ANTIBIOFILM POTENCY OF GINGER (*ZINGIBER OFFICINALE*) AND QUERCETIN AGAINST *STAPHYLOCOCCUS AUREUS* ISOLATED FROM URINARY TRACT CATHETERIZED PATIENTS

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Abstract. *Staphylococcus aureus* is a common cause of urinary tract infections associated with catheters. Biofilm is a community of microbial cells attached to a surface and is embedded in the extracellular polymeric substances. Catheters were collected from 157 patients; *S. aureus* was identified by molecular and conventional microbiological methods. *S. aureus* isolates showed a higher level of biofilm production, and all isolates showed biofilm production using Microtiter plate assay, while 89% of the isolates produced using Congo red agar method. All biofilm-producing isolates were positive for *icaC*, *icaD,* and *cna* genes, except isolate SA88, indicating the vital role of *ica* genes as markers of virulence in *S. aureus* infections. The difference in the inhibition of biofilm formation between the culture containing *Z. officinale* extract and the control was recorded. The high percentage of biofilm inhibition was 61.4 against SA04 at a concentration of 16%, whereas there is no effect on the formation of biofilm at concentrations 4, 2, 1, and 0.5% against each of SA36, SA48, and SA62. Quercetin lowers the formation of biofilm against SA36 of *S. aureus* at 32 μg/mL compared to a positive control (p= 0.0041), and the percentage of inhibition power reached to 92. At a concentration 32 μg/mL, the highest antibiofilm potential of ascorbic acid was 62 percent against SA86 strain, while at a concentration of 4 μg/mL, the minimum potency was recorded toward SA62 isolate.
Keywords: *bacterial isolates, catheters, biofilm-related genes, PCR*

Introduction

Staphylococcus aureus is responsible for causing a variety of human-acquired community and hospital-acquired infections worldwide. A significant number of *S. aureus* clinical isolates have evolved to be resistant to commonly used antibiotics (Jeong et al., 2019). The importance of nosocomial diseases caused by *S. aureus*, especially by methicillin resistant *S. aureus* (MRSA), is well known for its frequency, morbidity, mortality, and principally for its difficulty to treat (Shin et al., 2010; Saraiva et al., 2012). Resistance has also been reported against both newly introduced and last-resort drugs such as vancomycin, daptomycin, and linezolid used for the treatment of *S. aureus* infection. Therefore, new therapies against this series human pathogens need to be developed urgently (Nair et al., 2016). *S. aureus*, including antibiotic-resistant strains, are highly biofilm-producing bacteria and are dangerous causes of common infectious diseases in humans (Phuong et al., 2017; Wang et al., 2017). Due to genetic and metabolic adaptations of cells in films, the bacteria in biofilms are highly tolerant to antimicrobials. Biofilms consist of both the cells and the extracellular matrix produced by the cells (Shahmoradi et al., 2019), bacterial cell communities in a self-produced polymer matrix, and adhere to an inert or living surface (Onsare and Arora, 2015; Singh et al., 2015).

S. aureus is known to form biofilms, and it has been shown that reside in biofilms are highly resistant to antibiotics (Rodrigues et al., 2017), and also is a leading cause of skin

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structure infections and it is particularly associated with urinary tract catheters (Bayer et al., 2016; Trübe et al., 2019). *S. aureus* produces several virulence factors that enable it to colonize, adhere to surfaces and form biofilms, invade or escape the immune system, develop resistance to multiple antibiotics and cause host toxicity (Fey et al., 2003; Cheung et al., 2004). *S. aureus* is commonly observed, colonizing several parts of the body in healthy individuals (Kiedrowski and Horswill, 2011) and causing associated biofilm infections (Balamurugan et al., 2017). The ability of *S. aureus* to form an extracellular slime and constitutive a biofilm assists this organism to endure the host immune response, thus impairing clinical treatment since biofilm formation defends bacteria from antimicrobial agents (Foster, 2005). *Zingiber officinale* has been used for thousands of years as a culinary and medicinal herb. A recent study has shown that *Z. officinale* has antibacterial activity against *S. aureus,* and that is higher than the antibiotics on the market (Kim and Park, 2013). The rhizome is rich in secondary metabolites such as phenolic compounds (gingerol, paradol, and shogunal), volatile sesquiterpenes (zingiberene and bisabolene) and monoterpenoids (curcumin and citral). Among herbal extracts, the inhibitory effect of *Z. officinale* extract on microorganisms has been well documented (Ali et al., 2008). Previous studies have demonstrated that isolated compounds from *Z. officinale* possess potent antioxidant, antibacterial, antifungal, anticancer, and anti-inflammatory effects (Habib et al., 2008), as well as the impact of this extract on biofilm formation (Stoilova et al., 2007).

Quercetin, the most commonly studied flavonoid, has a wide range of biological activities, including antimicrobial activities (Hirai et al., 2010). Quercetin also influences quorum sensing, hence acts as an antibiofilm compound against *S. aureus*. It inhibits alginate production in a concentration-dependent manner, resulting in declination in the adherence during biofilm formation. It also reduces exopolysaccharide (EPS) production required for the initial attachment of bacteria. Some other reports also suggested that usinic acid show inhibitory effect on the *S. aureus* biofilm, and this has been hypothesized that this may be due to any interference in quorum sensing, but the exact mechanism of action is still indistinct (Roy et al., 2018). Based on quantification of the biofilm extracellular polymeric substances content and cell viability, quantitative proteome analyses and genome-scale metabolic modeling point to a vitamin C-dependent inhibition of the synthesis of polysaccharides that form the biofilm matrix. This proceeds *via* inhibition of the quorum sensing and other regulatory mechanisms, leading to repression of specific biosynthetic operons. Once the EPS content is reduced beyond a critical point, bacterial cells become exposed and more susceptible to killing by any external factors (Pandit et al., 2017).

Ascorbic acid has been shown to be an effective antioxidant, acting both directly through aqueous peroxyl radical's reaction and indirectly through the restoration of the antioxidant properties of fat-soluble vitamin E. Interestingly, ascorbic acid has been reported to increase the effectiveness of antibiotics *vs*. a wide range of bacteria through a synergistic effect, but this synergy's mechanism remains unclear (Helgadóttir et al., 2017).

The main objective of this study was using *S. aureus*, a model biofilm-forming microorganism, also investigated the effects of *Z. officinale* extract, quercetin, and ascorbic acid on biofilm formation using a static biofilm assay. The distinction in the inhibition of biofilm between the culture containing *Z. officinale* extract and the control.

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Materials and method

Clinical isolate repository

In the period from July 2016 to March 2017, 157 samples taken from catheter patients received from the Rizgary Urinary Unit and Artificial Kidney Hospitals were investigated in Erbil City, Erbil, Iraq after cutting catheter (inside parts of the body) into 2 cm pieces and then incubating in brain heart infusion broth for 24–48 hrs. with shaking at 250 rpm, after that, the positive growth was cultured on Nutrient, Blood agar (BA), MacConkey and Mannitol Salt Agar (MSA) (Oxoid, UK).

Culture and identification

Primarily, isolates were cultured on MSA and BA (Oxoid, UK) and incubated at 37ºC for 24 hrs. The assumed colonies of *S. aureus* were identified by using conventional methods; include gram staining, biochemical tests which comprise: catalase, oxidase, urease, coagulase using tube coagulase test (TCT) (Karasu and Rathish, 2014), gelatin liquefaction, hemolysis, staphyloxanthin and protease, tellurite reduction, lipase and lecithinase production, dextrose reduction, carbohydrate fermentation and tellurite reduction, and DNase with methyl green, followed by VITEK II Compact System (bioMérieux, Inc., France). Finally, identity of the isolates was confirmed *via* polymerase chain reaction (PCR) (Alpha PCRmax, UK) based on identifying *16S* rRNA and *nuc* genes. The sequences of both genes and PCR setup can be found in *Table 1*.

Table 1. *Sequences of oligonucleotide primers used for PCR amplification of biofilm-associated genes with 16S rRNA, nuc and mecA genes used in this study*

Gene name	Primers detail			References
	Primer Sequence (5´ – 3´) (Oligonucleotide)	Amplicon size (bp)	Cycling program	
16S rRNA	CAC CTT CCG ATA CGG CTA CC GTT GAC TGC CGG TGA CAA AC	372	95°C–30 s; 59°C–45 s; 72°C–1 min; 35 cycles	In this study
nuc	GCG ATT GAT GGT GAT ACG GTT AGC CAA GCC TTG ACG AAC TAA AGC	279	95°C–30 s; 53°C–45 s; 72°C–40 s; 40 cycles	(Blaiotta et al., 2004)
mecA	ATG TCT GCA GTA CCG GAG CTT T AAA AT CGA TGG TAA AGG TTG GC	533	94°C–30 s; 55°C–45 s; 72°C–1 min; 40 cycles	(Alli et al., 2015)
icaA	ACA CTT GCT GGC GCA GTC AA TCT GGA ACC AAC ATC CAA CA	188	94°C–30 s; 56°C–60 s; 72°C–45 s; 30 cycles	(Kouidhi et al., 2010)
icaB	CCC AAC GCT AAA ATC ATC GC ATT GGA GTT CGG AGT GAC TGC	1080	95°C–30 s; 58°C–30 s; 72°C–45 s; 40 cycles	(Gowrishankar et al., 2016)
icaC	CTT GGG TAT TTG CAC GCA TT GCA ATA TCA TGC CGA CAC CT	209	95°C–30 s; 55°C–40 s; 72°C–45 s; 40 cycles	(Nourbakhsh and Namvar, 2016)
icaD	ATG GTC AAG CCC AGA CAG AG CGT GTT TTC AAC ATT TAA TGC AA	198	94°C–30 s; 55°C–40 s; 72°C–45 s; 30 cycles	(Kouidhi et al., 2010)
cna	CGA TAA CAT CTG GGA ATA AA ATA GTC TCC ACT AGG CAA CG	716	95°C–30 s; 54°C–40 s; 72°C–45 s; 35 cycles	(Tang et al., 2011)
atl	GCC TGT TGC AAA GTC AAC AA CAC CGA CAC CCC AAG ATA AG	600	95°C–30 s; 56°C–30 s; 72°C–45 s; 40 cycles	In this study
fnbA	GAT ACA AAC CCA GGT GGT GG TGT GCT TGA CCA TGC TCT TC	191	95°C–30 s; 57°C–1 min; 72°C–1 min; 35 cycles	(Kouidhi et al., 2010)
fnbB	GAC CTG CTT CGC TAT CCA CA AGT CGT AAT GGC GAC AGG TG	980	95°C–30 s; 57°C–30 s; 72°C–1 min; 40 cycles	In this study

Antimicrobial susceptibility screening

According to the references of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2017), antimicrobial sensitivity testing was carried out against the following antimicrobials using disk diffusion method; Amikacin AK 30 μg, Azithromycin AZM

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15 µg, Ciprofloxacin CIP 5 µg, Clindamycin CD 2 µg, Erythromycin E 15 µg, Gentamicin G 10 µg, Levofloxacin LEV 5 µg, Netilmicin NET 30 µg, Nitrofurantoin NIT 300 µg, Norfloxacin NOR 10 µg, Oxacillin OX 1 µg, Penicillin P 10 U, Tetracycline TE 30 µg, Tobramycin TOB 10 µg, Trimethoprim+Sulfamethoxazole SXT 1.25+23.75 µg, and Vancomycin VA 30 µg (Bioanalyse, Turkey). A lawn of test *S. aureus* was prepared by evenly spreading 100 µL inoculums ($1.5*10^8$ CFU/ml) according to 0.5 McFarland (1907) standard solution with the sterilized swab on top of the entire surface of Mueller Hinton Agar plate (Oxoid, UK). The disks were resolutely applied onto the agar plates surface within 15 minutes of inoculation (Bimanand et al., 2018).

Assessment of biofilm synthesis by S. aureus

Congo red agar method

Phenotypical biofilm production in all *S. aureus* isolates was evaluated through culturing CRA plates and explained by (Szczuka et al., 2013; Khoramrooz et al., 2016). First of all, they prepared CRA plates by adding 0.8 g of Congo red (Merck, Germany) and 36 g of sucrose (Sigma, USA) to one liter of brain heart infusion agar (BHI) (Merck, Germany). The plates have been incubated for 24 hours at 37ºC. The morphology of colonies was then interpreted based on colony color as Bordeaux pink (red), almost black, black, and strong black. Strains with red colonies were classified as strains that are unable to produce biofilm, while nearly black color indicated a weak activity in biofilm production. Whereas, colonies that were very black and black were considered strong strains of biofilm producers.

Polystyrene microtiter plate assay

Biofilm production was quantitatively determined through an MTP method as defined by Yousefi et al. (2016). In short, bacterial isolates were grown with 0.5 percent glucose in trypticase soy broth (TSB) (Merck, Germany) and incubated at 37ºC overnight. Cultures with 0.5 percent glucose with 1:40 in fresh TSB were diluted (Sigma, USA). Two hundred µL of the diluted solution was added to Microtiter plate wells and incubated at 37ºC for 48 hrs. The negative control wells contained only 200 µL of TSB–0.5% glucose without bacterial suspension. Wells were gently washed with phosphate buffer saline (PBS) (pH 7.2) three times and fixed with methanol for 20 minutes, dried at room temperature, then stained with crystal violet 0.1 percent. The dye attached to the adhering cells was dissolved with 1 mL of 95% ethanol per well. Finally, optical density (OD) was obtained at 570 nm (A_{570}) for each well using ELISA reader (BioTek ELx800, USA). The average OD of negative control + 3 standard deviation (SD) of negative control was calculated for the optical density cut–off (ODc). Based on the absorbance of crystal violet stain linked to the adhered cells, biofilms formed by various strains have been analyzed and categorized (*Table 2*).

Table 2. *Classification of biofilm formation abilities by Microtiter plate method*

Cut-off value calculation	Mean of OD_{570} values results	Biofilm formation abilities
$OD > 4\times ODc$	$OD > 0.557$	Strong
$2\times ODc < OD \leq 4\times ODc$	$0.278 < OD \leq 0.557$	Moderate
$ODc < OD \leq 2\times ODc$	$0.139 < OD \leq 0.278$	Weak
$OD \leq 0.139$	$OD \leq 0.139$	None

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Genomic DNA extraction

According to the manufacturer's instructions, genomic DNA was extracted from pure cultures through the Presto™ Mini gDNA Bacteria Kit (Geneaid, Taiwan); extract was eluted with an elution buffer of 100 µL. Before running PCR, extracts were stored at -20°C. The NanoDrop 1000 spectrophotometer (ThermoFisher Scientific, USA) was used to evaluate DNA concentration and purity in which one µL of the genome DNA was used to define DNA concentration and purity.

PCR screening of biofilm genetic determinants

In our research, detection in *S. aureus* isolates for biofilm-related genes was carried out using the PCR technique. The final volume of the PCR reaction was 25 µL using 12.5 µL of 2x HotStart Taq Master Mix, one µL of the DNA template, one µL of each primer (20 pmol) and 9.5 µL of ddH_2O. DNA amplification was done in a thermocycler PCR. Primers and amplification conditions for PCR programs are mentioned in *Table 1*. Amplified products were subjected to electrophoresis using 1.2% agarose (GeNet Bio, Korea) gel containing 1x GelRed DNA stain.

Ferric Reducing Antioxidant Power (FRAP) assay

To be able to measure the ferric reduction activity of our samples, the test was performed based on Benzie and Strain (1996). The FRAP solution was freshly prepared by combination of acetate buffer (0.3 Mol/L) (pH= 3.6), 2, 4, 6-tripyridyltriazine (TPTZ) (0.01 Mol/L in HCl (0.04 Mol/L) and $FeCl_3$ (0.02 Mol/L) (10:1:1) by volume, respectively. The test was performed by placing 100 µL of the tested chemicals in a test tube (Conc. 1 mg / mL) and 2 mL of the FRAP reagent, the samples were continuously shaken and leave in the dark place for 30 min. Then the absorbance at 593 nm was recorded. The standard ascorbic acid curve was prepared for comparison using various concentrations, as shown in *Fig. 1*. The FRAP value calculated by *Equation 1* for each compound. In this study, the test solution's yellow color reduces ferric complex with TPTZ (less color) to ferrous complex with TPTZ (violet color) changes to different shades of violet depending on each compound's reduced power. The higher reduction potential was determined by higher absorbance of the reaction mixture.

$$FRAP\ value\ of\ sample\ (\mu M) = Abs.(sample) \times \frac{FRAP\ value\ of\ standard\ (\mu M)}{Abs.of\ standard} \quad \text{(Eq.1)}$$

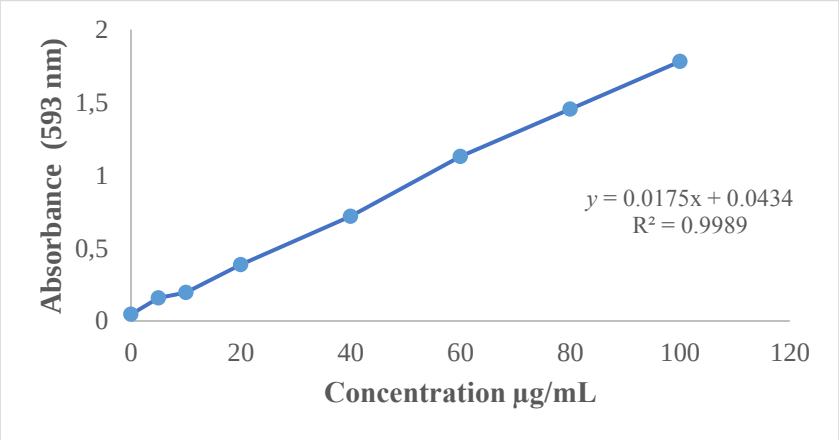

Figure 1. *The standard curve of ascorbic acid*

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Biofilm formation inhibition test

Preparation of Z. officinale extract

Z. officinale extract was prepared under the protocol described by Kim and Park (2013). In short, 150 g of shredded rhizome of *Z. officinale* was mixed with 300 mL toluene. The debris was allowed to settle at room temperature for 24 hrs. A Whatman No. 1 filter paper (pore size = 11 µm) had been used for filtration of the supernatant. Then the mixture was stirred for 24 hrs. at room temperature using a magnetic stirrer. Then the mixture was left to form phases of water and toluene. Using a pipette, the water phase was collected and filtered through a 0.22 µm microfilter. The filtrate (100% *Z. officinale* extract) was used to test if *Z. officinale* extract inhibits the formation of biofilms.

Antimicrobial activity of quercetin

The antimicrobial activity of quercetin was performed by the microdilution method, already described previously, according to the CLSI. The range of concentration of quercetin used in this study was 2 to 256 µg/ml (Sérgio et al., 2018). The experiment was performed in triplicate.

Preparation and dilution of ascorbic acid

One milligram of ascorbic acid was dissolved in 1 ml of TSB medium to get a stock solution of 1000 µg/ml. Final concentrations of 2, 4, 8, 16, 32, 64, 128, and 256 µg/ml were obtained through serial dilutions to explore the antibiofilm activity against *S. aureus*. Ascorbic acid was mixed for two minutes with TSB medium and vortex. It was covered with aluminum foil to prevent light. At the time of their use, all solutions were prepared.

Static biofilm formation assay

To test the effect of plant extract on biofilm formation, a modified crystal violet assay was used. In sterile 96 well plates containing 50 µL of nutrient broth per well, two-fold serial dilutions of compounds were made. The concentration range of the compounds tested is 2 to 256 µg/mL in separate wells. Each well was supplemented with a 50 µL fresh bacterial suspension (0.5 McFarland) with growth control (*cells + broth*). The biofilm biomass was tested using the crystal violet staining assay after incubation at 37°C for 48 h (Kim and Park, 2013). The biofilm inhibition percentage was calculated using *Equation 2*.

$$Biofilm\ inhibition\% = \frac{OD\ growth\ control - OD\ sample}{OD\ growth\ control} \times 100 \qquad \text{(Eq.2)}$$

Data analysis

For statistical analysis, the Social Science Statistical Package (SPSS 24.0) software (SPSS Inc., USA) was used. The statistical significance was assessed through *Turkey's multiple comparisons*; the test was used to analyze the association between phenotypic biofilm formation methods and biofilm-related genes, and also the significant difference in biofilm inhibition percentage between active, positive–controlled biofilm producers and p-value < 0.05 was considered statistically significant.

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Results

Isolation and characterization of S. aureus

Based on biochemical tests, isolates from one hundred catheter specimens (63.69%) were identified as *S. aureus* (*Table 3*). The strains were from samples belonging to 67 male patients (67%), and 33 female patients (33%). To support the identification of *S. aureus* isolates by a conventional method, VITEK II Compact System was performed, and all strains of *S. aureus* were reidentified by this system, some results contradicted the traditional tests and identified a different staphylococcal species than *S. aureus*. The present findings of the VITEK II system show that 83 isolates were identified as *S. aureus* among 100 isolates that were identified in the conventional method, and the remaining strains identified were *xylosus* (*n=6*), *sciuri* (*n=6*), *vitulinus* (*n=1*), *warneri* (*n=1*), *lentus* (*n=1*), *hemolytics* (*n=1*), and *gallinarum* (*n=1*). To further confirm the identity of the isolates, all *S. aureus* were examined for the presence of the *16S* rRNA and *nuc* genes to characterize and validate the *S. aureus*. All of the strains were confirmed as *S. aureus* by the occurrence of *16S* rRNA, and *nuc* genes (*Fig. 2*). About two-thirds of catheter-isolated bacteria were identified as *S. aureus*.

Table 3. *The morphological, cultural, biochemical, and molecular tests for the identification of S. aureus isolates*

Biochemical tests	**Positive *S. aureus***		**Positive% among catheter specimens (*n=157*)**
	Positive ***n.*** (%)	**Negative** ***n.*** (%)	
Mannitol fermentation	**100** (100)	**0** (0)	63.69
Coagulase HP	**91** (91)	**9** (9)	57.95
Coagulase RP	**96** (96)	**4** (4)	61.14
Catalase	**100** (100)	**0** (0)	63.69
Oxidase	**0** (0)	**100** (100)	0
Urease	**54** (54)	**46** (46)	34.39
Gelatinase	**100** (100)	**0** (0)	63.69
β Hemolysis	**71** (71)	….	45.21
α Hemolysis	**2** (2)	….	1.27
γ Hemolysis	**27** (27)	….	17.19
DNase	**100** (100)	**0** (0)	63.69
Staphyloxanthin	**85** (85)	**15** (15)	54.13
Caseinase	**82** (82)	**18** (18)	52.22
Tellurite reduction	**100** (100)	**0** (0)	63.69
Shiny colonies	**100** (100)	**0** (0)	63.69
Lipase activities	**64** (64)	**36** (36)	40.76
Lecithinase production	**62** (62)	**38** (38)	39.48
VITEK II System	**83** (83)	**17** (17)	52.86
16S rRNA gene	**100** (100)	**0** (0)	63.69
nuc gene	**100** (100)	**0** (0)	63.69

Susceptibility patterns of S. aureus against different antimicrobials

The results of the antimicrobial sensitivity test for all *S. aureus* isolates against 16 antimicrobials demonstrated various sensitivity patterns (*Table 4*). The highest resistant percentage recorded was against oxacillin (99%), followed by penicillin (97%). However, the lowest resistant 1% recorded was against each of gentamycin, nitrofurantoin, and trimethoprim-sulfamethoxazole.

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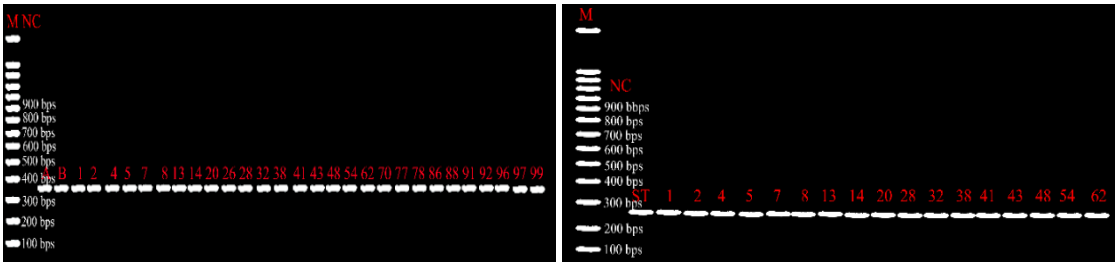

A **B**

Figure 2. *Agarose gel electrophoresis of PCR amplification products of S. aureus.* ***A****: 16S rRNA gene, M: The DNA marker (100 bp ladder), lane A and B: S. aureus ATCC 23925, lanes (1–99) positive amplification of 372 bp for 16S rRNA gene.* ***B****: nuc gene, M: The DNA marker (100 bp ladder), lane NC: S. epidermidis ATCC 22922, lane ST: S. aureus ATCC 23925, lanes (1–62) positive amplification of 279 bp for nuc gene*

Table 4. *Susceptibility patterns of S. aureus isolate toward antimicrobials*

Antimicrobials	Resistance ***n.*** (%)	Intermediate ***n.*** (%)	Sensitivity ***n.*** (%)
AK*	**1** (1)	**1** (1)	**98** (98)
AZM	**47** (47)	**4** (4)	**49** (49)
CIP	**11** (11)	**1** (1)	**88** (88)
CD	**30** (30)	**19** (19)	**51** (51)
E	**53** (53)	**6** (6)	**41** (41)
G	**1** (1)	**4** (4)	**95** (95)
LEV	**9** (9)	**0** (0)	**91** (91)
NET	**1** (1)	**3** (3)	**96** (96)
NIT	**21** (21)	**1** (1)	**78** (78)
NOR	**18** (18)	**34** (34)	**58** (58)
OX	**99** (99)	**0** (0)	**1** (1)
P	**97** (97)	**0** (0)	**3** (3)
TE	**23** (23)	**3** (3)	**74** (74)
TOB	**19** (19)	**3** (3)	**78** (78)
SXT	**1** (1)	**8** (8)	**91** (91)
VA	**38** (38)	**25** (25)	**47** (47)

*: AK: Amikacin, AZM: Azithromycin, CIP: Ciprofloxacin, CD: Clindamycin, E: Erythromycin, G: Gentamicin, LEV: Levofloxacin, NET: Netilmicin, NIT: Nitrofurantoin, NOR: Norfloxacin, OX: Oxacillin, P: Penicillin, TE: Tetracycline, TOB: Tobramycin, SXT: Trimethoprim+Sulfamethoxazole, and VA: Vancomycin

Biofilm formation through microtiter plate test

The ability to form a biofilm was evaluated using the MTP described elsewhere (Yousefi et al., 2016). In this study, OD_{570} mean of microplate readings after crystal violet staining ranged from 0.216 to 0.827. The mean of negative control was 0.054. An ODc_{570} of biofilm formation was defined as 0.139. The strains were divided into four groups: non–biofilm producer (−), $OD_{570} \leq 0.139$; weak biofilm producer (+), $0.139 < OD_{570} \leq 0.278$; moderate biofilm producer (++), $0.278 < OD_{570} \leq 0.557$; strong biofilm producer (+++), $0.557 \leq OD_{570}$. Our data shows that 100% of *S. aureus* isolates were positive for biofilms, 21% of which were recorded as a strong producer of biofilms (*n=21*), 71% as a moderate producer of biofilms (*n=71*) and 8% as a weak producer of biofilms (*n=8*).

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Biofilm formation determination by Congo red agar test

In vitro biofilm formation by the CRA method differs from MTP assay. Results show that 49% of the isolates (*n=49*) demonstrated strong biofilm formation (strong black), 15% were moderate biofilm producer, 25% weak biofilm producer, and 11% of the isolates were classified as non–biofilm producer. Statistically, there is not a significant difference (p= 0.8997) between the total percent formation in both MTP assay, and CRA methods for the detection of phenotypic biofilm formation among isolates of *S. aureus* was observed (*Table 5*). While there are highly significant differences among biofilm formation status when compared between both MTA assay and CRA method (*Fig. 3*).

Table 5. *Screening of S. aureus isolates from biofilm production by CRA and MPM assay*

Biofilm formation status	**Screening method**		***P*-value**
	CRA *n.* (%)	**MTP *n.*** (%)	
Strong	**49** (49%)	**21** (21%)	
Moderate	**15** (15%)	**71** (71%)	
Weak	**25** (25%)	**8** (8%)	=0.8997
None	**11** (11%)	**0** (0%)	
Total	**89** (89%)	**100** (100%)	

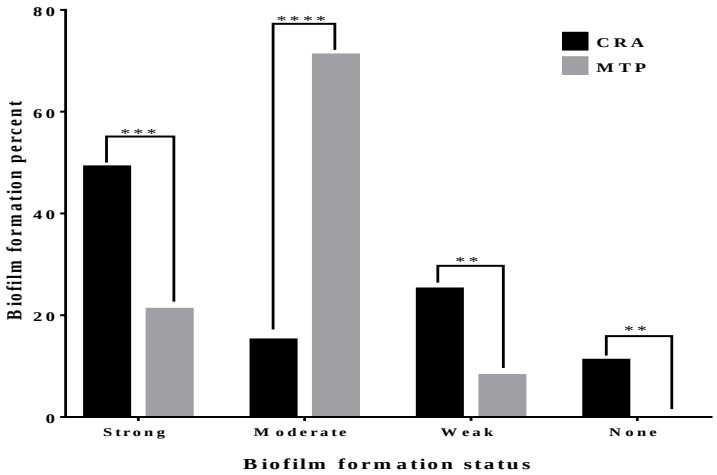

Figure 3. *Comparison of biofilm formation status*

Detection of genes involved in biofilm formation

PCR assay was used to detect *icaA*, *icaB*, *icaC*, *icaD*, *cna*, *atl*, *fnbA*, and *fnbB* genes among the primary intention of this study are the genotypically characterization of biofilm responsible genes. Ninety-six percent of *S. aureus* isolates have been selected for this purpose. We evaluated the relationship between the formation of biofilm and the eight genes associated with biofilm. The distribution of these genes in *S. aureus* isolates is illustrated in *Table 6*. All of the eight genes were detected among isolates with different frequencies. As can be seen, the majority of *S. aureus* isolates (96% [*n=24*]) were found to be positive for *the icaC* and *cna* gene (*Fig. 4*). The prevalence rates of the *icaA*, *icaB*, *icaD*, *atl*, *fnbA*, and *fnbB* genes were unswervingly found to be 76%, 68%, 88%, 92%, 84%, and 80%, respectively. Nine of the isolates (36%) (*n=9*) that were biofilm producers were shown to possess all of the eight genes aimed to detect in the current study. A

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significant association was only observed between the presence of *icaB* gene (p= 0.028) and phenotypic biofilm formation in *S. aureus* isolates, while non-significant association was noticed for each of *icaA* (p = 0.2085), *icaC* (p=0.9894), *icaD* (p= 0.7238), *cna* (p= 0.9894), *atl* (p= 0.9004), *fnbA* (p= 0.5207) and *fnbB* (p= 0.3421) and phenotypic biofilm formation features.

Table 6. *Relationships between biofilm-related genes and biofilm formation detection (MTP assay and CRA method) in S. aureus isolates*

Isolates	*In vitro* Adherence (MTP) Assay		CRA method		Presence of biofilm-related genes							
	Adherence A_{570} nm Mean	Adherence Ability	Biofilm phenotype	Biofilm producer	*icaA*	*icaB*	*icaC*	*icaD*	*cna*	*atl*	*fnbA*	*fnbB*
SA01	0.236	+	Black**	Producer	+	+	+	+	+	+	—	+
SA02	0.488	++	Strong Black	Producer	+	—	+	—	+	+	+	+
SA04	0.608	+++	Strong Black	Producer	+	—	+	+	+	+	—	+
SA05	0.379	++	Strong Black	Producer	+	+	+	+	+	+	+	+
SA07	0.578	+++	Black	Producer	—	+	+	+	+	+	+	+
SA08	0.402	++	Black	Producer	+	+	+	+	+	+	+	—
SA13	0.491	++	Almost Black	Producer	—	—	+	+	+	+	+	+
SA14	0.586	+++	Almost Black	Producer	+	+	+	—	+	+	+	—
SA20	0.216	+	Bordeaux pink	Nonproducer	+	+	+	+	+	+	+	+
SA26	0.552	++	Almost Black	Producer	+	+	+	+	+	+	—	+
SA28	0.297	++	Almost Black	Producer	+	+	+	+	+	+	+	+
SA32	0.36	++	Strong Black	Producer	—	+	+	+	+	+	+	+
SA38	0.318	++	Bordeaux pink	Nonproducer	—	—	+	+	+	+	+	+
SA41	0.379	++	Strong Black	Producer	+	+	+	+	+	+	+	+
SA43	0.317	++	Strong Black	Producer	+	+	+	+	+	+	+	+
SA48	0.667	+++	Strong Black	Producer	+	+	+	+	+	—	+	—
SA49	0.376	++	Bordeaux pink	Nonproducer	—	—	—	—	—	—	+	—
SA54	0.26	+	Black	Producer	+	—	+	+	+	+	—	+
SA62	0.458	++	Strong Black	Producer	+	+	+	+	+	+	+	+
SA70	0.349	++	Strong Black	Producer	+	+	+	+	+	+	+	+
SA77	0.36	++	Strong Black	Producer	+	—	+	+	+	+	+	+
SA78	0.383	++	Almost Black	Producer	+	+	+	+	+	+	+	+
SA86	0.425	++	Strong Black	Producer	+	+	+	+	+	+	+	—
SA88	0.352	++	Strong Black	Producer	—	—	+	+	+	+	+	+
SA93	0.284	++	Strong Black	Producer	+	+	+	+	+	+	+	+
Total positive	25		22		19	17	24	22	24	23	21	20
Percent	100		88		76	68	96	88	96	92	84	80
P-value			0.8997		0.2085	0.028	0.9894	0.7238	0.9894	0.9004	0.5207	0.3421

*: indicates the varied adhering ability of S. aureus isolates, where strong black colonies; strong biofilm producer, black colonies; moderate biofilm producer, almost black colonies; weak biofilm producer, Bordeaux pink colonies; non – biofilm producer, also +: weak biofilm producer. ++: moderate biofilm producer. +++: strong biofilm producer. [1]: ODC: mean + 3 standard deviation of negative control in microplate. ODC=0.139, 2ODC=0.278, 4ODC=0.556

FRAP reducing power

The antioxidant activity of the *Z. officinale*, quercetin, and ascorbic acid was measured by FRAP assay and has been presented in *Table 7*. It shows that electron-donating groups enhanced reducing power. Quercetin indicates that they are most effective electron donor and can reduce the oxidized intermediates highly reactive molecules like free radicals and reactive oxygen species of peroxidation processes and the FRAP value 10406.4 μM/g while the *Z. officinale* extract has low antioxidant activity (FRAP value = 5420.8 μM/g) when compared with both quercetin and ascorbic acid. Ascorbic acid usually used as a standard for preparing the standard curve and has high antioxidant activity (FRAP value = 8379.5 μM/g).

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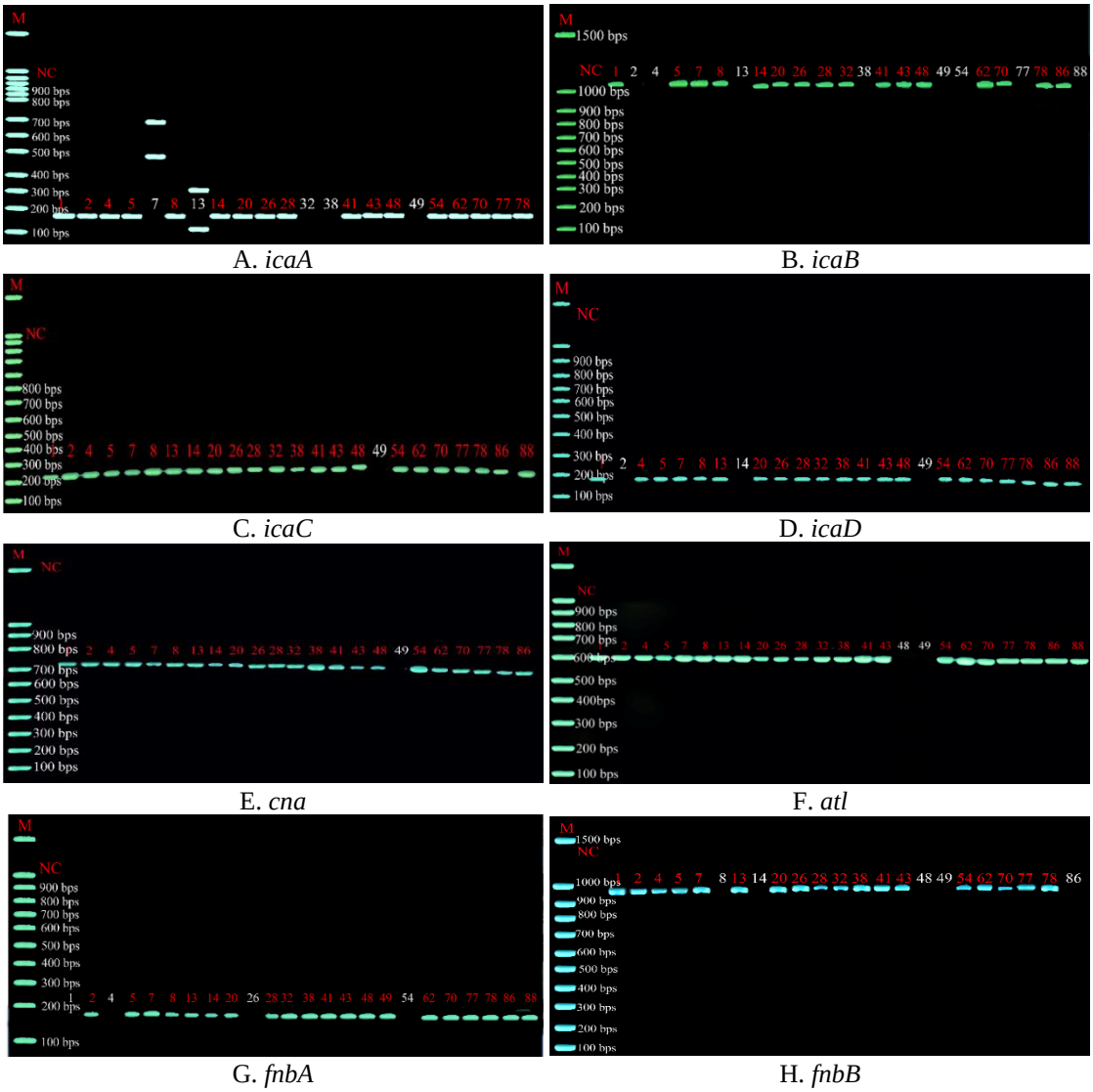

Figure 4. *Agarose gel electrophoresis graphic of PCR amplification for biofilm-related genes in S. aureus isolates*

Table 7. *Anti-oxidant activity of the Z. officinale, quercetin and ascorbic acid measured by FRAP assay*

Compounds	Absorbance	FERAP value		
		µM	µM/mg	µM/g
Z. officinale	1.507	54.208	542.08	5420.8
Quercetin	2.893	104.064	1040.64	10406.4
Ascorbic acid	2.327	83.705	837.05	8370.5

Effect of Z. officinale extract on biofilm formation

A static biofilm quantification assay was performed to evaluate the effect of *Z. officinale* extract on five strong biofilm isolates of *S. aureus*. *Table 8* shows the difference in biofilm inhibition between the culture containing *Z. officinale* extract and the control (i.e., no *Z. officinale* extract addition). The high biofilm inhibition percent

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were 61.4 at concentration 16% against SA04 while there is no any effect on biofilm formation at concentrations 4, 2, 1, and 0.5% against each of SA36, and SA62 isolates. Significantly, there were differences in biofilm reduction for *Z. officinale* extract at concentration 32, 16, and 8% (p= 0.0458, p= 0.0391, and p= 0.0247, respectively). In spite of decreasing of biofilm formation, there were no observed significant at concentrations 64, 4, 2, 1, and 0.5% (p= 0.1015, p= 0.1676, p= 0.8017, p= 0.8811, and p= 0.5925, respectively). The inhibition of *Z. officinale* extract to biofilm formation was not due to minor toluene contamination or something extracted from the glassware during the preparation of *Z. officinale* extract, which was confirmed by an experiment using mock extraction.

Table 8. *Antibiofilm potency of Z. officinale, quercetin and ascorbic acid*

Isolates	***% of Biofilm Inhibitors of Z. officinale* (%)**								**Growth Control**
	64	**32**	**16**	**8**	**4**	**2**	**1**	**0.5**	**(OD)**
SA04	54	59	61.4	57	41	41	47	9.7	0.131
SA36	14	28	36.4	36	0	0	0	0	0.109
SA48	36	47	43.1	45	30	0	17	43	0.108
SA62	47	46	45	45	46	0	0	0	0.143
SA86	47	45	47.4	46	46	30	0.9	40	0.157
P value	0.1015	0.0458	0.0391	0.0247	0.1676	0.8017	0.8811	0.5925	
Isolates	***% of Biofilm Inhibitors of Quercetin* (µg/mL)**								**Growth Control**
	256	**128**	**64**	**32**	**16**	**8**	**4**	**2**	**(OD)**
SA04	18	76	79.4	58	56	48	43	40	0.139
SA36	25	31	0	92	20	43	43	45	0.134
SA48	23	16	0	52	34	41	41	38	0.155
SA62	34	38	20.3	57	55	41	41	36	0.137
SA86	24	48	28.7	59	59	61	61	62	0.15
P value	0.0221	0.1426	0.6646	0.0041	0.0855	0.0221	0.0246	0.0369	
Isolates	***% of Biofilm Inhibitors of Vitamin C* (µg/mL)**								**Growth Control**
	256	**128**	**64**	**32**	**16**	**8**	**4**	**2**	**(OD)**
SA04	57	57	59	55	57	55	49	50	0.145
SA36	51	52	53	59	57	56	48	56	0.088
SA48	58	59	61.9	60	59	57	50	60	0.109
SA62	53	58	59.1	55	55	50	15	49	0.111
SA86	56	61	60	62	61	59	55	51	0.114
P value	0.0003	0.0062	0.071	0.0079	0.0271	0.240	0.840	0.406	

Antibiofilm activities of quercetin

Concerning the action of quercetin as antibiofilm, this molecule reduces biofilm formation against SA36 isolate of *S. aureus* at 32 µg/mL, and the inhibition potency percent reached to 92, and have a significant difference when compared to a positive control (p = 0.0041). Quercetin reduced biofilm formation at all concentrations against all five isolates except concentrations of 64 µg/mL against both SA36 and SA48 isolates. In particular, the lowest inhibited biofilm formation was 16%, and the highest was 92% (*Table 8*). Significant differences was recorded at concentrations 256, 8, 4, and 2 µg/mL when compared with positive control (p = 0.0221, p = 0.02, p = 0.0246, p = 0.0369) respectively.

Biofilm inhibitory activity of ascorbic acid

Once the antibiofilm efficacy of ascorbic acid has been determined, we analyze their potential action against *S. aureus* biofilm formation. MTP assessed this ability, and these results showed complete inhibition of biofilm formation by ascorbate when compared to

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growth control (*Table 8*). The highest antibiofilm potency of ascorbic acid was 62% against SA86 isolate at concentration 32 μg/mL, while the minimum power was recorded at concentration four μg/ml a toward SA62 isolate. In spite of the low inhibition percent of biofilm formation, while there are a significant differences in the decreasing of biofilm formation at concentrations 256, 128, 32, and 16 μg/mL (p = 0.0003, p = 0.0062, p = 0.0079, and p = 0.0271) respectively when compared with OD of growth control.

Discussion

All 100 *S. aureus* isolates were branded as Gram-positive; further cultural characteristics and biochemical test results were stated in *Table 3*. Out of these isolates, 91 isolates were coagulase-positive *S. aureus,* and nine were recorded as coagulase-negative. Results obtained from the conventional biochemical tests and PCR method match with a statistical confidence of 91% (The remaining 9% non―reluctant possibly due to analysis conditions and sample preparation) between the methods. This probably will be due to the concealing of the clumping factor by the capsular polysaccharides or due to misidentification of particular clumping factor producing CoNS; by this means, lessening its efficiency, reliability, and sensitivity (Subramanian et al., 2017). An additional test used *S. aureus* identification is the DNase test. All isolates of *S. aureus* have been positive for DNase activity in the present study. Parallel results have been reported previously with a lack of clarification; thus, additional tests are required for confirmation purposes.

In our study, 78 *S. aureus* isolates showed the ability to hydrolyze gelatin, thus confirming the presence of the gelatinase enzyme. However, 81, 51, and 48 strains of *S. aureus* secrete protease, lipase enzymes, and nonwhite pigmented colonies, respectively. Among the 100 strains of *S. aureus* showed β–hemolytic, γ–hemolytic and α–hemolytic properties, 71 strains of *S. aureus* showed the ability of β–hemolysin and only two isolates expressed α–hemolysin, and 27 isolates demonstrated the γ–hemolytic activity. Likewise, hemolytic activity among 57 different clinical sources of *S. aureus* exhibited vigorous hemolytic activities and five clinical strains that had no ability of blood hydrolysis on blood agar (Tang et al., 2013). All isolates of *S. aureus* were undergone PCR assay using universally conserved *16S* rRNA and *nuc* genes, specifically designed primers were used for discrimination of *S. aureus* isolates (*Table 1*), and all of the strains were positive for both genes (*Fig. 3*). Antimicrobial susceptibility carried out for 16 antimicrobials through disc diffusion methods, and the results are presented in *Table 6*. The highly resistant percentage was seen against oxacillin (99%) followed by penicillin (97%), and the lowest resistant 1% recorded was against each of gentamycin, nitrofurantoin, and trimethoprim–sulfamethoxazole. Based on the antimicrobial susceptibility reports by Boada et al. (2018), among the 765 assessed *S. aureus* isolates, the maximum resistance rates were observed to a penicillin (87.1%), followed by azithromycin (11.6%), erythromycin (11.2%) and clindamycin (9.7%). Oxacillin and methicillin resistance mechanism is by gaining a gene that encodes a PBP2 homolog called PBP2a that is not susceptible to drug deed, and this is due to the serine residue that is not reachable by β–lactams at the active site of the TP of PBP2a (Foster, 2017).

Different results in both methods used for β–lactamase detection in all *S. aureus* isolates, the positive result in an iodometric method was 91%, while 73 isolates could produce β–lactamase through Cefinase disk. Most strains of *S. aureus* were resistant to penicillin; nearly 20% of *S. aureus* remained susceptible to penicillin (Parija, 2014).

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Clinical *S. aureus* isolates were examined for biofilm formation *via* phenotypic and molecular assays, and any possible association between biofilm formation and bacterial genetic lineage was investigated. *S. aureus* remains the most recurrently encountered bacterial pathogen and is responsible for a variety of mild to life-threatening infections (El-Huneidi et al., 2006). The ability of *S. aureus* to adhesion and form biofilm makes them more resistant against antibiotics. Bacterial virulence factors such as adhesion play a vital role in catheter-related infections. Staphylococcus' ability to settle in artificial material is linked to two main mechanisms; polysaccharide slime production and host matrix protein adsorption on the biomaterial surface. When the biofilm is produced, it would be easy to run away from immune systems and to cause chronic infections. While PIA is essential for the formation of biofilm by *S. aureus*, in this study, we found in these isolates more than one gene of *ica* operon, i.e. *icaADBC*, *cna*, *atl*, *fnbA* and *fnbB* genes are associated with the production of biofilm in these isolates.

Although many genes and conditions are responsible for biofilm production, our results demonstrated that *icaA, icaB, icaC, icaD, cna, atl, fnbA,* and *fnbB* genes have a critical role in the production of biofilms. In this work we found that 89 out of 100 strains were biofilm producers developing almost black (*n=25*), black (*n=15*) or strong black colonies (*n=49*) on CRA plate, while in MTP assay, all isolates produce biofilm with ranging from weak (*n=8*), moderate (*n=71*) and strong biofilm production (*n=21*). Comparatively, similar results have been achieved elsewhere, stating that 50% of clinical *S. aureus* isolates to form a biofilm (Kouidhi et al., 2010). Out of 100 bacterial isolates, the formation of biofilm was recorded in 60% of isolates by CRA method, while PCR detection of biofilm-related genes, *icaA,* and *icaD*, revealed that both genes were present in 78% of the isolates (Salehzadeh et al., 2016). Discrepancies have been reported between phenotypic and genotypic methods for the detection of *S. aureus* producing biofilms (Yazdani et al., 2006). Statistical analysis revealed there is no significant difference between the biofilm formation using the MTP assay and the CRA method ($p = 0.8997$). Numerous studies have confirmed that some *S. aureus* strains need *icaADBC* to form biofilms (Johnson et al., 2008). On the other hand, adhesion to host cells requires genes such as *fnbA*, *fnbB*, and *cna* that encode microbial surface constituents known as adhesive matrix molecules that are different from those involved in the adhesion on abiotic surfaces (Arciola et al., 2005).

In the present study, the prevalence of the *icaC*, *icaD*, and *cna* (96%) and *icaA*, *icaB*, *atl*, *fnbA,* and genes were found to be% 80%, 72%, 80% and 86%, respectively. This result may reflect the role of these genes in the pathogenicity of *S. aureus* isolates. However, *fnbB* was found in a lower percentage (60%). The prevalence of twelve genes involved in biofilm production explains that numerous factors may be useful in various stages of biofilm formation due to the ability of all the strains identified to form biofilm at different levels, but the gene incidence was different. Further research to clarify the expression of these genes in *S. aureus* strains should be considered (Nourbakhsh and Namvar, 2016), and they stated that the prevalence of the genes involved in biofilm production was: *icaA* (34.2%), *icaB* (29.7%), *icaC* (69.3%), *icaD* (54.8%), *fnbA* (38.1%), *fnbB* (46.6%), and *can* (18.3%). Similar to our study, several other researchers have shown that the formation of slime and biofilm in *S. aureus* is associated with the presence of *icaA* and *icaD* genes (Cramton et al., 1999; Arciola et al., 2001; Vasudevan et al., 2003). Production of intercellular adhesion molecules, e.g., by *icaABCD* and other genes, plays an essential role in staphylococcal biofilm. Biofilm production may be the fundamental reason for the increasing antibiotic resistance of *S. aureus* strains. A

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significant association was observed between the presence of genes such as *icaA*, *icaB*, *icaC*, *icaD*, *can*, *atl*, *fnbA* and *fnbB*, and biofilm formation. Although not all of the genes were found simultaneously in all the isolates of the biofilm producers, at least one of the related genes was detected in the entire isolates of the biofilm producers (except one strain). The ability to form biofilm in negative strains for one or more genes may be associated with some other genes associated with biofilm.

In the present study, the prevalence of the *icaC*, *icaD*, and *cna* (96%) and *icaA*, *icaB*, *atl*, *fnbA*, and genes were found to be% 80%, 72%, 80% and 86%, respectively. This result may reflect the role of these genes in the pathogenicity of *S. aureus* isolates. However, *fnbB* was found in a lower percentage (60%). The prevalence of twelve genes involved in production of biofilm explains that numerous factors may be useful in different steps of biofilm formation because all of the strains identified could form biofilm at various levels, but the incidence of genes was different. Further research should be considered to elucidate the expression of these genes in *S. aureus* strains. We investigated the effects of *Z. officinale* extract, quercetin, and ascorbic acid on biofilm formation using a static biofilm assay and using *S. aureus* as a model biofilm-forming microorganism, and found that quercetin inhibits the formation of biofilm when compared with *Z. officinale* extract and Ascorbic acid through the percentage of biofilm inhibition.

Conclusion

In the current study, a significant association between the presence of *icaA*, *icaB*, *icaC*, *icaD*, *can*, *atl*, *fnbA*, and *fnbB* genes and biofilm formation were observed. Although all of the genes were not found in all of the biofilm producer isolates, however, in the entire biofilm producer isolates (except one isolate) at least one of the related genes was detected. The ability of biofilm formation in isolates negative for one or more genes might be related to some other biofilm-associated genes. The most critical future suggestions are to use all active components which present in the *Z. officinale* against biofilm formation in different bacterial species and genera.

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