Int.J.Curr.Microbiol.App.Sci (2018) *7*(2): 2921-2926

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 7 Number 02 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.702.354

Effect of Natural Compounds on Inhibition of Biofilm Formation of Multi Drug Resistant *Staphylococcus aureus* and *Staphylococcus epidermidis* - an *in vitro* Study

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Keywords

Biofilm inhibition, Natural compounds, Minimum biofilm inhibitory concentration, Multi drug resistance, *Staphylococci*

Article Info

Accepted:
26 January 2018
Available Online:
10 February 2018

A B S T R A C T

Biofilm formation by *Staphylococcus aureus* and *Staphylococcus epidermidis* is a common cause of chronic infection and intravascular device failure. Biofilms are resistant to almost all available drugs rendering them difficult to treat. Thus, there is a need to identify natural compounds which can inhibit biofilm formation. A total of 52 multi drug resistant isolates of *Staphylococcus aureus and Staphylococcus epidermidis* were isolated from various clinical samples. Isolates were identified by standard microbiological procedures. Biofilm formation was seen by modified tissue culture plate method. The in vitro inhibitory activity of reserpine, eugenol, linoleic acid, curcumin, chitosan and berberine in strong biofilm producing isolates with their minimum biofilm inhibitory concentration (MBIC) was calculated. Biofilm formation was seen in 70.4% isolates of *Staphylococcus epidermidis and* 48% isolates of *Staphylococcus aureus*. In isolates of *Staphylococcus aureus*, the maximum activity for biofilm inhibition was observed with reserpine (MBIC 0.0156 mg/ml) while in case of *Staphylococcus epidermidis* with eugenol (MBIC 0.0312 mg/ml). In our study, natural compounds were found effective in vitro for inhibition of biofilm formation in *Staphylococcus aureus* and *Staphylococcus epidermidis* with reserpine and eugenol most effective and curcumin least effective.

Introduction

Staphylococcus is known to produce biofilms on host surfaces. Biofilm linked infections are of great concern because biofilm associated bacteria can withstand host immune defences, antibiotics, biocides, and hydrodynamic shear forces. *Staphylococcus aureus* causes a range of infections, from minor skin infections to pneumonia, bacteraemia, sepsis, meningitis and surgical site infections. *Staphylococcus epidermidis* is a permanent and ubiquitous colonizer of human skin and the resulting high probability of device contamination during insertion (Otto, 2009).

Biofilms have been associated with a variety of persistent infections which respond poorly to conventional antibiotic therapy. Biofilms contains exopolysaccharide slime which causes a diffusion barrier by restricting the transport rate of drug molecule to the interior of the biofilm, or by chemically reacting with the molecules itself. Combinations of

antibiotics, silver nanoparticle, paramagnetic iron particles and mechanical methods like flushing, chlorination, and ultraviolet disinfection can be used to combat biofilms (Bose *et al.*, 2011).

Natural compounds of plant extracts like reserpine, berberine, curcumin, eugenol, essential oils (EO) like linoleic acid, and chitosan etc. can inhibit biofilm formation. Reserpine (an alkaloid derived from plant *Rouwolfia surpentine*) and berberine (an alkaloid isolated from *Berberis fremontii*) inhibit efflux pumps (Stavri *et al.*, 2007). Linoleic acid (a naturally occurring omega-6 essential fatty acid) is capable of inhibiting autoinducer-2 (Al-2) activity in *Escherichia coli* K-12 biofilm producing strain. Al-2 is a signal transduction molecule in biofilm (Soni *et al.*, 2008). Significant inhibitory activity of eugenol (oily extract from clover leaf) on MRSA and Methicillin- sensitive *Staphylococcus aureus (*MSSA*)* biofilm formation in vitro has been observed (Yadav *et al.*, 2015). Naturally occurring polysaccharide chitosan (partially deacetylated poly N-acetyl glucosamine derived from shells of shrimp and other sea crustaceans) resists biofilm formation by bacteria and fungi like *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa and Cryptococcus neoformans* (Martinez *et al.*, 2010). Curcumin (principal curcuminoid of turmeric) has been shown to inhibit biofilm formation and anti-adhesive activity in *Helicobacter pylori* (Pattiyathanee *et al.*, 2009). It shows inhibition of quorum sensing hence biofilm development by *Staphylococcus aureus*, uropathogens such as *Escherichia coli*, *Pseudomonas aeruginosa* PAO1, *Proteus mirabilis* and *Serratia marcescens* (Packiavathy *et al.*, 2014; Sardi *et al.*, 2017).

The present study was carried out to investigate the biofilm formation in Gram-positive aerobic bacteria *Staphylococcus aureus* and *Staphylococcus epidermidis.* Biofilm inhibitory effect of natural compounds reserpine, berberine, eugenol, linoleic acid, chitosan and curcumin on strong biofilm producing strains was detected.

Materials and Methods

The present study was conducted in the Department of Microbiology, Pt. B.D. Sharma Post Graduate Institute of Medical Sciences, Rohtak over a period of one year. A total of 52 multidrug resistant isolate comprising Gram positive bacteria *Staphylococcus aureus* (n=25) and *Staphylococcus epidermidis* (n=27) were isolated from pus, urine, blood and sputum samples by standard conventional microbiological techniques.

Detection of biofilm production by Modified Tissue Culture Plate method (MTCP) (Mathur *et al.*, 2006)

Isolates from fresh agar plates were inoculated in brain heart infusion (BHI) broth supplemented with 2% sucrose dispensed 2 ml in test tubes and tubes were incubated at 37˚C for 24 hours. Then the broth was diluted in the ratio of 1:100 with fresh BHI medium. Two hundred µl of this diluted culture broth was then added to 96 well- flat bottom, non-adherent, non-treated polystyrene tissue culture plates (HiMedia Laboratories, Mumbai, India). Uninoculated broth served as control to check sterility and non-specific binding of the medium while blank well served as control to check the quality of tissue culture plate.

These inoculated tissue culture plates were incubated for 24 hours at 37˚C. After incubation, the contents of the wells were removed by gently tapping the plates. The wells were then washed four times with 0.2 ml of phosphate buffered saline (PBS) to remove planktonic forms. Biofilms which remain

adhered to the wells were fixed with 2% sodium acetate for 30 minutes and stained with crystal violet (0.1% w/v) for 30 minutes. Excess stain was rinsed off with distilled water. After drying, the wells were then treated with 200 µl ethanol/ acetone (80: 20, v/v) for 15 min at room temperature, to evade interference with the stained matter at the liquid– air interface, which is not considered to be indicative of biofilm formation (Nahar *et al.,* 2013). Optical densities (OD) of stained adherent biofilms were then determined by an automated micro ELISA reader at wavelength of 570 nm. These OD values were considered as an index of bacterial adhesion and biofilm formation.

Quality control strains

American Type Culture Collection (ATCC) strains *Staphylococcus epidermidis* (ATCC 35984), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922) were used as control strains.

Strains with OD value >0.24 were strong, 0.12-0.24 moderate and <0.12 weak biofilm producers. Strong biofilm producing bacteria were tested for effect of natural compounds.

The commercially available compounds reserpine, berberine, eugenol, linoleic acid, chitosan and curcumin were dissolved at a concentration of 4 mg/ml in dimethyl sulfoxide (DMSO, 10%v/v) aseptically. Two fold serial dilutions of compounds were made to get concentration ranges from 0.0156 mg/ml to 4 mg/ml (Magesh *et al.,* 2013).

Determination of minimum biofilm inhibitory concentration (MBIC) of natural compounds (Magesh *et al.,* 2013)

The MBIC for each of the natural compounds were determined using modified tissue culture plate method. The strong biofilm isolates maintained in Brain Heart Infusion agar plate were inoculated into sterile BHI broth and incubated at 37^0C overnight without shaking. The overnight culture was diluted to 0.5 Mc farland standards in fresh BHI medium.

A 96 well microtitre plate was taken and in each well 100 µl of test compound was added to 100 µl BHI broth culture. The final concentration of the compounds was ranging from 2 mg/mL in the first well to 0.0078 mg/mL in the ninth well.

Broth inoculum was added in 10^{th} well to see biofilm formation. The "media control" only contained 100 µl sterile BHI broth was added in 11^{th} well. Microtitre plate was incubated at 37^0C in stationary condition for 24 hours.

After incubation, the contents of the wells were removed by gently tapping the plates. The wells were then washed four times with 0.2 ml of phosphate buffered saline (PBS) to remove planktonic forms.

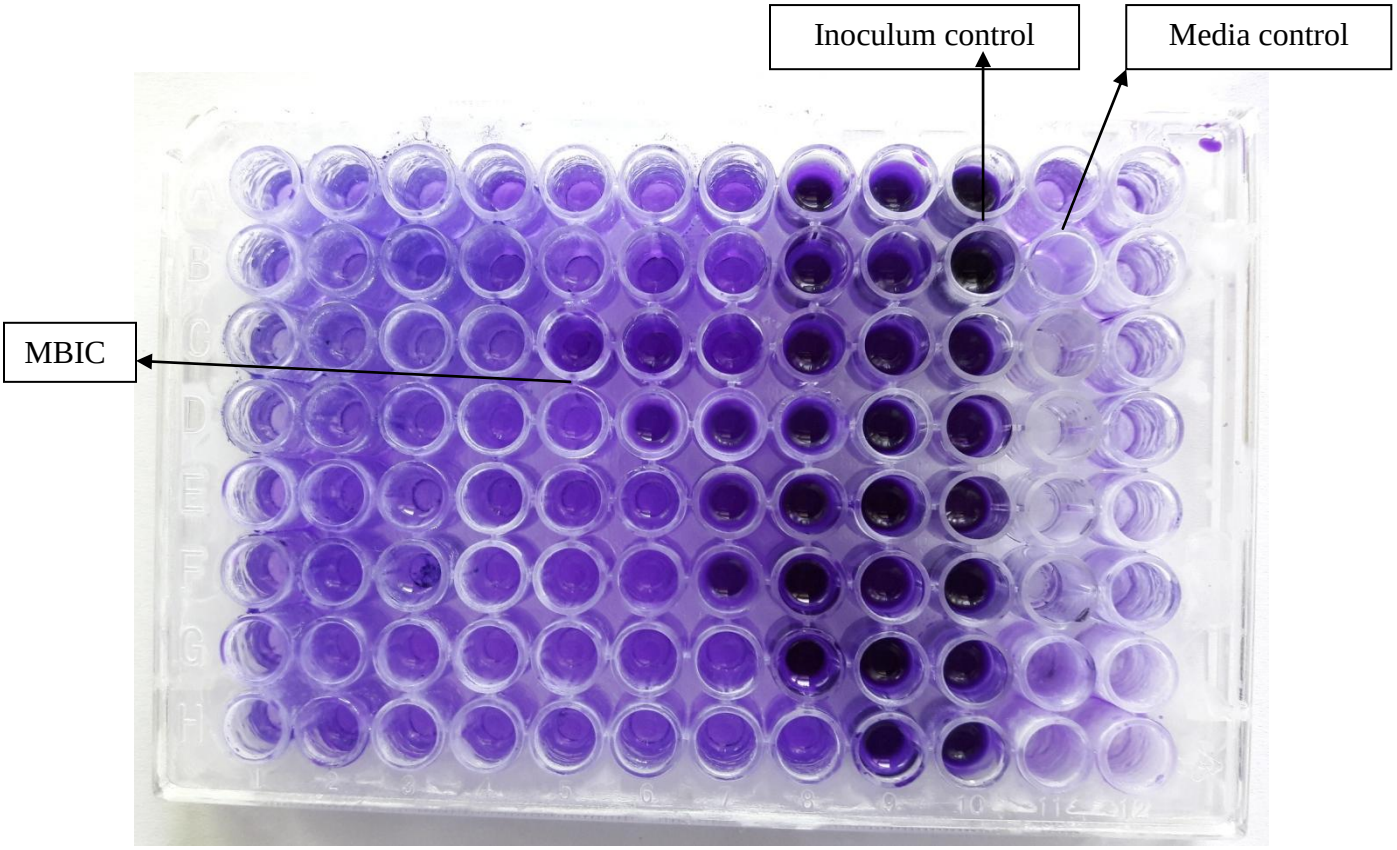
Biofilms which remain adhered to the wells were fixed with 2% sodium acetate for 30 minutes and stained with crystal violet (0.1% w/v) for 30 minutes. Excess stain was rinsed off with distilled water.

After drying, the wells were then treated with 200 µl ethanol/ acetone (80: 20, v/v) for 15 min at room temperature, to solubilize the dried crystal violet stain which was adherent to any biofilm. Optical densities (OD) of stained adherent biofilms were then determined by an automated micro ELISA reader at wavelength of 570 nm. The inhibitory effect of the compound on biofilm production was calculated by subtracting the media control. The minimum biofilm inhibitory concentration (MBIC) is the concentration of the natural compound at which biofilm formation was reduced to <0.12 OD value (Fig. 1).

Table.1 Minimum biofilm inhibitory concentration (MBIC ranging from 2 mg/ml to 0.0078 mg/ml) of natural compounds in various strong biofilm forming isolates

Organism	Reserpine (mg/ml)	Eugenol (mg/ml)	Curcumin (mg/ml)	Linoleic acid (mg/ml)	Chitosan (mg/ml)	Berberine (mg/ml)
Staphylococcus aureus	**0.0156**	0.0312	0.25	0.0625	0.25	0.125
Staphylococcus epidermidis	0.0625	**0.0312**	0.5	0.125	0.125	0.125

Fig.1 Biofilm inhibition by natural compounds



(MBIC- Minimum biofilm inhibitory concentration)

Results and Discussion

Out of 52 MDR isolates, maximum were obtained from pus (n=29) followed by blood culture (n=12), urine (n=8), and sputum (n=3) samples. Majority of MDR bacteria were isolated from samples received from indoor (n=36) than outdoor patients (n=16). Biofilm formation was seen in 19 (70.4%) isolates of *Staphylococcus epidermidis* and 12 (48%) isolates of *Staphylococcus aureus* by modified tissue culture plate method. Out of total 31 biofilm forming isolates, 12 (18%) were strong and 19 (32.5%) moderate biofilm producers. The rest were weak/non biofilm producers.

Biofilm detection can be included as routine diagnostic procedure, so that emergence of resistant isolates can be predicted at the earliest. Prevalence of biofilm formation in different bacteria helps to start empirical

therapy. In our study, biofilm formation was seen in 59.6% isolates. Samant *et al.,* found 42.7% isolates of *Staphylococcus* species to be biofilm producers (Samant *et al.,* 2012). Jayachandran *et al.,* studied *Staphylococci* spp. from various clinical isolates. It was observed that 46% of the isolates were biofilm producers.(Jayachandran *et al.,* 2016) In study done by Apoorva *et al.,* 52.6% of *Staphylococcus aureus* were able to produce biofilm (Apurva *et al.,* 2013).

In our study, natural compounds reserpine, berberine, curcumin, eugenol, linoleic acid and chitosan were found effective in inhibition of biofilm formation at MBIC ranging from 2 mg/ml to 0.0078 mg/ml *in vitro* (Table 1). The maximum activity for biofilm inhibition in case of *Staphylococcus aureus* isolates was for reserpine (MBIC 0.0156 mg/ml) while minimum activity was for curcumin and chitosan (MBIC 0.25 mg/ml). The maximum activity for biofilm inhibition in case of *Staphylococcus epidermidis* isolates was for eugenol (MBIC 0.0312 mg/ml) while minimum activity was for curcumin (MBIC 0.5 mg/ml).

It was found that MBIC of berberine to inhibit biofilm formation in *Staphylococcus epidermidis* was 0.125 mg/ml. In contrast, Chu *et al.,* in 2014 found that berberine significantly inhibited biofilm formation in *Staphylococcus epidermidis* at the concentration of 8 mg/ml. Whereas Wang *et al.,* found inhibitory effect at concentration of 30-45 mg/ml. In a study by Yadav *et al.,* in 2015, it was observed that eugenol significantly inhibited the growth of *Staphylococcus aureus* biofilms. At 0.05-0.2 mg/ml concentrations, biofilm formation was reduced in eugenol treated isolates, as compared to the control isolates. While in our study, it was inhibited at concentration of 0.0312 mg/ml. Asli *et al.,* observed that chitosan inhibited biofilm formation in *Staphylococcus aureus* at concentration 16 mg/ml while in our study much lower concentration 0.25 mg/ml was needed. No similar studies were available regarding the reserpine, linoleic acid and curcumin.

Staphylococcus aureus and *Staphylococcus epidermidis* are the commonly isolated multi drug resistant aerobic bacteria both in indoor and outdoor settings. Persistent infections occur due to biofilm formation by these bacteria. Early identification of infection caused by biofilm producing strains might help to modify treatment and outcome. In our study, natural compounds were found effective in vitro for inhibition of biofilm formation in *Staphylococcus aureus* and *Staphylococcus epidermidis* with reserpine and eugenol most effective and curcumin least effective.

Acknowledgments

Conflict of interest. The authors declare that they have no conflict of interest related to this article.

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How to cite this article:

Ankita Agrawal and Uma Chaudhary. 2018. Effect of Natural Compounds on Inhibition of Biofilm Formation of Multi Drug Resistant *Staphylococcus aureus* and *Staphylococcus epidermidis* - an *in vitro* Study. *Int.J.Curr.Microbiol.App.Sci.* 7(02): 2921-2926.
doi: https://doi.org/10.20546/ijcmas.2018.702.354