



Antibiofilm Activity of Chalcone in Methicillin Resistant *Staphylococcus aureus*

Entesar Taha Al- Musawi¹ , Kamil M. Aljobori¹ , Harith Jabbar Al-Mathkhury²,
Khiaria Jaber³

¹Institute of Genetic Engineering and Biotechnology for Postgraduate Studies / University of Baghdad / Baghdad / Iraq.

²Department of Biology/ College of Science / University of Baghdad / Baghdad / Iraq.

³Ministry of Health / Ibn Baladi Hospital.

Received: November 18, 2019 / **Accepted:** December 23, 2019 / **Published:** December 31, 2019

Abstract: Methicillin-resistant *Staphylococcus aureus* (MRSA) is known as a key agent implicated in infections resulting in significant morbidity and mortality among patients. MRSA can develop resistant structure named biofilm. Among the various naturally occurring classes, chalcones are important intermediates for the biosynthesis of flavonoids. Therefore, the current study aimed to investigate in vitro the inhibitory effect of chalcone on MRSA planktonic cells and biofilm. Around 111 *Staphylococcus aureus* locally isolated from different clinical specimens were collected in this study. Methicillin resistance was evaluated by detection *mecA* amplification by PCR assay. Minimum inhibitory concentration (MIC) of chalcone was estimated by broth microdilution method. Moreover, Atomic Force Microscopy (AFM) technique was employed for confirming the effect of chalcone on biofilm. The present findings revealed that MRSA constituted, nearly, (93.22 %) (55 isolates) of all *S. aureus* isolates. Most of MRSA isolates were able to form biofilms, around 70.9% have the capacity to form strong biofilm. Chalcone at MIC (5 µg/ml) significantly reduced the planktonic MRSA cells. Whereas at Minimum Biofilm Inhibitory Concentration (MBIC) (20 µg/ml) and sub MBIC (15 µg/ml) reduced biofilm formation significantly and this findings are confirmed by using Atomic force microscopy. In conclusion, suggest that chalcone could be a new agent for prevention of bacterial adherence to surfaces and could be identified as anew agent against *S. aureus* infection.

Keywords: Methicillin-resistant *Staphylococcus aureus*, *mecA*, Chalcone.

Corresponding author: (Email: alzahraa113@gmail.com).

Introduction

Staphylococcus aureus is one of the most common causes of indwelling device-associated, nosocomial, and community-acquired infections (1). The ability of *S. aureus* to colonize the host and the capacity of this bacterium to exchange and obtain genetic information reflect its success as a versatile pathogen. This contributes to the fact that *S. aureus* strains can express a variety of virulence factors that play key roles in their spread and proliferation in its human and animal

hosts. It is noteworthy that a virulence factor can be multifunctional in pathogenesis (2,3).

Methicillin-resistant *S. aureus* (MRSA) is one of the major pathogens in hospitals and the community. MRSA shows drug-resistance to all beta-lactam antibiotics. Resistance is caused by a modified penicillin binding protein, PBP2a which is encoded by *mecA* gene (4-6).

Biofilm can be defined as a microbially-derived community, typified by cells that are attached to a

substratum, interface, or to each other, are embedded in a matrix of extracellular polymeric substance, and exhibit an altered phenotype with regard to growth, gene expression and protein production (7). The main advantages of biofilm for *S. aureus*, it protects the bacterium from being washed or scraped away to enhance the survival rate and it helps *S. aureus* to escape from the host defense (8). Impedes delivery of antibiotics which may cause impairment in wound healing, once

antibiotic regimens are halted, these persisters are able to spontaneously shift out of their quiescent state and produce a reactivation of infection(9).

Chalcone is an open-chain favonoid with α , β -unsaturated carbonyl group and is one of the important compound groups of favonoid derived from nature which are considered as precursors for the preparation of various favonoids and exhibit interesting pharmacological activities(10)(Figure 1).

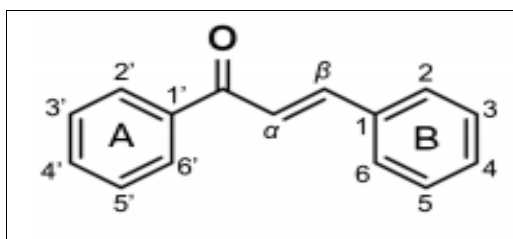


Figure (1): Structural and numerical representation of chalcone scaffold (11).

Chalcones and their synthetic derivatives possess extensive pharmacological properties, such as antihypertensive, antiplatelet, antidiabetic, antineoplastic, antiangiogenic, antiretroviral, antihistaminic, antitubercular, anti-invasive, and antiulcer properties (12, 13). It is worthwhile, the surface of *S. aureus* is decorated with proteins that are covalently anchored to the cell wall by sortases and then act important role in biofilm formation(14). During the process of secretion and anchoring to the cell wall peptidoglycan, studies suggests that plant natural product chalcone flavonoids have potential as sortase-specific oral biofilm inhibitors (15).

The AFM is a technique used to study microbial systems to provide a unique insight into their behavior and relationship with their environment (9). this research aim to study the impact of

chalcone on biofilm formation, determine MIC of chalcone on MRSA planctonic cells and MBIC of it on MRSA biofilm formation.

Materials and methods

Specimens collection

One hundred and eleven clinical specimens (wound swabs, mid-stream urine, blood, boils and abscesses swabs) were collected from National Center for Educational Laboratories, Ibn baladi and Imam Ali hospitals in Baghdad, for the period from May / 2018 to September /2018.

Isolation and identification of *Staphylococcus aureus*

All specimens were streaked on mannitol salt agar, as selective media as well as biochemical tests (16-18), and

then identification was confirmed by Vitek-2 Compact automated system.

Detection of methicillin resistance

Extracting and purifying of genomic DNA

Genomic DNA was extracted from *S. aureus* isolates by using a commercial Genomic DNA purification Kit

(Geneaid, Taiwan) according to instructions of the company. The DNA concentration and purity were determined using Nanodrop instrument.

Detection of *mecA*

In this study MRSA isolates were identified by molecular method using specific primer pair (Table 1).

Table (1): The sequence of primer and PCR Product Length used in the conventional PCR.

Primer	Sequence (5'-3')	Product size (bp)	Reference
<i>mecA</i>	F TGG CTA TCG TGT CAC AAT CG R CTG GAA CTT GTT GAG CAG AG	309 bp	(18)

PCR mixture was set up in a total volume of 25µl included 12.5 µl of Green Master Mix (1X) (promega), 2 µl of each primer (10 µM/ µl) and 3 µl of template DNA (50 ng/µl) have been

used. The rest volume was completed with free nuclease water. The adopted PCR protocol is demonstrated in Table (2).

Table (2): PCR Program for the amplification of *mecA* (19).

No.	Steps	Temperature (°C)	Time	cycles
1.	Pre-Denaturation	94°C	5min	1
2.	Denaturation	94°C	30 sec	30
3.	Annealing	52°C	30 sec	
4.	Extension	72°C	30 sec	
5.	Final extension	72°C	7 min	1

Biofilm formation assay

Quantification of biofilm formation by MRSA on abiotic surfaces was assessed by microtiter plate method as previously described by (20). In brief; 10 µl of the bacterial solution isolates, after adjusted turbidity to 0.5 McFarland standard (1.5×10^8 CFU/ml), was added into the 96-well flat-bottom polystyrene microtiter plates containing 290 ml of Brain-Heart infusion broth (BHI) and 3% (w/v) sucrose. The mixture was incubated for 18 hr at 37°C. After incubation, the liquid containing the bacteria and medium was removed, 100 µl of 10%

formaldehyde solution was added, left overnight at room temperature to fix the biofilm. Subsequently, the formaldehyde was removed, and each well was stained with 100 µl of 1% crystal violet for 30 min at room temperature. After rinsing with double distilled water and drying, 200 µl of 33% acetic acid was added to each well. The absorbance of the plates was subsequently read at 490 nm. All assays were carried out in triplicates. Classification of bacterial adherence summarized in Table 3, based on OD₄₉₀ values obtained for individual isolate of MRSA was used for the purpose of data simplification and calculation.

Table (3): Classification of bacterial adherence by microtiter plate method (MTP) (21).

Mean OD ₄₉₀	Biofilm intensity
OD ≤ OD _c *	Non-adherent
2OD _c > OD > OD _c	Weak
4 OD _c > OD > 2OD _c	Moderate
OD > 4 OD _c	Strong

*Cut off value (OD_c)= average OD of negative control + (3 *Standard Deviation).

Effect of Chalcone on Planktonic Cells

To determine the efficacy of chalcone on MRSA, broth microdilution method was employed in order to estimate the Minimum Inhibitory Concentration (MIC). Briefly, individual wells of sterile, polystyrene 96-well-flat bottom plates were filled with 50 µl of MRSA culture (being grown in BHI broth to an OD₆₀₀ of 0.6), 50 µl of BHIB and 100 µl of ten different concentrations of chalcone by using DMSO as a solvent (5, 10, 15, 20, 25, 30, 35, 40, 45, 50 µg/ml), incubated overnight, afterward, absorbance was read at 600 nm. All assays were carried out in triplicates (20,22).

Effect of Chalcone on MRSA biofilm

To determine the Minimum Biofilm Inhibitory Concentration (MBIC) and sub MBIC of chalcone on strong biofilm producing MRSA isolates, the same protocol performed for Planktonic

cells was followed, and then from fixing step, the aforementioned protocol in Biofilm formation assay was followed.

Atomic force microscopy

The isolate with the strongest biofilm formation capacity was treated with chalcone at sub MBIC. The same protocol described by (11) was followed for the biofilm formation assay, except for staining step that was skipped and then send to examination by Atomic force microscopy (Unico, USA).

Results and Discussion

Upon the results summarized in Table 4, only fifty-nine isolates (53.15%) were identified as *S. aureus* out of 111 collected clinical specimens. This primary identification was confirmed by Vitek 2 compact system (BioMerieux, France).

Table (4): Biochemical tests and their results of *Staphylococcus aureus*.

Test	<i>Staphylococcus aureus</i>	Other <i>Staphylococcus spp.</i>
Mannitol fermentation	Yellow colonies	Pink colonies without changing the color of the medium
Gram stain	Gram positive cocci	Gram positive cocci
Coagulase	+	-
Catalase test	+	+
Oxidase	-	+

+ and – denote to positive and negative results, respectively

Detection of *mecA*

DNA extracted from all isolates of *S. aureus* by DNA extraction kit (Geneaid, Taiwan). The extracted DNA

was confirmed and analyzed by the horizontal gel electrophoresis, where the DNA appears as compact bands, as shown in Figure (1).

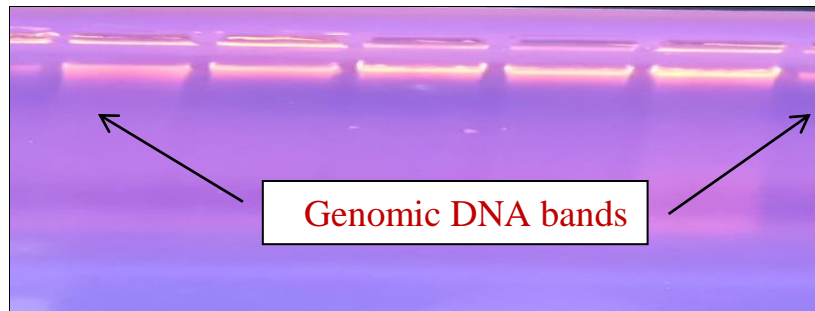


Figure (1): Gel electrophoresis of DNA extraction of *s. aureus* isolates using 1% Agarose for 45 min. at 100 volt.

Polymerase chain reaction technique used to detect *mecA* gene to identify MRSA. In this study, around 55

(93.22%) MRSA out of 59 *S. aureus* isolates were detected (Figure 2).

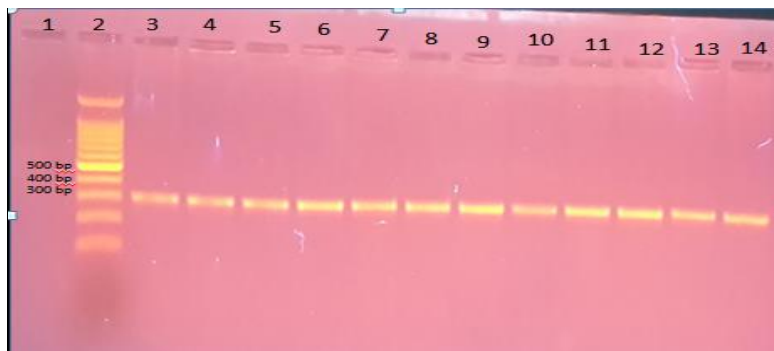


Figure (2): Agarose gel electrophoresis of PCR amplification products of *S. aureus*, *mecA* gene (309pb) at 1.5% agarose, 75 volts for 90 min. lane 1 represents ladder 100 bp. Lanes 2-13 represent *mecA* positive isolates.

The accurate and early determination of methicillin resistance is of key importance in the prognosis of infections caused by *S. aureus*. Although multiple methods of detection of this resistance have been developed, they are often too slow or not sufficiently sensitive or specific to ensure appropriate treatment of the MRSA-infected patients. Identification of the *mecA* gene is the most reliable method of detecting MRSA isolates

(23-25). *mecA* is the novel coding gene of penicillin-binding protein (PBP) 2a of MRSA, which is the key resistance factor of β -lactam (26).

Biofilm forming capacity

In the present work, MRSA isolates exhibited a wide spectrum of biofilm-forming capacities. Of interest, findings of this study revealed that most of isolates (70.9%) have the capacity to form strong biofilm (Table 5).

Table (5): Biofilm intensity of MRSA based on estimated cutoff value*of this study.

ID	Biofilm intensity	No. of isolates	Percentage
1	Non-biofilm producer	2	3.63%
2	Weak	5	9.09%
3	Moderate	9	16.36%
4	Strong	39	70.9%

cutoff value = 0.2712 (defined as the Mean of control OD₄₉₀ plus 3 Standard deviation).

Biofilm production is considered as a marker of virulence; several new methodologies have been recently developed for biofilm studies that have contributed to deeper knowledge on biofilm physiology, structure and composition (27).

Al-Musawi (28) referred that microtiter plate method (MTP) was considered the gold-standard test for the detection of biofilm formation, when compared with data from the test tube and Congo red agar methods.

Phenotypic Effect of Chalcone on MRSA isolates

Estimation of Chalcone minimum inhibitory concentration

The alarming increase in antibiotic-resistant *S. aureus* advocates the need to search for novel anti-infective drugs. Treatment of chalcone for overnight resulted in an inhibition in planktonic growth at 5 µg/ml as shown in figure 3. This MIC was insignificantly ($P < 0.01$) different from the higher concentrations (Figure 3).

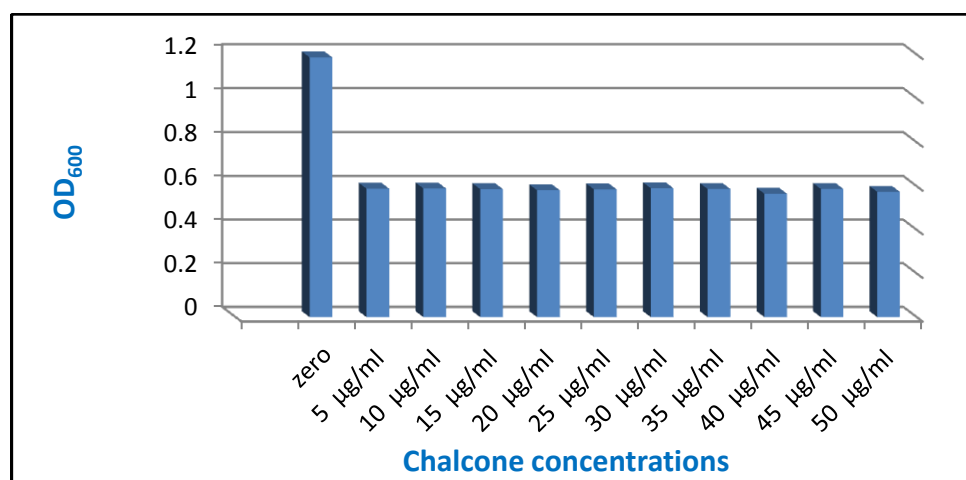


Figure (3): Effect of chalcone on growth of MRSA planktonic cells.

Mori *et al.* (29) demonstrated that flavonoids inhibit DNA synthesis and they can also affect RNA synthesis in *S. aureus*, they noted that many enzymes involved in metabolism of macromolecules (DNA, RNA, carbohydrates and proteins) can also be affected in *S. aureus*.

Effect of Chalcone on MRSA Biofilm Formation

The biofilm formation was inhibited at low concentrations of chalcone, at 20 µg/ml. Obviously, higher concentrations insignificantly ($P < 0.01$) differed from this concentration (Figure 4).

These concentrations of chalcone are very safe considering if we take into account that LD₅₀ of chalcone in male Albino-Swiss mice is 1048 mg/ kg (30).

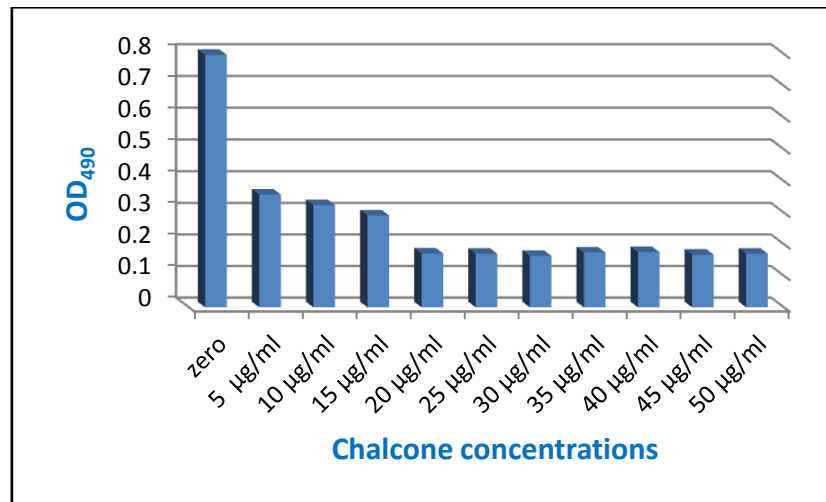


Figure (4): The effect of chalcone on biofilm formation by MRSA.

El-Messery *et al.*(31) reported that increased antibiotic tolerance has been promoted by biofilm formation to levels 1000 times greater than those observed in planktonic bacteria, and suggested that chalcone compounds could be used as good orally absorbed anti-biofilm agents. Cushnie and Lamb (32) reported that the inhibition of biofilm formation may be due to flavonoids.

Atomic force microscopy (AFM) examination of MRSA biofilm

Chalcone at 15 µg/ml reduced biofilm intensity to 36.58 % in MRSA

isolates, which was illustrated by AFM, that demonstrated the morphological differences between the control and chalcone treated isolate. The AFM image depicted in Figure 5, enlightens that the chalcone inhibits biofilm formation appeared as a reduction in tower heights (from 17.75 nm down to 5.73 nm). Moreover, the average of surface roughness analysis revealed that chalcone significantly reduced the roughness average of biofilm in comparison to the untreated biofilm; from 2.12 mm down to 1.18 mm, respectively.

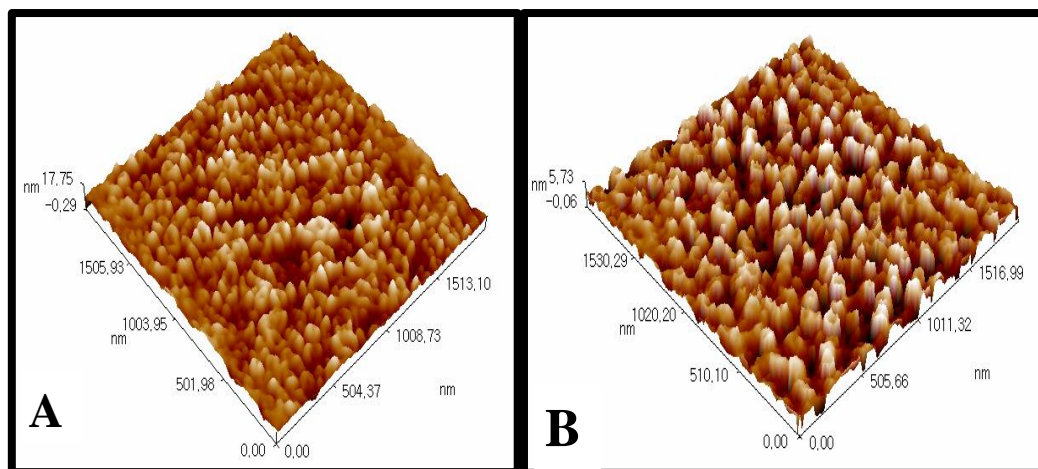


Figure (5): Atomic force micrograph of methicillin-resistant *S. aureus* biofilm. A- without chalcone and B- with chalcone.

Several microbiological studies have employed the AFM to examine the bacterial cells, biofilm and the effect of antibiotics on bacterial cells. Jaddoa and Al-Mathkhury (33) reported that the gentamicin reduced the height of biofilm towers of MRSA. Bazari *et al.* (34) elucidate that AFM is a useful tool to observe bacterial biofilm formation in their study about regarding the association between intercellular polysaccharides layer and biofilm aggregation of *S. aureus* isolates. While Neethirajan and DiCicco (35) reveal that the fosfomycin inhibits cell division, and prevents the adhesion on the surface discouraging the biofilm attachment on methicillin-resistant *Staphylococcus psudintermedius* by using AFM image analysis.

Conclusions

Findings of this study revealed that chalcone significantly reduced the planktonic MRSA cells. Whereas at MBIC and sub MBIC reduced biofilm formation significantly. Hereby, chalcone could be a new agent for prevention of bacterial adherence to surfaces.

References

1. Kirmusaoğlu, S. (2017). MRSA and MSSA: The Mechanism of Methicillin Resistance and the Influence of Methicillin Resistance on Biofilm Phenotype of *Staphylococcus aureus*, World's largest Science (chapter), Technology & Medicine Open Access book publisher, Chapter 2 FROM the book The Rise of Virulence and Antibiotic Resistance in *Staphylococcus aureus*.
2. Decker, C. (2008). Pathogenesis of MRSA infections. *Dis. Mon.*, 54:774-9.
3. Gordon, R. J. and Lowy, F. D. (2008). Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection. *Clin. Infect. Dis.*, 46(Suppl 5): S350–S359.
4. Monecke, S.; Muller, E.; Schwarz, S.; Hotzel, H. and Ehricht, R. (2012). Rapid microarray based identification of different *mecA* alleles in *Staphylococci*. *Antimicrob Agents Chemother*, 56: 5547–5554.
5. Stefani, S.; Campanile, F.; Santagati, M.; Mezzatesta, M.L.; Cafiso, V. and Pacini, G. (2015). Insights and Clinical Perspectives of Daptomycin Resistance in *Staphylococcus aureus*: A Review of the Available Evidence. *International Journal of Antimicrobial Agents*, 8: 1-12.
6. Alagely, H.S.; AL-hashimy, A.B. and Majeed, K.R. (2019). Characterization of Five Types of Staphylococcal Cassette Chromosomal *mec* Genes in Methicillin-Resistant *Staphylococcus aureus* (MRSA) Isolates from Iraqi Patients. *Iraqi Journal of Biotechnology*, 18(2):289-299.
7. Donlan, R.M. and Costerton, J.W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev.*, 15:167–193.
8. Otto, M. (2013). Staphylococcal Infections: Mechanisms of Biofilm Maturation and Detachment as Critical Determinants of Pathogenicity. *Annual Review of Medicine*, 64: 175-188.
9. Gurjala, A.N.; Geringer, M.R.; Seth, A.K. *et al.* (2011). Development of a novel, highly quantitative in vivo model for the study of biofilm-impaired cutaneous wound healing. *Wound Repair Regen*, 19(3): 400–410.
10. Božić, D.D.; Milenković, M.; Ivković, B. and Cirković, I. (2014). Newly-synthesized chalcones-inhibition of adherence and biofilm formation of methicillin-resistant *Staphylococcus aureus*. *Braz.J. Microbiol.*, 45: 263–270.
11. Gomes, N.; Muratov, E.N. ; Pereira, M.; , Josana C. Peixoto, J.C.; Rosseto, L.P. *et al.* (2017). Chalcone derivatives: promising starting points for drug design marcelo. *Molecules*, 22(8): 25.
12. Mahapatra, D.K.; Bharti, S.K. and Asati, V. (2015). Chalcone scaffolds as anti-infective agents: Structural and molecular target perspectives. *Eur. J. Med. Chem.*, 11:496–524.
13. Özdemir, A.; Altıntop, M.D.; Sever, B.; Gençer, H.K.; Kapkaç, H. A.; Atli ,O. *et al.* (2017). A new series of pyrrole-based chalcones: synthesis and evaluation of

- antimicrobial activity, cytotoxicity, and genotoxicity. *Molecules*, 22: 2112,16 pages.
14. Foster, T. J.; Geoghegan, J. A.; Ganesh, V. K. and Höök, M. (2014). Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. *Nat. Rev. Microbiol*, 12:49–62.
 15. Wallock-Richards, D.J.; Marles-Wright, J; Clarke, D.J.; Maitra, A.; Dodds, M.; Hanley, B., *et al.* (2015). Molecular basis of *Streptococcus mutans* sortase A inhibition by the flavonoid natural product trans-chalcone. *Chem. Commun.*, 51: 10483-10485.
 16. MacFaddin, J. F. (2000). *Biochemical tests for identification of medical bacteria*, 3rd ed. Lippincott Williams and Wilkins, Philadelphia, PA.
 17. Rasigade, J. and Vandenesch, F. (2014). *Staphylococcus aureus*: A pathogen with still unresolved issues. *Infection, Genetics and Evolution*, 21: 510-514.
 18. Tong, S.Y.C. ; Davis, J.S.; Eichenberger, E.; Holland, T.L. and Fowler, V.G. (2015). *Staphylococcus aureus* infections: Epidemiology, pathophysiology, clinical manifestations, and management. *Clinical Microbiology Reviews*, 28(3): 604-660.
 19. Prates, K.A. ; Torres, A.M.; Garcia, L.B.; Ogatta, S.F. ; Cardoso, C.L. and Tognim, M.C. (2010). Nasal carriage of methicillin-resistant *Staphylococcus aureus* in university students. *Braz J Infect Dis.*, 14:316-8.
 20. Zhang, B.; Teng, Z.; Li, X.; Lu, G.; Deng, X.; Niu, X. and Wang, J. (2017). Chalcone Attenuates *Staphylococcus aureus* Virulence by Targeting Sortase A and Alpha-Hemolysin. *Front. Microbiol*, 8:1715.
 21. Atshan, S.S.; Shamsudin, M.N.; Sekawi, Z.; Lung, L.T. Hamat, R.A.; Karunanidhi, A., *et al.* (2012). Prevalence of adhesin and regulation of biofilm-related genes in different clones of *Staphylococcus aureus*. *J Biomed Biotechnol.*, 6: 1-10.
 22. Balamurugan, P.; Krishna, V.P.; Bharath, D.; Lavanya, R.; Vairaprakash, P. and Princy, S.A. (2017). *Staphylococcus aureus* Quorum Regulator SarA Targeted Compound, 2-[(Methylamino)methyl]phenol Inhibits Biofilm and Down-Regulates Virulence Genes. *Frontiers in Microbiology*, 8: 1290.
 23. Nasution, G.S.; Suryanto, D. and Kusumawati, R.L. (2018). Detection of *mecA* gene from methicillin resistant *staphylococcus aureus* isolates of north sumatera, IOP Conf. Series: Earth and Environmental Science, 130(1) :12026.
 24. Cikman, A.; Aydin, M.; Gulhan, B.; Karakeçili, F.; Kurtoglu, M.G.; Yuksekkaya, S., *et al.* (2019). Absence of the *mecC* gene in methicillin-resistant *Staphylococcus aureus* isolated from various clinical samples: The first multi-centered study in Turkey. *J Infect Public Health*. 12(4): 528-533.
 25. Nwaogaraku, C.N.; Smith, S.I. and Badaki, J. A. (2019). Non detection of *mecA* gene in methicillin resistant *Staphylococcus aureus* isolates from pigs. *African Journal of Clinical and Experimental Microbiology*, 20(2): 159-163.
 26. Zhan, X.Y. and Zhu, Q.Y. (2018). Evolution of methicillin-resistant *Staphylococcus aureus*: Evidence of positive selection in a penicillin-binding protein (PBP) 2a coding gene *mecA*. *Infect Genet Evol.* 59:16-22.
 27. Azeredo, J.; Azevedo, N.F. ; Briandet, R.; Cerca, N.; Coenye, T. Costa, A.R., *et al.* (2017). Critical review on biofilm methods. *Critical Reviews in Microbiology*, 43(3): 313-351.
 28. Al- Musawi, H.T. (2018). The Effects of Iron Oxide Nanoparticles on Gene Expression for Biofilm Formation Genes of *Acinetobacter baumannii* Isolated from Clinical Samples. PhD Thesis. Genetic Engineering and Biotechnology Institute for Postgraduate Studies/University of Baghdad.
 29. Mori, A.; Nishino, C.; Enoki, N. and Tawata, S. (1987). Antibacterial activity and mode of action of plant flavonoids against *Proteus vulgaris* and *Staphylococcus aureus*. *Phytochemistry*, 26 :2231-2234.
 30. Alonso, J.; Navarro, E. (1993). Estudio toxicológico de una chalcona sintética ; Report to the Instituto Universitario de BioOrgánica., 13 pp.
 31. El-Messery, S.M.; Habib, E.E.; Al-Rashood, S.T.A. and Hassan, G.S. (2012). Synthesis, antimicrobial, anti-biofilm evaluation and molecular modelling study of new chalcone linked amines derivatives. *Journal of Enzyme Inhibition and Medicinal Chemistry*, 33(1): 818-832.
 32. Cushnie, T.P. and Lamb, A.J. (2011). Recent advances in understanding the antibacterial properties of flavonoids. *Int J Antimicrob Agents*, 38(2):99-107.
 33. Jaddoa, N. and Al-Mathkhury, H. J. F. (2018a). Biofilm Shows Independency form Hemolysin Genes Arsenal in Methicillin

- Resistant *Staphylococcus aureus*. Iraqi Journal of Science, [S.l.] :2184-2194.
34. Bazari, P.A.M.; Jahromy, S.H. and Karizi, S.Z. (2017). Phenotypic and genotypic characterization of biofilm formation among *Staphylococcus aureus* isolates from clinical specimens, an atomic force microscopic (AFM) study. Microb Pathog., 110:533–9.
 35. Neethirajan, S. and DiCicco, M. (2014). Atomic force microscopy study of the antibacterial effect of fosfomycin on methicillin-resistant *Staphylococcus pseudintermedius*. Applied Nanoscience, 4(6): 703–709.