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Green Synthesized Copper Oxide Nanoparticles Effect on Gene Expression for Biofilm Forming *E. Coli*

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Abstract

Biofilm bacteria pose a profound threat to human and animal health. This study helps to explore the potential anti-gene expression effect of the green copper nanoparticles (CuONPs) on biofilm genes in strong biofilm forming E. coli. The anti-gene expression of green synthesis of CuONPs in different concentrations (MIC and Sub MIC) were evaluated against E. coli biofilm genes (*luxS*, *mqsR*, *fliA*) with housekeeping *GapA* gene by Real-time quantitative polymerase chain reaction (RT- qPCR) after treatment in comparative with control. The results revealed that green CuONPs were significantly effective in MIC and Sub MIC concentration on biofilm gene expression by downregulating of luxS gene in fold change (1.2 and 4.92) in human E. coli isolates and (0.744 and 6.01) in animal E. coli respectively, in comparative with gene expression of control (bacteria without treatment) (14.870) and (12.616) respectively. The expression of *Mqsr* gene was down regulate after treatment with green CuONPs fold change (0.95 and 9.06) and (1.078 and 6.7) in human and animals in MIC and Sub MIC concentrations respectively with comparative control (13.9) and (18.8) respectively. Also, the expression of *Flia* gene was decrease after treatment with green CuONPs fold change (1.054 and 7.06) and (0.5 and 7.4) in human and animals of MIC and Sub MIC concentration respectively with comparative control (16.318) and (20.353) respectively. Conclusion: Green CuONPs showed an excellent inhibitory effect and anti-biofilm activity against *MDR* biofilm *E. coli genetically which* act as down-regulator of biofilm genes in strong biofilm forming *E. coli*.

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Introduction

Biofilms are bacterial communities that aggregate and adhere to living surfaces and are embedded in an extracellular polymeric substances matrix. Biofilms are notoriously resistant to conventional antimicrobial agents, making them difficult to eradicate. Disrupting biofilm formation and targeting biofilm-related genes are areas of active research [1].

Diarrheagenic Escherichia coli (*E. coli*) strains can indeed form biofilms, which are complex communities of bacteria attached to surfaces and encased in a matrix of extracellular polymeric substances (EPS). Biofilm formation is a survival strategy employed by many bacteria, including various pathogenic strains of *E. coli* [2]. Biofilm formation plays a crucial role in the pathogenicity of *E. coli* by enabling them to adhere to host tissues, resist immune responses, and persist in the environment. It also facilitates the spread of bacteria from contaminated surfaces to other hosts. *E. coli* biofilms that have been implicated in various infections, including urinary tract infections, catheter-associated infections, and gastrointestinal infections [3].

The ability of *E. coli* to form strong biofilms is attributed to several factors. One of the key components is the production of extracellular polymeric substances, which consist of polysaccharides, proteins, and DNA [4]. These substances provide structural support and protection to the bacteria within the biofilm. *E. coli* strains which are associated with diarrhea, such as enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC), possess specific adhesion factors called fimbriae or pili that aid in biofilm formation [5].

Additionally, the expression of certain genes, such as those involved in the quorum-sensing system, motility, and stress response, can influence the biofilm-forming capabilities of *E. coli*. Quorum sensing is a mechanism by which bacteria communicate with each other using chemical signaling, coordinating their behavior and promoting biofilm formation [4].

Understanding the mechanisms underlying biofilm formation in diarrheagenic *E. coli* strains is crucial for developing strategies to prevent and control infections caused by these bacteria. Efforts are being made to target biofilms with alternative antimicrobial agents or disrupt their formation to improve treatment outcomes and reduce the spread of infections. Hence, the aim of the current study is to investigate the anti- gene expression of green synthesis CuONPs against biofilm genes (LuxS, MqsR, and FliA).

Materials and methods:

Green synthesis of CuONPs:

The green synthesis of CuONPs was mediated by fig leaves extract according to previous research [6].

Characterization of Green synthesis of CuONPs:

All the characterization methods of green CuONPs by (FE-SEM and TEM) were listed in previous report [6].

Biofilm forming Bacterial isolates:

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Ten strong biofilm *E. coli* isolates were isolated from diarrheal human and animals (calves) (5 from each one) were detected as strong biofilm formation using Congo-red method according to previous research [6]. All these bacterial isolates were treated with green synthesized CuONPs to investigate the anti-genic expression of biofilm genes by real time PCR.

Study's Primers:

Real Time PCR primers were designed by [7]. These primers were provided by Macrogen/ Korea.

Table (1): The qPCR primers used in this study

Primer	Sequence (5'-3')	Product Size
***LuxS* Biofilm gene**	F 5′TGCCACACTGGTAGACGTTC-3′ R 5′-TGATTGGTACGCCAGATGAG-3′	116 bp
***MqsR* Biofilm gene**	F 5′-ACGCACACCACATACACGTT-3′ R 5′-TCCAAACCTAACTCATCTGCAT-3′	105 bp
***FliA* Biofilm gene**	F 5′-GCTGGCTGTTATTGGTGTCG-3′ R 5′-CAACTGGAGCAGGAACTTGG-3′	112 bp
GapA **Housekeeping gene**	*F 5′-GAAATGGGACGAAGTTGGTG-3′* R 5′-AACCACTTTCTTCGCACCAG-3′	104bp

Quantitative Reverse Transcription Real-Time PCR:

In order to quantify the expression, the biofilm in pathogenic ***E. coli*** isolates from humans and animals that form biofilms, the housekeeping (**GapA**) gene was used as a standard. The procedure was followed in accordance with [8] and included the following steps: Total RNA extraction according to (easy-BLUE™ Total RNA Extraction Kit), **Estimation of extracted total RNA** using Nano-drop (Thermo Scientific Nano Drop Lite UV Visible Spectrophotometer. USA**:** checked the RNA purity at absorbance (260 /280 nm), DNase I treatment**.**

The qRT-PCR analysis was performed by OptimaseProtocolWriter™ according to manufactured protocol. The protocol conditions were Initial Denaturation, 95c for 10 min. to one repeat, denaturation 95c for 20 sec. 40 cycle, initial annealing/ extension detection 60c for 30 sec. 40 cycle, melting 65-95c to one cycle.

Statistical analysis of qRT-PCR results:

qRT-PCR results for target and housekeeping gene were analyzed (fold change) using the (Livak method) described by [9] as the follows:

ΔCT (Test) = CT (target gene, test) – CT (HKG gene, test)

ΔCT (Control) = CT (target gene, control) – CT (HKG gene, control)

ΔΔCT= ΔCT (Test)– ΔCT (Control)

Fold change (target / HKG) $=2^{-\Delta\Delta^{CT}}$

Statistical analysis:

Statistical analysis of the results was done by using computer program (SPSS), Version 23 one-way analysis of variance (ANOVA), the difference was considered significant at ($P \leq 0.05$).

Results and discussion:

According to the optimization of green synthesis CuONPs results in previous report [6], the size of CuONPs was 80 nm.

The in vitro study of the effect of green-synthesized copper oxide nanoparticles on gene expression for biofilm-forming E. coli isolated from diarrheal human and calf samples is an investigation into the potential antimicrobial properties of nanoparticles.

All ten strong biofilm forming human and animals *E.coli* (5 to each one) were treated with green CuONPs in different concentrations according to Macrotiter tube method (MIC 62.5 µg/mL and Sub MIC 31.25 µg/mL) with control (biofilm forming *E.coli* without treating with the green CuONPs) mentioned in previous report [6]. Each treated and untreated bacteria with green CuONPs in different concentrations (MIC and Sub MIC), have evaluated their biofilm gene expression depending on three genes (*LuxS, MqsR, and FliA*). The genes were expressed to protein encoded biofilm forming in *E. coli* using real time PCR. The expression of all biofilm genes was estimated according to expression of house-keeping gene (*GapA*). Selection of strong biofilm forming bacteria has been a challenge to determine the powerful antigenic activity of green CuONPs. Little local studies have been conducted about antigenic effect of nanoparticles against biofilm bacteria. A good quality and quantity of mRNAs were obtained by this strategy. Additionally, cDNA was created, and the relative gene expression was assessed using RT-PCR and gene-specific primers. The information shows statistically significant downregulation of expression related to (*LuxS, MqsR, and FliA* genes).

Estimation *LuxS* gene expression:

The expression of *luxS* gene in biofilm forming *E. coli* without treating with green CuONPs (control) was (14.87 and 12.616) in human and animals respectively, while the treating biofilm forming *E. coli* with green CuONPs in MIC concentration, the expression was (1.20 and 0.74 fold change in human and animals *E. coli* respectively) and the Sub MIC (4.92 and 6.01 fold change human and animals respectively) (Table 2) and (Fig. 1 and 2), that was down regulated as a result of anti-genic activity of green CuONPs which most likely resulted in a statistically significant decrease in biomass. These disparate results on the expression of the *luxS* gene may be caused by

variations in the physical characteristics of nanoparticles, such as their high surface area to volume ratios and penetration powers [10]. Bacterial cells create exopolysaccharides during the early stages of adhesion to surfaces in biofilm process [11], then, the expression of genes encoding exopolysaccharides synthesis was monitored. *E. coli activates the luxS gene to synthesize polysaccharide which encourages and promote the adhesion of cells to surfaces to initiate biofilm formation* [12]. *LuxS*-like synthase is involved in the synthesis of AI-2 and is transported outside the cell after completion. Extracellular AI-2 can be taken in by the cell during the stationary phase of cell-cycle development using the *luxS* controlled transporter proteins LsrABCD, an ATP-binding cassette (ABC) transporter [13]. *luxS* gene is AI-2 synthase, via detection and the secret of chemical signaling molecules AI-2 identify in *E coli* and in wide variety of bacteria.

The *luxs* gene has a role in regulating the quorum sensing process in biofilm and considers a part of the QS regulatory mechanism as *LuxS/AI-2* system, this mechanism make bacteria more free to express many genes [14]. As a component of the bacterial metabolism, LuxS not only contributes to the synthesis of AI-2 signaling molecules but also plays a crucial function in activating the methyl cycle [14]. A key mechanism for bacteria to recover methyl groups, LuxS is primarily in charge of hydrolyzing S-adenosyl homo cysteine to S-adenosyl methionine (SAMe) as a methyl donor. LuxS also plays a significant role in bacterial vitamin production and polyamine *creation* [15].

The extracellular concentration of S-ribosylhomocysteine (SAMe with *LuxS* function), which is affected by the *luxS* mutation, can also alter. Numerous experimental findings the biological importance of QS system by demonstrating the role of *LuxS*/AI-2 in different vital process in bacteria such as biofilm formation, and antibiotic resistance [14].

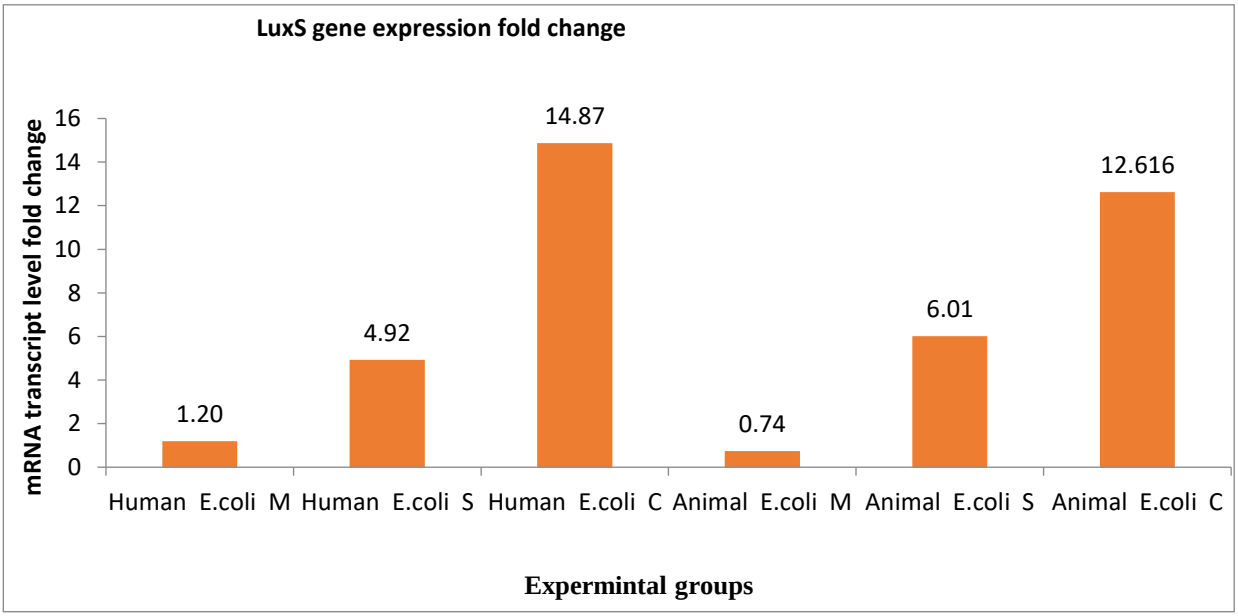

Figure 1. Fold change of expression of *luxS* gene in strong biofilm forming *E. coli* isolates after treatment with M: MIC, S: Sub MIC of green CuONPs in comparative with control.

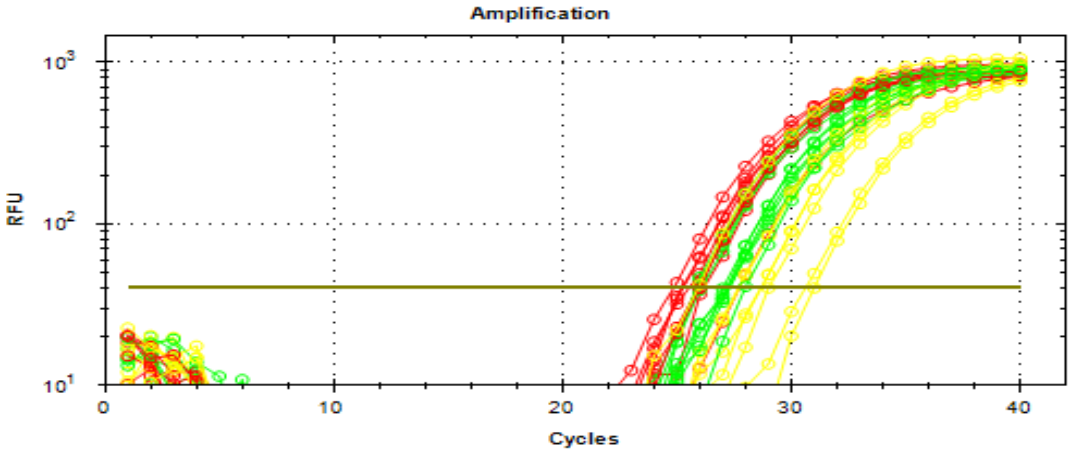

Figure 2. RT- qPCR amplification biofilm formation genes (*Luxs*) in treated and untreated (control) where red blot= control, green plot= sub MIC and yellow plots = MIC

Table 2. The statistical analysis of fold change *Luxs* gene expression to human and animal biofilm forming *E. coli*.

Treatment	Mean ± SE
Human *E.coli* MIC	1.20 ±8.17 A
Human *E.coli* SUBMIC	4.92 ±0.61 B
Human *E.coli* control	14.87 ±2.5 C
Animal *E.coli* MIC	0.74 ±0.42 A
Animal *E.coli* SUB MIC	6.01 ±0.8 B
Animal *E.coli* control	12.61 ±2.08 C
LSD P ≤ 0.05	4,10

Deferent letters denote to the significant difference of $p < 0.05$

The statistical analysis revealed that it was a significant difference among experimental groups in human and animal's isolates while there was no significant difference between human and animal's isolates.

Estimation *MqsR* gene Expression:

The expression of biofilm gene (*MqsR*) in normal state (without treating with green CuONPs) was (13.95 and 18.833) human and animals respectively, while their gene expression effect was treated by green CuONPs. The

result showed a down-regulate to fold change luxs expression based on the activity of green CuONPs MIC concentration (0.95 and 1.08 in human and animal E. coli respectively) and while their gene expression was affected when treated with green CuONPs SUB MIC concentration (9.06 and 6.70 in human and animals E. coli respectively) (Table 3). Thus, copper nanoparticles down-regulated or inhibited the factors that control polysaccharide synthesis of biofilm bacteria on surface coatings with copper would therefore be particularly valuable in medical settings, where controlling microbial contamination and preventing the spread of infections are critical. Copper-containing surface coatings have been explored for various applications, such as on hospital equipment, door handles, and touch surfaces, where they can help reduce the risk of healthcare-associated infections (fig. 3 and 4). Both inter-and intra-species bacterial QS are caused by AI-2. AI-2 has been demonstrated to improve biofilm biomass by activating the motility QS regulator (*MqsR*) and improving flagellar motion and motility by activating *MotA* [16]. *MqsR* controls flageller movement via the QseBC two component system, in which *qseB* encodes the response regulator and *qseC* causes the sensor kinase to be synthesized. *MqsR* activates QseB, which controls *E. coli* motility via the master regulon flhDC, which then stimulates *MotA* and *FliA*, resulting in biofilm formation. *MqsR* also increases motility and induces curli expression via crl and csrA [17].

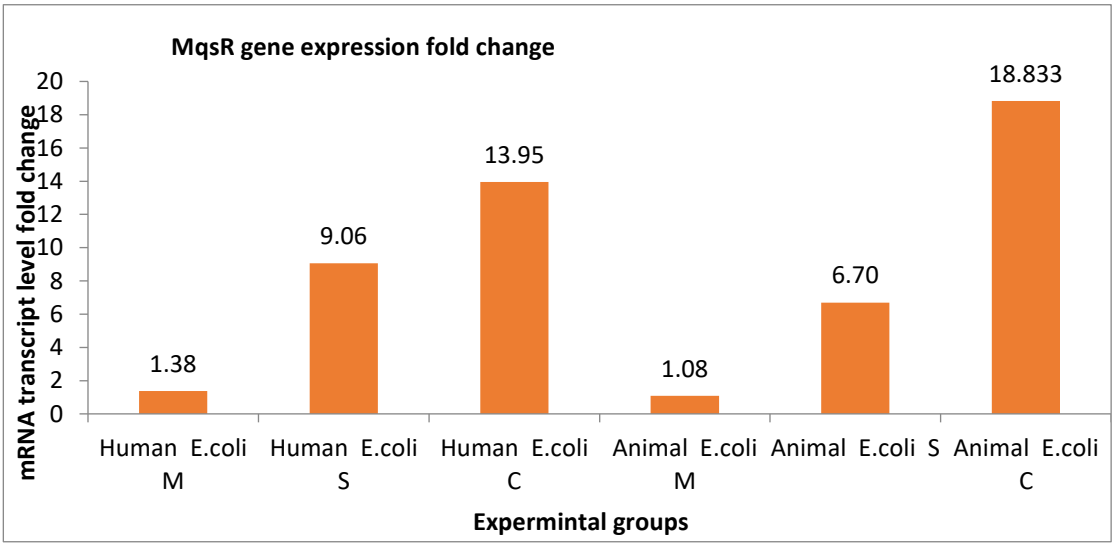

Figure 3. Fold change of expression of *MqsR* gene in strong biofilm forming *E. coli* isolates after treatment with M: MIC, S: Sub MIC of green CuONPs in comparative with control.

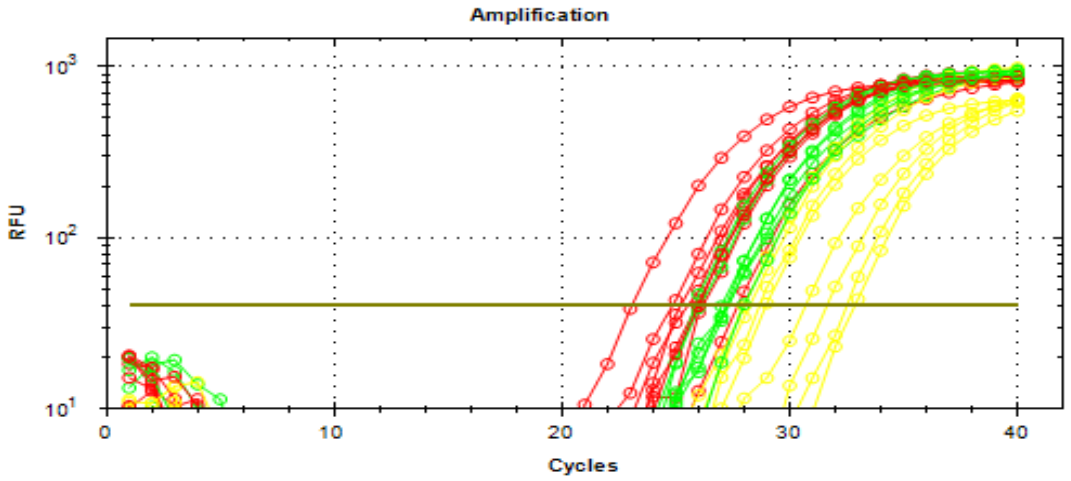

Figure 4. RT- qPCR amplification biofilm formation genes (*mqsr*) in treated and untreated (control) where red blot= control, green plot= sub MIC and yellow plots = MIC

The statistical analysis (Table 3) revealed that expression of *MqsR* gene was different in significant value between MIC value of green CuONPs and sub-MIC and control in human biofilm *E. coli* while in animal biofilm *E. coli* the MIC value significantly different with control**.**

Table 3. The statistical analysis of fold change *MqsR* gene expression to human and animal biofilm forming *E. coli*.

Treatment	Mean ± SE
Human *E.coli* MIC	1.38 ±0.37 A
Human *E.coli* SUBMIC	9.06 ±2.83 BC
Human *E.coli* control	13.95 ±2.2 BD
Animal *E.coli* MIC	1.08 ±0.70 A
Animal *E.coli* SUB MIC	6.7 ±0.95 AC
Animal *E.coli* control	18.83 ±4.15 D
LSD P ≤ 0.05	6.70

Deferent letters denote to the significant difference of $p < 0.05$

Estimation *FliA* gene Expression:

In an earlier study, according to the expression of *FliA* gene, CuONPs interact with bacterial surfaces, copper ions were released then bind and cause DNA dissociation [18]. The result revealed that the gene expression of the biofilm gene *FliA* in normal state (without treating with green CuONPs was (16.32 and 20.353) human and animals respectively while gene-expression effect treating by green CuONPs had a significant effect on the *FliA* (DNA polymerase l) gene activity MIC concentration (1.05 and 0.52 log fold decrease in human and animals respectively) (Fig. 5 and 6), and in sub MIC concentration of green CuONPs, the gene expression log fold decrease (7.06 and 7.43) in human and animals E. coli respectively (Table 4).

The current study proves that CuONPs have an impact on various cellular targets. The current study proves that CuONPs have an impact on various cellular targets. The impacts of CuONPs and ionic Cu at the level of the transcriptome of biofilm genes in biofilm-forming E. coli isolated from humans and animals diarrhea. *FliA* (also known as σ28) is a sigma factor protein involved in regulating the expression of other genes related to bacterial motility like flagellar biosynthesis, which considers the transcription factor dual regulator FlhDC and the master regulator of the transcriptional network that drives expression to functioning flagella and movement. In addition to triggering the transcription of the minor sigma factor *FliA* (RpoF, sigma28), the proteins FlhD and FlhC form a heterohexameric complex that activates the transcription of a number of genes crucial for early flagellar assembly [19]. *FliA* is a transcription factor that influences the genes involved in chemotaxis and late stage flagellar assembly in its own downstream regulon [20]. Inhibition or modulation of *FliA* gene expression could potentially affect the formation or maintenance of biofilms in certain bacteria [16].

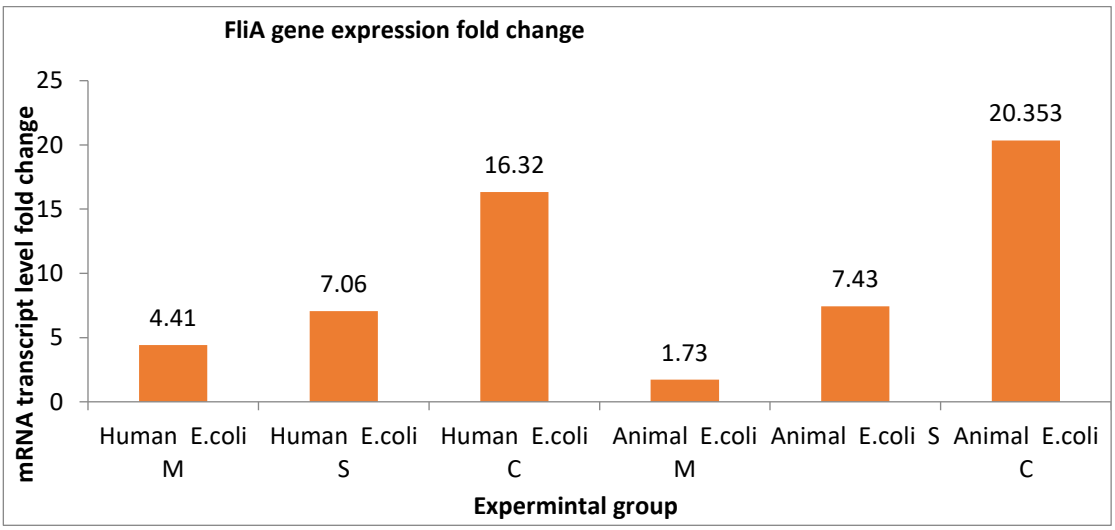

Fig 5. Fold change of expression of *FliA* gene in strong biofilm forming *E. coli* isolates after treatment with M: MIC, S: Sub MIC of green CuONPs in comparative with control.

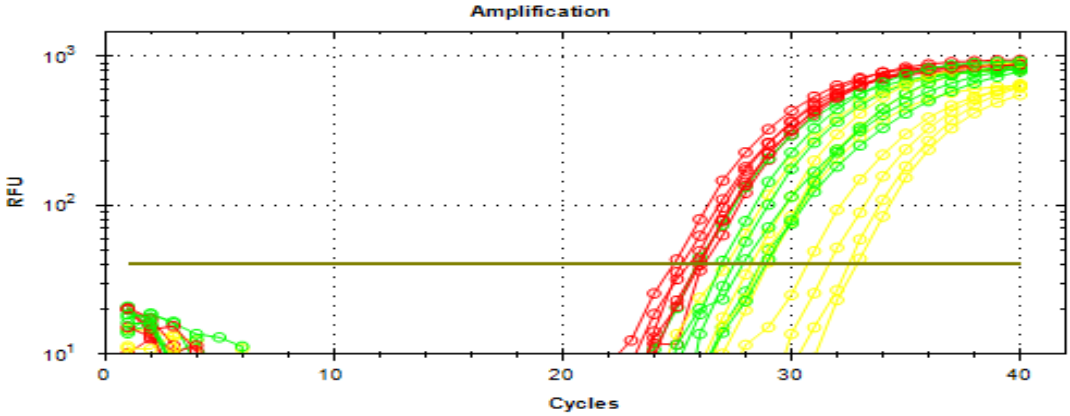

Figure 6. RT- qPCR amplification biofilm formation genes (*FliA*) in treated and untreated (control) where red blot= control, green plot= sub MIC and yellow plots = MIC.

In the table below, the expression of the FliA gene is significantly different in expression according to the MIC and Sub MIC concentrations of green CuONPs with control in human and animal biofilm E. coli, instead, there is no significant difference between the two groups (human E. coli and animal E. coli).

Table 4. The statistical analysis of fold change *FliA* gene expression to human and animal biofilm forming *E. coli*.

Treatment	Mean ± SE
Human *E.coli* MIC	4.41 ±1.53 A
Human *E.coli* SUBMIC	7.06 ±2.46 A
Human *E.coli* control	16.32 ±3.8 B
Animal *E.coli* MIC	1.73 ±1.13 A
Animal *E.coli* SUB MIC	7.43 ±2.14 A
Animal *E.coli* control	20.35 ±4.63 B
LSD $P \leq 0.05$	8,45

Deferent letters denote to the significant difference of $p < 0.05$

Green-synthesized copper oxide nanoparticles were produced using environmentally friendly methods, typically involving plant extracts. These nanoparticles have been shown to possess antibacterial properties, making them potential candidates for use as a disinfectant or antibacterial agent. Although there is limited research specifically on the effects of CuONPs on biofilm gene expression, the current study has shown that these nanoparticles can

interfere with microbial gene expression in special manner (biofilm formation). Copper ions released from CuONPs can interact with cellular components and affect gene regulation. They can potentially modulate the expression of genes involved in biofilm formation, virulence factors, and stress responses.

In this study, biofilm-forming *E. coli* isolated from diarrheal human and calf were exposed to varying concentrations of green-synthesized copper oxide nanoparticles. The expression of biofilm formation genes significantly affected with green synthesis CuONPs.

The findings of the study could believe insight into the impact use of green-synthesized copper oxide nanoparticles as an alternative to traditional antibacterial agents. However, it is important to note that in vitro studies may not necessarily reflect the true effects of nanoparticles in vivo, and further research is needed to fully understand their potential applications and risks.

Conclusion:

The study's results could provide valuable insights into the potential use of CuONPs as an antimicrobial agent against biofilm-forming E. coli strains. Understanding the effect of CuONPs on the gene expression of these strains could also shed light on their mode of action and help researchers develop more effective strategies to combat them in addition to using the CuONPs as alternative antibiotics to overcome the world's problem of antibiotic resistance.

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