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Inhibition of virulence of *Staphylococcus aureus* – a food borne pathogen – by squalene, a functional lipid

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ABSTRACT

Increased resistance of *Staphylococcus aureus* – a pathogen responsible for hospital and food related infections – to several antibiotics has become a major concern. Staphyloxanthin, a carotenoid, is attributed to be the major virulent factor contributing to the pathogenicity of *S. aureus*. The present study investigated squalene for its ability to render *S. aureus* avirulent and consequent effect on staphyloxanthin. Squalene inhibited staphyloxanthin synthesis in *S. aureus* as confirmed by spectral profiling and HPLC analysis. It also reduced the haemolytic ability of the pathogen in a dose dependent manner (0.05–0.75 mM). Squalene pretreatment increased the susceptibility of the pathogen to oxidants by 48% and reduced the neutrophil resistance by about 82%. Further, biofilm forming ability of *S. aureus* was also affected by squalene. This is the first evidence of the antivirulent effects of squalene, a functional lipid, and, provides an alternate approach for treating *S. aureus* infections.

1. Introduction

Staphylococcus aureus, a bacterial pathogen, has emerged as a threat associated with community related and hospital acquired infections globally (Karamatsu, Thorp, & Brown, 2012) and is also responsible for staphylococcal food borne diseases worldwide, responsible for 241,000 illnesses annually in the United States alone (Kadariya, Smith, & Thapaliya, 2014). The repeated and long term usage of bactericidal or bacteriostatic antibiotics have resulted in multidrug resistant strains of *S. aureus* (Cegelski, Marshall, Eldridge, & Hultgren, 2008) and numbers of epidemic outbreaks spreading due to antibiotic resistant *S. aureus* are on the rise (Shoham, 2011). Further, increasing spread of methicillin resistant *S. aureus* (MRSA) in hospital acquired and community related infections has become a clinical challenge to cope up with staphylococcal infections (Karamatsu et al., 2012). Though newer therapies are prescribed,

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Chemical compounds: Staphyloxanthin (PubChem CID: 56928085); Squalene (PubChem CID: 1105); Glutaraldehyde (PubChem CID: 3485); Ethyl acetate (PubChem CID: 8857); Dimethyl sulphoxide (PubChem CID: 679); Acetone (PubChem CID: 180).

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recent reports indicate that MRSA is becoming resistant to the newer therapies as well, raising concerns about such therapies in clinical practice (Johnson & Woodford, 2002). The rising minimum inhibitory concentration (MIC) of vancomycin in the treatment of *S. aureus* infection apart from emergence of less-susceptible strains, toxicity and risk of high dosage (Rodvold et al., 2006) has made researchers look for newer alternatives. Thus, strategies involving substances that reduce the virulence without affecting the growth and viability of the pathogenic bacterium are gaining increased research interest. Antivirulent compounds provide advantages over classic antibiotics in two key ways – firstly, they target pathways essential for host pathogen interaction instead of those essential for basic metabolism; and, secondly, they are specific in their effect i.e., they do not affect the normal flora (Escaich, 2008).

S. aureus is known to harbour several virulent factors with staphyloxanthin, a carotenoid, being the major factor (Liu et al., 2008) apart from haemolysin, enterotoxins, coagulase, TSST-1, and protein A (Pantrangi, Singh, Wolz, & Shukla, 2010). Staphyloxanthin, a gold coloured pigment, acts as an antioxidant due to its conjugated double bonds thereby conferring *S. aureus* the resistance to reactive oxygen species during host neutrophil based killing (Liu et al., 2008). Recently several antivirulent strategies involving synthetic drugs have been proposed including inhibition of staphyloxanthin by lipid metabolism inhibitors (Sakai et al., 2012), sesquiterpene farnesol (Kuroda, Nagasaki, & Ohta, 2007), phosphonosulphates (Song et al., 2009) apart from inhibition of haemolysins by β-cyclodextrins (Karginov et al., 2007), inhibition of enterotoxin by plant derived extracts (Braga et al., 2005) and prevention of biofilm formation by engineered polymers (Chung et al., 2007).

Squalene, a natural triterpene hydrocarbon finds many applications in clinical, cosmeceutical and daily usage products (Bhattacharjee & Singhal, 2003). It is also a well documented biochemical intermediate in the synthesis of cholesterol and other steroids (Reddy & Couvreur, 2009). Previously, sebum containing squalene as a fraction of its constituents has been tested for its antibacterial effect against gram positive bacteria including *S. aureus* (Wille & Kydonieus, 2003). However, no reports attributing bactericidal and fungicidal activity to squalene alone are available and more so in the case of *S. aureus*. Unlike many synthetic anti-infectious agents used for the treatment of *S. aureus* infections, no toxic doses of squalene or adverse effects have been detected in the human body (Reddy & Couvreur, 2009). Against this background, we tested a probable hypothesis for inhibition of staphyloxanthin by squalene as it has structural similarity with some of the intermediates of staphyloxanthin biosynthesis. The possible mechanism of antivirulent properties of squalene was evaluated by assessing the effect of squalene on staphyloxanthin synthesis, haemolysis and biofilm formation in *S. aureus*.

2. Materials and methods

2.1. Antimicrobial agents and chemicals

Squalene (>98% purity) was purchased form M/s Sigma-Aldrich (St. Louis, MO, USA). Tryptic soy broth (TSB), tryptic soy agar (TSA), de-Man Rogosa & Sharpe (MRS) broth, MRS agar, Mueller-Hington agar, nutrient broth, antibiotic octa disc and crystal violet dye were from M/s Hi-media (Mumbai, Maharashtra, India). Dimethyl sulphoxide (DMSO), ethanol, glycerol and glutaraldehyde were form M/s Merck (Billerica, MA, USA). Analytical grade hydrogen peroxide (H_2O_2), methylene blue, ethyl acetate, sodium chloride and anhydrous sodium sulphate were procured from M/s Merck-India (Mumbai, India). Catalase enzyme was from M/s MP Biomedicals (Solon, OH, USA). All the chemicals used during experiments were of analytical grade unless mentioned otherwise.

2.2. Bacterial strains and growth conditions

Several food borne pathogens and beneficial bacterial cultures were tested for the effect of squalene. The staphylococcal strains used in this study were *S. aureus* subsp. *aureus* MTCC1430 (human plural fluid isolate) and *S. aureus* subsp. *aureus* ATCC27217 (nasal isolate). The other pathogenic strains used for antibacterial assay were *Escherichia coli* MTCC108, *E. coli* MTCC433, *Listeria monocytogenes* ATCC19111, *Bacillus cereus* ATCC49064, *Salmonella sp* type B MTCC1163 and *Yersinia enterocolitica* MTCC859. The lactic acid bacteria (LAB) strains, used as beneficial bacteria, were *Enterococcus faecium* MTCC5691, *E. faecium* NCIM 5367 and *Pediococcus acidilactici* NCIM5368. All the stock cultures were maintained in 50% glycerol and preserved at −80 °C. The cultures were repeatedly revived in appropriate medium and sub-cultured once a month to avoid contamination and stored under refrigeration condition (4 °C). The bacteria were cultured in TSB at 37 °C overnight for use in this study except LAB which were cultured in MRS broth.

All the bacterial cell growth measurements were conducted at 600 nm using a UV–visible spectrophotometer (Shimadzu UV-1800, M/s Shimadzu, Kyoto, Japan) and viability was enumerated as log colony forming units (cfu) per millilitre. All the experiments were conducted in triplicate by using different concentrations of squalene ranging from 0.05 mM, 0.25 mM, 0.50 mM, 0.75 mM, 1.00 mM to 5.0 mM (Lee et al., 2013).

2.3. In-vitro antibacterial assay

In-vitro antimicrobial assays (Ahuja, Kaur, & Sharma, 2015) to evaluate the activity of squalene against several food borne pathogens and LAB were carried out. Briefly, antibacterial activity against the pathogens and LAB cultures were carried out by agar well diffusion method on Muller-Hington and MRS agar respectively with a standard reference control (antibiotic octa disc). Different concentrations of squalene (0.05, 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 mM) dissolved in DMSO were tested. One per cent DMSO used as a solubilizer formed the positive control.

2.4. Growth curves

Cell growth experiments were conducted to check whether or not to ascertain the effect of squalene on growth of *S. aureus*. The inoculum was prepared as mentioned above (section 2.2). *S. aureus* strains were grown in the presence and absence of squalene at different concentrations ranging from 0.05, 0.25, 0.50 to 0.75 mM for 48 hours. The optical density values were