Original Article

Effect of Green Gold Nanoparticles on The Expression of Efflux Pump Genes *AcrAv* and *NorA* for MDR Uropathogenic *Escherichia coli* and *Staphylococcus epidermidis*

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Abstract

Background: With each day, the danger of antibiotic-resistant bacterial strains is increasing worldwide due to the development of resistance mechanisms, including efflux pumps, that are used to expel harmful substances (such as antibiotics) outside bacterial cells. Currently, gold nanoparticles (AuNPs) are regarded as viable substitutes for eradicating microorganisms. **Aim of Study:** This study aims to investigate the effect of green AuNPs on the gene expression of two efflux pump genes, the *norA* gene for *Staphylococcus epidermidis* and the *acrA* gene for *Escherichia coli*. **Materials and Methods:** The green AuNPs were synthesized using pomegranate peel extract and characterized by scanning electron microscope (SEM), ultraviolet–visible spectrophotometry, X-ray diffraction, and Fourier transform infrared spectroscopy. The genes responsible for the expression of multi-drug resistance (MDR) efflux pumps, namely, *acrA* in *E. coli* and *norA* in *S. epidermidis*, have been identified by polymerase chain reaction (PCR), and the expression of the abovementioned efflux pump genes of both bacteria exposed to different concentrations of AuNPs (minimum inhibitory concentration [MIC] and sub-MIC concentrations) was assessed using RT-PCR. **Results:** All bacterial isolates of *E. coli* possess the *acrA* gene, and those of *S. epidermidis* possess the *norA* gene. The results demonstrated a significant downregulation in the expression of efflux pump genes *acrA* and *norA* when treated with green AuNPs, with significant differences represented by *P* value (≥0.05) 0.009 for *S. epidermidis* and 0.011 for *E. coli*, as compared to the bacteria untreated with green AuNPs in standardizing with housekeeping genes. **Conclusions:** Green AuNPs have shown the ability to reduce bacterial resistance by destroying or disabling resistance pathways.

Keywords: *acrA* gene, *E. coli*, efflux pump, gene expression, gold nanoparticles, MDR, *norA* gene, *S. epidermidis*

Introduction

Antibiotic resistance (ABR) has become a considerable and urgent public health issue in the twenty-first century. It arises due to bacterial alterations that reduce the effectiveness of medicines.[1] Every year, in the United States, there are about 2.8 million illnesses caused by microorganisms that are resistant to antimicrobial treatments, leading to the death of more than 35,000 individuals.[2,3]

Staphylococcus epidermidis is a Gram-positive bacterium that may cause infection in humans. While mostly benign, it is the primary source of nosocomial infections. Treatment of *S. epidermidis* infections often involves use of antibiotics. However, the increasing prevalence of antibiotic-resistant strains has posed challenges in effectively treating these infections[4] *Escherichia coli* is a Gram-negative bacteria that is, a common pathogen mostly causing urinary tract infections (UTIs). Uropathogenic *Escherichia coli* is a primary contributor to both community-acquired UTIs (80%–90%) and hospital-acquired UTIs (30%–50%).[5-7]

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Discovery of ABR efflux pumps in the 1990s marked a significant advancement in the field of microbiology, enabling the identification and study of many efflux pumps in Gram-negative and Gram-positive bacteria.[8,9] Efflux pumps are proteinaceous transporters that have significant functions in metabolism, pathogenesis of bacteria, and resistance to many drugs by reducing the levels of foreign substances (disinfectants, detergents, and antibiotics) inside the cell, hindering it from achieving its biological goals. Efflux pumps significantly contribute to the growing problem of antimicrobial resistance, rendering many treatments ineffective.[10] A significant proportion of bacteria have developed resistance to multiple drugs as a result of insufficient dosing and improper use of current antimicrobial agents.[11] Efflux pumps found on the cell wall of bacteria are responsible for ABR and are classified into six main families: the main facilitator superfamily, the proteobacterial antimicrobial compound efflux superfamily, the resistance nodulation division family, the small multidrug resistance superfamily, the adenosine triphosphate (ATP)-binding cassette superfamily (ABC), and the multidrug and toxic compound extrusion superfamily. ABC efflux pumps work as major active transporters that use the hydrolysis of ATP as a source of energy to expel foreign substances.[12,13]

Biomedical applications have lately focused on metallic nanoparticles. The exponential progress in scientific research in this domain has resulted in the synthesis of many functional nanoparticles (NPs). Gold nanoparticles (AuNPs) have garnered significant interest among many metallic nanoparticles because they have unique surface plasmon resonance properties, are easy to produce, can be changed in size, and have many different functions, while still having clear properties.[14,15] These materials are highly adaptable, chemically unreactive, compatible with living organisms, and often resistant to change. In past experiences, doctors and alchemists recognized the distinctive therapeutic qualities of colloidal gold solutions.[15-17] Based on the increase in the burden of ABR, bacteria have posed a severe concern on public health worldwide and are expected to be the first etiology of morbidity and mortality; in addition, a new technology called "green synthesis" has emerged as an alternate strategy to produce biocompatible nanoparticles. Green synthesis methods are significant due to their environmentally benign application. Green synthesis methods utilize natural bioresources and avoid the use of hazardous chemicals and have significant medical uses; especially, AuNPs are used as an antibacterial alternative to traditional antibiotics. So, this study aimed to investigate the effect of green AuNPs on the expression of efflux pump genes in multi-drug resistance (MDR) bacteria isolated from UTI cases.

Materials and Methods

Bacterial isolates and green AuNPs

The bacterial isolates (*E. coli* and *S. epidermidis*) were isolated from UTI clinical cases. The identification and antibiotic sensitivity testing were carried out by the VITEK 2 system (Biomerieux/France) as well as green synthesis of AuNPs and its optimization according to previous research.[18] Green AuNPs were synthesized using pomegranate peel extract and characterized by ASM, UV spectrophotometry, X-ray diffraction, and Fourier transform infrared spectroscopy.

The polymerase chain reaction technology

The polymerase chain reaction (PCR) approach was used to identify the presence of efflux pump genes in MDR uropathogenic *S. epidermidis* and *E. coli*.

DNA extraction of both *E. Coli* and *S. epidermidis* isolates

After identifying antibiotic-resistant bacterial isolates of both *E.coli* and *S. epidermis*, bacteria were cultured on neutron broth medium at a constant temperature of 37°C for a duration of 24 h. The genomic DNA of the bacterial isolates was then extracted using the Presto™ Mini gDNA Bacteria Kit (Geneaid Biotech Ltd, a company, Taiwan). To validate the isolates, genotypic identification was conducted using the norA gene for *S. epidermidis* and the acrA gene for *E.coli*. This was achieved by using specific primers as given below for optimizing the PCR conditions [Table 1]. Following the PCR, the resulting PCR product was subjected to electrophoresis on 2% agarose gel and visualized using a UV transilluminator.

Molecular detection of efflux pump genes

The PCR Master Mix for all genes was prepared using the (GoTaq ®Green PCR master kit, Promega Corporation, USA), following the manufacturer's instructions. A polymerase chain reaction was conducted using a final volume of 25 µL. The reaction mixture consisted of 12.5 µL of GoTaq ®Green PCR master mix (Promega Corporation, USA), 2 µL of the forward primer (10

Table 1: PCR detection efflux pumps gene primers with their nucleotide sequences

PCR primer		Sequence (5′–3′)	Product size (bp)	NCBI reference code
S. epidermidis norA gene	F	GCTATTATCGGTGGAGGCGT	508	AY566250.1
	R	CCACCTCGTCCCCAAAACTT		
E.coli acrA gene	F	GAGTACGATCAGGCTCTGGC	423	NC_000913.3
	R	GTTCGGGAAGATAGCGCGTA		

pmol), 2 µL of the reverse primer (10 pmol), 5 µL of the DNA template (5–50 ng), and 3.5 µL of PCR water and thereafter inserted into the T100 PCR Thermocycler (manufactured by BioRad, USA). PCR products are subjected to electrophoresis on 2% agarose gel. The bands were then visualized by photography using a UV transilluminator.

Real-time PCR technology

The qPCR approach was used to estimate the expression for efflux pump genes present in the MDR *E. coli* and *S. epidermidis*.

Minimum inhibitory concentration test for AuNPs

The minimum inhibitory concentration (MIC) of AuNPs was used to assess their antibacterial activity. After dilution, the minimum inhibitory concentration was obtained, and the viability of the cells was evaluated after a period of 24 h. We cultivated bacterial isolates in a nutrient-rich broth. Various concentrations of green AuNPs (1000, 500, 250, 125, 62.5, and 31.2 µg/mL) were added to tubes during the logarithmic growth phase after 3–4 h of incubation. After that, the tubes were incubated in the 37°C incubator for 24 h. To assess the bacterial concentration, optical density was measured at 600 nm. For gene expression analysis, sub-MIC values were recorded after finding the MIC.

RNA extraction and cDNA synthesis of bacterial isolates

Total RNA from *S. epidermidis* and *E. coli* isolates treated with green synthesized AuNPs and untreated was extracted, during the logarithmic growth phase. This was achieved using the easy-BLUE™ Total RNA Extraction Kit (iNtRON Biotechnology, Inc., South Korea). Genotypic identification was conducted using specific primers targeting the norA gene in *S. epidermidis* and the acrA gene in *E. coli* [Table 2]. The total RNA obtained was assessed using the NanoDrop (Thermo Scientific NanoDrop Lite UV Visible Spectrophotometer, USA) to determine the RNA concentration (ng/µL) and verify the RNA quality by measuring the absorbance at 260/280 nm. The extracted RNA underwent treatment with DNase-I enzyme in order to eliminate any residual genomic DNA present in the eluted total RNA. This was achieved by using samples from the DNase-I enzyme kit. RNA samples processed with DNase-I were used for cDNA synthesis by using the M-MLV Reverse Transcriptase kit. The qPCR master mix was prepared using the GoTaq® qPCR Master Mix Kit (Promega Corporation, USA), which uses SYBR green dye amplification in a Real-Time PCR system. Additionally, a separate qPCR master mix was prepared specifically for efflux pumps. The target genes and housekeeping gene were prepared as follows. The experimental setup involves adding 5 µL of the cDNA template (10 ng/µL), 1 µL of forward primer, 1 µL of reverse primer, 10 µL of qPCR Master Mix, and 3 µL of nuclease-free water. The total volume of the mixture should be 25 µL. Subsequently, this reaction is transferred to the CFX96 Real-Time PCR system. The qPCR thermocycler conditions were performed in accordance with the instructions provided by the qPCR kit, using the Clinical Protocol Writer™. The temperature for the first denaturation is 59°C/10 min at a rate of one cycle, Denaturation occurs at a temperature of 95°C 20 s at 40 cycles. Annealing and extension detection (scans) were performed at 60°C 30 s, with 40 cycles and a melting temperature range of 65°C–95°C for one cycle. The data findings of qPCR for the target gene and housekeeping gene were obtained. The expression analysis (fold change) was performed using the Livak technique.

Data analysis using statistical programs

All data were analyzed by statistical package for the social sciences, version 24 (IBM Corporation, USA), with a P value of <0.05. The gene expression of the efflux pump was analyzed using a one-way analysis of variance on the linear $2^{-\Delta\Delta CT}$ data set.

Ethical approval

The study was carried out according to the manufacturer's protocol, and the subject information and the consent form were reviewed and approved by the Al-Qasim Green

Table 2: qPCR detection gene primers with their nucleotide sequences

qPCR primer		Sequence (5′–3′)	Product size (bp)	NCBI reference code
S. epidermidis norA gene	F	AAGTCACGCCGTAAAGAAGC	89	AY566250.1
	R	TCCCCAAAACTTGCTCTGTC		
S. epidermidis housekeeping *gyrB* gene	F	ACGGGTATTGGAGGAGAATTCG	87	NZ_CP035288.1
	R	ATGTGCACCATCAACATCGG		
E. coli acrA gene	F	AAAGCTGCCGTTGAAACTGC	150	NC_000913.3
	R	ATCAAGTTGCTGCACGGTTG		
E. coli housekeeping *rpsL* gene	F	AAACGTGGCGTATGTACTCG	100	NC_000913.3
	R	CTTCGAAACCGTTAGTCAGACG		

University Ethical Committee according to document number M311 on November 23, 2022.

Results

In accordance with the prior study,[18] 10 bacterial isolates were chosen for each strain of *E. coli* and *S. epidermidis*, which were most frequent in MDR.

The microtiter dilution technique is used to calculate both the minimum inhibitory concentration and the sub-minimum inhibitory concentration

Three bacterial isolates exhibiting the highest resistance to antibiotics were chosen for each type: *E. coli* and *S. epidermidis*. Every bacterial isolate was subjected to a different set of concentrations of green AuNPs (1000, 500, 250,125, 62.5, and 31.25 µg/mL). The findings indicated that tube No. (1) represents the MIC, which is the percentage of bacterial growth for the total number of isolates for both bacterial kinds, at 0% owing to the high concentration (1000 µg/mL), whereas tube nos. 2, 3, and 4 represent sub-MICs with varying percentages of bacterial growth because they contain low concentrations (500, 250, and 125 µg/mL) [Figure 1].

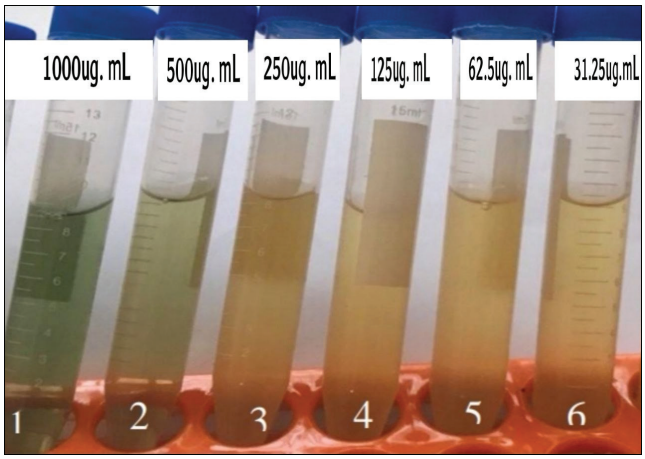

Figure 1: Using microtiter tube technology: determination of MIC and sub-MIC through the effect of gold nanoparticles on bacterial isolates of both *E. coli* and *S. epidermidis*

Molecular detection of the efflux pump (*acrA*) gene in *E. coli* and the (*norA*) gene in *S. epidermidis* using (PCR technology)

Based on the microbial susceptibility test findings, 10 MDR bacterial isolates from each species of *E. coli* bacteria and *S. epidermidis* were selected according to previous research[18] based on the resistance of *E. coli* to cefotaxime and that of *S. epidermidis* to tetracycline, which was determined to be their resistance caused by efflux pump mechanism. The MDR bacterial isolates were diagnosed using the polymerase chain reaction (PCR) technology to verify the existence of efflux pumps genes for *E. coli* (*acrA*) and *S. epidermidis* (*norA*). The findings indicated that all MDR *E. coli* isolates exhibited the presence of the (*acrA*) gene at a 100% rate, with an amplification size of 400 bp. Similarly, all MDR *S. epidermidis* isolates demonstrated the presence of the (*norA*) gene at a 100% rate, with an amplification size of 350 bp, as seen in the [Figure 2].

Evaluating the gene expression ratio of efflux pump genes after and before treatment with green AuNPs using real-time PCR technology

The presence of the *acrA* gene in all *E. coli* MDR isolates, as well as the *norA* gene in all *S. epidermidis* MDR isolates, was evaluated before the gene expression was verified. The effect of green synthesized AuNPs at different concentrations (MIC and sub-MIC) on efflux pump gene expression in MDR bacteria was investigated in combination with the most resistant antibiotics associated with efflux pumps; in order to activate efflux pumps (cefotaxime *in E. coli* and tetracycline in *S. epidermidis*) by the normalization method with the *rpsL* gene of *E. coli* and *gyrB* gene of *S. epidermidis*, they are expressed as reference genes. After RNA extraction and cDNA synthesis, the results are expressed through curve-analysis, as in Figure 3. The expression ratio of genes (*acrA in E.*

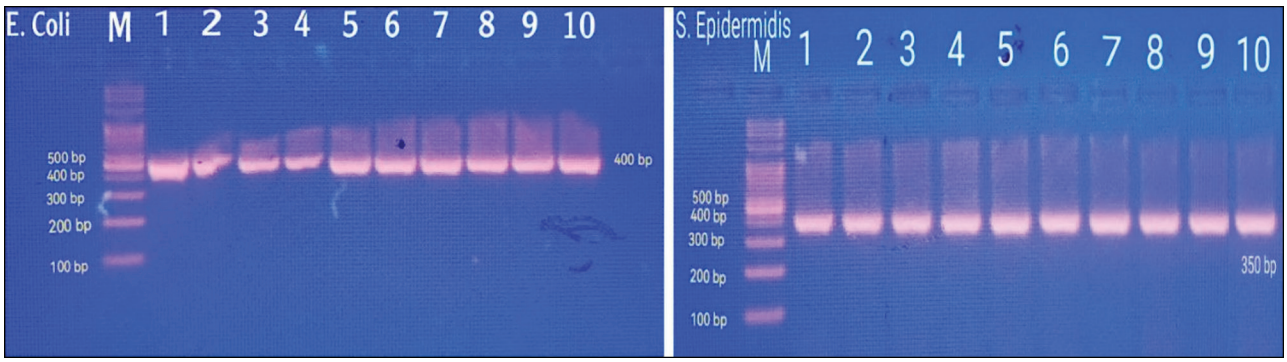

Figure 2: Gel electrophoresis shows gene replication for the *AcrA* gene of *E. coli* with amplified size 450 bp and *NorA* of *S. epidermidis* with amplified size 350 bp

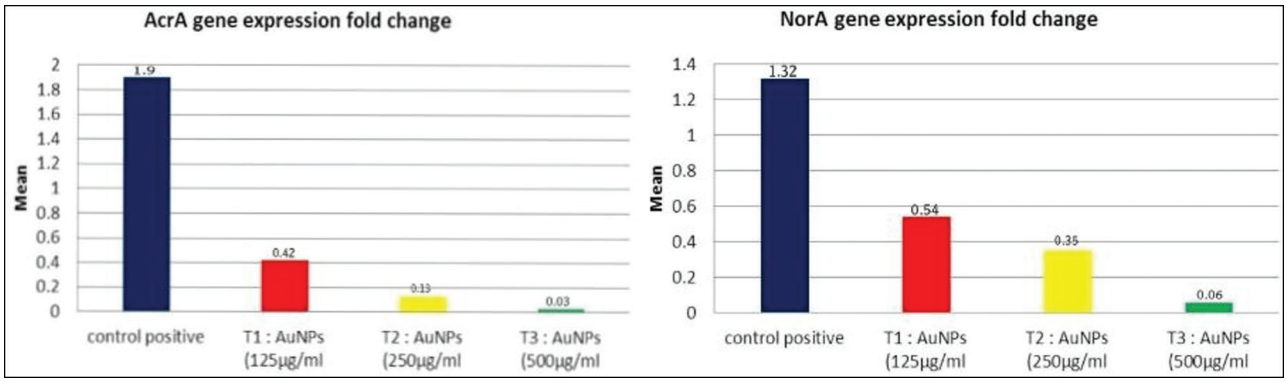

Figure 3: Mean fold change in gene expression of *norA* for *S. epidermidis* and *acrA* for *E. coli*, which have been treated with different concentrations of AuNPs (T1:125, T2: 250, and T3:500 μg/mL) and C: bacteria without treatment where *P* value 0.009 for *S. epidermidis* and 0.011 for *E. coli*

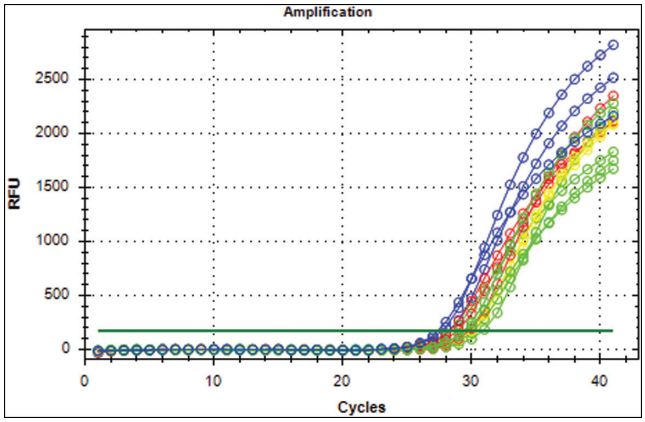

Figure 4: Real-time amplification plots for the efflux pump *acrA* gene in experimental and control resistance *E. coli* isolates. The blue plots C: control positive only cefotaxime, the red plots T1: gold nanoparticle 125 μg/mL, the yellow plots T2: gold nanoparticle 250 μg/mL, and the green plots T3: gold nanoparticle 500 μg/mL

coli and *norA* in *S. epidermidis*) that were treated with green AuNPs was fully low, compared to those that were not treated (control).

The gene expression of the two efflux pump genes (*acrA* and *norA*) in all MDR *E. coli* and *S. epidermidis* isolates treated with T1 (with concentration 125 µg/mL from green AuNPs) exhibited an increase in the mean fold change level (0.42 ± 0.38) for *acrA* and (0.54 ± 0.28) for *norA*. The isolates treated with T2 (with concentration 250 µg/mL from green AuNPs) exhibited a significant reduction in the fold change in the gene expression level of two genes, *acrA* (with mean value of 0.13 ± 0.05) and *norA* (with mean value of 0.35 ± 0.27). The fold change in the gene expression level for both genes treated with T3 (with concentration 500 µg/mL from green AuNPs) exhibited a reduction compared to those treated with T1 and T2, with a mean value of (0.03 ± 0.01) for the gene *acrA* and mean value of (0.06 ± 0.07) for the gene *norA*. The control group (which represents the bacteria in a normal state) had the highest fold change in gene expression levels with a mean value (1.9 ± 0.46) for the *acrA* gene and (1.32 ± 0.53) for the norA gene compared to the groups treated with T1, T2, and T3. The results of this study indicate that the inhibitory impact of AuNPs on the *acrA* gene is increased compared to that of the *norA* gene, which can be seen in Table 3 and Figures 4 and 5. This suggests that there is a significant difference between the two groups, with a *P* value of 0.009 for *S. epidermidis* and 0.011 for *E. coli*, in relation to the reduced levels of *norA* and *acrA* gene fold changes, respectively, shown in Table 3.

Discussion

Elimination of antibiotic-resistant bacteria has become almost impossible with antibiotics due to the continuous development of resistance mechanisms toward chemical compounds found in antibiotics. This has made researchers look for alternative methods that are safer for human health and more effective against microbes. Among these methods are those that specialize in nanoscience,[19] which has become a pioneer in this era through its unique properties., particularly its small size, and hence now widely used in medicine.[20] The efflux pump genes of antibiotic-resistant bacterial isolates were detected using PCR technology. The results showed that all *E. coli* isolates possessed the *acrA* gene by 100%. This result was consistent with those of the previous study, which confirmed that all *E. coli* isolates possessed this gene.[21] The *acrA* gene is found in the expulsion of a wide range of antibiotics such as tetracycline, fluoroquinolones, and β-lactams, in addition to disinfectants and organic solvents.[22] the results also showed that all *S. epidermidis* isolates possessed the *norA* gene 100%. This result is consistent with those of the previous study, which showed that all *S. epidermidis* isolates possess the *norA* gene in a similar proportion.[23] The *norA* gene uses the proton motive force as an energy source, which plays an important role in the expulsion of antibiotics, especially fluoroquinolones, and the rest other antimicrobials.[24] Using real-time PCR technology, the gene expression of efflux pumps was evaluated, where a significant decrease

Table 3: Impact of green AuNPs on the expression of the *acrA* gene in *E. coli* and the *norA* gene in *S. epidermidis*

Isolates NO	Mean± SD of AcrA gene (*E. coli*)	*P* value	Mean± SD of NorA gene (*S. epidermidis*)	*P* value
C	1.9 ± 0.46	0.011	1.32 ± 0.53	0.009
T1	0.42 ± 0.38		0.54 ± 0.28	
T2	0.13 ± 0.05		0.35 ± 0.27	
T3	0.03 ± 0.01		0.06 ± 0.07	
$LSD_{0.05}$	7.428		7.959	

Significant difference ($P < 0.05$), SD: standard deviation

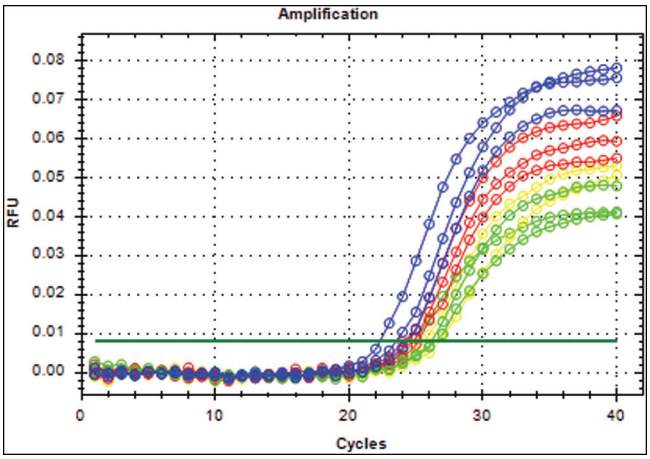

Figure 5: Real-time amplification plots for the efflux pumps *norA* gene in experimental and control resistance *S. epidermidis* isolates. The blue plots C: control positive only tetracycline, the red plots T1: gold nanoparticle 125 μg/mL, the yellow plots T2: gold nanoparticle 250 μg/mL, and the green plots T3: gold nanoparticle 500 μg/mL

was observed in the *acrA* gene and then in the *norA* gene compared to untreated isolates. Green AuNPs have shown their effectiveness in reducing gene expression due to their small size (diameter 70 nm), which makes it easier for the nanomaterial to penetrate the bacterial cell and manipulate its components. Due to the lack of research on the effect of green AuNPs on efflux pump genes compared to other nanoparticles, one study confirmed the effect of green AuNPs on the gene expression of efflux pumps in the bacteria *Pseudomonas aeruginosa*, where there was a significant decrease in expression of efflux pump (*mexA* and *mexB*) genes when compared to untreated isolates.[25] Evidence from a research suggests that green AuNPs in combination with the antibiotic ciprofloxacin resulted in decreased expression of the NorA and B genes for efflux pumps in *Staphylococcus aureus*. This, in turn, leads to the enhancement of the antibiotic's efficacy in inhibiting the growth of antibiotic-resistant bacteria.[26,27] Efflux pumps can be inhibited through two mechanisms: the inhibitory molecule binds directly to the specific site of the efflux pumps on the bacterial cell membrane, preventing the release of the antibiotic from the cytosol and leading to bacterial cell death. This is considered a competitive inhibitor strategy through which a special efflux pump is disabled, which results in the antibiotic remaining inside the cell for as long as possible,[28] or by disrupting the kinetics of efflux pumps, such as genes found in antibiotic-resistant bacteria, which can be inactivated using nanoparticles that are effective against those genes. This makes bacteria sensitive to antibiotics by disrupting the dynamics of efflux pumps.[29] Inactivation of the efflux pump kinetics occurs in two ways: either by turning off the proton gradient, resulting in a reduction of the proton motive force, or by a decrease in the membrane potential,[30] or by stopping the full propulsion force used to activate the flow pumps. One study showed that due to the small size of the nanomaterial, which makes it interact with a very complex flow pump system, copper nanoparticles were effective in inhibiting the *norA* gene through copper ions. Copper ions have an important role in inhibiting efflux pumps by disrupting efflux pumps' dynamics and energy levels.[31]

Conclusions

Green AuNPs synthesized using pomegranate peel extract are an effective alternative to killing bacteria by inhibiting or reducing resistance mechanisms such as efflux pumps by reducing the gene expression (*acrA* and *norA*). Due to their small size of less than 100 nm (70 nm), they are safer and non-toxic, as several different concentrations were used. The effectiveness of the nanomaterial in decreasing the gene expression in active efflux pumps increases with its concentration.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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