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EFFECT OF CHALCONE ON THE FORMATION OF BIOFILMS AND EXPRESSION OF VIRULENCE GENES IN METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

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ABSTRACT : MRSA is one of the major pathogens in hospitals and the community, which have the ability to produce biofilm as a virulence factor, the impact of chalcone on biofilm formation, the synergism effect of chalcone and antibiotic in both *in vitro* and *in vivo* experiments, the gene expression of virulence genes (*srtA*, *fnbA*, *fnbB*) before and after treatment of it on MRSA biofilm cells *in vitro*, all these were the prime aims of this study. Chalcone at MBIC (20 µg/ml), significantly reduced the biofilm formation to 21.45% and at sub MBIC (15 µg/ml) to 36.58 %. While, Chalcone at MIC(5 µg/ml) reduced MRSA planktonic cells to 49.61%. Susceptibility of MRSA isolates against eight antibiotics showed that all isolates were sensitive to vancomycin and none of the isolates developed susceptibility to erythromycin. The combinatorial effect of chalcone at 5 µg/ml and vancomycin at MIC of (1 µg/ml) on MRSA planktonic cells was reduced it from 70 to 23.3% , and in combination with erythromycin at 32 µg/ml, was decreased from 53.1% to 22% and the effect of chalcone at sub MBIC (15 µg/ml) when combined with vancomycin was reducing the biofilm formation from 87% to 27.6 and with erythromycin from 55.1% to 23.8%. Combinatorial phenotypic effect of the antibiotics and chalcone (at sub MBIC), *in vitro* came in line with the result of *in vivo* experiment and the results showed decrease in the expression of *fnbA*, *fnbB* and *srtA* genes in tested isolates in the presence of chalcone at sub MBIC. In our study, we demonstrated that chalcone exhibited significant effect in biofilm formation of MRSA strains, which can be considered as promising antimicrobial agents that can be used for prevention of MRSA adherence or as adjunct to antibiotics in conventional therapy.

Key words : MRSA, chalcone, biofilm, *fnbA*, *fnbB* and *srtA* genes.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of hospital-acquired infections in the world. MRSA is one of the common threatening pathogens due to its multi-drug resistance and strong biofilm-production capacity (Vaishampayan *et al*, 2018). *mecA* is the novel coding gene of penicillin-binding protein (PBP) 2a of MRSA, which act as a key for resistance factor of β-lactams (Zhan and Zhu, 2018).

Bacterial cells, in order to colonize biotic and abiotic surfaces, initially produce large amounts of factors which facilitating the interaction with host extracellular ligands (Kot *et al*, 2018) and develop biofilm that define as a multilayered structure comprising of bacterial communities embedded into the extracellular hydrated polymeric matrix (Paharik and Horswill, 2016). In most Gram-positive bacteria Sortase A encoded by *srtA* gene is a cysteine transpeptidase enzyme, this enzyme is responsible for the anchorage of many surface protein virulence factors to the bacterial cell wall layer (Liu *et al*, 2015; McCarthy *et al*, 2015). FnBPA and FnBPB are important adhesins for *S. aureus* to fibrinogen, elastin and fibronectin. Both proteins (FnBPA and FnBPB) are coded by two closely linked genes, *fnbA* and *fnbB*, respectively (Shinji *et al*, 2011; Murai *et al*, 2016).

It's necessary to develop new strategies to inhibit MRSA biofilm prodiction. Chalcones or (E)-1,3-diphenyl-2-propene-1- ones, it is an important intermediates for the biosynthesis of flavonoids, which have a wide range of biological activities, chalcone structures reveal nowadays interesting perspectives in various clinical fields which have high pharmacological activities (Koudokpon *et al*, 2018; Verma *et al*, 2018). This research aim to study the impact of chalcone on biofilm formation and the role of it on the expression of *fnbA*, *fnbB* and *srtA* genes in biofilm cells.

MATERIALS AND METHODS

Specimen's collection

One hundred and eleven clinical specimens were collected Baghdad hospitals. The identification was depending on microscopic examination after Gram staining, culturing on selective media (Mannitol salt agar), as well as biochemical tests along with Vitek-2 tests.

Biofilm formation assay

Quantification of biofilm formation by MRSA on abiotic surfaces was assessed bymicrotiter plate method as previously described by Zhang *et al* (2017). In brief; 10 ìl 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 ìl 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 ìl of 1% crystal violet for 30 min at room temperature. After rinsing with double distilled water and drying, 200 ìl 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.

Antibiotic susceptibility

Disk diffusion method : Antibiotic susceptibility assay was performed to determine the drug resistance of MRSA to various antibiotics (Ciprofloxacin, Clindamycin, Gentamicin, Erythromycin, Levofloxacin, Tetracycline and Trimethoprim), while Vancomycin susceptibility was determined by agar dilution method. 100 µl of bacterial suspension of each isolate was swabbed onto Mueller-Hinton agar plates and left to dry for 10 min. Antibiotic disks were placed on the media plates and incubated for 24 hrs. at 37°C. After the incubation period,the inhibition zone was measured and interpreted in accordance to the guidelines of Clinical Laboratory Standards Institute (Balamurugan *et al*, 2017).

Agar Dilution method : Agar dilution test is the reference methods for antimicrobial susceptibility testing. For the agar dilution method, MRSA suspensions were adjusted to a turbidity equivalent to a 0.5 McFarland standard and 10 µl of these suspensions was inoculated onto Mueller-Hinton agar containing a twofold dilution series of antibiotics (Nayef, 2016). The results were interpreted in accordance to the guidelines of Clinical Laboratory Standards Institute 2016.

Determination the Minimum Inhibitory Concentration (MIC) : The MIC of the selected antibiotics (in which MRSA bacteria showed sensitivity and resistance) was determined by agar dilution method.

Combinatorial tests of chalcone and antibiotics : TheMinimum Inhibitory Concentration (MIC), MBIC (Minimum biofilm inhibitory concentration) and sub MBIC were assessed as described by Zhang *et al* (2017). The combinatorial studies were carried out between selected antibiotics (Sensitive and Resistant) and chalcone. MRSA isolates were cultured in Brain-Heart infusion broth (BHIB) along with chalcone (at the fixed MIC, MBIC and sub MBIC concentration) and MIC of antibiotics, the same protocol of Zhang *et al* (2017) was followed.

In vivo study

For the establishment of skin infections in murine model, 20 male mice aged 6-8 weeks and weighing 25±1 g were used, hair was shaved on the back of mice with 12 scalpel blade for one cm long until a red area appears (just below the blood draw). Wound blood is removed with sterile cotton. 50 ìl of the bacterial suspension was adjusted to 0.5 McFarland standards ($1\text{-}1.5 \times 10^8$ CFU/ml) and added to the wound sites. Presence of gray-white abscesses was used to confirm the occurrence of bacterial infection (Chung *et al*, 2017). Animals were divided into four groups (5 per each):

- **Group A :** control positive which received only MRSA isolate
- **Group B :** control negative which received 150µl of chalcone at sub MBIC (15 µg/ml).
- **Group C :** Animals treated with 150µl of erythromycin at MIC.
- **Group D :** Animals treated with sub MBIC of chalcone and erythromycin at MIC.

Quantitative Real-time PCR Assay (qRT-PCR) : MRSA isolates were grown in BHIB in the presence and absence of chalcone at sub MBIC, incubation overnight at 37°C. RNA was extracted from biofilm cells of MRSA isolates byRNA extraction kit (GENEzol Tri RNA Pure Kit-Geneaid, Taiwan). cDNA was synthesized using HiSenScript™ RH(-) RT PreMix kit by adding 5 ìl of RNA to RT premix tube then volume was completed up to 20 ìl with nuclease free water. Subsequently,the reaction step was Reverse Transcription at 50°C at 1hr and inactivation of samples at 85°C for 10 min. In order to assess the gene expression of the target genes, the results were normalized using *16SrRNA*. All Primers

Table 1 : The primers used in this study.

Primer	Sequence (5´-3´)	Reference	Annealing temperature (°C)
16SrRNA	F GCT GCC CTT TGT ATT GTC	Ming *et al* (2017)	60°C
	R AGA TGT TGG GTT AAG TCC		
srtA	F TCG CTG GTG TGG TAC TTA TC	Schwan *et al* (2017)	55°C
	R CAG GTG TTG CTG GTC CTG GA		
fnbA	F CCA GGT GGT GGT CAG GTT AC	Yin *et al* (2017)	60°C
	R TGT GCT TGA CCA TGC TCT TC		
fnbB	F ACG CTC AAG GCG ACG GCA AAG	Pereyra *et al* (2016)	60°C
	R ACC TTC TGC ATG ACC TTC TGC ACC T		

listed in Table 1. This process was undertaken on the Luna Universal qPCR Master Mix kit using SYBR green fluorescent dye. The reaction mixture in a totalvolume of 20ì l, consisted 10ì l of Luna Universal qPCR Master Mix, forward and reverse primers (1 ì l each), 4 ì l of nuclease free water and 4 ì l of cDNA. Thermocycler Program for Quantitative RT-qPCR conditions included an initial denaturation at 95°C for 10 mins, followed by 40 cycles of denaturation (95°C for 15 sec), annealing (55 °C for *strA*, 60°C for *fnbA*, *fnbB* and *16SrRNA*) for 20 sec and Melting curve analysis at 60-95°C for 1 sec / step.

Statistical analysis

The differences were analyzed using the Statistical Analysis System (SAS, 2012) program. The statistical significance was assessed using Least Significant Difference –LSD test and the differences were considered statistically significant when P < 0.01.

RESULTS

Identification of MRSA and Biofilm formation

Fifty-five isolates were identified as MRSA out of 111 collected clinical specimens. This study revealed that most of isolates (70.9%) have the capacity to form strong biofilm. Nonetheless, isolates No. Sa 9, Sa 27, Sa 31, Sa 35 and Sa 38 developed the strongest biofilm; hence, they were selected for further experiments.

Antibiotic susceptibility

Disk diffusion

The results revealed that often MRSA clinical isolates have a high level of resistance to some antibiotics as presented in Table 2. Noticeably, isolates varied in their susceptibility to the indicated antibiotics.

Agar Dilution method

The results showed sensitivity of all isolates to vancomycinat 2 µg/ml, Agar dilution test was also used to estimate the MIC for erythromycin. Even though, this antibiotic developed variable results by the disk diffusion

Table 2 : The susceptibility (Resistance, Intermediate and Sensitive) for MRSA isolates against antibiotics.

Isolation No.	CN	CIP	TE	TMP	E	DA	LVX
Sa 9	R	R	S	R	R	S	R
Sa 27	S	S	R	S	R	S	S
Sa 31	S	S	R	R	R	S	S
Sa 35	R	R	S	R	I	S	S
Sa 38	I	S	R	R	I	S	S

R, I and S denoted to Resistance, Intermediate and Sensitive, respectively.

assay ranging from resistance to intermediate resistance, none of isolates showed sensitivity to this antibiotic, as shown in Table 2. While by Agar dilution test, all isolates showed resistance to erythromycin with concentration 64 µg/ml.

Combinatorial studies of vancomycin or erythromycin with chalcone

Planktonic MRSA

Findings of this work established that there was a minor effect of vancomycin (30%) on planktonic cells; whereas erythromycin exhibited a moderate influence (46.8%) in comparison to zero concentration. Further, effect of chalcone with an antibiotic at MIC (1 µg/ml for vancomycin and 32 µg/ml for erythromycin) against MRSA was tested by combinatorial usage of both in a 96-well microtiter plates. Both antibiotics showed a significant reduction (P< 0.01) in growth of planktonic MRSA when used in combination with 5 µg/ml of chalcone as shown in Fig. 1 and Table 3. Notably, the study isolates developed significant variation (P< 0.01) when treated with antibiotic alone; nevertheless, such variation was absent in combinatorial treatment.

MRSA Biofilm

The effect of vancomycin and erythromycin individually and combinatorial effect with chalcone on biofilm formation was investigated. The results of the present study are summarized in Table 4, which revealed an interesting insignificant differences (P < 0.01) regarding

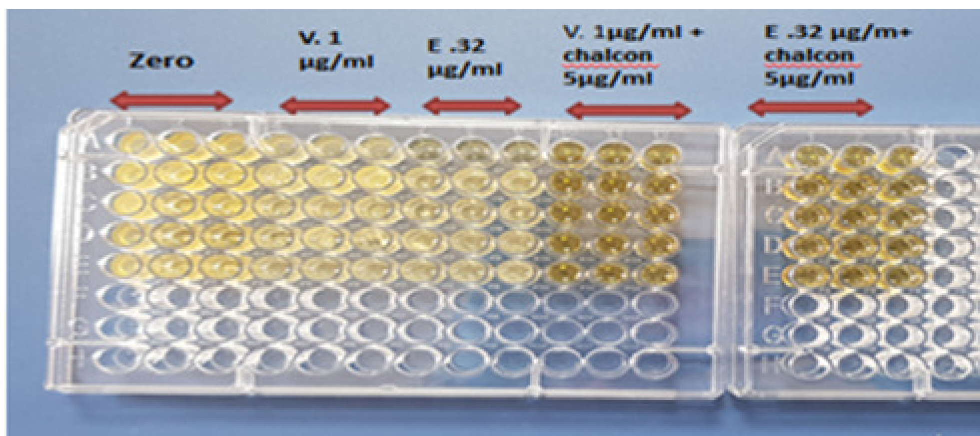

Fig 1 : The combinatorial effect of the antibiotics and chalcone on planktonic cells. V and E stand for vancomycin and erythromycin, respectively.

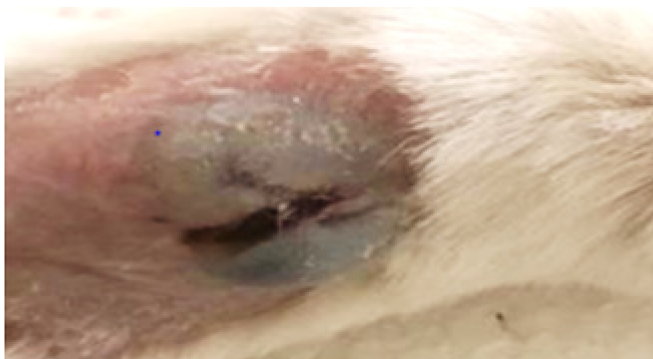

Fig. 2 : contaminated wounds on mouse skin by MRSA isolate.

to combinatorial effect. Such finding suggests the possibility of using sub MBIC of chalcone with the same effect of MBIC to reduce biofilm formation. Nevertheless, chalconepresence significantly influences the biofilm inhibition in comparison to control or to individual antibiotic treatment.

In vivo study

For wound healing experiments, the study was conducted on 20 mice in four groups, five for each group. Each wound was inoculated with MRSA. Presence of gray-white abscesses was used to confirm the occurrence of bacterial infection,that appeared three days post experimental infection, as shown in Fig. 2.

All groups of a mouse model of MRSA infection were treated except the first group. The treatment period lasted 16 days. During this period; 3, 2, 1 and 1 death were recorded in mice groups A, B, C and D, respectively. The survived mice examined via routine histological analyses Fig. 3, the results were as follows:

Group A (control positive which received only MRSA isolate)

The sections showed that the epidermis was destroyed and covered by a thick fibrin clot. There was heavily invasion of clot with mononuclear leukocytes and there were no signs of re-epithelization. The dermis was noticed as very thick layer with marked fibrogenesis; given that, it revealed much of fibroblasts and fibrocytes with immature collagen bundles without sebaceous glands and hair follicles (Figure 3a).

Group B (control negative which received chalcone at sub MBIC)

The sections showed that the epidermis appeared normally and composed of a thin of non-keratinized stratified squamous epithelium. The dermis revealed irregular dense collagenous connective tissue and had more sebaceous glands and hair follicles (Fig. 34a, b).

Group C (Mice treated with erythromycin at MIC)

The sections showed that the epidermis composed of a thick non-keratinized stratified squamous epithelium. The dermis was very thick layer constituting of irregular dense collagenous connective tissue and had sebaceous

Table 3 : The effect of antibiotics alone and in combination with chalcone on MRSA planktonic cells.

Antimicrobial (concentration)	Sa 9	Sa 27	Sa 31	Sa 35	Sa 38	Percentage	LSD
None	0.804	0.872	1.413	1.446	1.148	100%	0.218**
Vancomycin (1 µg/ml)	0.546	0.762	1.197	0.919	0.594	70%	0.278**
Erythromycin (32 µg/ml)	0.384	0.672	0.668	0.716	0.581	53.20%	0.176**
Vancomycin (1 µg/ml) + chalcone (15 µg/ml)	0.262	0.274	0.279	0.270	0.265	23.30%	NS
Erythromycin (32 µg/ml) + chalcone (15 µg/ml)	0.249	0.267	0.269	0.263	0.252	22.00%	NS
LSD	0.0371**	0.2118**	0.1531**	0.2071**	0.1096**		—

** (P<0.01), NS: Non-Significant.

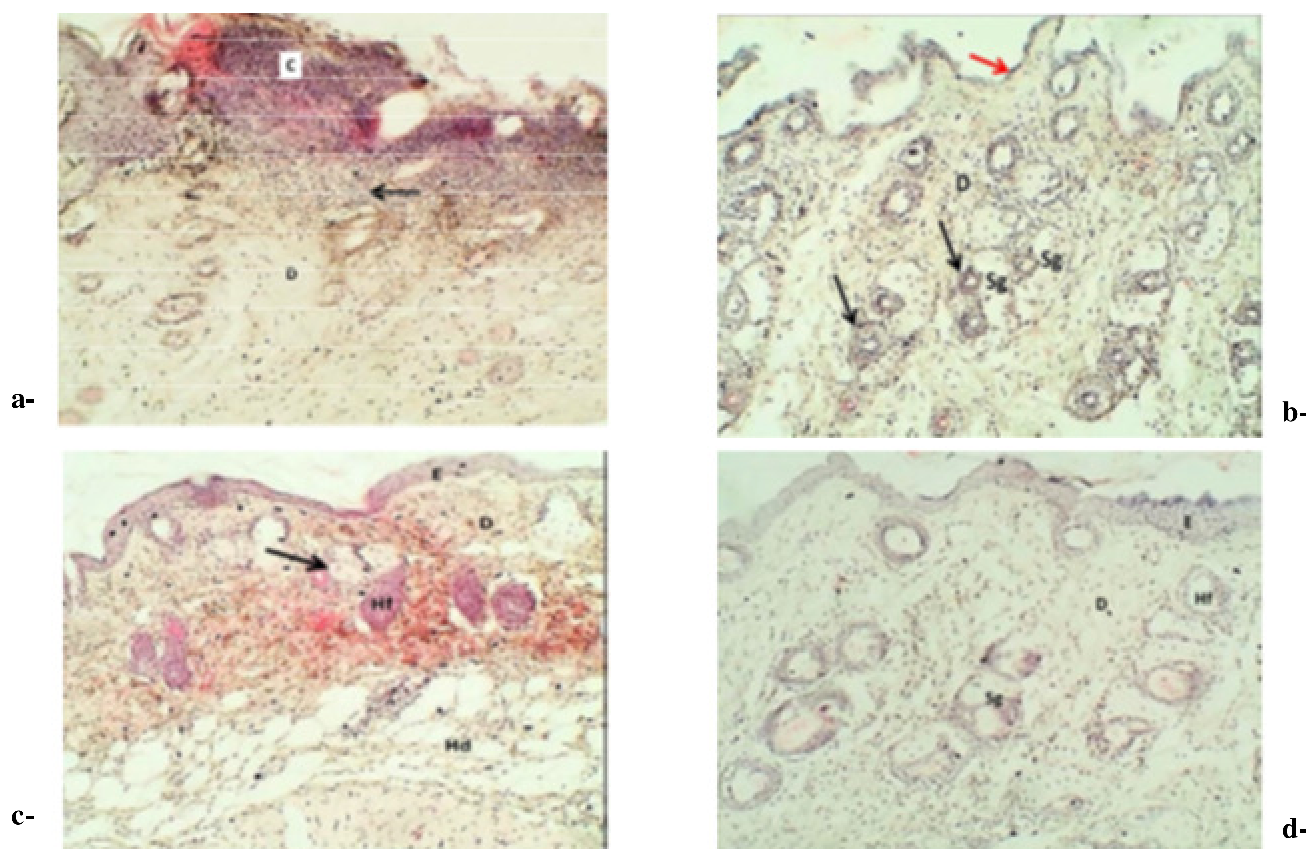

Fig. 3 : Section of mice skin with H and E stain. In general, c denotes to Fibrin clot, dermis (D), sebaceous glands (Sg), Epidermis (E), hair follicles (Hf) and hypodermis (Hd).

Table 4 : Biofilm Inhibition activity of antibiotics alone and in combination with chalcone (P<0.01).

Antimicrobial	Sa 9	Sa 25	Sa 31	Sa 35	Sa 38	Percentage	LSD
None	0.529	0.866	1.026	1.126	1.186	100%	0.133 **
Vancomycin (1 µg/ml)	1.101	0.523	0.805	0.535	1.148	86.90%	0.298 **
Erythromycin (32 µg/ml)	0.245	0.447	0.867	0.648	0.402	55.10%	0.207 **
Vancomycin (1 µg/ml) + chalcone (15 µg/ml)	0.219	0.268	0.239	0.299	0.279	27.60%	NS
Erythromycin (32 µg/ml) + chalcone (15 µg/ml)	0.176	0.231	0.233	0.237	0.251	23.80%	NS
Vancomycin (1 µg/ml) + chalcone (20 µg/ml)	0.173	0.248	0.222	0.259	0.273	24.82%	NS
Erythromycin (32 µg/ml) + chalcone (20 µg/ml)	0.189	0.251	0.240	0.261	0.226	24.65%	NS
LSD	0.1052**	0.1090**	0.1011**	0.1088**	0.0733**		—

** (P<0.01), NS: Non-Significant.

glands and hair follicles. The hypodermis has composed of adipose connective tissue (Fig. 3c).

Group D (Mice treated with sub MBIC of chalcone and erythromycin at MIC)

The sections showed that the epidermis appeared normally and composed of keratinized stratified squamous epithelium. The dermis was very thick layer constituting of irregular dense collagenous connective tissue and had sebaceous glands and hair follicles (Fig. 3d).

Gene expression

The study of gene expression was conducted by selecting two MRSA isolates that showed the highest biofilm formation. *16SrRNA* used as housekeeping gene. The C_t of *16SrRNA* gene did not change at a high range (29.65 to 30.49). There were insignificant differences between isolates before and after treatment with chalcone. The present study focused on the effect of exposing MRSA biofilm to chalcone at sub MBIC on the expression of *fnbA*, *fnbB* and *srtA* genes, a decrease in mRNA level was observed upon chalcone treatment as shown in Table 5.

Table 5 : Fold change of *fnbA* ,*fnbB* and *srtA*genes in MRSA biofilm cells.

Type of genes	Isolate code	Sample with Chalcone			Calibrator (without Chalcone)				
		Ct of target gene	Ct 16srRNA	ÄCt	Ct of target gene	Ct 16srRNA	ÄCt	ÄÄCt	Fold change
fnbA	Sa 9	20.1	29.65	-9.55	15.01	29.89	-14.88	5.33	0.024
	Sa 27	19.57	29.63	-10.06	14.07	30.49	-16.42	6.36	0.012
fnbB	Sa 9	18.8	29.65	-10.85	13.21	29.89	-16.68	5.89	0.017
	Sa 27	19.55	29.63	-10.08	13.4	30.49	-17.09	7.01	0.007
srtA	Sa 9	18.25	29.65	-11.4	13.06	29.89	-16.83	5.43	0.023
	Sa 27	18.46	29.63	-11.17	13.19	30.49	-17.3	6.13	0.014

DISCUSSION

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become an important MRSA pathogens in hospitals and the community, which shows drug-resistance to all beta-lactam antibiotics (Monecke *et al*, 2012; Stefani *et al*, 2015). MRSA biofilm cells are more resistant to antibiotics than planktonic cells due to their lower level of metabolic activity and cell division (Stewart and Franklin, 2008).

In the present study, the results of the antibiotic susceptibility test showed a variation in the sensitivity of the isolates to antibiotics (Table 2). Clearly, isolate Sa 9 resisted the highest number of antibiotic in question. Furthermore, clindamycin is the only antibiotic being effective in inhibiting all the tested antibiotics. On the other hand, isolates Sa 9, Sa 31 and Sa 35 showed multi drug resistance. Such drug resistance MRSA isolates greatly necessitates more attention. Moreover, erythromycin showed intermediate resistance and resistance. Yet none of the isolates developed susceptibility; therefore, it was chosen for further experiments. Results show all isolate were sensitive to vancomycin. Similarly, Jaddoa and Al-Mathkhury (2018b) stated that all MRSA isolates were sensitive to vancomycin. Ghosh and Banerjee (2016) also showed that 100% of MRSA isolates were sensitive to vancomycin while 81.8% were resistance to erythromycin.

Results show that there was a minor effect of Vancomycin at MIC on planktonic cells. Othman *et al* (2019) reported that the decreased vancomycin susceptibility in MRSA isolates might be related to MIC creep in some isolates. Chang *et al* (2015) also referred to the same result when they suggested this shift of vancomycin MIC within the susceptible range may be associated with an increasing probability of treatment failure. It should be noted that CLSI lowered pre-2006 vancomycin MIC breakpoints by broth microdilution from ≤ 4 to ≤ 2 μg/mL for susceptible strains of *S. aureus*.

The effect of erythromycin showed uneven results between isolates varied from average effect to simple effect on MRSA planktonic cells. Krut *et al* (2004) reported that erythromycin suppresses the cytotoxic activity of *S. aureus* but many of these will only suppress when the antibiotic pressure is maintained. Except for rifampicin, intracellular *S. aureus* will regain its cytotoxic effect and kill the host cells following withdrawal of antibiotics.

While the combinatorial effect of the antibiotics (MIC) and chalcone at MIC on MRSA plankton was very clear through the decreased in the reading results, and this came in line with many studies that dealt with the effect of chalcone and its derivatives with antibiotics on MRSA planktonic, Bo•iæ *et al* (2014) reported that the synergistic effect of chalcones with antibiotics, significantly enhanced the efficacy of Ciprofloxacin, Gentamicin and Trimethprim.

In general, antibiotics reduced biofilm formation; however, many studies showed that the antibiotics could significantly induce biofilm formation according to antibiotics class and the bacterial strain (Hemati *et al*, 2016; Narasnna *et al*, 2017). Through the results of the synergistic effect of antibiotics with chalcone, there is a significant decrease in values. Although, vancomycin was chosen as a drug of choice for MRSA treatment, but MRSA showed high resistance of isolates to vancomycin in particular (Garoy *et al*, 2019). Concerns around the use of high doses of vancomycin and the associated toxicity and high level of vancomycin resistance have led to the study of compound that reduce the use of high doses of vancomycin (Lambert, 2011; Holmes *et al*, 2015; Monteiro *et al*, 2018). Results in this study revealed clearly the effect of chalcone when combined withvancomycin in reducing the biofilm, which Leading to suggest its effect to decrease the dosage ofvancomycin. Very close results have been showed the effect of chalcone in combination with erythromycin. Zhou *et al* (2017) showed that erythromycin monotherapy did not inhibit MRSA growth

and had no activity in bone infection,but the combination of erythromycin and curcumin lead to stronger efficiency against MRSA induced osteomyelitis in rats than monotherapy.

Histopathological results of mice skin showed significant synergistic effect when chalcone was combined with erythromycin, this result agreed with *in vitro* tests and with Gaur *et al* (2015) as chalcone derivatives lowered the systemic bacterial load in blood, liver, kidney, lung and spleen tissues in systemically infected Swiss albino mice model.

Therapeutic agents targeting virulence factors genes, such as *srtA*, (which do not threaten survival), may not lead to the development of resistance as quickly as conventional antibiotics typically do, and this will have important implications for *S. aureus* infection (Liu *et al*, 2015; Zhou *et al*, 2015; Zhang *et al*, 2016). Sortase A has attracted significant interest as a potential drug target because earlier studies have shown that inactivation of the *srtA* gene had a significant impact upon the pathogenesis of infections caused by several clinically relevant Gram positive pathogens. These include *S. aureus* (Maresso and Schneewind, 2008).

One of the most important stages in the occurrence of infection caused by *S. aureus*, it's the ability to adhere of the bacterium to the cells and the extracellular matrix. Among adhesins, two fibronectin binding proteins (*fnbA* and *fnbB*). The presence of these genes indicate a significant association between adhesion and biofilm formation, thus these genes play a prerequisite for surface colonization. That, fibronectin-adhesins are present in the most of the MRSA clinical isolates encourages the development of strategies to specifically block the interaction of this bacterium with fibronectin by antagonist molecules (Mirzaee *et al*, 2015; Dai *et al*, 2019).

The present study focused on the effect of exposing MRSA biofilm to chalcone at sub MBIC on the expression of *fnbA*, *fnbB* and *srtA* genes, a decrease in mRNA level was observed upon chalcone treatment, suggesting a possible inhibiting effect of chalcone on transcription of virulence factor gene. Thus, it can be concluded that chalcone affected *fnbA*, *fnbB* and *srtA* production and then biofilm production. In addition, these results are compatible with phenotypic biofilm production, which was significantly reduced by addition of chalcone. Accordingly, it can be suggested that chalcone acted as a repressor and down regulated these genes expression in biofilm cells.The combination of conventional anti-MRSA therapy with chalcone could be a new method for prevention of bacterial adherence to surfaces or for treatment of staphylococcal infections.

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