INHIBIOTORY EFFECT OF TRYPTOPHAN ON BIOFELIM DEVELOPMENT AND VIABILITY IN METHICILLIN RESISTANT *Staphylococcus aureus*

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ABSTRACT

This study was aimed to evaluate the antimicrobial and antibiofilm activity of tryptophan against methicillin-resistant *S. aureus* (MRSA). A 326 samples were obtained from patients attending Al-Karama hospital\Baghdad city with various infections including: blood, vagina, acne, nasal cavity, wounds, gums and skin. Only 100 isolates were identified as *S. aureus*, according to conventional and molecular methods. Detection of *mecA* and *icaAD* gene was performed using polymerase chain reaction (PCR) and the results showed 70% of isolates harbored *mecA* gene (MRSA) and 61% of isolates harbored *icaAD* gene. The results of detecting quorum sensing (QS) genes, include *agr1*, *agr2*, *agr3* and *agr4*, using PCR were revealed, that 27. 14%, 18. 6% and 41. 43 of MRSA isolates were carrying *agr1*, *agr2* and *agr3* genes, respectively. The antimicrobial and antibiofilm activity of different concentrations of tryptophan were estimated using colony-forming unit (CFU) assays. The findings revealed that the FSA96 isolate was showed greater ability to resist 2mg/ml of tryptophan in percentage 25. 6% than other isolates (FSW61, FSW69 and FSB76 in percentage (16, 16. 25 and 16. 7) % respectively. The biofilms were showed significantly decrease in their percentages (2.17, 1.64, 3.63 and 1. 49) % at concentration 2 mg/ml of tryptophan for FSW61, FSW69, FSB76 and FSA96 isolates. The present study examined the role of QS (*agr1* and *agr2*) genes in the production of biofilm *ica*AD gene for three selected MRSA isolates using qPCR. The results indicate the significant influence for the expression of *ica*AD gene which is associated with the expression of a QS genes (*agr1* and *agr2*) which significantly downregulated after being treated with 2. 0 mg/ml of tryptophan compared with the control that expressed. The results indicate the significant differences for the expression of *ica*AD gene which is associated with the expression of a QS genes (*agr1* and *agr2*) when showed down regulation after being treated with 2.0 mg/ml of tryptophan compared with the control that expressed.

Keywords: *agr* gene, *ica* gene, QS gene, antibiotic resistance, crystal violet

التأثير المثبط للتريبتوفان على تطور الغشاء الحيوي والحيوية في المكورات العنقودية الذهبية المقاومة للميثيسيلين

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المستخلص

هدفت هذه الدراسة لتقييم النشاط المضاد للميكروبات والنشاط المضاد للغشاء الحيوي للتربتوفان ضد المكورات العنقودية المقومة للميثيسيلين. تم الحصول على 326 عينة من مرضى يرتادون مستشفى الكرامة \ مدينة بغداد ويعانون من التهابات مختلفة شملت: الدم, المهبل,حب الشباب وتجويف الانف والجروح, والجلد. تم التعرف على 100 عزلة فقط على انها مكورات عنقودية ذهبية (*S. aureus*) اعتمادًا على الطرق التقليدية والجزيئية. تم اجراء الكشف عن جين (*mecA*) وجين (*nuc*) باستخدام تفاعل البلمرة المتسلسل واظهرت النتائج ان 70% من العزلات تحمل جين (*mecA*) وهي (MRSA) و61% من العزلات تحمل جين (*icaAD*). كشفت نتائج جينات التعبير الجيني والتي شملت جينات (*agr1* و*agr2* و*agr3* و*agr4*) باستخدام تقنية (PCR) ان 27.14 و18.6 و41.43% من عزلات (MRSA) تحمل جينات (*agr1* و*agr2* و*agr3*)، على التوالي. تم تقييم النشاط المضاد للميكروبات والنشاط المضاد للأغشية الحيوية لتراكيز مختلفة من التربتوفان باستخدام تحليل الوحدة المكونة للمستعمرة (CFU). كشفت النتائج ان العزلة (FSA96) قد أظهرت اعلى قابلية على تحمل (2 mg/ml) من التربتوفان بنسبة 25.6% اكثر من باقي العزلات (FSW61 وFSW69 وFSB76) بنسب 16 و16.25 و16.7%، على التوالي. أظهرت الاغشية الحيوية انخفاضًا معنويًا في نسبها (2.17 و1.46 و3.63 و1.49) عن تركيز (2 mg/ml) للتربتوفان لكل من العزلات (FSW61 وFSW69 وFSB76 وFSA96)، على التوالي. اختبرت الدراسة الحالية دور جينات استشعار النصاب QS (*agr1* و*agr2*) في انتاج جين (*icaAD*) لإنتاج الأغشية الحيوية لثلاث عزلات (MRSA) منتخبة باستخدام (qPCR). اشارت النتائج ان هناك تأثيرًا واضحًا للتعبير الجيني لجين (*icaAD*) والذي على صلة بالتعبير الجيني لمورثات استشعار النصاب (*agr1* و*agr3*) والتي قل تعبيرها معنويًا بعد معالجتها بتركيز (2 mg/ml) مقارنة بالسيطرة التي تعبر عنها. واظهرت النتائج الاختلافات المعنوية للتعبير الجيني لـ*icaAD* والمرتبط بالتعبير الجيني لمورثات QS (*agr1* و *agr3*) والذي اظهر انخفاض ملحوظ بمستوى التعبير الجيني بعد المعاملة ب 2.0 ملغم / مل من التربتوفان مقارنة بالسيطرة التي تم التعبير عنها.

كلمات مفتاحية: جين *agr*، جين*ica*، *المكورات العنقودية*، البنفسجي البلوري.

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INTRODUCTION

The *Staphylococcus aureus* species is the most opportunistic pathogenic bacterial species, can cause various infections in patients with immune compromised or immune competent. Nasal cavity of human is the major habitat for this species, and it also presents in oral cavity, on skin, inside skin glands, in gastrointestinal tract. About 60% of individuals may be carry transient *S. aureus*, 20% of individuals may be have asymptomatic *S. aureus* and 20% is rare or non-colonized bacteria (39, 44). Methicillin-resistant *Staphylococcus aureus* (MRSA is a subset of this genus that shares the same bacterial makeup as other *S. aureus* strains but differs genetically. *S. aureus* strains that are susceptible to methicillin are known as MSSA (16, 20). The (*mecA*) gene, which is responsible for methicillin resistance and is present on the Staphylococcal chromosomal Cassette mec (SCCmec) (48), which is acquired by methicillin-resistant *Staphylococcus aureus* (MRSA), lowering its affinity for penicillin and β-lactam medicines (26). Indeed, biofilm associated infections are linked to higher mortality and morbidity with the infected indwelling medical devices in the hospital (35, 42). Biofilms can be defined as bacterial aggregations that are immersed in an extracellular matrix produced by the bacteria itself and made up of Exopolysaccharides (EPSs), Proteins, and Micro-molecules like DNA. They can develop on both biological and non-biological surfaces (28). The operon *ica* is a part of the accessory genes, it is not present in all isolates of *Staphylococcus*, this operon is composed of the *icaR* regulatory gene and *icaADBC* biosynthetic genes (29). *Staphylococcus* biofilm mechanism was characterized as an *ica*-dependent biofilm pathway (1). However, there is a biofilm pathway that is not dependent on *ica* (43). Several types of bacteria have been demonstrated to use intercellular signaling—also known as quorum sensing—during biofilm growth (18, 25). The accessory gene regulator (*agr*) locus encodes the quorum-sensing system of *S. aureus* (7, 23). However, the specific involvement of the *agr* system varies with the kind of infection model studied (46). The *agr* system contributes to virulence in model biofilm-associated infections such as endocarditis and osteomyelitis. Multiple natural and synthetic substances have been shown to suppress microbial biofilm development in laboratory settings. Like the aromatic amino acid tryptophan, which has been shown to effectively suppress biofilm formation by *Pseudomonas aeruginosa* (36). Tryptophan is an essential amino acid for all forms of life, yet mammals lack the enzyme machinery necessary to create it from simpler molecules. Primary producers like bacteria, fungi, and plants are responsible for driving the tryptophan flow up the food chain (27,41), since they synthesis tryptophan from compounds like phosphoenol pyruvate. *Staphylococcus aureus* strains 655 with and without tryptophan biosynthesis enzyme mutations were tested (38). The objective of this study was evaluating the inhibitory effects of tryptophan in the development of biofilm and the viability of clinical methicillin-resistant *Staphylococcus aureus* isolates, in addition to investigate their relationship with expression of quorum sensing genes.

MATERIALS AND METHODS

Collection, isolation and identification of clinical specimens: A total of three hundred twenty-six clinical samples were obtained from patients with various infections, including blood, vagina, acne, nasal cavity, wounds, gums and skin, with ensure gained an approval the College of Science Research Ethics Committee based on (CSEC/0122/0045) during the period from February 2022 to April 2022. All these samples were supplied from AL – Karama Hospital in Baghdad, then streaked on blood agar and mannitol salt agar plates (Himedia/India) and incubated at 37^{o}C for 24h. The morphological and biochemical examination were performed in order to identify the bacterial isolates of *S. aureus* (3). In addition, the molecular diagnosis was carried out using PCR technique for detection of *nuc* gene to confirm the isolates of *S. aureus* (37) and for detection of *mecA* to distinguish the MRSA (49).

Molecular detection of (*nuc*, *mecA* and *icaAD)* genes

DNA extraction: Extraction of DNA from samples was carried out in accordance with the guidelines provided by the manufacturer using

a PrestoTM Mini gDN Bacteria Kit (Geneaid/Korea). then the concentration and purity were measured and the integrity of genomic DNA was investigated using gel electrophoresis method.

Molecular detection of *nuc* gene

The detection of *nuc* gene was done by using PCR technique. The presence of *nuc* gene confirmed the diagnosis of *S. aureus* isolates which was designed according to the method described by Brakstad et al., (3). The primer sequence and its amplicon size were listed in Table (1). While the program that was used for *nuc* gene amplification was listed in Table (2).

Molecular Detection of *mecA* gene

Primers specific for the mecA gene of *S. aureus* isolates were designed for use with methicillin-resistant *Staphylococcus aureus* (MRSA) according to (48), allowing for the detection of *mecA* gene using polymerase chain reaction. Table (1) detailed the amplicon size and primer sequence. Table (2) shows the results of the *mecA* amplification programme.

Molecular detection of biofilm formation

To detect the ability of *S. aureus* to form biofilm, the molecular detection of *icaAD* genes were performed according to (30) using PCR technique, as shown in Table (1). A 2% agarose gel electrophoresis was performed for monitoring the amplification of these genes at 7 v/cm for 90 min. The sequence of primers and their sizes of amplicon are shows in Table (1) and the RCR program was carried out as in Table (2)

Table 1. Primer's sequence of (*nuc*, *mecA* and *icaAD*) genes and amplicon sizes

Primers	Sequences (5'-3')	Amplicon size (bp)
***nuc*–F**	**GCGATTGATGGTGATACGGTT**	**279**
***nuc*–R**	**AGCCAAGCCTTGACGAACTAAAGC**	
***mecA* -F**	**GTGAAGATATACCAAGTGATT**	**147**
***mecA* -R**	**ATGCGCTATAGATTGAAAGGAT**	
***icaAD*-F**	**TATTCAATTTACAGTCGCAC**	**470**
***icaAD*-R**	**GATTCTCTCCCTCTCTGCCA**	

Table 2. The PCR analysis program for the (*nuc*, *mecA* and *icaAD)* primers.

The steps	Temperatures	Period	No. of cycles
Initial Denaturation	**95**	**5 minutes**	**1**
Denaturation	**94**	**45 second**	
Annealing	**56*, 52****	**1 minutes**	**30**
Extensions	**72**	**45 second**	
Final extensions	**72**	**5 minutes**	**1**

*** For *nuc* and *mecA* genes, ** For *icaAD* gene.**

Molecular detection of quorum sensing genes: For detection of the presence of quorum sensing genes, the molecular detection of *agr1*, *agr2*, *agr3* and*agr4* genes were performed according to (2) using PCR technique. The sequence of primers and their sizes of amplicon are shows in Table (3), and the RCR program was carried out as in Table (4).

Table 3. Primer's sequence of quorum sensing genes and amplicon sizes

Primer	Sequence	Product size
***Agr* 1**	**F: 5- ATG CAC ATG GTG CAC ATG C-3** **R: 5- GTCACAAGTACTATAAGCTGCGAT-3**	**441**
***Agr* 2**	**F: 5- TCAAACGGTGATAGCTTAATTCCA-3** **R:5- CTTTAAGGGTGAAAAGCGACATTA-3**	**103**
***Agr* 3**	**F: 5-ATG CAC ATG GTG CAC ATG C-3** **R:5-GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G-3**	**323**
***Agr* 4**	**F: 5- ATG CAC ATG GTG CAC ATG C-3** **R: 5- CGA TAA TGC CGT AAT ACC CG-3**	**659**

Table 4. The PCR analysis program for the primers of quorum sensing genes.

The steps	Temperatures	Period	No. of cycles
Initial Denaturation	**95**	**5 minutes**	**1**
Denaturation	**94**	**30 second**	
Annealing	**56**	**1 minutes**	**30**
Extensions	**72**	**30 second**	
Final extensions	**72**	**5 minutes**	**1**

Polymerase chain reaction (PCR)

The reaction mixture was offered as follows: 12.5µl from the Go Taq®Green Master-Mix provided by (Biolabs-England) that contains *Taq* DNA polymerase, MgCl2, deoxynucleosides (dNTP), reactions buffer, and two dyes (yellow and green) that allow monitoring of progress throughout electrophoresis, 0.7µl of each (10 pmol) primer, 1µl of template-DNA, and adding sterile D.W to obtain a total volume of 25 microliter. All amplification reactions were carried out in an aseptic laminar air flow cabinet. The negative control reaction had all of the components but no DNA template so that any contaminating DNA in the reaction would be amplified and detected on an agarose gel (Thermo, USA).

Assay for biofilm formation by microtiter plate: For the biofilm experiment, *S. aureus* isolates were diluted 1:100 from their nutrition broth media-grown overnight cultures into brain heart infusion broth supplemented with 1% glucose. Each isolate had two copies of its culture diluted to 200 l placed on a 96 Microtiter plate. After diluting the cultures, they spent 24 hours in a 37°C incubator. The bacterial cultures were discarded after 24 hours, and the Microtiter plate rinsed with distilled water to eliminate any cells that did not adhere. The layer of cells that had adhered to the bottom of each well was stained with 200 µl of 0.1% crystal violet. After two hours (17) the plate had dried after having the excess stain emptied out and rinsed with distilled water. Each well was treated with 200µl of 30% acetic acid for 10 minutes to solubilize the dye before biofilm measurement. A fresh Microtiter plate was used to store the crystal violet solution. Each well's optical density was evaluated using a micro-ELISA auto reader at 590 nm, with a blank and background of 200 l of 30% acetic acid (10). A duplicate was performed and the average optical density and standard deviation were calculated. OD590< 0. 2 a non- biofilm forming cell, 0. 2 < OD590 > 0. 9 a moderate biofilm forming cell, OD590 ≥ 0. 9 a strong biofilm forming cell.

Estimation of antimicrobial activity of tryptophan against *S. aureus* using colony forming unit (CFU) assay: Colony forming unit (CFU) assay was performed to estimate the antimicrobial activity to tryptophan on viability of *S. aureus* isolates. Each experimental set's CFU was calculated after incubation (4).

Estimation of antibiofilm formation activity of tryptophan against *S. aureus* using crystal violet assay: The crystal violet (CV) assay was carried out according to (5) the specified methodology to determine the effect of tryptophan on *S. aureus*'s capacity to produce biofilms. The optical density (OD) of the CV stain at 630 nm was then used to further evaluate the intensity of color it produced using the ELISA Reader.

Estimation of quorum sensing genes' expression

Extraction of RNA: The method of total RNA extraction with Trizol LS Reagent was carried out according to the protocol provided by the manufactured company (Invitrogen USA), then the concentration and purity were measured using Nanodrop (Invitrogen USA).

Synthesis of complementary DNA from Mrna: The first step is done through synthesis of cDNA from RNA for genes and *16S rRNA* transcripts and protoscript cDNA synthesis kit (NEB, UK). The procedure was achieved in a volume of reaction (20µl) according to manufactured instructions.

Real time quantitative PC for estimation of expression of quorum sensing genes: The goal of the quantitative PCR technique was to determine the cycling threshold (Ct) using ProtoScript® first strand cDNA synthesis kit (NEB/UK). The threshold cycle was calculated using the software used by real-time cycler (labenet/USA). Each sample was executed twice and the values of mean were estimated. The expression analysis of required genes was normalized against the housekeeping gene. Results were reported as a folding change in gene expression based on the ΔΔCt method's recommendations for data processing (24). For each sample, the difference between the CT values (ΔCt) for each gene of target and the housekeeping gene was calculated, as follow:

$\Delta Ct_{control} = CT_{gene} - CT_{Housekeeping\ gene}$

$\Delta Ct_{gene\ of\ interest} = CT_{gene} - CT_{Housekeeping\ gene}$

The difference in ΔCt values represented as (ΔΔCt) for the genes of Interest was calculated as follow: $\Delta\Delta Ct = \Delta Ct_{gene\ of\ interest} - \Delta Ct_{control}$

The fold-change in gene expression was calculated as follow: **Fold change=$2^{-\Delta\Delta Ct}$**

Statistical Analysis: SPSS-28, a freely available statistical software, was used for the analysis (Statistical Packages for Social Sciences- version 28). If there were more than two independent means to compare, an analysis of variance (ANOVA) was used to see if the differences were statistically significant. Pearson's Chi-square test (2-test) with Yate's adjustment or Fisher's exact test was used to determine if statistically significant differences existed between sets of percentages (qualitative data).

RESULTS AND DISCUSSION

Collection isolation and identification of clinical specimens: A total of three hundred twenty-six clinical isolates were collected from AL-Karama hospital in Baghdad, which distributed as follows: 95% wound swabs, 52% blood samples, 17 % acne swabs, 64% skin, 25(%) nasal cavity, 32% vaginal swabs, 41% gum. Various studies were reported that wounds are common source for isolate this bacterium (21,33). Gramme stain, coagulase, oxidase, and catalase assays, as well as growth on mannitol salt agar, confirmed the results. Isolates thought to be *S. aureus* showed Gramme positivity, positive coagulase and oxidase results, and positive catalase and mannitol results. Hence, the results of examination were revealed 100 (50. 8%) of isolates were *S. aureus*. Only *S. aureus* isolates were recognised by the presence of *nuc* gene using particular primer, and this was confirmed by the development of thermostable nuclease, the major product for *nuc* gene (10). Positive findings for certain gene sequences (*nuc* gene) were seen in the PCR products of all 100 *S. aureus* isolates, indicating that all 100 isolates were indeed *S. aureus* (Figure 1). Based on these findings, it seems that all of the *S. aureus* isolates in this investigation have a common ancestor in the thermo stable nuclease-encoding *nuc* gene.

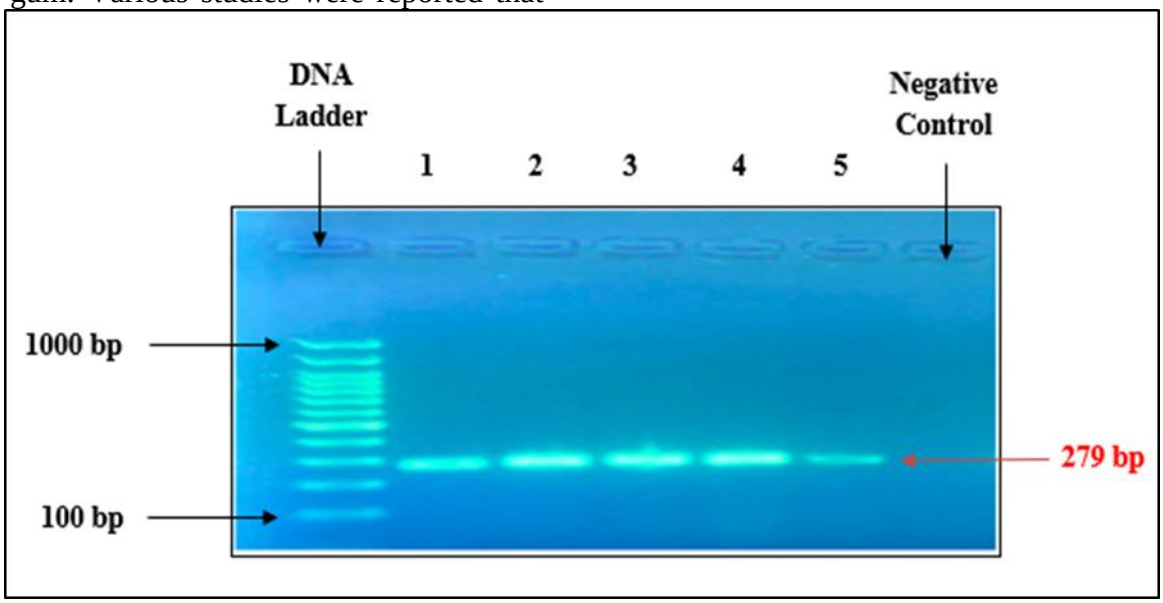

Figure 1. Gel electrophoresis in agarose for (279 bp) for *nuc* gene amplification using PCR among *S. aureus* isolates in in agarose (2%), for 90minutes under 70 volts

Among 100 isolates of *S. aureus*, 70 isolates (70%) were MRSA (*mecA* positive) while 30 isolates (30%) were MSSA (*mecA* negative) (Figure 2). This finding is agreed with (24) who reported that *mecA* gene were presented in 98.7% of MRSA.

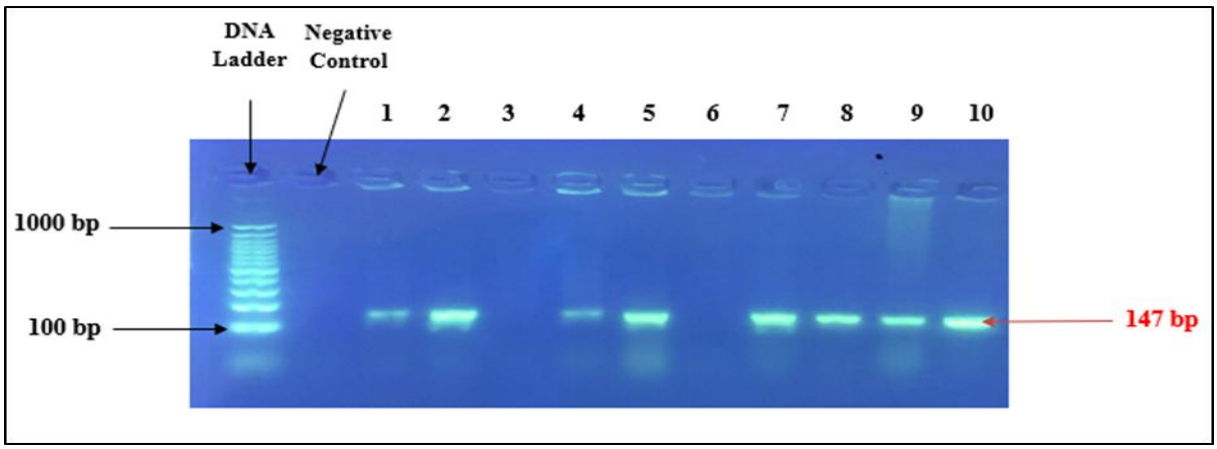

Figure 2. Gel electrophoresis for *mecA* gene (147bp) amplification using PCR among isolates of *S. aureus* in agarose (2%) for 90minutes under 70 volts. Lane (1): negative control, lanes (2,3,5,6,8,9,10 and 11): isolates with positive of *mecA* gene and lanes (4,7): isolates with negative result of *mecA* gene

Molecular detection of *icaAD* gene

The molecular detection of *icaAD* gene was performed with specific primers using PCR technique. This assay was performed for all MRSA isolates, whereas the results showed only 61% of isolates harbored *icaAD* gene, which appeared in single band with molecular size (470 bp), include 44. 3% of wound, 27. 1% of blood, 4. 29% of skin and 1. 43% of acne, as shown in Figure 3. These findings matched those of (19) who also found widespread ica expression in *S. aureus* bacteria. The *icaAD* gene has been shown to be widely dispersed across *S. aureus* isolates, but this has not been shown to correlate with the ability of these strains to produce biofilms or slime layers in culture. Both the *icaA* and *icaD* genes have been linked to *S. aureus* biofilm- associated infections (11). Extracellular components and characteristics, such as adhesion and biofilm formation, are responsible for a specific *S. aureus* strain's pathogenesis (9). The surface of a wound is a fertile breeding ground for bacteria, which may easily adhere, proliferate, and persist as an early biofilm component. The capacity of *S. aureus* to build biofilms is blamed for chronic or persistent infections (8,14), because it allows the bacterium to live in the host's hostile environment. Several investigations have linked the presence of the *icaA* and *icaD* genes to the development of slime and biofilm in *S. aureus* and *S. epidermidis* that cause catheter-associated and nosocomial infections, respectively (1). The study shows that *icaAD* is highly prevalent among *S. aureus* isolates; nevertheless, its presence is not necessarily linked to invitro slime or biofilm production. As a result of producing damaging enzymes and toxins and fostering a persistent inflammatory state, bacteria in biofilm are shielded from opsonophagocytosis and antimicrobial agents (13,22).

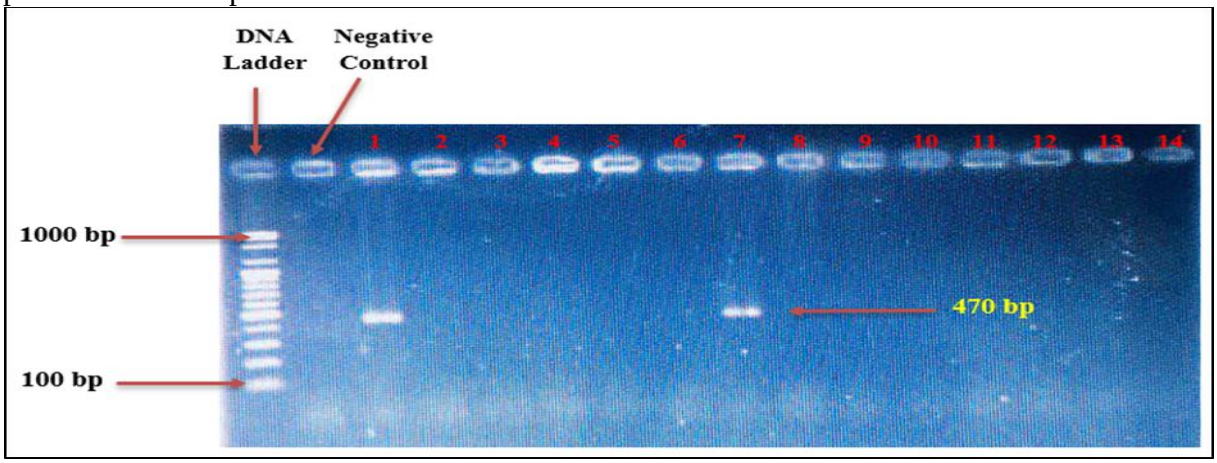

Figure 3. Gel electrophoresis of amplified PCR product of *icaAD* gene (470 bp) in 2% agarose gel at 70v for 90min Followed by Red Safe dye Staining for 20 min. then visualized by UV light. DNA ladder (100bp). C: negative control, Lines (1, 7): positive result for *S. aureus*. Line (3-6, 8-14): negative result for *S. aureus*.

Molecular detection of quorum sensing

Molecular detection of quorum sensing genes was performed using PCR technique. Based on the results, 27.14%, 18.6% and 41.43 of MRSA isolates were carried *agr1*, *agr2* and *agr3* genes, respectively, in varied distributions among these isolates. The results were summarized as shown in Table (5) and Figure (4), Figure (5) that 18.6, 5.7 and 22. 9% of isolates from wound samples were give positive for *agr1*, *agr2*and *agr3* genes, in addition, 7. 1, 2. 9 and 11. 4% of isolates from blood samples were give positive for *agr1*, *agr2* and *agr3* genes, while 2. 9, 7. 1 and 5. 7% of isolates from acne sources were give positive for *agr1*, *agr2* and *agr3*. The isolates from skin samples (1. 43%) were give positive for *agr2*, while 2. 9% of isolates from nasal cavity were positive for *agr3*.

Table 5. The prevalence of quorum sensing genes among MRSA isolates.

Source			*agr1*		*agr2*		*agr3*	
	N=70	(%)	No.	(%)	No.	(%)	No.	(%)
Wound	32	45. 7	13	18. 6	4	5. 7	16	22. 9
Blood	22	31. 43	5	7. 1	2	2. 9	8	11. 4
Acne	5	7. 1	2	2. 9	5	7. 1	4	5. 7
Skin	2	2. 86	0	0	1	1. 43	0	0
Nasal Cavity	3	4. 29	0	0	0	0	2	2. 9
Vaginal Cavity	2	2. 86	0	0	0	0	0	0
Gum samples	4	5. 71	0	0	0	0	0	0

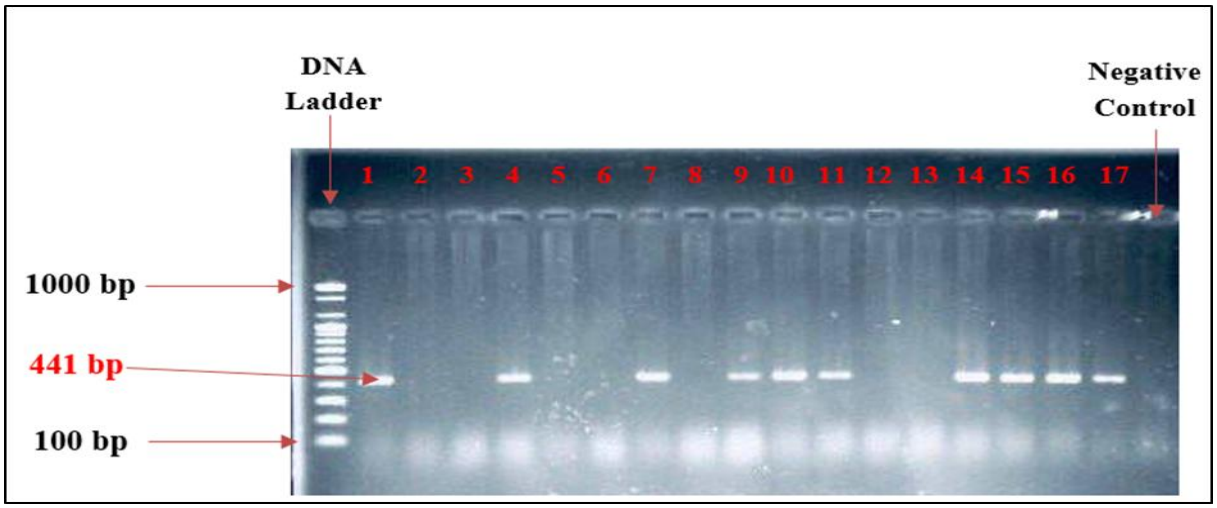

Figure 4. Gel electrophoresis of amplified PCR product of *agr1* gene (441 bp) in 2% agarose gel at 70v for 90min followed by Red Safe dye Staining for 20 min, then visualized by UV light. DNA ladder (100bp). Lines (1, 4, 7, 9, 10, 11, 14, 15, 16 and 17): positive result for *S. aureus*. Line (2, 3, 5, 6, 8, 12 and 13): negative result for *S. aureus*

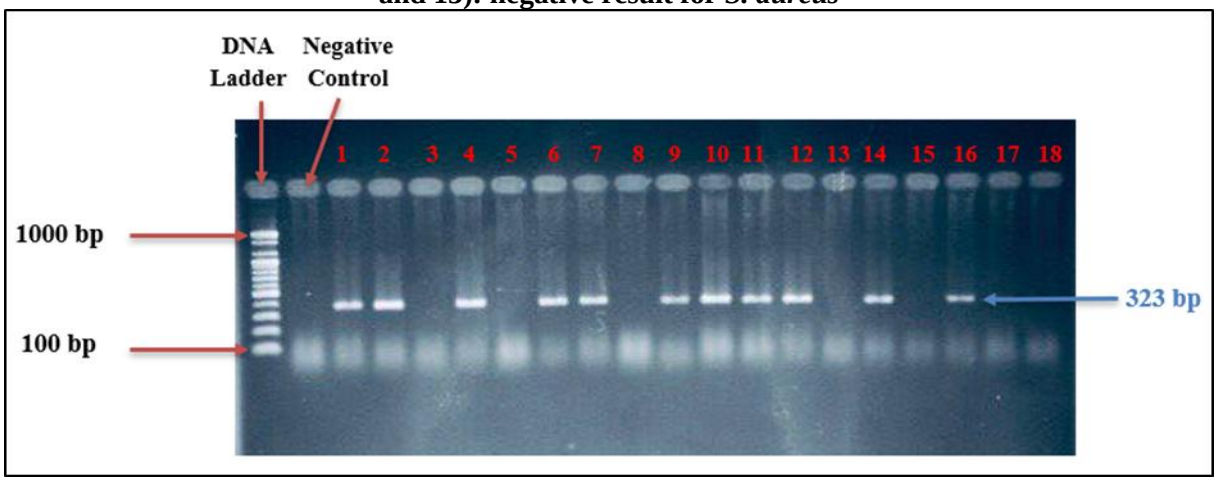

Figure 5. Gel electrophoresis of amplified PCR product of *agr3*gene (323 bp) in 2% agarose gel at 70v for 90min Followed by Red Safe dye Staining for 20 min. then visualized by UV light. DNA ladder (100bp). Lines (1, 2, 4, 6, 7, 9-12, 14 and 16): positive result for *S. aureus*. Line (3, 5, 8, 13, 15, 17 and 18): negative result for *S. aureus*

Staphylococci have evolved quorum-sensing systems, which allow cell-to-cell communication and modulation of multiple colonization and virulence factors, to increase their capacity to cause such a wide range of human diseases and to inhabit such a wide variety of niches within the host. During the shift from late-exponential growth to stationary phase in vitro, the staphylococcal accessory gene regulator (*agr*) quorum-sensing system reduces the expression of numerous cell surface proteins and enhances the expression of several secreted virulence factors (32). Quorum sensing describes bacterial intercellular signaling and is related to biofilm development. Reports showing moderate to poor biofilm development in both *agr*-negative and -positive strains suggest that the *agr* genes play a variable role in biofilm formation, with the degree of biofilm formation perhaps depending on the level of expression of the *agr* genes. This indicates that quorum sensing can be targeted, and measures to improve the *agr* system, to treat chronic or device-related *S. aureus* infections. Furthermore, the presence of the icaAD gene does not completely correlate with biofilm development in vitro, and ica-independent biofilm formation emphasizes the need for additional genetic studies (40). Murine subcutaneous abscesses (n=7), murine arthritis (n=3), and rabbit endocarditis (n=11) were all infection models in which *agr* expression was demonstrated to contribute to staphylococcal pathogenesis. It indicates that *agr* expression is also involved in epithelial cell invasion and apoptosis (45). Diseases seem to cluster around certain *agr* subgroups, which may be identified by their propensity to produce and respond to particular secreted signals (31). A deeper knowledge of this phenomena may help us better comprehend the epidemiology of staphylococcal illnesses (46), although the reasons for this link between *agr* group and infection type are not yet known. Out of the MRSA isolates, only four isolates were selected for estimation of biofilm formation ability of MRSA and their capability to resist the QS inhibitors, tryptophan, in order to investigate the association between biofilm formation and gene expression of QS genes. Two of these isolates (FSW69 and FSB76) were obtained from wounds, one from blood (FSW61) and one from acne (FSA96).

Antimicrobial activity of tryptophan against *S. aureus:* Four selected isolates of MRSA were subjected to different concentrations of tryptophan (62. 5, 1. 25, 250, 500, 1000 and 2000 µg/ml) for estimated their inhibitory effect on the viability of bacterial cells. After being incubated for 24hr After being incubated for 24hr, the number of colony-forming units (CFU) in both tryptophan-treated and untreated growth conditions were compared. Based on the Figure (5), the FSA96 isolate was showed greater resisted to 2000 µg/ml of tryptophan in percentages 25.6%, than other isolates, include FSW61, FSW69 and Based on the Figure (6), the FSA96 isolate was showed greater resisted to 2 µg/ml of tryptophan in percentages 25. 6%, than other isolates, include FSW61, FSW69 and FSB76in percentages (16, 16. 25 and 16. 7%), respectively. Effective antibiofilm activity without harming the relevant microorganism is a key feature of a prospective antibiofilm agent (5,6). It has been reported that the tryptophan (at concentrations 5, 10, 25 and 50 µg/mL) did not exhibit antimicrobial activity on *S. aureus* isolates (36).

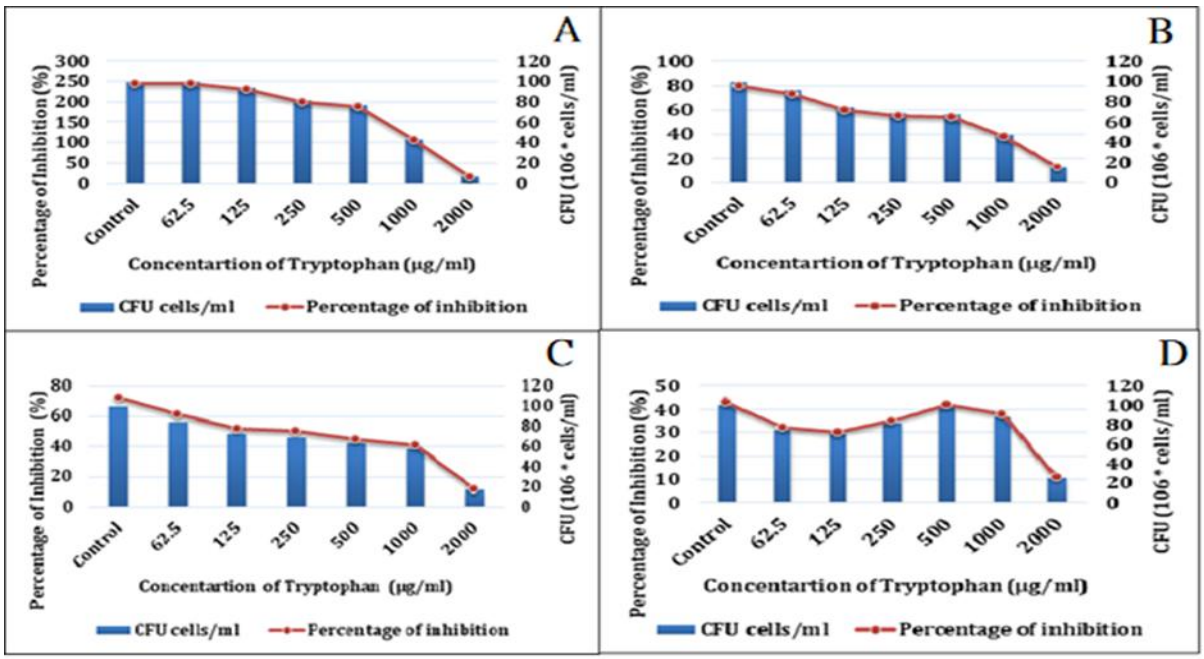

Figure 6. The antimicrobial activity of various concentrations of tryptophan on the clinical MRSA isolates (A: FSW61, B: FSW69, C: FSB76 and D: FSA96)which isolated from various sources using CFU assay.

Antibiofilm activity of tryptophan

The inhibitory effect of various concentrations of tryptophan (62. 5, 125, 250, 500, 1000 and 2000) on four selected MRSA isolates was examined using crystal violet method. The results show that there are significant antibiofilm activity of tryptophan against these isolates. The biofilms, which formed by all isolates, were showed significantly decrease in their percentages by approximately 2.17, 1.64, 3.63 and 1. 49% using 2000 µg/ml of tryptophan for FSW61, FSW69, FSB76 and FSA96 MRSA isolates, as shown in Figure (7).

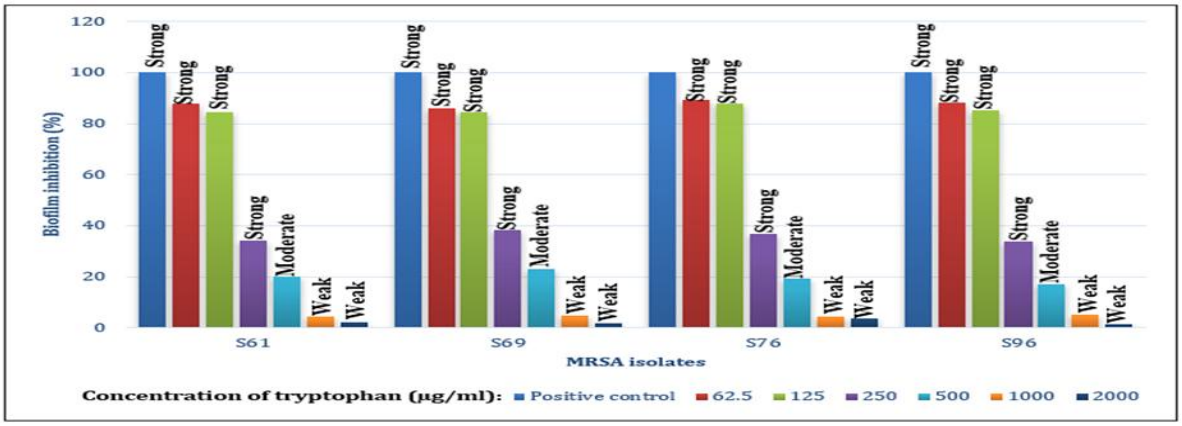

Figure 7. The antibiofilm activity of tryptophan concentrations on the clinical MRSA isolates which isolated from various sources

The *S. aureus* biofilm-linked infections are associated with a higher risk of death and morbidity (17). It has been noted that microbial biofilm is more challenging to handle due to the presence of high levels of antibiotic resistance in the biofilm itself (15). Amino acids have been found to be effective agents in combating the development of microbial biofilm (4). When exposed to the same amino acid, however, various *S. aureus* strains have been found to have varying degrees of biofilm-forming capacity (12). D-amino acids, such as D-tryptophan, were discovered to effectively inhibit *S. aureus* biofilm growth (12). However, contradictory results were reported when testing the same amino acid (D-tryptophan) on *S. aureus* (34), indicating that it lacked antibiofilm action. It has been found that L-tryptophan effectively suppressed biofilm formation in *Pseudomonas aeruginosa* (5), *Pseudomonas mendocina* (12), and other bacteria. However, in some other

reports, it has been noticed that instead of biofilm inhibition, the same amino acid was found to promote the biofilm formation of *C. sakazakii* (23) and *S. aureus* (12).

Gene expression of biofilm formation gene and quorum sensing genes: Quantitative Real Time PCR was utilized for determining the gene expressions of quorum sensing genes (*agr1* and *agr3*) of non-treated isolates (controls) and tryptophan-treated isolates of *S. aureus*. SYBER green dye was utilized in this experiment as an indicator for expression of genes by binding with double stranded DNA and release green light that measured at the end of each cycle by qPCR and the amplification results of each cycle was called CT (cycling threshold). The expression of genes was normalized to the scale of a housekeeping gene *16S rRNA* and quantified by the (ΔCt) value and folding ($2^{-\Delta\Delta Ct}$). In this experiment, three isolates were used, including FSW61, FSW69 and FSA96. Two tubes for each isolate, including control tube (free-tryptophan tube), tube treated with 2. 0 mg/ml of tryptophan. The analysis of results and determination the fold of expression for studied genes for each sample was performed according to the equations of (29). According the result as shown in Table (6), the relative expressions ($2^{-\Delta\Delta Ct}$) of *icaAD* gene in FSW61, FSW69 and FSA96 isolates of *S. aureus* were down regulated in (0.289172, 0.138696 and 0.273573) fold after treated with 2.0 mg/ml of tryptophan, respectively and showed significant difference ($P < 0.05$ - 0.0239) in folding expression of *ica* gene between the control (without treated) and treated isolate.

Table 6. Gene expression of *icaAD* gene among tryptophan-treated isolates and non-treated isolates (control) of *S. aureus*

Isolates	Means Ct Ica gene	Means Ct of Ica	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$	*p*-value	Mean of differences	SD of differences
				Sensitive (Control)				
FSW61	18. 72		2. 37	0	1			
FSW69	16. 9	16. 35	0. 56	0	1			
FSW96	18. 36		2. 01	0	1			
Treated isolates				After treated with 2. 0 mg /L Tryptophan				
FSW61	20. 51		4. 16	1. 79	0. 289172	0. 0039 Significant	-0. 7662	0. 08274
FSW69	19. 76	16. 35	3. 41	2. 85	0. 138696			
FSW96	20. 23		3. 88	1. 87	0. 273573			

C: Control (*SrRNA* gene; HKG), ΔCt: Means Ct of *icaAD* – Means Ct of HKG

The operon *ica* is a part of the accessory genes, it is not present in all isolates of staphylococcus. The *Staphylococcus* biofilm mechanism was characterized as an *ica*-dependent biofilm pathway (1). However, there is a biofilm pathway that is not dependent on *ica* (43). According to the result in Table (6), the relative expressions ($2^{-\Delta\Delta Ct}$) of *agr1*gene in FSW61, FSW69 and FSA96 isolates of *S. aureus* revealed down regulation in (0.222211,0.255253 and0.239816) fold after treated with 2.0 mg/ml of tryptophan, respectively comparison with control .The finding of current study was identified significant difference($P<0.05$ - 0.0235) in folding expression of *agr1*gene between the control (without treated) and treated isolate as showed in table (7).

Table 7. Gene expression of *agr1* gene among tryptophan-treated isolates and non-treated isolates (control) of*S. Aureus*

Isolates	Means Ct	Means Ct of agr1	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$	*P*-value	Mean of differences	SD of differences
				Sensitive (Control)				
FSW61	18. 72	16. 35	2. 37	0	1			
FSW69	16. 9		0. 56	0	1			
FSW96	18. 36		2. 01	0	1			
Treated isolates				After treated with 2. 0 mg /L Tryptophan				
FSW61	20. 89	16. 35	4. 54	2. 17	0. 222211	0. 0039 Significant	-0. 7662	0. 08274
FSW69	18. 88		2. 53	1. 97	0. 255253			
FSW96	20. 42		4. 07	2. 06	0. 239816			

C: Control (*SrRNA* gene; HKG), ΔCt: Means Ct of *agr1* – Means Ct of HKG

According the result in Table (8), the relative expressions ($2^{-\Delta\Delta Ct}$) of *agr3*gene in FSW61, FSW69 and FSA96 isolates of *S. aureus* were down regulation in (0.348686, 0.231647 and 0.400535) fold after treated with 2.0 mg/ml of tryptophan, respectively compression with fold of control (1). And showed significantly differences ($P < 0.05$ - 0.0055) in all three isolates which effect on protein synthesis for this gene comparison with control (1).

Table 8. Gene expression of *agr3* genes among tryptophan-treated isolates and non-treated isolates (control) of *S. aureus*

Isolates	Means ct	Means ct of agr3	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$	*P*-value	Mean of differences	SD of differences
				Sensitive (Control)				
FSW61	18. 72		2. 37	0	1			
FSW69	16. 9	16. 35	0. 56	0	1			
FSW96	18. 36		2. 01	0	1			
Treated isolates				Aftertreatedwith2. 0 mg /L Tryptophan				
FSW61	20. 24		3. 89	1. 52	0. 348686	0. 0055		
FSW69	19. 02	16. 35	2. 67	2. 11	0. 231647	Significa nt	-0. 6730	0. 08652
FSW96	19. 68		3. 33	1. 32	0. 400535			

C: Control (*SrRNA* gene; HKG), ΔCt: Means Ct of *agr3* – Means Ct of HKG

The results document differences in bacterial gene expression throughout *S. aureus* infections. Ica AD transcripts level was very low related with low level of expression for QS genes which is affected when treated with tryptophan due to effect synthesis of protein, and a significantly decrease was then progressively observed in the *ica AD* gene that is responsible for biofilm formation. the expression of *ica AD* gene was associated with the expression of a QS genes. The findings of present study detect the expression of *ica AD* is controlled by quorum sensing systems. The findings of present study detect the expression of *ica AD* is controlled by quorum sensing several kinds of bacterial quorum sensing have been connected to biofilm development, an example of intercellular signalling. *S. aureus* has a quorum-sensing mechanism that is encoded at the accessory gene regulator (agr) locus (32). It is widely known that the agr system contributes to virulence in model biofilm-associated diseases such endocarditis and osteomyelitis, but the precise participation of the agr system differs depending on the infection model used (46). Several substances, both natural and synthetic, have been shown in studies to prevent the production of microbial biofilms. Tryptophan (an aromatic amino acid) has been shown to effectively suppress biofilm formation in *Pseudomonas aeruginosa* (36).

CONCLUSIONS

The increasing prevalence of illnesses associated with microbial biofilms poses significant threats to global public health. Biofilm attenuating agent/s research may give a long-term answer to this problem. Tryptophan was discovered to inhibit Staphylococcal cells and biofilms, according to the current study. Tryptophan, via interfering with microbial biofilm development, may be utilized to control the intensity of pathogenic.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received a fund.

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