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ANTI-BIOFILM ACTIVITY OF CYNANCHUM ACUTUM LEAVES EXTRACT AND ASSESSING THEIR EFFECT AGAINST THE VIRULENCE GENE SEG OF STAPHYLOCOCCUS AUREUS BACTERIA

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Abstract:
Due to the ability of bacteria to develop many mechanisms of resistance to multiple drugs and antibiotics, and the emergence of strains with acquired resistance through horizontal transfer, and due to the excessive or underuse of antibiotics, and their spread in all countries of the world. This study aimed to evaluate the effect of the ethanolic extract of Cynanchum acutum leaves as an anti-biofilm formation agent and to study the gene expression of the virulence gene SEG that contributes to the production of enterotoxins in resistant Staphylococcus aureus bacteria after treatment. Methods: The disk diffusion method was used to determine the extent of bacteria's resistance to 12 antibiotics. A 96-well microtiter plate and ELISA technology were also used to verify the ability of bacteria to form biofilms, as well as the ability of the ethanolic extract to inhibit their formation. RNA treated with ethanolic extract was extracted using the triazole method to study its effect on gene expression of the virulence gene SEG. Results: The results showed that all isolates produced biofilms that ranged from very to moderately strong with high to moderate adhesion. The concentrations of the extract in inhibiting biofilms ranged between 62,500 - 31,200 µg/ml. Gene expression results after treatment showed a significant increase in the virulence gene, with the exception of one isolate with down regulation. Conclusions: The results revealed that there is an inverse relationship between inhibitory activity and gene expression after treatment with the extract, as it has inhibitory activity on biofilms and is considered a stimulator of a gene that contributes to the production of intestinal toxins.

Keywords: Cynanchum acutum, Staphylococcus aureus, Antibiotic, anti-biofilm activity,and Gene expression

Introduction

Multidrug-resistant pathogens can develop resistance to various antimicrobials by horizontal gene transfer and by genetic mutations as a result of drug exposure. It is exacerbated by the misuse of antibiotics, which has led to the emergence of these resistant bacteria and their spread everywhere. Therefore, there is a strong demand for developing new strategies that can deal with these problems 1.

Staphylococcus aureus produces an astounding array of virulence factors that allow to survive extreme conditions in human and promote tissue colonization, tissue damage, and ensues life-threatening systemic infections. These include a plethora of toxins and immune evasion factors, and a vast array of protein and non-protein factors that enable host colonization during infection

2.
Bioactive compounds isolated from medicinal plants are considered safe new drugs, so their study has been focused on as they are a key to finding low-cost treatments with fewer complications to treat various diseases. Traditional ethnomedicinal applications of a wide range of plants are used as a powerful tool to this end 3. Cynanchum acutum is a climbing vine that belongs to the family Asclepiadaceae (milkweed family) which includes about 2900 species in 315 genera 4 . It is native to Asia, Africa and southern Europe. The ethanolic extract of C. acutum leaves could be used as hypotensive, in treatment of cardiac arrhythmia, against skin infections, for improving respiration in asthma and anti-spasmodic 5; 6; 7 . Latex-producing plants have been reported as a valuable medicinal resource in many countries due to their representative latex components. Latex has a defensive purpose in plants, it may have strong antimicrobial activity and thus plants can provide a good source of antimicrobial compounds 8 . This study aims to inhibit the production of biofilms and study the gene expression of the virulence gene SEG after treatment with the ethanolic extract of C. acutum leaves against resistant S. aureus bacteria.

Material and method

Preparation of ethanolic leaves extract

The leaves were soaked in ethanol: water at a ratio of 3:1, dry material was 100 g and placed in 1000 ml of the solvent at 40°C for 24 hours with stirring. The filtration process was carried out using filter paper, and the chlorophyll pigment was removed by adding activated charcoal and filtered using a Buchner funnel. This step repeated two times, then the extract was evaporated in a rotary evaporator, then poured into glass Petri dishes and dried in a convection oven at 40 °C and stored at 4 °C until usage 9; 10 .

Ethical issues

The approval of the Ministry of Science and Technology / Department of Environment and Water for Scientific Research Ethics was obtained by Administrative Order No. 3035 T.H. on 11/28/2022.

Collection of bacterial isolates

The isolates were obtained from the Ministry of Science and Technology and local laboratories. The isolates were transferred to the biological laboratory under sterile conditions for cultivation on manitol salt agar, nutrient agar and Blood agar and incubated at 37°C for 24 hours for further confirmatory testing.

Detection of bacteria

Selective media such as manitol salt agar and blood agar were prepared to check for the presence of the isolates targeted in this study, and then incubated for 24 h at 37°C. The following tests were used: 1- Morphological characteristics 2- PCR technology by detecting the SEG virulence gene of S. aureus

Disc Diffusion Method (DDM)

This was done based on the Kirby-Bauer test described by (CLSI, 2022) as follows:

Colonies were transferred to 5 ml of normal saline from nutrient agar overnight in Petri dish

culture, and the turbidity was adjusted to 0.5 McFarland equivalent to 1.5 × 108 CFU/ml. The bacterial suspension was inoculated into Muller Hinton agar Petri dishes and left for 10 minutes before use antibiotic tablets, with a maximum of 4 to 6 tablets per Petri dish placed on the agar using sterile forceps, and then incubated at 37°C for 18-24 hours 11; 12 . The resulting zones of inhibition were then measured in millimeters (mm) and the isolate was interpreted as either susceptible, intermediate, or resistant to a particular antibiotic in comparison to the CLSI 2022 breakpoints 13 .

Primer

The primer applied in this research was newly designed in cooperation with Macrogen Korea, as shown in table (1).

Table (1): The newly designed Staphylococcus aureus virulence gene SEG primer that was applied in this study.

Primer for *S. aureus*		Primer sequence F (5'→3') R (5'→3')	Product size	Reference
SEG	F	5'-ACCCGATCCTAAATTAGACGAACT-3'	107 bp	Newly designed in current study
	R	5'- TTCCTTCAACAGGTGGAGACG- 3'		

R: Reverse primer, F: Forward primer, bp: base pair.

Molecular Detection of the SEG virulence gene of Staphylococcus aureus

This step was carried out by adding 12.5 µl from OneTaq (NEB®) mastermix, 3 µl of DNA sample, 1 µl 10 pmol/µl from each primer and 7.5 µl of free-nuclease water. The reaction done under the optimal PCR conditions for gene 14; 15, as shown in table (2).

Table (2): PCR conditions for SEG gene virulence gene of Staphylococcus aureus

ID	Virulence gane	Cycle No.	Stage	Temp.	Time
S. aureus	*SEG*	1	Initial Denaturation	94 ºC	5 mins
		38x	Denaturation Annealing Extension	94 ºC 54 ºC 72 ºC	30 sec. 45 sec. 45 sec.
		1	Final Extension	72 ºC	7 mins.

Biofilm Formation Assays

A quantitative screening method was used to evaluate the biofilm formation of each of the S. aureus isolates described by 16 using MTP, where all isolates were cultured in brain heart infusion

broth at 37°C for 18-24 h. Next, adjusted to a 0.5 McFarland standard (1.5×108 CFU/ml), 20 µl of the overnight bacterial suspension was used to inoculate the wells of a sterile flat-bottomed 96-well microplate containing 180 µl of Brain- Heart infusion broth (B.H.I.B) contains 1% glucose. Negative control wells contained 180 µl of B.H.I.B and 20 µl of normal saline. The dishes were covered with their lids and incubated under aerobic conditions at 37°C for 18–24 h. After incubation, the plate was gently washed twice with distilled water and left to dry at room temperature for 15 minutes. The plate was stained with 200 µl of a 0.1% solution of crystal violet for 15 minutes at room temperature, then the excess stain was rinsed with distilled water. The plate was dried for 30 minutes at room temperature and the adherent cell was dissolved in 200 µL of 99% ethanol and left for 10 minutes. The optical density of each well was obtained by measuring the absorbance of each well at 600 nm using a microplate reader. The experiments were performed in three replicates. Blanks are un incubated wells containing sterile MHB supplemented with 1% glucose with 20 µl normal saline, which are called negative controls as it is used to determine whether isolates form biofilms or not 17; 18; 19 . The OD of each isolate was obtained by formula (5) 20 . ODc: Average OD of negative control + (3 × standard deviation (SD) of negative control) ……..(4)

OD isolate: Average OD of isolate – ODc…….. (5)

Classification of bacterial adherence is summarized in (table 3) based on OD values obtained for individual bacterial 21; 22 .

Table (3): Classification of bacterial adherence by microtiter plate method

Mean OD	Biofilm intensity
OD <= ODc	Non – adherent
2ODc> OD> ODc	Weak
4ODc> OD> 2ODc	Moderate
OD > 4 ODc	Strong

Anti-biofilm activity of leaves ethanolic extract

The anti-biofilm activity of the extract was performed based on the method 23 with some modifications using microplates containing 180 µl of Brain- Heart infusion broth (BHIB). The broth containing 1% glucose was inoculated with 10 µl of the bacterial suspension and 10 µl of MIC leaves extract at concentrations (125,000, 62,500, 31,200 µg/ml), the positive control was selected, which includes (180 µl BHIB and 20 µl leaves extract), while the negative control includes (180 µl BHIB and 20 µl normal salin), and the plates were incubated. Minutes for 24 hours at 37°C, the liquid medium was discarded,

the adhesion cells were washed twice with distilled water, the wells were dried at 37°C for half an hour, then 200 µl of methanol was added to fix the biofilm on it, walls and leave for 15 minutes. Then wash once with distilled water and leave to dry for half an hour. 200 µL of 0.1% crystal violet was added and left for 15 minutes. The violet-stained wells were washed (2-3) times. After drying the wells of the microplate in the incubator for an hour, 200 µL of 99% ethanol was added

to dissolve the biofilm dye lining the walls of the microplate. It was left for 10 minutes and the microplate was then measured at 580 nm with an ELISA reader. The percentage of biofilm inhibition was calculated by equation (6) 24.

% Biofilm inhibition = [(OD Control- OD Sample)/OD Control] ×100……(6).

RNA isolation by TRIzol™

One milliliter of bacterial growth was added to a 1.5 ml tube and centrifuged at 13,000 × g. For 2 min, the supernatant was discarded, 0.5 ml Triazole was added, and the pellet was vortexed. Then incubate for five minutes and add chloroform to 0.15 ml of TRIzol™ reagent used for lysis, then incubate for 2-3 minutes. The sample was then placed in a centrifuge for 15 minutes and the mixture was then separated into a phenol-chloroform red substrate, an interstitial phase, and a colorless upper aqueous phase. The aqueous phase containing the RNA was transferred to a new tube and the RNA was precipitated by adding 0.45 mL of isopropanol to the aqueous phase and keeping the mixture for 10 minutes. The mixture was then incubated for 10 min and then centrifuged for 10 min at 12,000 × g. While the total RNA precipitates, forming white, jelly-like granules at the bottom of the tube. The supernatant was removed and the pellet was resuspended in 0.75 ml of 75% ethanol, a vortex was used to dissolve the pellet and centrifugation for 5 minutes, then the supernatant was discarded. The tube was opened for 15 minutes to dry the RNA pellet, the pellet was resuspended in 20 µl of RNase-free water and incubated at 60°C for 15 minutes and the total RNA samples were stored at -20. Celsius. RNA concentration was measured by Qubit 4.0.

RT-qPCR protocol

This step is divided into two stages, the first is the synthesis of cDNA from RNA through the transcription-specific primer SEG, 16S rRNA and a cDNA synthesis kit. Five microliters of each extracted RNA sample was added to a fresh sample PCR tube, initial reaction mixed containing dNTPs, buffer and others basic components were added to 10ul per sample, MuLV enzyme was added to the reaction at a rate of 2µl each sample and add 2-4 µl of random oligo, and the volume of 20 µl was completed by adding nuclease-free water, this mixture was incubated for 1 h at 42°C using thermal cycling followed by 80°C to inactivate the cDNA. The product is also quantified by Qubit 4.0 and stored until the second step (relative quantitative PCR) is performed. The second part of this protocol is done by selecting a cDNA sample from each sample in the same run, where each sample has PCR tubes, and one tube for each gene, which is considered the home of gene conservation in this study. Quantitative detection depends on the fluorescent power of Syber. Green, the reaction mixture consists of the components in their quantities as shown in table 4.

Table 4 : shown the components of the reaction mixture

Component	20 ul
Luna Universal qPCR Master	10 ul
Forward primer (10 µM)	1 ul
Reverse primer (10 µM)	1 ul
Template DNA	5 ul
Nuclease-free Water	3 ul

The PCR tubes were rapidly rotated to remove bubbles and collected liquid, and then the real-time PCR program was set up using the indicated thermal cycling protocol , as shown in table 5.

Table 5: shows the thermal cycling protocol.

Cycle Step	Temp.	Time	Cycles
Initial Denaturation	95°C	60 seconds	1
Denaturation Extension	95°C 60°C	15 seconds 30seconds (+plate read)	40-45
Melt Curve	60-95°C	40 minutes	1

The result was collected and analyzed by Livak formula(Gol: SEG, References: 16s rRNA).

Statistical Analysis

The Statistical Analysis System- SAS (2018) program was used to detect the effect of difference factors in study parameters. Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significant compare between means in this study.

Results

Staphylococcus aureus was identified and isolated when grown on selective media, where it appeared in Mannitol salt agar has the ability to aerobically ferment mannitol and convert it from red to yellow 25 , and on Blood agar showed a light golden yellow stain with lysis of red blood cells 26, as for Nutrient agar there were golden colonies 25.

Antibiotic Susceptibility Assays

The sensitivity of bacterial isolates to antibiotics was conducted by selecting the most resistant isolates from among the 50 isolates and testing them on 12 antibiotics. It was selected based on the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2022) using tablets of origin (Liofilchem/Italy) and were as follows: azithromycin, cefoxitin, ciprofloxacin, chloramphenicol, doxycycline, erythromycin, gentamicin, levofloxacin, nitrofurantion, ofloxacin, rifampicin, Tetracycline by disk diffusion method, the results are as in table (6).

Table (6): Antibiotic test results by inhibition zone diameter of Staphylococcus aureus restricted zones according to 27

Id	Antimicrobial agent	Inhibition zone (mm)			Inhibition zone S1	Inhibition zone S2	Inhibition zone S73
		S	I	R			
1	Azithromycin	≥ 18	14-17	≤13	0	0	0
2	Cefoxitin	≥ 22	-	≤21	0	15	16
3	Chloramphenic	≥ 18	13-17	≤12	18	28	21
4	Ciprofloxacin	≥ 21	16-20	≤15	0	0	11
5	Doxycyline	≥ 16	13-15	≤14	16	11	23
6	Erythromycin	≥ 23	14-22	≤13	0	17	17.5
7	Gentamicin	≥ 15	13-14	≤12	0	0	0
8	Levofloxacin	≥ 19	16-18	≤15	0	9	11
9	Nitrofurantoin	≥ 17	15-16	≤14	25	27	20
1	Ofloxacin	≥ 18	15-17	≤14	0	0	7
1	Rifampicin	≥ 20	17-19	≤16	0	0	28
1	Tetracycline	≥ 19	15-18	≤14	16	15	18
P-value		0.0091 **	0.0086 **	0.0082 **	0.0001 **	0.0001 **	0.0001 **
** (P≤0.01).							

*Significant

Molecular detection of the SEG virulence gene of Staphylococcus aureus

The results of the polymerase chain reaction (PCR) on bacterial samples showed amplification of the SEG gene, and using agarose gel electrophoresis to confirm this amplification of this region, using the Red Safe dye, the fragment size of 107 bp was successfully amplified for all samples using a single band. As shown in the figure (1)

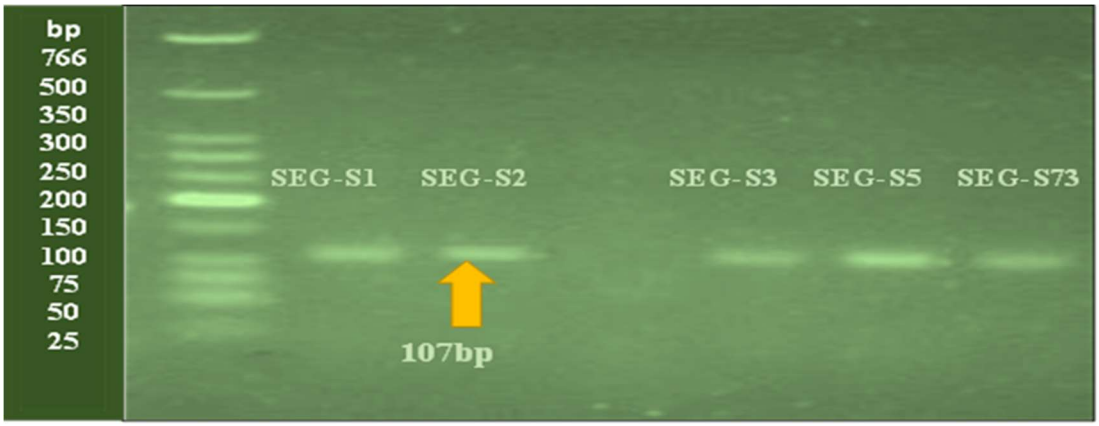

Figure (1): Shows the results of electrophoresis of the SEG gene for Staphylococcus aureus PCR reaction at a PCR product size of 107 bp. A DNA specific ladder (25-766 bp) and a 2% agarose gel at 70 v for 90 min were used.

Detection of the Bacterial Ability for Biofilm Formation

The results were divided into two different categories: (a) About 33.3% were very strong, producing biofilms with very strong adhesion. (B) About (66.6%) of the S. aureus isolates were moderately high to medium (high-moderate) .

Detection of anti-biofilm activity using the Microtiter Plate Method (MTP (method.

The effect of the extract on biofilm production or inhibition of strong biofilm-generating S. aureus isolates was studied using MIC and Sub-MIC inhibition concentrations, are shown in table (7).

Table (7): Shows the biofilm inhibition results of Staphylococcus aureus isolates after treatment with the MIC and Sub-MIC of Cynanchum acutum ethanolic leaves extract, using an ELISA reader.

Isolate	Negative control	Positive control	Concentration 125.000 µg/ml of the extract with bacteria	Concentration 62.500 µg/ml of the extract with bacteria	Concentration 31.200 µg/ml of the extract with bacteria
Control concentration of the extract			125.000 µg/ml	62.500 µg/ml	31.200 µg/ml
			0.137	0.144	0.102
S1	0.110	0.348	0.00	0.271	0.237
S2	0.110	0.351	0.00	0.289	0.391
S73	0.110	0.730	1.109	0.524	0.00
LSD (P-value)	0.00 NS (1.00)	0.288 * (0.0352)	0.327 ** (0.0031)	0.308 * (0.0447)	0.291 ** (0.0097)
* (P≤0.05), ** (P≤0.01).					

gene expression

The quantitative RT-qPCR test used to analyze the mRNA expression of the SEG gene showed variation in the gene expression of the S. aureus isolates after treatment, where the gene expression was high (overexpressed) about 9.8 in isolate S73 and the ΔΔCt value was about (-3.34) and also in isolate S1 the expression was high Relatively, it reached (4.9), and its ΔΔCt value was about (-1.67). As for isolate S2, it showed low expression (down-regulation), with the value of ΔΔCt reaching about 2.25, giving a value of 0.21, as gene expression, as shown in table (8).

Table 8: The gene expression of the SEG gene of Staphylococcus aureus in isolates: S1, S2, and S73, depending on the 2-ΔΔCt method (Fold of expression).

Groups	Isolate code	Means Ct of *SEG*	Means Ct of HKG	ΔCt Means (Ct of *SEG* - Ct of HKG)	ΔΔCt Means (ΔCt T - ΔCt C)	Fold of gene expression $2^{-\Delta\Delta Ct}$
After treatment	S1	19.36	16.54	2.82	- 1.67	3.03 ±0.28 c
	S2	41.02	19.59	21.43	2.25	0.21 ±0.09 d
	S73	16.37	12.04	4.33	- 3.34	9.8 ±1.06 a
Before treatment (control)	S1	17.81	13.32	4.49	0	1.00 ±0.00 d
	S2	36.99	17.81	19.18	0	1.00 ±0.00 d
	S73	24.34	16.67	7.67	0	1.00 ±0.00 d
LSD (P-value)	--	--	--	--	--	1.694 ** (0.0001)
Means having with the different letters in same column differed significantly, ** (P≤0.01).						

Discussion

The ethanolic extract may cause irreparable damage to the outer membrane of Gram-positive bacteria, leading to their eventual death. The large molecules, which include the active substances in the extract, are flavonoids, which amounted to 78.14%, and terpenes, which amounted to 21.87%, according to the results of gas chromatographic analysis GC-MS 9 . These molecules may have anti-biofilm activity, as they can penetrate the outer layer (which consists of a very thick layer of peptidoglycan), causing cellular functional and metabolic disorders, and loss of cellular components, and thus leading to the death of bacteria .

Test results showed that the isolates were highly resistant to antibiotics, and were resistant (100%) to azithromycin, gentamicin, ofloxacin, ciprofloxacin, levofloxacin, and cefoxitin), followed by erythromycin, tetracycline, and rifampicin (85%, 82%, 70%), as for the groups sensitive to each of the antibiotics Nitrofurantion, Chlorophenicol, and Doxycycline, the percentage reached (100%, 70%), respectively.

This confirms that all S. aureus isolates are multidrug resistant (MDR), and these results are consistent with 29 . Since it was 100% resistant to the antibiotics cefoxitin and oxacillin, it is considered methicillin-resistant bacteria (MRSA), as mentioned in the study 30 .

Biofilm inhibition results were calculated according to equation (6). The results showed a significant decrease in optical density after treatment with the ethanolic extract of C. acutum leaves, as it was found to have a strong inhibitory activity on biofilms. The highest value reached 0.237 at a concentration of 31,200 µg/ml in isolate S1, followed by Isolate S2 reached 0.289 µg/ml at a concentration of 62,500 µg/ml, and the lowest value was recorded at 0.524 µg/ml in isolate S73 at the same concentration. The conclusion was that concentrations of 62,500 and 31,200 µg/ml gave good inhibitory activity for three resistant isolates. Gene expression results when treated with ethanolic extract at a value sub- MIC showed a significant increase in the regulation of virulence genes (SEG) involved in the production of enterotoxins for most isolates, with the exception of one isolate, S2, which recorded a significant decrease in its gene expression (down regulation).

Conclusion

The conclusion from the results of this study is that the ethanolic extract of C. acutum leaves has an inhibitory effect on the growth and formation of biofilms of S. aureus bacteria, through many previous studies, it was found that the plant contains phytochemical compounds such as flavonoids, terpenes, steroids, tannins, and other resin compounds, and perhaps their presence may cause increased oxidative stress in microbial cells, this causes damage to large molecules inside cells, which leads to their death.

The difference in gene expression indicates that the bacteria are constantly mutating and changing in their resistance, so it is difficult to find materials that break this resistance.

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Not Applicable.

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