



## Down-regulation of *abaI*, *abaR*, *Bap* and *OmpA* genes in *Acinetobacter baumannii* by ethanol extract of *Glycyrrhiza glabra* after toxicity assessment

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### ABSTRACT

*Acinetobacter baumannii* is a pathogen that has caused rising concerns within healthcare facilities in recent years. As antibiotic overuse and resistance rise, natural remedies with the potential have received attention as antibiotics that might have fewer side effects and lower resistance. *Glycyrrhiza glabra* was used to investigate its effects on *A. baumannii*'s quorum sensing and biofilm production abilities. In this study, the toxicity assessment of *Glycyrrhiza glabra* L. extract on rats, the phytochemical analysis and the quantitative measurement for the association of the biofilm reduction with the active components in the plant was determined. The results indicated ciprofloxacin and gentamicin were the most effective antibiotics and that various capabilities of biofilm-productions were demonstrated, only four percent of the samples established robust biofilm, while 40% to 56% demonstrated weak to moderate biofilm production, respectively. Phytochemical qualitative testing of ethanol leaf extracts from *Glycyrrhiza glabra* showed the existence of flavonoids, alkaloids, phenolic, tannic acid, and terpenoids, but no saponins. Assessment of toxicity revealed a low hazard, with an LD<sub>50</sub> of 4.95 g/Kg. Our results showed that the extract's SICs elucidated a substantial quantitative decrease in biofilm production by the bacterial isolates, including the reference ATCC strain, which is known to be a potent biofilm producer. As a conclusion, biofilm creation in *Acinetobacter baumannii* has been shown to be greatly reduced by *G. glabra* extract.

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### Introduction

Medicinal plants found in the wild have been used by many different cultures, dating all the way back to ancient times. A growing percentage of the world's population, especially in underdeveloped regions, relies on plants to meet their most basic medical needs (1). Natural and traditional remedies are essential. Some forms of medicine have been practiced for centuries and refined into standardized, reliable systems of health care in specific regions of the world (2). *Glycyrrhiza glabra* (*G. glabra*), a member of the Leguminosae family, is a common plant found worldwide. The properties of *G. glabra* Linn include those of an antibacterial, antioxidant, anti-inflammatory, and anti-hyperglycemic agent, antiulcer, antiviral, and antifungal effects have also been investigated (3). Resistance among pathogenic microbial species is rising and spreading in modern times due to the widespread use and misuse of antibiotics (4, 5). The emanation of bacteria that are resistant to antibiotics has complicated efforts to treat infectious diseases. In light of this, efforts to find novel sources of antibiotic compounds have increased researchers are therefore scouring the natural world for compounds with potential as anti-infective therapy (6). Since quite some time ago, infectious disease treatment has taken into account and made use of medicinal plants considering their

antimicrobial features (7).

An elevation of antibiotic-resistant Gram-negative bacteria prevalence has been detected along with biofilm-forming activity, especially among healthcare-associated pathogens (8). Possessing various virulence factors, like the ability to create a slimy layer and survive in an exsiccated ambience situation, has caused difficulties in the health care setting, particularly for patients in intensive care units of hospitals (9). *AbaI* and *AbaR*, a two-component system, have recently been reported as making up *A. baumannii*'s quorum sensing (QS) system. Acyl homoserine lactone (AHL) signal synthesis is catalyzed by autoinducer synthases, which are encoded by the *abaI* gene. The receptor protein encodes by *abaR* gene that links to AHLs and functions as a transcriptional controller factor; 3-hydroxy-C12-homoserine lactones are the highest common AHLs made by *A. baumannii* (10). The biofilm production capabilities of *A. baumannii*-associated infections are counted among the many important causes of drug resistance, and this biofilm formation is linked to quorum sensing (QS) (11). Biofilm-associated protein gene, *Bap*, is known to be translated into a broad extend variability protein. Most *A. baumannii* strains have been sequenced they possess the *Bap* gene. However, many of these strains appear to possess disordered or shortened *Bap* sequences, which may be due to chimeric events typical of the highly tandem elements

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of *Bap* coding sequences (12). Outer membrane protein A (*OmpA*) is a highly abundant and exceedingly described virulence factor possessed by *A. baumannii*, and it is also the most abundant OMP produced by *A. baumannii* (13). The aim of this research was to examine *G. glabra* ethanol extract efficacy against *A. baumannii* strains that had been isolated from patients.

## Materials and Methods

This work was implemented in agreement to Helsinki Declaration rules and was accepted by the Ethics Committee of Science College at Salahaddin University Erbil (No:4S/458; date, September 9, 2020; Erbil, Iraq).

### Plant gathering

*Glycyrrhiza glabra L.* leaf parts were taken in the Spring of 2021 from the Gomaspan area in the Northern part of Iraq. Mr. Abdullah Shukur, Ph.D. in the plant taxonomy at the Biology Department of Education College/ Salahaddin University, assisted in identifying plants under investigation using information about Iraqi flora (14).

### Sample collections and sources

Fifty *A. baumannii* isolates were gathered from a variety of patient specimens at various hospitals in Erbil, Northern part of Iraq, including cerebrospinal fluid (CSF), blood, pus, sputum, and wound swabs.

The samples were cultivated on acumedia MacConkey agar medium (Neogen, USA) and kept overnight at 37°C. Next, by using several conventional biochemical and diagnostic procedures as explained previously by Tille (15), singular colonies were determined as *A. baumannii*. VITEK 2 automatic technique (Biomérieux, France) was applied to identify bacterial isolates. Various antimicrobials were used in order to test the diagnosed isolates for their susceptibility, the antimicrobial agents included; Amikacin, Amoxicillin/Clavulanic Acid, Ampicillin, Ampicillin/Sulbactam, Azithromycin, Imipenem, Levofloxacin, Meropenem, Cefepime, Cefoxitin, Ceftazidime, Ceftriaxone, Cefuroxime, Ciprofloxacin, Colistin, gentamicin, Piperacillin, Piperacillin/Tazobactam, Tazobactam, Tetracycline, Aztreonam, Cefazolin, Trimethoprim/Sulfamethoxazole). For more study, 1000 µl of a sterilized Tryptic Soy Broth (TSB) (Oxoid) having 30% glycerol were inoculated with identified colonies and stored at -70°C. To serve as the study's control, an ATCC strain of *A. baumannii* (19606) was bought from Medya Diagnostic Center.

### Plant extraction

The *G. glabra L.* leaves were harvested, cleaned, and then meticulously dried until they gained constant weight at forty to fifty °C. After being finely powdered over time by grinders, they were then placed in a specific bottle for storage. Leaf powder of *G. glabra* (30g) was used for preparing the extract, the powder was stirred at constant intervals consuming 300ml of 99.9% ethanol for 72 hours at room temperature. The extract was then purified by two layers of muslin fabric and Whatman no. 1 filter paper. Next, a rotovaps at 45 °C and lower pressure condensed the filtrated extract (16).

### Assessment of the toxicity of *G. glabra L.* extract in rats

The present investigation was conducted at Salahaddin University, Education College, Biology Department. Female rats (*Rattus norvegicus*), with 180–200 g weight were used. All of them were kept in a typical setting that included 27±2°C temperature, the cycles of light and dark periods were set as light for 12 hours and 12 hours dark, regular feeds, and unrestricted feasible tap water access. The animals were housed in standard plastic crates and were divided into several clusters at random (n=5). The untreated (control) group was set as the first cluster and given only DMSO, whereas other specified clusters received only one dosage of the ethanol extract of *G. glabra* at doses of 0.5, 1, 2, 4, 4.5, and 5 g/kg based on previous studies(17). Depending on the animals size, the maximum quantity of extract solution which could be administered orally was 1000 µl/100 gram body weight. Animals were administered the extract (16–18) hours behind being only food underprivileged and allowed to drink. The LD50 value was calculated from the observed registered mortality after seven days (17).

### Phytochemical qualitative screening for *G. glabra* extract

A Stock solution was created by dissolving the undiluted crude extract of plant leaf parts in basic solvent (mg/ml), then was employed for phytochemical screening.

### Detection of alkaloids

After adding HCl (1%) and a small amount of the Dragendroff reagent to the plant extract solution, alkaloid existence was detected by the precipitate (18).

### Detection of flavonoid

The extract was treated by gradually adding sodium hydroxide, a few drops at a time. When diluted acid was introduced in small amounts, flavonoids produced a bright yellow color that vanished almost instantly (19).

### Detection of phenolic compounds

A small amount of lead acetate solution was combined with a plant extract solution. The white precipitate indicated phenolic compounds existence (19).

### Tannin detection

To establish this detection, 500mg of extract was dissolved in distilled water (10 mL), and stirred. Adding of FeCl<sub>3</sub> to the filtrate caused the color to change to blue-black or blue-green, which was employed as a confirmatory test for tannin (18).

### Detection of terpenoids

With the addition of chloroform and 2 ml H<sub>2</sub>SO<sub>4</sub>, 500 mg of extract was extracted by 2000 µl of acetic acid. Terpenoids are indicated by the presence of a reddish-brown color (20).

### Detection of saponins

To create a stable froth, 5000 µl of distilled water and 500mg extract were combined, and then vigorously shaken. The frothing was combined then three drops of extra olive oil were added and vigorously mixed before being checked for foam colloid development (20).

**Table 1.** Primer sequences of quorum sensing and biofilm genetic targets.

Genetic Target	Primer Sequence (5'–3')		Product size	Ref.
	Forward	Reverse		
<i>abaI</i>	AAAGTTACCGCTACAGGG	CACGATGGGCACGAAA	435	(25)
<i>abaR</i>	TCCTCGGGT CCAATA	AAATCTACCGCATCAA	310	(25)
<i>Bap</i>	AATGCACCGGTACTTGATCC	TATTGC CTGCAGGGTCA GTT	205	(26)
<i>OmpA</i>	ATGAAAAGACAGCTATCGCGATTGCA	CACCAAAAGCACAGCGCCCAGTTG	136	(26)

### Quantitative biofilm formation assay

With minor changes, the microtiter plate technique, which was established by Limban et al.(21), was utilized to assess the isolates of *A. baumannii*'s capacity in producing the slime layer, biofilm. Overnight cultures (15µl) of desirable bacteria were cultured into the microtiter plate wells (Citotest Labware, China) that contain 0.2mL of sterilized Nutrient broth (NB) (Neogen, USA) augmented with 2% glucose. Wells with NB alone were considered as control. Next, MTP was kept in a stationary state at 37°C for 24 hours. This step was followed by washing the wells three times with sterile PBS, planktonic broth culture was removed, and all plates were desiccated at 50 °C for twenty minutes. After adding crystal violet staining solution (200 µl of 1% concentration) to each well, the plate was left for ten minutes at room temperature. Then, wells were rinsed with PBS, dehydrated and eluted with ethanol 95% and the produced biofilm was quantified by ELISA (Epson, Biotek, UK) at 490 nm. The analysis included three biological samples. For further experiments throughout the study, two isolates which were the highest biofilm builder and weakest antibiotic susceptibility were chosen.

### Minimum inhibitory and sub-inhibitory concentrations

The minimum inhibitory concentration (MIC) for of *G. glabra* extract in counter to MDR *A. baumannii* isolates was calculated by a broth microdilution way (22). From a stationary phase 10 µL of *A. baumannii* cells were equilibrated to OD550 0.5 and added to 0.1 mL NB with different extract concentrations (1–30 mg/ml) in a 96-well polystyrene MTP. Cultures were incubated in the presence of oxygen at 37 °C for 24h. The last concentration, where visible growth was not detected, was defined as MIC. For sub-inhibitory concentration (SIC), concentrations lower than MICs value were recorded and furthermore used for evaluation of anti-virulence and anti-biofilm functions amongst the isolated *A. baumannii* strains. Three biological samples were analyzed on separate occasions.

### Sub-MIC influence of *G. glabra* on biofilm formation by the isolates

The plant extract effects on biofilm production were performed through applying a polyvinyl chloride biofilm builder assay. Briefly, 24-hour cultures of *A. baumannii* were activated in fresh NB medium in the - and + of SICs of plant extract and incubated overnight in a stationary status at 37 °C. The extra fluid of the media in the wells was discarded, and the wells were cleaned thrice with PBS. Then 1% crystal violet reagent was used for staining, rinsed with distilled water and quantified by solubilizing the dye in ethanol. The capacity of adhesion to a non-viable exterior was identified by colored suspension density measures to an Elisa reader (Epson, Biotek, UK) at 490 nm

(23). Triplicate biological trials were investigated on separate occasions, and the calculation of standard error was done.

### RNA extraction and quantification of QS and biofilm genetic targets

Real-time PCR (RT-qPCR) was applied so that the effects of *G. glabra* ethanol extract at SIC values could be determined on the quantitative scales of cell-to-cell communication genetic targets (*abaI*, *abaR*), biofilm attachment protein (*Bap*), and outer membrane protein A (*OmpA*). Whole RNA was isolated as stated by the leaflet supplied by the total RNA kit (Favorgen Biotech, Taiwan) producer. Isolated RNA was used to synthesize cDNA by means of AddScript cDNA synthesis kit based on the company procedure (addbio, Korea). Finally, RT-qPCR reactions using the RealQ Plus 2x Master Mix Green (Ampliqon, Denmark) in PCRmax Eco 48 RT-qPCR.  $\Delta\Delta C_t$  method was used to analyze the results (24). Primer sequences are described in Table 1.

### Statistical analysis

GraphPad Prism 8.0 software was used. The two-way analysis of variance (ANOVA) method was applied for multiple comparisons. Data revealed as mean  $\pm$  SE.

### Results

#### Estimation of the toxicity of *G. glabra* L. extract in rats and calculation of the lethal dose.

The toxicity of the *G. glabra* ethanol leaf extract on rats was assessed, and the lethal dose was calculated, 4.95 g/kg was recorded as the LD<sub>50</sub> value.

#### Phytochemical qualitative test for *G. glabra* L. extract

According to the results of qualitative phytochemical screening for *G. glabra*, ethanol leaf extract showed the availability of alkaloids, flavonoids, phenolic, tannic acids and terpenoids while saponins were not found.

In this present study, we screened the antimicrobial resistance patterns. the isolates exhibited different resistant patterns, 48(96%) of the isolates were resistant to CIP, 45(90%) were resistant to CN, and 44(88%) were resistant to SXT. Whereas, 1(2%) were sensitive to each of AMC, AMP, AMC, and CAZ. A full description of antimicrobial resistance patterns is presented in Figure 1. The ability of biofilm formation was assessed and this pathogen fortunately depicted that only 4% of the total isolates possessed a potent biofilm production ability, then 56% and 40% of the isolates displayed moderate and weak biofilm development competencies respectively, all results were compared to control (Table 2).

In order to evaluate the effects of *G. glabra* ethanol leaf

**Table 2.** Biofilms formation abilities of *A. baumannii*.

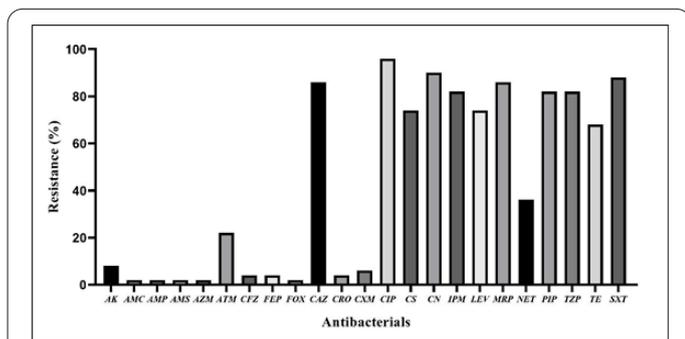
Degree of biofilm	No. of isolates	Percentage (%)
Weak	20	40%
Moderate	28	56%
Strong	2	4%
<b>Total</b>	<b>50</b>	<b>100%</b>

**Table 3.** The Minimum Inhibitory Concentrations and Sub-MICs of *G. glabra* extract against MDR *A. baumannii*.

Bacterial isolates	MIC (mg.ml <sup>-1</sup> )	SUB-MIC (mg.ml <sup>-1</sup> )
<i>ATCC</i>	10	5
<i>AB-1</i>	10	5
<i>AB-2</i>	8	5

**Table 4.** Transcription profile via RT-qPCR of *A. baumannii* QS and biofilm genes expression treated with SIC of *G. glabra* extract. Data expressed as  $\Delta\Delta Ct$ .

Bacterial isolates	<i>abaI</i>	<i>abaR</i>	<i>Bap</i>	<i>OmpA</i>
<i>ATCC</i>	0.000081	0.00002969	0.006302	0.001514
<i>AB-1</i>	0.000966254	0.002191631	0.004787	0.003545
<i>AB-2</i>	0.026303395	0.015017466	0.251879	0.015727

**Figure 1.** Antibacterial resistance patterns of pathogenic *A. baumannii* isolates. AK, Amikacin; AMC, Amoxicillin/ Clavolanic acid; AMP, Ampicillin; AMS, Ampicillin/Sulbactam; AZM, Azithromycin; ATM, Aztreonam; CFZ, Cefazolin; FEP, cefepime; FOX, Cefoxitin; CAZ, Ceftazidime; CRO, Ceftriaxone; CXM, Cefuroxime; CIP, Ciprofloxacin; CS, Colisitn; CN, Gentamicin; IPM, Imipenem; LEV, Levofloxacin; MRP, Meropenem; NET, Netlmicin; PIP, Pipracillin; TZP, Pipracillin/Tazobactam; TE, Tetracycline; SXT, Trimethoprim/Sulfamethoxazole.

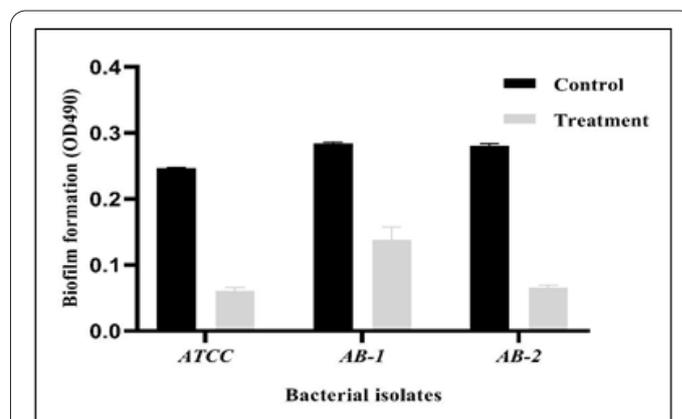
extracts on QS and biofilm formation, the MICs and SICs of *G. glabra* were determined and the results are shown in Table 3. Then the SIC values were used to treat two chosen isolates that exhibited the highest resistance towards antibiotics and were most potent to build the slime layers. Intriguingly, we found that the plant under investigation has the ability to significantly reduce the quantitative capabilities of these three isolates to form biofilm. The strong effect was pronounced by the ATCC strain, results are shown in Figure 2. Table 4 shows the significant effects of *G. glabra* ethanol leave extract on quantitatively down-regulation of bacterial cellular communication genes, *abaI*, and *abaR*, which consequently influenced biofilm builder genes, *Bap* and *OmpA*.

## Discussion

Hospital infections with the *A. baumannii* pathogen

have grown common, which could modulate the virulence components they have through QS, such as slime layer formation, motility, catalase, antibiotic resistance, and superoxide dismutase (SOD) (27).

The development and high prevalence of multi-drug resistant pathogenic bacteria are becoming a huge threat to community health. In recent years, various medicinal plants have taken great attention from researchers to uncover novel drug candidates against different diseases owing to the existence of significant bio-active components within plants that possess antimicrobial and antioxidant characteristics, phenolic and flavonoid bioactive components have always been examined for human health improvement of disease prevention (28). Successful attenuations of bacterial virulence genes were achieved with the consumption of phytochemicals. In this study, *G. glabra* was selected for detecting their impacts on the *A. baumannii* abilities for QS and biofilm production. Prior to starting the experiments, *G. glabra* toxicity was assessed, we found that the LD<sub>50</sub> of the ethanolic leaves extract is considered to be low toxic and according to Schorderet (1992), a substance is

**Figure 2.** Quantitative reduction of *A. baumannii* biofilm capability by SIC of *G. glabra* extract. Results are described as mean  $\pm$  SE. Significance is considered at  $P < 0.0001$ .

categorized as with low toxicity when the value of LD<sub>50</sub> is greater than 5000 mg/kg (29).

Phytochemical screening revealed that the ethanolic leaves extract of the plant under investigation is with valuable biomolecules such as flavonoid, in addition to phenolic, tannins and terpenoids that could be used as anti-oxidant, antibacterial, antifungal, and therapeutic activities (30, 31). Here we found that this bacterium exhibited different and high resistance to most of the antimicrobials used in this investigation (Figure 1). This robust resistance is attributed to biofilm formation, which is facilitated by QS, and could alter the bacterial tolerance to antibiotics (32), thus quenching QS would target the ability of this pathogen to resist antibacterial agents. One of three possible mechanisms could be responsible for the resistance to multi-drugs; slower antimicrobial penetration through the biofilm matrix, physiological growth changes due to biofilm, and alteration of growth rate of biofilm producer organisms (33). This work looked at 50 bacterial isolates' capacity to build biofilm on abiotic surfaces. The results (Table 2) showed that these isolates had different capabilities in regards to biofilm formation, where most had moderate potency to grow and colonize abiotic surface at 37°C. It has been reported that this bacterium may form stronger biofilm when kept for a longer time and when the temperature is unfavorable for them at 25° C than that at 35-37°C(34), although more biofilm could be formed at 37°C which is the physiological temperature and condition of the human body, but still the capability of biofilm production on abiotic surface at 25°C is more potent and could be related to severe infections happening in clinical settings and facilitate the pathogen spread (35).

The two chosen isolates plus the control strain of *A. baumannii* from ATCC were used for pursuing additional tests. *G. glabra* ethanol leaves extract MICs and SICs anti-microbial activities were recorded against MDR *A. baumannii* isolates. *G. glabra* extract fraction rich with flavonoid, an active bio-compound that exist in plant cells, was reported to significantly decrease biofilm building and motility in *A. baumannii* and this reduction was not due to inhibition of bacterial cell growth as the fraction showed no toxicity to the cells (27).

The *G. glabra* ethanol leaves extract was used to investigate the bioactive components on the building of the slime layers. Our results revealed that SICs of the extract elucidated a significant quantitative reduction in biofilm production by the bacterial isolates including the standard ATCC strain which is known for being a potent biofilm producer, similar effects were demonstrated against *P. aeruginosa* biofilm production where an inhibition in biofilm production was reported with increasing in the concentration of *G. glabra* extract (36).

It has been clarified, that medicinal plant extracts produce a diversity of bioactive secondary compounds such as flavonoids, phenolic acid, saponins, phenols, tannic acid, quinones, terpenoids, alkaloids, and polyacetylenes (37), and that these compounds have been reported in inhibiting the formation of harmfulness factors like making pyocyanin, protease, swarming and twitching motility in pathogenic bacteria could result in a decrease in biofilm (38).

*Acinetobacter baumannii* cellular communication system predominantly included *abaI* and *abaR* genes (39) where recent research has linked the development of bio-

film to QS (32). In this study, our studied plant fortunately revealed a significant ability to downregulate QS genes expression, *abaI* and *abaR*, and QS-mediated biofilm production genes, *Bap* and *OmpA*, when compared to control, interestingly the downregulation was most pronounced in the ATCC strain when compared to the other two isolates. Comparable results were achieved by (27). where they proposed that quorum quenching by *G. glabra* crude extracts and flavonoid fraction in *A. baumannii* could result from repressing the expression of autoinducer synthase, that's the *AbaR*-N-acyl homoserine lactone complex formation would be affected with the inhibition of AHL production, it has been demonstrated that eliminating this complex might have a significant role in the reduction of surface motility, twitching, and biofilm building. Another study showed that curcumin and other flavonoids have a prospected role in adjusting biofilm building up and other harmful factors by *A. baumannii* (40). It has been clarified that different biological extracts or natural plant yields can diminish communication between *A. baumannii* cells and affect biofilm and antibiotic resistance (41).

Discovering new biomolecules from plants targeting QS network and biofilm formation is urgent in order to control multi-drug resistant bacterial infections such as *A. baumannii*.

## Conclusion

We demonstrated that the ability to form biofilm varied among these isolates most having a moderate ability, and it has been found that an extract fraction from *G. glabra* that is high in flavonoids significantly reduces biofilm formation in *A. baumannii*. Fortunately, our investigated plant demonstrated a significant ability to downregulate the expression of cell-to-cell connecting genetic targets, *abaI* and *abaR*, and QS-mediated biofilm production genetic targets, *Bap* and *OmpA*, compared to control. Interestingly, the downregulation was most pronounced in the ATCC strain compared to the other isolates. Finally, *G. glabra* has a promising activity counter to *A. baumannii* QS and biofilm formation, indicating its possible application as an antibacterial agents.

## Conflicts of Interest

The authors declare no conflict of interest.

## Author's contribution

Akhter Ahmed & Jwan Rahman designed research; Akhter Ahmed & Jwan Rahman conducted research, Akhter Ahmed analysed data; Trefa Salih, Akhter Ahmed, Aryan Ganjo and Jwan Rahman wrote and edited the paper; Akhter Ahmed, Jwan Rahman, Aryan Ganjo, Trefa Salih had primary responsibility for final content. All authors read and approved the final manuscript.

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