evolved within specific lineages, potentially via horizontal gene transfer or plasmid-mediated mechanisms (9). The emergence of resistant isolates in genetically distinct clusters, such as ECOR21 and ECOR22, indicates independent acquisition of resistance determinants.

All in all, the integrated phenotypic and genotypic analyses underscore substantial heterogeneity among *E. coli* O157 isolates and emphasize the importance of combining molecular typing with antibiotic-resistance profiling to elucidate bacterial evolution, mechanisms of resistance dissemination, and to guide the development of novel antimicrobial strategies.

## Conclusion

This study provides an integrated evaluation of both phenotypic antibiotic resistance and genotypic diversity among *Escherichia coli*

O157 isolates, with special emphasis on the antibacterial activity of *Melissa officinalis* (lemon balm) extract. The findings revealed significant heterogeneity among isolates, forming five distinct genotypic clusters with variable resistance profiles. The correlation between clustering patterns and antibiotic susceptibility suggests that genetic diversity strongly influences phenotypic resistance behavior.

The aqueous-alcoholic extract of *M. officinalis* exhibited a potent inhibitory effect (32 mm inhibition zone), comparable to or greater than several standard antibiotics. This highlights the potential of lemon balm as a promising natural antimicrobial source, capable of targeting multidrug-resistant *E coli O157 strainsE. coli* strains. Its bioactive phytochemicals, such as rosmarinic acid and flavonoids, may interfere with bacterial cell wall synthesis, enzyme activity, or quorum sensing, offering an alternative therapeutic approach.

From a public health perspective, the results emphasize the growing importance of integrating molecular typing with antimicrobial profiling to monitor the spread of resistant clones and to identify effective natural alternatives as well. Future studies shouldneed to focus on purifying the active compounds in *M. officinalis* and assessing their synergistic effects with with conventional antibiotics.

## Recommendations

Investigation into the molecular mechanisms underlying the antibacterial effects of *M. officinalis* particularly the roles of rosmarinic acid, flavonoids, and terpenoids should be prioritized to better understand their interaction with bacterial membranes and enzymes.

Evaluating synergistic interactions between *M. officinalis* extract and conventional antibiotics could lead to the development of combination therapies that enhance bacterial inhibition and reduce antibiotic dosage.

In vivo animal model experiments are necessary to confirm the therapeutic potential, bioavailability, and safety of *M. officinalis* extracts observed in vitro.

Further purification, structural identification, and formulation of active compounds from *M. officinalis* should be carried out to develop standardized natural herbal antimicrobial products.

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## Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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