Determination of Inhibitory Effects of *Allium sativum* Extract on Biofilm Production by Clinical *Staphylococcus aureus* Isolates

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ABSTRACT

Objectives: To determine anti-biofilm effect of fresh garlic extract (FGE) on *Staphylococcus aureus* biofilm production and the relationship between methicillin resistance and biofilm production intensity.

Methods: Clinical *S. aureus* isolates were identified methicillin resistant *S. aureus* (MRSA) by cefoxitin disc diffusion method. The anti-biofilm effects of FGE on *S. aureus* biofilm biomass determination was done using crystal violet assay.

Results: Among 71 *S. aureus* isolates, MRSA were 37 (52.1%). Among biofilm producers, comparison of biofilm biomass (absorbance at 492 nm) showed no significant differences in biofilm formation ability between MRSA and MSSA ($p=0.325$). Use of 10% FGE decreased biofilm production in MRSA and MSSA by 40.4% ($p<0.001$) and 48.1% ($p<0.001$) respectively. Detachment assay using sodium dodecyl sulfate showed that control group biofilm biomass was decreased by 41.2%, while test group was decreased by 61.7% ($p<0.001$).

Conclusion: Garlic extracts has potency as an anti-biofilm agent and could be developed and used to manage different *S. aureus* biofilm related infections.

Key words: Fresh garlic extract, biofilms, MRSA, *Staphylococcus aureus,* Nepal

INTRODUCTION

Staphylcoccus aureus, is a pathogen with its natural reservoir in humans**.** This pathogen causes skin, wound and burn infections, septicaemia and endocarditis (Tong et al 2015)**.** Between 63-65% of hospital acquired infections (HAI) are due to antibiotics resistant bacteria (Cassini et al 2018), antibiotics resistant strains of *S. aureus* is one of the major causative agents (Wu et al 2021). Vancomycin resistant *S. aureus* (VRSA) has been reported from various parts of the world (Rossi et al 2014; Hasan et al 2016; Azhar et al 2017; Shekarabi et al 2017; ElSayed et al 2018; Wu et al 2021).

Unlike in nutrient rich conditions of laboratory where bacteria grow planktonically, bacteria growing naturally form complex aggregated structures called biofilms (Costerton et al 2005). The ability to form a biofilm, gives the pathogen's ability to produce chronic diseases such as chronic osteomyelitis, chronic cystitis, chronic prostatitis, chronic otitis media, chronic pneumonia in patients with cystic fibrosis and dental plaques (Lebeaux et al 2013). In addition, biofilm producing microorganisms also cause infections of various organs by producing biofilms on implanted biomedical devices (Mack et al 2004). Various microbial pathogens growing as biofilms are resistant to most antimicrobial agents, whereas same pathogens growing planktonically are sensitive to virtually all antibiotics tested (Olson et al 2002).

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Usually, the compounds that kill or inhibit bacterial growth are used routinely to reduce production of biofilm, but application of these compounds at sub-inhibitory levels may stimulate biofilm formation (Nucleo et al 2009). Because of these reasons, compounds that inhibit biofilm formation without affecting bacterial growth are getting attentions.

Garlic is being used as therapeutic and prophylactic agent for a long time. The principal organosulphur compound in intact garlic cloves is alliin (+ S-allyl-L-cysteine sulfoxide). Upon chopping or crushing garlic, allinase enzymes activates and converts alliin to form 2-propenesulfenic acid, which self-condenses to form allicin (diallyl thiosulfinate). Allicin is only present in fresh, raw garlic and raw garlic preparation contains about 3.1 mg/g of allicin (Block 1992). Allicin readily diffuses across both artificial and natural phospholipid membranes (Miron et al 2000). The antimicrobial effects of allicin is due to interaction with thiol- containing enzymes and at slightly higher concentrations other enzymes, such as dehydrogenases or thioredoxin reductases, might be affected which could be lethal to microorganisms (Ankri and Mirelman 1999).

Garlic extracts showed excellent antibacterial activity against wound pathogens such as *S. aureus* and *S. epidermidis* (Nidadavolu et al 2012). Likewise, application of fresh garlic ointment lead to more organized and rapid wound healing due to activation of fibroblasts by allicin (Alhashim and Lombardo 2018). Ratthawongjirakul and Thongkerd (2016) reported significant reduction in biofilm formation of *S. aureus* under chemopreventive and chemotherapeutic conditions.

Natural products are used as an alternative medicine for treatment of various diseases because of less side effects, inexpensiveness and better patient tolerance. In this study we determined the biofilm production intensity of *S. aureus* isolates, identified the inhibitory effect of fresh garlic extract (FGE) on *S. aureus* biofilm production and assessed the effects of FGE on biofilm detachment of *S. aureus*.

METHODS

Research design

This was a cross-sectional quantitative study and primary data were collected from May to December 2018. Garlic bulbs were collected and tested against biofilm of *S. aureus*.

Collection of garlic bulbs and test bacteria

The garlic samples were collected from the local market of Lalitpur and disinfected at the laboratory. Seventy-one *S. aureus* isolates from the clinical samples collected during May to August 2018 were kindly provided by Clinical Microbiology Laboratory of the Alka hospital, Lalitpur. Amies transport medium was used for bacterial isolates transportation and stored at -20°C for further processing.

Preparation of garlic extract

A 100 gram of the bulb of garlic was squeezed using mortar and pestle. The squeezed sample was sucked at 200ml distilled water for overnight with shaking at 30°C. Then the extractions were filtered through muslin cloth and through Whatman no.1 filter paper. The aqueous extract was kept in sterile bottle in refrigerator at -20°C until use (Suleria et al 2012).

Re-confirmation and characterization of clinical isolates of *S. aureus*

S. aureus isolates first streaked on blood agar (BA) and incubated at 37°C for 24 hours. Round, raised, opaque and β- hemolytic colonies of size 1-2 mm growing on BA was grown on nutrient broth (NB) for about 3 hours at 37°C. Then organisms from NB were streaked on freshly prepared mannitol salt agar (MSA) and incubated at 37°C for 24 hours. Primary characterization of isolates was done on the basis of fermentation of mannitol, catalase, oxidase, coagulase (slide and tube) and DNase tests.

Phenotypic detection of MRSA

Identified *S. aureus* isolates were subjected to modified Kirby-Bauer's disc diffusion test as recommended by CLSI guidelines (CLSI 2014). The cefoxitin (30 mcg) disc (Hi-media) was used to detect MRSA. The inoculums were prepared by transferring 2-3 identical colonies from nutrient agar to sterile normal saline. The turbidity of the inoculums were made equivalent to 0.5 McFarland standard. The lawn culture of the test inoculums was prepared by swabbing MHA with a sterile cotton swab dipped into inoculums. Cefoxitin (30 mcg) disc was applied to the inoculated MHA plate and incubated at 35ºC for 18 hours. After incubation, the zone of inhibition of ≤21 mm around the disc was identified as MRSA. The MRSA COL strain was used as positive control and *S. aureus* ATCC 25923 as negative control.

Screening of biofilm producing *S. aureus* strains

Tube method, a qualitative method was applied for biofilm detection. A loopful of test organisms were inoculated with 2 ml of tryptone soya broth (TSB) with 1% glucose in test tubes. The tubes were incubated at 37°C for 48 hours. After incubation, tubes were decanted and washed with phosphate buffered saline (pH 7.4) and air dried (for 30 minutes) in inverted position. Tubes were stained with

crystal violet (1%) and washed to remove excess stain with distilled water. Tubes were dried in inverted position for 18-24 hours. Biofilm formation was considered positive when a visible thick film lined the bottom of the tube.

Static biofilm formation assay and determination of inhibitory effects of fresh garlic extract on biofilm formation of *S. aureus*

S. aureus was grown on TSB for overnight at 37°C. The culture was diluted (1:20) with fresh TSB. The diluted cultures (150 µl) without garlic extract were aliquoted into 96-well microtiter plate as controls. The diluted cultures (150 µl) with FGE (10%) were aliquoted into 96-well microtiter plates as tests. Along with controls and tests, un-inoculated and FGE free TSB and un-inoculated and FGE-supplemented TSB was applied on 96 microtiter plates adjacently. Three replicate wells for each treatment were performed. The plate's surface was sealed by applying paraffin tape and incubated at 37°C for 24 hours. After incubation, the bacterial culture solutions were discarded, and the wells were thoroughly washed three times with phosphate buffered saline (PBS) (pH=7.4). The plates were subsequently dried at 60°C for 30 minutes. The adherent biofilms in each well were stained with 175 µl of a 0.1% (w/v) solution of crystal violet in water at room temperature for 15 minutes. The plates were rinsed three times with water by submerging in a tub of water and tapping vigorously on a paper towel to completely remove all excess cells and dye. The plates were dried overnight at room temperature. Approximately 175 µl of ethanol (99.9%) was added to each well to solubilize the crystal violet. Then the absorbance at 492 nm was measured using an ELISA plate reader (O'Neill et al 2007).

The mean OD_{492} values for the control and tested wells were subtracted from the mean OD_{492} values obtained from the un-inoculated FGE-free and un- inoculated FGE-supplemented wells, respectively to calculate biofilm inhibitory effect of FGE.

Determination of effect of fresh garlic extract on biofilm detachment of *S. aureus*

S. aureus was grown on TSB overnight at 37°C. Culture was diluted (1:20) with fresh TSB. The diluted cultures (150 µl) without garlic extract were aliquoted into 96-well microtiter plate as controls. The diluted cultures (150 µl) with FGE (10%) were aliquoted into 96-well microtiter plates as tests. Along with controls and tests, un-inoculated-Sodium dodecyl sulfate (SDS) (5 µl) present-FGE free TSB and un-inoculated-SDS (5 µl) present-FGE supplemented TSB was applied on 96 microtiter plates adjacently. Three replicate wells for each treatment were performed. The plate's surface was sealed by applying paraffin tape and incubated at 37°C for 24 hours. Then 5 µl of 10% SDS was added to each well, and the mixture was incubated for 30 minutes. After incubation, the bacterial culture solutions were discarded, and the wells were thoroughly washed three times with phosphate buffered saline (PBS) (pH=7.4). The plates were subsequently dried at 60°C for 30 minutes. The adherent biofilms in each well were stained with 175 µl of a 0.1% (w/v) solution of crystal violet in water at room temperature for 15 minutes. The plates were rinsed three times with water by submerging in a tub of water and tapping vigorously on a paper towel to completely remove all excess cells and dye. The plates were dried at room temperature overnight. Approximately 175 µl of ethanol (99.9%) was added to each well to solubilize the crystal violet. Absorbance at 492 nm was measured using an ELISA plate reader (O' Neill et al 2007).

The mean OD_{492} values of the control and tested wells were subtracted from the mean OD_{492} values of un-inoculated-SDS (5 µl) present-FGE free TSB and un-inoculated-SDS (5 µl) present-FGE supplemented TSB wells, respectively to calculate biofilm detachment capacity of FGE.

Statistical analysis

The data were analyzed using SPSS software version 25.0. The biofilm absorbance data were expressed as a mean± standard deviation (S.D.). In order to determine p-value for association, Chi-square test and Mann-Whitney U-test were used.

RESULTS

Rate of MRSA and MSSA and biofilm production

Of 71 *S. aureus* isolates, 37 (52.1%) were MRSA and 34 (47.8%) were MSSA. Out of 71 *S. aureus* strains, 87.3% (n=62) were biofilm producers and 12.6% (n=9) were biofilm non-producers.

Among 37 MRSA strains examined, 59.4% (n=22) were moderate biofilm producers. The weak biofilms were produced by 32.4% (n=12) of MRSA strains, while 8.1% (n=3) of strains were strong biofilm producers. Similarly, out of 34 MSSA strains, 41.1% (n=14) were moderate biofilm producers, followed by 29.4% (n=10) weak biofilm producers, 26.4% (n=9) no biofilm producers and 2.9% (n=1) strong biofilm producers (Table 1).

The average absorbance of biofilm biomass of MRSA and MSSA groups were 0.4329±0.2566 and 0.3696±0.1925, respectively (Figure 1). Comparison of average absorbance of biofilm biomass showed that there was no statistically

significant difference in biofilm forming ability between MRSA and MSSA strains (p=0.325).

Effect of fresh garlic extract on biofilm formation of *S. aureus* isolates

The average absorbance of biofilm biomass of FGE-untreated and FGE-treated groups were 0.4074±0.2333 and 0.2315±0.1428, respectively (Figure 2). The average absorbance of biofilm biomass in FGE treated isolates were significantly lower than those in the control FGE untreated group (p<0.001). The biofilm formation for the experimental group (i.e. cultures containing FGE) was 43.1% less than the amount of biofilm formation in the control group (Figure 2).

The average absorbance of biofilm biomass of FGE-untreated MRSA and FGE-treated MRSA groups were 0.4329±0.2566 and 0.2581±0.1571, respectively. The biofilm biomass in FGE-treated MRSA isolates were significantly lower than those in the control FGE-untreated MRSA group (p=0.001). Similarly, the average absorbance of biofilm biomass of FGE-untreated MSSA and FGE-treated MSSA group were 0.3696±0.1925 and 0.1920±0.1100, respectively. The biofilm biomass in FGE-treated MSSA isolates were significantly lower than those in the FGE-untreated MSSA group (p<0.001). The biofilm biomass was decreased by 40.3% and 48.1% in MRSA and MSSA test groups respectively (Figure 3).

Effect of fresh garlic extract on biofilm detachment of *S. aureus* isolates

The average absorbance of biofilm biomass of the control SDS treated-FGE untreated group was 0.2394±0.1482, while that of SDS treated-FGE treated group was 0.1559±0.1198, with statistically significant difference (p<0.001). The biofilm biomass in SDS-FGE-untreated groups were decreased by 41.2% and those in SDS-FGE-treated groups were decreased by 61.7% (Figure 4).

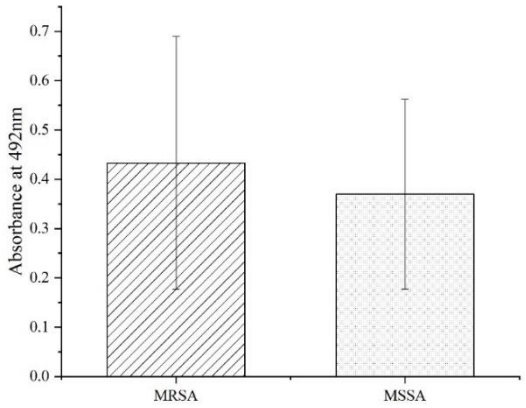

Figure 1: Comparison of average absorbance of biofilm formation by MRSA and MSSA

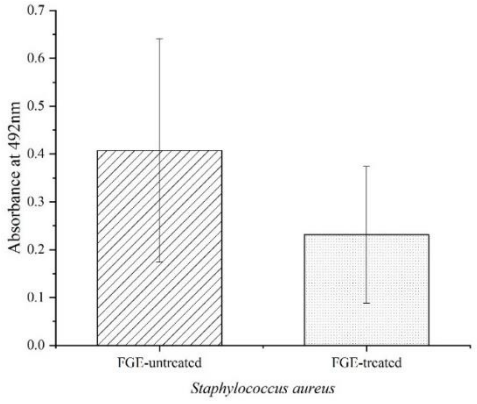

Figure 2: Comparison of average absorbance of values of biofilm biomass of FGE-untreated isolates and FGE-treated isolates

Table 1: Biofilm production among MRSA and MSSA

S. aureus Phenotypes	Strong biofilm producers (SBP) %	Moderate biofilm producers (MBP) %	Weak biofilm producers (WBP) %	No biofilm producers (NBP) %	Total (%)
MRSA	3 (4.2)	22 (30.9)	12 (16.9)	0 (0.0)	37 (52.1)
MSSA	1 (1.4)	14 (19.7)	10 (12.6)	9 (12.6)	34 (47.8)
Total	**4 (5.6)**	**36 (50.7)**	**22 (30.9)**	**9 (12.6)**	**71 (100.0)**

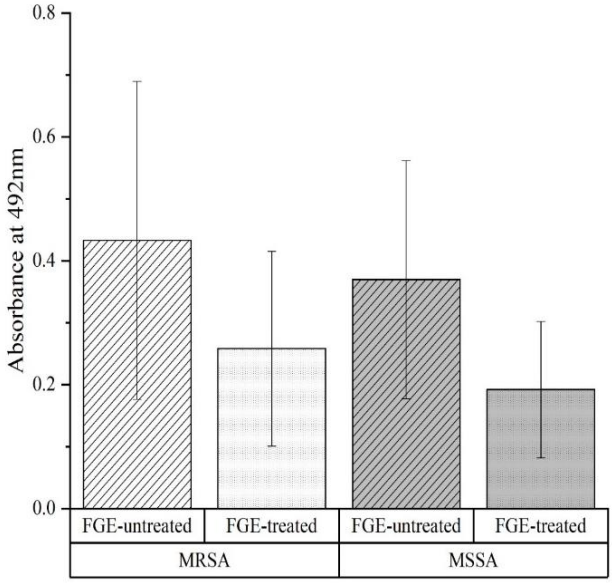

Figure 3: Comparison of an average values of biofilm biomass of FGE-untreated isolates of MRSA and MSSA with their FGE treated counterparts

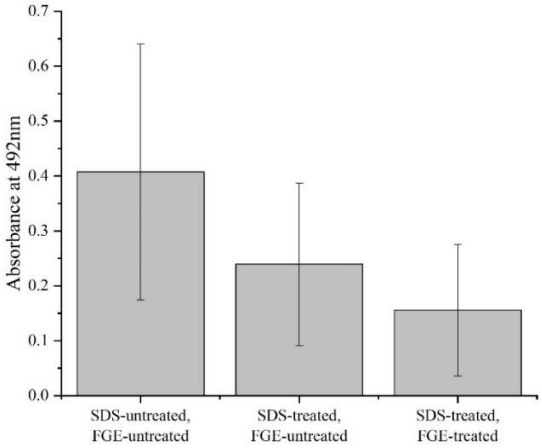

Figure 4: Comparison of average values of biofilm biomass of SDS untreated-FGE untreated isolates, SDS treated-FGE untreated isolates and SDS treated-FGE treated isolates

DISCUSSION

The MRSA in this study was found to be 52.1%. The reported prevalence of MRSA in Nepal was 21.1% to 68% in previous studies (Kumari et al 2008; Khanal and Jha 2010; Shrestha 2013; Shahi et al 2018; Khanal et al 2018). The rate of MRSA in hospitals of other countries was also similar (Wangai et al 2019; Hussein et al 2019) but different from some studies (Tariq and Javed 2019; Garoy et al 2019; Joshi et al 2013; Rajesh et al 2018; Dulon et al 2011; Adam and Abomughaid 2018; Omuse et al 2014). Hence, the prevalence of MRSA is variable among different countries and also between different regions of the same country. The various factors which affect intra- and intercountry variation in the prevalence of MRSA include differences in types of specimen, study population and study duration. Studies relying on genotypic detection by PCR tend to report the lower MRSA prevalence compared to phenotypic detection procedures such as cefoxitin disc diffusion test (Nwankwo and Nasiru 2011).

In this study, 100% MRSA isolates (37/37) produced biofilm whereas only 73.5% (25/34) MSSA possessed biofilm producing ability. The number of biofilm producers among MRSA is statistically significantly higher compared to MSSA strains (p= 0.001). There was no significant difference in biofilm production intensity between biofilm producing MRSA and MSSA isolates. The comparison of an average absorbance of biofilm biomass of MRSA and MSSA isolates showed no statistically significant differences. This result is broadly comparable to previous studies (Ghafourian et al 2013; Ghasemian et al 2016). The presence of large number of both strains of *S. aureus* as moderate biofilm producers (more than 55%) may help explain the high dissemination and the infection rate of *S. aureus* in healthcare facilities. Also the presence of large number (87.3%) of biofilm producing isolates in this study indicates the possibility of increased drug resistance in patients which may lead to treatment failures. However, other studies reported that biofilm production capacity is stronger in MRSA compared to MSSA (Manandhar et al 2018; Piechota et al 2018). MSSA strains produce NaCl induced biofilm whereas MRSA biofilms were glucose induced. A study of large collection of *S. aureus* isolates (114 MRSA and 98 MSSA) sampled from device-related infections containing 5 clonal complexes (CC5, CC8, CC22, CC30 and CC45) found that there is a significant relationship between *SarA* regulated PIA/PNAG and MSSA biofilm development. The biofilm development in MRSA is *ica* independent and involves a protein adhesion (s) regulated by *Sar A* and *Agr* (O'Neill et al 2007).

The FGE treatment significantly decreased the intensity of biofilm formation in both MRSA and MSSA isolates *in vitro*.

There are also reports of antibacterial effects of garlic extract from the previous studies (Ratthawongjirakul and Thongkerd 2016; Ninyio et al 2016; Farrang et al 2019; Wu et al 2015), anti-biofilm effects (Ninyio et al 2016), used as remedy for cardiovascular diseases (Rahman and Lowe 2006), cancer (Roy et al 2016) and chemically induced hepatotoxicity (Ademilugi et al 2013). Raw FGE is used in this study because raw garlic contains large amount of allicin, which exhibits broad spectrum antimicrobial activity against Gram positive and Gram negative bacteria (Wallock-Richards et al 2014; Wu et al 2015; Reiter et al 2017) in addition to its anti-biofilm activity (Lihua et al 2013; Rasmussen et al 2005).

In this study, FGE (10%) inhibiting effects on biofilm was analyzed under chemo preventive conditions i.e. the *S. aureus* isolates were grown on microtiter plates in the presence of FGE. So, the reduced biofilm formation in the presence of FGE may be due to combination of killing of planktonic cells, reducing cell attachment to the surface and disturbing maturation of biofilms.

The toxic effects of garlic have been tested in a mouse model. The study suggested that garlic extract didn't exhibit toxicological effects at the hematological and the histological levels, but instead provided protective effects (Farrang et al 2019). Garlic extracts can be coated on biomedical devices, used as ointments for wound infections and used orally to combat pathogenic biofilm related bacteria.

The detachment assay performed in this study showed that detachment efficiency for *S. aureus* biofilm cells with FGE treatment was higher compared to biofilm cells without FGE. This result suggests that FGE treated biofilm cells are loosely attached to the surface than those untreated biofilm cells. It has industrial and commercial applications as garlic extracts can be used with surfactants in order to remove unwanted biofilms present in water pipeline, membrane filters used in water treatment plant, mixing tanks and vats in food industries etc.

This study possesses certain limitations. Due to limited resources molecular characteristics of *S. aureus* isolates could not be determined. This made the distinction of HA-MRSA and CA-MRSA impossible, making the source of infection uncertain.

CONCLUSION

There are no statistically significant differences between MRSA and MSSA in their biofilm producing ability. The study also shows that FGE inhibits biofilm production by both MRSA and MSSA isolates significantly, in addition to their ability to detach biofilms effectively.

This suggests that garlic extracts have potency as an anti-biofilm agent and could be developed and used to manage different *S. aureus* biofilm related infections.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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