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Biosynthesis of AgNPs Using *Teucrium polium* and Effect on TEM gene of *E. coli*

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A B S T R A C T

Objective: *Escherichia coli* is a prevalent causative agent of nosocomial and urinary tract infections, with the presence of *β-Lactamase* enzymes encoded by the *blaTEM* gene contributing to enhanced resistance against antimicrobial agents. Consequently, this study aimed to evaluate the antibacterial efficacy of silver nanoparticles (AgNPs) biosynthesized via *Teucrium polium* extract against the *TEM* gene-positive *E. coli* strains responsible for nosocomial and community-acquired UTIs.

Methods: In this investigation, first, the synthesis of AgNPs was performed under optimal conditions by *T. polium* extract. Then the production of AgNPs was determined by the color change of extract to dark brown in the presence of silver nitrate and UV-visible absorbance in the 300–650 nm using a spectrophotometer. Then, 155 *E. coli* isolates obtained from urinary infection samples referred to the Dr. Shariati Medical Diagnostic Laboratory in Jiroft during the years 2023–2024.The isolates identified and their antibiotic susceptibility profiles determined. Among these isolates, *E. coli* strains producing *β-Lactamase* enzymes detected. Finally, the antibacterial activity of AgNPs against *β-Lactamase* positive *E. coli*, was assessed through disc diffusion and broth microdilution methods. Furthermore, the impact of AgNPs on *TEM* gene expression was quantified by real-time PCR analysis.

Results: results showed that among 155 *E. coli* isolates, 100 *E. coli* strains producing *β-Lactamase* enzymes. And PCR assays revealed the presence of the *TEM* gene in 60% isolates. Then the highest absorption at 450nm indicates surface plasmon resonance characteristic of synthesized AgNPs. Also, the biosynthesized AgNPs exhibited significant bacteriostatic and bactericidal effects against *E. coli* strains harboring *β-Lactamase* enzymes. Importantly, treatment with AgNPs resulted in a 34% reduction in *TEM* gene expression.

Conclusion: Based on these findings, future research may explore the potential application of *T. polium* extract-derived AgNPs as an adjunct or alternative therapeutic modality to conventional antibiotics in the management of multidrug-resistant *E. coli* infections.

Keywords: *Teucrium polium*, *β-Lactamase*, *TEM* gene, AgNPs, Urinary Tract Infection

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Introduction

Urinary tract infections (UTIs), which reduce the quality of life of patients and increase the social burden on society, are the second most common type of infectious disease (after respiratory tract infections) in hospitals and communities [1]. *E.coli* (UPEC) accounts for 80–90% and 30–50% of community-acquired and hospital-acquired UTIs respectively, and have been classified into four major UPEC phylogroups (A, B1, B2, and D) based on chromosomal Pathogenicity Islands (PAI) [2]. This opportunistic pathogen has acquired plasmid-encoded extended-spectrum *β-Lactamase* (ESBLs), which provide resistance to a wide range of β-lactam antibiotics, including extended-spectrum cephalosporins. This complicates therapeutic management [3]. These enzymes inactivate oxyimino β-lactams such as penicillins and cephalosporins by hydrolyzing the β-lactam ring [4]. The *blaTEM* gene encodes the TEM-type β -lactamase, the most prevalent form among Enterobacteriaceae, responsible for over 90% of ampicillin resistance in *E. coli* strains [5, 6]. TEM was the first β-Lactamase isolated from *E. coli* and is frequently detected in both *E. coli* and *K. pneumoniae*. To date, more than 130 TEM variants have been identified, differing in amino acid substitutions at the enzyme's active site, which affects substrate specificity[7]. The genes encoding ESBLs induce cross-resistance to multiple antibiotics, severely limiting therapeutic options. Molecular detection of predominant *ESBL* genes and characterization of antibiotic resistance profiles in ESBL-producing bacteria provide essential epidemiological and antimicrobial stewardship data[8, 9]In the current decade, the search for antibiotic alternatives has become one of the most quintessential issues due to the massive expansion of antibiotic resistance. One of the newest strategies to fight and prevent diseases is nanotechnology. Silver nanoparticles (AgNPs), clusters of silver atoms ranging from 1 to 100 nanometers in diameter, exert antimicrobial effects by binding to sulfur-containing proteins on bacterial membranes [10]. This interaction alters the membrane's morphology and permeability, disrupts the respiratory electron transport chain, and interferes with bacterial cell division, ultimately causing cell death [11, 12]. Numerous studies have reported the biosynthesis of AgNPs and their potent antimicrobial activities. For instance, AgNPs synthesized using aqueous extracts of *Sambucus nigra* (elderberry), *Citrus aurantium* (bitter orange), and *Satureja hortensis* (savory) have demonstrated inhibitory effects against *E. coli* and *Staphylococcus aureus* [13]. AgNps have proved a good antibacterial and antioxidant activity against *E. coli* and *K. pneumoniae* isolates [14]. Moreover, it was demonstrated that AgNPs have a powerful toxic action on ampicillin resistant *K. pneumoniae* genes and bacterial proteins as well as bacterial membrane damage and oxidative stress [15].

Teucrium polium (commonly known as Kalpoureh or Golden Germander) is a perennial herbaceous plant from the Lamiaceae family, with a history of use dating back over two millennia. Traditionally, it has been employed to treat rheumatism, promote wound healing, mitigate inflammation, regulate glycemia, and manage gastrointestinal disorders. Pharmacological studies have confirmed its anti-inflammatory, analgesic, antipyretic, anticonvulsant, and antioxidant properties [16, 17].*T. polium* is a medicinal plant rich in bioactive compounds, with the most significant being flavonoids and essential oils. These compounds contribute to its antioxidant, anti-inflammatory, antidiabetic, and antimicrobial properties. Traditionally, it has been used to lower blood sugar, protect the liver, relieve pain, and treat infections. However, excessive consumption may cause hepatotoxicity, requiring cautious use under professional supervision ~~[16, 18, 19]~~.The antimicrobial phytochemicals present in *T. polium* is

especially noted for its significant antibacterial and antifungal activity [17, 20].

Due to the increasing difficulty in treating infections caused by plasmid-mediated ESBL-producing bacteria and the rapid dissemination of these organisms leading to a surge in UTI prevalence worldwide, investigating antibiotic susceptibility patterns and the prevalence of the *blaTEM* gene in urinary *E. coli* isolates is critical [21, 22].

To our knowledge, there is limited research on the antibacterial activity of AgNPs biosynthesized against ESBL bacteria in Iran. This study aims to evaluate the antimicrobial activity of *T. polium* extract-derived AgNPs in southern Kerman province on the *TEM* gene expression of *E. coli* that causes urinary tract infections, *in vitro* for the first time in Iran. It is hoped that the use of synthesized AgNPs can treat antibiotic-resistant microbial infections, which are one of the major problems in the treatment of these infections.

Materials and Methods

Isolation and Identification

A total of 155 *E. coli* strains isolated from urine samples of patients diagnosed with UTIs at Dr. Shariati Medical Diagnostic Laboratory, Jiroft, during the years 2023–2024. Identification was performed via Gram staining, oxidase testing, and selective culturing on Triple Sugar Iron Agar (TSI), Simon's Citrate Agar, MR/VP broth, Eosin Methylene Blue (EMB) agar and SIM agar. Mueller-Hinton broth and agar utilized for antibiogram assays. Following biochemical testing and IMViC confirmation, isolates stored in Tryptic Soy Broth (TSB) supplemented with 15% glycerol at –20°C for subsequent analyses. Frozen *E. coli* cultures suspended in TSB with 15% glycerol thawed to room temperature and inoculated on MacConkey agar and EMB agar, incubated at 37°C for 24 h. Suspected colonies underwent standard biochemical and microbiological assays.

Antibiotic Susceptibility Testing

Antibiotic susceptibility was evaluated using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar, following Clinical and Laboratory Standards Institute (CLSI) guidelines and utilizing antibiotic disks obtained from Padten Teb Company. The screening for Extended-Spectrum β -lactamase (ESBL) production was conducted using the combined disk test (CDT) in accordance with CLSI version 33 protocols. In brief, Mueller-Hinton agar plates inoculated with a 0.5 McFarland bacterial suspension (approximately 1.5 × 10^8 CFU/mL) using a lawn culture technique. Disks of cefotaxime (30 µg), cefotaxime-clavulanic acid (30/10 µg), ceftazidime (30 µg), and ceftazidime-clavulanic acid (30/10 µg) placed at least 2.5 cm apart on the plates. The plates then incubated at 37°C for 24 h. A confirmed ESBL production was indicated by a ≥5 mm increase in the zone diameter for ceftazidime-clavulanic acid compared to ceftazidime alone, or a ≥3 mm increase for cefotaxime-clavulanic acid compared to cefotaxime alone β -lactam resistance and antibiotic susceptibility profiles evaluated using Mueller-Hinton agar and the disk diffusion method. The antibiotics tested included carbenicillin (100 µg), penicillin (25 µg), ampicillin (10 µg), ofloxacin (5 µg), streptomycin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), nitrofurantoin (300 µg), norfloxacin (10 µg), and ciprofloxacin (5 µg) [23]. Bacterial suspensions prepared to match the 0.5 McFarland standard and uniformly inoculated onto Mueller-Hinton agar plates. Antibiotic disks placed aseptically, positioned 1.5 cm from the edge of the plate and 2.5 cm apart from each other. The plates then incubated for 18 to 24 h at 37°C. After incubation, the zones of inhibition measured using a calibrated ruler and interpreted according to CLSI guidelines to classify the isolates as sensitive or resistant.

Collection of *T. polium* and Extraction

T. polium plant was collected from mountainous regions surrounding Jiroft, Kerman Province. Botanical authentication was confirmed by the Faculty of Pharmacy, Kerman University. Aqueous extracts prepared from dried flowers and leaves through maceration. The plant material was initially disinfected in 5% sodium hypochlorite for 5 min, rinsed three times with sterile distilled water, and then treated with 70% ethanol for 2 min, followed by another three rinses with sterile water. After air-drying at room temperature in the shade, the material was ground into a fine powder. Five portions of 100 g each (totaling 500 g) macerated with 100 mL of distilled water and boiled for 10 min. The extract was then filtered using Whatman No. 1 filter paper with 25-micron pores (Figure 1) and centrifuged at 4000 rpm for 20 min to remove particulate matter. The supernatant was stored at –4°C for future use.

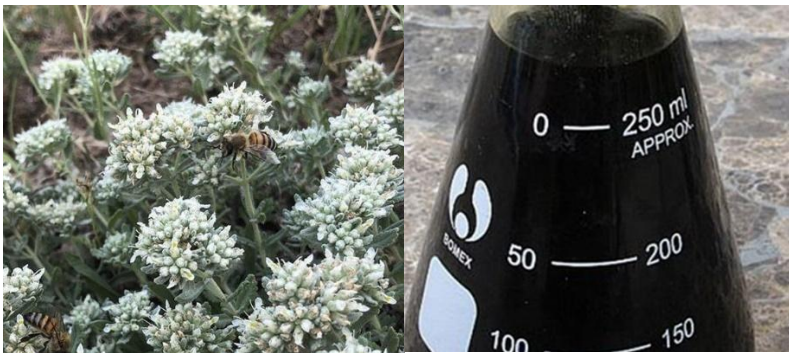


Figure 1: Extraction stage of the *T. polium* plant (straightening the extract after boiling)

Synthesis of AgNPs

For the biosynthesis of AgNPs, a 50 g/L aqueous *T. polium* extract was prepared by mixing 0.5 g dried extract with 10 mL distilled water. To each 10 mL extract sample at room temperature, 90 mL of 1 mM silver nitrate solution was added. A control sample with extract but no AgNO3 was included. After 24 h, samples visually inspected for color changes and UV-visible absorbance was measured in the 300–650 nm range using a spectrophotometer.

Confirmation Tests for AgNPs

The formation of AgNPs was indicated by the development of a brownish coloration corresponding to the surface plasmon resonance peak at approximately 450 nm. Increasing particle formation intensified the brown color. The spectral peak between 400–450 nm confirms nanoparticle synthesis due to localized surface plasmon resonance induced by free electrons on the particle surface. Parameters influencing synthesis (pH, extract volume, AgNO3 concentration, reaction time) optimized referencing previous studies on green tea extract-mediated synthesis.

DNA Extraction

From 100 β-lactamase-positive isolates identified via disk diffusion, 10 strains with the highest level of antibiotic resistance selected. Each was cultured in 5 mL TSB for 16–18 h at 37°C with shaking. Cultures centrifuged at 7,000 rpm for 5 min, after which the supernatants discarded. The pellets then resuspended in 100 µL of distilled water. Samples boiled at 100°C

for 10 min in a thermoblock, followed by centrifugation at 10,000 rpm for 10 minminutes. The supernatants, which contained bacterial DNA, transferred to new tubes and stored at –20°C [24].

PCR

Extracted DNA was amplified using a specific primer set targeting the *blaTEM* gene (primer sequences detailed in Table 1) to confirm the presence of the *TEM β-Lactamase* encoding gene in isolated strains.

Table 1: Specific primer sequences of *blaTEM* β-Lactamase genes

Gene Name/Primer Name	Primer Name (5′-3′)
TEM/ BlaTEM	(F) ATG AGT ATT CAA CAT TTC CG (R) CCA ATG CTT AAT CAG TGA GG

DNA extraction was performed using the KiaGene kit, followed by multiplex PCR assays to detect *β-Lactamase* genes, specifically *blaTEM*, employing specially designed primers for targeted amplification. The PCR reaction mixture included the following reagents and volumes: 0.5 µL of template DNA, 12.5 µL of master mix, 1 µL of forward and reverse primers (each at 10 pmol/µL), and 11.5 µL of double-distilled water, resulting in a total volume of 25 µL. The multiplex PCR protocol was carried out using a thermocycler.

Table 2: Steps and conditions for performing PCR for the *E. coli TEM* gene

Stage	Cycle	Tm (°C)	Time
Initial denaturation	1	94	5 min
Denaturation	35	94	1 min
Renaturation	35	55.5	45 sec
extension	35	72	1 min
Final extension	1	72	5 min

After completing the PCR amplification, electrophoretic separation was performed. The PCR products resolved on 1% agarose gel and subsequently stained with ethidium bromide for visualization. Size comparisons of PCR amplicons carried out using a 1000 bp DNA ladder marker. Five microliters of each PCR product loaded into the agarose wells. The *E. coli* standard strain ATCC 25923, obtained from the Pasteur Institute of Iran, was used as a quality control.

Antimicrobial Activity of Biosynthesized AgNPs

To evaluate the antimicrobial efficacy of AgNPs synthesized using *T. polium* extract against *E. coli* isolates collected from the Jiroft Diagnostic Laboratory, both agar diffusion (disk diffusion) and broth dilution methods employed:

Disk Diffusion Assay

A bacterial suspension of *E. coli* equivalent to the 0.5 McFarland standard was prepared and cultured on Mueller-Hinton agar plates, which incubated at 37°C. Sterile, autoclaved 6-mm blank discs (from Padten Teb Company) impregnated with biosynthesized AgNPs at concentrations of 5, 10, 15, 20, 25, 30, 35, and 40 µg/mL, suspended in deionized water. After a 24-h incubation at 37°C, the zones of inhibition measured in millimeters. Tetracycline antibiotic discs (30 µg/mL, Padten Teb Company) used as positive controls and culture medium or extract without nanoparticles used for negative controls.

Broth Dilution Method (Tube Dilution Assay)

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of biosynthesized AgNPs determined using the broth dilution technique. Eight sterile tubes prepared, each containing 0.1 mL Mueller-Hinton broth. In the first tube, 0.1 mL of silver nanoparticle solution at 512 µg/mL concentration (prepared with *T. polium* extract) was added and thoroughly mixed. Subsequently, 0.1 mL was serially transferred to the next tube, continuing this twofold dilution process through to the penultimate tube. The final tube contained 0.1 mL of sterile distilled water, serving as a negative control for the nanoparticles, and was mixed thoroughly. Next, 0.1 mL of *E. coli* suspension was added to each tube (refer to Table 3). The tubes then incubated at 37°C for 24 h (see Figure 2) Turbidity was visually assessed to determine bacterial growth. The lowest nanoparticle concentration exhibiting complete clarity with no observable turbidity was designated as the MIC.

Table 3: Titration method of aqueous extracts of the plant *T. polium* to determine MIC

Tube No. / Solution (mL)	1	2	3	4	5	6	7	8
Mueller-Hinton Broth (mL)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	—
Biosynthesized AgNPs (mL)	—	—	—	—	—	—	—	0.1

Sterile Distilled Water (mL)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Microbial Suspension (mL)	128	64	32	16	8	4	2	—
Final Concentration (µg/mL)	128	64	32	16	8	4	2	—

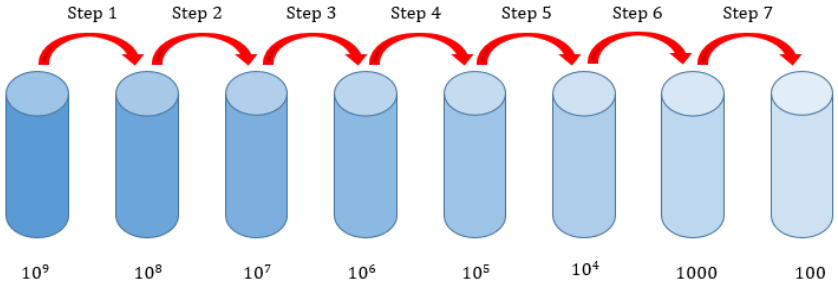


Figure 2: Schematic view of the tube dilution method

Determination of MBC

From the clear tubes in the MIC assay where bacterial growth was inhibited, aliquots aseptically streaked with a sterile loop onto Mueller-Hinton agar plates and incubated at 37°C for 24 h. The lowest concentration of AgNPs at which no viable bacterial colonies recovered was designated as the MBC.

Expression Analysis of the *blaTEM* Gene

To evaluate the modulatory impact of AgNPs synthesized via *T. polium* extract on *TEM* gene expression, six *E. coli* isolates harboring the *blaTEM* gene analyzed. Relative quantification of *TEM* mRNA levels was conducted following RNA extraction and cDNA synthesis, using quantitative real-time PCR (qRT-PCR). Data interpretation encompassed relative gene expression calculation, statistical analysis, and graphical representation.

For treatment, 100 µL of peptone broth culture containing *E. coli* at a 0.5 McFarland turbidity standard was incubated with 100 µL of *T. polium*-derived AgNPs at the respective MBC concentration for 24 h under controlled conditions. Post-exposure, gene expression levels of *TEM* quantified by qRT-PCR. The experiment employed an assumed 100% amplification efficiency, and data from treated and untreated groups statistically compared using the independent samples t-test. The control group consisted of bacterial cultures treated with 100 µL peptone broth devoid of AgNPs. Mean gene expression from the six isolates served as the baseline control, while treated groups received varying nanoparticle dosages. Additionally, to validate the gene expression results obtained via qRT-PCR, the Pfaffl method was employed as a confirmatory analysis.

Molecular Detection and Melting Curve Analysis

Molecular assays targeting specific bacterial resistance genes through dedicated primer design allow for

sensitive and specific identification of bacterial species [25]. Among the most sensitive rapid identification tools is the melting curve analysis performed during real-time PCR [24]. This technique relies on the correlation between temperature escalation and DNA denaturation profiles, yielding characteristic sigmoidal melting curves. Such precise discrimination is based on DNA strand dissociation dynamics with increasing temperature [26].

Statistical Analysis Methodology

All experiments conducted in triplicate, and results are presented as mean ± standard deviation. Data analyzed using one-way analysis of variance (ANOVA), followed by independent sample t-tests for mean comparisons at a significance level of $p<0.05$. Relative gene expression was quantified using the Pfaffl analytical method. Statistical calculations and graphical presentations carried out using SPSS and Microsoft Excel software, respectively.

Protein Sequence Retrieval

The amino acid sequences of blaTEM from *E. coli* (GenBank: KX462012.1) (Figure 3) and oleander methyltransferase mRNA from *T. polium* (Apigenin (PubChem CID: 5280443))(Figure 4) retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/). UniProt (https://www.uniprot.org/) is considered one of the most comprehensive and reliable resources for protein information [27].

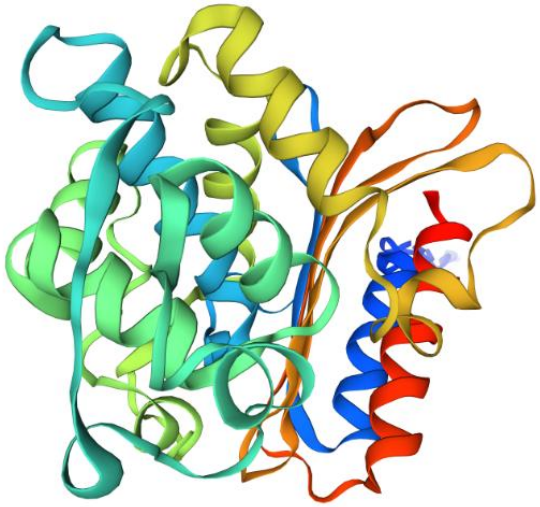


Figure 3: 3D structure of bla*TEM* protein (*E. coli* (GenBank: KX462012.1))

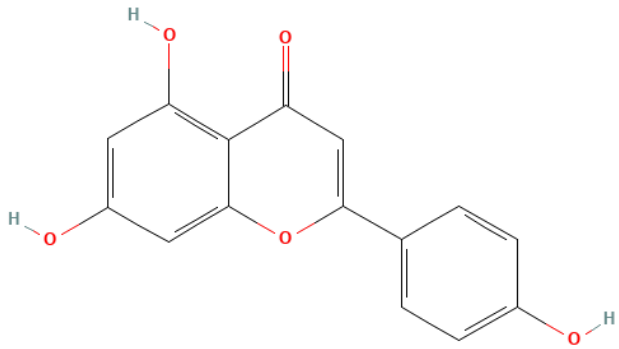


Figure 4: **2D structure of Apigenin (*T. polium* (PubChem CID: 5280443))**

Results

Isolation and Identification

A total of 155 *E. coli* isolates obtained from urine specimens of patients diagnosed with UTIs, referred to the Jiroft Medical Diagnostic Laboratory between the years 2023 and 2024. Isolation was achieved using selective and differential culture media. Biochemical characterization revealed positive catalase, triple sugar iron (TSI) reaction, indole production, and methyl red tests, while urease activity, Simon's citrate utilization, and hydrogen sulfide production tests negative.

E. coli demonstrated slow growth on simple media, motility via peritrichous flagella, lactose fermentation, and produced characteristic metallic green sheen colonies on eosin methylene blue (EMB) agar (Figure 5). The isolates exhibited lysine decarboxylase activity, could utilize citrate as the sole carbon source, and hydrolyzed tryptophan to indole.

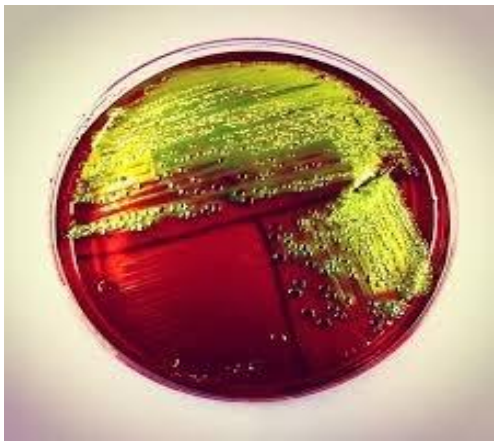


Figure 5: **Culturing *E. coli* on EMB medium and metal polish**

Antibiotic Susceptibility testing

Among 155 *E. coli* isolates, 100 strains (equivalent to 65%) phenotypically confirmed as *β-Lactamase* producers (Figure 6). Analysis of the antibiotic resistance profiles of *E. coli* strains isolated from urinary tract infection samples revealed the highest resistance frequencies against ampicillin (10 µg) with 74% resistance, carbenicillin (100 µg) exhibiting 61%

resistance, and chloramphenicol (30 µg) showing 53% resistance rates. Furthermore, of the 100 β-lactamase-positive *E. coli* isolates identified by phenotypic assays, 10 isolates subjected to PCR for the detection of the *blaTEM* gene. Among these, 6 isolates (60%) harbored the *blaTEM* gene, indicating that 60% of the examined *E. coli* strains contained this specific β-*lactamase*-encoding genetic determinant.

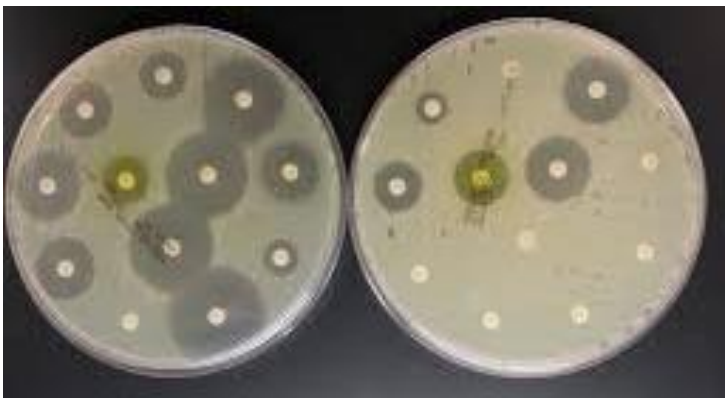


Figure 6: **Antibiogram using the disk method on *E. coli***

Synthesis of AgNPs

Initially, specimens of *T. polium* harvested and taxonomically authenticated (Figure 7). Within two h of mixing the aqueous plant extracts with silver nitrate solution, a color change from yellow to brown was observed in the reaction mixture, signaling the beginning of silver nanoparticle biosynthesis. This color transformation continued and stabilized after 24 h, confirming the complete formation of AgNPs (Figure 8).



Figure 7: T. polium plant collected from the mountainous areas of Jiroft County, Kerman Province



Figure 8: Color change of *T. polium* plant extract to dark brown in the presence of silver nitrate and production of synthesized nanoparticles

The obtained results are illustrated in Figure 9. The detection of a characteristic plasmon resonance peak at 450 nm wavelength for AgNPs corresponded congruently with findings reported by other researchers [28].

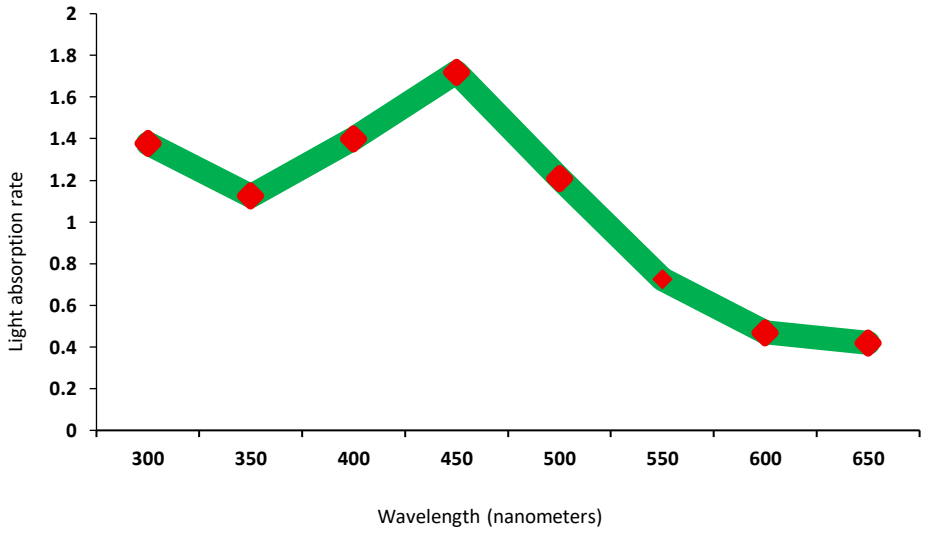


Figure 9: Optical absorption diagram of AgNPs synthesized with ***T. polium*** **aqueous extract** at different wavelengths

PCR Results

Among 10 *E. coli* isolates carrying *β-Lactamase* enzymes, six isolates (60%) tested positive for the *blaTEM* gene. Hence, the prevalence of the *TEM* gene among tested isolates was 60%. Molecular identification and sequencing of *TEM*-positive strains corroborated these findings (Figure 11).

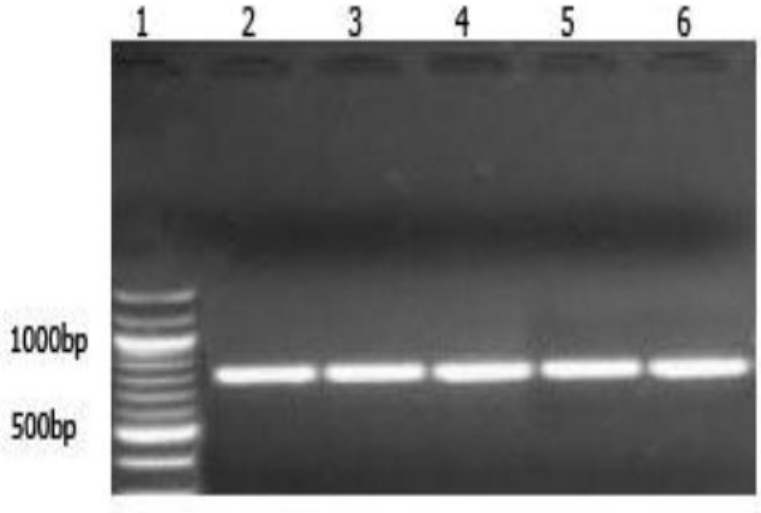


Figure 10: Electrophoretic Bands of *blaTEM* Genes (Lane 1: DNA ladder; Lane 2: Positive control; Lanes 3-6: *blaTEM* gene amplicons (~850 bp))

Antimicrobial effect of nanoparticles against E. coli containing the TEM gene

The results of disking tests in E. coli strains containing the TEM gene treated with biosynthesized silver nanoparticles are shown in Figure 11. According to these results, the diameter of the growth inhibition zone for silver nanoparticles synthesized by the extract of Colpore was observed at a concentration of 25 to 40 µg / mL. This result indicated the inhibitory effect of silver nanoparticles synthesized by Colpore on E. coli strains. In addition, with increasing concentration of nanoparticles, the growh inhibition zone and antimicrobial effect increased. The highest growth inhibition zone was observed at a concentration of 40 µg / mL (Figure 11).

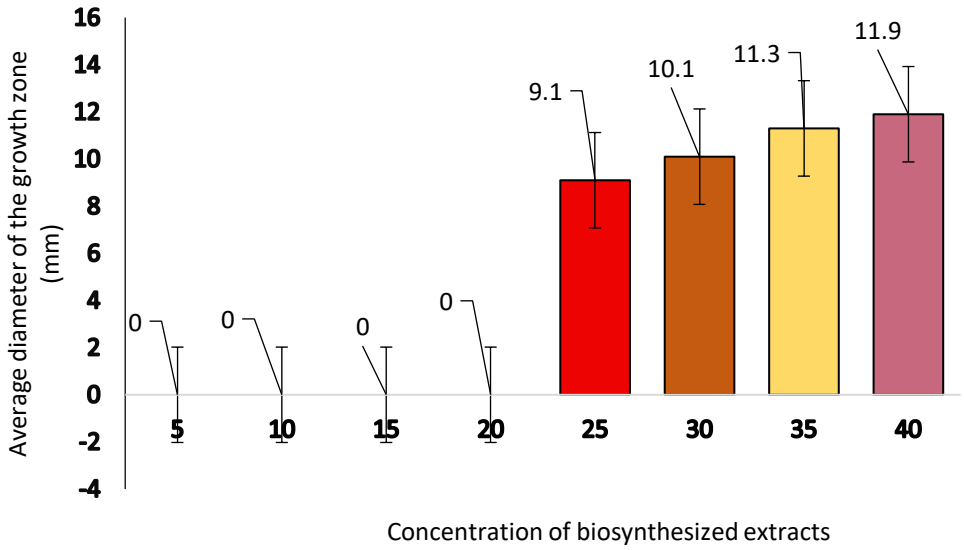


Figure 11: Diameter of the growth inhibition zone of bacteria cultured in the presence of the *T. polium* aqueous extract in millimeters by agar diffusion method

MIC Determination

The MIC values for growth inhibition of *E. coli* using AgNPs biosynthesized with *T. polium* aqueous extract are presented in Table 4. As indicated, the MIC for the *T. polium* extract-synthesized nanoparticles was 32 µg/mL (Figure 10).

Table 4: MIC results of *E. coli* growth inhibition via broth dilution assay using varying concentrations of agnps synthesized with *t. polium* aqueous extract

Plant	**Extract concentration (µg/mL)**							
T. polium	2	4	8	16	32	64	128	Control
	-	-	-	-	+	+	+	-

Legend: "+" indicates absence of turbidity (growth inhibition), "–" indicates turbidity (growth present).

MBC Determination

It has been illustrated the MBC results of *E. coli* treated with AgNPs derived from *T. polium* extract. The MBC was determined to be 64 µg/mL (table 5).

Table 5: MBC Results of *E. coli* Using Broth Dilution with Various Concentrations of *T. polium* Extract-Synthesized AgNPs

Plant	**Extract concentration (μg/mL)**							
T. polium	2	4	8	16	32	64	128	Control
	*	*	*	*	*	+	+	-

Legend: "*" indicates not tested; "+" indicates no turbidity with bactericidal effect.

Effect on *blaTEM* Gene Expression Post-Treatment

Six *blaTEM*-positive *E. coli* isolates analyzed for changes in *TEM* gene expression following exposure to *T. polium*-derived AgNPs using quantitative real-time PCR (qRT-PCR). Results are summarized in Table 6 and Figure 12.

The data indicated a mean *TEM* gene expression reduction from control to treated groups by approximately 34% (mean expression 0.363 post-treatment vs. control; $p < 0.05$). This significant downregulation highlights the inhibitory impact of *T. polium*-derived AgNPs on *blaTEM* gene expression in multidrug-resistant *E. coli*. Further validation was conducted via the Pfaffl method, confirming the qRT-PCR gene expression results (Figures 13, 14, and 15; Table 7).

Table 6: Relative Expression of *TEM* Gene in *E. coli* Before and After Treatment

	control	E1	E2	E3	E4	E5	E6	16SrRNA
Calpore (64 μg/mL)		0.41	0.35	0.3	0.43	0.3	0.39	0.8
Before treatment	0.55	0.51	0.60	0.54	0.54	0.59	0.52	0.8

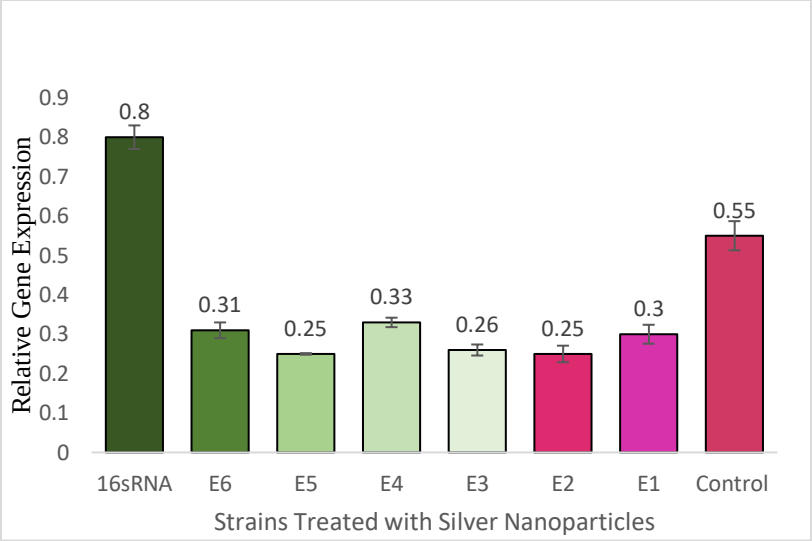


Figure 12: Comparative analysis of *TEM* gene expression in six *E. coli* isolates treated with biosynthesized AgNPs versus untreated controls

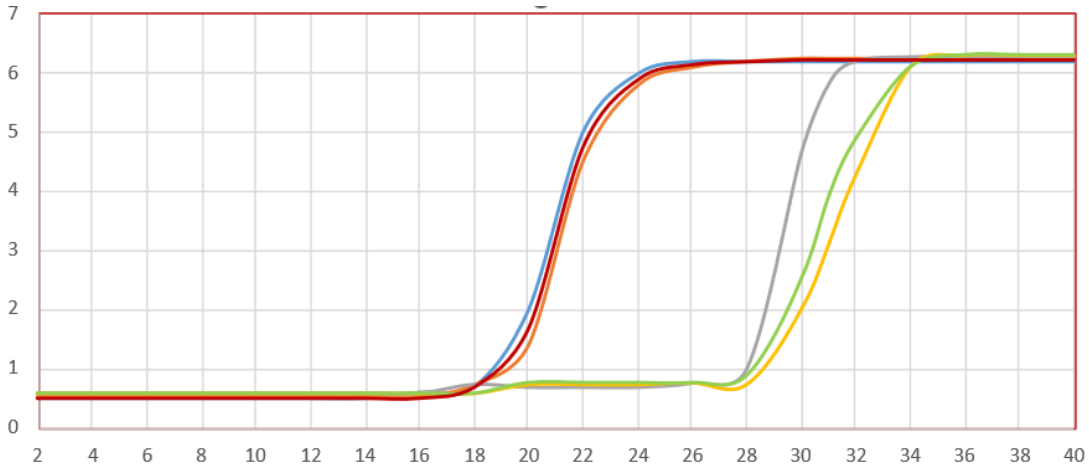


Figure 13: Real-time PCR amplification plots depicting *TEM* gene and 16S rRNA (housekeeping gene) before and after nanoparticle treatment. The melting curves reveal distinct, single-peak profiles at ~86°C, indicating amplification specificity

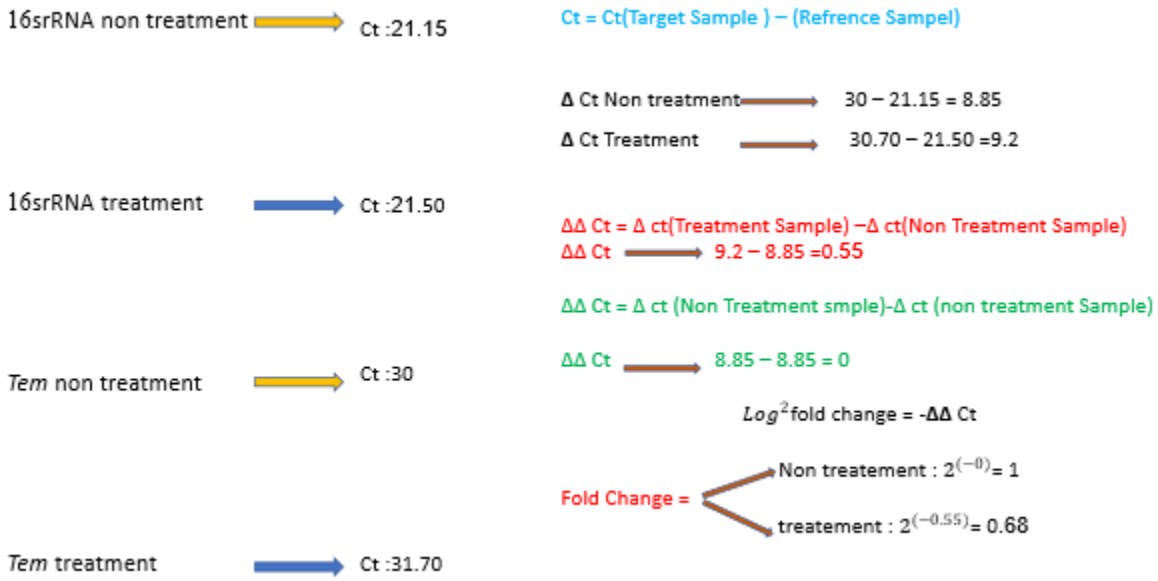


Figure 14: Quantitative gene expression analysis of *blaTEM* in *E. coli* isolates post-treatment with biosynthesized nanoparticles using Pfaffl method

Table 7: ΔCt and ΔΔCt values demonstrating *TEM* gene expression reduction after nanoparticle exposure

Sample	ΔCt Control	ΔCt Treated	ΔΔCt (Control)	ΔΔCt (Treated)	Fold Change (2^-ΔΔCt)
1	2.9	8.85	0.68	0.55	~0.68

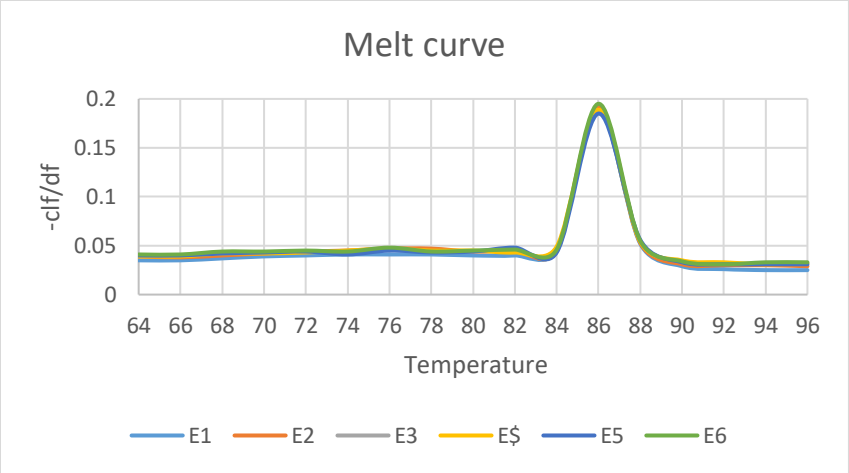


Figure 15: The main peak in the melting curve of the Tem gene in the *E. coli* strain treated with *T. polium*-derived nanoparticles showed a temperature of 86°C

Based on the PROCHECK statistics, the protein model exhibits excellent stereochemical quality, as evidenced by its Ramachandran plot and G-factor scores. An impressive 89.1% of non-glycine and non-proline residues are located in the most favored regions of the Ramachandran plot. The remaining 10.9% are found in additional allowed regions, with no residues in either generously allowed or disallowed regions. This distribution indicates a very good backbone conformation. Furthermore, the overall G-factor average of -0.06 is well above the threshold for being considered unusual (-0.5), with only the omega torsion angle score (-0.93) standing out as a minor anomaly, while all other parameters concerning dihedral angles and main-chain covalent forces are within normal or favorable ranges (Figure 16).

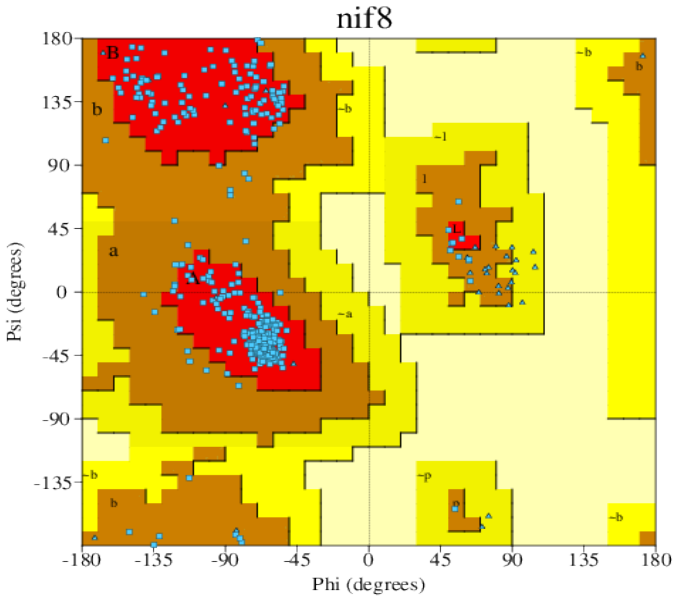


Figure 16: Ramahcnadran chart for 3D structure of **docking between ligand (**Apigenin (*Teucrium polium* (PubChem CID: 5280443) **and target (**blaTEM**)**

Based on the provided sequence, the docking simulation between the *β-Lactamase* enzyme *blaTEM* from *E, coli* and the flavonoid Apigenin from *T. polium* suggests a potential inhibitory interaction (Figure 17). The *blaTEM* sequence reveals key structural motifs, including several conserved active site residues (e.g., Ser70, Lys73, Ser130, Glu166, Asn170, and Lys234) (Figure 18) that are critical for its antibiotic hydrolysis function. Apigenin, a known *β-Lactamase* inhibitor, is predicted to bind within or near this active site pocket, likely forming hydrogen bonds and hydrophobic interactions with residues in the conserved regions around Ser70 and the omega loop, thereby sterically hindering the enzyme's ability to bind and degrade β-lactam antibiotics. This molecular docking result supports the experimental evidence that Apigenin can effectively counteract *blaTEM*-mediated antibiotic resistance (Figure 19).

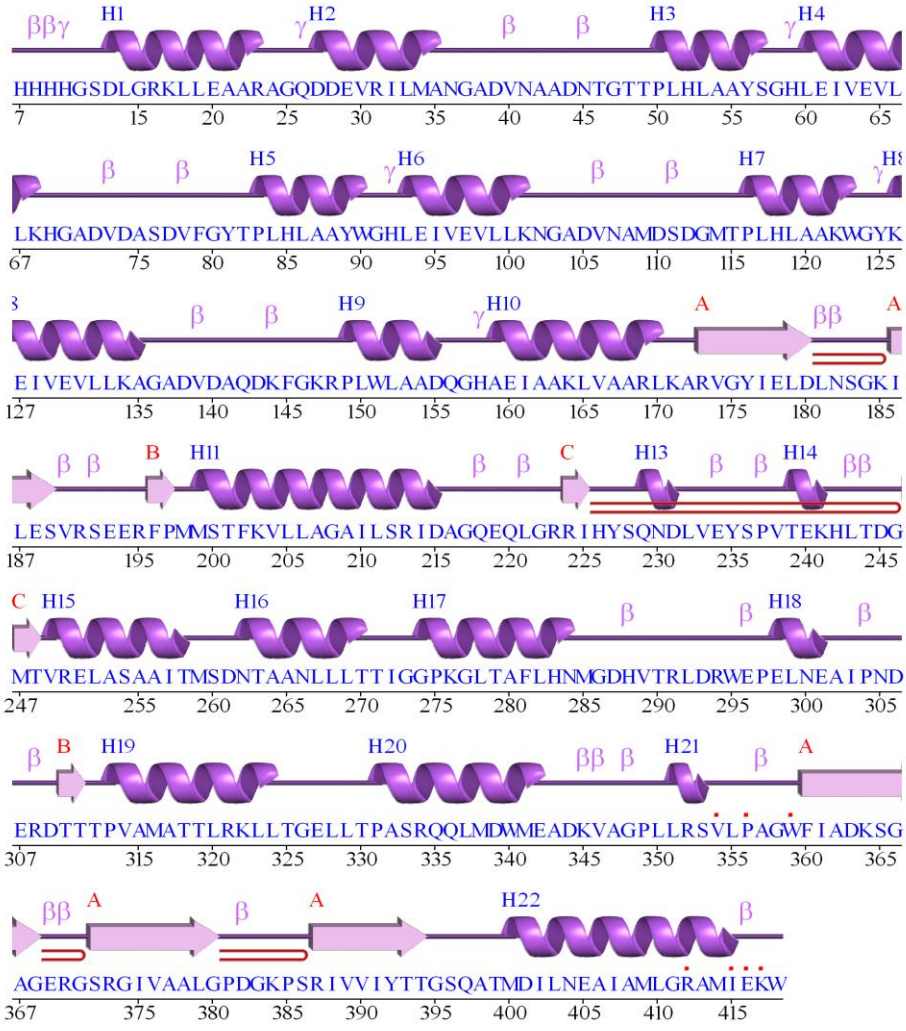


Figure 17: Structure, motif and residue of the docking between ligand (Apigenin (T. polium (PubChem CID: 5280443))) and target (blaTEM). (Helices labelled H1, H2, ... and strands by their sheets A, B, ... Helix Strand); Motifs: beta turn, gamma turn, beta hairpin ; Residue contacts: to ligand

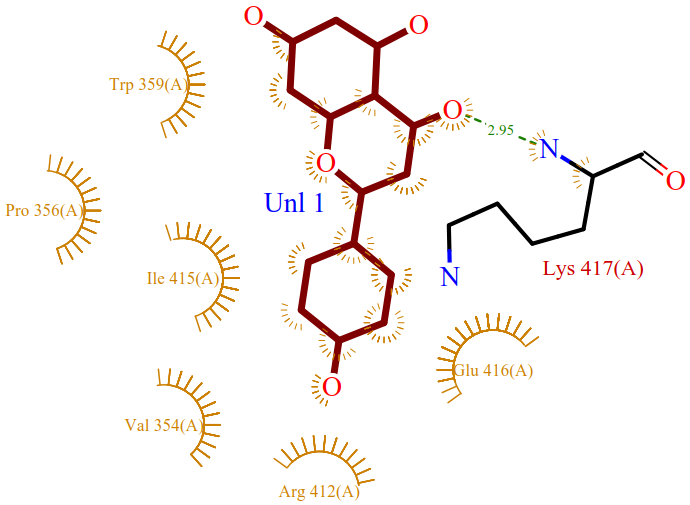


Figure 18: The chemical links between between ligand **(**Apigenin (*T. polium* (PubChem CID: 5280443))) and target **(***blaTEM***)**

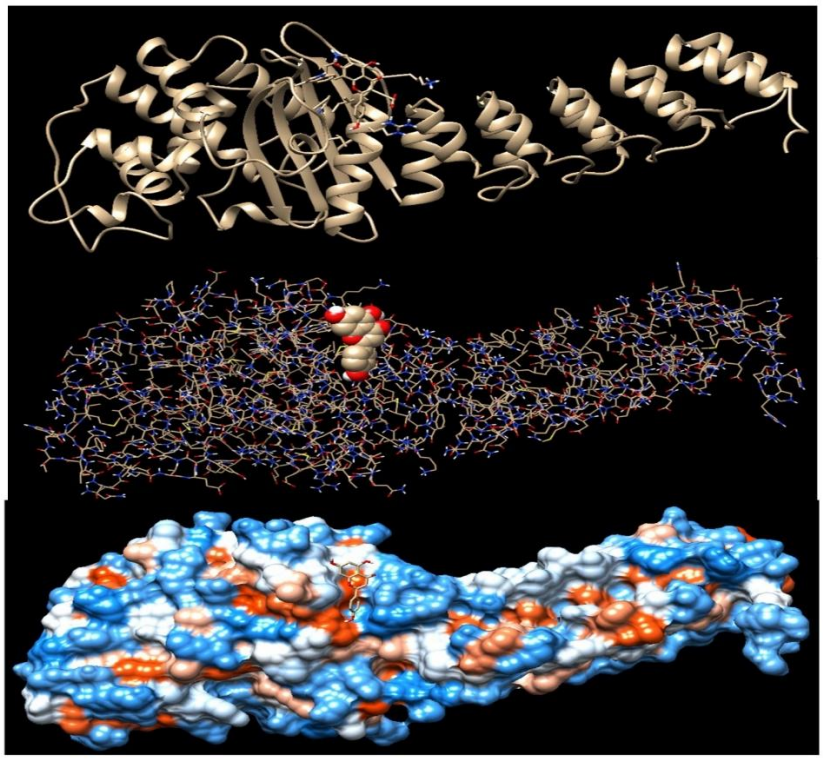


Figure 19: The 3d structure and the location of docking between ligand **(**Apigenin (*T. polium* (PubChem CID: 5280443))) and target **(***blaTEM***)**

Discussion

E. coli is the predominant pathogen causing UTIs, accounting for approximately 90% of cases among young women. [29-31].

The *blaTEM* gene is among the most prevalent ESBL genes, frequently plasmid-mediated, facilitating horizontal transfer among Enterobacteriaceae. Its global prevalence and temporal variation necessitate continuous surveillance. Our investigation of 155 *E. coli* urinary isolates identified 65% as *β-Lactamase* producers according to CLSI standards. Of these, 60% harbored the *blaTEM* gene, consistent with phenotypic and molecular data from regional and international studies. Biosynthesized AgNPs have emerged as promising alternatives due to their broad-spectrum antimicrobial activity and ability to inhibit resistance gene expression. The present study demonstrates the capacity of *T. polium*-based AgNPs to diminish *TEM* gene expression and impede growth of resistant *E. coli* strains [32, 33]. Given the simplicity, cost-effectiveness, and environmental sustainability of green synthesis methods, coupled with notable antimicrobial efficiency, these nanoparticles have potential for development as adjunct or replacement therapies against resistant bacterial infections[34].

Due to the high prevalence of *E. coli* in UTIs and the escalating dissemination of antibiotic resistance, numerous investigations have been conducted to assess the antimicrobial resistance patterns of this pathogen across various regions of Iran and internationally. Epidemiological data collected in Iran between 2007 and 2011 revealed that the prevalence of Extended-Spectrum *B-Lactamase*(ESBL)-producing *E. coli* strains ranged from 15.62% to 64% [35]. Hang Fang *et al.*, during 2001–2006 in Sweden, demonstrated that among 11 phenotypically identified ESBL-producing *E. coli* isolates, 12% harbored the *TEM*-type *β-Lactamase* gene [36]. Similarly, Shakil and colleagues in India reported that 29.3% (91 out of 311 isolates) of *E. coli* strains ESBL producers [37]. A 2015 study by Espan et al., evaluating Enterobacteriaceae isolates from clinical samples, documented a 58% prevalence of the TEM gene [38]. In a 2005 Turkish study of intestinal bacteria collected from hospitalized patients, the TEM-1 gene incidence was reported at 52.7% [31].

In our study, from 155 urine specimens collected from patients with UTIs, 100 *E. coli* isolates (approximately 65%) identified as *β-Lactamase* producers using the CLSI standard phenotypic methodology. This finding aligns with research by Eslami et al., who, through phenotypic and molecular analysis of *REB*, *TEM*, and *VEB β-lactamases* in *E. coli* strains from Tehran, observed that 90% *ESBL* producers [39]. Of these 100 isolates, 10 analyzed by PCR for the presence of the *TEM* gene, revealing a 60% prevalence (6 isolates), corroborating Haghhi et al.'s 2012 findings in Zanjan, where 33% of 200 isolates exhibited ESBL phenotype and 52.2% carried the *TEM* gene detected via molecular assays [40].

Antimicrobial resistance against infectious pathogens currently poses a critical challenge in medical science, largely driven by irrational and self-prescribed antibiotic use. The escalation in antibiotic resistance inflates healthcare costs and prolongs convalescence durations, underscoring the pressing necessity for novel antimicrobial agents and alternatives to conventional antibiotics [41].

Antibiotic susceptibility profiling of *E. coli* isolates derived from uropathogenic samples revealed the highest resistance rates against ampicillin discs (10 µg) at 74%, carbenicillin (100 µg) at 61%, and chloramphenicol (30 µg) at 53%. These resistance patterns are consistent with reports from other countries; for instance, a 2011 study by Lee et al. observed 60% resistance to ampicillin [42], and a contemporaneous study in Greece indicated 50% resistance to the same antibiotic [43].

Among nanomaterials employed in biomedical applications, AgNPs are extensively studied due to their potent antimicrobial properties. Silver ions exhibit affinity for electron-donating functional groups such as hydroxyl, oxygen, and nitrogen moieties in biomolecules [44]. AgNPs disrupt bacterial outer membrane integrity, inducing uncontrolled release of components like lipopolysaccharides and purines from the cytoplasm; upon internalization, they bind to sulfhydryl groups of bacterial proteins, disabling critical enzymes and damaging DNA, culminating in bacterial eradication [45]. Notably, cytotoxicity and toxicological profiles of silver ions are concentration-dependent [46]. While diverse physical and chemical synthesis techniques exist for nanoparticle fabrication, their potential environmental hazards have prompted adoption of greener biosynthesis methods, which are cost-effective and environmentally benign [47]. In this study, the aqueous extracts of *T. polium* leaves and flowers prepared using a maceration technique at ambient temperature and dark conditions for 24 h. A pronounced color change from pale yellow to deep brown indicated effective reduction of silver ions, demonstrating the extract's high potential for silver nanoparticle biosynthesis.

The UV-Vis spectrophotometry peak at approximately 450 nm indicates surface plasmon resonance characteristic of AgNPs, attributed to the excitation of free electrons. Enhanced optical absorbance corresponds to silver ion reduction and nanoparticle agglomeration, validating the method's suitability for bulk nanomaterial production [48]. Similar biosynthesis successes utilizing extracts from *Pelargonium*, *Medicago sativa*, *Pinus*, and *Platanus* species have been documented. The stability of the biosynthesized AgNPs is attributed to bioactive metabolites such as alkaloids, polyphenols, and polysaccharides, serving as reducing and capping agents [25].

Antimicrobial activity evaluation of biosynthesized AgNPs against *TEM*-positive *E. coli* via disk diffusion assay demonstrated that lower concentrations effectively inhibited bacterial growth, with larger zones of inhibition observed at increased nanoparticle dosages. The minimum average inhibition zone diameter for *T. polium* extract at 25 µg/mL confirmed the nanoparticles' inhibitory efficacy ($p < 0.05$) [49]. Furthermore, broth microdilution assays established a MIC of 32 µg/mL for AgNPs synthes ized with *T. polium* extract against *E. coli* ($p < 0.05$), consistent with preceding reports [49]. Variations observed are attributable to differences in plant parts utilized for extraction, metabolite content, and geographic or climatic influences on plant chemistry. MBC was determined as 64 µg/mL via tube dilution method ($p < 0.05$).

Notably, this investigation revealed that biosynthesized AgNPs from *T. polium* aqueous extracts exert a significant downregulatory effect on the expression of the *TEM* gene in tested *E. coli* strains under in vitro conditions. This parallels findings by Walid Albraidi et al. (2020) who reported that AgNPs inhibit *blaTEM* gene expression in β-lactam-resistant *E. coli* [26]. Similarly, Ahmad Rasheed et al. demonstrated that AgNPs suppress *mecA* gene expression in methicillin-resistant *Staphylococcus aureus* isolates by diminishing gene transcription [50]. Independent t-test analysis indicated a 34% reduction (mean expression level = 0.363, decrease of 0.186

relative to control, $p < 0.05$) in *TEM* gene expression following AgNP treatment synthesized from *T. polium* extracts. The Pfaffl method further validated a 32% decline in *TEM* transcript levels. Quantitative real-time PCR with melting curve analysis confirmed specific and efficient amplification of the TEM gene across all samples, as evidenced by single melting peaks at 86°C, ensuring assay reliability.

Future perspectives include evaluating the antimicrobial efficacy of *T. polium* -based AgNPs against *E. coli* strains harboring other *β-Lactamase* genes beyond *TEM* and extending this inquiry to other Enterobacteriaceae family pathogens. Moreover, assessment of other indigenous medicinal plants for modulation of antibiotic resistance gene expression remains a compelling research avenue. The unique phytochemical constituents of *T. polium* provide a robust bioreductive milieu facilitating silver nanoparticle synthesis with potent antimicrobial properties. These nanoparticles effectively decrease *blaTEM* gene expression and bacterial viability in resistant *E. coli* isolates. Future research should extend to (i) evaluating the impact of these nanoparticles on other resistance determinants beyond *TEM*, (ii) assessing efficacy against diverse members of Enterobacteriaceae, and (iii) exploring synergistic potential with existing antimicrobials. Incorporation of indigenous medicinal plants in nanoparticle biosynthesis constitutes a sustainable avenue for combating multidrug-resistant pathogens affecting both clinical and agricultural sectors.

Conclusion

T. polium possess unique bioreductive capacity enabling facile biosynthesis of AgNPs endowed with antimicrobial properties. Due to their rich secondary metabolite profile, *T. polium* extracts are promising bioreagents for green synthesis of AgNPs exhibiting potent antimicrobial activity. This is the first study in Jiroft County examining the impact of *T. polium* aqueous extract-derived AgNPs on *TEM* gene expression in *E. coli* isolates from outpatients with UTIs. The results showed that the biosynthesized AgNPs exhibited significant bacteriostatic and bactericidal effects against *E. coli* strains harboring *β-Lactamase* enzymes. Importantly, treatment with AgNPs resulted in a 34% reduction in *TEM* gene expression. Based on these findings, future research may explore the potential application of *T. polium* extract-derived AgNPs as an adjunct or alternative therapeutic modality to conventional antibiotics in the management of multidrug-resistant *E. coli* infections that warranting further investigation with larger sample sizes.

Footnote

Conflict of Interests

All authors declare no conflict of interest.

Ethics approval and consent to participate

No human or animals used in the present research.

Ethical issues

Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors. The authors have adhered to ethical standards, including avoiding plagiarism, data fabrication, and double publication.

Statements and Declarations

Consent for publication

All authors read and approved the final manuscript for publication.

Availability of data and material

All the data are embedded in the manuscript.

Authors' contributions

Conceptualization: N. H.

Data curation: Z. F.

Formal analysis: B. F-N.

Investigation: M. Sh.

Methodology: A.S., B. F-N.

Project administration: Z. F.

Resources: All authors.

Supervision: B. F-N.

Validation: N. H.

Visualization: Z. F.

Writing–original draft: N. H.

Writing–reviewing & editing: All authors.

Informed Consent

The authors declare not used any patients in this research.

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