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Antibacterial and anti-biofilm activities of *Dicranopteris linearis* leaf extracts

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Abstract

Biofilms are defined as cells that are irreversibly attached to a surface and embedded in an exopolysaccharide matrix. Biofilms can grow on living tissues and indwelling medical devices, which can threaten human health. Biofilms are more resistant to antibacterial agents and are difficult to eradicate. Moreover, the presence of non-multiplying cells in biofilms further complicates antibacterial treatments as they are able to tolerate extremely high doses of antibacterials. *Staphylococcus aureus* is one of the most frequent causes of biofilm-associated infections. Due to the various challenges in biofilm treatments, there is a need to search for effective compounds for biofilm treatments.

Dicranopteris linearis or "resam" has been used in traditional medicine to treat fever, constipation and burns. The objective of this study was to determine the antibacterial and anti-biofilm activities of *D. linearis* against the non-multiplying cells and biofilms of *S. aureus.* Methanol crude extraction (MCE) and sequential solvent extraction (SSE) of *D. linearis* was conducted. The extracts were assessed for antibacterial and anti-biofilm activities.

Through broth microdilution assay, antibacterial activity against *S. aureus* was observed for MCE of *D. linearis* leaves (MCE(L)), MCE of *D. linearis* roots (MCE(R)) and methanol (MeOH) fraction of SSE. The minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) for MCE(L) and MCE(R) were at 2.5 – 5.0 mg/ml while MeOH fraction had MIC and MBC values at 5 mg/ml and 10 mg/ml, respectively. Furthermore, time-kill assay against non-multiplying cells of *S. aureus* was also conducted, by testing against *S. aureus* cultures that were growth arrested through nutrient depletion, cold temperature and protein synthesis inhibition. MCE(L) demonstrated bactericidal activity at 20 mg/ml against the growth arrested cultures of *S. aureus* caused by nutrient depletion and protein synthesis inhibition, and was not effective against culture growth arrested at cold temperature.

For anti-biofilm activity, the water (H_2O) fraction and hexane (HEX) fraction was the most effective for biofilm inhibition activity and biofilm disruption activity, respectively, when tested against five *S. aureus* biofilm strains. The H_2O fraction demonstrated biofilm inhibition activity at 0.31 – 2.5 mg/ml while HEX fraction showed biofilm disruption activity at 0.07 – 5 mg/ml. This is the first study to report on the anti-biofilm activity of *D. linearis*. Both H_2O fraction and HEX fraction did not inhibit cell growth, thus the anti-biofilm effect observed was only due to the biofilm structure itself or the genes that codes for the biofilm.

Additionally, H_2O fraction was able to inhibit *S. aureus* biofilm formation on various polymer materials commonly used in medical settings: polystyrene (85-93% inhibition), polyvinyl chloride (76-

91% inhibition), polyethylene (68-90% inhibition); polypropylene (52-93% inhibition), silicone rubber (68-94% inhibition). The presence of various phytochemicals such as flavonoids terpenoids, tannins, cardiac glycosides, phenols, quinones and saponins were identified in H_2O fraction. However, further purification and isolation of H_2O fraction was not conducted due to difficulties in identifying the specific phytochemical responsible for the biofilm inhibition effect.

HEX fraction was able to disrupt about 42-75% of *S. aureus* biofilms. Through scanning electron microscopy, HEX fraction demonstrated destruction of the biofilm structure and scant biofilms were observed, with only few bacterial cells. Few phytochemicals were identified in HEX fraction, and thus, HEX fraction was selected for further purification and isolation process. Purification of HEX fraction had yielded Fraction A and based on nuclear magnetic resonance spectroscopy and liquid chromatography-mass spectrometry data, the compound from Fraction A was identified as α-tocopherol.

α-Tocopherol was tested for anti-biofilm activity and was found to exhibit biofilm disruption activity against *S. aureus* biofilms at 0.01 – 0.5 mg/ml. Currently, there has not been any study reported on the biofilm disruption effect of α-tocopherol. This will be the first study to report on the biofilm disruption activity of α-tocopherol against *S. aureus* biofilms or any other bacterial biofilms.

Further investigation revealed that α-tocopherol affects the biofilm matrix and not the cells within biofilms. α-Tocopherol was also effective in disrupting *E. faecalis* biofilm (23% disruption) and *E. coli* biofilm (31% disruption), and the polymicrobial biofilms of *S. aureus* + *E. faecalis* (22-25% disruption) at 0.01 – 0.5 mg/ml. The combination of α-tocopherol with vancomycin had mostly showed indifferent effect towards the disruption of biofilm biomass. The combination of α-tocopherol and vancomycin was indifferent to the presence of each other in reducing the biofilm biomass of *S. aureus* and would not cause a greater effect in disrupting biofilm as compared to using either α-tocopherol and vancomycin alone. Furthermore, the combination of α-tocopherol and vancomycin at low concentrations (4 µg/ml of α-tocopherol + 0.008 µg/ml of vancomycin) was shown to affect the viability of cells within *S. aureus* biofilms.

In conclusion, findings from this study demonstrated the antibacterial and anti-biofilm activities of *D. linearis*, with α-tocopherol being the active constituent for biofilm disruption activity. Further work on the biofilm disruption effect of α-tocopherol is necessary to explore its potential use in anti-biofilm therapies.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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Date: 31st July 2016

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List of abbreviations

ACN	acetonitrile
CFU/ml	colony forming unit (CFU) per millilitre
CLSI	Clinical and Laboratory Standards Institute
DCM	dichloromethane
EA	ethyl acetate
EPS	extracellular polymeric substances
FBEC	fractional biofilm eradication concentration
H_2O	water
HEX	hexane
HPLC	high performance liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
MCE	methanol crude extract/extraction
MeoH	methanol
MBC	minimum bactericidal concentration
MBDC	minimum biofilm disruption concentration
MBIC	minimum biofilm inhibition concentration
MIC	minimum inhibitory concentration
NMR	nuclear magnetic resonance
OD	optical density
PBS	phosphate buffer saline
SSE	sequential solvent extraction
TSA	tryptic soy agar
TSB	tryptic soy broth

α	alpha
β	beta
δ	delta
γ	gamma
°X	degrees Celsius
%	percent
cm	centimetre
g	gram
µg	microgram
µl	microliter
mg	milligram
ml	millilitre
mm	millimetre
nm	nanometre
rpm	revolutions per minute
SD	standard deviations

Chapter 1 Literature review

1.1 Introduction

Bacteria can exist in a planktonic state, in which single cells are freely suspended in a liquid medium, or as biofilms, which are sessile bacterial communities (Cabarkapa *et al.*, 2013). Most of the antibacterial treatments have been developed for planktonic bacteria (Lennox, 2011; Lynch and Abbanat, 2010). However, the majority of bacteria in natural environments grow as biofilms and they differ greatly from their planktonic counterparts (Cos *et al.*, 2010; Hernandez-Jimenez *et al.*, 2013). Bacteria in biofilms are capable of developing resistance towards conventional antibacterial treatments rapidly (Skogman, 2012) and therefore, there is a need to meet the demands for effective anti-biofilm therapies.

1.2 Planktonic bacteria

Planktonic bacteria are single cells which are floating freely in a liquid medium and are designed to colonize new niches. Planktonic bacteria differ from bacteria in biofilms in terms of their external structures, whereby planktonic bacteria are individual microorganisms while a biofilm is a well-connected organization of millions of them. The first stage of a pathogenic infection is typically caused by planktonic bacteria that are detached from the biofilm structure. Bacteria from the outermost layer of a mature biofilm detach and produce planktonic cells that are able to escape and colonize new surfaces (Cos *et al.*, 2010; Hernandez-Jimenez *et al.*, 2013). Bacterial attachment is crucial to colonize the niche and in developing a new biofilm structure, and with bacteria in the planktonic mode posing less challenges to the immune system, this enables them to colonize new niches easier (Hernandez-Jimenez *et al.*, 2013).

Until now, most of microbiological and immunological studies have been developed using bacteria inoculums in planktonic state and have obtained results that might not be reproducible with biofilms. Traditional antibiotics have been designed for and tested on bacterial cells in the planktonic state and thus, planktonic bacteria are more susceptible to antibacterial agents but they are ineffective against bacteria existing in a biofilm (Cunningham *et al.,* 2010; Hernandez-Jimenez *et al.*, 2013). With planktonic bacteria having a lower chance of survival, bacteria in a biofilm provides a more secure way for bacteria to reproduce and survive, which in part, explains the increased tolerance of biofilms against antibacterial treatments (Hoiby *et al.,* 2011; Hernandez-Jimenez *et al.*, 2013).

1.3 Biofilms

1.3.1 Biofilms defined

In the past 30 years, the definition of a biofilm has been constantly changing with new studies building on the existing knowledge on formation, structure, maturation and resistance of biofilms. Nowadays, the generally accepted definition of biofilms takes into consideration the observable characteristics, physiological and genetic properties of biofilms (Cos *et al.*, 2010). Donlan and Costerton (2002) defined a biofilm as a microbial-derived sessile community, which is characterized by cells that are irreversibly attached to a surface, an interface or to each other. These cells are embedded in a matrix of self-produced extracellular polymeric substances (EPS) and they exhibit altered phenotypes with respect to growth rate and gene transcription. True biofilms must fulfil these three criteria of a biofilm, involving the matrix formation, surface growth, and showing the distinct biofilm phenotype (Donlan and Costerton, 2002).

1.3.2 Stages of biofilm development

Biofilm formation is a complex process which occurs in several stages: adhesion, aggregation, maturation and dispersion (Park *et al.*, 2011). Figure 1-1 shows the stages of bacterial biofilm development.

1.3.2.1 Surface Conditioning

Some studies describe surface conditioning prior to microbial adhesion as the first step in biofilm formation, even though conditioning represents the interaction of the surface and its environment without the involvement of the bacteria (Cos *et al.*, 2010; Donlan, 2002; Dunne, 2002; Lindsay and Von Holy, 2006). In the natural environment, bacteria do not adhere to the substrate themselves but stick onto this so-called conditioned layer, which is formed on most substrates as a result of chemical surface modification. Molecules including polysaccharides, DNA, salts and plasma proteins can bind to surfaces and change the physiochemical properties of surfaces (Cos *et al.*, 2010). For example, the native surface of medical devices implanted in the bloodstream is modified by the absorption of molecules such as albumin, complement factors and fibronectin (Dexter *et al.*, 2001). Bacterial adhesion will depend on the compatibility of macromolecules of the conditioned surface and bacterial surface properties, which can influence bacterial adhesion onto the substrate. The conditioned layer permanently alters the properties of the surface, and to aid as the source of nutritive substances and important microelements (Cabarkapa *et al.*, 2013; Cos *et al.*, 2010; Dunne, 2002).

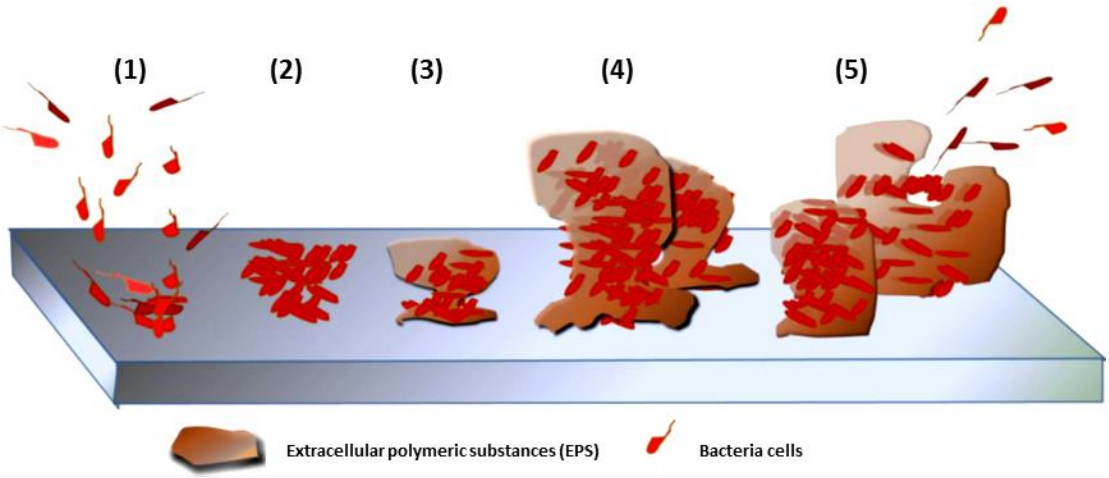


Figure 1-1: General overview of bacterial biofilm development: (1) reversible adhesion of bacteria; (2) irreversible adhesion of bacteria; (3) aggregation - production of extracellular polymeric substance and formation of micro-colonies; (4) maturation; (5) dispersion. After dispersion of the biofilm, bacteria move to other surfaces and stages (1)–(5) occurs again (modified from Park *et al.*, 2011).

1.3.2.2 Adhesion

Adhesion of bacteria onto a surface plays the key role in biofilm formation. The adhesion process involves two stages: primary adhesion (reversible binding) and secondary adhesion (irreversible binding) (Figure 1-1(1) and (2)). During primary adhesion, a reversible contact between the conditioned surface and planktonic bacteria is achieved. Primary adhesion depends mainly on the attractive and repulsive forces generated between the bacterial and conditioned surfaces, which includes Van der Waals forces, electrostatic and hydrophobic interactions, steric hindrance, temperature and hydrodynamic forces (Cos *et al.*, 2010; Dunne, 2002; Liu and Tay, 2001). Once the attractive forces become stronger than repulsive forces, irreversible binding is achieved. Secondary adhesion involves the anchoring or locking phases and surface attachment is mediated by binding between specific adhesins and the surface. Reversibly bound bacteria began producing EPS that forms a complex with surface material and/or receptor specific ligands on pili or fimbriae (Cos *et al.*, 2010; Dunne, 2002).

1.3.2.3 Aggregation

Aggregation is the second stage of biofilm development (Figure 1-1(3)) and is the result of simultaneous accumulation, growth and multiplication of bacteria. Bacteria began to multiply while initiating the quorum sensing mechanism and furthering EPS production (Cabarkapa *et al.*, 2013). Quorum sensing refers to the phenomenon whereby the chemical signals emitted by bacteria accumulates in the surrounding environment, thus, enabling a single bacterial cell to sense the cell density, so that the bacterial population can make a co-ordinated response as a whole (Cos *et al.*,

2010). The EPS matrix enables bacteria cells to aggregate by forming micro-colonies and further strengthens the adhesion of biofilm to the surface.

1.3.2.4 Maturation

The third stage involves biofilm maturation (Figure 1-1(4)). The biofilm develops to become denser and complex as a result of adherence of new planktonic cells in combination with the continuous growth of already bonded cells and EPS production. Within the formed micro-colonies, bacteria are bonded with intercellular bonds, with the surrounding EPS matrix binding them together. Typical water channels are also formed within the matrix layer enabling the exchange of nutrients and discharge of waste metabolic products (Cabarkapa *et al.*, 2013). Biofilm maturation is dependent on factors such as nutrient availability, elimination of waste metabolic products, internal pH, and oxygen perfusion (Cos *et al.*, 2010). The biofilm development process is relatively slow and can be evaluated as mature after several days (Cabarkapa *et al.*, 2013).

1.3.2.5 Dispersion

Dispersion is the final stage of biofilm development, whereby bacteria detach from the biofilm structure (Figure 1-1(5)). Bacteria from the outermost layer detach and produce planktonic cells that are able to escape and colonize new surfaces (Cos *et al.*, 2010). Dispersion can occur as an adaptive response to a changed environmental condition. The dispersion mechanism can be actively initiated by the bacterial cells themselves or can be a passive mechanism mediated by shear forces or abrasion (Cabarkapa *et al.*, 2013).

1.3.3 Biofilm-associated infections

Biofilms have been increasingly recognized as being an important feature in human diseases. Biofilms can grow on living tissues, and on indwelling medical devices and implants, leading to infections that can threaten human health (Chen *et al.*, 2013; Davies, 2003). Recent research showed that biofilms are involved in 80% of microbial infections in the body such as urinary tract infections, endocarditis, catheter associated infections, formation of dental plaque, and infections of permanent indwelling devices such as joint prostheses and heart valves (Figure 1-2) (Lebeaux *et al.*, 2013). Biofilm-associated infections are usually difficult to treat as the body's immune system is incapable of penetrating biofilms and destroying biofilm cells, and antibacterial treatments is only effective against their planktonic counterparts as biofilms are far more resistant (Agarwal *et al.*, 2010; Kaali *et al.*, 2011).

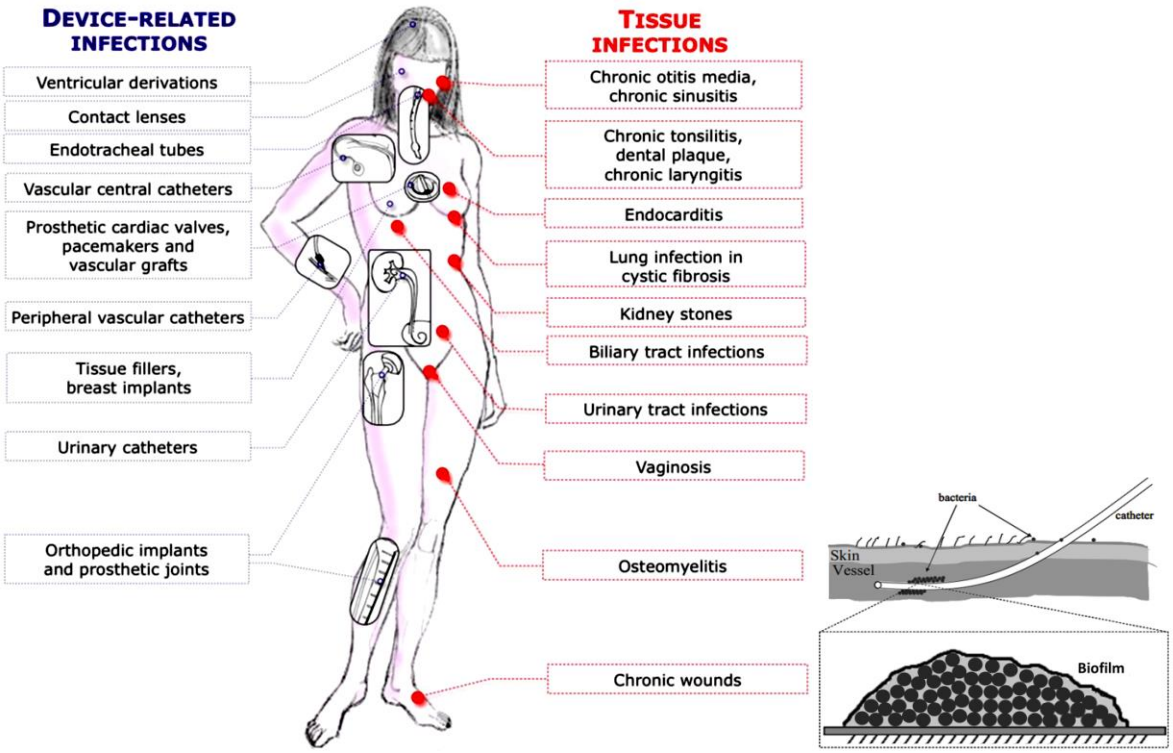


Figure 1-2: (A) Biofilm-associated infections of human body surfaces and medical devices (Lebeaux *et al.*, 2013); (B) Bacterial colonization on catheter, leading to biofilm formation (modified from Treter and Macedo, 2011).

1.3.4 Biofilm resistance

Biofilms confer major advantages to adherent bacteria as they are continuously provided with the appropriate environment for growth and survival, and protected against unfavourable conditions such as heat, UV radiation and host immune defences (Cos *et al.*, 2010). Another important advantage is that biofilm organisms are far more resistant to antibacterial agents as compared to their planktonic counterparts (Dunne, 2002). Treatment of biofilms with antibacterial agents often results in incomplete killing, allowing unaffected bacteria to cause recurrent infection following the withdrawal of antibacterial treatments (Figure 1-3) (Davies, 2003). Several mechanisms have been proposed to explain the increased resistance of bacteria biofilms to antibacterial agents and this will be discussed in Sections 1.2.4.1 to 1.2.4.3 (Willey *et al.*, 2014). Figure 1-4 represents the different properties of biofilm contributing to biofilm resistance.

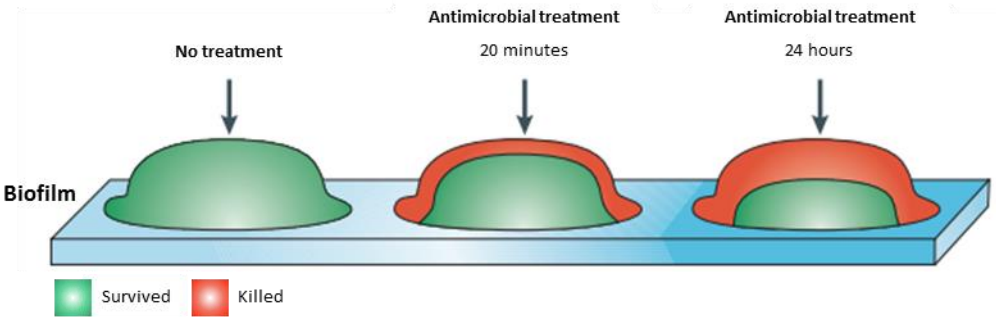


Figure 1-3: Biofilm resistance to antibacterial treatment. Treatment of bacteria biofilms with antibacterial agents often results in incomplete killing (even with 24 hours of treatment), allowing unaffected bacteria to cause re-infection following the withdrawal of antibacterial therapy (modified from Davies, 2003).

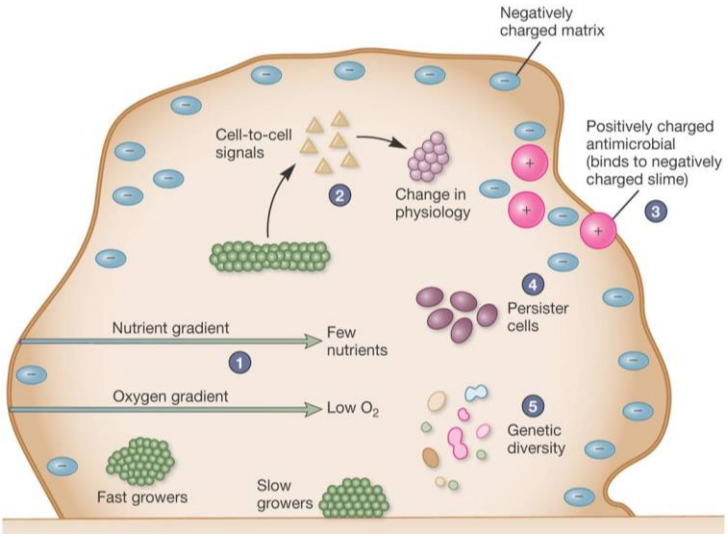


Figure 1-4: Schematic representation of different properties of biofilm contributing to antibacterial resistance (Willey *et al.*, 2014).

1.3.4.1 Poor penetration through the biofilm matrix

Antibacterial agents are unable to penetrate the full depth of biofilm and diffusion of antibacterial agents in EPS is relatively low (Kaali *et al.*, 2011). The biofilm EPS matrix blocks the transport of antibacterial agents by interacting with the antibacterial agents and inactivating them. For example, the negatively-charged matrix binds and reduces entry of positively-charged antibacterial agents (Figure 1-4(3)) (Stewart and Costerton, 2001; Willey *et al.*, 2014). The biofilm matrix acts as a diffusion barrier, and is highly efficient in protecting biofilms against antibacterial agents of larger molecular mass. Diffusion of an antibacterial agent through the biofilm matrix towards deeper layers causes the concentration of the antibacterial agent to reduce and thus, only surface biofilm bacteria are exposed to lethal concentrations. The biofilm matrix also slows down the penetration of antibacterial agents, which also allows time for the development and establishment of antibacterial resistance in deeper layers (Cabarkapa *et al.*, 2013).

1.3.4.2 Altered microenvironment and slow growth

The development of biofilm changes the conditions of the microenvironment surrounding the bacteria: - in terms of the availability of nutrients, oxygen and waste products (Cabarkapa *et al.*, 2013). Due to extremely limited availability of nutrients and oxygen in the deeper layers of biofilm, bacteria grow at slower rates, or become dormant (Figure 1-4(1)) (Willey *et al.*, 2014). These slow growing bacteria are known as non-multiplying bacteria. These non-multiplying bacteria are capable of slowing down their metabolism, leading to arrested growth and multiplication, making them less susceptible to antibacterial treatment as compared to surface biofilm bacteria or their planktonic counterparts (Cos *et al.*, 2010; Hu *et al.*, 2010). Since antibacterial agentss usually target rapidly growing cells, slower growth rates contributes majorly towards biofilm resistance to antibacterial agents as many antibacterial agents are incapable in destroying the slow growing or dormant cells (Kaali *et al.*, 2011).

1.3.4.3 Biofilm phenotype

A small part of the biofilm population known as persister cells (Figure 1-4(4)), are distinctly different and intrinsically more resistant to antibacterial agents (Willey *et al.*, 2014). Despite long-term exposure to high dosages of antibacterial agents, they are capable of remaining alive and upon withdrawal of treatment, can quickly re-colonize the biofilm leading to recurrent infections. The mechanism responsible for their emerging and existence has remained unknown but is assumed to be related to the dormant nature of persisters. By slowing down its metabolism, persisters avoid damages by antibacterial agents and thus, making them resistant (Cabarkapa *et al.*, 2013; Cos *et al.*, 2010).

1.4 Biofilm producing bacteria

1.4.1 *Staphylococcus aureus*

Staphylococcus aureus is a gram positive and ubiquitous bacterial species. *S. aureus* is recognized as one of the most frequent causes of biofilm-associated infections, including on indwelling medical devices. This is because *S. aureus* are commensal bacteria on the human skin and mucous surfaces and thus, are among the most likely bacteria to infect any medical devices that penetrate the skin, such as when inserted during surgery (Otto, 2008). *S. aureus* biofilms have been associated with chronic wound infections such as diabetic foot ulcers, venous ulcers and pressure sores (Archer *et al.,* 2011). *S. aureus* causes about 40-50% of prosthetic heart valve infections and about 50-70% catheter associated infections (Agarwal *et al.*, 2010). *S. aureus* biofilms are inherently resistant to antibacterial treatment. *S. aureus* is also known for evolving and spreading antibiotic resistance mechanisms, consequently causing further challenges in biofilm treatments.

1.4.2 *Enterococcus faecalis*

Enterococcus faecalis is a gram positive bacterium and are natural inhabitants of the oral cavity, normal intestinal microflora, and female genital tract of both human and animals. *E. faecalis* is an opportunistic pathogen and is one of the leading causes of nosocomial infections such as severe urinary tract infections (UTIs), surgical wound infections, bacteremia, and bacterial endocarditis (Duggan and Sedgley, 2007; Mohamed and Huang, 2007; Toledo-Arana *et al.*, 2001). It was reported that *E. faecalis* and *Enterococcus faecium*, the other frequently encountered enterococcal pathogen, together accounted for 16.0% of central line-associated bloodstream infections, 14.9% of catheter-associated UTIs, and 11.2% of surgical site infections (Frank *et al.,* 2013; Hidron *et al.,* 2008). *E. faecalis* is known to form biofilms and is often isolated from surfaces of medical devices such as ureteral stents, intravascular catheters, biliary stents and silicone gastrostomy devices (Mohamed and Huang, 2007). Furthermore, *E. faecalis* is intrinsically resistant to numerous antibiotics and has the tendency to acquire antibiotic resistance via horizontal gene transfer (Dale *et al.,* 2015).

1.4.3 *Escherichia coli*

Escherichia coli is a predominant species among facultative anaerobic bacteria of the gastrointestinal tract and is the most frequent microorganism involved in UTIs. *E. coli* is known to form biofilms and has been the cause of various medical device associated infections in devices such as prosthetic grafts and joints, shunts, and urethral and intravascular catheters (Beloin *et al.*, 2008; Sharma *et al.,* 2016; Soto, 2014). The formation of *E. coli* biofilms on catheters makes catheter-associated urinary tract infections (CAUTI) as one of the most frequent nosocomial infections (Sharma *et al.,* 2016). *E. coli* is responsible for most infections in patients with indwelling bladder catheters, with 10–50 % of patients undergoing short-term catheterization developing UTI and essentially all patients with an indwelling urinary catheter in place for more than a month will have UTIs (Ferrieres *et al.,* 2007).

The development of antibiotic resistance in these biofilm producing bacteria had further led to various challenges in treating biofilm associated infections. The conventional treatment of biofilm infections would be to remove the infected devices, but for some implants such as joint prostheses, or biofilm growth on host tissue, removal of the infected devices is not always an option (Kiedrowski and Horswill, 2011). The cost for implant removal can be exorbitant and some critically ill patients may not be able to endure the surgical procedures involved in implant removal. Therefore, there is an ongoing need for continuous development of treatment options for preventing biofilm growth on

medical devices and for effective removal of established biofilms on infected sites (Connaughton *et al.,* 2014; Kiedrowski and Horswill, 2011).

1.5 Anti-biofilm strategies

Understanding the mechanisms of biofilm formation is of fundamental importance in identifying the methods to prevent biofilm growth and removal of established biofilms. Biofilm development proceeds through multiple stages and therefore, it is important to understand factors involved in each stages of biofilm development, so that the various methods to prevent biofilm formation and remove established biofilms can be identified (Kumar *et al.*, 2010). According to the stages of biofilm formation, possible anti-biofilm strategies should be based on: (1) inhibition of bacterial adhesion to the surface and bacterial colonization; (2) interference with the signal molecules that modulates biofilm development; (3) disaggregation of the biofilm matrix (Figure 1-5) (Francolini and Donelli, 2010).

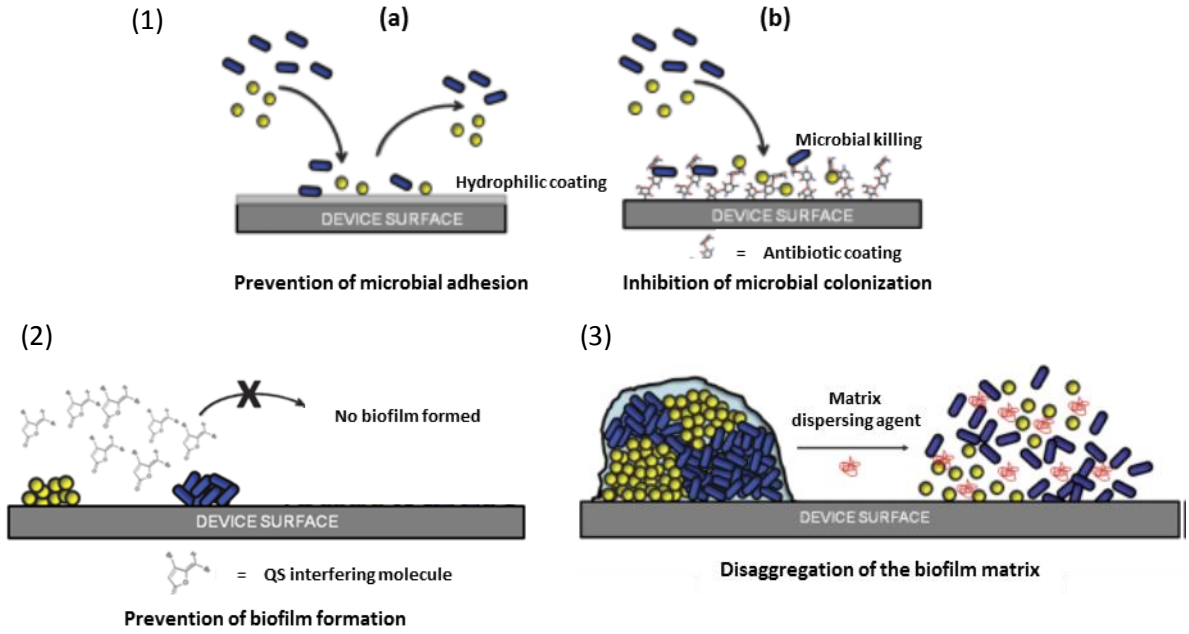


Figure 1-5: The possible anti-biofilm strategies: (1)(a) Prevention of microbial adhesion and (b) prevention of microbial colonization by coating device surfaces; (2) Prevention of biofilm formation using agents that interfere with quorum sensing (QS); (3) Disaggregation of biofilm matrix using matrix dispersing agent (modified from Francolini and Donelli, 2010).

1.5.1 Inhibition of bacterial adhesion to the surface and bacterial colonization

The first anti-biofilm strategy targets the adhesion stage of bacterial biofilm development, in which it involves the modification of physiochemical properties of surfaces to create anti-adhesive surfaces, and/or the incorporation of antibacterial agents to surfaces to prevent bacterial colonization (Rodrigues, 2011).

Since microbial surfaces are hydrophobic in nature, prevention of bacterial adhesion can be achieved through surface coating with hydrophilic polymers (Figure 1-5(1a)). It was reported that hydrophilic polymers such as hyaluronic acid (Cassinelli *et al.,* 2000) and poly-*N*-vinylpyrrolidone (Boelens *et al.,* 2000), used for polyurethane catheters coating and silicon elastomer shunts coating, respectively, were able to reduce the adhesion of bacteria such as *Staphylococcus epidermidis.* Hydrophilic coatings reduce bacterial adhesion by altering the physiochemical properties of surfaces to prevent surface conditioning and to prevent favourable interaction between bacteria and surfaces (Francolini and Donelli, 2010; Rodrigues, 2011).

For microorganisms that have already adhered to a surface, the use of antibiotics as coatings on surfaces can prevent colonization (Figure 1-5(1b)). Antibiotic coatings on the surface of central venous catheter (CVC) have helped to significantly reduce the risk of infection as compared to uncoated standard CVC. The two most effective antibacterial-coated CVCs are the minocycline-rifampin (M/R)-coated CVCs and the chlorhexidine-silver sulfadiazine (CHX/SS)-coated CVCs. M/R-coated CVCs exhibited significant activity against various gram positive and gram negative bacteria except *Pseudomonas aeruginosa* and *Candida* spp. (Francolini and Donelli, 2010; Raad *et al.,* 2012; Sampath *et al.*, 2001). The combination of CHX-M/R-coated CVCs provided better activity, whereby it was able to completely inhibit biofilm colonization of methicillin resistant *S. aureus* (MRSA), vancomycin resistant Enterococci, *P. aeruginosa* and *Candida* spp., as compared to that of M/R- and CHX-coated catheters alone (Raad *et al.,* 2012). However, it has been reported that CHX and M/R coated catheters are more expensive when compared to standard CVCs (Harron *et al.,* 2016; Wassil *et al.,* 2007).

1.5.2 Interference with the signal molecules that modulates biofilm development

The second anti-biofilm strategy targets quorum sensing (QS), the regulatory mechanism that allows bacteria to respond to needs related to cell density, through the expression of specific genes. The use of molecules that interferes with quorum sensing is a promising strategy to counteract bacterial adaptation to surfaces and prevent the development of biofilm (Figure 1-5(2)) (Francolini and Donelli).

Naturally occurring QS inhibitors have been reported from a large number of organisms such as prokaryotes, plants, marine organisms and fungus. *Streptomyces* sp. strain Y33-1 produces Siomycin I, which inhibits gelatinase and gelatine biosynthesis activating pheromone, resulting in the disruption of *Enterococcus faecalis* biofilm (Nakayama *et al*., 2007). Hamamelitannin, from the bark of *Hamamelis virginiana* (witch hazel) inhibits the QS regulator RNAIII in *Staphylocoocus* spp. to prevent biofilm formation and cell attachment *in vitro*, without affecting the growth of *Staphylocoocus* spp. (Kiran *et al.,* 2008). The marine alga, *Delisea pulchra* produces halogenated

furanones, which inhibits QS mediated activities in bacteria by competing with cognate N-acyl homoserine lactones (AHLs) signals for their receptor site (LuxR) and resulting in the rapid turnover of the receptor (Kalia, 2013; Manefield *et al.,*2002). Natural pigments produced by the fungus, *Auricularia auricular* had the ability to act as QS inhibitors when it inhibited violacein production in *Chromobacterium violaceum* (Zhu *et al.,* 2011).

The major limitation with natural QS inhibitors is the small concentrations in which these inhibitors are produced. Besides that, some QS inhibitors may be toxic in certain cases. For example, halogenated furanones are too reactive and thus, presumably too toxic for treatment of bacterial infections in humans (Chenia, 2013; Kalia, 2013). Besides naturally occurring QS inhibitors, synthetic QS inhibitors have also been synthesized. Synthetic furanone such as (Z)-4-bromo-5-(bromomethylene)furan-2(5H)-one and (Z)-5-(bromomethylene)furan-2(5H)-one were effective in inhibiting QS mediated biofilm formation when tested in mouse models of chronic *Pseudomonas aeruginosa* lung infection (Wu *et al.,* 2004).

1.5.3 Disaggregation of the biofilm matrix

The third anti-biofilm strategy targets the EPS matrix of biofilms (Figure 1-5(3)). This involves the use of substances that are able to destroy the physical integrity of the biofilm matrix. With the subsequent loss of the highly protective biofilm matrix, the sessile microbial cells are exposed to treatment by antibiotics (Francolini and Donelli, 2010). An enzyme called dispersin B, produced by *Actinobacillus actinomycetemcomitans*, can dissolve mature biofilms produced by *S. aureus* and *S. epidermidis* by degrading polysaccharide intercellular adhesion (PIA). PIA is an important component in both *S. aureus* and *S. epidermidis* biofilms and thus, could be an ideal target for anti-biofilm drugs (Boles and Horswill, 2011; Schillaci, 2011). Other matrix dispersing agents are such as Dnase I, proteinase K and trypsin (Boles and Horswill, 2011).

Although effective, there are several limitations in the usage of these matrix dispersing enzymes. Different enzymes have their own specific mechanism of action. It is difficult to identify effective enzymes against all different types of biofilms and thus, this complicates their practical application to control biofilms. Moreover, the high cost and low commercial accessibility of different enzymes also limits their usage in biofilm control strategies (Sadekuzzaman *et al.*, 2015).

1.6 Plants as an alternative source for biofilm control

Antibacterial products have been the main agent used to control unwanted biofilms. Although this method is widespread in biofilm control, there are no standardized antibiotic with reliable efficacy. This is due to the rapid development of bacterial resistance to all classes of antibiotics, rendering them ineffective in treating biofilm-associated infections. Therefore, there is a

need to search for newer and effective control methods for biofilms, and one that can be much less susceptible to the emergence of resistance compared to conventional antibiotics (Simoes, 2011).

Plants are a good source for discovering anti-biofilm compounds because of their therapeutic values in traditional medicine. Besides that, they have potent broad-spectrum of activity (Budzyńska *et al.*, 2011). There has also been increasing interest in their ecological role in the regulation of interactions between microorganisms. Unlike humans and mammals that possess immune systems to defend against invading pathogens, plants lack such sophisticated immune response and therefore, rely on the cellular and biochemical defence systems for protection against biofilms (Koh *et al.*, 2013; Villa and Cappitelli, 2013). Therefore, it is of interest to discover these plant chemicals or phytochemicals, which had protected them against biofilms.

Studies on the bioactivities of medicinal plants and phytochemicals have identified plant extracts and plant compounds which exhibits anti-biofilm properties. Table 1-1 and Table 1-2 lists some plant extracts and plant compounds that exhibit anti-biofilm effect against bacterial biofilms, and the effective concentrations for the anti-biofilm activities.

Table 1-1: Plant extracts reported to exhibit anti-biofilm activity against bacterial biofilms

Plant extracts	Anti-biofilm effect	Concentrations	Reference
Methanolic extract from *Capparis spinosa* Linn.	Inhibit biofilm formation and motility, decrease bio-surfactant and EPS production and disrupt mature biofilms of *Pseudomonas aeruginosa*, *E. coli*, *Proteus mirabilis* and *Serratia marcescens.*	2.0 mg/ml	Abraham *et al.* (2011)
Crude extract and ethanolic fraction from *Emblica officinalis*	Inhibit biofilm formation and obliterate biofilm structure of *Streptococcus mutans.*	0.04 – 0.08 mg/ml	Hasan *et al.* (2012)
Ethanolic extracts from *Hydrastis canadensis* L. (Ranunculaceae)	Anti-quorum sensing activity against *S. aureus.*	0.04 – 0.08 mg/ml	Cech *et al.* (2012)
n-hexane extract of *Dalbergia trichocarpa*	Inhibit biofilm formation, motility and virulence factor production of *P. aeruginosa.*	0.3 mg/ml	Rasamiravaka *et al*. (2013)
Polyphenol rich extract from *Rosa rugosa* tea	Inhibition of swarming motility and biofilm formation of *E. coli* and *P. aeruginosa*. Reduce violacein production in C*hromobacterium violaceum*.	0.6 – 1.2 mg/ml	Zhang *et al.* (2014)
Extract from wheat bran	Anti-quorum sensing activity, inhibition of biofilm formation and eradication of preformed biofilms of *Pseudomonas fluorescens* and *S. aureus*.	0.6 mg/ml	González-Ortiz *et al*. (2014)
Extracts from *Chamaemelum nobile* (Chamomile)	Inhibit biofilm formation and swarming motility of *P. aeruginosa* and clinical isolates from different types of infections.	6.3 – 25.0 mg/ml	Kazemian *et al.* (2015)

Table 1-1: Plant extracts reported to exhibit anti-biofilm activity against bacterial biofilms (*Continued*)

Plant extracts	Anti-biofilm effect	Concentrations	Reference
Ethanolic extract from *Amomum tsaoko*	Inhibition of violacein production of *C. violaceum*. Inhibition of swarming motility and biofilm formation of *S. aureus*, *P. aeruginosa* and *Salmonella enterica* serovar Typhimurium.	4.0 mg/ml	Rahman *et al.* (2015)
Methanolic extracts from *Castanea sativa* (European chestnut)	Anti-quorum sensing effect resulting to inhibition of haemolytic activity, harmful exotoxin production and inhibition of biofilm formation of *S. aureus.*	0.0016 – 0.025 mg/ml	Quave *et al.* (2015)
n-hexane (HEX) and dichloromethane (DCM) extracts of *Liriodendron hybrid* barks	DCM extract inhibit violacein production of *C. violaceum*. HEX and DCM extracts inhibit biofilm formation of MRSA.	0.1 – 4.0 mg/ml 0.2 mg/ml	Tan *et al.* (2015)
Crude extract and methanolic fraction from *Zingiber officinale*	Inhibition of biofilm formation and reduction of adherence of *S. mutans*.	0.064 – 0.12 mg/ml	Hasan *et al.* (2015)
Methanolic extracts rich in tannin from *Phyllanthus emblica, Terminalia bellirica, Terminalia chebula, Punica granatum, Syzygium cumini*, and *Mangifera indica*	Anti-quorum sensing activity against *S. aureus* and *C. violaceum.*	0.1 – 0.2 mg/ml	Shukla and Bathena (2016)
Extract of *Rosmarinus officinalis* (rosemary), *Mentha piperita* (peppermint) and *Melaleuca alternifolia*	Inhibit biofilm formation and disrupt the biofilms of *Listeria monocytogenes.*	1.0 mg/ml	Sandasi *et al.* (2010)
Ethanolic extract from *Piper betle*	Inhibit biofilm formation and pyocyanin production, and reduce swarming of *P. aeruginosa*. Inhibit biofilm formation and eradicate biofilms of *S. mutans.*	0.02 – 0.20 mg/ml 1.50 – 6.25 mg/ml	Datta *et al.* (2016) Teanpaisan *et al.* (2016)
Butanol fraction from *Quercus cerris*	Inhibit biofilm formation of *S. aureus.*	0.2 mg/ml	Hobby *et al.* (2012)
Extracts of *Commiphora leptophioeos, Bauhinia acuruana* and *Pityrocarpa moniliformis*	Inhibit biofilm formation of *Staphylococcus epidermidis.*	0.4 mg/ml and 4.0 mg/ml	Trentin *et al.* (2011)
Methanolic extract from *Cuminum cyminum*	Inhibit biofilm formation of *P. mirabilis*, *P. aeruginosa* and *S. marcescens*	2.0 mg/ml	Issac Abraham *et al.* (2012)
Polyphenols from grape marc extract and pine bark extract	Inhibit biofilm formation of oral bacteria such as *S. mutans* and *Streptococcus sobrinus*	2.0 mg/ml	Furiga *et al.* (2008)

Table 1-2: Plant pure compounds reported to exhibit anti-biofilm activity against bacterial biofilms

Plant pure compounds	Anti-biofilm effect	Concentrations	Reference
Carvacrol from oregano and thyme	Inhibit biofilm formation of *C. violaceum, S. aureus* and *S. enterica* serovar Typhimurium.	0.01 – 0.10 mg/ml	Sara *et al.* (2014)
Ellagic acid , esculetin and fisetin from green tea, fruits and legumes	Ellagic acid inhibits biofilm formation of *Streptococcus dysgalactiae.*	0.004 mg/ml	Durig *et al.* (2010)
	Esculetin inhibit biofilm formation of *S. aureus.*	0.130 mg/ml	
	Fisetin inhibit biofilm formation of *S. dysgalactiae* and *S. aureus.*	0.016 mg/ml	
Glabranine and flavanone from plants of *Tephrosia* genus	Inhibition of biofilm formation and reduction of biofilm biomass of *S. aureus.*	0.02 – 0.10 mg/ml	Manner *et al.* (2013)
Salvipisone from *Salvia sclarea* L.	Prevent bacterial adhesion and biofilm formation of *S. aureus* and *S. epidermidis.*	0.009 – 0.018 mg/ml	Rozalki *et al.* (2007)
Ellagic acid derivatives from *Rubus ulmifolius*	Inhibit biofilm formation of *S. aureus.*	0.05 – 0.20 mg/ml	Quave *et al.* (2012)
Lectins	Inhibit adherence and biofilm formation of *S. mutans.*	0.1 – 0.5 mg/ml	Islam *et al.* (2009)
Catechin, epigallocatechin gallate and tannic acid from green tea	Inhibit biofilm formation of *P. aeruginosa.*	0.004 mg/ml	Jagani *et al.* (2009)
Methyl eugenol from C*uminum cyminum*	Reduce biofilm formation of *P. aeruginosa* and inhibit violacein production of *C. violaceum.*	0.01 mg/ml	Issac Abraham *et al.* (2012)
Zingerone from ginger root	Reduce biofilm formation, decrease swimming, swarming and twitching motility, and interfere with the production of virulence factors of *P. aeruginosa*	10 mg/ml	Kumar *et al.* (2015)
Hamamelitannin	Anti-quorum sensing effect and increases the *in vitro* and *in vivo* biofilm susceptibility of MRSA to antibiotic treatment.	0.12 mg/ml	Brackman *et al.* (2016)
Eugenol	Inhibition of violacein production, elastase, pyocyanin and biofilm formation, interference with quorum sensing systems of *P. aeruginosa, E. coli* and *C. violaceum.*	0.02 – 0.06 mg/ml	Zhou *et al.* (2013)
Saponin	Inhibit biofilm formation and reduces biofilm biomass of *E.coli*	3.2 mg/ml	Monte *et al.* (2014)

1.7 *Dicranopteris linearis*

Dicranopteris linearis, belongs to the Gleicheniaceae family (Figure 1-6). It is commonly known as scrambling fern or false staghorn and is locally known as “resam” in Malaysia. *D. linearis* is an Old World tropical and subtropical species and is one of the most common ferns in South East Asia. Table 1-3 describes the traditional medicinal uses (Chin, 1992; de Winter *et al.*, 2003), the biological activities (Lai *et al.*, 2009; Ponnusamy *et al.*, 2015; Zakaria *et al.*, 2007; Zakaria *et al.*, 2008;

Zakaria *et al.*, 2011) and the phytochemicals (de Winter *et al.*, 2003; Jaishee and Chakraborty, 2015; Li *et al.*, 2008; Raja *et al.*, 1995) identified in *D. linearis*.

(a)  (b) 

Figure 1-6: (a) *D. linearis* in its natural habitat; (b) Leaves of *D. linearis*

Table 1-3: Information on *D. linearis*: the traditional medicinal uses, the biological activities studied and the list of phytochemicals identified.

Traditional medicinal uses	Biological activities studied	Phytochemicals identified
❖ to combat fever ❖ to cure constipation ❖ to cure chest complaints such as asthma and cough ❖ to cure burns, bruises and sprains ❖ to treat insomnia ❖ to treat bad skin rash in children ❖ used as an anthelmintic	❖ Antimicrobial ❖ Antinociceptive ❖ Anti-inflammatory ❖ Antipyretic ❖ *In vitro* cytotoxic ❖ Antioxidant ❖ *In vitro* wound healing	❖ Tannins (3.8%) ❖ Essential oils (0.03%) ❖ Saponins ❖ Clerodane glycosides ❖ Terpenoids ❖ Flavonoids (flavonol 3-O-glycosides) ➢ afzelin ➢ quercitrin ➢ isoquercitrin ➢ astragarin ➢ rutin ➢ kaempferol

As far as we know, the anti-biofilm properties of *D. linearis* against *S. aureus* have not been reported.

1.8 Overall objectives

The overall objectives of this study are:

I. To obtain crude extracts from the leaves of *D. linearis*

II. To determine if the crude extracts have antibacterial activity and anti-biofilm activity
   a. Note: On the basis of the preliminary results, the decision was made to focus solely on the anti-biofilm activity of the extract(s). The following objectives reflect this decision.

III. To purify and identify the active compound(s)

IV. To characterize the anti-biofilm activity of the purified active compound(s)

Chapter 2 Antibacterial activity of *D. linearis*

2.1 Introduction

Biofilms can consist of multiplying cells and non-multiplying cells. Biofilms are more resistant to antibacterial agents and thus, are difficult to be eradicated. The presence of non-multiplying cells in biofilms further complicates treatment with antibacterial agents as they are able to tolerate extremely high doses of antibacterial agents. *S. aureus* infections such as endocarditis and osteomyelitis consist predominantly of both non-multiplying and biofilm cells of *S. aureus*, which leads to treatment problems (Quave *et al.*, 2008). Therefore, there is a need for the discovery of compounds that are effective against both non-multiplying bacteria and biofilms.

2.1.1 Non-multiplying bacteria

Bacteria consist of at least two populations that exist simultaneously in two states, multiplying and non-multiplying. Non-multiplying bacteria are common among human infections and antibacterial agents with a killing mode of action are needed to assist the host to rapidly kill bacterial pathogens (Coates and Hu, 2007; Podos *et al.*, 2012). Antibacterial agents are capable of killing actively multiplying bacteria but are very inefficient in killing non-multiplying bacteria (Figure 2-1) (Hu *et al.*, 2010).

Non-multiplying bacteria are capable of entering a dormant state where all growth and reproduction ceases, enabling them to tolerate extremely high doses of antibacterial agents. Non-multiplying bacteria do not overtly cause diseases but act as a pool from which multiplying bacteria emerge to cause recurrent disease (Hu *et al.*, 2010). Antibacterial agents are then repeatedly administered for treatment, leading to prolonged treatment periods (Figure 2-1). This prolonged treatment can result in the emergence of antibacterial resistance in non-multiplying bacteria (Coates *et al.*, 2002; Lewis, 2010). Besides that, coping with environmental stresses such as lack of nutrients, adverse temperatures and low oxygen levels, can also lead to non-multiplying states with significantly increased resistance towards antibacterial agents (Podos *et al.*, 2012).

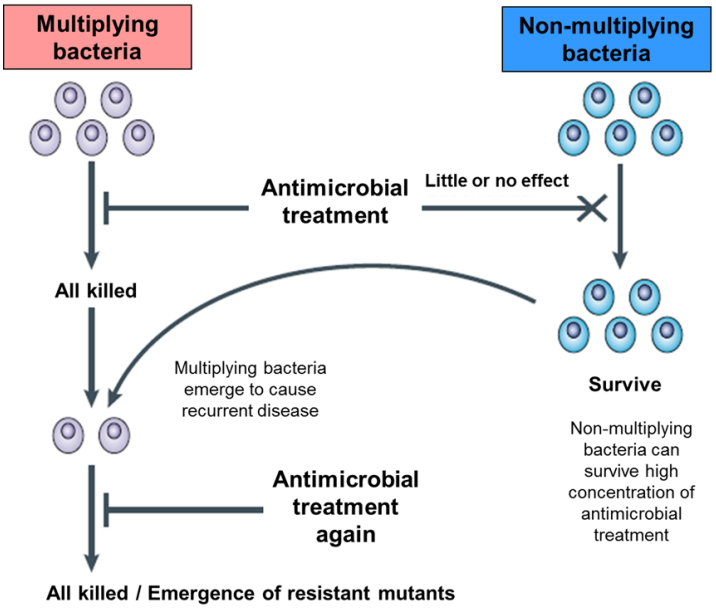


Figure 2-1: Multiplying bacteria are killed by antibacterial agents while non-multiplying bacteria survive even at high doses of antibacterial agents. Non-multiplying bacteria act as a pool for multiplying bacteria to emerge to cause recurrent disease, and may also give rise to resistant strains when prolonged treatments are administrated (modified from Coates *et al.*, 2002).

Studies in relations to non-multiplying cells of *S. aureus* had involved investigations on commercially available antibacterial compounds to yield bactericidal compounds, and some had reported on successful activities against non-multiplying *S. aureus,* such as HT61 (Hu *et al.*, 2010), daptomycin (Mascio *et al.*, 2007) and ACH-702 (Podos *et al.*, 2012). Thus far, no studies have been reported on the use of plant-derived compounds against non-multiplying *S. aureus*. Studies on *D. linearis* have reported findings on its antimicrobial activity (Lai *et al.*, 2009; Zakaria *et al.,* 2007) and therefore, it is of interest to investigate the potential use of *D. linearis* extract against non-multiplying cells of *S. aureus*.

2.2 Objectives

The objectives of this chapter were:

I. To perform methanol crude extraction (MCE) on *D. linearis* leaves and roots

II. To screen MCE for antibacterial activity against:

   a. Both multiplying and non-multiplying cells of *S. aureus* using broth microdilution assay

   b. Non-multiplying cells of *S. aureus* using time-kill assays

III. To perform sequential solvent extraction (SSE) on *D. linearis* leaves.

IV. To screen the SSE fractions for antibacterial activity

2.3 Methodology

The summary of methodology for the investigation of the antibacterial activity of *D. linearis* is presented in Figure 2-2.

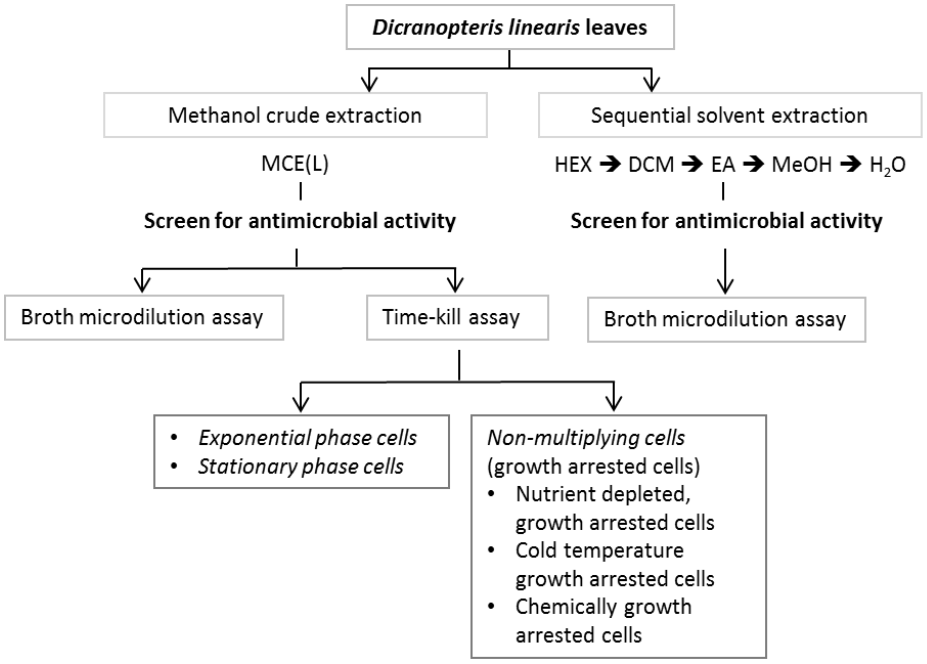


Figure 2-2: Summary of methodology for antibacterial studies on *D. linearis*

2.3.1 Bacterial strains

Five *S. aureus* strains were used, as listed in Table 2-1. *S. aureus* strains were cultured in tryptic soy broth (TSB) and incubated at 37°C.

Table 2-1: The characteristics of each *S. aureus* strain

Strain	ATCC number	Antibiotic resistance	Biofilm production
Staphylococcus aureus	6538P	Methicillin susceptible	Strong
Staphylococcus aureus	43300	Methicillin resistant	Moderate to strong
Staphylococcus aureus	33591	Methicillin resistant	Weak to moderate
Staphylococcus aureus	29213	Methicillin susceptible	Weak to moderate
Staphylococcus aureus	700699	Methicillin resistant	Weak

*Weak → $OD_{570} < 2$; Moderate → $2 \leq OD_{570} \leq 4$; Strong → $OD_{570} > 4$ (The characteristics for *S. aureus* biofilm production was determined based on the results obtained in this study and categorised in reference to Stepanović *et al.*, 2007).

2.3.2 Plant material

Fresh leaves of *D. linearis* were collected from its natural habitat in Genting Highland, Selangor (GPS co-ordinate: N 03.40357°, E 101.78545°) and identified by Miss Nor Ezzawanis, a botanist from Forest Research Institute Malaysia (FRIM). A voucher specimen was kept at Monash University.

2.3.3 Methanol crude extraction

The leaves of *D. linearis* were washed and rinsed with water to remove all dirt and unwanted particles and then blotted on tissue to dry. The leaves were then cut into small pieces and soaked in methanol (MeOH). The mixture was sonicated for 15 minutes, and then filtered under reduced pressure. The leaves were repeatedly extracted two more times until the filtrate was light coloured. The filtrates were pooled and the solvent was evaporated off using a rotary evaporator. The extract was freeze-dried and then stored at -20°C prior to analysis (Lai *et al.*, 2009). Extraction from roots of *D. linearis* was also conducted. The methanol crude extracts of *D. linearis* leaves and roots were abbreviated as MCE(L) and MCE(R), respectively.

2.3.4 Sequential solvent extraction

The leaves of *D. linearis* were washed and rinsed with water to remove all dirt and unwanted particles and then blotted on tissue to dry. The leaves were then freeze-dried. After that, the freeze-dried leaves were subjected to sequential solvent extraction. The leaves were soaked in hexane (HEX). The mixture was sonicated for 15 minutes, and then filtered under reduced pressure. The leaves were extracted two more times until the filtrate was light coloured. These steps were continued by sequentially soaking the leaves and extracting in dichloromethane (DCM), ethyl acetate (EA), methanol (MeOH) and water (H_2O). The solvents of the HEX, DCM, EA, MeOH and H_2O filtrates were evaporated off using a rotary evaporator. The extracts were freeze-dried and then stored at -

20°C prior to analysis (Lai *et al.*, 2009). Table 2-2 lists the five fractions obtained from sequential solvent extraction.

Table 2-2: The abbreviation assigned for each fraction obtained through sequential solvent extraction

Fraction	Abbreviation	Polarity
Hexane	HEX	Non-polar
Dichloromethane	DCM	↓
Ethyl acetate	EA	
Methanol	MeOH	
Water	H_2O	Polar

2.3.5 Determination of antibacterial activity

2.3.5.1 Broth microdilution assay

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined using broth microdilution assay according to Clinical and Laboratory Standards Institute (CLSI) standard methods (CLSI, 2012). Broth microdilution assay uses overnight bacterial cultures, which are a mixture of actively multiplying and non-multiplying cells. The concentrations of MCE tested ranges at 0.3125 – 40 mg/ml while the concentrations of SSE fractions tested ranges at 0.15 - 10 mg/ml. MIC was defined as the minimum inhibitory concentration of extract that inhibits growth while MBC was recorded as the lowest concentration that kills bacteria.

2.3.5.2 Time-kill assay

In order to determine if MCE can affect non-multiplying cells specifically and to determine how they do so, time-kill assays were conducted using *S. aureus* as the test organism. Three different methods were used to create bacterial cultures consisting solely on non-multiplying *S. aureus*, i.e. by nutrient depletion (Section 2.3.5.2.2), reducing the temperature (Section 2.3.5.2.3) and inhibiting protein synthesis (Section 2.3.5.2.4). Time-kill assays against exponential and stationary phase cells (Section 2.3.5.2.1) were also performed for comparison.

The time-kill assays were conducted according to the methods by Mascio *et al.* (2007) and Podos *et al.* (2012), and tested against one strain, *S. aureus* ATCC 29213. Basically, bacterial suspension cultures in TSB or phosphate buffer saline (PBS) were treated with various concentrations of MCE. For comparison, a growth control, i.e. cell suspension without the addition of extract, and treatment with daptomycin (positive control) was also included. The time kill assays were performed at 37°C with shaking at 150 rpm. At different time points after incubation, culture aliquots (100 µl) were removed and serially diluted in TSB or PBS. Then, 100 µl of every dilution was plated on tryptic soy agar (TSA) and incubated at 37°C for 24 hours. Cell viability was assessed by determining the colony forming unit (CFU) per mililiter (CFU/ml). Bactericidal activity was defined as a ≥3 log reduction with

extract treatment compared with the untreated control at the beginning of each assay (Mascio *et al.*, 2007). Bacteriostatic activity was defined as maintenance of, or a ≤2-log reduction of cell count compared with the original inoculum (Bantar *et al.*, 2008; Lin *et al.*, 2005). The time-kill experiments were repeated three times and each experiment was performed in triplicate.

2.3.5.2.1 Time-kill assays against exponential- and stationary-phase S. aureus

Both time-kill assays were performed based on the methods by Mascio *et al.* (2007) and Podos *et al.* (2012), with several modifications. For time-kill assay against exponential-phase *S. aureus*, an overnight culture was diluted into fresh TSB and grown with shaking at 37°C for 4 hours before treatment at approximately 10^8 CFU/ml with MCE at 1X MIC, 2X MIC and 4X MIC. For time-kill assay against stationary-phase *S. aureus*, a 20-hour stationary phase culture (10^9 CFU/ml) was directly treated with MCE at similar concentrations. An untreated control and a positive control (treatment with daptomycin) were also included. The cultures were incubated at 37°C with shaking and viable cell counts were assayed at various time points. Figure 2-3 summarizes the methodology for time-kill assays against exponential and stationary phase cultures of *S. aureus*.

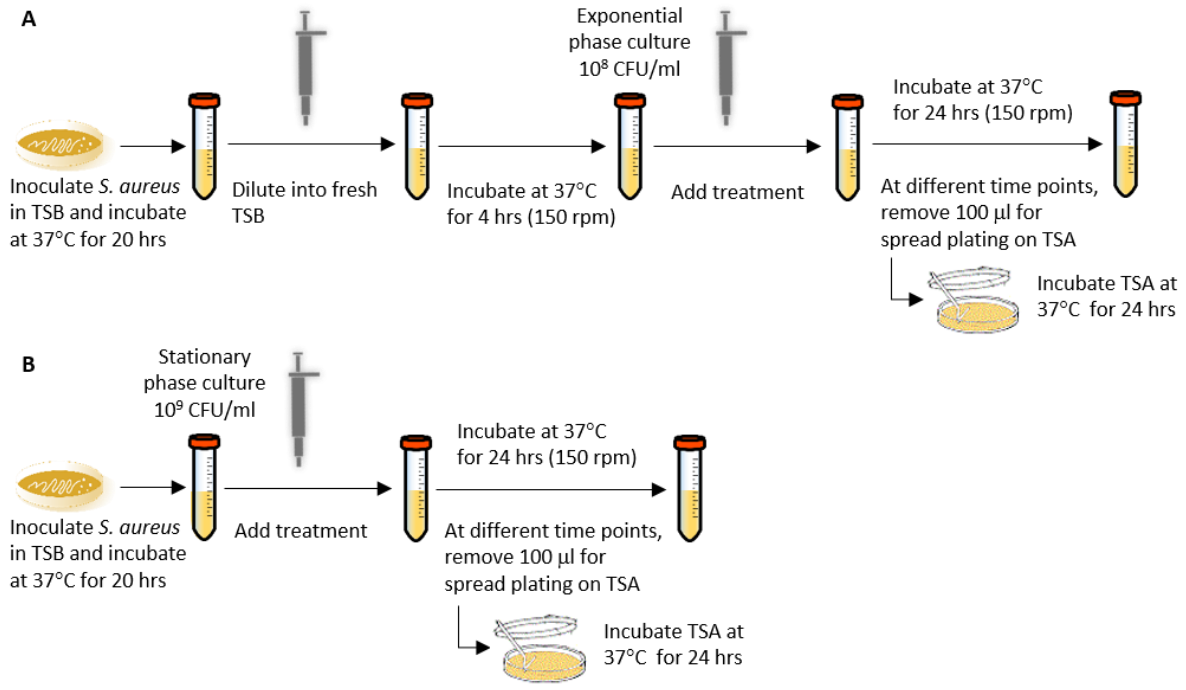


Figure 2-3: Summary of methodology for time-kill assays against (A) exponential phase culture and (B) stationary phase culture of *S. aureus*

2.3.5.2.2 Time-kill assay against nutrient depleted, growth arrested (non-multiplying stationary phase) S. aureus

In order to establish the stationary phase model, *S. aureus* was grown in TSB at 37°C with shaking (150 rpm) for 10 days (Figure 2-4 (A)). Viability was determined by determining the CFU/ml at

various time points. Based on the stationary phase model, a 24-hour culture was chosen to test the bactericidal activity of the MCE against nutrient depleted, growth arrested *S. aureus*.

To induce the bacterial cells into a non-multiplying state, the 24-hour culture was diluted and washed with PBS to 10^7 CFU/ml, which served as the cell suspension for the time-kill assay. The cell suspension was treated with MCE at 1X MIC, 2X MIC and 4X MIC. For comparison, a control (the 24-hour culture that was washed and diluted with PBS but without any treatment added) and a positive control (daptomycin) were also included. At different time points after incubation, the cultures were washed twice with PBS, re-suspended in the original volume and viable cell counts were determined (Hu *et al.*, 2010; Mascio *et al.*, 2007). Figure 2-4 (B) summarizes the methodology for time-kill assay against nutrient depleted, growth arrested culture of *S. aureus*.

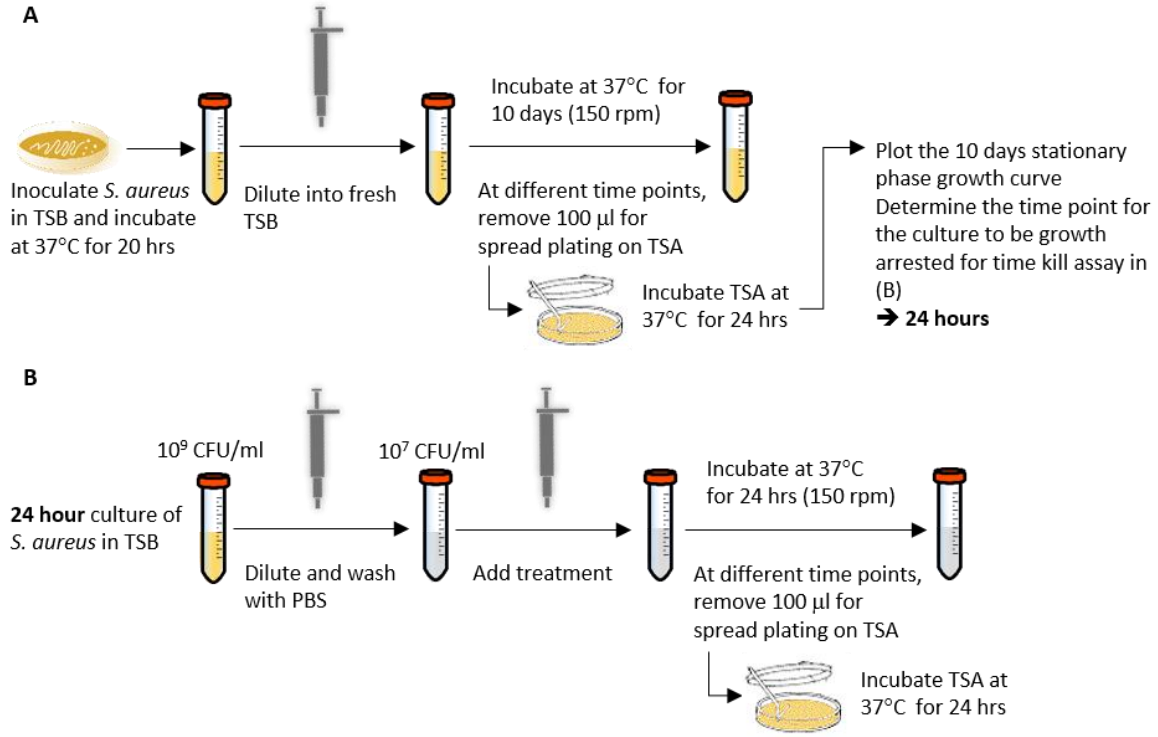


Figure 2-4: (A) Summary for the 10 days growth curve of *S. aureus*. (B) Summary of methodology for time-kill assay against nutrient depleted, growth arrested culture of *S. aureus*

2.3.5.2.3 Time-kill assay against cold-temperature growth arrested S. aureus

Exponentially growing *S. aureus* cultures were arrested by chilling on ice for 1 hour while shaking (150 rpm), prior to treatment with MCE at 1X MIC, 2X MIC and 4X MIC. A control (culture incubated in ice without any treatment added) and a positive control (daptomycin) were also included. The treated cultures were incubated on ice with shaking for 24 hours. After 24 hours, the cultures were then grown at 37°C for 2 hours. Viable cell counts were assayed at various time points (Mascio *et al.*, 2007; Podos *et al.*, 2012). Figure 2-5 summarizes the methodology for time-kill assay against cold-temperature growth arrested culture of *S. aureus*.

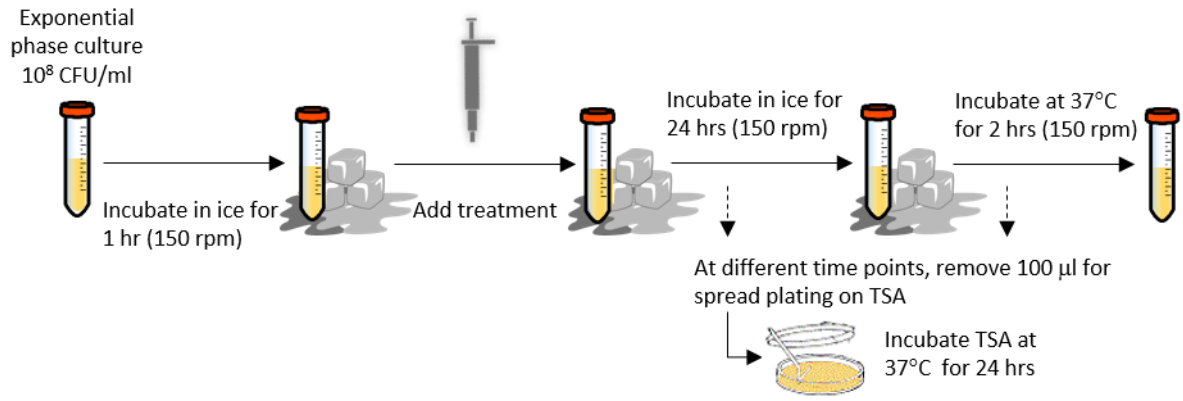


Figure 2-5: Summary of methodology for time-kill assay against cold-temperature growth arrested culture of *S. aureus*

2.3.5.2.4 Time-kill assay against chemically growth arrested S. aureus

Exponentially growing *S. aureus* cultures were arrested by the protein synthesis inhibitor, erythromycin (ERY) (4 µg/ml) for 1 hour at 37°C while shaking (150 rpm) to allow growth arrest. After the 1 hour pre-treatment, the arrested cells were treated with MCE at 1X MIC, 2X MIC and 4X MIC for 24 hours. An untreated control, ERY control (culture added with ERY to arrest growth prior to treatment) and a positive control (daptomycin) were also included. Viable cell counts were determined at various time points (Mascio *et al.*, 2007; Podos *et al.*, 2012). Figure 2-6 summarizes the methodology for time-kill assay against chemically growth arrested culture of *S. aureus*.

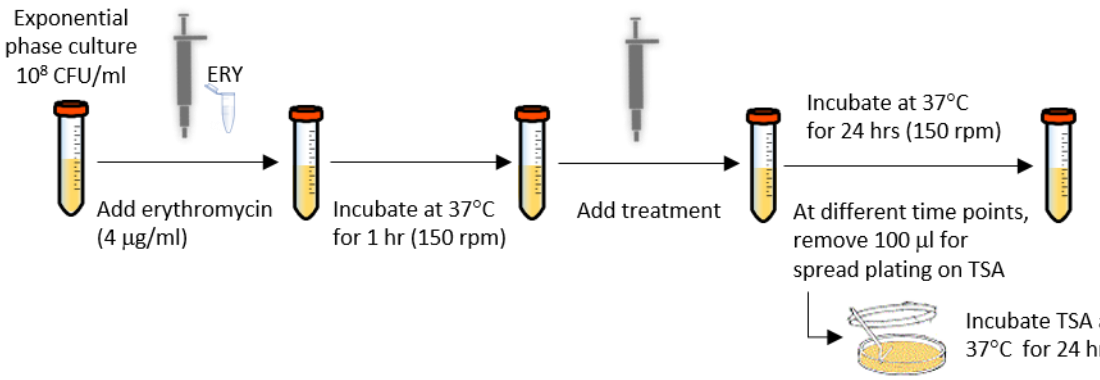


Figure 2-6: Summary of methodology for time-kill assay against chemically growth arrested culture of *S. aureus*

2.3.6 Data analysis

Statistical analysis was conducted using the One-way Analysis of Variance (ANOVA) test for comparing mean scores of more than two groups, with significance at $p<0.05$. The IBM SPSS Statistics 20 software was used (Kerekes *et al.*, 2013). All graphs were generated using the GraphPad Prism 6 software.

2.4 Results and discussion

2.4.1 Antibacterial activity of MCE

Methanol crude extraction (MCE) was performed on the leaves and roots of *D. linearis.* The MCE(L) and MCE(R) were screened for their antibacterial activity.

2.4.1.1 Broth microdilution assay

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values for MCE(L) and MCE(R) determined through broth microdilution assay are as presented in Table 2-3.

Table 2-3: Antibacterial activity of MCE determined by broth microdilution assay

Strains	MCE(L)		MCE(R)	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
S. aureus ATCC 6538P	5.0	5.0	2.5	2.5
S. aureus ATCC 43300	2.5	5.0	2.5	2.5
S. aureus ATCC 33591	5.0	5.0	2.5	2.5
S. aureus ATCC 29213	5.0	5.0	2.5	5.0
S. aureus ATCC 700699	2.5	5.0	2.5	2.5

*Minimum inhibitory concentration (MIC) & minimum bactericidal concentration (MBC) in mg/ml

Based on Table 2-3, the MIC and MBC values of MCE(L) were at 2.5 – 5.0 mg/ml and 5.0 mg/ml, respectively, while for MCE(R), the MIC and MBC values were at 2.5 and 2.5 – 5.0 mg/ml, respectively when tested against all five strains of *S. aureus*. Since *S. aureus* can cause wound infections, the susceptibility of *S. aureus* towards the extracts justifies its traditional use in treating wounds and skin rash. In addition, studies by Lai *et al.* (2009), Thomas *et al.* (2007), Zakaria *et al.* (2007) and Zakaria *et al.* (2010) had also reported on the antibacterial activity of *D. linearis* methanol extract against *S. aureus*.

Therefore, it was of interest to identify whether MCE would also be effective against non-multiplying cells of *S. aureus*. However, due to the higher yield of MCE(L) as compared to MCE(R) obtained during methanol crude extraction, subsequent screening for antibacterial time-kill assays was performed only using MCE(L).

2.4.1.2 Time-kill assay

Time-kill assays of MCE(L) was conducted to evaluate its bactericidal activity against the multiplying and non-multiplying cells of *S. aureus* within certain time points. The time-kill assays were conducted against *S. aureus* ATCC 29213. The MIC and MBC for antibiotics used as controls for the time kill assays against *S. aureus* ATCC 29213 were determined as in Table 2-4.

Table 2-4: Antibacterial activity of antibiotics used as controls for the time-kill assays against *S. aureus* ATCC 29213

Strains	Daptomycin		Erythromycin	
	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)
S. aureus ATCC 29213	64	128	0.5	1.0

*Minimum inhibitory concentration (MIC) & minimum bactericidal concentration (MBC) in μg/ml

In this study, daptomycin was selected as the control for comparison with MCE(L) to assess its bactericidal activity against *S. aureus*. Daptomycin works by disrupting membrane function and causing leakage of essential potassium ions, leading to the loss of membrane potential and ultimately cell death. Daptomycin has been reported to exhibit bactericidal activity against stationary phase and non-multiplying cells of *S. aureus* and does not require cell division or active metabolism to manifest its bactericidal activity (Mascio *et al.*, 2007). Therefore, in time-kill assays against non-multiplying cells of *S. aureus*, daptomycin was used as the positive control.

For time kill assays against the non-multiplying cells of *S. aureus*, three different models were used to create a bacterial suspension consisting of non-multiplying *S. aureus*. These non-multiplying models involve subjecting *S. aureus* cultures under growth arrest condition by: nutrient depletion, cold temperature and chemical treatment by erythromycin, a protein synthesis inhibitor (Mascio *et al.*, 2007).

The nutrient depleted non-multiplying model was used to determine the effects of changes in cellular physiology towards the effectiveness of MCE(L) while cold temperature non-multiplying model was used to evaluate the effects of changes in temperature towards the activity of MCE(L). Chemically arrested non-multiplying model was used to determine the effects of artificial metabolic arrest and the requirement for active protein synthesis on the bactericidal effect of MCE(L) against non-multiplying cells (Mascio *et al.*, 2007; Podos *et al.*, 2012). Results from these three models would provide suggestions on the possible mechanism of action involved to exhibit bactericidal activity by MCE(L) against non-multiplying cells.

Nevertheless, as a basis for comparison with time-kill assays of growth arrested cultures, the effects of MCE(L) were first evaluated against exponential phase and stationary phase cultures of *S. aureus.*

2.4.1.2.1 Time-kill assays against exponential- and stationary-phase S. aureus

The effects of MCE(L) was evaluated against exponential phase culture of *S. aureus* by adding MCE(L), at 1X MIC (5 mg/ml), 2X MIC (10 mg/ml) and 4X MIC (20 mg/ml) to exponentially growing cultures of *S. aureus*. Treatment against daptomycin at 128 μg/ml (MBC) served as the positive control. Figure 2-7 shows the time kill curve of MCE(L) against exponential phase culture of *S. aureus*.

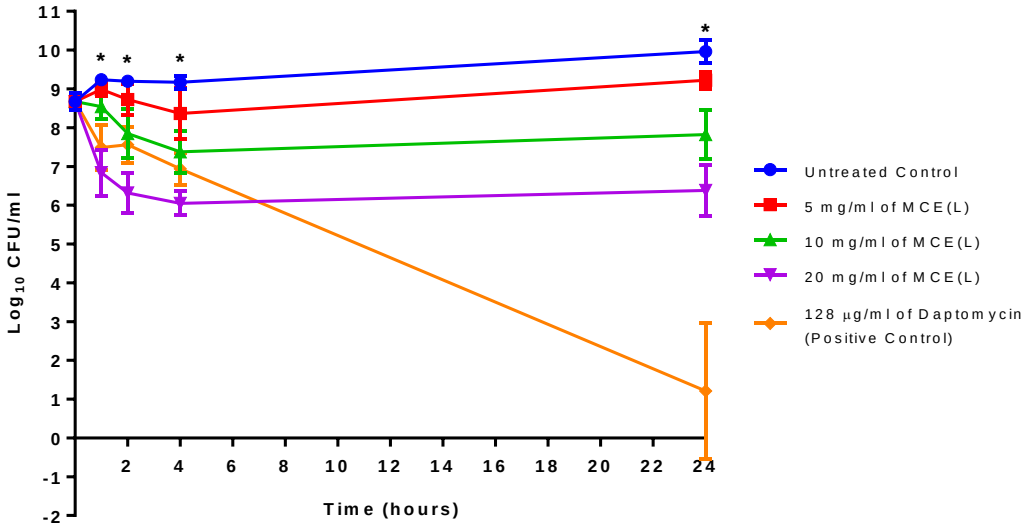


Figure 2-7: Effects of MCE(L) and daptomycin against exponential phase *S. aureus* ATCC 29213. Mean log CFU/ml ± SD plotted against time. * denotes the statistically significant difference between each treatment compared with untreated control for each time point at $p<0.05$. All treatments showed significant difference at each time points.

Based on Figure 2-7, the untreated control continued to grow throughout the experiment and cultures treated with MCE(L) and daptomycin showed statistically significant difference when compared with untreated culture for all time points at $p<0.05$. Treatment with 5 mg/ml of MCE(L) did not show any reduction in viable count. Treatment with 10 mg/ml of MCE(L) resulted in a 1-log reduction (90% kill rate) in viable count at the 4th hour and this remained until the 24th hour. For treatment with 20 mg/ml of MCE(L), a 2-log reduction (99% kill rate) in viable count was observed at the 4th hour and this continued until the 24th hour, indicative of a bacteriostatic effect against exponential culture. Bacteriostatic activity was defined as a ≤2-log reduction of cell count compared with the original inoculum while bactericidal activity was defined as a ≥3 log reduction with treatment compared with the untreated control at the beginning of each assay (Bantar *et al.*, 2008; Lin *et al.*, 2005; Mascio *et al.*, 2007). Treatment with daptomycin showed significant ≥3-log reduction (≥99.9% kill rate) in viable count after 24 hours incubation, demonstrating a strong bactericidal effect.

To determine whether MCE(L) also had an effect on stationary phase cultures, *S. aureus* ATCC 29213 was grown for 20 hours to create an inoculum of 1 X 10^9 CFU/ml, and then subjected to treatments with similar concentrations. Figure 2-8 shows the time kill curve of MCE(L) against stationary phase culture of *S. aureus*.

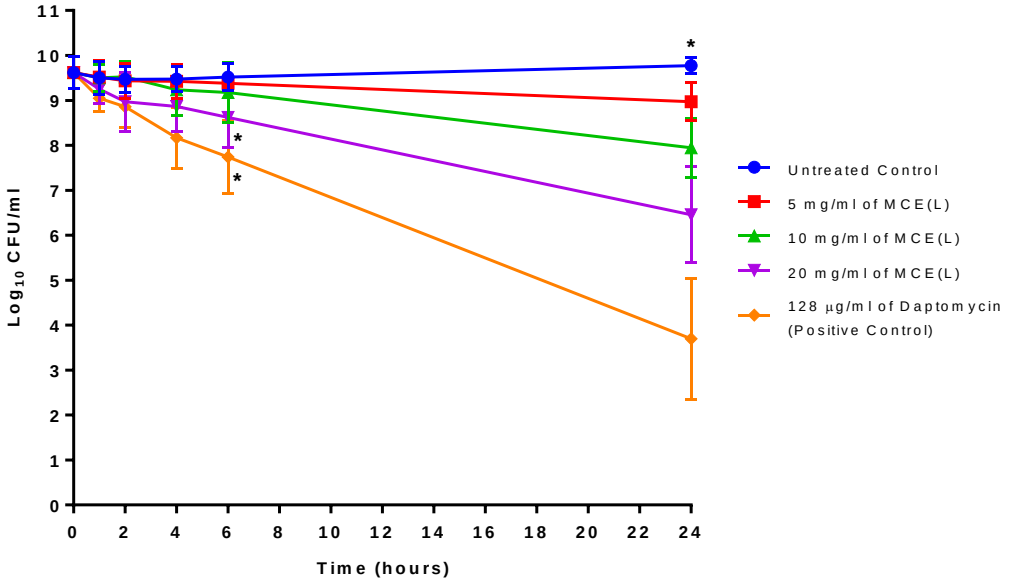


Figure 2-8: Effects of MCE(L) and daptomycin against stationary phase *S. aureus* ATCC 29213. Mean log CFU/ml ± SD plotted against time. * denotes the statistically significant difference between each treatment compared with untreated control for each time point at $p<0.05$. Only at the 24th hour, all treatments showed significant difference to untreated control.

Based on Figure 2-8, *S. aureus* cells was determined to be in stationary phase because the untreated culture showed a flat viability curve throughout the experiment, confirming that the untreated cells did not multiply. From the 1st to the 4th hour, no significant cell reduction was observed for all treated cultures compared with the untreated culture. Treatment with 5 mg/ml of MCE(L) showed a slight reduction (<1-log reduction) in viable count while 10 mg/ml of MCE(L) showed a significant 1-log reduction (99% kill rate) in viable count after 24 hours. Unlike in treatment against exponentially growing *S. aureus* cells (Figure 2-7), treatment with 20 mg/ml of MCE(L) showed a bactericidal effect after 24 hours, having a significant >3-log reduction in viable count (>99.9% kill rate). Daptomycin had a strong bactericidal effect, with a significant ≥3-log reduction (>99.9% kill rate) in viable count after incubation for 24 hours.

Overall, treatment with 20 mg/ml of MCE(L) against exponential phase cultures showed bacteriostatic activity while treatment against stationary phase cultures showed bactericidal activity, after 24 hours incubation. In addition, a dose dependent effect was observed when the cultures were treated with MCE(L), whereby killing effect increased with increasing extract concentration. Killing of cells were observed to be more effective against stationary phase cultures using 20 mg/ml of MCE(L). Bacterial cells in stationary phase culture have limited growth whereby, the growth rate and death rate are equal and thus, the cells may be more susceptible to killing by MCE(L). Moreover, the bactericidal activity exhibited by daptomycin was as expected against both cultures as

Mascio *et al.* (2007) had reported that high concentration (100 µg/ml) of daptomycin was bactericidal against exponential and stationary cultures.

Although it was determined that the MBC value for *S. aureus* ATCC 29213 was 5 mg/ml (Table 2-3), the MBC value does not finalize that a bactericidal activity must be achieved at 5 mg/ml of MCE(L). Results obtained from broth microdilution assay have no correlation with time-kill assays due to the effect of initial inoculum size used in each assay (Podos *et al.*, 2012). A high inoculum density can deter the effectiveness of antibacterial agents, in which reduced killing occurs with higher initial cell numbers (Mascio *et al.*, 2007). Broth microdilution assay was conducted using a standard inoculum size of 10^5 CFU/ml (CLSI, 2012) while in the time-kill assays for exponential and stationary phase cultures, an inoculum size of 10^8 CFU/ml and 10^9 CFU/ml were used, respectively.

2.4.1.2.2 Time-kill assay against nutrient depleted, growth arrested (non-multiplying stationary phase) S. aureus

Prior to determining the effect of MCE(L) towards nutrient depleted, growth arrested *S. aureus* culture, a stationary phase growth curve was established to determine the time point for the culture to be growth arrested. Based on Figure 2-9, *S. aureus* growth remained constant at 10^9 CFU/ml between 24- to 48-hour, and then gradually decreasing after the 48-hour, indicating bacterial death. Therefore, a 24-hour culture was chosen to test the bactericidal activity of MCE(L) against nutrient depleted, growth arrested *S. aureus* cells. The 24-hour culture may represent a mixed population with a dynamic balance of cell division and cell death.

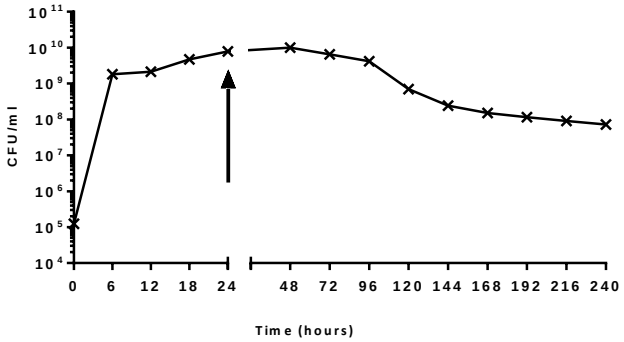


Figure 2-9: Growth curve of *S. aureus* ATCC 29213. *S. aureus* was grown in TSB at 37°C with shaking (150 rpm) for 10 days (240 hours). The arrow indicates the time point when the culture was used for time-kill assay against nutrient-depleted, growth arrested cells.

To induce the *S. aureus* cells into a non-multiplying stage, the 24-hour culture (10^9 CFU/ml) was diluted and washed with PBS to 10^7 CFU/ml, which served as the cell suspension for the time-kill assay. The cell suspension was treated with MCE(L) and daptomycin (positive control), and viable cell counts were determined. Figure 2-10 shows the time-kill curve of MCE(L) against nutrient depleted, growth arrested *S. aureus* culture.

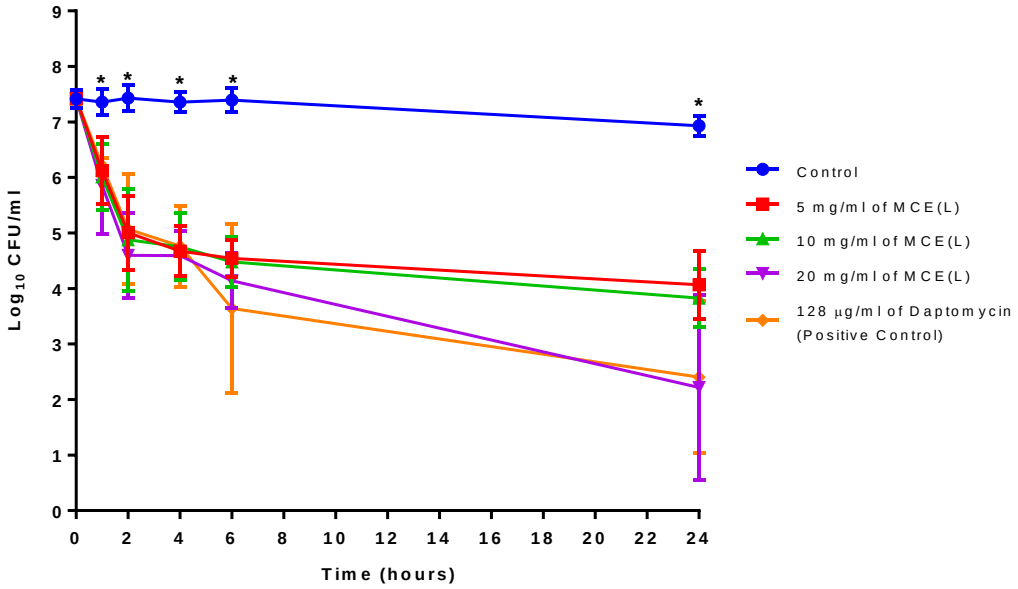


Figure 2-10: Effect of MCE(L) against nutrient depleted, growth arrested *S. aureus* ATCC 29213. Mean log CFU/ml ± SD plotted against time. Control refers to the 24-hour culture that was washed and diluted with PBS but without any treatment added. * denotes the statistically significant difference between treatment as compared with control for each time point at $p<0.05$. At every time point, significant difference was observed for each treatment when compared with control.

Based on Figure 2-10, no growth was observed for control culture (cells incubated in PBS), indicating that *S. aureus* were still in a non-multiplying state throughout the whole experiment. It was observed that treatment with MCE(L) at all three concentrations reduced the viable count significantly from 1st to 24th hour when compared with the control culture. There was no significant difference between each MCE(L) concentrations and daptomycin for each time points. Bactericidal activity was achieved by the 24th hour, with a 3-log reduction (99.9% kill rate) in viable count for treatment with 5 mg/ml and 10 mg/ml of MCE(L). For treatment with 20 mg/ml of MCE(L), rapid bactericidal activity was achieved at the 6th hour.

Upon the lack of nutrients for growth, bacteria will adjust their metabolism from one that supports growth to one that permits its survival in the absence of nutrients. The bactericidal activity of antibacterial agents against stationary phase cultures that have resulted from nutrient depletion is often used to assess its efficacy against non-multiplying cells (Ooi *et al.*, 2010). In this study, the bactericidal activity of MCE(L) at 5 to 20 mg/ml showed no significance difference when compared with daptomycin, indicating that the efficacy of MCE(L) was comparable to daptomycin in killing non-multiplying (nutrient depleted, growth arrested) *S. aureus* cells. The rapid bactericidal activity exhibited by MCE(L) at both low and high concentrations suggests that altered cell physiology may have influenced the efficacy of MCE(L) against the non-multiplying cells (Mascio *et al.*, 2007; Podos *et al.*, 2012).

2.4.1.2.3 Time-kill assay against cold-temperature growth arrested S. aureus

To determine the effect of MCE(L) on cold-temperature growth arrested cultures of *S. aureus* ATCC 29213, exponentially growing cultures were chilled in ice prior to treatment. Figure 2-11 shows the time-kill curve of MCE(L) against cold-temperature growth arrested *S. aureus* cultures.

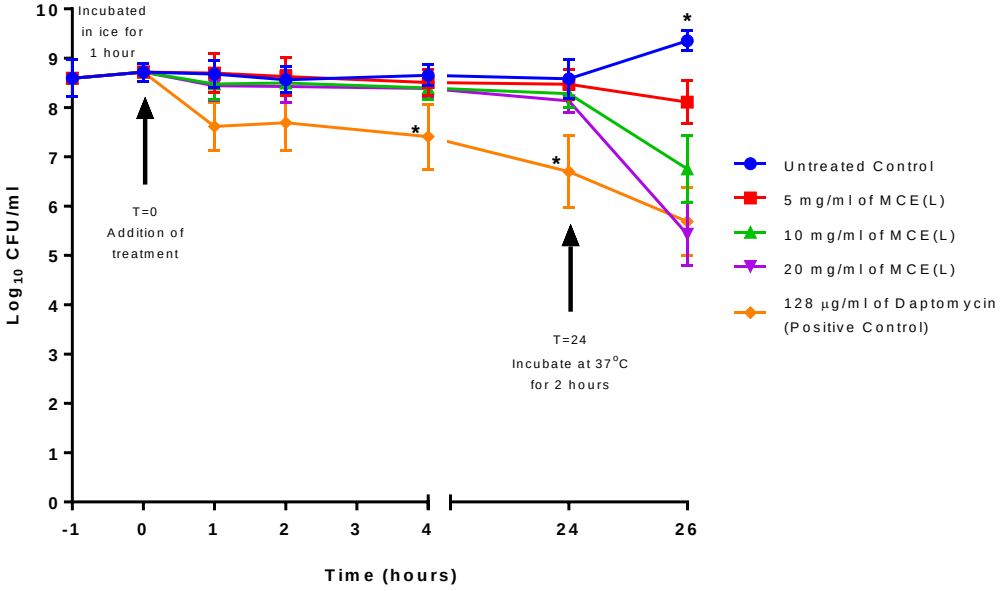


Figure 2-11: Effect of MCE(L) against cold-temperature, growth arrested *S. aureus* ATCC 29213. Mean log CFU/ml ± SD plotted against time. Untreated control refers to culture incubated in ice and without any treatment added. Treatment was added at time 0 hour. * denotes the statistically significant difference between treatment as compared with untreated control for each time point at $p<0.05$. At the 4th and 24th hour, only daptomycin showed significant difference in viable count while at the 26th hour, all treatments were significantly different in viable count when compared with the untreated control.

Based on Figure 2-11, treatment with MCE(L) at all three concentrations resulted in no significant changes in viability throughout the 24 hour incubation, similar to the control. After the temperature shift to 37°C, the untreated control showed significant bacterial growth while all treated cultures showed significant reduction in viability. Treatments at 10 mg/ml of MCE(L) and daptomycin significantly reduced viable counts by 1-log (90% kill rate) after 2 hours incubation in 37°C while treatment with 20 mg/ml of MCE(L) showed bactericidal activity with significant 3-log reduction (99.9% kill rate) in viable counts.

Under cold temperatures, bacteria will enter a slow-growing state due to the activation of stress responses that decrease membrane fluidity and reduce enzymatic activity within the cell. Cold temperatures will influence the susceptibility of bacteria to antibacterial agents that target intracellular metabolic targets by decreasing their rates of diffusion and molecular interaction with the bacteria (Ooi *et al.*, 2010). However, in this study, no bactericidal activity was observed for

MCE(L) at all concentrations, throughout the 24 hours incubation in ice. Bactericidal activity was only demonstrated after temperature shift to 37°C for treatment with 20 mg/ml of MCE(L). This observation was also reported by Mascio *et al.* (2007), in which killing of *S. aureus* by antibiotics such as ciprofloxacin and nafcillin was prevented by cold treatment. It can be suggested that cold arrest had also protected the non-multiplying cells against the effects of MCE(L) and that MCE(L) has no efficacy at cold temperatures. The bactericidal activity of MCE(L) was temperature dependent and will have an effect at 37°C.

2.4.1.2.4 Time-kill assay against chemically growth arrested S. aureus

To determine the effect of artificial metabolic arrest and the requirement for active protein synthesis on the bactericidal effect of MCE(L) against non-multiplying cells, exponentially growing cells were treated with the bacteriostatic protein synthesis inhibitor, erythromycin (4 µg/ml) prior to treatment. Erythromycin remained in the cultures to arrest growth throughout the 24 hour experiment. Figure 2-12 shows the time-kill curve of MCE(L) against chemically growth arrested *S. aureus* cultures.

Based on Figure 2-12, treatment with erythromycin alone effectively stopped growth throughout the 24 hour incubation period while the untreated control continued to grow. The viable count was significantly different between untreated and erythromycin treated culture. Treatment with 5 mg/ml of MCE(L) showed no significant reduction in viable counts throughout the 24 hour period while treatment with 10 mg/ml of MCE(L) showed 1-log reduction (90% killed) in viable counts at the 24^{th} hour. Treatment with 20 mg/ml of MCE(L) and daptomycin both showed significant reduction in viable count at the 24^{th} hour, with ≥3-log reduction (≥99.9% killed) observed, indicating strong bactericidal activity of 20 mg/ml of MCE(L).

Erythromycin inhibits protein synthesis by reversibly binding to the 50S subunit of the bacterial ribosome and hampers with the elongation cycle of the peptidyl chain (Siibak *et al.*, 2009). *S. aureus* cells growth arrested by chemical treatment using erythromycin had exhibited strong bactericidal activity when treated with 20 mg/ml of MCE(L). The bactericidal effect of 20 mg/ml of MCE(L) was not blocked by protein synthesis inhibition by erythromycin and this suggests the possibility that active protein synthesis may not be required in the mechanism of action of MCE(L) (Podos *et al.*, 2012).

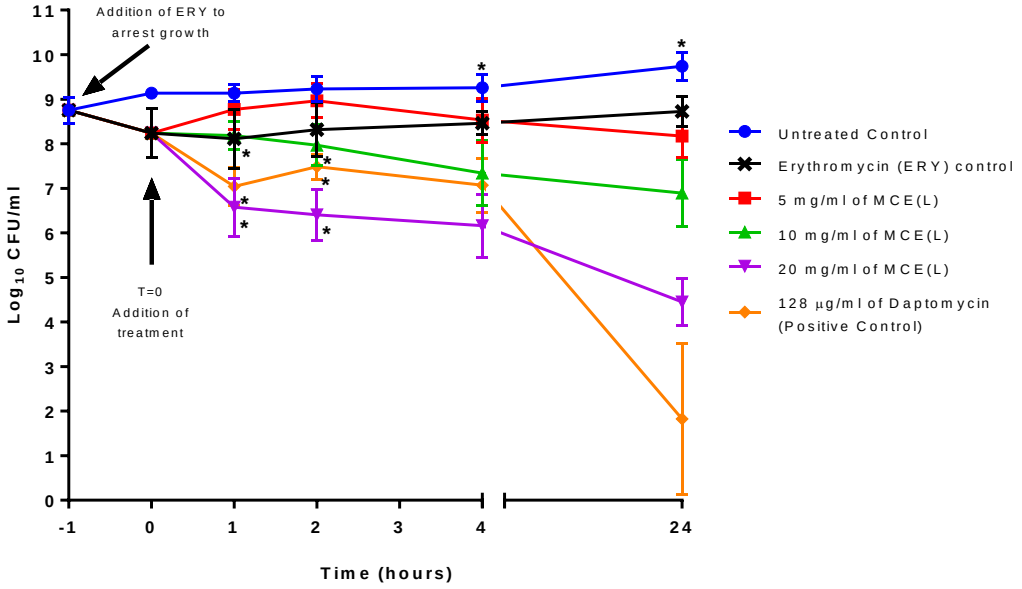


Figure 2-12: Effect of MCE(L) against chemically growth arrested *S. aureus* ATCC 29213. Mean log CFU/ml ± SD plotted against time. Untreated control refers to culture without treatment while ERY control refers to culture added with erythromycin (4 µg/ml) to arrest growth prior to treatment. Treatment was added at time 0 hour. * denotes the statistically significant difference between treatment as compared with the untreated control for each time point at $p<0.05$. At the 1st and 2nd hour, only treatment with MCE(L) at 10 mg/ml and 20 mg/ml, and daptomycin showed significant difference when compared with the untreated control.

2.4.1.2.5 Overall discussion for non-multiplying models

Overall findings from the three non-multiplying models suggested that altered cell physiology may have influenced the effectiveness of MCE(L) against non-multiplying cells since rapid bactericidal activity was observed at both low and high concentrations of MCE(L). Cold arrest had protected the non-multiplying cells against activity by MCE(L), suggesting that its bactericidal activity was temperature dependent and has no efficacy at cold temperatures. The bactericidal effect of MCE(L) was not blocked by protein synthesis inhibition by erythromycin, suggesting the possibility that active protein synthesis may not be required in the mechanism of action of MCE(L).

The mechanism of action of antibacterial agents generally involves inhibition of cell wall synthesis, inhibition of protein synthesis, inhibition of nucleic acid synthesis, inhibition of cell membrane function and inhibition of other metabolic processes. For example, beta-lactams targets bacterial cell wall synthesis while some quinolones requires targets RNA and protein synthesis for bactericidal activity (Jenssen *et al.*, 2006). Since altered cell physiology may have influenced the activity of MCE(L) and active protein synthesis was not required for its action, the only possible mechanism of action involved in MCE(L)'s ability to affect non-multiplying cells would be the inhibition of cell membrane function.

Compounds such as essential oils and flavonoids have been reported to inhibit cytoplasmic membrane function. Phytochemical studies on *D. linearis* by other researchers have identified the presence of these compounds in *D. linearis* (de Winter *et al.*, 2003; Jaishee and Chakraborty, 2015; Li *et al.*, 2008; Raja *et al.*, 1995). The hydrophobic nature of essential oils allows them to interact well with the lipid membrane of bacteria, resulting in the leakage of the inner cell components of the cell as well as affecting the potassium ion reflux, eventually leading to cell death (Bajpai *et al.*, 2013). Some flavonoids have also been reported to cause cytoplasmic membrane damage and potassium leakage (Cushnie and Lamb, 2005). In addition, the fact that MCE(L) had no efficacy at cold temperatures was not surprising as there are antibacterial compounds that are inactive at low temperatures. Further work on the mechanism of action of MCE(L) against non-multiplying *S. aureus* cells and the identification of the exact compound exhibiting the activity are of interest.

2.4.2 Antibacterial activity of SSE fractions

Since screening with MCE had shown some antibacterial activity, it was of interest to study the extract in depth in case any compounds were not extracted out by MCE. Therefore, sequential solvent extraction (SSE) was performed on the leaves of *D. linearis*. The plant material was fractionated according to the solubility in solvents ranging from non-polar hydrophobic compounds to polar hydrophilic compounds, i.e. with hexane, dichloromethane, ethyl acetate, methanol and water. The five SSE fractions obtained were screened for antibacterial activity.

2.4.2.1 Broth microdilution assay

The MIC and MBC values for each SSE fractions determined using broth microdilution assay are as presented in Table 2-5. Only the MeOH fraction exhibited antibacterial activity, with MIC and MBC values of 5 mg/ml and 10 mg/ml, respectively.

Table 2-5: Antibacterial activity of SSE fractions by broth microdilution assay

Strains	HEX		DCM		EA		MeOH		H_2O	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus ATCC 6538P	>10	>10	>10	>10	>10	>10	**5**	**10**	>10	>10
S. aureus ATCC 43300	>10	>10	>10	>10	>10	>10	**5**	**10**	>10	>10
S. aureus ATCC 33591	>10	>10	>10	>10	>10	>10	**5**	**10**	>10	>10
S. aureus ATCC 29213	>10	>10	>10	>10	>10	>10	**5**	**10**	>10	>10
S. aureus ATCC 700699	>10	>10	>10	>10	>10	>10	**5**	**10**	>10	>10

*Minimum inhibitory concentration (MIC) & minimum bactericidal concentration (MBC) in mg/ml

Since extraction was conducted sequentially starting with hexane and then dichloromethane, many of the hydrophobic compounds would have been extracted out earlier into HEX and DCM fractions. The more polar compounds would be found in MeOH and H_2O fractions.

Among all SSE fractions, only MeOH fraction exhibited antibacterial activity. Therefore, it can be deduced that the MeOH fraction was made up mostly by polar compounds, and these polar compounds might be responsible for the antibacterial activity observed. Compounds that can be extracted by methanol, such as tannins and polar flavonoids, had been reported to exhibit antibacterial activity (Cushnie and Lamb, 2005; Min *et al.*, 2008) and these compounds had been identified to be present in *D. linearis* by other researchers (de Winter *et al.*, 2003; Jaishee and Chakraborty, 2015; Li *et al.*, 2008; Raja *et al.*, 1995).

In comparison between the MIC and MBC values of the MeOH fraction to those obtained with MCE(L), not much improvement was observed as the MIC and MBC values of the MeOH fraction was similar or higher to those obtained with MCE(L). The antibacterial activity did not improve after fractionation with SSE. SSE ensures the extraction of active compounds according to their polarity, and can also reduce any antagonistic effect of compounds in the extract (Jeyaseelan *et al.*, 2012) and thus, the antibacterial activity was expected to be improved as there would be less interfering non-polar substances in the MeOH fraction.

In several plant extracts studies, the MIC value of 100 µg/ml was used as a threshold for significant antibacterial activity (Borges-Argáez *et al.*, 2007; Jimenez-Arellanes *et al.*, 2003; Kuete, 2010; Molina-Salinas *et al.*, 2006). In reference to this criterion, the MIC value of MeOH fraction was not as good as 100 µg/ml and therefore, further characterization of MeOH fraction and its antibacterial activity was not conducted.

2.5 Conclusion

In conclusion, the MIC and MBC values of MCE(L) were at 2.5 – 5.0 mg/ml and 5.0 mg/ml, respectively, while for MCE(R), the MIC and MBC values were at 2.5 and 2.5 – 5.0 mg/ml, respectively when tested against *S. aureus*. Based on time-kill assays, MCE(L) had exhibited bactericidal activity, when tested against growth arrested cultures caused by nutrient depletion and protein synthesis inhibition. MCE(L) was not effective at cold temperatures and active protein synthesis may not be required in the mechanism of action of MCE(L) against non-multiplying cells.

Among the five fractions obtained through SSE, only the MeOH fraction had exhibited antibacterial activity, with MIC and MBC values of 5 mg/ml and 10 mg/ml, respectively. The antibacterial activity of *D. linearis* did not improve after fractionation with SSE and therefore, further characterization of MeOH fraction and its antibacterial activity was not conducted.

Chapter 3 Anti-biofilm activity of *D. linearis* against *S. aureus*

3.1 Introduction

Biofilms are bacterial communities embedded in a self-produced extracellular polymeric substance (EPS) matrix. Bacteria have the capacity to adhere to catheters and other indwelling medical devices, to form biofilms and this had led to the emergence of biofilm-associated infections. Biofilm-associated infections are more difficult to eradicate with antibacterial treatment as biofilms are more resistant to antibacterial agents as compared to planktonic cells (Podos *et al.*, 2012). Therefore, there is a need to discover new compounds which are effective against biofilms.

3.1.1 Anti-biofilm properties of plant extracts and the possible mechanism of action involved

Several studies have identified some medicinal plants that exhibit anti-biofilm properties. Crude extracts of *Leopoldia comosa* (tassel grape hyacinth) and *Arundo donax* (giant reed) exhibited anti-biofilm activity against methicillin resistant *S. aureus* (Quave *et al.*, 2008). The extracts of *Rosmarinus officinalis* (rosemary) and *Mentha piperita* (peppermint) demonstrated biofilm inhibition and disruption activities against *Listeria monocytogenes* (Sandasi *et al.*, 2010). Furthermore, there have also been reports of antibacterial compounds demonstrating anti-biofilm properties, such as the essential oils (Gursoy *et al.*, 2009), phenolic compounds (Carneiro *et al.*, 2011; Lee *et al.*, 2011), and glycosides (Ye *et al.*, 2010).

Besides identifying for the presence of anti-biofilm activities, it is also important to identify the mechanism of action involved in preventing biofilm. Understanding the mechanisms of biofilm formation is of fundamental importance in identifying the methods for anti-biofilm strategies (Figure 1-5). Biofilm development proceeds through multiple stages and therefore, it is important to understand the factors involved in each stages of biofilm development in order to identify the various methods to prevent biofilm formation and remove established biofilms. Figure 3-1 shows the various factors affecting biofilm formation and thus, suggests the possible factors to be targeted in identifying methods to prevent biofilm formation (Nandakumar *et al.*, 2013).

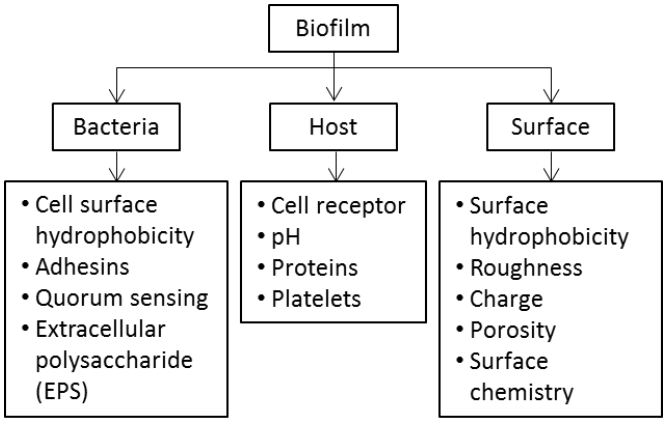


Figure 3-1: Factors affecting biofilm formation (Nandakumar *et al.*, 2013)

Several studies have been conducted on the mechanism of action of phytochemicals against bacterial biofilms. The possible mechanism of action in inhibiting biofilm formation that have been reported are such as prevention of bacterial adhesion, quorum sensing inhibition, inhibition of interspecies so-aggregation, and inactivation of matured single and multi-species biofilms. Quorum sensing molecules from *Medicago truncatula* and garlic (*Allium sativum*) demonstrated the potential to inhibit quorum sensing process (Simoes, 2011). Methanol extracts of *Capparis spinosa* (Abraham *et al.*, 2011) and *Pongamia pinnata* (Abraham *et al.*, 2012) reduced EPS matrix production to inhibit biofilm.

There are also studies on the ability of plant extracts to disrupt biofilms. The use of substances that are able to destroy the physical integrity of the biofilm matrix is an attractive anti-biofilm strategy, as the subsequent loss of the highly protective EPS matrix exposes the sessile microbial cells to treatment by antibiotics (Francolini and Donelli, 2010). As the biofilm matrix is composed of DNA, proteins, and extracellular polysaccharides, some studies have been focusing on the disruption of biofilm structure via the degradation of individual biofilm components by various enzymes. However, with recent findings on the biofilm disruption activities of plant extracts, there is the potential to discover more phytochemicals with effective biofilm disruption activity for newer treatment strategies. Several findings of plant extracts with biofilm disruption activity are such as *Terminalia catappa* (bengal almond), casbane diterpene extracted from *Croton nepetaefolius* bark and *Boesenbergia pandurate* (finger root) oil (Taraszkiewicz *et al.*, 2012).

3.2 Objectives

The objectives of this chapter were:

I. To perform methanol crude extraction (MCE) and sequential solvent extraction (SSE) on *D. linearis* leaves

II. To screen MCE(L) and SSE fractions for anti-biofilm activity:
   a. Biofilm inhibition activity
   b. Biofilm disruption activity

III. To characterize the extract with most effective biofilm inhibition activity
   a. To identify the minimum biofilm inhibition concentration (MBIC)
   b. To confirm that biofilm inhibition activity does not affect cell growth
   c. To evaluate biofilm inhibition activity on various polymer materials commonly used in medical devices
   d. To investigate the possible mechanism of action involved in biofilm inhibition effect
   e. To identify the phytochemicals present

IV. To characterize the extract with most effective biofilm disruption activity
   a. To identify the minimum biofilm disruption concentration (MBDC)
   b. To investigate the effect of extract on cell growth of *S. aureus*
   c. To investigate the effect of extract on the biofilm structure of *S. aureus*
   d. To identify the phytochemicals present

3.3 Methodology

The summary of methodology for the investigation of the anti-biofilm activity of *D. linearis* is presented in Figure 3-2.

3.3.1 Bacterial strains

Five *S. aureus* strains were used, as listed in Table 2-1. *S. aureus* strains were cultured in tryptic soy broth (TSB) and incubated at 37°C. In Table 2-1, the characteristics for biofilm production for each *S. aureus* strain was determined based on the results obtained in this study and categorised in reference to Stepanović *et al.* (2007).

3.3.2 Plant material

Fresh leaves of *D. linearis* were collected as described in Section 2.3.2.

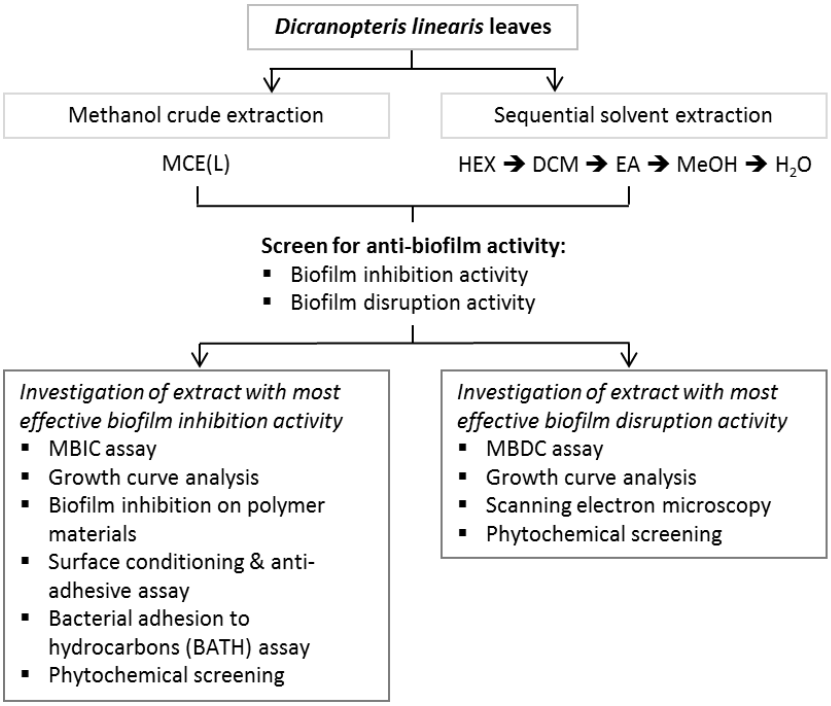


Figure 3-2: Summary of methodology for anti-biofilm studies on *D. linearis*

3.3.3 Methanol crude extraction

Methanol crude extraction of *D. linearis* leaves was performed as described in Section 2.3.3.

3.3.4 Sequential solvent extraction

Sequential solvent extraction of *D. linearis* leaves was performed as described in Section 2.3.4.

3.3.5 Determination of anti-biofilm activity: Biofilm inhibition activity

3.3.5.1 Preparation of inoculum suspension for anti-biofilm assay

Overnight cultures of *S. aureus* grown in TSB at 37°C for 20 hours were adjusted to 0.5 McFarland turbidity standard (0.08-0.1 at $OD_{625\ nm}$), corresponding to approximately 10^8 CFU/ml. The suspension was further diluted 1:100 in TSB supplemented with 1% glucose, resulting in a final inoculum suspension of ~10^6 CFU/ml (Stepanović *et al.*, 2007).

3.3.5.2 Initial screening for biofilm inhibition activity

Biofilm inhibition assay was performed to determine the effectiveness of extract in inhibiting the formation of *S. aureus* biofilms. Sub-MIC was used for initial screening with extract. Inoculum suspension was prepared as described in Section 3.3.5.1. One hundred microliter of extract was added with 100 µl of inoculum suspension into a 96-well microtiter plate to obtain the sub-MIC of the extract. Solvent control refers to treatment with solvents used to dissolve the extract while treatment with 1% sodium hypochlorite served as positive control. Negative control (treatment with water) was added as a comparison to solvent control. The plates were incubated at 37°C for 24

hours. Inhibition of biofilm formation was quantified using crystal violet staining (Section 3.3.5.3) (Kwasny and Opperman, 2010; Stepanović *et al.*, 2007). The experiment was performed in triplicates. Figure 3-3 summarizes the methodology for biofilm inhibition assay.

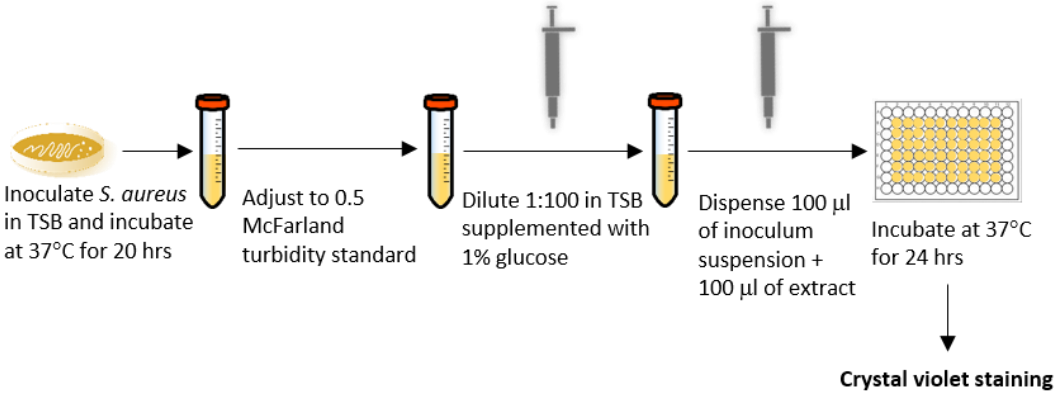


Figure 3-3: Summary of methodology for biofilm inhibition assay

3.3.5.2.1 Minimum Biofilm Inhibition Concentration (MBIC) assay

Upon observation of biofilm inhibition activity, the minimum biofilm inhibition concentration (MBIC) assay was performed. MBIC was defined as the lowest concentration of extract in a serial two-fold dilution series that inhibits biofilm formation as compared with the solvent control. Inoculum suspension was prepared as described in Section 3.3.5.1. The procedure was as previously described in Section 3.3.5.2, with a two-fold dilution series of various concentrations of extract used for treatment. The plates were incubated at 37°C for 24 hours and quantified using crystal violet staining (Section 3.3.5.3) (Kwasny and Opperman, 2010). The experiment was performed in triplicates.

3.3.5.3 Crystal violet staining

Crystal violet staining was performed to quantify the biofilm biomass after treatment with and without extract. Crystal violet stains both the bacteria cells (living and dead cells) and the extracellular matrix in the wells. After treatment for 24 hours, liquid cultures were removed from the microtiter plate by inverting and decanting the liquid by gentle flicking. Using a multichannel pipette, the wells were rinsed three times with 300 µl of sterile water to remove unattached cells. Following each washing step, the wells were emptied by flicking the plates. After washing, the remaining attached bacteria were heat fixed at 60°C for 1 hour. The wells were stained with 200 µl of 0.1% crystal violet for 15 minutes. Excess stain was removed by washing the wells three times with sterile water. The plates were air dried. The bound dye was eluted from attached cells with methanol. The amount of crystal violet bound in each well was measured at OD_{570} nm using a microplate reader (Kwasny and Opperman, 2010; Peeters *et al.*, 2008; Stepanović *et al.*, 2007). Figure 3-4 summarizes the crystal violet staining assay.

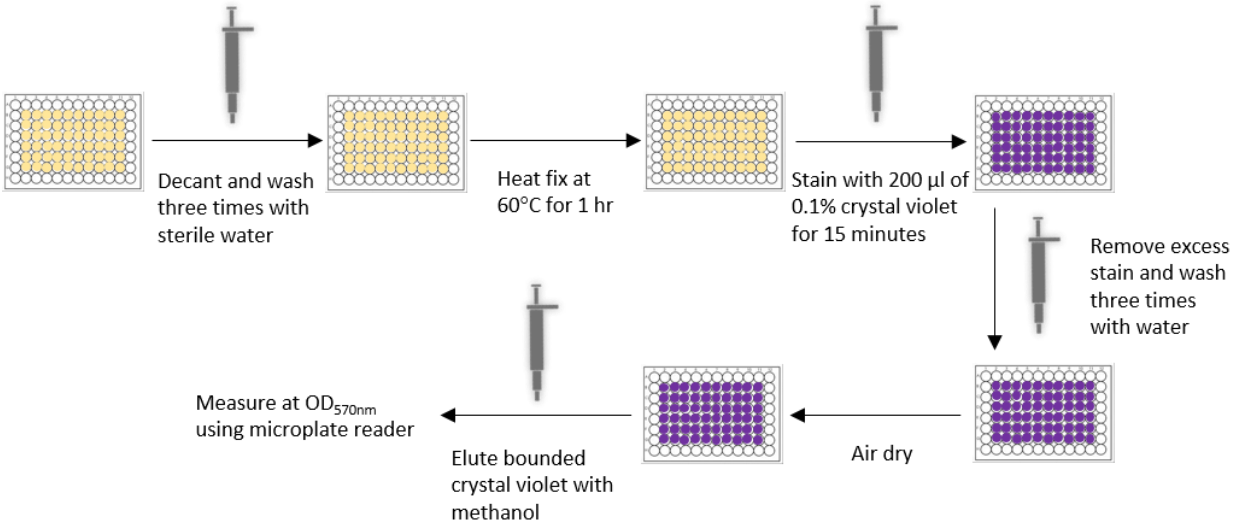


Figure 3-4: Summary of methodology for crystal violet staining assay

3.3.6 Investigation of extract with most effective biofilm inhibition activity

3.3.6.1 Growth curve analysis

Growth curve analysis was performed to confirm biofilm inhibition activity and to demonstrate that extract does not affect the growth of *S. aureus* cells. The procedure was as described for MBIC assay (Section 3.3.5.2.1) except that the plates were incubated at 37°C for 24 hours in a microplate reader and measured hourly at $OD_{600\ nm}$ to trace bacterial growth. The growth curve was plotted and the growth rate of *S. aureus* for each treatment was calculated (Kim and Park, 2013). The experiment was performed in triplicates.

3.3.6.2 Biofilm inhibition on polymer materials

Biofilm inhibition activity on different polymer surfaces was evaluated according to the methods by Tran *et al.* (2012), with modifications. Several polymer materials were chosen to mimic the materials used in medical devices. Poly-vinyl chloride tubing, polyethylene tubing, silicone rubber were cut into 1 cm length sizes while polypropylene caps of micro centrifuge tubes were cut from the tubes. Each piece was sterilized by soaking in 70% ethanol and air dried (Kadurugamuwa *et al.*, 2003). Each material was placed in 24-well plates and served as the attaching surface for the cells. Inoculum suspension was prepared as described in Section 3.3.5.1. Two hundred microliters of extract was added with 1.8 ml of inoculum suspension to obtain the desired concentration of extract in a 24 well microtiter plate. Negative control refers to treatment with solvents used to dissolve the extract while 1% sodium hypochlorite served as positive control. The plates were incubated at 37°C for 24 hours. Quantification was done using crystal violet staining, as described in Section 3.3.5.3. The experiment was performed in triplicates.

3.3.6.3 Surface conditioning and anti-adhesive assay

To determine whether the extract can modify the surface properties of an abiotic substrate, the extract was deposited onto the surface of polystyrene wells and the ability of the conditioned surface to prevent attachment by *S. aureus* was tested. A 96-well polystyrene microtiter plate was filled with 200 µl of extract and the concentration used was 1.25 mg/ml. Water was used as negative control. The plate was incubated at 37°C for 24 hours and then washed once with sterile water. Overnight cultures of *S. aureus* grown in TSB at 37°C for 20 hours were adjusted to 0.5 McFarland turbidity standard (0.08-0.1 at $OD_{625\ nm}$), corresponding to approximately 10^8 CFU/ml. After 24 hours conditioning, 200 µl of adjusted suspension was added to the wells. The plate was incubated at 37°C for another 24 hours. Unattached bacteria were removed by washing the wells three times with water. Crystal violet staining was conducted, as described in Section 3.3.5.3 (Bendaoud *et al.*, 2011; Zeraik and Nitschke, 2010). The experiment was performed in triplicates.

3.3.6.4 Bacterial adhesion to hydrocarbons (BATH) assay

The effect of extract on cell surface hydrophobicity was measured by BATH as described by Rosenberg *et al.* (1980), with modifications. *S. aureus* were grown for 20 hours and then subjected to centrifugation for 5 minutes at 4000 rpm. The pelleted cells were retained and washed twice with 0.85% sodium chloride before re-suspended in 0.85% sodium chloride and adjusted to achieve $OD_{400\ nm}$ = 1.0. Extract was transferred to a test tube and added with an equal volume of adjusted bacterial suspension. The mixture was agitated on a vortex for 1 minute and incubated for 24 hours at 37°C. After 24 hours incubation, the initial OD of the mixture was measured at 400 nm. One millilitre of hexane was added to the mixture and the mixture was agitated on a vortex for 1 minute. The mixture was left to stand for 30 minutes for phase separation, whereby the hydrocarbon phase will rose completely above the aqueous phase. The aqueous phase was then measured (OD after addition of hexane) at 400 nm. Sodium chloride (0.85%) was used as a blank and the experiments were performed in triplicates. The calculation of hydrophobicity index is as below:

$$\text{Hydrophobicity index (\%)} = \frac{\text{Initial OD-OD after addition of hexane}}{\text{Initial OD}} \times 100$$

3.3.6.5 Phytochemical screening

Phytochemical screening was performed to identify the phytochemicals present in the extract, which may be responsible for the biofilm inhibition effect. The detailed standard procedures for phytochemical screening in Table 3-1 are described in Appendix I.

Table 3-1: Lists of phytochemicals tested and assays used

Phytochemical of interest	Test	Reference
Flavonoids	Shinoda test	Jones and Kinghorn (2006)
Terpenoids	Salkowski test	Neelam *et al.* (2014); Ugochukwu *et al.* (2013);
	Liebermann-Buchard test	Jones and Kinghorn (2006)
Tannins	Ferric chloride test	Jones and Kinghorn (2006)
	Gelatin test	
Alkaloids	Wagner's reagent test	Jones and Kinghorn (2006)
Cardiac glycoside	Keller Kelliani's test	Ugochukwu *et al.* (2013);
	Kedde's test	Jones and Kinghorn (2006)
Phenols	Ferric chloride test	Jones and Kinghorn (2006)
Sterols	Liebermann-Buchard test	Jones and Kinghorn (2006)
Quinones	Quinones test	Ugochukwu *et al.* (2013)
Saponin	Foam test	Jones and Kinghorn (2006);
❖ Bio-surfactant properties	Emulsification capacity test	Batista *et al.* (2006);
	Drop collapse assay	Berti *et al.* (2007)

3.3.7 Determination of anti-biofilm activity: Biofilm disruption activity

3.3.7.1 Initial screening for biofilm disruption activity

Biofilm disruption assay was performed to determine the effectiveness of extract in disrupting pre-formed biofilms of *S. aureus*. For initial screening, 10 mg/ml of MCE(L) and 5 mg/ml of SSE fractions were used. Inoculum suspension was prepared as described in Section 3.3.5.1. Two hundred microliters of inoculum suspension was added to the wells of microtiter plates and incubated for 24 hours to allow the formation of biofilms. After 24 hours, the planktonic cells were decanted and the wells were washed three times with sterile water. Two hundred microliters of extract was applied to the wells. Solvent control refers to treatment with solvent used to dissolve extract while treatment with 1% sodium hypochlorite served as positive control. Negative control (treatment with water) was added as a comparison to solvent control. The plates were further incubated at 37°C for 24 hours. Disruption of pre-formed biofilms was quantified using crystal violet staining (Section 3.3.5.3) (Dusane *et al.*, 2008; Shakeri *et al.*, 2007). The experiment was performed in triplicates. Figure 3-5 summarizes the methodology for biofilm disruption assay.

3.3.7.1.1 Minimum biofilm disruption concentration (MBDC) assay

Upon observation of biofilm disruption activity, the minimum biofilm disruption concentration (MBDC) assay was performed. MBDC was defined as the lowest concentration of extract that disrupts pre-formed biofilms of *S. aureus* as compared with the solvent control. The procedure was as previously described in Section 3.3.7.1, whereby the biofilm was allowed to form first before adding a two-fold dilution series of various concentrations of extract for treatment. Disruption of

pre-formed biofilms was quantified using crystal violet staining (Section 3.3.5.3) (Dusane *et al.*, 2008; Shakeri *et al.*, 2007). The experiment was performed in triplicates.

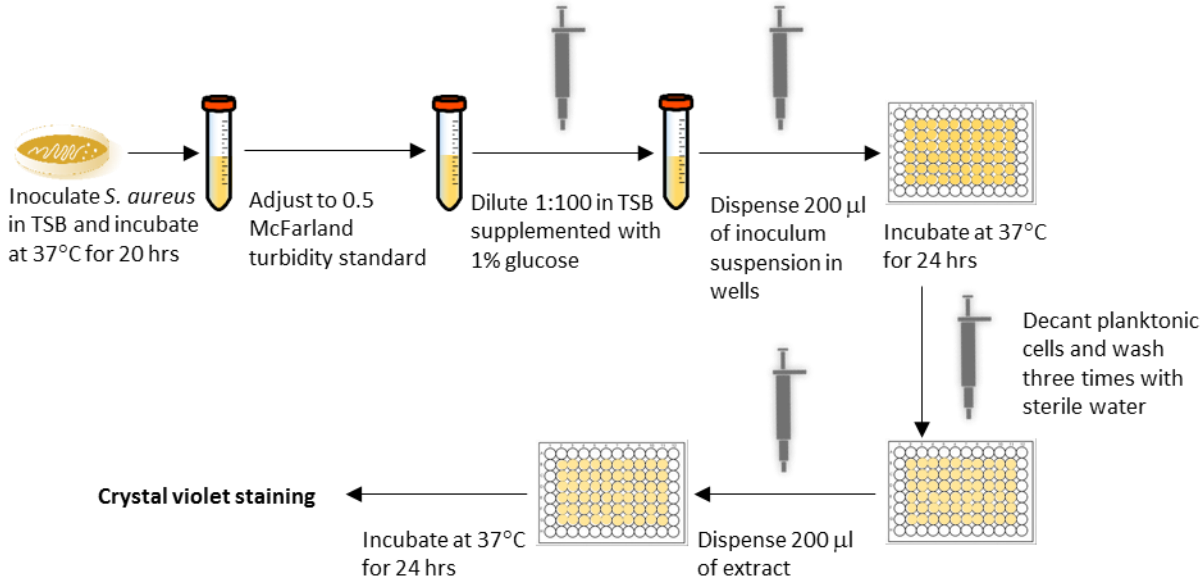


Figure 3-5: Summary of methodology for biofilm disruption assay

3.3.8 Investigation of extract with most effective biofilm disruption activity

3.3.8.1 Growth curve analysis

Growth curve analysis was performed to demonstrate that extract at MBDC does not affect the growth of *S. aureus* cells. The procedure was as described for growth curve analysis in Section 3.3.6.1. The plate was incubated at 37°C for 24 hours in a microplate reader and measured hourly at $OD_{600\ nm}$ to trace bacterial growth (Kim and Park, 2013). The experiment was performed in triplicates.

3.3.8.2 Scanning electron microscopy (SEM)

Scanning electron microscopy was used to investigate the structural modifications of biofilms after treatment with extract at MBDC. The method was performed according to Kerekes *et al.* (2013), with modifications. Inoculum suspension was prepared as described in Section 3.3.5.1. Sterile polystyrene discs were placed in the wells of a 24-well plate containing the inoculum suspension and served as the attaching surface for *S. aureus* cells. The plate was incubated for 24 hours at 37°C to allow the formation of biofilms on the polystyrene discs. Then, the polystyrene discs were carefully washed with phosphate buffered saline (PBS) and placed into a new 24-well plate containing treatment and control solutions. For treatment, extract at MBDC was used. Negative control (water), solvent control (solvent used to dissolve extract) and positive control (1% sodium hypochlorite) were included. Then, the plate was incubated for 24 hours at 37°C. After incubation, the discs were washed with PBS. The preparation of the discs for electron microscopy was

performed with the following procedure: soaking of the discs in filtered 2.5% glutaraldehyde in PBS for 4 hours at room temperature, soaking in PBS for 10 minutes, and then followed by ethanol dehydration of concentrations: 20, 40, 60, 70, 80, 90, 95, 100%. Each ethanol treatment lasted for 10 minutes at room temperature. The discs were then placed in a desiccator overnight. The discs were sputter coated with gold and examined with a scanning electron microscope.

3.3.8.3 Phytochemical screening

Phytochemical screening was performed to identify the phytochemicals present in the extract, which may be responsible for the biofilm disruption effect. The detailed standard procedures for phytochemical screening in Table 3-2 are described in Appendix I.

Table 3-2: List of phytochemicals tested and assays used

Phytochemical of interest	Test	Reference
Flavonoids	Shinoda test	Jones and Kinghorn (2006)
Terpenoids	Salkowski test	Neelam *et al.* (2014); Ugochukwu *et al.* (2013);
	Liebermann-Buchard test	Jones and Kinghorn (2006)
Alkaloids	Wagner's reagent test	Jones and Kinghorn (2006)
Cardiac glycoside	Keller Kelliani's test	Ugochukwu *et al.* (2013);
	Kedde's test	Jones and Kinghorn (2006)
Phenols	Ferric chloride test	Jones and Kinghorn (2006)
Sterols	Liebermann-Buchard test	Jones and Kinghorn, 2006
Quinones	Quinones test	Jones and Kinghorn (2006);

3.3.9 Data analysis

Statistical analysis was conducted using the One-way Analysis of Variance (ANOVA) test for comparing mean scores of more than two groups, with significance at $p<0.05$. Independent-samples t-test was performed to compare the mean scores of two different groups, with significance at $p<0.05$. The IBM SPSS Statistics 20 software was used (Kerekes *et al.*, 2013). All graphs were generated using the GraphPad Prism 6 software.

3.4 Results and discussion

3.4.1 Anti-biofilm activity of MCE(L)

Methanol crude extraction (MCE) was performed on the leaves of *D. linearis.* MCE(L) was screened for anti-biofilm activity, which includes biofilm inhibition and biofilm disruption activities. Crystal violet assay was used to evaluate anti-biofilm activity. Crystal violet assay quantifies the biofilm biomass by staining both biofilm bacteria cells (living and dead cells) and the biofilm matrix (Peeters *et al.*, 2008).

3.4.1.1 Initial screening for biofilm inhibition activity of MCE(L)

For initial screening for biofilm inhibition activity, MCE(L) was tested at sub-MIC values i.e. half the MIC. Antibacterial extracts used at sub-MIC values were often assumed to not impact bacterial growth (Brackman *et al.*, 2009; Packiavathy *et al.*, 2012). Therefore, by using sub-MIC values, if biofilm inhibition effect was observed, it can be concluded that the inhibitory effect was not due to dead or dying cells that were unable to produce biofilm but due to the extract causing an as-yet undetermined anti-biofilm activity. Figure 3-6 shows the presence of biofilm inhibition activity at sub-MIC values for MCE(L).

Based on Figure 3-6, MCE(L) demonstrated effective biofilm inhibition activity at sub-MIC against all five strains of *S. aureus* tested, as shown by the significant reduction in biofilm formation (biofilm biomass) when compared with the solvent control. The solvent control (5% MeOH) showed no significance difference in biofilm formation when compared with the negative control (water). The biofilm inhibition effect of MCE(L) was comparable to the positive control (1% sodium hypochlorite) used.

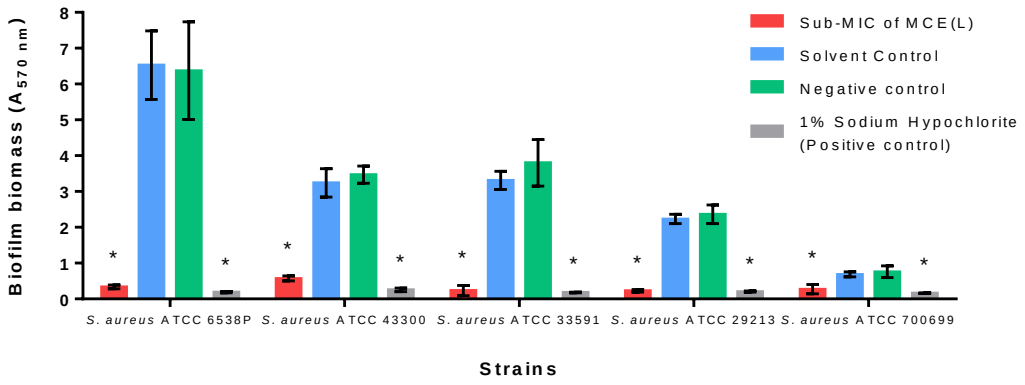


Figure 3-6: Biofilm inhibition activity at sub-MIC of MCE(L) against five strains of *S. aureus*. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against *S. aureus* strains. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm inhibition activity when compared with the solvent control (5% MeOH). Biofilm inhibition activity was observed at sub-MIC against all five strains of *S. aureus.*

3.4.1.1.1 Minimum biofilm inhibition concentration (MBIC) of MCE(L)

Upon observation of the biofilm inhibition effect at sub-MIC, the minimum biofilm inhibition concentration (MBIC) assay was done to obtain the minimum concentration of MCE(L) needed to exhibit biofilm inhibition activity. Figure 3-7 shows the biofilm inhibitory effect of MCE(L) at various concentrations and Table 3-3 summarizes the MBIC for each *S. aureus* strain.

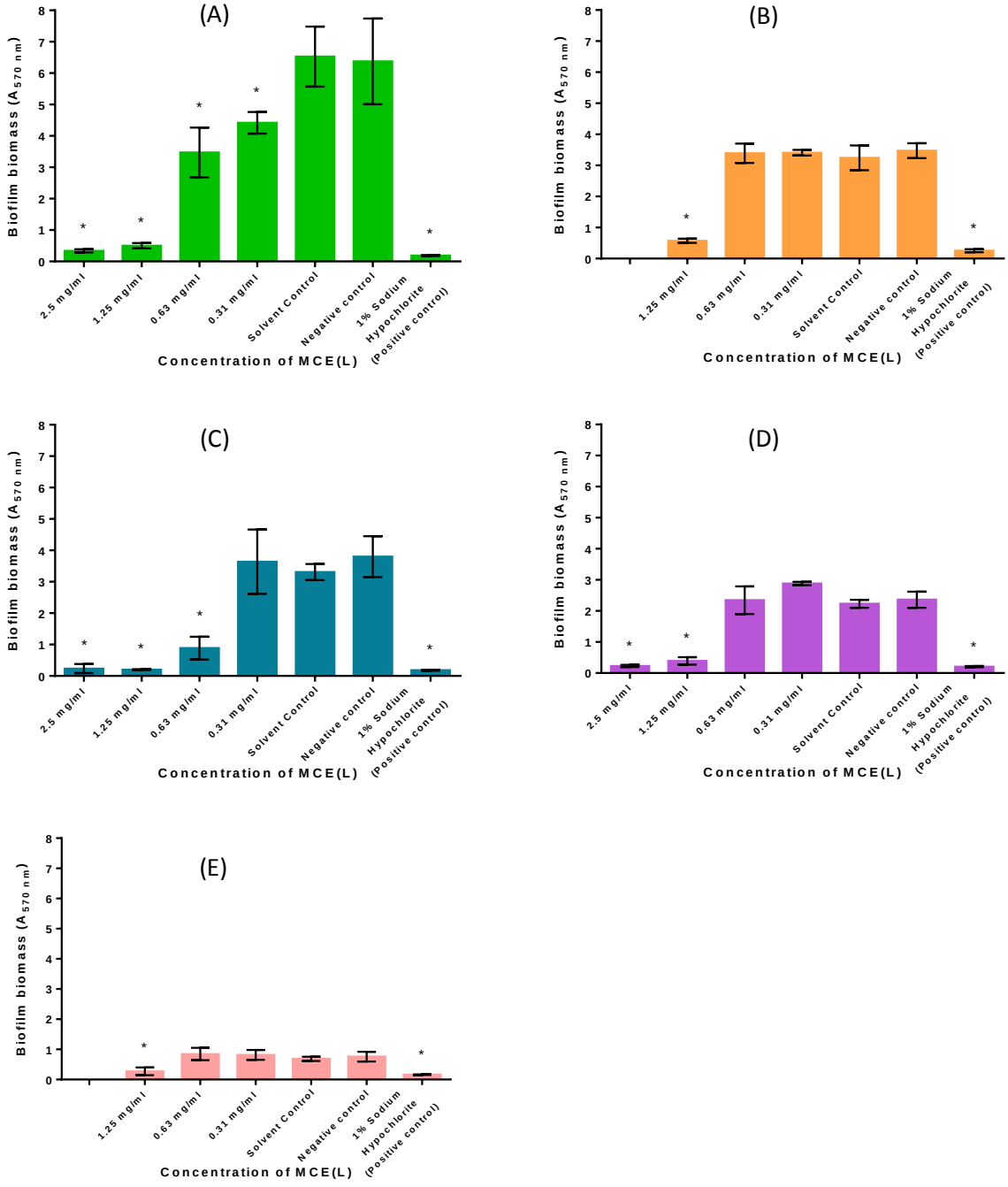


Figure 3-7: Biofilm inhibition activity of MCE(L) at various concentrations against (A) *S. aureus* ATCC 6538P; (B) *S. aureus* ATCC 43300; (C) *S. aureus* ATCC 33591; (D) *S. aureus* ATCC 29213; (E) *S. aureus* ATCC 700699. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against concentrations of MCE(L). * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm inhibition activity when compared with the solvent control (5% MeOH).

Table 3-3: Summary of the minimum biofilm inhibition concentration (MBIC) of MCE(L) against *S. aureus*

Strains	MIC (mg/ml)	Sub-MIC (mg/ml)	MBIC (mg/ml)
S. aureus ATCC 6538P	5.0	2.5	0.31
S. aureus ATCC 43300	2.5	1.25	1.25
S. aureus ATCC 33591	5.0	2.5	0.63
S. aureus ATCC 29213	5.0	2.5	1.25
S. aureus ATCC 700699	2.5	1.25	1.25

*Minimum inhibitory concentration (MIC) (Table 2-3), sub-MIC & minimum biofilm inhibition concentration (MBIC) in mg/ml

Based on the MBIC values determined (Table 3-3), concentrations ranging from 0.31 - 1.25 mg/ml were able to inhibit biofilm formation in *S. aureus* tested. For *S. aureus* strains ATCC 29213, ATCC 33591 and ATCC 6538p, effective biofilm inhibition was achievable at concentrations lower than the sub-MIC while for *S. aureus* strains ATCC 700699 and ATCC 43300, effective biofilm inhibition was achievable only up to the sub-MIC tested.

In other studies, compounds such as essential oils, flavonoids, terpenoids and glycosides have been found to have anti-biofilm activity (Carneiro *et al.*, 2011; Gursoy *et al.*, 2009; Lee *et al.*, 2011; Ye *et al.*, 2010). These compounds have been reported to be present in *D. linearis* and therefore, might be the possible active compound responsible for the biofilm inhibition effect observed for MCE(L). More analysis would need to be conducted to determine the specific compound(s) responsible for the biofilm inhibition activity of MCE(L).

Currently, there are no studies in literature on the anti-biofilm effect of *D. linearis*, especially on its biofilm inhibition activity. With the biofilm inhibition activity observed for MCE(L), therefore, this study is the first to report on the biofilm inhibition activity of *D. linearis.*

3.4.1.2 Initial screening for biofilm disruption activity of MCE(L)

For initial screening, MCE(L) was tested for biofilm disruption activity at 10 mg/ml. Figure 3-8 shows the presence of biofilm disruption activity of MCE(L) at 10 mg/ml.

Based on Figure 3-8, MCE(L) showed no biofilm disruption activity against all five strains of *S. aureus* tested, as shown by the lack of significant decrease in pre-formed biofilm (biofilm biomass) when compared with the solvent control. The solvent control did not show any significant difference in biofilm biomass when compared with the negative control, indicating that the solvent used to dissolve MCE(L) did not affect the biofilm and thus, should not affect any biofilm disruption activity by MCE(L), if activity was present. Due to the lack of biofilm disruption activity by MCE(L) at 10 mg/ml, the minimum biofilm disruption concentration (MBDC) assay was not conducted.

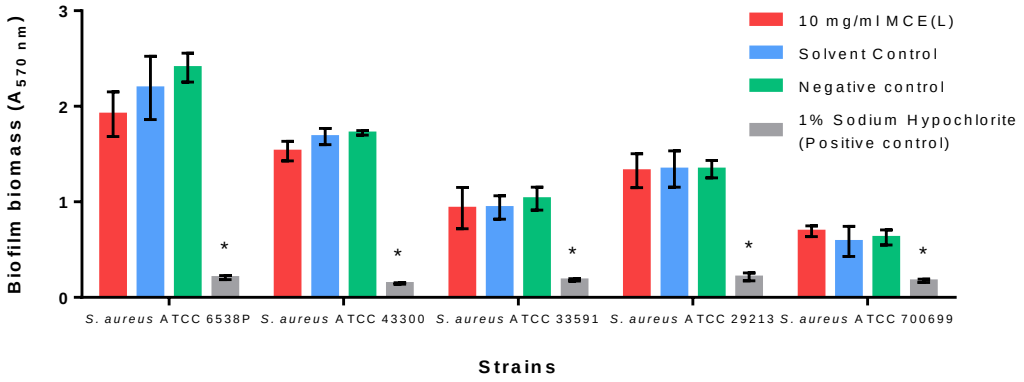


Figure 3-8: Biofilm disruption activity at 10 mg/ml of MCE(L) against five strains of *S. aureus*. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against *S. aureus* strains. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm disruption activity when compared with the solvent control (5% MeOH). No biofilm disruption effect was observed.

The lack of biofilm disruption activity by MCE(L) was not surprising as there are not many reports on the biofilm disruption activity of plant extracts as compared to the various findings on biofilm inhibition activity of plant extracts. The presence of the exopolysaccharide matrix on pre-formed biofilms may have restricted penetration by MCE(L) and thus, preventing any disruption effect by MCE(L). Besides that, the lack of biofilm disruption activity but the ability to inhibit biofilm formation by MCE(L) suggests that MCE(L) may have targeted the attachment stage (initial stage) of biofilm formation when inhibiting biofilm formation because disruption of pre-formed (mature) biofilms would require more complex mechanisms (Donlan and Costerton, 2002).

3.4.2 Anti-biofilm activity of SSE fractions

Since screening with MCE(L) had shown promising results with biofilm inhibition activity present, it was of interest to study the extract in depth in case there were compounds not being extracted out by MCE. Sequential solvent extraction (SSE) was performed on the leaves of *D. linearis* with hexane, dichloromethane, ethyl acetate, methanol and water. The five SSE fractions obtained were screened for anti-biofilm activity, which includes biofilm inhibition and biofilm disruption activities.

3.4.2.1 Initial screening for biofilm inhibition activity of SSE fractions

For initial screening, the SSE fractions were tested for biofilm inhibition activity at sub-MIC values i.e. half the MIC. As mentioned previously, antibacterial extracts used at sub-MIC values are often assumed to not impact bacterial growth (Brackman *et al.*, 2009; Packiavathy *et al.*, 2012). Therefore, by using sub-MIC values, if biofilm inhibition effect was observed, it can be concluded that the inhibition effect was not due to dead or dying cells that are unable to produce biofilm but due to the extract causing an as-yet undetermined anti-biofilm activity.

The HEX, DCM, EA and H_2O fractions had MIC values of >10 mg/ml (Table 2-5) and when screening for biofilm inhibition activity, the concentration used was at sub-MIC of 5 mg/ml. MeOH fraction had an MIC of 5 mg/ml (Table 2-5) and thus, the concentration used for screening of biofilm inhibition activity was at sub-MIC of 2.5 mg/ml. Table 3-4 summarises the SSE fractions exhibiting biofilm inhibition activity at sub-MIC values.

Table 3-4: Biofilm inhibition activity observed at sub-MIC of SSE fractions

Strains	Biofilm production	HEX	DCM	EA	MeOH	H_2O
S. aureus ATCC 6538P	Strong	×	×	×	√	√
S. aureus ATCC 43300	Moderate to strong	×	×	×	√	√
S. aureus ATCC 33591	Weak to moderate	×	×	×	√	√
S. aureus ATCC 29213	Weak to moderate	×	×	×	√	√
S. aureus ATCC 700699	Weak	×	×	×	×	√

*HEX, DCM, EA and H_2O fractions tested at sub-MIC = 5 mg/ml; MeOH fractions tested at sub-MIC = 2.5 mg/ml
*√ = presence of activity; × = absence of activity

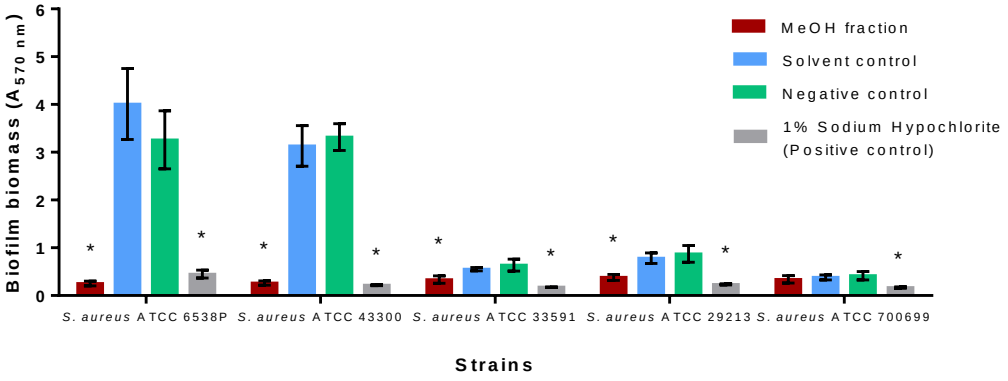


Figure 3-9: Biofilm inhibition activity at sub-MIC of MeOH fraction against five strains of *S. aureus*. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against *S. aureus* strains. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm inhibition activity when compared with the solvent control (5% Tween 80). Biofilm inhibition activity was observed against four strains of *S. aureus.*

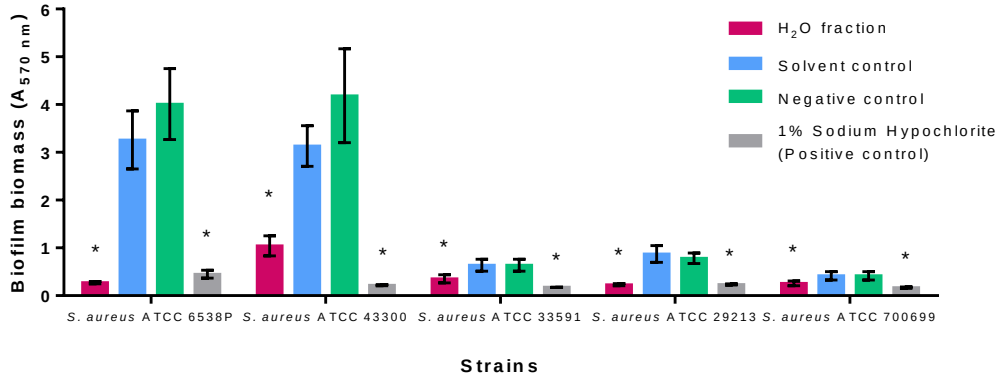


Figure 3-10: Biofilm inhibition activity at sub-MIC of H_2O fraction against five strains of *S. aureus*. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against *S. aureus* strains. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm inhibition activity when compared with the solvent control (water). Biofilm inhibition activity was observed against all five strains of *S. aureus*.

Figure 3-9 and Figure 3-10 demonstrates the presence of biofilm inhibition activity at sub-MIC for MeOH and H_2O fractions, respectively, as shown by the significant reduction in biofilm formation (biofilm biomass) when compared with the solvent control. The H_2O fraction was effective against all five *S. aureus* strains tested while the MeOH fraction was only effective against four strains (Table 3-4). Biofilm inhibition activity was not observed with HEX, DCM and EA fractions (Table 3-4; Appendix II: Figure A 1 to Figure A 3). The biofilm inhibition effect observed for MeOH and H_2O fractions supports the findings found previously on the biofilm inhibition activity of MCE(L). These findings further confirm the presence of biofilm inhibition activity in *D. linearis*.

The presence of biofilm inhibition activity only for the polar fractions, i.e. MeOH and H_2O fractions indicates that the bioactive compound(s) responsible for the activity, to be highly polar, as strongest activity was in the H_2O fraction. Several studies that have reported on the biofilm inhibition activity of plant extracts, are such as the methanol extracts of *Capparis spinosa* (Abraham *et al.*, 2011), *Pongamia pinnata* (Abraham *et al.*, 2012) and *Alnus janponica* (Lee *et al.*, 2013), and the water extracts of *Calendula offinialis* flowers (Ghaima *et al.*, 2013) and *Solidago virgaurea* (Chevalier *et al.*, 2012).

As the H_2O fraction was more effective as compared to the MeOH fraction, therefore, only the H_2O fraction was chosen for further characterization of biofilm inhibition activity. This will later be discussed in Section 3.4.3.

3.4.2.2 Initial screening for biofilm disruption activity of SSE fractions

For initial screening, the SSE fractions were tested for biofilm disruption activity at 5 mg/ml. Table 3-5 summarizes the SSE fractions exhibiting biofilm disruption activity.

Table 3-5: Biofilm disruption activity observed for SSE fractions at 5 mg/ml

Strains	Biofilm production	HEX	DCM	EA	MeOH	H_2O
S. aureus ATCC 6538P	Strong	√	×	×	×	×
S. aureus ATCC 43300	Moderate to strong	√	√	×	×	×
S. aureus ATCC 33591	Weak to moderate	√	√	×	×	×
S. aureus ATCC 29213	Weak to moderate	√	√	√	×	×
S. aureus ATCC 700699	Weak	√	√	√	×	×

*√ = presence of activity; × = absence of activity

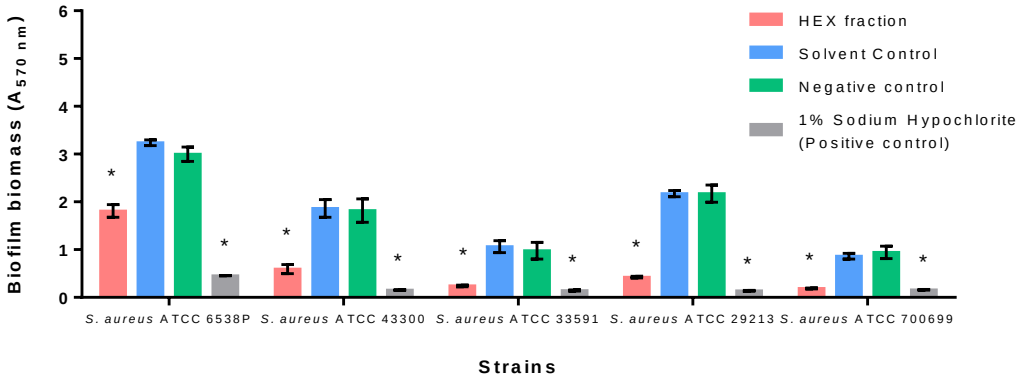


Figure 3-11: Biofilm disruption activity at 5 mg/ml of HEX fraction against five strains of *S. aureus*. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against *S. aureus* strains. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm disruption activity when compared with the solvent control (0.125% of Tween 80 in 2.5% acetonitrile) and negative control. Biofilm disruption activity was observed against all five strains of *S. aureus*.

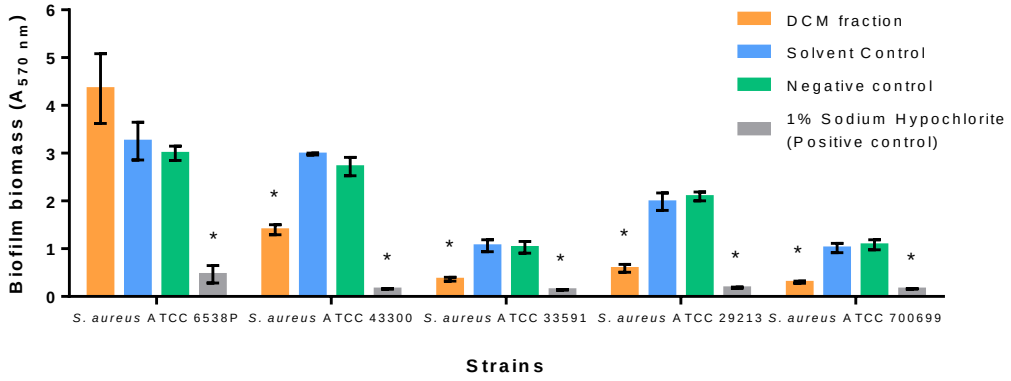


Figure 3-12: Biofilm disruption activity at 5 mg/ml of DCM fraction against five strains of *S. aureus*. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against *S. aureus* strains. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm disruption activity when compared with the solvent control (0.125% of Tween 80 in 2.5% acetonitrile) and negative control. Biofilm disruption activity was observed against four strains of *S. aureus*.

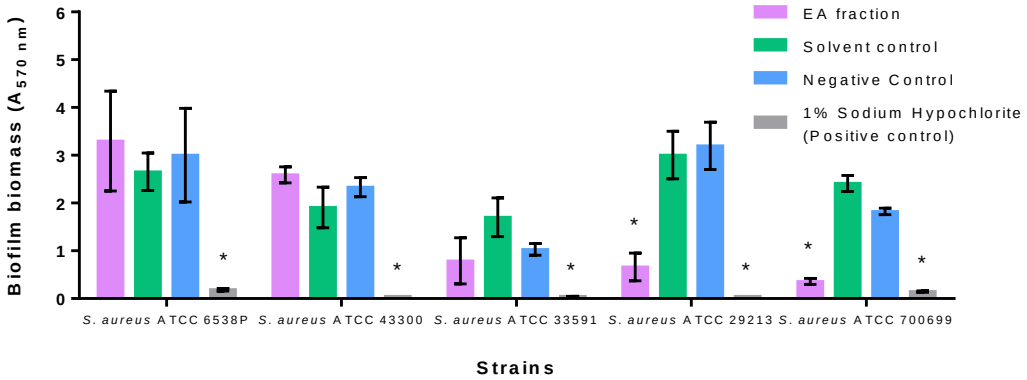


Figure 3-13: Biofilm disruption activity at 5 mg/ml of EA fraction against five strains of *S. aureus*. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against *S. aureus* strains. * denotes statistically significant difference at $p<0.05$ and exhibitings biofilm disruption activity when compared with the solvent control (5% Tween 80) and negative control. Biofilm disruption activity was observed against two strains of *S. aureus*.

Based on Table 3-5, anti-biofilm activity for disruption of pre-formed biofilm was observed for HEX, DCM and EA fractions. Figure 3-11, Figure 3-12 and Figure 3-13 shows the presence of the biofilm disruption activity of HEX, DCM and EA fractions respectively, at the concentration of 5 mg/ml, as shown by the significant decrease in pre-formed biofilm (biofilm biomass) when compared with the solvent control. The HEX fraction was effective against all five *S. aureus* strains while the DCM and EA fractions were effective against only four and two strains, respectively. Biofilm disruption activity was not observed with the MeOH and H_2O fractions (Table 3-5; Appendix III: Figure A 4 and Figure A 5).

As stated previously, when screening was conducted with MCE(L), no biofilm disruption activity was observed for MCE(L) (Figure 3-8). However, through SSE, biofilm disruption activity was observed for HEX, DCM and EA fractions. The presence of biofilm disruption activity only for the more non-polar fractions, i.e. HEX, and DCM fractions, and EA (intermediate polarity) indicates that the active compound(s) responsible for the activity was highly non-polar, since strongest activity was observed for the HEX fraction. The lack of biofilm disruption activity observed for MCE(L) might be due to the fact that methanol (polar solvent) was unable to extract the highly non-polar active compound(s) during MCE.

Currently, there are no studies in literature on the anti-biofilm effect of *D. linearis*, especially on its biofilm disruption activity. With biofilm disruption activity observed for the HEX, DCM and EA fractions, therefore, this study is the first to report on the biofilm disruption activity of *D. linearis.*

There are few findings on disruption activity of biofilms due to their resistance to antibacterial agents compared to planktonic cells. Few studies that have reported on biofilm disruption activity by plant extracts are such as the eradication of *Propionibacterium acnes* biofilms by the water extracts of *Epomedium brevicornum* and *Polygonum cuspidatum* (Coenye *et al.*, 2012), the eradication of *Pseudomonas aeruginosa* and *S. aureus* biofilms by the essential oils of cassia (*Cinnamomum aromaticum*), Peru balsam (*Myroxylan balamum*) and red thyme (*Thymus vulgaris*) (Kavanaugh and Ribbeck, 2012), and the disruption of *P. aeruginosa*, *Escherichia coli*, *Serratia marcescens* and *Proteus mirabilis* biofilms by the methanol extract of *Capparis spinosa* (Abraham *et al.*, 2011).

As the HEX fraction was more effective than DCM and EA fractions, therefore, only the HEX fraction was chosen for further characterization of biofilm disruption activity. This will later be discussed in Section 3.4.4.

3.4.3 Biofilm inhibition activity of H_2O fraction

As stated in Section 3.4.2.1, only the H_2O fraction was chosen for further characterization, as it showed biofilm inhibition activity against all five strains of *S. aureus* when screened at sub-MIC of 5 mg/ml.

3.4.3.1 Growth curve analysis and MBIC assay

The minimum biofilm inhibition concentration (MBIC) assay was conducted to obtain the minimum concentration needed for H_2O fraction to exhibit biofilm inhibition activity. In order to fully confirm that the activity observed was due to an anti-biofilm effect and not growth inhibition effect, growth curve analysis with H_2O fraction was performed simultaneously with MBIC assay. If biofilm inhibition occurred at concentrations that impacted growth, this would suggest that the inhibition of biofilm occurred primarily through growth inhibition. In contrast, if the concentration did not affect growth, this would suggest that the biofilm inhibition effect was of growth independent mechanisms (Starner *et al.*, 2008).

Figure 3-14 shows the biofilm inhibition effect of various concentrations of H_2O fraction against all five strains of *S. aureus* tested. Since water was used to dissolve the H_2O fraction, all results were compared with the negative control (treatment with water), which also represents the solvent control. The growth curves for each *S. aureus* strain treated with the various concentrations of H_2O fraction are presented in the Appendix IV (Figure A 6 to Figure A 10) and the growth rates obtained for each strain are shown in Table 3.6.

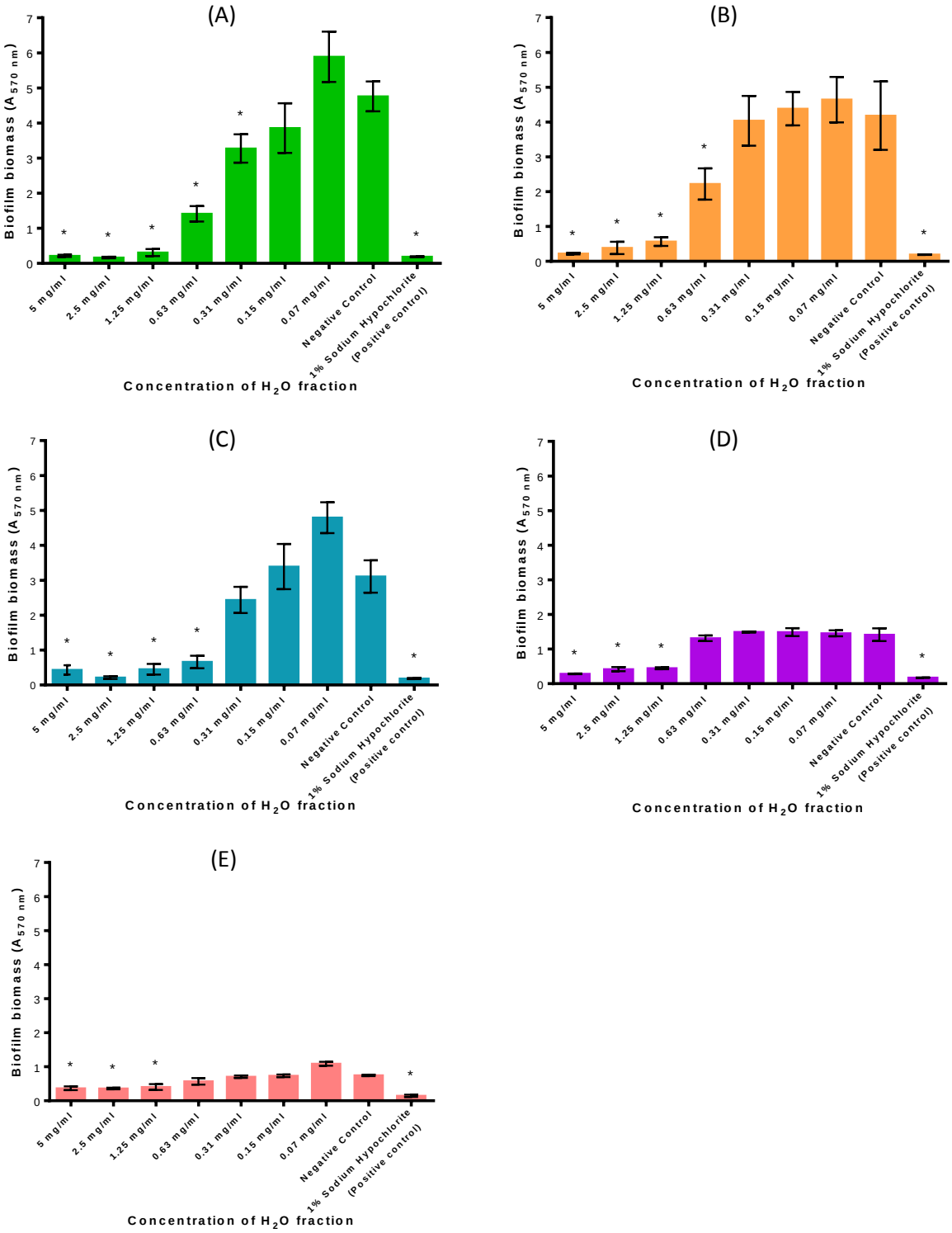


Figure 3-14: Biofilm inhibition activity of H_2O fraction at various concentrations against (A) *S. aureus* ATCC 6538P; (B) *S. aureus* ATCC 43300; (C) *S. aureus* ATCC 33591; (D) *S. aureus* ATCC 29213; (E) *S. aureus* ATCC 700699. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against various concentrations of H_2O fraction. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm inhibition activity when compared with the negative control.

Table 3-6: Growth rates of each *S. aureus* strain at various concentrations of H_2O fraction

Concentration of H_2O fraction	Growth rates of *S. aureus* strains (hour per generation)				
	S. aureus ATCC 6538P	*S. aureus* ATCC 43300	*S. aureus* ATCC 33591	*S. aureus* ATCC 29213	*S. aureus* ATCC 700699
5.0 mg/ml	12.51 ± 0.67*	10.24 ± 0.51*	12.19 ± 3.96*	12.00 ± 0.93*	12.52 ± 1.00*
2.5 mg/ml	**1.21 ± 0.08**	11.99 ± 1.91*	**1.93 ± 0.05**	9.34 ± 0.90*	11.86 ± 1.47*
1.25 mg/ml	**1.10 ± 0.05**	**1.34 ± 0.07**	**1.74 ± 0.20**	3.70 ± 0.33*	5.31 ± 0.92*
0.63 mg/ml	**1.09 ± 0.04**	**1.36 ± 0.05**	**1.82 ± 0.11**	4.44 ± 0.33*	4.91 ± 0.59*
0.31 mg/ml	**1.28 ± 0.12**	1.25 ± 0.08	2.00 ± 0.17	4.24 ± 0.61*	3.62 ± 0.12*
0.15 mg/ml	0.83 ± 0.13	1.05 ± 0.09	1.67 ± 0.21	1.05 ± 0.18	2.56 ± 0.34
0.07 mg/ml	0.94 ± 0.22	1.09 ± 0.14	1.50 ± 0.23	0.95 ± 0.15	2.11 ± 0.54
Negative control	0.84 ± 0.34	1.15 ± 0.24	2.22 ± 0.56	0.96 ± 0.12	2.15 ± 0.61
Positive control	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*	0.00 ± 0.00*

*Treatment with significant difference in growth rate when compared with the negative control ($p<0.05$)
Concentration of H_2O fraction exhibiting biofilm inhibition activity without growth inhibition effect (**values in bold and shaded**)

Table 3-7: Minimum biofilm inhibition concentration (MBIC) of H_2O fractions against *S. aureus* strains

Strains	Biofilm production	MBIC (mg/ml)
S. aureus ATCC 6538P	Strong	0.31
S. aureus ATCC 43300	Moderate to strong	0.63
S. aureus ATCC 33591	Weak to moderate	0.63
S. aureus ATCC 29213	Weak to moderate	-
S. aureus ATCC 700699	Weak	-

*Minimum biofilm inhibition concentration (MBIC) in mg/ml

The growth rate represents the time interval required for *S. aureus* cells to divide. Treatment with H_2O fraction showing high growth rate values will indicate growth inhibition effect by H_2O fraction since a longer generation time is required for cell division. Based on the growth rates in Table 3-6, inhibition of growth was observed only at 5 mg/ml for *S. aureus* ATCC 6538P and *S. aureus* ATCC 33591, and at 2.5 – 5 mg/ml for *S. aureus* ATCC 43300. This indicates that the biofilm inhibition effect observed at 0.31 mg/ml – 2.5 mg/ml for *S. aureus* ATCC 6538P (Figure 3-14 (A)), at 0.63 – 2.5 mg/ml for *S. aureus* ATCC 33591 (Figure 3-14 (C)), and at 0.63 mg/ml – 1.25 mg/ml for *S. aureus* ATCC 43300 (Figure 3-14 (B)), were due to a mechanism unrelated to inhibition of growth and were only due to an anti-biofilm effect. Therefore, at these concentrations, the H_2O fraction had exhibited biofilm inhibition effect without bacteriostatic or bactericidal activity against the three *S. aureus* strains. The MBIC values (the minimum concentration needed for H_2O fraction to exhibit biofilm inhibition activity) for these three strains were summarized in Table 3-7.

Inhibition of growth by H_2O fraction was observed in treatments from 0.31 – 5 mg/ml for *S. aureus* ATCC 29213 and from 0.63 – 5 mg/ml for *S. aureus* ATCC 700699 (Table 3-6), as shown by the high growth rate values. The biofilm inhibition effect observed against *S. aureus* ATCC 29213 and ATCC 700699 at 1.25 – 5 mg/ml (Figure 3-14 (D) and Figure 3-14 (E)) may be due to inhibition of growth and not due to an anti-biofilm effect. Both *S. aureus* ATCC 29213 and ATCC 700699 are much

weaker biofilm producers compared to the other three strains tested and this might have contributed to the growth inhibition effect observed on its biofilm inhibition activity. The MBIC values could not be determined and these two strains were not used for further characterization of biofilm inhibition activity.

Further characterization with the H_2O fraction for biofilm inhibition activity on *S. aureus* strains ATCC 6538P, ATCC 33591 and ATCC 43300 was standardised to 1.25 mg/ml of H_2O fraction as stronger biofilm inhibition effect was observed at 1.25 mg/ml as compared to at MBIC.

3.4.3.2 Biofilm inhibition activity on polymer surfaces

Medical devices such as prostheses, implants and catheters are composed by polymers made mostly of biomaterials such polyurethane, polyethylene, silicone rubber and poly vinyl-chloride. These surfaces are susceptible to bacterial colonization, which pose an important public health concern. When these devices are implanted, they become a site for bacterial adhesion, colonization and infection. There is a need for preventive measures in combating the problem of bacterial colonization on medical devices (Kaali *et al.*, 2011; Treter and Macedo, 2011).

In order to access the effects of H_2O fraction in preventing biofilm formation on various types of polymer materials, five different polymer materials were selected: polystyrene, poly-vinyl chloride, polyethylene, polypropylene, and silicone rubber. These polymer materials are used to make medical devices as listed in Table 3-8.

Table 3-8: The medical devices made using the various types of polymers (Agarwal *et al.*, 2010)

Polymer materials	**Medical devices**
Polystyrene	catheter, ureteral stents
Poly-vinyl chloride	catheter, tracheostomy tubes
Polyethylene	catheter, knee implants
Polypropylene	catheter, suture, mechanical heart valves
Silicone rubber	catheter, pacemaker,

Biofilm inhibition activity of 1.25 mg/ml of H_2O fraction on these polymer materials was conducted against *S. aureu*s ATCC 6538P, *S. aureus* ATCC 43300 and *S. aureus* ATCC 33591, and presented in Figure 3-15. Table 3-9 shows the percentage biofilm inhibition by H_2O fraction for each polymer material.

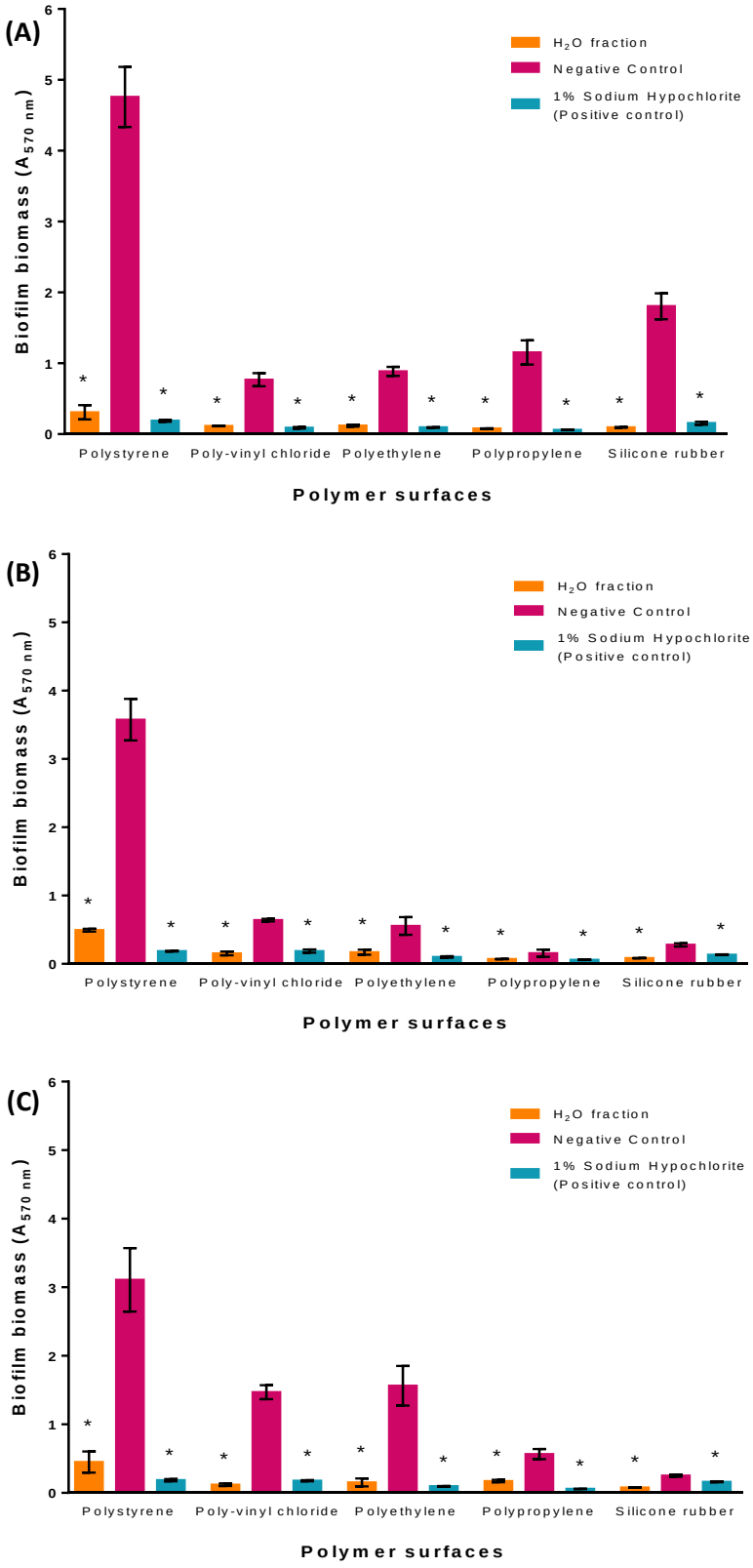


Figure 3-15: Biofilm inhibition activity of 1.25 mg/ml of H_2O fraction conducted on polymer surfaces such as polystyrene, poly-vinyl chloride, polyethylene, polypropylene, and silicone rubber against (A) *S. aureus* ATCC 6538P; (B) *S. aureus* ATCC 43300; (C) *S. aureus* ATCC 33591. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against various polymer surfaces. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm inhibition activity when compared with the negative control.

Based on Figure 3-15, against all three *S. aureus* strains tested, it was observed that H_2O fraction was effective in inhibiting biofilm formation on all five polymer materials, as shown by the significant reduction in biofilm formation (biofilm biomass) when compared with the negative control. In terms of percentage biofilm inhibition, based on Table 3-9, H_2O fraction was able to inhibit *S. aureus* biofilm formation on polystyrene with 85-93% inhibition, polyvinyl chloride with 76-91% inhibition, polyethylene with 68-90% inhibition; polypropylene with 52-93% inhibition, and silicone rubber with 68-94% inhibition.

Table 3-9: Percentage biofilm inhibition by H_2O fraction against polymer materials

Strains	Percentage biofilm inhibition (%)				
	Polystyrene	**Polyvinyl chloride**	**Polyethylene**	**Polypropylene**	**Silicone rubber**
S. aureus ATCC 6538P	93.6 ± 1.8	84.7 ± 2.1	86.2 ± 2.2	93.2 ± 0.7	94.7 ± 0.3
S. aureus ATCC 43300	86.1 ± 1.8	76.0 ± 3.7	68.5 ± 8.8	52.5 ± 12.3	70.0 ± 1.6
S. aureus ATCC 33591	85.1 ± 6.3	91.8 ± 1.3	90.4 ± 2.4	68.8 ± 7.7	68.9 ± 3.4

S. aureus is one of the primary colonizers on these five polymer materials, which explains the high percentage of biofilm-associated infections caused by *S. aureus* on medical devices (Agarwal *et al.*, 2010). With H_2O fraction being effective in inhibiting biofilm formation on these five polymer materials, this indicates that H_2O fraction may be beneficial for preventing *S. aureus* biofilm formation on the medical devices.

3.4.3.3 Possible mechanism of action involved

In order to identify the possible mechanism of action involved in H_2O fraction inhibiting biofilm formation, the effects of H_2O fraction on the factors affecting biofilm formation has to be explored.

3.4.3.3.1 Surface conditioning & anti-adhesive assay

Bacterial adhesion to surfaces is one of the initial steps leading to biofilm formation. Adhesion to surfaces can be prevented if the surface properties become altered. To determine whether H_2O fraction can modify the surface properties of an abiotic substrate, 1.25 mg/ml of H_2O fraction was deposited onto the surface of polystyrene wells and the ability of the conditioned surface to prevent adhesion by *S. aureus* was tested.

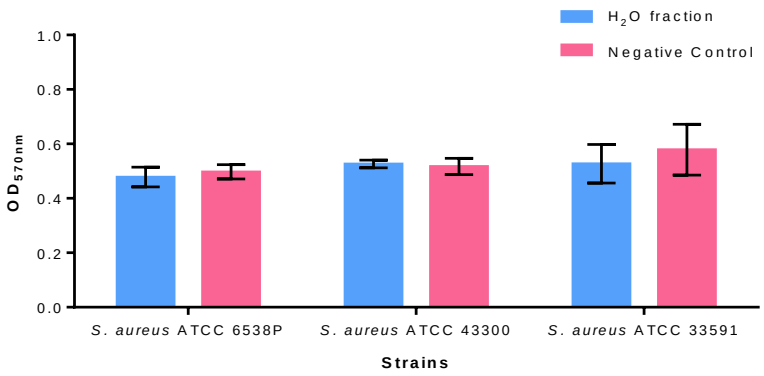


Figure 3-16: Effect of surface conditioning by 1.25 mg/ml of H_2O fraction on *S. aureus* adhesion to polystyrene. Mean $OD_{570\ nm}$ plotted against *S. aureus* strains. * denotes statistically significant difference between treatments. No statistically significant difference in *S. aureus* adhesion was observed between surface conditioned with H_2O fraction and water as negative control.

One of the most convenient methods to prevent biofilm formation is to interfere with the early stages of bacterial adhesion by modifying the surface properties of materials (Kumar *et al.*, 2010). Figure 3-16 shows effect of the conditioned surface by H_2O fraction on *S. aureus* adhesion to polystyrene. There was no statistically significant difference in bacterial adhesion to polystyrene observed for surfaces conditioned with H_2O fraction when compared with the negative control. Based on this result, the H_2O fraction does not modify surface properties to inhibit biofilm formation on polystyrene.

3.4.3.3.2 Measurement of cell surface hydrophobicity

Bacterial adhesion to hydrocarbon (BATH) assay determines the bacterial cell surface hydrophobicity based on the ability of bacterial cells to adhere to hydrocarbon, which was hexane. Table 3-10 shows the hydrophobicity index of *S. aureus* cells before and after treatment with 1.25 mg/ml of H_2O fraction.

Table 3-10: Hydrophobicity index of *S. aureus* cells before and after treatment with 1.25 mg/ml of H_2O fraction

Strains	Hydrophobicity index (%)	
	Untreated	H_2O fraction
S. aureus ATCC 6538P	71.15 ± 12.61	40.53 ± 7.67*
S. aureus ATCC 43300	76.70 ± 5.08	33.80 ± 7.11*
S. aureus ATCC 33591	79.66 ± 2.27	28.89 ± 12.47*

Mean percentage inhibition ± SD from triplicate independent experiments are shown.
* indicates statistically significant difference ($p<0.05$) in hydrophobicity when compared with untreated cells.

The cell surface hydrophobicity of untreated control was >70% for all three strains and after treatment with H_2O fraction, cell surface hydrophobicity decreased to 28 – 40% (Table 3-10). Reduction of the level of hydrophobicity index has often been associated with inhibition of biofilm formation. Thus, the reduction of cell surface hydrophobicity after treatment with H_2O fraction

indicates the modification of bacterial cell surface, which had resulted in reduced colonization and therefore, had contributed to the biofilm inhibition effect (Jiang *et al.*, 2011). It can be deduced that the biofilm inhibition activity observed may be attributed to changes in cell surface hydrophobicity. This deduction was further supported by the detection of bio-surfactants in the H_2O fraction when phytochemical screening was conducted in the following section (Table 3-11). Surfactants can influence the interaction between hydrocarbon and microorganisms and can also alter the outer hydrophobic surface of the cell wall of microorganisms (Kaczorek *et al.*, 2008).

Although it was deduced that the H_2O fraction may affect cell surface hydrophobicity to inhibit biofilm formation, H_2O fraction may also affect other factors involved in biofilm formation (Figure 3-1). Therefore, more analysis has to be conducted to fully elucidate the mechanism of action of H_2O fraction in inhibiting biofilm formation.

3.4.3.4 Phytochemical screening

Phytochemical screening was conducted on H_2O fraction and Table 3-11 shows the phytochemicals identified. Phytochemical screening conducted by previous studies on *D. linearis* had identified the presence of tannins, essential oils, saponins, clerodane glycosides, terpenoids and flavonoids (Table 1-3) (de Winter *et al.*, 2003; Jaishee and Chakraborty, 2015; Li *et al.*, 2008; Raja *et al.*, 1995).

In the H_2O fraction, the presence of flavonoids (flavanones or flavonol), terpenoids, tannins, cardiac glycosides with deoxysugar characteristic of cardenolides, phenols, quinones, and saponins were identified (Table 3-11). Bio-surfactant properties were also observed.

Table 3-11: Phytochemical screening of H_2O fraction

Phytochemicals of interest	Present (√)/Absent (×)
Flavonoids	√
Terpenoids	√
Tannins	√
Alkaloids	×
Cardiac glycoside	√
Phenols	√
Sterols	×
Quinones	√
Saponins	√

Various polyphenols and saponins have been reported to exhibit anti-biofilm activity (Bink *et al.*, 2011; Raut *et al.*, 2013). Phenolics such as epigallocathechin gallate can inhibit biofilm formation by *Pseudomonas aeruginosa* and *Escherichia coli*, and decrease EPS production by *S. aureus*. Hamamelitannin, a polyphenol belonging to the family tannins, and extracted from *Hamamelis virginiana*, reduces the biofilm metabolic activity of different microorganisms (Savoia, 2012).

Terpenoids and saponins have been reported to exhibit biofilm inhibition activity against *Candida albicans* biofilms (Bink *et al.*, 2011; Raut *et al.*, 2013).

At present, it was difficult to identify the specific phytochemical responsible for the biofilm inhibition effect. The phytochemical screening assay had helped to narrow down the various types of phytochemicals present in H_2O fraction, and will be a useful tool for further bioactive compound analyses. Further purification and isolation process has to be conducted to identify the specific compound(s) responsible for the biofilm inhibition activity by H_2O fraction.

3.4.4 Biofilm disruption activity of HEX fraction

As stated in Section 3.4.2.2, only the HEX fraction was chosen for further characterization, as it showed biofilm disruption activity against all five strains of *S. aureus* tested when screened at 5 mg/ml.

3.4.4.1 MBDC assay

The minimum biofilm disruption concentration (MBDC) assay was conducted to obtain the minimum concentration of HEX fraction needed to disrupt pre-formed biofilms. Figure 3-17 shows the biofilm disruption effect by HEX fraction at various concentrations against five *S. aureus* biofilms. Table 3-12 shows the average percentage of biofilm disruption by HEX fraction, calculated from concentrations that showed significant biofilm disruption activity. The MBDC of HEX fraction was also determined and presented in Table 3-12. The negative control used was water while the solvent control was 0.125% of Tween 80 in 2.5% acetonitrile.

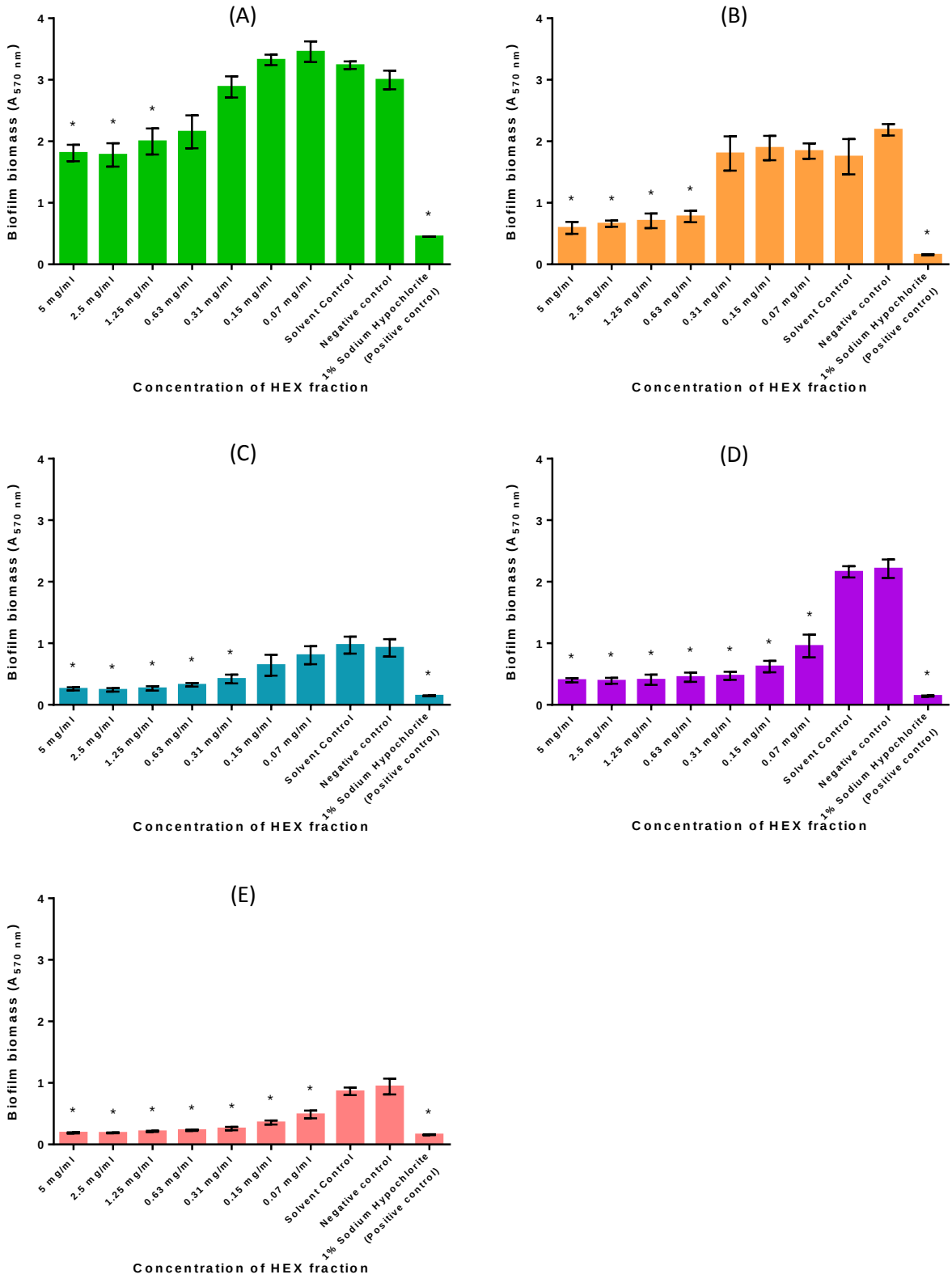


Figure 3-17: Biofilm disruption activity of HEX fraction at various concentrations against (A) *S. aureus* ATCC 6538P; (B) *S. aureus* ATCC 43300; (C) *S. aureus* ATCC 33591; (D) *S. aureus* ATCC 29213; (E) *S. aureus* ATCC 700699. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against various concentrations of HEX fraction. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm disruption activity when compared with the solvent control.

Table 3-12: Percentage biofilm disruption by HEX fraction and the minimum biofilm disruption concentration (MBDC) against *S. aureus* strains

Strains	Biofilm production	Percentage biofilm disruption (%)	MBDC (mg/ml)
S. aureus ATCC 6538P	Strong	42.5 ± 4.7	1.25
S. aureus ATCC 43300	Moderate to strong	60.2 ± 8.3	0.63
S. aureus ATCC 33591	Weak to moderate	68.0 ± 7.5	0.31
S. aureus ATCC 29213	Weak to moderate	75.5 ± 4.0	0.07
S. aureus ATCC 700699	Weak	68.1 ± 2.5	0.07

*Percentage biofilm disruption (%) = average percentage of biofilm disruption by HEX fraction from concentrations that showed significant biofilm disruption activity
*Minimum biofilm disruption concentration (MBDC) in mg/ml

Based on Figure 3-17, HEX fraction showed significant biofilm disruption activity at 1.25 – 5 mg/ml for *S. aureus* ATCC 6538P, at 0.63 – 5 mg/ml for *S. aureus* ATCC 43300, at 0.31 – 5 mg/ml for *S. aureus* ATCC 33591, and at 0.07 – 5 mg/ml for both *S. aureus* ATCC 29213 and *S. aureus* ATCC 700699. The average percentage of biofilm disrupted by HEX fraction from concentrations that showed significant biofilm disruption activity was calculated and it was determined that HEX fraction was able to disrupt about 42-75% of *S. aureus* biofilms (Table 3-12). Moreover, the percentage of biofilm disruption varied between *S. aureus* strains. Biofilms of *S. aureus* ATCC 6538P and *S. aureus* ATCC 43300, which were stronger biofilm producers had less percentage of disruption, with 42% and 60% disruption, respectively. In contrast, the weaker biofilm producers had larger percentage of biofilm disruption: both *S. aureus* ATCC 33591 and *S. aureus* ATCC 700699 (68% disruption), and *S. aureus* ATCC 29213 (75% disruption). Additionally, the MBDC determined also showed a strain variation effect. The two stronger biofilm producers had higher MBDC values, indicating that these pre-formed biofilms were much harder to disrupt, requiring higher concentrations of HEX fraction. The weaker biofilm producers were easier to be disrupted even with a concentration as low as 0.07 mg/ml. It was concluded that the difference in biofilm production among these five strains might have influenced the disruption effect by HEX fraction.

3.4.4.2 Growth curve analysis

Growth curve analysis was performed to identify whether HEX fraction affects the growth of *S. aureus*. Figure 3-18 shows the growth curve of *S. aureus* ATCC 6538P with and without HEX fraction tested at MBDC i.e. 1.25 mg/ml of HEX fraction (Table 3-12). The growth rates obtained were shown in Table 3-13.

Based on Figure 3-18, 1.25 mg/ml of HEX fraction did not negatively affect cell growth of *S. aureus* ATCC 6538P and had showed no significant difference in terms of growth rate (Table 3-13) when compared with the negative control. Therefore, it can be deduced that at this concentration, HEX fraction affects the biofilms specifically and not by inhibiting or killing the cells.

The solvent control used to dissolve 1.25 mg/ml of HEX fraction was 0.125% of Tween 80 in 2.5% acetonitrile. Based on Figure 3-18 and Table 3-13, the solvent control showed no significant difference in growth when compared with the negative control, indicating that the solvent used to dissolve HEX fraction does not affect cell growth and did not influence the biofilm disruption effect observed for the HEX fraction.

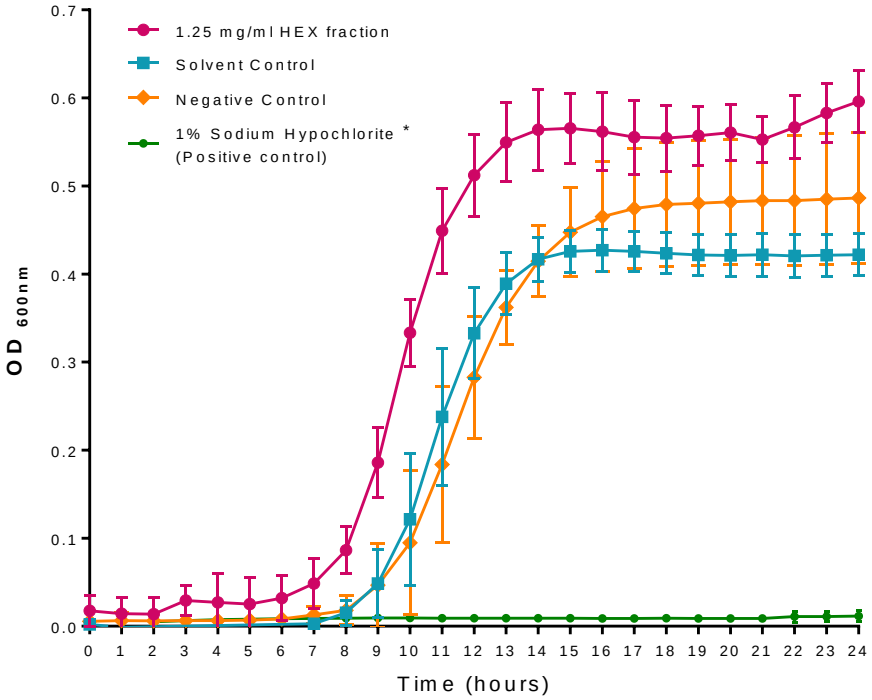


Figure 3-18: The effect of HEX fraction on the growth of *S. aureus* ATCC 6538P. Mean growth at OD_{600nm} ± SD plotted against time. * denotes statistically significant difference in growth and negatively affects cell growth ($p<0.05$). Treatment with 1.25 mg/ml of HEX fraction did not negatively affect cell growth of *S. aureus* ATCC 6538P.

Table 3-13: Growth rates of *S. aureus* ATCC 6538P treated with 1.25 mg/ml HEX fraction

Treatment	Growth rate (hour per generation)
1.25 mg/ml HEX fraction	2.30 ± 0.42
Solvent control	2.56 ± 0.72
Negative control	2.23 ± 1.64
Positive control	0.00 ± 0.00*

*Treatment with significant difference in growth rate when compared with the negative control ($p<0.05$)

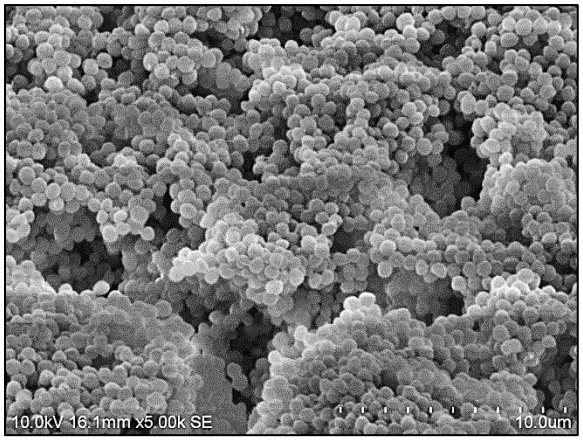
3.4.4.3 Scanning electron microscopy (SEM)

In order to investigate the structural modifications of biofilms after treatment with HEX fraction, scanning electron microscopy was performed. Scanning electron microscopy (SEM) has been widely used to visualise the structure of biofilms. Through SEM, the morphology of bacteria adhered on a material surface, the morphology of the material surface and the relationship between them can be observed. It is a key technique that provides information about the morphology of biofilm, the thickness of biofilm and the presence of EPS. SEM is also one of the many methods available to visualize the effects of anti-biofilm compounds on the biofilm structure and morphology (Kerekes *et al.*, 2013; Khan and Ahmad, 2012; Neto *et al.*, 2014).

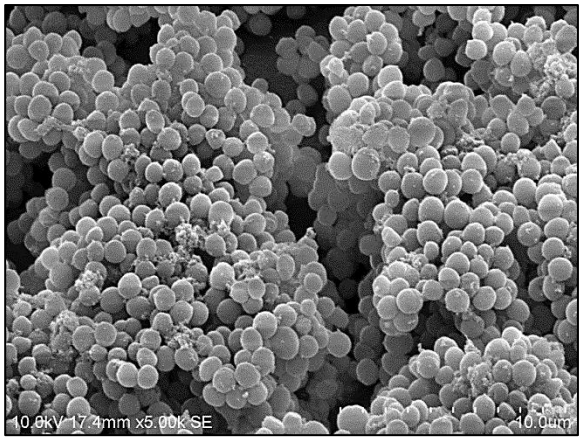
Figure 3-19 shows the scanning electron microscopy images of *S. aureus* ATCC 6538P after treatment with 1.25 mg/ml HEX fraction (MBDC) and the structural modifications of its biofilms after treatment. As stated previously, 1.25 mg/ml of HEX fraction had been shown to not affect cell growth (Figure 3-18, Table 3-13) and thus, can be deduced to only affect the pre-formed biofilm.

In Figure 3-19, based on the image of solvent control, the solvent used to dissolve HEX fraction does not affect cell morphology and biofilm structure of *S. aureus*, as there was no difference in cell morphology and biofilm structure when compared with the negative control. This was further supported by the growth curve assay results, whereby the solvent control showed no significant difference in growth when compared with the negative control (Figure 3-18, Table 3-13).

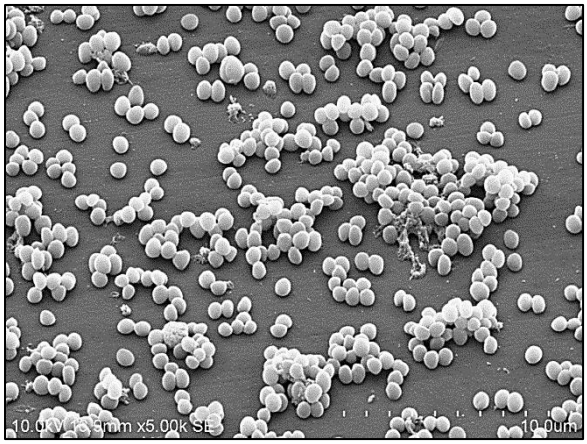
In Figure 3-19, treatment with 1.25 mg/ml HEX fraction demonstrated destruction of the biofilm structure and reduced biofilms attached to the surface. Scant biofilms were observed, with only few bacterial cells. The SEM images proves that the HEX fraction had an effect on pre-formed biofilms and confirms the results obtained with crystal violet assay (Figure 3-17 (A)), which demonstrated reduction in biofilm biomass of *S. aureus* after treatment.



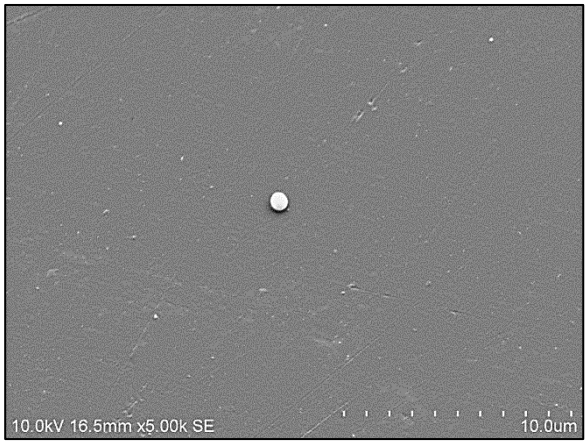
Negative control



Solvent control



HEX fraction



1% Sodium Hypochlorite (Positive control)

Figure 3-19: Scanning electron microscopy images of *S. aureus* ATCC 6538P. Negative control refers to treatment with water (no effect on cell morphology and biofilm structure), solvent control refers to treatment with 0.125% Tween 80 in 2.5% acetonitrile, the solvent used to dissolve HEX fraction. Positive control refers to treatment with 1% sodium hypochlorite. Treatment with HEX fraction was at 1.25 mg/ml.

3.4.4.4 Phytochemical screening

Phytochemical screening was conducted on HEX fraction and Table 3-14 shows the phytochemicals identified.

Table 3-14: Phytochemical screening of HEX fraction

Phytochemicals of interest	Present (√)/Absent (×)
Flavonoids	×
Terpenoids	√
Alkaloids	×
Cardiac glycoside	×
Phenols	×
Sterols	√
Quinones	×

As mentioned previously, phytochemical screening conducted by previous studies on *D. linearis* had identified the presence of tannins, essential oils, saponins, clerodane glycosides, terpenoids and flavonoids (Table 1-3) (de Winter *et al.*, 2003; Jaishee and Chakraborty, 2015; Li *et al.*, 2008; Raja *et al.*, 1995). In the HEX fraction, the presence of terpenoids and sterols were identified (Table 3-14).

Raut *et al.* (2013) had reported on the eradication of mature *Candida albicans* biofilms by terpenoids. Disruption of biofilm by sterols has not been reported. However, biofilm disruption activity by plant extract containing sterols has been reported. The methanol and aqueous branch extracts of *Juniperus* species have been reported to exhibit biofilm disruption activity and phytochemical screening had revealed the presence of sterols in the extracts, among others (Marino *et al.*, 2010).

The biofilm disruption activity by HEX fraction may be attributed to the presence of terpenoids and/or sterols. However, the activity observed could also be attributed to other types of phytochemicals not screened in this study. Therefore, further purification and isolation process would have to be conducted to identify the specific compound(s) responsible for the biofilm disruption activity of HEX fraction.

3.5 Conclusion

In conclusion, screening with MCE(L) exhibited only biofilm inhibition activity. No biofilm disruption activity by MCE(L) was observed. Screening with the SSE fractions exhibited both biofilm inhibition and biofilm disruption activities. Biofilm inhibition activity was observed in MeOH and H_2O fractions, with H_2O fraction being the most effective in inhibiting biofilms. Biofilm disruption activity was observed in HEX, DCM and EA fractions, with HEX fraction being the most effective in disrupting biofilms.

Currently, there are no studies in literature on the anti-biofilm effect of *D. linearis*. With MCE(L), MeOH fraction and H_2O fraction exhibiting biofilm inhibition activity, and HEX, DCM and EA fractions exhibiting biofilm disruption activity, this will be the first study to report on the anti-biofilm activity of *D. linearis*, for both biofilm inhibition and biofilm disruption activities.

Further characterization on H_2O fraction had demonstrated biofilm inhibition effect of H_2O fraction without affecting cell growth at 0.31 mg/ml – 2.5 mg/ml, 0.63 – 2.5 mg/ml and 0.63 mg/ml – 1.25 mg/ml against *S. aureus* ATCC 6538P, *S. aureus* ATCC 33591, and *S. aureus* ATCC 43300, respectively. H_2O fraction was able to inhibit *S. aureus* biofilm formation on various polymer materials commonly used in medical settings: polystyrene (85-93% inhibition), polyvinyl chloride (76-91% inhibition), polyethylene (68-90% inhibition); polypropylene (52-93% inhibition), silicone rubber (68-94% inhibition). Furthermore, H_2O fraction does not modify surface properties of polystyrene to

prevent adhesion but may be involved in changing the cell surface hydrophobicity of *S. aureus* to inhibit biofilm formation on surfaces. The presence of various phytochemicals such as flavonoids terpenoids, tannins, cardiac glycosides, phenols, quinones and saponins were identified in H_2O fraction.

Further characterization on HEX fraction had demonstrated that HEX fraction was able to disrupt about 42-75% of *S. aureus* biofilms. The MBDC of HEX fraction ranges between 0.07-1.25 mg/ml against *S. aureus* biofilms. The biofilm disruption effect of HEX fraction was able to go as low as 0.07 mg/ml against weaker biofilm producers of *S. aureus* and would require higher concentrations against much stronger biofilm producers. Growth curve analysis demonstrated no negative effect on cell growth at 1.25 mg/ml HEX fraction against *S. aureus* ATCC 6538P. Through scanning electron microscopy, the biofilm disruption effect from treatment with HEX fraction demonstrated destruction of the biofilm structure and reduced biofilms attached to the surface. Among the phytochemicals screened, the presence of terpenoids and sterols were identified in HEX fraction.

Chapter 4 Purification & identification of active compound(s)

4.1 Introduction

The disaggregation of the biofilm matrix is one of the possible anti-biofilm approaches (Figure 1-5(3)). The use of substances that are able to destroy the physical integrity of the biofilm matrix is an attractive anti-biofilm approach, as the subsequent loss of the highly protective EPS matrix exposes the sessile microbial cells to treatment by antibiotics (Francolini and Donelli, 2010).

In Chapter 3, preliminary screenings on the extracts of *D. linearis* have shown the presence of anti-biofilm activity, with the (hexane) HEX fraction exhibiting the strongest biofilm disruption activity among the extracts tested. Due to its effectiveness against *S. aureus* biofilms, it is of interest to purify and identify the active compound(s) in HEX fraction responsible for the biofilm disruption effect. Furthermore, with only few phytochemicals being present in HEX fraction, the purification and isolation process for HEX fraction would be easier.

4.1.1 Purification of active compound(s)

4.1.1.1 Bioassay guided fractionation for compound isolation

Bioassay guided fractionation involves the use of various purification techniques to separate mixtures of compounds in extracts or fractions and then followed by the testing of bioactivity of each separated compound (Figure 4-1) (Koehn and Carter, 2005). The objective of this approach is to isolate compounds responsible for the activity based on their biological activity.

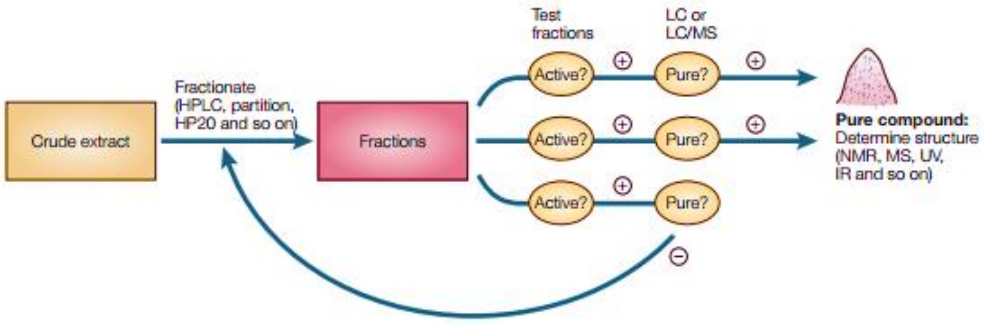


Figure 4-1: Generic scheme for bioassay guided fractionation. Several cycles of fractionation are usually needed to obtain a pure compound (Koehn and Carter, 2005).

4.1.1.2 Purification techniques

Chromatography is one of the most useful techniques for the isolation and purification of compounds from extracts. The methods of separation in chromatography are based on the distribution of the components in a mixture between a fixed (stationary) and a moving (mobile) phase. It is available for both preparative and analytical separation of compounds. The purpose of

preparative chromatography is to separate sufficient quantities of a mixture for further study, rather than analysis. An example of preparative chromatography is column chromatography. Analytical chromatography is performed with smaller quantities of sample and is used to measure the relative proportions of analytes in a mixture. Examples of analytical chromatography include thin-layer chromatography (TLC) and High performance liquid chromatography (HPLC) (Reid and Sarker, 2005; Sarker and Nahar, 2012).

4.1.1.2.1 Column Chromatography

Column chromatography is a technique used for preparative separation of compounds from mixtures on scales from micrograms up to kilograms. It is a solid - liquid technique, whereby the stationary phase (adsorbent) is a solid and the mobile phase (eluent) is a liquid. The principle of column chromatography is based on differential adsorption of substance by the adsorbent. The most common adsorbent for column chromatography is silica gel, followed by alumina. The mobile phase is either a pure solvent or a mixture of solvents (Çitoğlu and Acıkara, 2012; Reid and Sarker, 2005).

In silica gel column chromatography, the mixture to be separated is dissolved in a suitable solvent and introduced at the top of the column. The mobile phase is added to allow the mixture to pass through the column. As the mixture moves down through the column, the components are adsorbed at different regions depending on their ability for adsorption. The components with greater adsorption power will be strongly retained by silica and thus, eluted later, while the weakly adsorbed components will be weakly retained and are eluted more rapidly. The different fractions are collected separately and can be used for further analysis (Reid and Sarker, 2005).

4.1.1.2.2 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) is a technique widely used for analytical separation, enabling qualitative and quantitative determination of compounds in extracts or fractions (Sarker and Nahar, 2012). There are various modes of HPLC: normal phase, reversed-phase, gel permeation chromatography and ion exchange chromatography. The modes are determined by the stationary phase, the column used and the eluting solvents used. Reversed-phase HPLC has mostly contributed to the purification of most classes of natural products. It is usually the first technique used to analyse and attempt to purify compounds from a complex mixture, especially for unknown compounds (Latif and Sarker, 2012).

In reversed-phase HPLC, the stationary phase is more non polar than the eluting solvent. The eluent used usually comprises of a mixture of water and miscible organic solvents. The three most commonly used organic solvents are acetonitrile (ACN), methanol (MeOH) and tetrahydrofuran (THF). In order to assess the number of compounds and identify the compound of interest, analysis

has to be performed on the complex mixture to develop the suitable method and solvent system (elution). Gradient elution can be used to separate the complex mixture over a range of polarities while isocratic elution separates the complex mixture with the solvent mixture being kept constant throughout. Once a suitable solvent system and method has been established, it is then scaled-up to the preparative-HPLC system (Figure 4-2). Preparative-HPLC involves the use of prep columns, larger sample loading and high flow rates in a HPLC system with the aim to purify and collect the compound of interest in larger quantities using the fraction collector (Latif and Sarker, 2012).

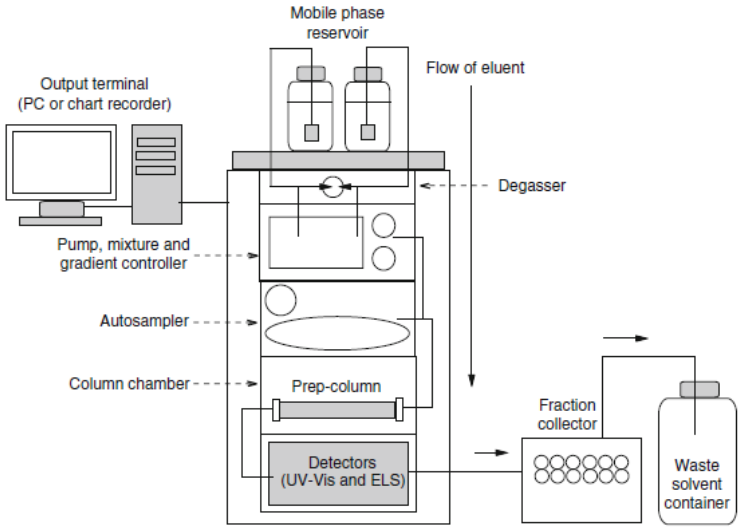


Figure 4-2: A typical preparative-HPLC system (Latif and Sarker, 2012).

4.1.2 Identification of active compound(s)

Structure elucidation and identification of compounds has been successfully accomplished with the aid of data obtained from techniques such as liquid chromatography mass spectrometry (LC–MS) and nuclear magnetic resonance (NMR) spectroscopy.

4.1.2.1 Liquid Chromatograph- Mass Spectrometry (LC–MS)

Liquid chromatography-mass spectrometry (LC–MS) or HPLC–MS, refers to the coupling between an LC and an MS (Figure 4-3). The separated samples that emerge from the HPLC column can be identified on the basis of their mass spectral data. A switching valve helps make a working combination of the two techniques. LC-MS combines the chemical separation power of LC and the ability of a mass spectrometer to selectively detect and confirm molecular identity. It is a highly sensitive and selective method of molecular analysis, providing information on the molecular weight and the fragmentation pattern of the analyte molecule. Data obtained from MS is helpful in confirming the identities of compounds, especially for known compounds. It is also possible to reconstruct an unknown compound based on qualitative analysis of MS data (Sarker and Nahar, 2012).

MS utilizes different ionization methods and may be equipped with different types of analyzers. Two most widely used, especially in relation to natural product analysis, are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Both can generate the ions essential for mass spectrometric analysis for >90% of analytes, ranging from amino acids to proteins and nucleic acids (Koehn and Carter, 2005). Various types of analyzers such as quadrupole, ion trap or time-of-flight can be used, with each offering various degrees of mass accuracy and resolution (Sarker and Nahar, 2012).

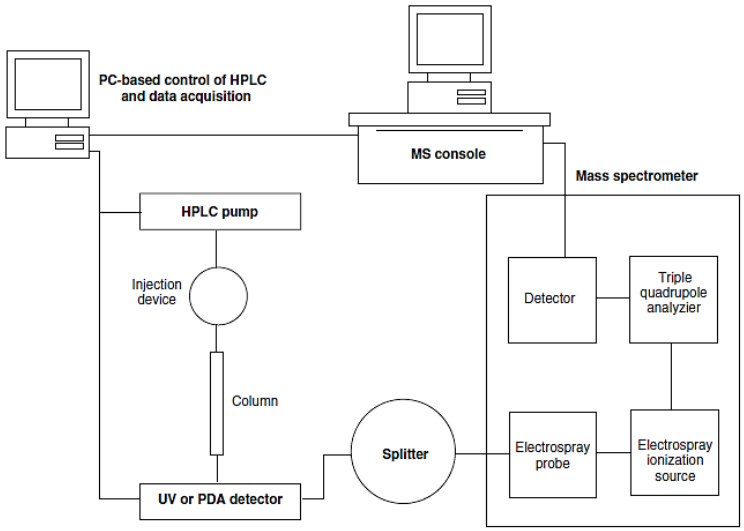


Figure 4-3: An LC-MS system with an electrospray ionization interface (Sarker and Nahar, 2012).

4.1.2.2 Nuclear magnetic resonance (NMR) spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a well-established and most commonly used method to elucidate the molecular structures of natural products. NMR is based on the application of pulse sequences, which involves precisely timed radio-frequency and magnetic-field gradient pulses (on the microsecond and millisecond timescale) that are designed to excite the atomic nuclei of molecules. This will produce diagnostic signals that can be analysed to determine the connectivity of the atomic nuclei of the molecule (Koehn and Carter, 2005). Figure 4-4 shows the schematic operation of a basic NMR spectrometer (Bruice, 2007). NMR consists of one-dimensional (1D) NMR and two-dimensional (2D) NMR experiments.

The number and types of atoms in a molecule can be obtained from 1D NMR experiments, whereby information on hydrogens are obtained using ^{1}H NMR spectroscopy, carbons using ^{13}C NMR spectroscopy, fluorine using ^{19}F NMR spectroscopy, nitrogen using ^{15}N NMR, and phosphorus using ^{31}P NMR spectroscopy. From the 1D NMR spectra, three types of spectral parameters are obtainable, which are the chemical shifts, spin-spin couplings, and intensities (Clayden *et al.*, 2001).

The 2D NMR spectra provide more information about the molecule as compared to 1D NMR spectra. It is particularly useful for structural determination of molecules that are too complicated

to work with using 1D NMR. The 2D NMR allows researchers to better resolve signals that would normally overlap in 1D NMR experiments, which may obscure structure interpretation. Common 2D NMR experiments include COSY (Correlated Spectroscopy), which provides connectivity information based on proton-proton interactions through covalent bonds, HSQC (Heteronuclear Single Quantum Coherence) spectroscopy, which provides connectivity information relating specific carbon atoms and the protons bound to those carbons, and HMBC (Heteronuclear Multiple Bond Correlation) spectroscopy, which provides long-range connectivity between protons and carbon atoms separated by 2-4 covalent bonds (Clayden *et al.*, 2001; Koehn and Carter, 2005).

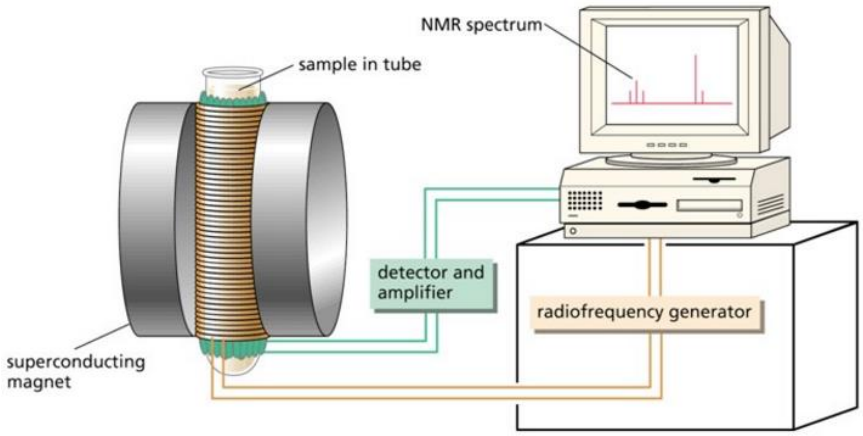


Figure 4-4: Schematic operation of a basic NMR spectrometer (Bruice, 2007).

4.2 Objective

The objectives of this chapter were:

I. To purify the active compound(s) from HEX fraction
II. To identify the purified active compound(s)

4.3 Methodology

4.3.1 Purification of active compound(s)

Bioassay-guided fractionation method was employed in the purification of the active compound(s) from HEX fraction. Fraction(s) obtained from every step of the fractionation process were evaluated for its biofilm disruption activity using the method described in Section 3.3.7.1 against *S. aureus* ATCC 6538P at 1 mg/ml (Dusane *et al.*, 2008; Shakeri *et al.*, 2007). Figure 4-5 briefly summarizes the purification methods employed.

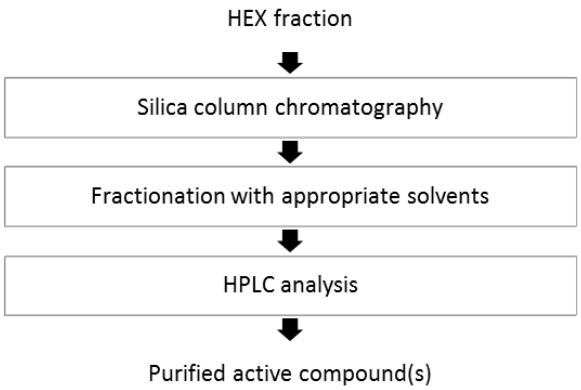


Figure 4-5: Flow chart for the purification of active compound(s) from HEX fraction. Biofilm disruption activity was evaluated for each fraction obtained after every step of the fractionation process.

4.3.1.1 Silica column chromatography

A slurry containing 100 g of silica gel 60 (0.040 – 0.063 mm) was prepared into the column. HEX fraction was dissolved in hexane at 150 mg/ml and was added to the top of the column. The mobile phase was 70% hexane: 30% ethyl acetate. This mixture of solvent was continuously added at the top to allow the sub-fractions to elute down the column and collected at the bottom. The mobile phase was finally changed to 100% methanol to elute the final fraction. Six sub-fractions were collected based on separation by colour: F1 (orange), F2 (dark green), F3 (light green), F4 (light yellow), F5 (yellow), F6 (light yellow). Figure 4-6 summarizes the procedure for silica column chromatography.

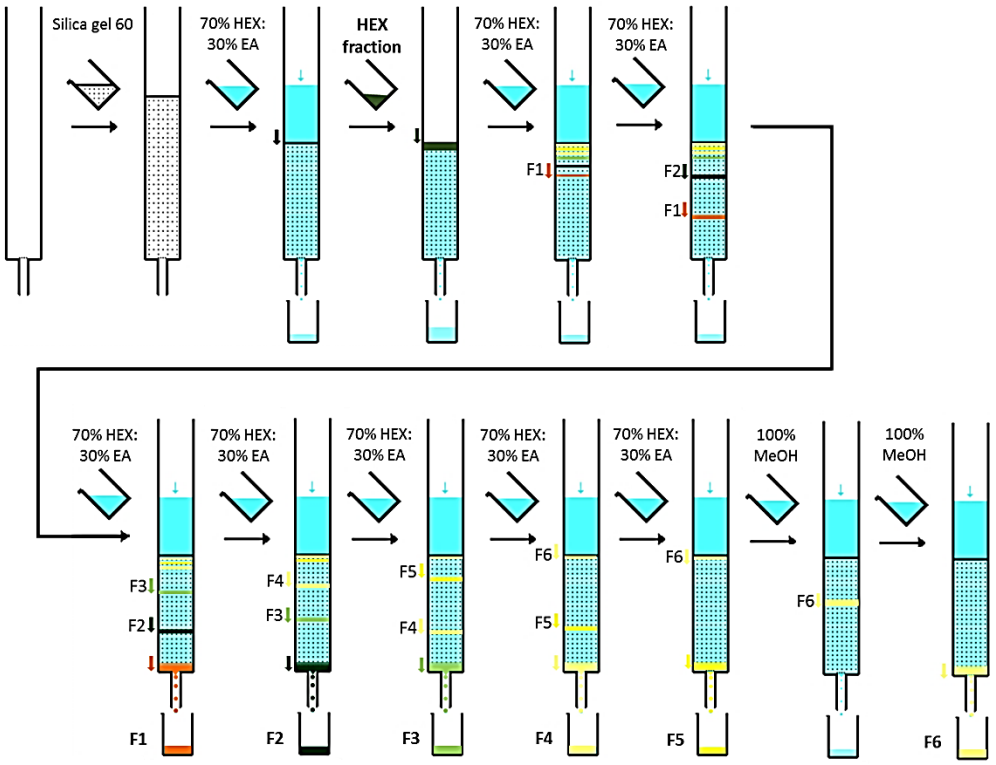


Figure 4-6: Procedure for the separation of HEX fraction using silica column chromatography. Six sub-fractions were collected based on separation by colour: F1 (orange), F2 (dark green), F3 (light green), F4 (light yellow), F5 (yellow), F6 (light yellow).

4.3.1.2 Fractionation with acetonitrile

F1 was fractionated three times with acetonitrile, assisted with sonication. The acetonitrile soluble fraction obtained from each fractionation was combined and subsequently evaporated using the rotary evaporator. The acetonitrile soluble fractions were designated as F1_ACN.

4.3.1.3 High performance liquid chromatography (HPLC)

The chemical profile of F1_ACN was evaluated by high performance liquid chromatography (HPLC) analysis. F1_ACN was dissolved in acetonitrile and chromatographic separation was attained on a COSMOSIL Guard column 5C-18-MS-II (10ID $\times$ 20 mm). The mobile phase consisted of 5% water (solvent A) and 95% acetonitrile (solvent B) at isocratic conditions (mobile phase composition remains constant) for 10 minutes. The flow rate was at 20 ml/min. Detection was performed at 210 nm and 450 nm. From the HPLC chromatogram, two peaks were identified and collected via preparative HPLC: Fraction A and Fraction B.

4.3.2 Identification of purified active compound(s)

Elucidation of the structure and identity of the purified active compound(s) was carried out with the help of nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography-mass spectrometry (LC-MS) data.

4.3.2.1 Nuclear Magnetic Resonance (NMR) spectroscopy

One dimensional (1D) NMR and two dimensional (2D) NMR experiments of the compound(s) dissolved in chloroform-d were performed on an Ascend^{TM} 700 NMR spectrometer with ^{1}H NMR (700 Mhz) and ^{13}C NMR (176 MHz). For 1D NMR experiments, ^{1}H NMR and ^{13}C NMR, including ^{13}C DEPT (Distortionless Enhancement by Polarisation Transfer) were performed. The 2D NMR experiments such as ^{1}H-^{1}H Correlated Spectroscopy (COSY), Heteronuclear Single Quantum Correlation (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC) experiments were included to facilitate the acquisition of all the structural information about the compound(s).

4.3.2.2 Liquid Chromatography-Mass Spectrometry (LC-MS)

The LC chromatogram was obtained using AcquityTM Waters Ultra Performance Liquid Chromatography (UPLC) with the ACQUITY UPLC BEH C18 (1.7 µm, 2.1 × 50 mm) column. The solvent system consisted of 5% of water + 0.1% formic acid (solvent A) and 95% of ACN + 0.1% formic acid (solvent B) at isocratic conditions for 15 minutes. ESI-MS(+) was obtained using a Synapt High Definition Mass Spectrophotometer quadrupole-orthogonal acceleration, time-of-flight detector.

4.3.3 Data analysis

Statistical analysis was conducted using the One-way Analysis of Variance (ANOVA) test for comparing mean scores of more than two groups, with significance at $p<0.05$. The IBM SPSS Statistics 20 software was used (Kerekes *et al.*, 2013). All graphs were generated using the GraphPad Prism 6 software.

4.4 Results and Discussion

4.4.1 Purification of active compound(s)

In order to purify the active compound(s), several purification methods were used. Figure 4-7 summarizes the overall purification methods employed and the fractions obtained from every step of the purification process. The evaluations for the biofilm disruption activity of these fractions are further discussed in the following sections. These fractions were tested against *S. aureus* ATCC 6538P at 1 mg/ml.

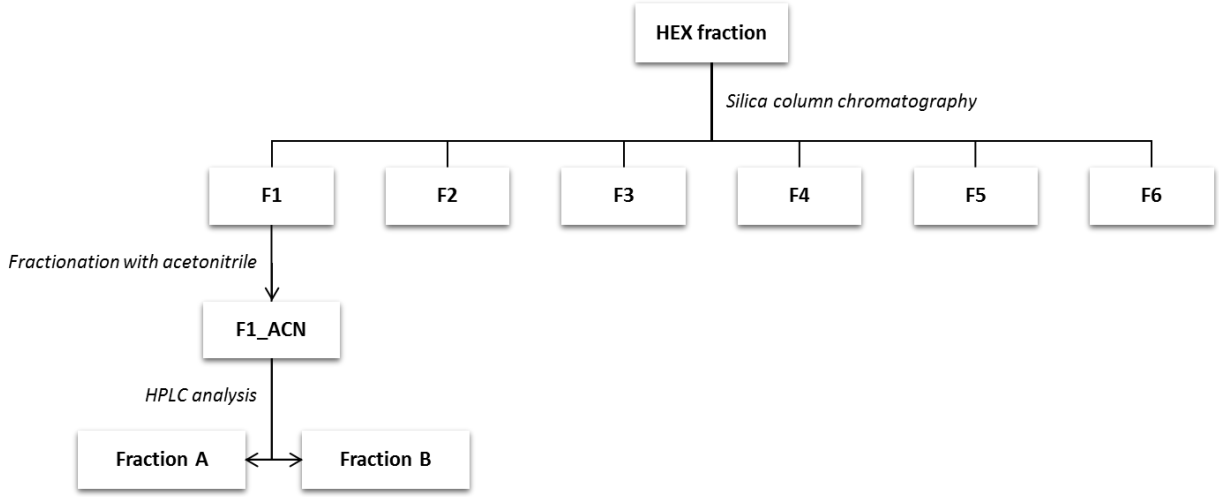


Figure 4-7: Summary of the purification methods employed and the fractions obtained from every step of the purification process.

4.4.1.1 Silica column chromatography

For the isolation of non-polar compounds, column chromatography is a convenient method. Silica gel is the most extensively used adsorbent for non-polar and medium polar compounds (Çitoğlu and Acıkara, 2012). Therefore, silica column chromatography was first used to fractionate the HEX fraction. Six sub-fractions were collected based on separation by colour and were tested for biofilm disruption activity as shown in Figure 4-8.

Based on Figure 4-8, it can be observed that the sub-fractions F1, F2 and F3 showed biofilm disruption activity, as shown by the significant decrease in pre-formed biofilm (biofilm biomass) when compared with the solvent control. Furthermore, both F1 and F2 showed comparable activity to HEX fraction. Since the active compound(s) were non-polar compounds, it was expected for them to be eluted early and to be mostly fractionated within these two fractions as non-polar compounds are weakly retained by silica gel and would be eluted early (Sarker *et al.*, 2005). Due to time constraints, only F1 was selected for the next step of purification process.

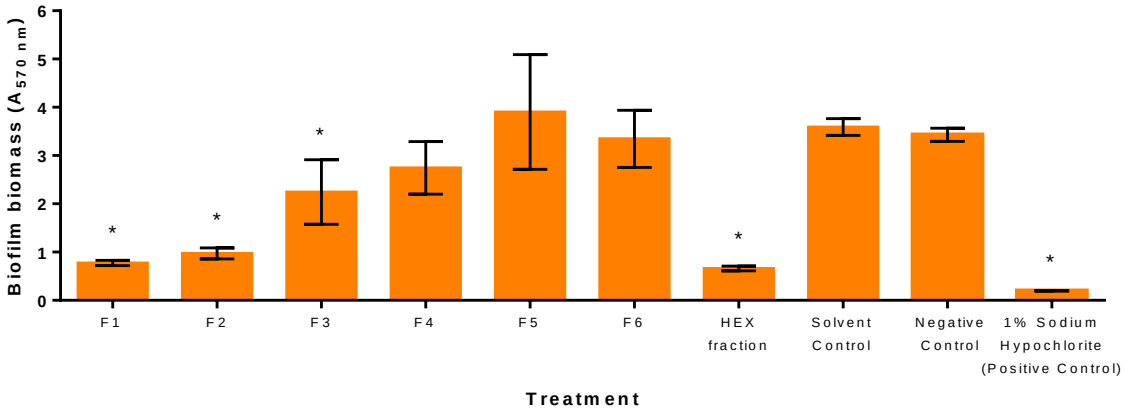


Figure 4-8: Biofilm disruption activity of the six sub-fractions obtained through silica column chromatography against *S. aureus* ATCC 6538P at 1 mg/ml. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against various treatments. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm disruption activity when compared with the solvent control (0.125% of Tween 80 in 2.5% acetonitrile).

4.4.1.2 Fractionation with acetonitrile

F1 was further fractionated with acetonitrile and the fraction obtained, F1_ACN was tested for its biofilm disruption activity as shown in Figure 4-9.

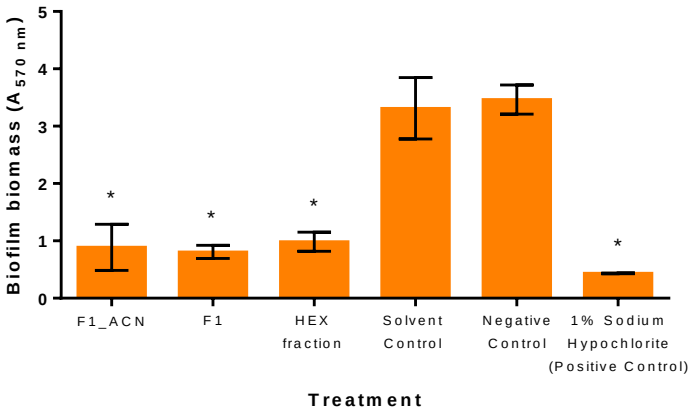


Figure 4-9: Biofilm disruption activity of F1_ACN against *S. aureus* ATCC 6538P at 1 mg/ml. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against various treatments. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm disruption activity when compared with the solvent control (0.125% of Tween 80 in 2.5% acetonitrile).

Fractionation with acetonitrile (polar solvent) will enable further analysis using HPLC, which employs a polar solvent system. Based on Figure 4-9, it can be observed that F1_ACN showed biofilm disruption activity which was comparable to F1 and HEX fraction. Therefore, F1_ACN was subjected to the next step of purification, which was HPLC analysis.

4.4.1.3 High performance liquid chromatography (HPLC) analysis

F1_ACN was analysed using HPLC and the HPLC chromatogram obtained are as shown in Figure 4-10. Two pure chromatographic peaks were observed (labelled in Figure 4-10) and collected via preparative HPLC column. The two fractions were tested for biofilm disruption activity as shown in Figure 4-11.

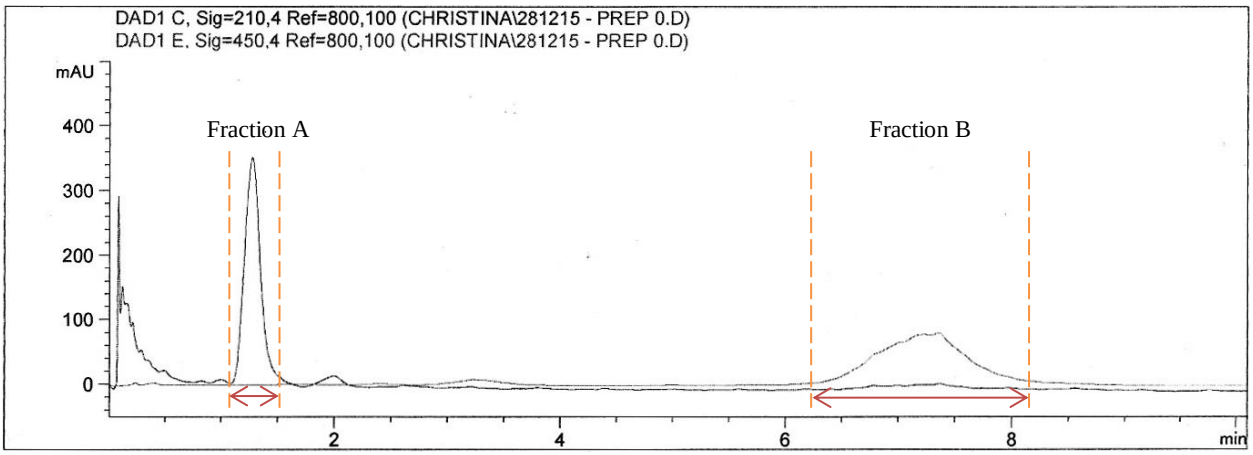


Figure 4-10: HPLC chromatogram of F1_ACN. Two pure chromatographic peaks were collected (↔) via preparative HPLC column and designated as Fraction A and Fraction B.

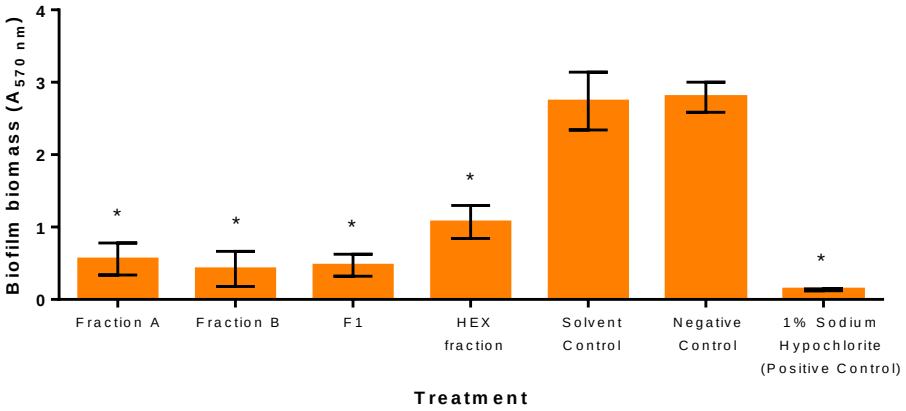


Figure 4-11: Biofilm disruption activity of the pure fractions against *S. aureus* ATCC 6538P at 1 mg/ml. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against various treatments. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm disruption activity when compared with the solvent control (0.125% of Tween 80 in 2.5% acetonitrile).

Based on Figure 4-11, it can be observed that both Fractions A and B showed biofilm disruption activities. Their disruption effect was comparable to F1 and better than HEX fraction. Although both showed activity, due to the low yield of Fraction B, only Fraction A was selected for further structure elucidation and identification analyses.

4.4.2 Identification of purified active compound(s)

Elucidation of the structure and identity of the compound from Fraction A (referred as Compound A onwards) was conducted based on nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography-mass spectrometry (LC-MS) data.

4.4.2.1 Nuclear Magnetic Resonance (NMR) spectroscopy

Compound A was identified as α-tocopherol, by comparison to the ^{1}H and ^{13}C NMR data reported in literature (Baker and Myers, 1991; Matsuo and Urano, 1976). The ^{1}H and ^{13}C NMR spectra of Compound A are presented in Figure 4-13 and Figure 4-14, respectively. The structure of Compound A (α-tocopherol) is presented in Figure 4-15. The ^{1}H and ^{13}C NMR chemical shift assignment of Compound A and its comparison to reported chemical shift assignment for α-tocopherol are presented in Table 4-1 and Table 4-2, respectively. It can be observed that the values matched the ^{1}H and ^{13}C NMR data reported by Baker and Myers (1991) and ^{13}C NMR data reported by Matsuo and Urano (1976).

4.4.2.2 Liquid Chromatography-Mass Spectrometry (LC-MS)

Following NMR analyses, the identity of Compound A was further confirmed using mass spectrometry data. The mass spectrum of Compound A is presented in Figure 4-12.

The molecular formula of Compound A was deduced to be $C_{29}H_{50}O_2$ based on the mass spectrum data and the combination of ^{1}H and ^{13}C NMR data. It has been reported in literature that the principle ions of α-tocopherol were of m/z 165, 205, 429, 430 and 431 (De Leenheer *et al.*, 1978; Scheppele *et al.*, 1972). The peaks corresponding to these ions were observed in the mass spectrum of Compound A (Figure 4-12), further supporting its identity as α-tocopherol.

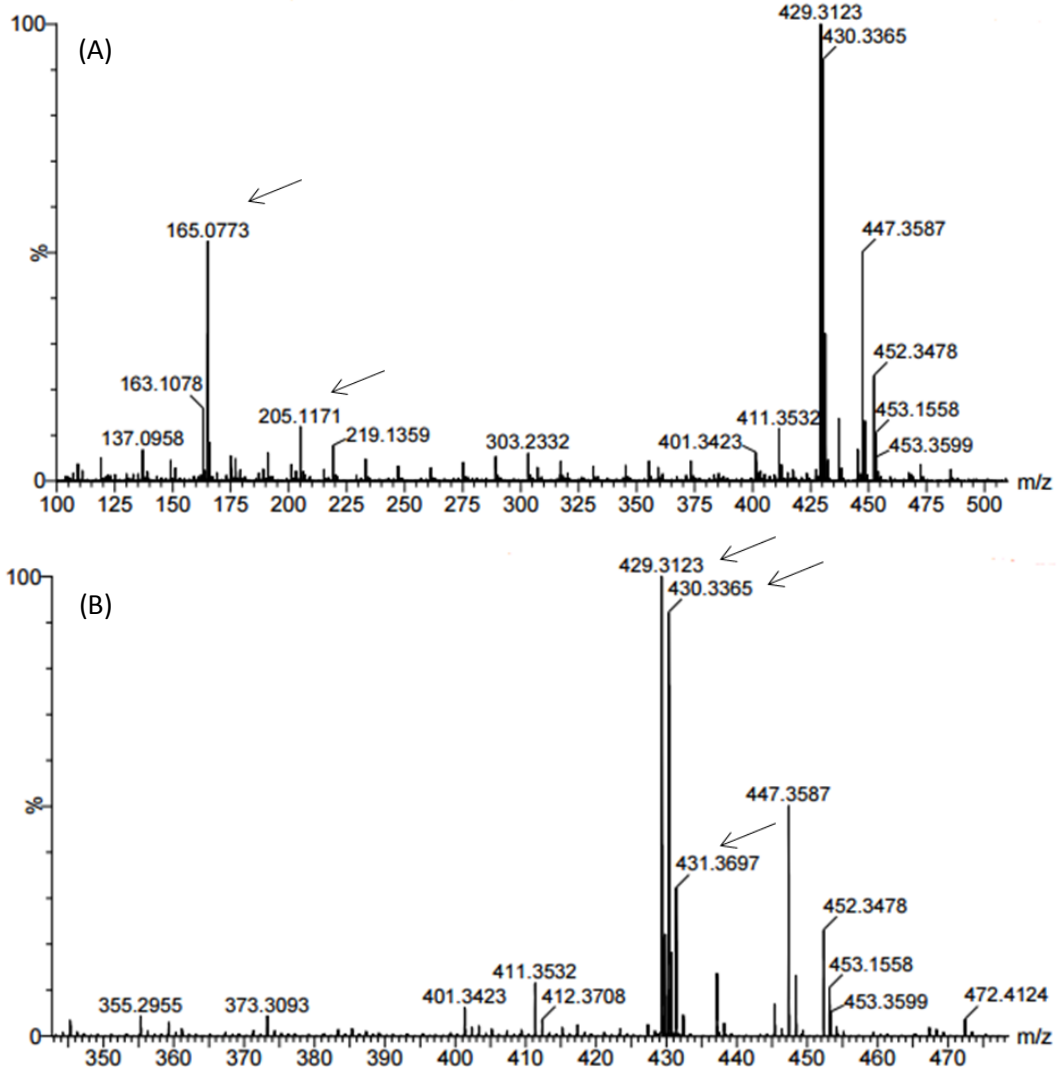


Figure 4-12: (A) ESI-MS(+) spectrum of Compound A. (B) Zoomed in image of ESI-MS(+) spectrum of Compound A. The arrows indicate the peaks corresponding to m/z 165, 205, 429, 430 and 431, which are the principle ions of α-tocopherol.

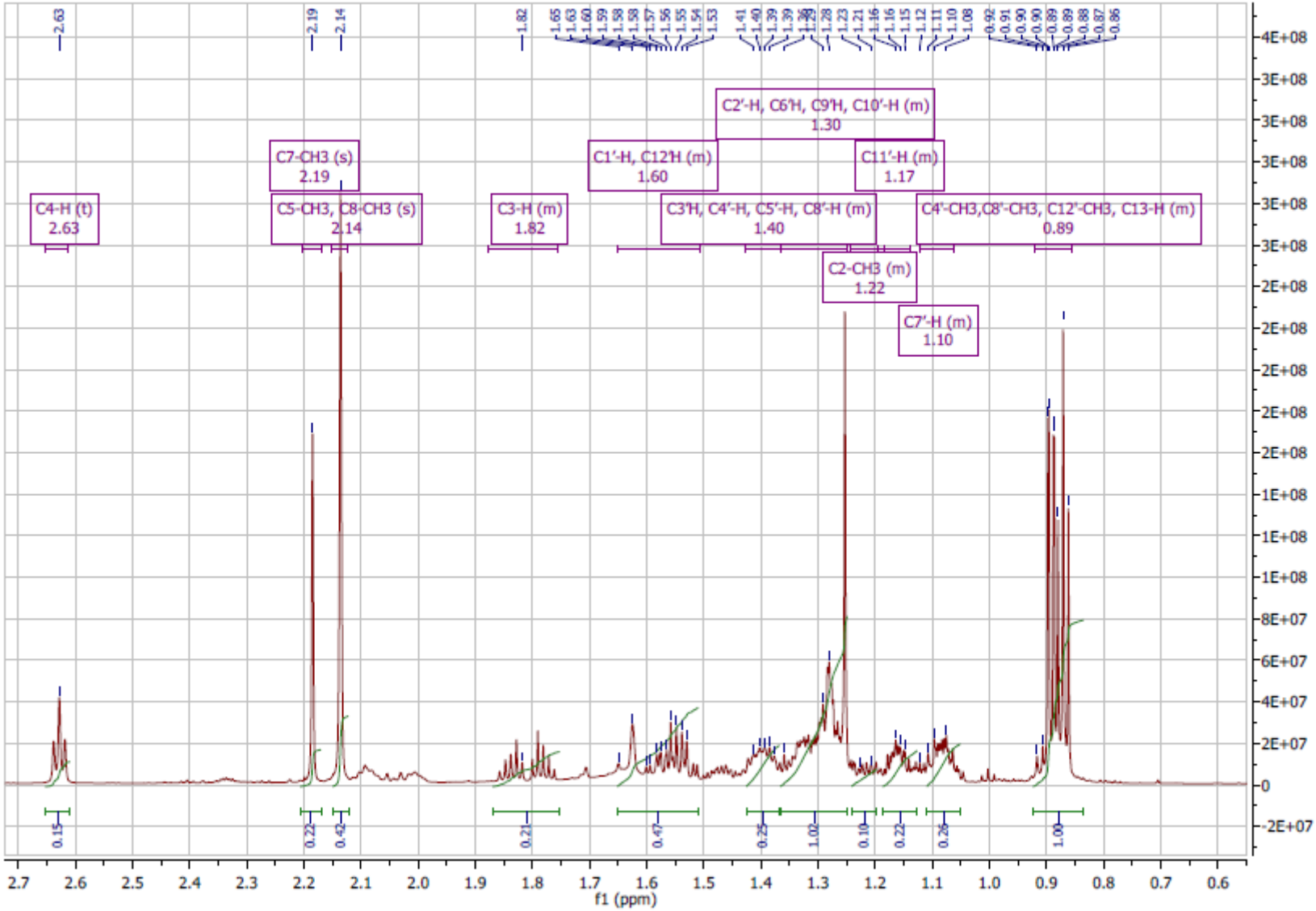


Figure 4-13: ^{1}H NMR spectrum of Compound A in chloroform-d (7.29 ppm-peak not shown in spectrum).

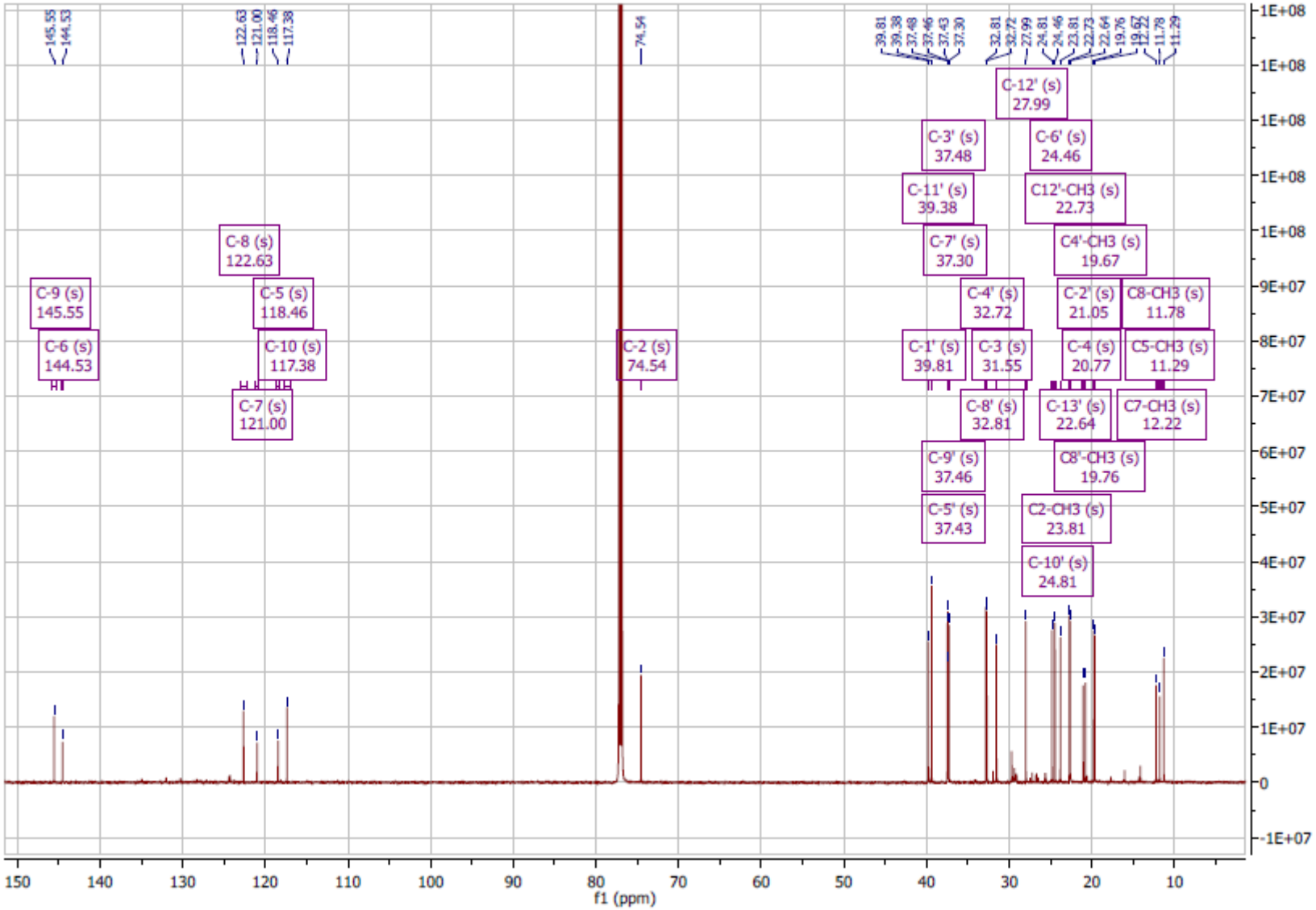


Figure 4-14: ^{13}C NMR spectrum of Compound A in chloroform-d (77 ppm).

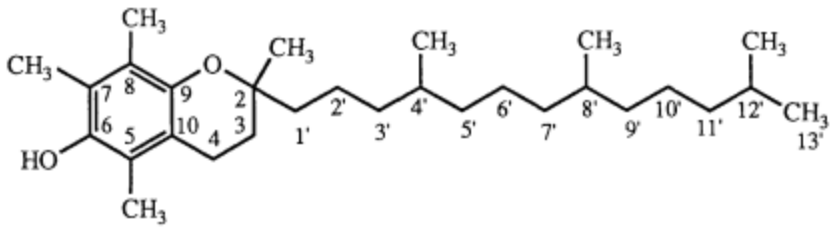


Figure 4-15: Structure of Compound A (α-tocopherol) with numbering system.

Table 4-1: ^{1}H NMR chemical shift assignments for Compound A and α-tocopherol (Baker and Myers, 1991)

Position	Compound A	α-Tocopherol
	Thesis data	Baker and Myers (1991)
C3-H	~1.82 ,m	~1.8
C4-H	2.63 ,t	2.6
C1'-H	~1.60 ,m	~1.5
C2'-H	~1.31 ,m	~1.3
C3'-H	~1.40 ,m	~1.4
C4'-H	~1.40 ,m	~1.4
C5'-H	~1.40 ,m	~1.4
C6'H	~1.31 ,m	~1.3
C7'-H	~1.10 ,m	~1.1
C8'-H	~1.40 ,m	~1.4
C9'H	~1.31 ,m	~1.4
C10'-H	~1.31 ,m	~1.3
C11'-H	~1.17 ,m	~1.2
C12'H	~1.60 ,m	~1.5
C2-CH_3	1.21 ,m	1.22
C5-CH_3	2.14 ,s	2.11
C7-CH_3	2.19 ,s	2.15
C8-CH_3	2.14 ,s	2.11
C4'-CH_3	~0.89 ,m	0.84
C8'-CH_3	~0.89 ,m	0.83
C12'-CH_3	~0.89 ,m	0.88
C13'-H	~0.89 ,m	0.85

Table 4-2: ^{13}C NMR chemical shift assignments for Compound A and α-tocopherol (Baker and Myers, 1991; Matsuo and Urano, 1976).

Position	Compound A	α-Tocopherol	
	Thesis data	Baker and Myers (1991)	Matsuo and Urano (1976)
C-2	74.54	74.5	74.3
C-3	31.55	31.5	31.6
C-4	20.77	20.8	20.8
C-5	118.46	118.5	188.5
C-6	144.53	144.5	144.4
C-7	121.00	121.0	121.0
C-8	122.63	122.6	122.3
C-9	145.55	145.6	145.4
C-10	117.38	117.3	117.0
C-1'	39.81	39.8	39.8
C-2'	21.05	21.1	21.0
C-3'	37.48	37.6	37.5
C-4'	32.72	32.7	32.7
C-5'	37.43	37.4	37.5
C-6'	24.46	24.5	24.5
C-7'	37.30	37.3	37.5
C-8'	32.81	32.8	32.7
C-9'	37.46	37.5	37.5
C-10'	24.81	24.8	24.8
C-11'	39.38	39.4	39.4
C-12'	27.99	28.0	28.0
C-13'	22.64	22.6	22.6
C2-CH_3	23.81	23.8	23.8
C5-CH_3	11.29	11.3	11.2
C7-CH_3	12.22	12.2	12.1
C8-CH_3	11.78	11.8	11.8
C4'-CH_3	19.67	19.7	19.7
C8'-CH_3	19.76	19.7	19.7
C12'-CH_3	22.73	22.7	22.6

4.4.2.3 α-Tocopherol

α-Tocopherol is (2R)-2,5,7,8-Tetramethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-3,4-dihydro chromen-6-ol (IUPAC name). α-Tocopherol is a type of tocopherol or vitamin E. It has a molecular formula of $C_{29}H_{50}O_2$ and molecular weight of 430.71 g/mol. It is found in many plants, especially in plant oils, germinating wheat grains, nuts and leafy green vegetables (Munné-Bosch and Alegre, 2002).

Natural vitamin E comprises of eight different forms, the α-, β-, γ-, and δ-tocopherols and the α-, β-, γ-, and δ-tocotrienols. Among these, α-tocopherol is the most biologically active form of vitamin E

and is the most important for human health. This is because the α-form is the only tocopherol species that can be absorbed and transported in the human body (Munné-Bosch and Alegre, 2002; Zingg, 2007).

4.4.2.3.1 The functions of α-tocopherol

α-Tocopherol is well known for its antioxidant effect. It is known to function as a lipid-soluble antioxidant, which involves the scavenging of peroxyl radicals that propagate chain reactions in the non-enzymatic lipid peroxidation. α-Tocopherol functions as a chain-breaking antioxidant in the reaction of its hydroxyl group with a peroxyl radical ($ROO^{\cdot}$), either by hydrogen transfer or by sequential electron, and then proton transfer to form a lipid hydroxyperoxide and the tocopheroxyl radical (Preedy and Watson, 2007; Munné-Bosch and Alegre, 2002; van Haaften *et al.*, 2003).

Besides that, α-tocopherol also regulates key cellular events by mechanisms that are not related to its antioxidant functions, referred as the non-antioxidant functions of α-tocopherol. It is known that α-tocopherol causes the inhibition of vascular smooth muscle cell proliferation by the inhibition of protein kinase C (PKC). Inhibition of PKC is due to dephosphorylation by protein phosphatase 2A, which is activated by α-tocopherol (Azzi *et al.*, 2002). PKC inhibition by α-tocopherol also diminishes the age-dependent increase of collagenase expression in human skin fibroblasts (Ricciarelli *et al.*, 1999). α-Tocopherol also regulates the expression of several genes such as CD36, scavenger receptor class A, intercellular adhesion molecule-1 (ICAM-1) and some integrins (Azzi *et al*., 2002). Further functions of α-tocopherol are listed in Table 4-3.

Table 4-3: Summary of function of α-tocopherol (modified from Preedy and Watson, 2007)

Function	Reference
Antioxidant function	Munné-Bosch and Alegre (2002);
• Scavenging of peroxyl radicals	van Haaften *et al.* (2003)
Non-antioxidant function	
• Inhibition of vascular smooth muscle cell proliferation	Azzi *et al*. (2002)
• Diminution of age-dependent increase of collagenase in skin fibroblast	Ricciarelli *et al.* (1999)
• Regulation of gene expression such as CD36, scavenger receptor class A, etc.	Azzi *et al*. (2002)
• Reduction of age associated increase in PGE_2 production and development of atherosclerosis	Wu *et al.* (2001)
• Inhibition of blood clotting cascade	Dowd and Zheng (1995)
• Diminution of testosterone plasma levels	Barella *et al.* (2004)

4.4.2.3.2 Research on the anti-biofilm effect of α-tocopherol

As for the anti-biofilm effect of α-tocopherol, only a few studies related to the testing of the biofilm inhibition effect of α-tocopherol have been reported. Jagani *et al.* (2009) had tested the effects of α-tocopherol against *Pseudomonas aeruginosa* biofilm and had reported on its lack of biofilm inhibition activity. Banche *et al.* (2011), Gomez-Barrena *et al.* (2011), Molina-Manso *et al.* (2010) and Williams *et al.* (2015) had incorporated vitamin E to polyethylene to decrease oxidation that may cause material degradation. These studies hypothesized that bacteria may have increased affinity to adhere to oxidized polyethylene surfaces and form biofilms, and thus, the addition of vitamin E may reduce oxidation and result in a reduction of biofilm formation on the surface. However, it was reported that bacteria adhesion was only reduced by 10% or less (Banche *et al.,* 2011; Gomez-Barrena *et al.,* 2011; Molina-Manso *et al*., 2010) while Williams *et al.* (2015) reported that the incorporation of vitamin E did not reduce biofilm formation by methicillin resistant *S. aureus* (MRSA). To conclude, it can be deduced from these studies that α-tocopherol exhibit poor biofilm inhibition activity.

In terms of the biofilm disruption effect of α-tocopherol, there has not been any study reported with regards to its testing or activity. In this study, α-tocopherol was identified as the active compound in HEX fraction that is responsible for the biofilm disruption activity observed against *S. aureus*. The biofilm disruption effect against *S. aureus* by α-tocopherol is a new finding and therefore, this study is the first to report on the biofilm disruption activity of α-tocopherol. Further discussions on the biofilm disruption activity of α-tocopherol are presented in Chapter 5.

4.5 Conclusion

The purification process of HEX fraction using silica column chromatography, acetonitrile fractionation and HPLC analysis had yielded Fraction A, with good biofilm disruption activity against *S. aureus*. Based on NMR spectroscopy and LC-MS data, the compound from Fraction A was identified as α-tocopherol. Therefore, α-tocopherol was the active compound present in HEX fraction that was responsible for the biofilm disruption activity observed against *S. aureus*.

Chapter 5 α-Tocopherol as a biofilm disrupting agent

5.1 Introduction

α-tocopherol or vitamin E, is well known for its antioxidant effect. It functions as a lipid-soluble antioxidant, which involves the scavenging of peroxyl radicals that propagate chain reactions in the non-enzymatic lipid peroxidation (Preedy and Watson, 2007). Besides that, α-tocopherol also regulates key cellular events by mechanisms that are not related to its antioxidant functions, referred to as the non-antioxidant functions of α-tocopherol. Some examples of its non-antioxidant functions are as follows: the inhibition of vascular smooth muscle cell proliferation, diminution of age-dependent increase of collagenase in skin fibroblast and the regulation of gene expression such as CD36 and scavenger receptor class A (Table 4-3) (Azzi *et al*., 2002; Ricciarelli *et al.,* 1999). The full description on the functions of α-tocopherol was discussed previously in Section 4.4.2.3.1.

As previously discussed in Chapter 4, α-tocopherol was isolated and identified as the active compound present in HEX fraction of *D. linearis* that was responsible for the biofilm disruption activity observed against *S. aureus* biofilms. Thus far, there has been no study reporting the biofilm disruption activity of α-tocopherol, especially against *S. aureus* biofilms. In fact, only a few studies on the testing of the biofilm inhibition effect of α-tocopherol have been reported, with these studies reporting poor biofilm inhibition activity (Banche *et al.,* 2011; Gomez-Barrena *et al.,* 2011; Molina-Manso *et al*., 2010 and Williams *et al.*, 2015).

Since there are no findings yet on the biofilm disruption activity of α-tocopherol, it is of interest to investigate the biofilm disruption effect of α-tocopherol against *S. aureus* biofilms. Furthermore, characterization of α-tocopherol and its potential application for anti-biofilm therapies are also explored.

5.1.1 Monomicrobial versus polymicrobial biofilms

Microbial biofilms formed by a single species are referred as monomicrobial biofilms while biofilms formed by multiple species are referred as polymicrobial biofilms. Previously, most diseases were characterized as being monomicrobial in nature, probably due to the extensive use of culture dependent isolation techniques. However, with the recent use of culture-independent community analysis techniques, several diseases have been recognized to be polymicrobial in nature. Polymicrobial

growth can be seen in almost everywhere in the human body, particularly in mucosal surfaces, where different species of microorganisms such as bacteria, fungi, and viruses coexist as communities. Thus, polymicrobial biofilms are defined as a variety of organisms (bacteria, fungi and viruses) coexisting as communities on a surface and embedded in an exopolysaccharide matrix. Examples of human polymicrobial infections includes diseases of the oral cavity, otitis media, diabetic foot wound infections, and chronic infection in the cystic fibrosis lung (Manavathu and Vazquez, 2015; Nair *et al.,* 2014; Peters *et al.*, 2012).

S. aureus is one of the most common pathogens found in polymicrobial infections. In polymicrobial infections, *S. aureus* is able to interact with microorganisms such as *Candida albicans*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae* and *Enterococcus faecalis*. *S. aureus* in polymicrobial infections behaves differently with respect to its monomicrobial growth and displays enhanced persistence and antibiotic tolerance. Thus, *S. aureus* in polymicrobial infections represents a greater clinical challenge compared to that of *S. aureus* in monomicrobial infections (Nair *et al.,* 2014; Peters *et al.*, 2012). Therefore, therapeutic options for the treatment of biofilm infections would need to be effective not only against monomicrobial biofilms, but especially against polymicrobial biofilms.

5.1.2 Combination therapy with antibiotics

Combination therapies represent a therapeutic option in the treatment of infections, as a result of the increasing appearance of multi-resistant microorganisms. Combination treatments are used to avoid the appearance of antibacterial resistance and to enhance the effect of individual agents through synergistic interactions (Monzón *et al.*, 2001).

The biofilm matrix degrading enzyme, dispersin B, is a glycoside hydrolase produced by *Actinobacillus actinomycetemcomitans*. Dispersin B can dissolve mature biofilms of many strains of *S. aureus* and *Staphylococcus epidermidis* by degrading polysaccharide intercellular adhesin (PIA), which is composed of poly-β-1,6-linked-N-acetylglucosamine (PNAG). Dispersin B acts by hydrolysing the glycosidic linkages of PNAG (Boles, 2011; Donelli *et al.,* 2007; Kaplan, 2010). Studies on dispersin B had reported on its broad spectrum activity and dispersin B had showed synergistic action when combined with the antibiotic molecule cefamandole nafate (CEF). It was reported that while exerting its anti-slime effect, dispersin B had also improved the diffusion of CEF into bacterial clusters and had promoted the reaching of antibiotic cell targets (Donelli *et al.,* 2007; Schillaci, 2011). However, since not all clinically relevant staphylococcal biofilm infections produce significant amounts of PIA, a significant drawback to

the therapeutic use of dispersin B is that it will only have excellent activity against PIA producing biofilms strains (Boles, 2011; Kaplan, 2010).

Another biofilm matrix degrading enzyme, lysostaphin, is a glycine endopeptidase produced by *Staphylococcus stimulans*. Treatment with lysostaphin disrupts established biofilms of *S. aureus* and *S. epidermidis* by degrading the pentaglycine bridge in the staphylococcal cell wall (Boles, 2011; Wu *et al.,* 2003). Studies on combination treatment of lysostaphin with antibiotics had reported lysostaphin to be additive to vancomycin and was favourable in eradicating methicillin resistant *S. aureus* (MRSA) biofilms. In addition, the combined use of lysostaphin with oxacillin had increased the susceptibility of biofilm cells to oxacillin (Walencka *et al.*, 2006) while lysostaphin combined with doxycycline or levofloxacin had showed high synergistic effect against MRSA and methicillin sensitive *S. aureus* (MSSA), respectively (Aguinaga *et al.*, 2011).

Despite showing effective biofilm control, there are several limitations in the usage of these biofilm matrix degrading enzymes. The specific mechanism of action of different enzymes and the difficulties in identifying effective enzymes against all different types of biofilms complicates their practical application to control biofilms. Moreover, the high cost and low commercial accessibility of different enzymes also limits their usage in biofilm control strategies (Sadekuzzaman *et al.*, 2015).

Nevertheless, the use of biofilm disrupting agents in combination with currently used antibiotics is a promising and highly effective method for the eradication of biofilms.

5.2 Objectives

The objectives of this chapter were:

I. To assess the anti-biofilm activity of α-tocopherol against four strains of *S. aureus* biofilms
   a. Biofilm disruption activity
      i. α-Tocopherol isolated from *Dicranopteris linearis*
      ii. Commercially available synthetic α-tocopherol
      iii. To identify and compare the minimum biofilm disruption concentration (MBDC)
   b. Biofilm inhibition activity

II. To characterise the biofilm disruption effects of α-tocopherol
   a. Effect against biofilm matrix
   b. Effect against bacterial cells within biofilm matrix

III. To identify the treatment time needed for α-tocopherol to exert its biofilm disruption effects

IV. To evaluate the biofilm disruption effect of α-tocopherol against other bacterial biofilms
   a. Monomicrobial biofilms*: Enterococcus faecalis* biofilms and *Escherichia coli* biofilms
   b. Polymicrobial biofilms: *S. aureus-E. faecalis* biofilms

V. To evaluate the effects of combination of α-tocopherol with antibiotics

5.3 Methodology

The summary of methodology for the investigation of α-tocopherol anti-biofilm activity is in Figure 5-1.

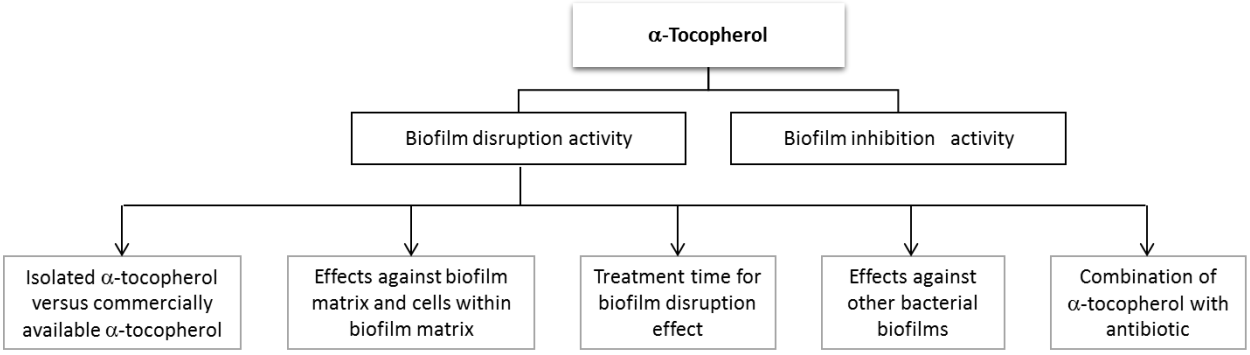


Figure 5-1: Flow chart summarizing the analysis on α-tocopherol activity

5.3.1 Bacterial strains

Bacterial strains as listed in Table 5-1 were used. All strains were cultured in tryptic soy broth (TSB) and incubated at 37°C.

Table 5-1: The characteristics of each strain used

Bacterial name	ATCC number	Antibiotic resistance	Biofilm production
Staphylococcus aureus	6538P	Methicillin susceptible	Strong
Staphylococcus aureus	43300	Methicillin resistant	Moderate to strong
Staphylococcus aureus	33591	Methicillin resistant	Weak to moderate
Staphylococcus aureus	29213	Methicillin susceptible	Weak to moderate
Enterococcus faecalis	700802	Vancomycin resistant	-
Escherichia coli	25922	-	-

*Weak ➔ $OD_{570} < 2$; Moderate ➔ $2 \leq OD_{570} \leq 4$; Strong ➔ $OD_{570} > 4$ (The characteristics for *S. aureus* biofilm production was determined based on the results obtained in this study and categorised in reference to Stepanović *et al.*, 2007)

5.3.2 Assessment of the anti-biofilm activity of α-tocopherol against *S. aureus* biofilms

5.3.2.1 Biofilm disruption activity of α-tocopherol

The biofilm disruption activity of α-tocopherol isolated from the HEX fraction of *D. linearis* (Chapter 4) and a commercially available synthetic α-tocopherol (Sigma-Aldrich, USA) was evaluated and compared. The minimum biofilm disruption concentration (MBDC) was determined according to the procedure as previously described in Section 3.3.7.1.1 (Dusane *et al.*, 2008; Shakeri *et al.*, 2007). The range of concentrations tested was from 0.01 – 0.5 mg/ml α-tocopherol. The experiment was performed in triplicates. MBDC was defined as the lowest concentration of α-tocopherol that disrupts pre-formed biofilms of *S. aureus* as compared with the solvent control. The negative control refers to treatment with water while positive control refers to treatment with 1% sodium hypochlorite. Solvent control refers to the solvent used to dissolve α-tocopherol, which was 0.03% of Tween 80 in 0.6% acetonitrile. These controls were used for all subsequent experiments.

5.3.2.2 Biofilm inhibition activity of α-tocopherol

The biofilm inhibition activity of α-tocopherol was evaluated. The minimum biofilm inhibition concentration (MBIC) was determined according to the procedure as previously described in Section 3.3.5.2.1 (Kwasny and Opperman, 2010). The range of concentrations tested was from 0.01 – 0.5 mg/ml α-tocopherol. The experiment was performed in triplicates. MBIC was defined as the lowest concentration of α-tocopherol in a serial two-fold dilution series that inhibits biofilm formation as compared with the solvent control.

5.3.3 Characterization of the biofilm disruption effect of α-tocopherol

5.3.3.1 Dimethyl methylene blue (DMMB) assay

Dimethyl methylene blue (DMMB) assay was performed to evaluate the effects of α-tocopherol on the biofilm matrix of *S. aureus*. DMMB forms a complex with the polysaccharides in the biofilm matrix, which can be measured spectrophotometrically to give an indirect amount of the biofilm matrix. *S. aureus* biofilm was allowed to form first before adding a two-fold dilution series of α-tocopherol from 0.01 – 0.5 mg/ml for treatment (Section 3.3.7.1.1). After 24 hours of incubation, the plates were rinsed with water. DMMB complexation solution was added and the plates were incubated in dark for 30 minutes. The plates were then centrifuged, washed to remove unbound DMMB and added with the decomplexation solution before further incubation for 30 minutes. Absorbance was measured at 620 nm. The experiment was performed in triplicates. Figure 5-2 summarizes the procedure for DMMB assay (Peeters *et al.*, 2008; Tote *et al.*, 2010).

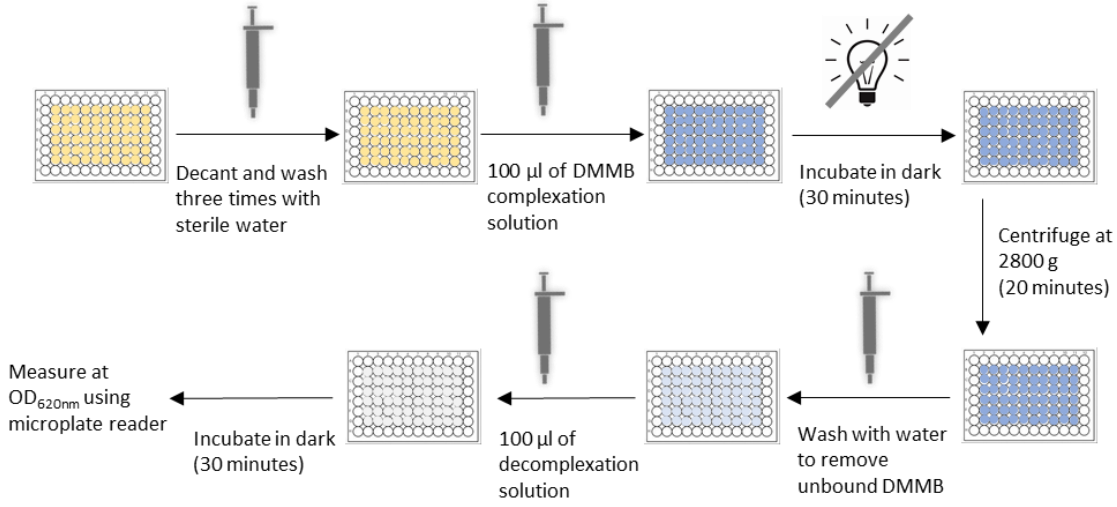


Figure 5-2: Summarized procedure for DMMB assay.

5.3.3.2 Resazurin assay

Resazurin assay was performed to evaluate the effects of α-tocopherol on the viability of cells within biofilms. The non-fluorescent resazurin is reduced to the fluorescent resorufin by metabolically active cells and the fluorescence measured would reflect the amount of viable cells within biofilms. *S. aureus* biofilm was allowed to form first before adding a two-fold dilution series of α-tocopherol from 0.01 – 0.5 mg/ml for treatment (Section 3.3.7.1.1). After 24 hours of incubation, the microtiter plates were rinsed with water. Then the wells were filled with 180 µl of water + 20 µl of 0.05 mg/ml resazurin. After

2 hours of incubation at 37°C, fluorescence (λ_{ex}: 560 nm and λ_{em}: 590 nm) was measured (Peeters *et al.*, 2008). The experiment was performed in triplicates.

5.3.4 Time-course experiment for biofilm disruption activity

In order to identify the treatment time needed for α-tocopherol to exert its activity, the biofilm disruption activity of α-tocopherol was evaluated every two hours within a 24-hour time period. The procedure was as described in Section 3.3.7.1.1 with activity tested at 0. 1 mg/ml of α-tocopherol (Dusane *et al.*, 2008; Shakeri *et al.*, 2007). The experiment was performed in triplicates.

5.3.5 Biofilm disruption activity against other bacterial biofilms

Biofilm disruption activity of α-tocopherol was evaluated against the biofilms of other bacteria, involving biofilms formed by a single species (monomicrobial biofilm) or multiple species (polymicrobial biofilms). The range of concentration tested was from 0.01 – 0.5 mg/ml α-tocopherol and the MBDC values were also determined.

For the monomicrobial biofilm assay, the biofilm disruption activity of α-tocopherol was evaluated against *Enterococcus faecalis* and *Escherichia coli* biofilms, according to the procedure described in Section 3.3.7.1.1 (Dusane *et al.*, 2008; Shakeri *et al.*, 2007). *E. faecalis* biofilms was cultivated with TSB supplemented with 2% glucose (Seneviratne *et al.*, 2013) while *E. coli* biofilms was cultivated with diluted TSB (Skyberg *et al.*, 2007). The experiment was performed in triplicates.

For the polymicrobial biofilm assay, biofilms consisting of *S. aureus* and *E. faecalis* were used. The procedure was as previously described in Section 3.3.7.1, with modifications. One hundred microliters of *S. aureus* inoculum suspension and 100 µl of *E. faecalis* inoculum suspension were added into the wells of microtiter plates, and incubated for 24 hours to allow the formation of polymicrobial biofilms. After 24 hours, the planktonic cells were decanted and the wells were washed three times with sterile water. Two hundred microliters of α-tocopherol was applied to the wells. The plates were further incubated at 37°C for 24 hours. Disruption of pre-formed polymicrobial biofilms was quantified using crystal violet staining (Section 3.3.5.3) (Dusane *et al.*, 2008; Seneviratne *et al.,* 2013; Shakeri *et al.*, 2007). The experiment was performed in triplicates.

5.3.6 Combination of α-tocopherol with antibiotic

In order to determine the possible synergistic activity between α-tocopherol and an antibiotic, the combinational effects of α-tocopherol and vancomycin in disrupting biofilms was assessed using the checkerboard assay. A synergistic activity is achieved when the effect of a combination of two

compounds is greater than the individual effect of a compound, in which one compound increases the effectiveness of another compound. The *S. aureus* biofilm was allowed to form first before adding treatment (Section 3.3.7.1). Dilutions for α-tocopherol only (4 – 130 µg/ml (8×MBDC)) and vancomycin only (0.008 – 4 µg/ml), and for the combination of α-tocopherol and vancomycin were prepared according to Figure 5-3. After treatment was added to the pre-formed biofilms, the plates were incubated for 24 hours before quantified using crystal violet staining, as described in Section 3.3.5.3 (Dusane *et al.*, 2008; Shakeri *et al.*, 2007). The experiment was performed in triplicates.

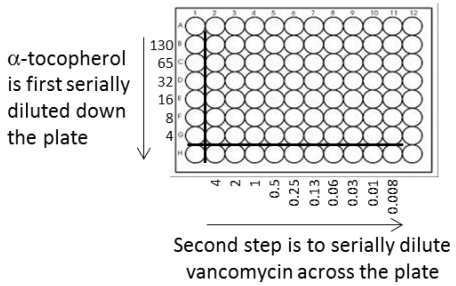


Figure 5-3: The dilution procedure for checkerboard assay.

The MBDC values for agents used separately and used in combination were determined. The mode of action of the two agents was expressed as the sum of their concentration fractions according to the equation: FBEC index = $\frac{Ac}{Aa} + \frac{Bc}{Ba}$, in which, *Aa* and *Ba* were the MBDC values of α-tocopherol and vancomycin when used separately, whereas *Ac* and *Bc* were the MBDC of α-tocopherol and vancomycin when used in combination. According to the equation, the interaction established by the FBEC index was considered as synergistic at ≤0.5, additive at >0.5 - 1.0, indifferent at >1.0 – 2.0 and antagonistic at >2.0 (Campeau and Patel, 2014; Krychowiak *et al.*, 2014; Rogers *et al.,* 2010).

The combination effects of α-tocopherol and vancomycin towards the cells within *S. aureus* biofilms were also assessed using the checkerboard assay. Cell viability was quantified using resazurin assay (Section 5.3.3.2) (Peeters *et al.*, 2008) and by determining the colony forming unit (CFU) per milliliter (CFU/ml). Serial dilutions were made with the aliquots from the microtiter wells, plated out on tryptic soy agar (TSA) and incubated at 37°C for 24 hours. The colonies formed were counted and used to determine CFU/ml (Rogers *et al.,* 2010). The experiment was performed in triplicates.

5.3.7 Data analysis

Statistical analysis was conducted using the One-way Analysis of Variance (ANOVA) test for comparing mean scores of more than two groups, with significance at $p<0.05$. The IBM SPSS Statistics 20 software was used (Kerekes *et al.*, 2013). All graphs were generated using the GraphPad Prism 6 software.

5.4 Results and Discussion

5.4.1 Anti-biofilm activity of α-tocopherol against *S. aureus* biofilms

The anti-biofilm activity of α-tocopherol was investigated against *S. aureus* biofilms, with regards to the biofilm disruption and biofilm inhibition activities.

5.4.1.1 Biofilm disruption activity of α-tocopherol

5.4.1.1.1 α-Tocopherol isolated from Dicranopteris linearis

As previously discussed in Chapter 4, α-tocopherol was isolated and identified as the active compound present in HEX fraction of *D. linearis* that was responsible for the biofilm disruption activity observed against *S. aureus* biofilms. In order to identify the biofilm disruption effect of the isolated α-tocopherol, activity testing was conducted against four strains of *S. aureus* biofilms and the minimum biofilm disruption concentration (MBDC) for each strain was determined. Figure 5-4 shows the biofilm disruption effect of the isolated α-tocopherol and Table 5-2 presents the MBDC values determined for each strain.

Based on Figure 5-4, biofilm disruption activity by α-tocopherol isolated from HEX fraction was observed against *S. aureus*, as shown by the significant decrease in pre-formed biofilm (biofilm biomass) when compared with the solvent control. The solvent control (0.03% of Tween 80 in 0.6% acetonitrile) showed no significant difference when compared with the negative control (water) indicating that the solvent used to dissolve the isolated α-tocopherol did not affect the biofilms and thus, does not influence the biofilm disruption effect observed. α-tocopherol was able to disrupt all four strains of *S. aureus* biofilms.

MBDC represents the lowest concentration of isolated α-tocopherol within the tested range, which was able to disrupt pre-formed biofilms of *S. aureus*. Based on Table 5-2, the MBDC for the isolated α-tocopherol was 0.01 mg/ml for all four strains tested. The isolated α-tocopherol was effective for biofilm disruption at low concentrations regardless of the difference in biofilm production among the strains, unlike the HEX fraction, which had required higher concentrations to disrupt stronger biofilm producers (Section 3.4.4.1). It can be seen that biofilm disruption activity had improved tremendously with the purification of the active compound.

The isolated α-tocopherol was extracted from a plant source and thus, is a natural form of α-tocopherol. This natural form of α-tocopherol occurs in nature as a single stereoisomer and is known as *D*-α-tocopherol or more correctly, *RRR*-α-tocopherol. In contrast, synthetic α-tocopherol is a mixture of

eight stereoisomers of α-tocopherol (*RRR, RSR, RRS, RSS, SRR, SSR, SRS, SSS*) in equal amounts. Synthetic α-tocopherol is commonly referred to as *DL*-α-tocopherol or *all-rac*-α-tocopherol. At present, only four stereoisomers of α-tocopherol (*RRR, RSR, RRS, RSS*) are considered to possess vitamin E activity and thus, the biological activity of synthetic α-tocopherol is only half of the natural form of α-tocopherol (Driskell, 2007; Litwack, 2007; Stone *et al.,* 2003).

Since the isolated α-tocopherol was shown to exhibit biofilm disruption activity, evaluations on the activity of synthetic α-tocopherol was performed to determine whether it would demonstrate similar biofilm disruption effect. The assessment for the biofilm disruption activity of synthetic α-tocopherol is discussed in Section 5.4. 1.1.2.

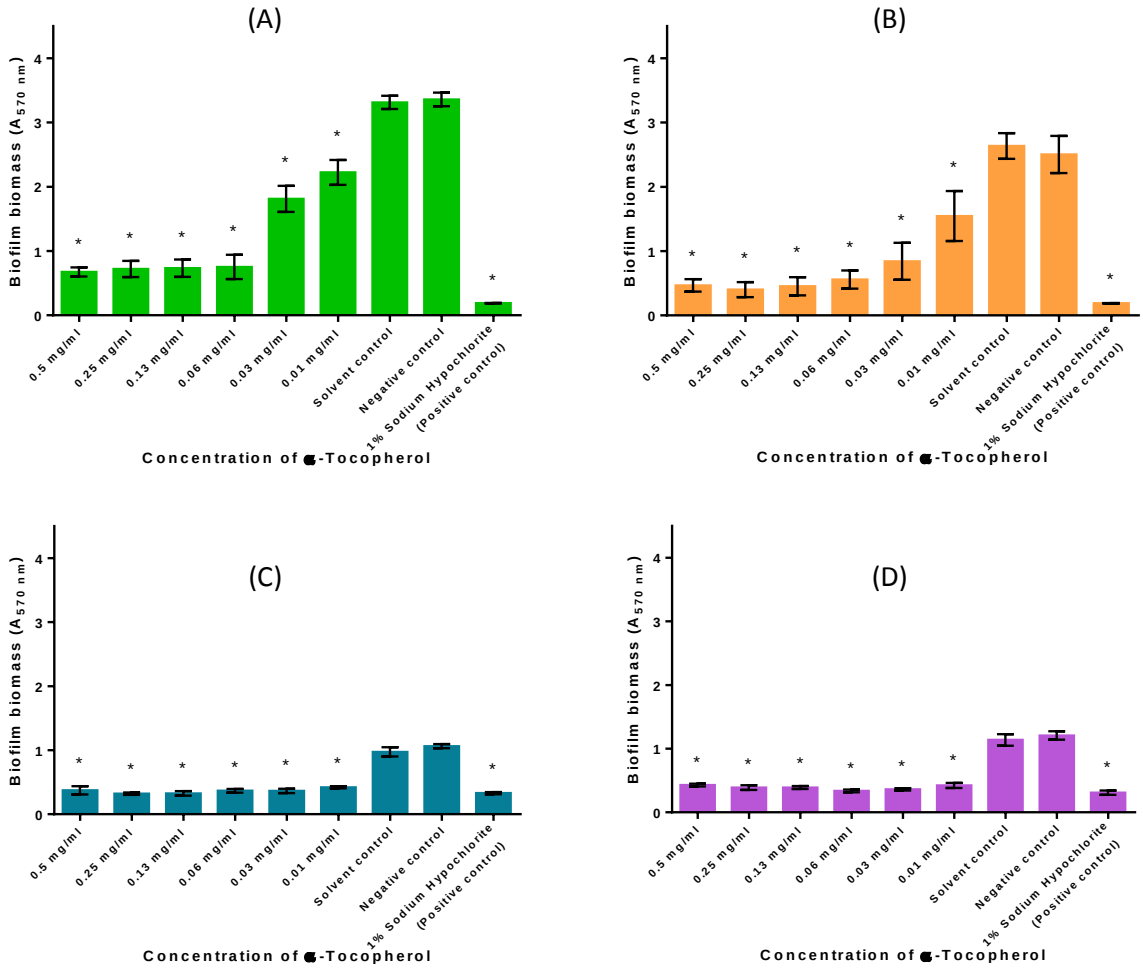


Figure 5-4: Biofilm disruption activity of α-tocopherol isolated from HEX fraction of *D. linearis* at various concentrations against (A) *S. aureus* ATCC 6538P; (B) *S. aureus* ATCC 43300; (C) *S. aureus* ATCC 33591; (D) *S. aureus* ATCC 29213. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against various concentrations of isolated α-tocopherol. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm disruption activity when compared with the solvent control. Biofilm disruption activity was observed for all four strains.

Table 5-2: Minimum biofilm disruption concentration (MBDC) of α-tocopherol against *S. aureus* strains

Strains	Biofilm production	MBDC (mg/ml)	
		α-Tocopherol isolated from *D. linearis*	Synthetic α-tocopherol
S. aureus ATCC 6538P	Strong	0.01	0.01
S. aureus ATCC 43300	Moderate to strong	0.01	0.01
S. aureus ATCC 33591	Weak to moderate	0.01	0.01
S. aureus ATCC 29213	Weak to moderate	0.01	0.01

*Minimum biofilm disruption concentration (MBDC) in mg/ml

5.4.1.1.2 Synthetic α-tocopherol

The biofilm disruption activity of a commercially available synthetic α-tocopherol was assessed and the MBDC values determined was compared to the isolated α-tocopherol. Figure 5-5 shows the biofilm disruption effect of synthetic α-tocopherol against four strains of *S. aureus* biofilms, with the MBDC values presented in Table 5-2.

Based on Figure 5-5, biofilm disruption activity by synthetic α-tocopherol was observed against *S. aureus*, as shown by the significant decrease in pre-formed biofilm (biofilm biomass) when compared with the solvent control. The solvent control was not significantly different when compared with the negative control indicating that the solvent used to dissolve synthetic α-tocopherol was not influencing the biofilm disruption effect observed. Synthetic α-tocopherol was able to disrupt all four *S. aureus* biofilm strains.

As for the MBDC values, similar results of MBDC to the isolated α-tocopherol was observed. MBDC of synthetic α-tocopherol was also 0.01 mg/ml for all four *S. aureus* strains, indicating that the biofilm disruption activity of synthetic α-tocopherol was comparable to the isolated α-tocopherol (Table 5-2). Differences in the stereoisomeric form between synthetic α-tocopherol (*RRR, RSR, RRS, RSS, SRR, SSR, SRS, SSS*) and the isolated α-tocopherol (*RRR*) did not cause any variation in biofilm disruption activity.

Currently, there has not been any study reported on the biofilm disruption effect of α-tocopherol. This will be the first study to report on the biofilm disruption activity of α-tocopherol against *S. aureus* biofilms.

For further analysis onwards, synthetic α-tocopherol will be used instead of the isolated α-tocopherol. This is because synthetic α-tocopherol is more readily available (no extraction process is needed), cheaper and activity had been proven to be similar to the isolated α-tocopherol. Synthetic α-tocopherol will be referred simply as α-tocopherol in the following sections onward.

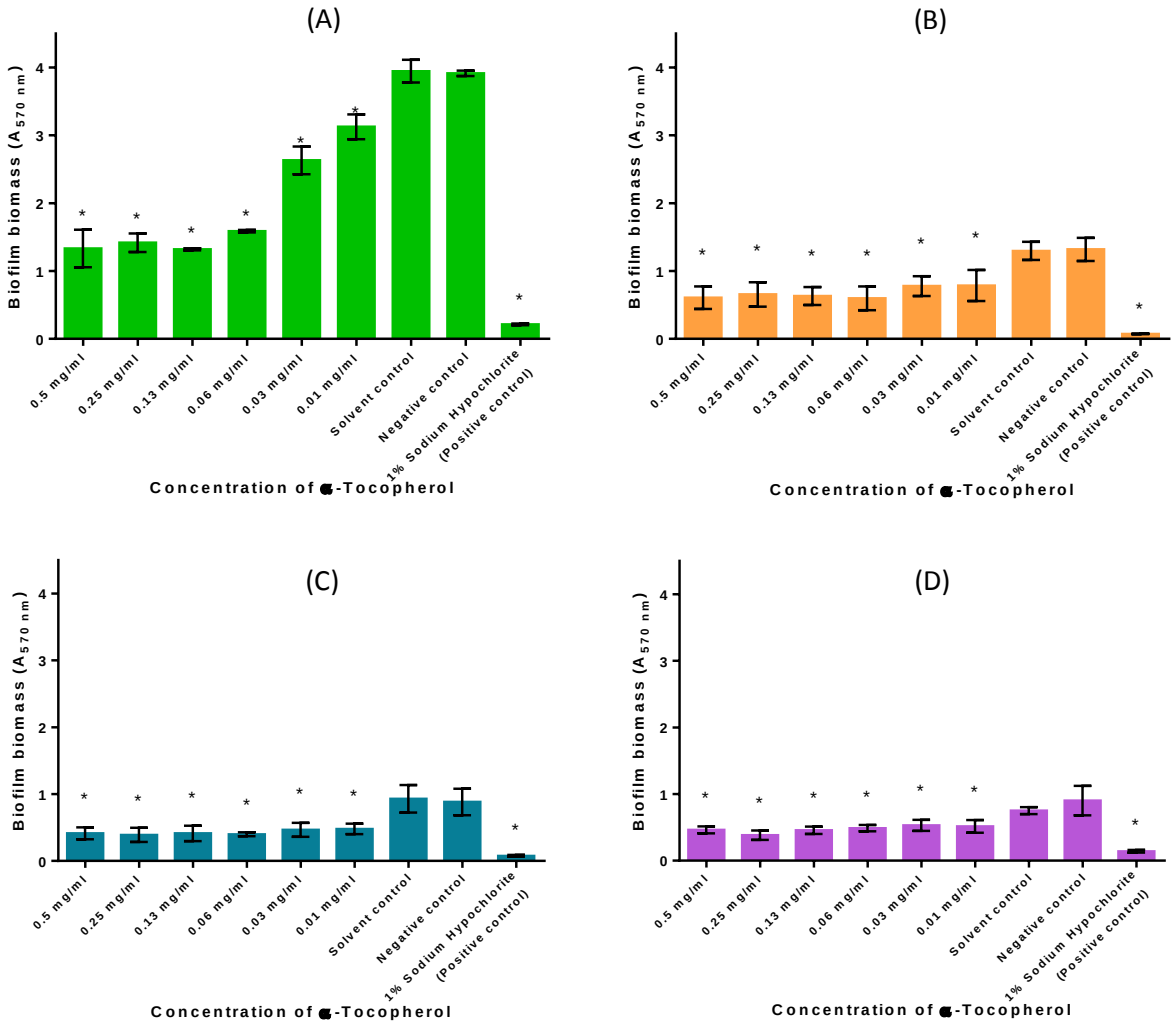


Figure 5-5: Biofilm disruption activity of commercially available synthetic α-tocopherol at various concentrations against (A) *S. aureus* ATCC 6538P; (B) *S. aureus* ATCC 43300; (C) *S. aureus* ATCC 33591; (D) *S. aureus* ATCC 29213. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against various concentrations of commercially available synthetic α-tocopherol. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm disruption activity when compared with the solvent control. Biofilm disruption activity was observed for all four strains.

5.4.1.2 Biofilm inhibition activity of α-tocopherol

In a previous study, Jagani *et al*. (2009) had reported on the lack of biofilm inhibition activity of α-tocopherol when tested against *Pseudomonas aeruginosa* biofilm. Therefore, it was of interest to verify whether the lack of biofilm inhibition effect by α-tocopherol also applies to *S. aureus* biofilms. In order to identify the effectiveness of the α-tocopherol in inhibiting *S. aureus* biofilms, it was tested against four strains of *S. aureus* biofilms. Figure 5-6 shows the biofilm inhibition effect by α-tocopherol.

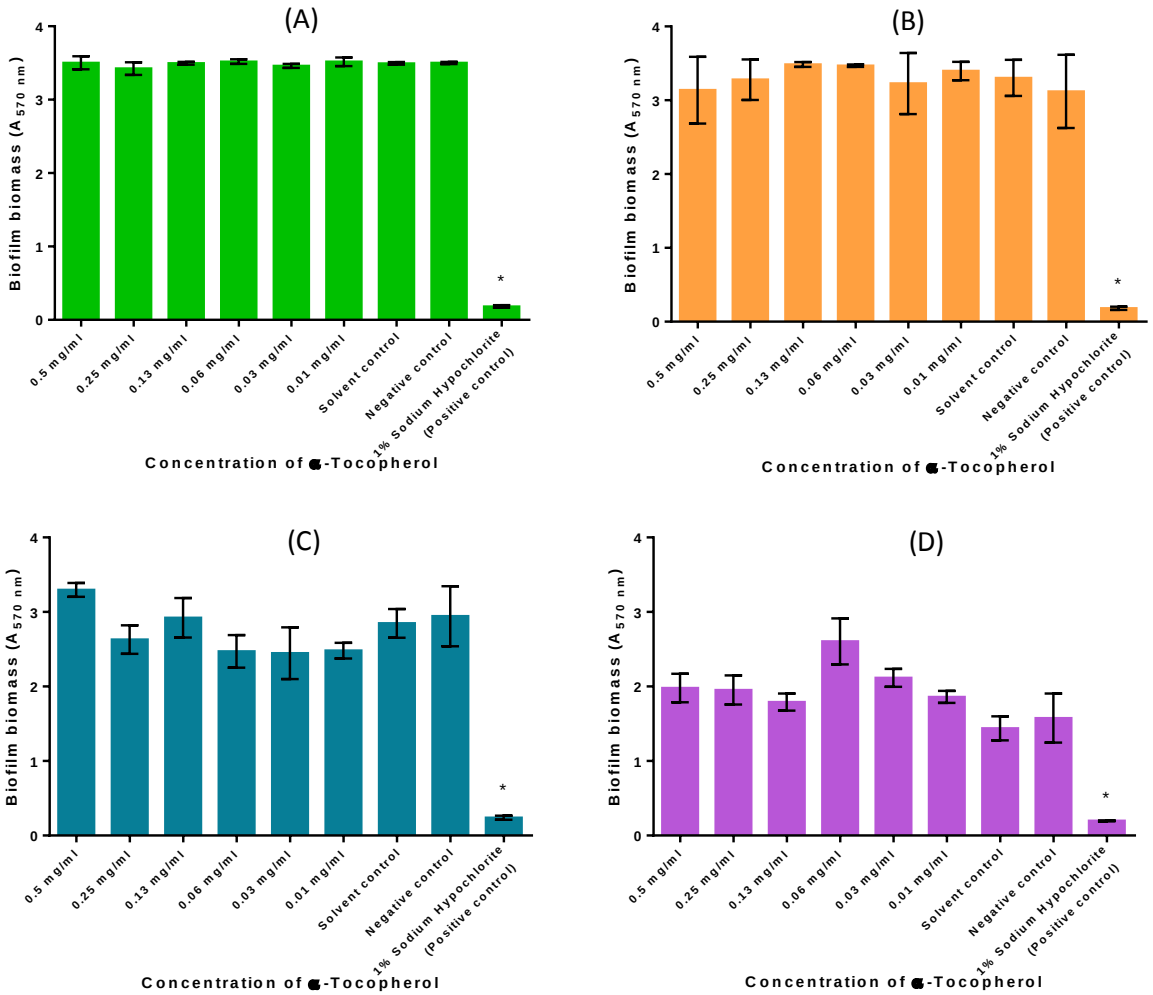


Figure 5-6: Biofilm inhibition activity of α-tocopherol at various concentrations against (A) *S. aureus* ATCC 6538P; (B) *S. aureus* ATCC 43300; (C) *S. aureus* ATCC 33591; (D) *S. aureus* ATCC 29213. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against various concentrations of α-tocopherol. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm inhibition activity when compared with the solvent control. No biofilm inhibition effect by α-tocopherol was observed.

Based on Figure 5-6, α-tocopherol had no biofilm inhibition effect against all four *S. aureus* strains tested, as shown by the lack of significant reduction in biofilm formation (biofilm biomass) when compared with the solvent control. Therefore, it was concluded that α-tocopherol does not exhibit biofilm inhibition activity and this result is similar to those previously reported by Jagani *et al*. (2009).

Overall, investigation on the anti-biofilm activity of α-tocopherol demonstrates the presence of biofilm disruption activity of α-tocopherol and confirms its lack of biofilm inhibition activity. Further analysis will focus on the characteristics and potential application of α-tocopherol as a biofilm disrupting agent.

5.4.2 Characterization of the biofilm disruption effect of α-tocopherol

The biofilm disruption effect of α-tocopherol on the biofilm matrix and cells within biofilms of *S. aureus* were assessed using dimethyl methylene blue (DMMB) assay and resazurin assay, respectively.

5.4.2.1 Dimethyl methylene blue (DMMB) assay

Dimethyl methylene blue (DMMB) assay is based on the consideration that the main constituent of *S. aureus* biofilm matrix is the polysaccharide intercellular adhesion (PIA), which is composed of poly-β-1,6-linked-N-acetylglucosamine (PNAG). DMMB is commonly used to detect specifically glycosaminoglycans (GAGs) in biological samples, and since there is structural similarity between PIA and GAGs, DMMB has been used for specific detection of *S. aureus* biofilm matrix (Peeters *et al.*, 2008). The complex between DMMB and PIA can be quantified spectrophotometrically to give the indirect amount of matrix biofilm and can reflect whether α-tocopherol affects the biofilm matrix of *S. aureus*. Figure 5-7 shows the quantification of the biofilm matrix after treatment with α-tocopherol against *S. aureus* biofilms.

Based on Figure 5-7, it can be observed that the biofilm matrix was reduced when treated with α-tocopherol for all four *S. aureus* strains, as shown by the significant reduction in biofilm matrix when compared with the solvent control. The solvent control was not significantly different when compared with the negative control indicating that the solvent used to dissolve α-tocopherol was not influencing the observed reduction in biofilm matrix by α-tocopherol. Therefore, it can be deduced that α-tocopherol had affected the biofilm matrix, leading to the biofilm disruption effect observed.

It can also be assumed that α-tocopherol affects a specific component of the biofilm matrix, which is the polysaccharides, as DMMB reflects the amount of polysaccharides present in the matrix (Peeters *et al.*, 2008). However, it is undetermined on how α-tocopherol affects the polysaccharides in the biofilm matrix and thus, this will need to be further studied. Besides that, there is also a possibility that α-tocopherol affects other components of the biofilm matrix besides polysaccharides, such as DNA and proteins (Boles *et al.,* 2011). Analyses are needed to verify the possible effect of α-tocopherol on these other biofilm matrix components.

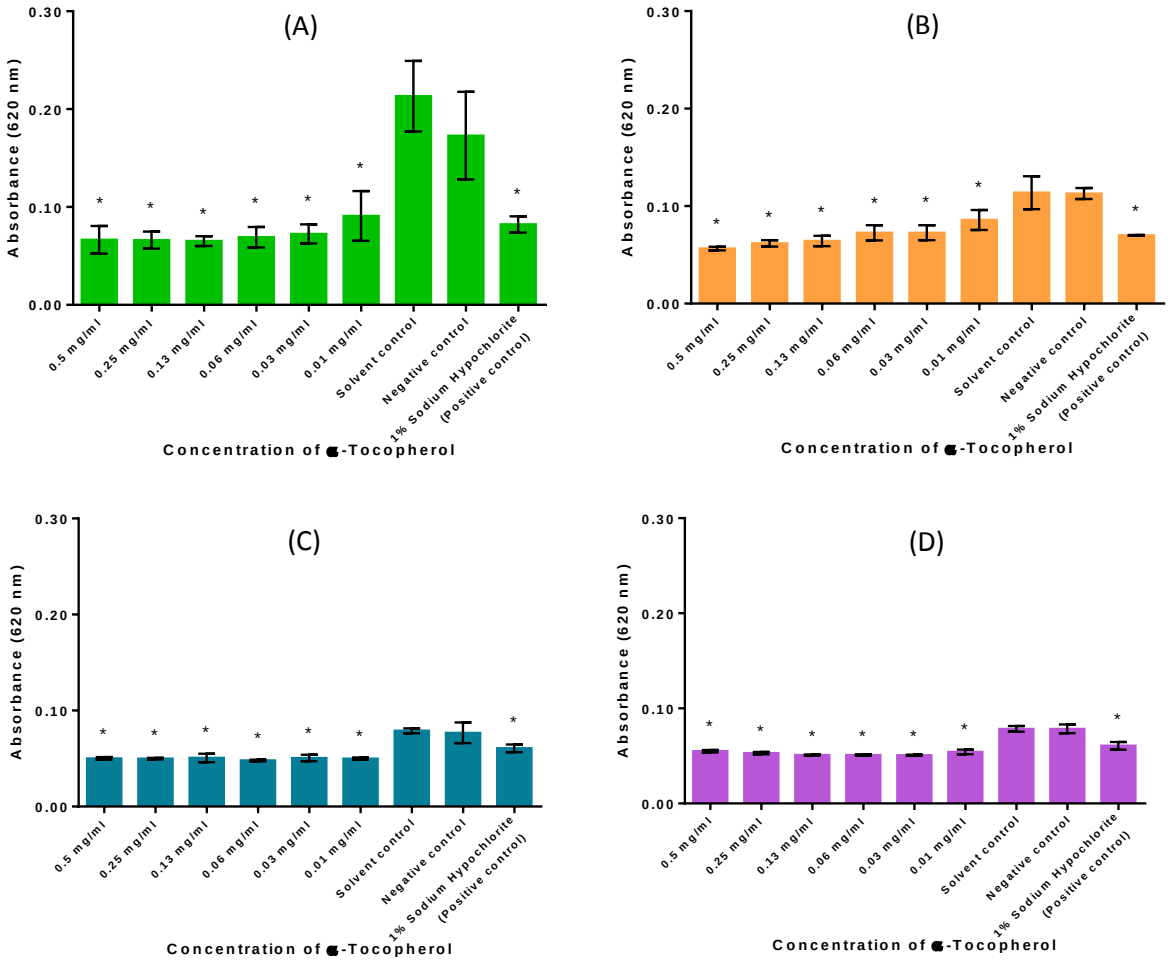


Figure 5-7: Quantification of the biofilm matrix after treatment with α-tocopherol at various concentrations against (A) *S. aureus* ATCC 6538P; (B) *S. aureus* ATCC 43300; (C) *S. aureus* ATCC 33591 and (D) *S. aureus* ATCC 29213. Mean absorbance (620 nm) ± SD plotted against various concentrations of α-tocopherol. * denotes statistically significant difference at $p<0.05$ and shows reduction in biofilm matrix when compared with the solvent control. Reduction in biofilm matrix was observed for all four strains.

An example of an antioxidant that disrupts biofilms is glutathione (GSH). In *Pseudomonas aeruginosa* biofilms, pyocyanin intercalates directly with extracellular DNA, to confer structural integrity to the biofilm. The antioxidant glutathione (GSH) reacts with pyocyanin, directly interfering with pyocyanin's ability to intercalate with extracellular DNA and thus, resulting in the disruption of biofilms (Das *et al.,* 2016; Klare *et al.,* 2016).

Besides that, N-acetyl cysteine (NAC) is a potent thiol-containing antioxidant that disrupts disulfide bonds in mucus and competitively inhibits amino acid (cysteine) utilization. Several *in vitro* studies have reported that NAC decreases biofilm formation by a variety of bacteria such as

S. epidermidis, Escherichia coli and *E. faecalis,* and may reduce the production of extracellular polysaccharide matrix while promoting the disruption of mature biofilm (Dinicola *et al.,* 2014; Perez-Giraldo *et al.,* 1997; Quah *et al.,* 2012; Silveira *et al.,* 2013). Few explanations were suggested to explain the reduction in the amount of extracellular polysaccharide in the presence of NAC. The more direct effects of NAC include disrupting disulfide bonds in enzymes involved in extracellular polysaccharide production or excretion, causing the molecules to be less active, or NAC competitively inhibiting cysteine utilization. Besides that, it was also suggested that NAC may interfere with control or signalling systems involved in extracellular polysaccharide production (Dinicola *et al.,* 2014; Olofsson *et al.,* 2003).

Since α-tocopherol is also an antioxidant, there is a possibility that the antioxidant property of α-tocopherol might be involved directly or indirectly in affecting the biofilm matrix. As it is currently unknown whether α-tocopherol targets one or more of the biofilm matrix components, and how it specifically affects the biofilm matrix, more assays will need to be done to identify the exact mechanism of action of α-tocopherol in affecting the biofilm matrix.

5.4.2.2 Resazurin assay

In the resazurin assay, the non-fluorescent resazurin is reduced to the fluorescent resorufin and the fluorescence measured is proportional to the amount of metabolic active cells present (Peeters *et al.*, 2008). This allows for the quantification of the viable cells within the biofilm matrix and thus, reflects whether α-tocopherol specifically affects the cells within the biofilm matrix, which could not be identified when stained with crystal violet. Figure 5-8 shows the quantification of viable cells within biofilms after treatment with α-tocopherol against four strains of *S. aureus* biofilms.

Based on Figure 5-8, it can be observed that the cells within the biofilms of all four *S. aureus* strains remained viable after treatment with various concentrations of α-tocopherol, as shown by the lack of significant reduction in cell viability when compared with the solvent control. This is advantageous for α-tocopherol as an anti-biofilm agent because it only has a biofilm disruption effect without any antibacterial effect. With no killing or inhibition effect by α-tocopherol towards the cells, selective pressure is much weaker and thus, the likelihood for the development of resistance towards the anti-biofilm agents is low.

Overall, it can be concluded from these two assays that α-tocopherol does not affect the cells within biofilms but instead affects the biofilm matrix in order to disrupt *S. aureus* biofilms.

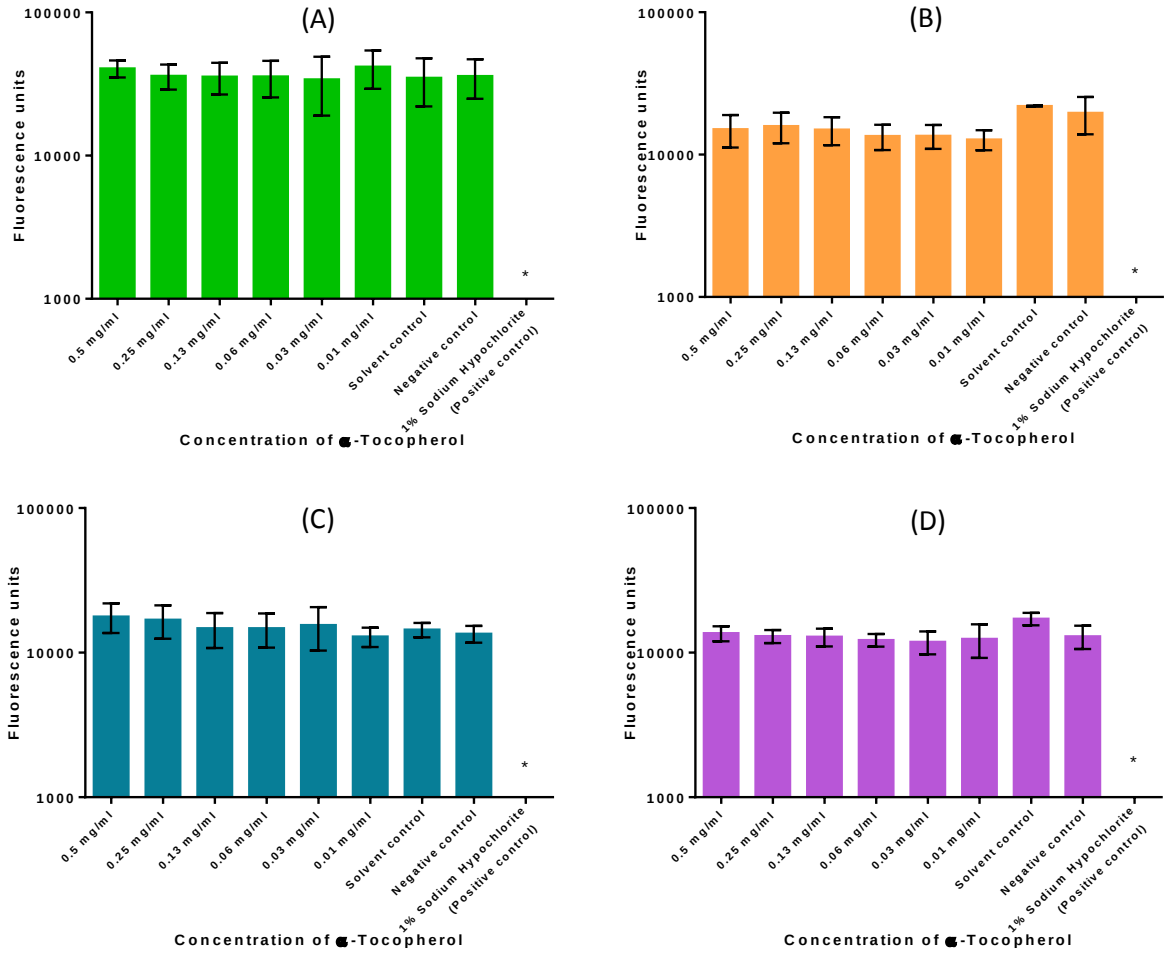


Figure 5-8: Quantification of viable cells within biofilm after treatment with α-tocopherol at various concentrations against (A) *S. aureus* ATCC 6538P; (B) *S. aureus* ATCC 43300; (C) *S. aureus* ATCC 33591 and (D) *S. aureus* ATCC 29213. Mean fluorescence units ± SD plotted against various concentrations of α-tocopherol. * denotes statistically significant difference at $p<0.05$ and shows reduction in cell viability when compared with the solvent control. No reduction in cell viability was observed when treated with α-tocopherol.

5.4.3 Time-course experiment for biofilm disruption activity

In previous assays, the procedure for biofilm disruption assay had required 24 hours of incubation for treatment. However, it was not known whether α-tocopherol causes rapid or slow biofilm disruption effect, requiring less or more incubation time, or whether 24 hours of treatment was really required for effective disruption. Therefore, the time-course experiment was conducted to determine the actual treatment time needed for α-tocopherol to disrupt biofilms. Activity was assessed at 0.1 mg/ml (8xMBDC) of α-tocopherol as stronger biofilm disruption effect was observed at 0.1 mg/ml as compared to at MBDC. Figure 5-9 shows the biofilm disruption activity of 0.1 mg/ml of α-tocopherol

over time within 24 hours for all four *S. aureus* strains while Table 5-3 indicates the treatment time for α-tocopherol to disrupt each *S. aureus* biofilms.

Table 5-3: The treatment time for α-tocopherol to disrupt *S. aureus* biofilms

Strains	Biofilm production	Time range of biofilm disruption (hours)
S. aureus ATCC 6538P	Strong	12 – 24
S. aureus ATCC 43300	Moderate to strong	10 – 24
S. aureus ATCC 33591	Weak to moderate	16 – 24
S. aureus ATCC 29213	Weak to moderate	12 – 24

Based on Figure 5-9 and Table 5-3, the biofilm disruption effect of α-tocopherol against all four *S. aureus* strains were considered to be slow as it had required an average of ~12 hours for the disruption effect to occur. The biofilm disruption of α-tocopherol requiring ~ 12 hours, was considered slow when compared to lysostaphin. Lysostaphin was reported to have a short response time, showing measurable disruption within 20 minutes of application and requiring about 3 hours for complete disruption effect (Wu *et al.,* 2003). In contrast, the biofilm disruption of α-tocopherol was considered shorter if compared to both oxacillin and vancomycin, which have been reported to require about 24 hours or more to disrupt biofilms (Amorena *et al.,* 1999; Wu *et al.,* 2003).

Besides that, a strain variation effect was observed, in terms of the treatment time of α-tocopherol. The variation in minimum treatment time was not due to the amount of biofilm produced by each strain. *S. aureus* ATCC 6538P, a stronger biofilm producer and *S. aureus* ATCC 29213, a weaker biofilm producer both showed biofilm disruption effect after 12 hours of treatment, while *S. aureus* ATCC 43300, a moderate biofilm producer and *S. aureus* ATCC 33591, a weak biofilm producer, required 10 hours and 16 hours of treatment, respectively. It is currently unknown as to why the treatment time varies between strains and to why the amount of biofilm produced does not influence the treatment time needed.

Although α-tocopherol was slow acting, this does not diminish its effectiveness in disrupting *S. aureus* biofilms. As shown previously, α-tocopherol does not affect the viable cells in biofilms under 24 hours of treatment (Section 5.4.2.2). Therefore, although the minimum treatment time of 10 – 16 hours was considered slow, α-tocopherol would still effectively disrupt biofilms without causing development of resistance for the biofilm cells. Nevertheless, to standardize treatment time and to achieve a reliable disruption effect, utilizing 24 hours of treatment would be considered as the best treatment time.

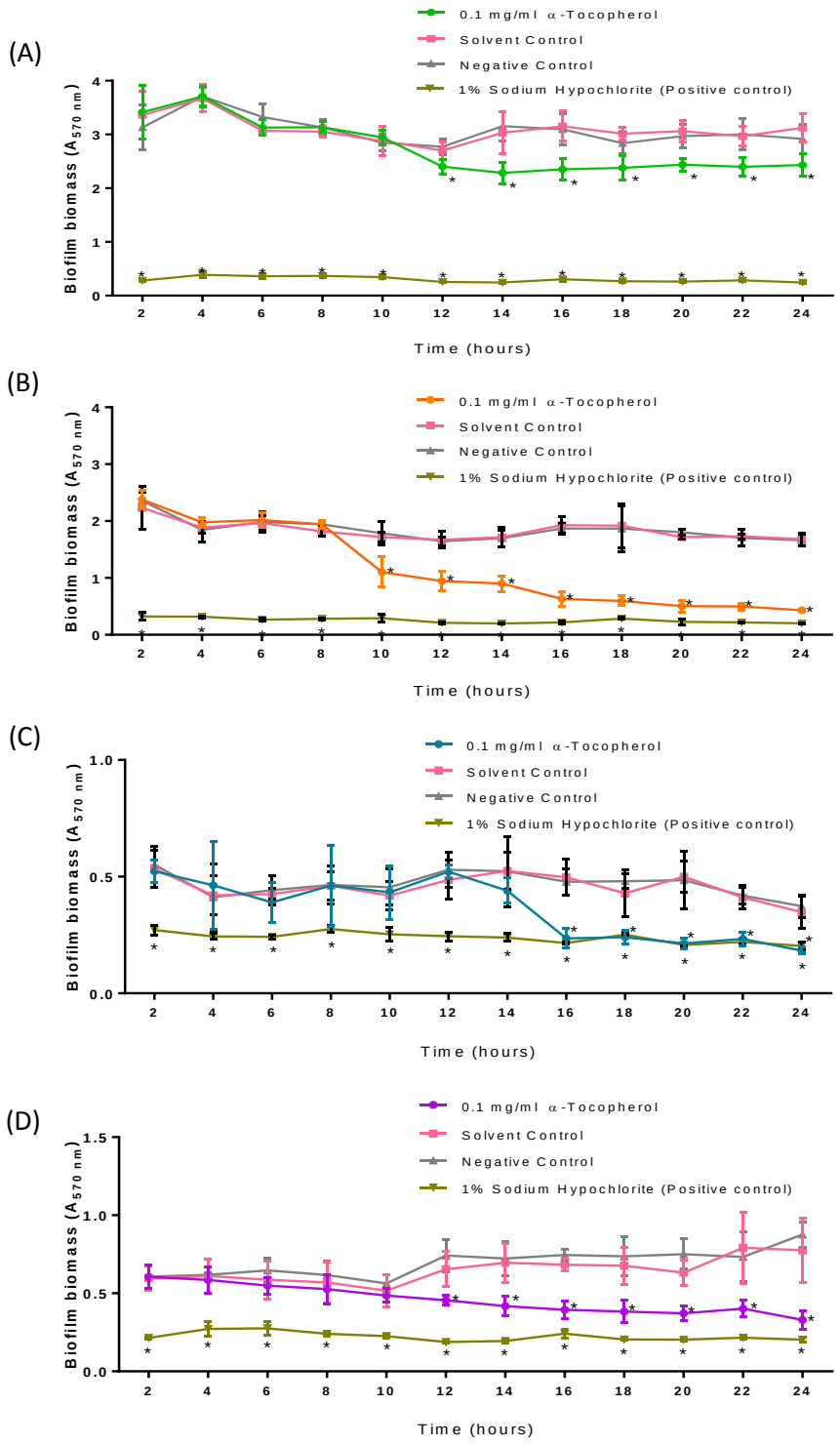


Figure 5-9: Biofilm disruption activity of α-tocopherol against (A) *S. aureus* ATCC 6538P; (B) *S. aureus* ATCC 43300; (C) *S. aureus* ATCC 33591 and (D) *S. aureus* ATCC 29213 evaluated every 2 hours within a 24 hour treatment period. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against time. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm disruption activity when compared with the solvent control. Biofilm disruption activity was observed from 12-24 hours of treatment.

5.4.4 Biofilm disruption activity of α-tocopherol against other bacterial biofilms

Biofilms can be monomicrobial (single species) or polymicrobial (multiple species). Since it has been shown that α-tocopherol was effective against *S. aureus* in monomicrobial biofilms, α-tocopherol was also investigated to determine whether it would be effective against other monomicrobial biofilms and against *S. aureus* in polymicrobial biofilms.

5.4.4.1 Monomicrobial biofilms: Enterococcus faecalis and Escherichia coli biofilms

Enterococcus faecalis is a gram positive bacterium and are natural inhabitants of the oral cavity, normal intestinal microflora, and female genital tract of both human and animals. They are opportunistic pathogens that can cause urinary tract infections, surgical wound infections, bacteremia, and bacterial endocarditis. *E. faecalis* is known to form biofilms and is often isolated from biofilms on the surfaces of various indwelling medical devices (Kristich *et al.*, 2004; Mohamed and Huang, 2007; Toledo-Arana *et al.*, 2001). *Escherichia coli* is a predominant species among facultative anaerobic bacteria of the gastrointestinal tract and is the most frequent microorganism involved in urinary tract infections (UTI). *E. coli* is known to form biofilms and it is one of the most frequently isolated pathogens from catheter-associated UTI (Beloin *et al.*, 2008; Soto, 2014).

Therefore, α-tocopherol was evaluated to determine whether it would be effective in disrupting *E. faecalis* and *E. coli* in monomicrobial biofilms. Figure 5-10 shows the biofilm disruption activity of α-tocopherol against *E. faecalis* and *E. coli* biofilms at various concentrations. Table 5-4 shows the average percentage of biofilm disruption by α-tocopherol against *E. faecalis* and *E. coli* biofilms, calculated from concentrations that showed significant biofilm disruption activity. The MBDC of α-tocopherol against *E. faecalis* and *E. coli* biofilms were also determined and presented in Table 5-4.

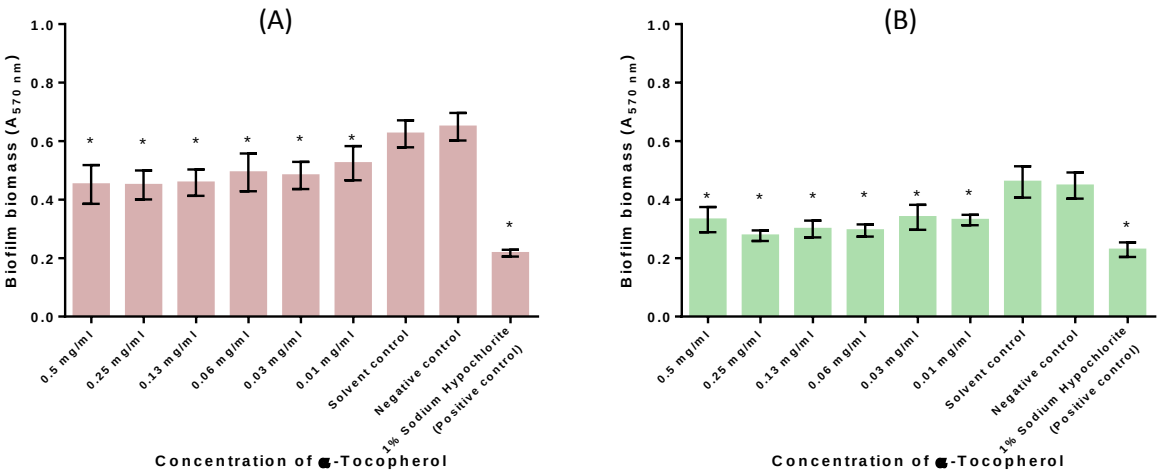


Figure 5-10: Biofilm disruption activity of α-tocopherol at various concentrations against (A) *E. faecalis* ATCC 700802; (B) *E. coli* ATCC 25922. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against various concentrations of α-tocopherol. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm disruption activity when compared with the solvent control. Biofilm disruption activity was observed against both bacteria biofilms.

Table 5-4: Percentage biofilm disruption by α-tocopherol and the minimum biofilm disruption concentration (MBDC) against *E. faecalis* and *E. coli* biofilms

Strains	Percentage biofilm disruption (%)	MBDC (mg/ml)
E. faecalis ATCC 700802	23.5 ± 9.5	0.01
E. coli ATCC 25922	31.5 ± 9.6	0.01

*Percentage biofilm disruption (%) = average percentage of biofilm disruption by α-tocopherol from concentrations that showed significant biofilm disruption activity
*Minimum biofilm disruption concentration (MBDC) in mg/ml

Based on Figure 5-10, biofilm disruption activity of α-tocopherol was observed against *E. faecalis* and *E. coli* biofilms, at 0.01 – 0.5 mg/ml, as shown by the significant decrease in pre-formed biofilm (biofilm biomass) when compared with the solvent control. The average percentage of biofilm disrupted by α-tocopherol from concentrations that showed significant biofilm disruption activity was calculated and it was determined that α-tocopherol was able to disrupt ~23% of *E. faecalis* biofilm and ~31% of *E. coli* biofilm (Table 5-4). The MBDC of α-tocopherol against *E. faecalis* and *E. coli* biofilms were determined to be 0.01 mg/ml (Table 5-4), and this was similar to the MBDC determined for *S. aureus* biofilms (Section 5.4.1.1), indicating that α-tocopherol can disrupt other bacteria biofilms besides *S. aureus* biofilms.

DMMB assay and resazurin assay would need to be conducted to further evaluate the effects of α-tocopherol against *E. faecalis* and *E. coli* biofilms. More analyses towards other strains of *E. faecalis* and *E. coli* biofilms have to be conducted to assess whether α-tocopherol can also be effective against

them. Furthermore, analyses on more monomicrobial biofilms, such as *Candida albicans, Pseudomonas aeruginosa* and Streptococcus species should also be conducted to evaluate the effectiveness of α-tocopherol against a variety of monomicrobial biofilms (Nair *et al.*, 2014).

5.4.4.2 Polymicrobial biofilms: S. aureus-E. faecalis biofilms

Since it has been shown that α-tocopherol can disrupt both *S. aureus* and *E. faecalis* in monomicrobial biofilms, the effectiveness of α-tocopherol in disrupting *S. aureus* and *E. faecalis* grown as polymicrobial biofilms was investigated. Although α-tocopherol was also effective against *E. coli* in monomicrobial biofilm, *E. coli* was not studied in polymicrobial biofilm study due to difficulties encountered in growing *S. aureus* and *E. coli* together as polymicrobial biofilms.

The primary site of colonization of *S. aureus* is the nose (anterior nares). However, at low concentrations, the intestinal tracts are co-colonized by both *S. aureus* and *E. faecalis* in some healthy humans. Both *S. aureus and E. faecalis* normally co-exist as commensals, but they can turn into opportunistic pathogens to cause polymicrobial infections such as urinary tract infections, bacteremia, and infective endocarditis (Franchi *et al.,* 1999; Nair *et al.*, 2014; Ray *et al.,* 2003).

For initial screening of activity, α-tocopherol was tested for biofilm disruption activity against the polymicrobial biofilms of *S. aureus* + *E. faecalis* at 0.5 mg/ml α-tocopherol. Four strains of *S. aureus* were each grown with *E. faecalis* to form the polymicrobial biofilms and the presence of biofilm disruption activity of α-tocopherol at 0.5 mg/ml against these polymicrobial biofilms were presented in Figure 5-11. Upon observation of activity at 0.5 mg/ml, MBDC assay would be conducted only for polymicrobial biofilms that showed activity.

Based on Figure 5-11, initial screening for biofilm disruption activity of α-tocopherol at 0.5 mg/ml showed activity against three polymicrobial biofilms of *S. aureus* + *E. faecalis*, which were *S. aureus* ATCC 43300 + *E. faecalis* ATCC 700802, *S. aureus* ATCC 33591 - *E. faecalis* ATCC 700802 and *S. aureus* ATCC 29213 + *E. faecalis* ATCC 700802, as shown by the significant decrease in pre-formed polymicrobial biofilm (biofilm biomass) when compared with the solvent control.

No biofilm disruption activity was observed against the polymicrobial biofilm of *S. aureus* ATCC 6538P + *E. faecalis* ATCC 700802. The interaction between *S. aureus* ATCC 6538P and *E. faecalis* ATCC 700802 in producing polymicrobial biofilm might have led to the development of more-persistent *S. aureus* or *E. faecalis* strains, with altered colony morphology, increased virulence and antibiotic resistance (Nair, 2014). This enhanced persistence in the polymicrobial biofilm might have contributed to the lack of biofilm disruption activity by α-tocopherol.

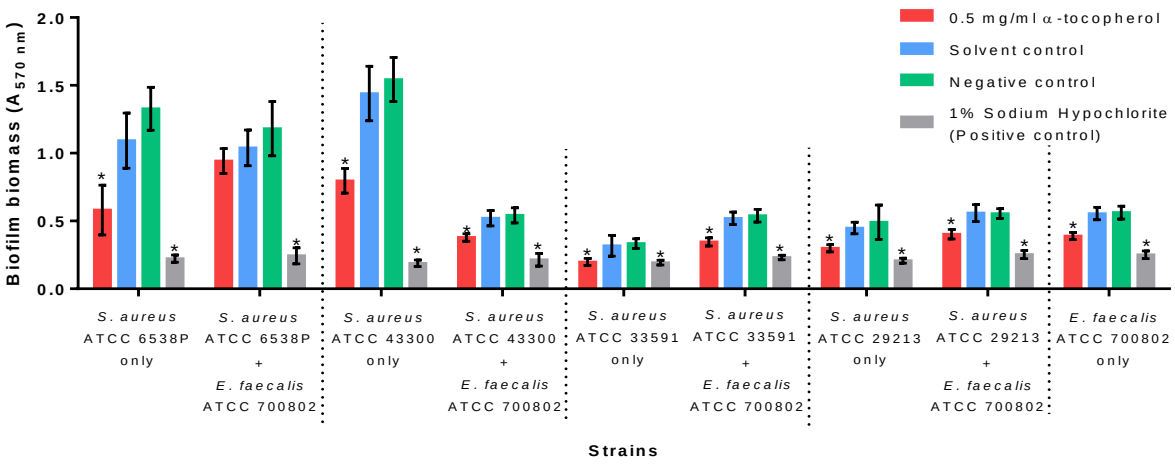


Figure 5-11: Biofilm disruption activity of α-tocopherol tested at 0.5 mg/ml against four polymicrobial biofilms of *S. aureus* + *E. faecalis*. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against various strains. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm disruption activity when compared with the solvent control.

The biofilm biomass for polymicrobial biofilm was expected to be higher than the biomass of either *S. aureus* or *E. faecalis* grown alone, or higher than the additive masses of both monomicrobial biofilms (Peters *et al.,* 2013; Sztajer *et al.,* 2014). This was observed for all expect for the polymicrobial biofilm of *S. aureus* ATCC 43300 + *E. faecalis* ATCC 700802. In Figure 5-11, the biofilm biomass of the polymicrobial biofilm of *S. aureus* ATCC 43300 + *E. faecalis* ATCC 700802 was fairly lower than the biofilm biomass of *S. aureus* ATCC 43300 grown alone and was similar to *E. faecalis* ATCC 700802 grown alone. Polymicrobial biofilm with less biomass indicates a possible competitive or antagonistic interaction between *S. aureus* ATCC 43300 and *E. faecalis* ATCC 700802, which may have subsequently hindered biofilm production. Antagonistic interaction may have occurred as a result of nutrient competition (Birkenhaur *et al.,* 2014; Nair, 2014).

Since α-tocopherol showed activity against the three polymicrobial biofilms of *S. aureus* + *E. faecalis* mentioned previously, these three polymicrobial biofilms (*S. aureus* ATCC 43300 + *E. faecalis* ATCC 700802, *S. aureus* ATCC 33591 - *E. faecalis* ATCC 700802 and *S. aureus* ATCC 29213 + *E. faecalis* ATCC 700802) were then tested at various concentrations to determine the MBDC. MBDC assay for the polymicrobial biofilm of *S. aureus* ATCC 6538P + *E. faecalis* ATCC 700802 was not conducted as no biofilm disruption activity was shown during initial screening at 0.5 mg/ml α-tocopherol. Figure 5-12 shows the biofilm disruption effect of α-tocopherol at various concentrations against the three polymicrobial biofilms. Table 5-5 shows the average percentage of biofilm disruption by α-tocopherol against the polymicrobial biofilms, calculated from concentrations that showed significant biofilm disruption activity, and the MBDC value determined.

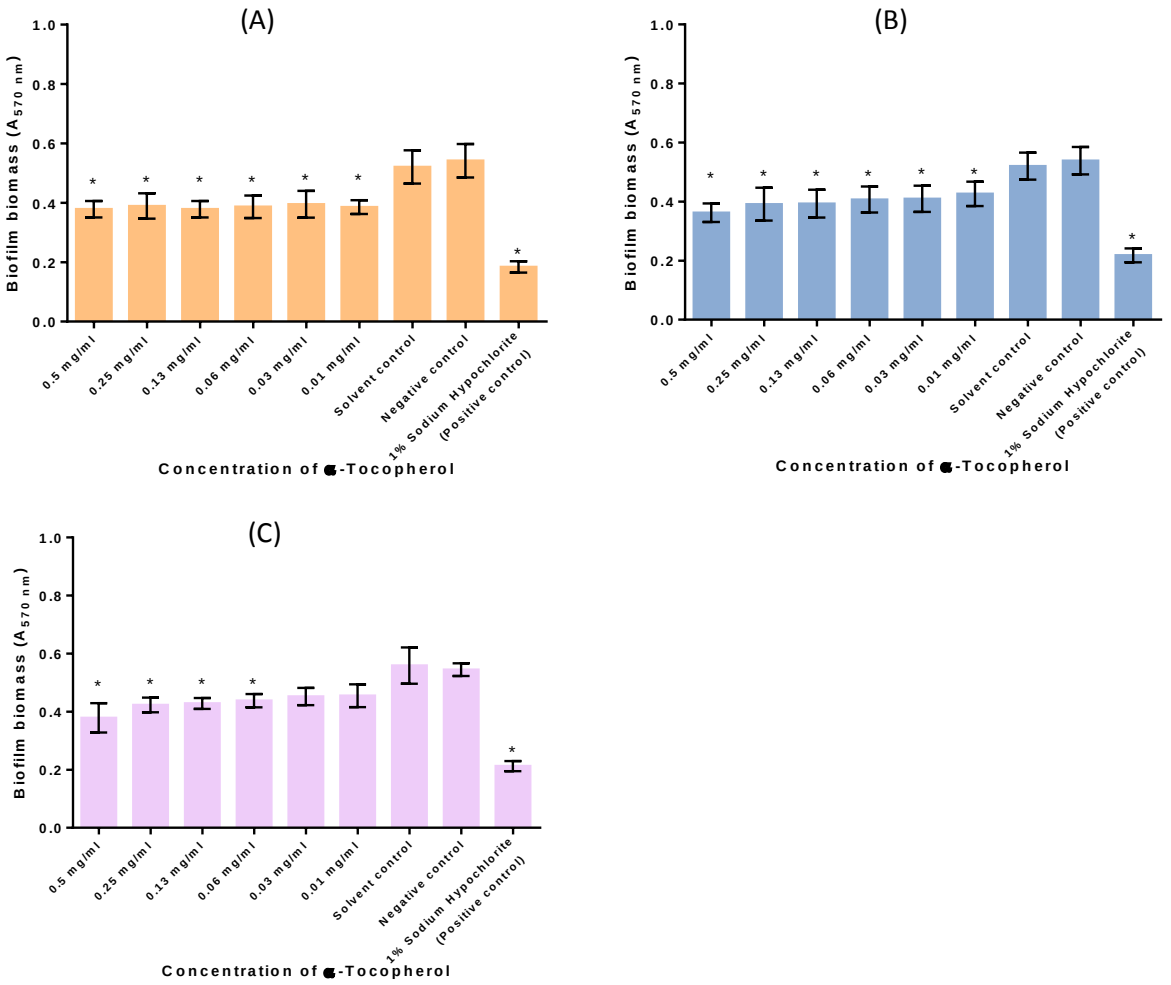


Figure 5-12: Biofilm disruption activity of α-tocopherol at various concentrations against polymicrobial biofilms: (A) *S. aureus* ATCC 43300 + *E. faecalis* ATCC 700802; (B) *S. aureus* ATCC 33591 + *E. faecalis* ATCC 700802 and (C) *S. aureus* ATCC 29213 + *E. faecalis* ATCC 700802. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against various concentrations of α-tocopherol. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm disruption activity when compared with the solvent control.

Table 5-5: Percentage biofilm disruption by α-tocopherol and the minimum biofilm disruption concentration (MBDC) against polymicrobial *S. aureus* + *E. faecalis* biofilms

Polymicrobial biofilm	Percentage biofilm disruption (%)	MBDC (mg/ml)
S. aureus ATCC 43300 + *E. faecalis* ATCC 700802	25.0 ± 12.7	0.01
S. aureus ATCC 33591 + *E. faecalis* ATCC 700802	22.8 ± 11.7	0.01
S. aureus ATCC 29213 + *E. faecalis* ATCC 700802	22.8 ± 6.4	0.06

*Percentage biofilm disruption (%) = average percentage of biofilm disruption by α-tocopherol from concentrations that showed significant biofilm disruption activity

*Minimum biofilm disruption concentration (MBDC) in mg/ml

Based on Figure 5-12, α-tocopherol showed significant biofilm disruption activity at 0.01 – 0.5 mg/ml for both polymicrobial biofilms of *S. aureus* ATCC 43300 + *E. faecalis* ATCC 700802 and *S. aureus* ATCC 33591 + *E. faecalis* ATCC 700802, and at 0.06 – 0.5 mg/ml for *S. aureus* ATCC 29213 + *E. faecalis* ATCC 700802. The average percentage of biofilm disrupted by α-tocopherol from concentrations that showed significant biofilm disruption activity was calculated and it was determined that α-tocopherol was able to disrupt 22-25% of the polymicrobial biofilms of *S. aureus* and *E. faecalis* (Table 5-5).

The MBDC for α-tocopherol against the polymicrobial biofilms of *S. aureus* ATCC 43300 + *E. faecalis* ATCC 700802 and *S. aureus* ATCC 33591 + *E. faecalis* ATCC 700802 was 0.01 mg/ml, while the MBDC for *S. aureus* ATCC 29213 + *E. faecalis* ATCC 700802 was 0.06 mg/ml (Table 5-5). From these results, it can be deduced that effective disruption of polymicrobial biofilms consisting of *S. aureus* and *E. faecalis* can be achievable with a concentration as low as 0.01 mg/ml of α-tocopherol. Since strain variation effect was observed for the MBDC values, it is important to further test with more strains of *S. aureus* and *E. faecalis* as the MBDC may differ between strains. Besides that, other polymicrobial biofilms consisting of *S. aureus* with other bacteria such as *C. albicans, P. aeruginosa* and *S. pneumoniae* should also be tested to assess the efficacy of α-tocopherol against these polymicrobial biofilms (Nair *et al.*, 2014).

5.4.5 Combination of α-tocopherol with antibiotic

Vancomycin is a glycopeptide antibiotic used for the treatment of gram positive bacterial infections, including *S.* aureus (Gardete and Tomasz, 2014). However, vancomycin is a large molecule with poor penetration into *S. aureus* biofilms (Deresinski, 2009; Jefferson *et al.*, 2005; Singh *et al.*, 2010). Vancomycin is able to disrupt biofilms, but only with very little effect and requiring about 24 hours or more to disrupt *S. aureus* biofilms (Amorena *et al.,* 1999; Wu *et al.,* 2003).

Based on Section 5.4.2, it has been shown that α-tocopherol affects the biofilm matrix in order to disrupt *S. aureus* biofilms but does not affect the cells within the biofilms. Therefore, hypothetically, in combining α-tocopherol and vancomycin for treatment, α-tocopherol would function to disrupt the biofilm matrix and with very little disruption effect by vancomycin, would allow increased penetration of vancomycin to target the cells within biofilms. It was of interest to observe whether a greater biofilm disruption effect can be achieved at a lower dosage when α-tocopherol and vancomycin are used in combination, which will also reduce the chances for the emergence of antibacterial resistance.

In this study, the checkerboard assay was used to evaluate the effects of α-tocopherol and vancomycin when used in combination against *S. aureus* biofilms, using crystal violet assay to assess biofilm disruption activity. The biofilm disruption activity of α-tocopherol and vancomycin used in combination against four *S. aureus* biofilms were presented in Figure 5-13 to Figure 5-16.

Based on Figure 5-13 to Figure 5-16, biofilm disruption activity was observed, as shown by the significant reduction in biofilm biomass when compared with the solvent control. The solvent control was not significantly different when compared with the negative control indicating that the solvent used to dissolve α-tocopherol and vancomycin was not influencing the biofilm disruption effect observed. From each of these figures, the MBDC for each *S. aureus* strains were determined and used for the calculation of the fractional biofilm eradication concentration (FBEC) index. The combinational effects of α-tocopherol and vancomycin was expressed as the FBEC index and the interaction established by the FBEC index. Table 5-6 shows the FBEC index and the established interaction between α-tocopherol and vancomycin towards the four *S. aureus* biofilms.

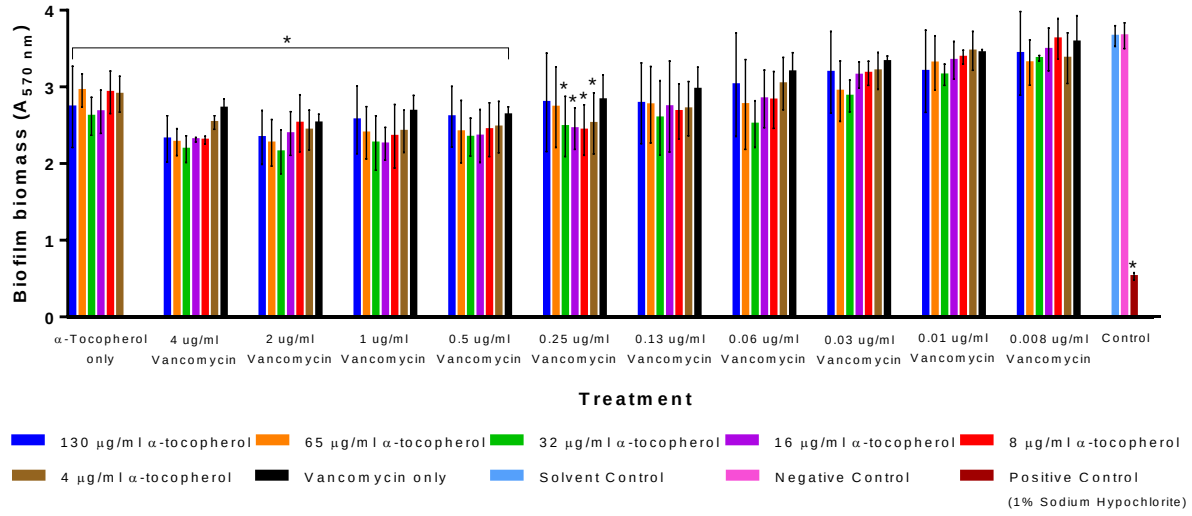


Figure 5-13: The combination effects of α-tocopherol and vancomycin towards the biofilm biomass of *S. aureus* ATCC 6538P determined using checkerboard assay. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against various treatments. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm disruption activity (reduction in biofilm biomass) when compared with the solvent control. The MBDC of α-tocopherol, Aa was 4 µg/ml while vancomycin, Ba was 0.5 µg/ml. The MBDC of α-tocopherol and vancomycin used in combination, Ac and Bc were 4 µg/ml and 0.25 µg/ml, respectively. FBEC index = Ac/Aa + Bc/Ba = 4/4 + 0.25/0.5 = 1.5 (indifferent interaction).

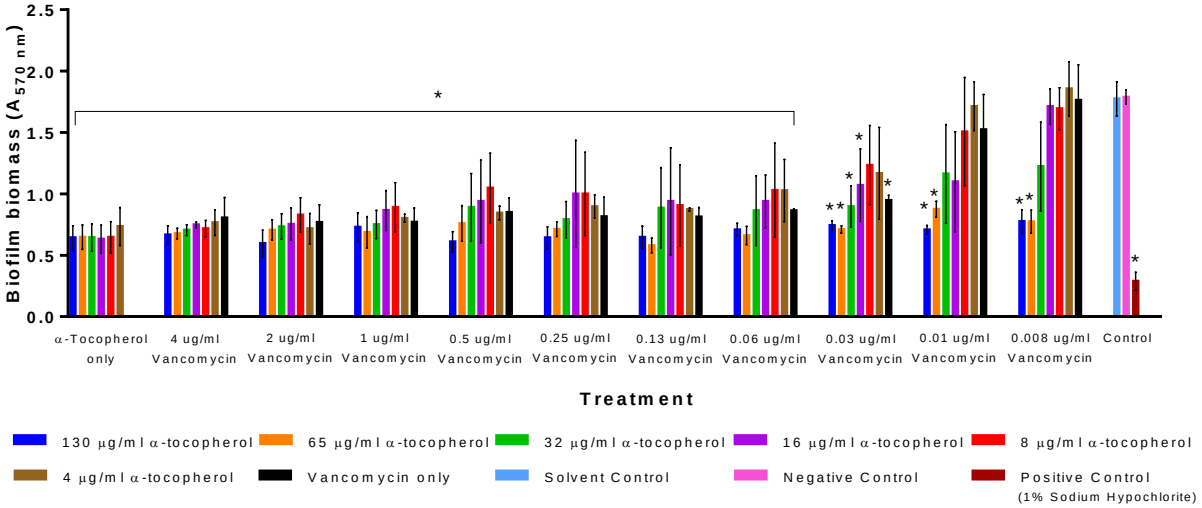


Figure 5-14: The combination effects of α-tocopherol and vancomycin towards the biofilm biomass of *S. aureus* ATCC 43300 determined using checkerboard assay. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against various treatments. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm disruption activity (reduction in biofilm biomass) when compared with the solvent control. The MBDC of α-tocopherol, Aa was 4 µg/ml while vancomycin, Ba was 0.03 µg/ml. The MBDC of α-tocopherol and vancomycin used in combination, Ac and Bc were 16 µg/ml and 0.03 µg/ml, respectively. FBEC index = Ac/Aa + Bc/Ba = 16/4 + 0.03/0.03 = 4 + 1 = 5 (antagonistic interaction).

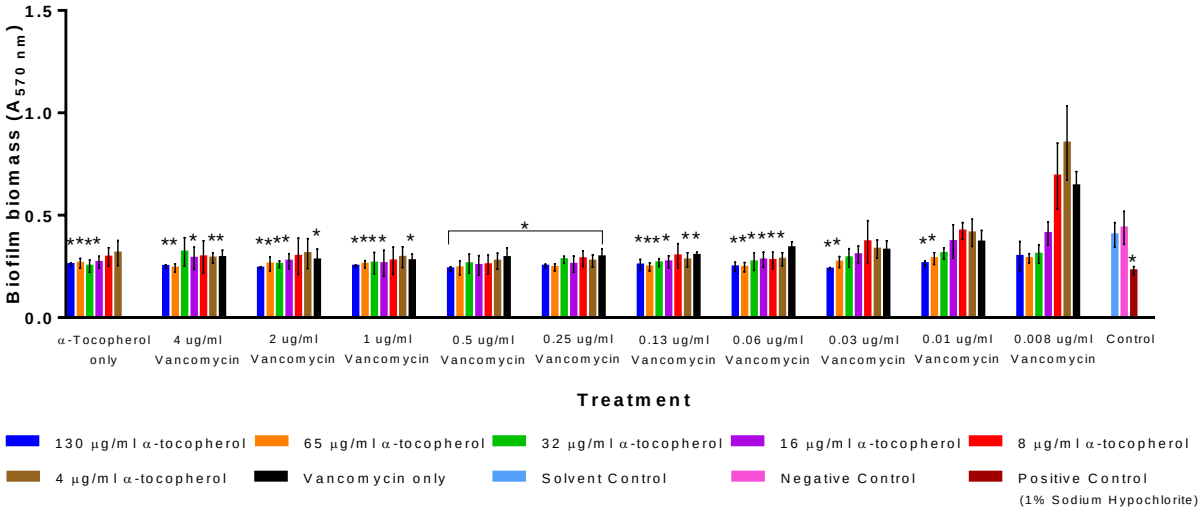


Figure 5-15: The combination effects of α-tocopherol and vancomycin towards the biofilm biomass of *S. aureus* ATCC 33591 determined using checkerboard assay. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against various treatments. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm disruption activity (reduction in biofilm biomass) when compared with the solvent control. The MBDC of α-tocopherol, Aa was 4 µg/ml while vancomycin, Ba was 0.13 µg/ml. The MBDC of α-tocopherol and vancomycin used in combination, Ac and Bc were 4 µg/ml and 0.06 µg/ml, respectively. FBEC index = Ac/Aa + Bc/Ba = 4/4 + 0.06/0.13 = 1 + 0.4 = 1.4 (indifferent interaction).

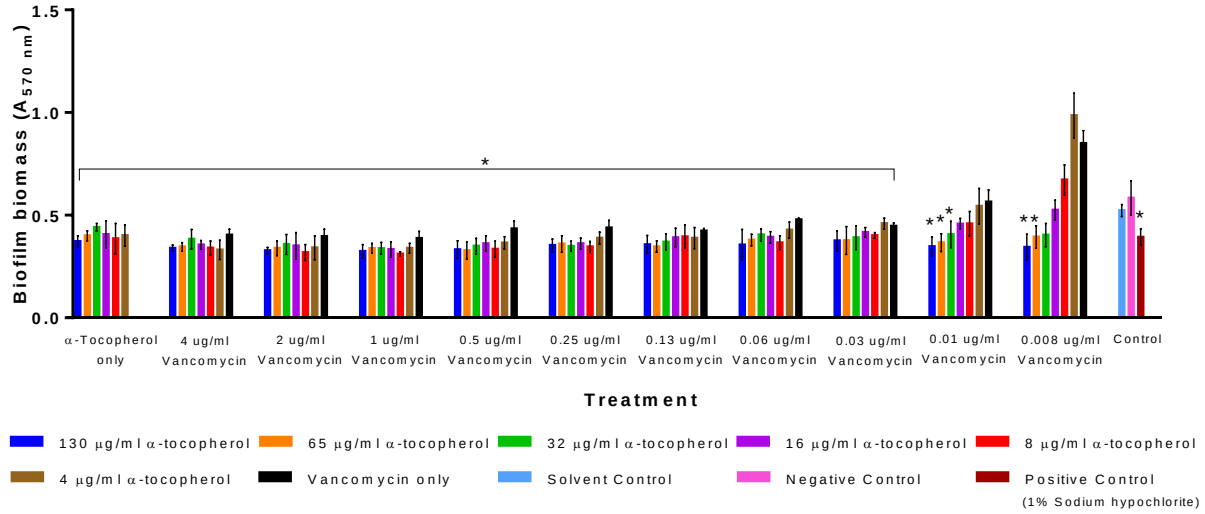


Figure 5-16: The combination effects of α-tocopherol and vancomycin towards the biofilm biomass of *S. aureus* ATCC 29213 determined using checkerboard assay. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against various treatments. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm disruption activity (reduction in biofilm biomass) when compared with the solvent control. The MBDC of α-tocopherol, Aa was 4 µg/ml while vancomycin, Ba was 0.03 µg/ml. The MBDC of α-tocopherol and vancomycin used in combination, Ac and Bc were 4 µg/ml and 0.03 µg/ml, respectively. FBEC index = Ac/Aa + Bc/Ba = 4/4 + 0.03/0.03 = 1 + 1 = 2 (indifferent interaction).

Table 5-6: The FBEC index and the established interaction between α-tocopherol and vancomycin towards *S. aureus* biofilms.

Strains	Combination		FBEC index	Interaction
	Ac/Aa	Bc/Ba		
S. aureus ATCC 6538P	1.0	0.5	1.5	Indifferent
S. aureus ATCC 43300	4.0	1.0	5.0	Antagonistic
S. aureus ATCC 33591	1.0	0.4	1.4	Indifferent
S. aureus ATCC 29213	1.0	1.0	2.0	Indifferent

* FBEC index = $\frac{Ac}{Aa} + \frac{Bc}{Ba}$, whereby *Aa* and *Ba* are the MBDC values of α-tocopherol and vancomycin when used separately whereas *Ac* and *Bc* are the MBDC of α-tocopherol and vancomycin when used in combination

* FBEC index is defined as synergistic at ≤0.5, additive at >0.5 - 1.0, indifferent at >1.0 – 2.0 and antagonistic at >2.0

In combination therapy, the combination is considered to be synergistic if the effect of the combination of agents is greater than the effect of either agent when used alone or greater than the total effects of the individual agents. The interaction is additive when the effect of the combination of agents is the sum of the effects of the individual agents. The interaction is indifferent when the combination of agents provides an effect equal to the effect of either agent used alone; i.e., the more active agent is indifferent to the presence of a second agent. The combination is considered as

antagonistic if the combination is less effective than either agent when used alone, or less than the total effects of the individual agents (Marymont and Marymont, 1981; Sweeney and Zurenko, 2003).

Based on Table 5-6, the FBEC index for the combination of α-tocopherol and vancomycin against *S. aureus* ATCC 6538P, *S. aureus* ATCC 33591 and *S. aureus* ATCC 29213 was 1.5, 1.4 and 1.0, respectively, indicative of an indifferent interaction. This indicates that the combination of α-tocopherol and vancomycin had provided an effect equal to the effect of either α-tocopherol and vancomycin when used alone, whereby α-tocopherol and vancomycin are indifferent to the presence of each other. However, the FBEC index against *S. aureus* ATCC 43300 was 3.5, indicating an antagonistic interaction. The combination of α-tocopherol and vancomycin against *S. aureus* ATCC 43300 was less effective than either α-tocopherol and vancomycin when used alone or less than the sum of the effects of α-tocopherol and vancomycin.

It can be deduced that the combination of α-tocopherol and vancomycin was indifferent to the presence of each other in reducing the biofilm biomass of *S. aureus*. The combination of α-tocopherol and vancomycin would not cause a greater effect in disrupting biofilm as compared to using either α-tocopherol and vancomycin alone. Since vancomycin can only cause very little effect in disrupting biofilms, the effect observed when α-tocopherol and vancomycin were used in combination might be due mostly to the disruption effect by α-tocopherol.

However, there was also the possibility of the combination effect being antagonistic as shown by its effect towards *S. aureus* ATCC 43300. Therefore, more analyses have to be conducted to verify whether indifferent or antagonistic effects would be applicable when α-tocopherol is combined with other antibiotics. Besides that, more strains could be tested to verify whether the indifference or antagonistic effect observed was strain specific.

As stated previously, hypothetically, in combining α-tocopherol and vancomycin for treatment, α-tocopherol would function to disrupt the biofilm matrix and with very little disruption effect by vancomycin, would allow increased penetration of vancomycin to target the cells within biofilms. Cells within biofilms are 1000-fold more resistant to antibiotics and represents a significant hurdle for antibiotic treatment (Rogers *et al.,* 2010). Therefore, the effects of α-tocopherol and vancomycin when used in combination was assessed against cells within biofilms, using resazurin assay to quantify cell viability. Figure 5-17 to Figure 5-20 shows the quantification of viable cells within biofilms after treatment with α-tocopherol and vancomycin when used alone and in combination against four *S. aureus* strains.

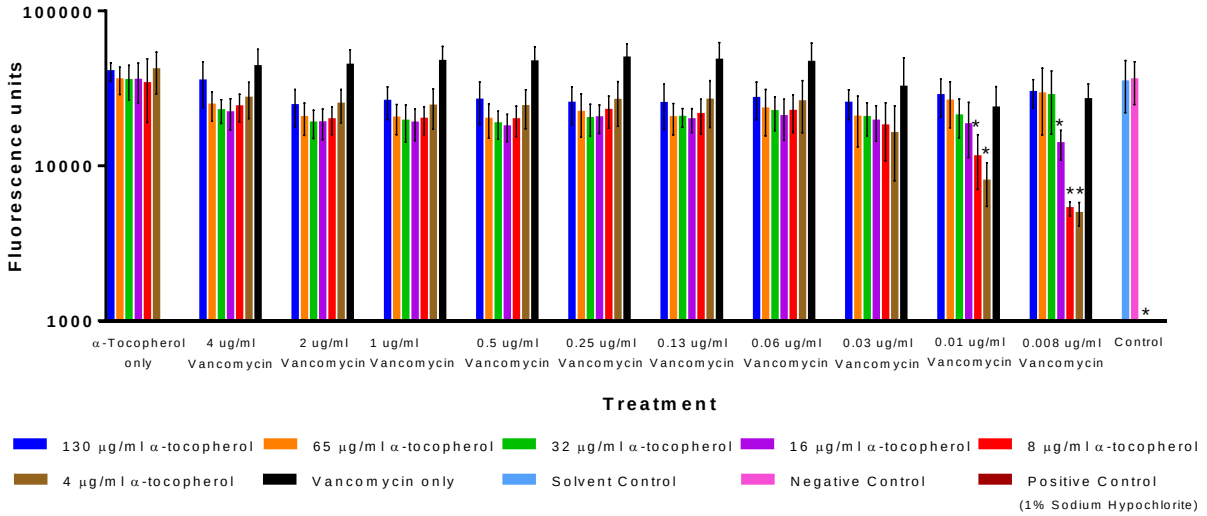


Figure 5-17: Quantification of viable cells within *S. aureus* ATCC 6538P biofilm for combinational treatment of α-tocopherol and vancomycin using checkerboard assay. Mean fluorescence units ± SD plotted against various treatments. * denotes statistically significant difference at $p<0.05$ and shows reduction in cell viability when compared with the solvent control. Almost all treatments showed no reduction in cell viability except for five combinations of α-tocopherol and vancomycin at lower concentrations, which showed slight reduction in cell viability.

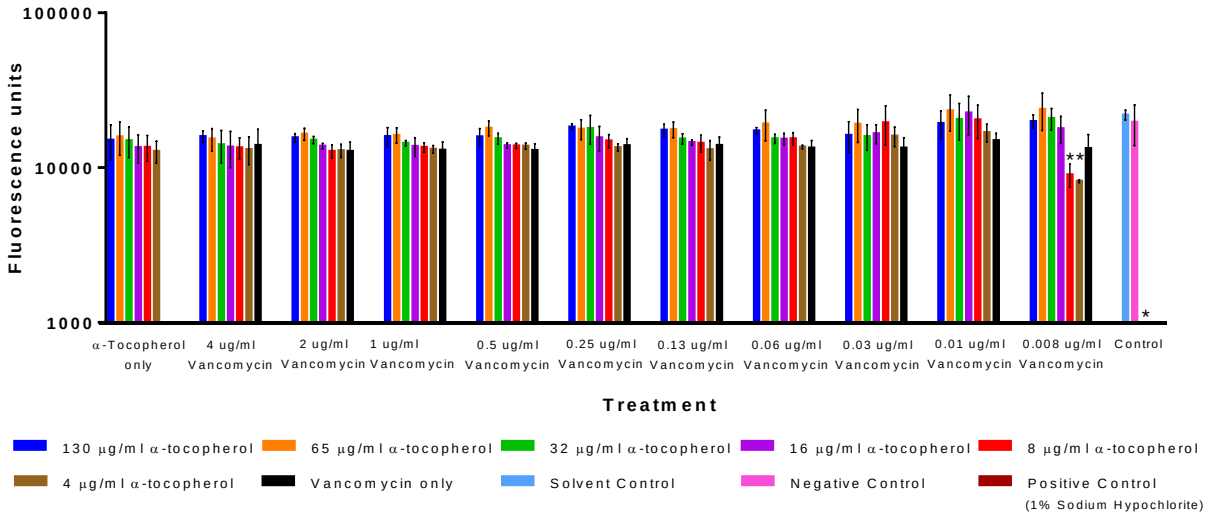


Figure 5-18: Quantification of viable cells within *S. aureus* ATCC 43300 biofilm for combinational treatment of α-tocopherol and vancomycin using checkerboard assay. Mean fluorescence units ± SD plotted against various treatments. * denotes statistically significant difference at $p<0.05$ and shows reduction in cell viability when compared with the solvent control. Almost all treatments showed no reduction in cell viability except for two combinations of α-tocopherol and vancomycin at lower concentrations, which showed slight reduction in cell viability.

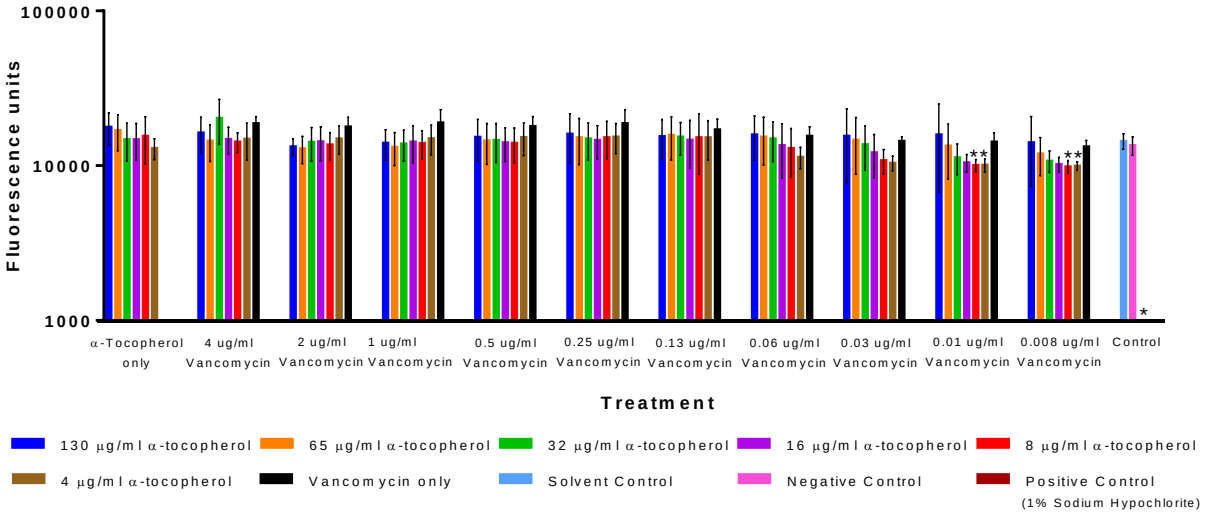


Figure 5-19: Quantification of viable cells within *S. aureus* ATCC 33591 biofilm for combinational treatment of α-tocopherol and vancomycin using checkerboard assay. Mean fluorescence units ± SD plotted against various treatments. * denotes statistically significant difference at $p<0.05$ and shows reduction in cell viability when compared with the solvent control. Almost all treatments showed no reduction in cell viability except for four combinations of α-tocopherol and vancomycin at lower concentrations, which showed slight reduction in cell viability.

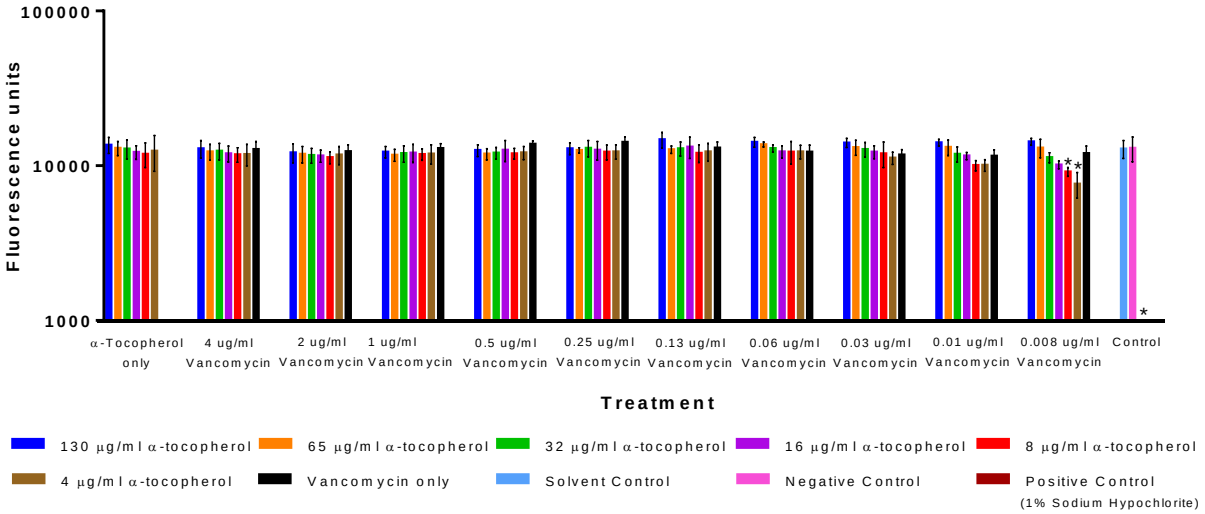


Figure 5-20: Quantification of viable cells within *S. aureus* ATCC 29213 biofilm for combinational treatment of α-tocopherol and vancomycin using checkerboard assay. Mean fluorescence units ± SD plotted against various treatments. * denotes statistically significant difference at $p<0.05$ and shows reduction in cell viability when compared with the solvent control. Almost all treatments showed no reduction in cell viability except for two combinations of α-tocopherol and vancomycin at lower concentrations, which showed slight reduction in cell viability.

Based on Figure 5-17 to Figure 5-20, it can be observed that when α-tocopherol and vancomycin were each used alone, there was no reduction in cell viability, indicating that the cells within biofilms were not killed or inhibited. This agrees to previous results on α-tocopherol not affecting the cells within biofilms (Section 5.4.2.2) and to reports of vancomycin having reduced penetration through *S. aureus* biofilms (Jefferson *et al.,* 2005; Singh *et al.,* 2010). For combination treatment, almost all showed no reduction in cell viability. This indicates that the combination of α-tocopherol and vancomycin had not allowed the increased penetration of vancomycin to target the cells within biofilms. However, it was observed that a few combinations of α-tocopherol and vancomycin at lower concentrations, had showed significant reduction in cell viability.

One of the combination treatments that showed significant reduction in cell viability was the combination of 4 μg/ml of α-tocopherol + 0.008 μg/ml of vancomycin. In order to further confirm the observed reduction in cell viability, this combination treatment was subjected for colony count measurement to determine the colony forming unit (CFU) per milliliter (CFU/ml).

Besides that, the highest and lowest concentrations for single treatments, i.e. 130 μg/ml (8×MBDC) and 4 μg/ml of α-tocopherol, and 4 μg/ml and 0.008 μg/ml of vancomycin, and the combination of 130 μg/ml of α-tocopherol + 4 μg/ml of vancomycin were also included for comparisons. These treatments did not show reduction in cell viability. Table 5-7 presents the viable count for single and combination treatments of α-tocopherol and vancomycin against four *S. aureus* biofilm strains.

Quantification with resazurin is fast, inexpensive and is an indirect method to measure cell viability since it is based on the reduction of the resazurin dye to detect bacterial metabolic activity (Peeters *et al.,* 2008). Colony count measurement to determine the CFU/ml provides visual appearance of viable cells (Yousef and Carlstrom, 2003), enabling direct counting of viable cells and therefore, can support results obtained from quantification by resazurin assay.

Table 5-7: Viable count (CFU/ml) for selected combinational treatments of α-tocopherol and vancomycin.

Treatment	Viable count (CFU/ml)			
	***S. aureus* ATCC 6538P**	***S. aureus* ATCC 43300**	***S. aureus* ATCC 33591**	***S. aureus* ATCC 29213**
130 µg/ml α-tocopherol (8×MBDC)	$5.9 \pm 1.2 \times 10^7$	$6.1 \pm 0.9 \times 10^6$	$5.4 \pm 1.3 \times 10^7$	$2.8 \pm 0.9 \times 10^6$
4 µg/ml α-tocopherol	$3.9 \pm 2.4 \times 10^7$	$2.5 \pm 1.1 \times 10^6$	$8.3 \pm 3.7 \times 10^7$	$2.7 \pm 0.3 \times 10^6$
4 ug/ml vancomycin	$6.6 \pm 1.6 \times 10^7$	$2.0 \pm 0.8 \times 10^6$	$1.8 \pm 0.8 \times 10^8$	$2.7 \pm 0.2 \times 10^6$
0.008 ug/ml vancomycin	$8.0 \pm 1.7 \times 10^7$	$1.5 \pm 0.8 \times 10^6$	$7.9 \pm 2.5 \times 10^7$	$3.5 \pm 2.0 \times 10^6$
130 µg/ml α-tocopherol + 4 ug/ml vancomycin	$3.0 \pm 1.3 \times 10^7$	$2.0 \pm 0.6 \times 10^6$	$3.2 \pm 0.3 \times 10^7$ *	$2.3 \pm 0.4 \times 10^6$
4 µg/ml α-tocopherol + 0.008 ug/ml vancomycin	$1.9 \pm 1.7 \times 10^5$ *	$2.3 \pm 1.5 \times 10^4$ *	$7.2 \pm 6.7 \times 10^6$ *	$5.4 \pm 3.7 \times 10^5$ *
Negative Control	$8.0 \pm 0.8 \times 10^7$	$2.7 \pm 1.2 \times 10^6$	$1.7 \pm 0.3 \times 10^8$	$1.7 \pm 0.1 \times 10^6$
Solvent Control	$3.9 \pm 0.7 \times 10^7$	$3.9 \pm 1.7 \times 10^6$	$6.1 \pm 1.9 \times 10^7$	$3.3 \pm 0.9 \times 10^6$
Positive Control	0.0 ± 0.0 *	0.0 ± 0.0 *	0.0 ± 0.0 *	0.0 ± 0.0 *

Mean CFU/ml ± SD from triplicates of an independent experiment. * indicates statistically significant difference and shows reduction in viable count when compared with the solvent control ($p<0.05$)

The combination of 4 µg/ml of α-tocopherol + 0.008 µg/ml of vancomycin had showed reduction in cell viability when quantified using the resazurin assay (Figure 5-17 to Figure 5-20). These results were further supported by the significant reduction in viable count when the CFU/ml was determined (highlighted in Table 5-7). Besides that, as expected, treatment with α-tocopherol alone (130 µg/ml and 4 µg/ml) and vancomycin alone (4 µg/ml and 0.008 µg/ml) does not reduce viable count and this agrees with the cell viability results quantified using resazurin assay.

The effect of the combination of 0.13 mg/ml α-tocopherol + 4 ug/ml vancomycin did not cause any decrease in viable count, which matches the results quantified using resazurin assay. However, there was one discrepancy in result, in which this combination caused a slight reduction in viable count for the strain, *S. aureus* ATCC 33591. This contradicts the results quantified using the resazurin assay, which showed no reduction in cell viability.

The reduction in cell viability for the combination of 4 µg/ml of α-tocopherol + 0.008 µg/ml of vancomycin was unexpected as there was no reduction in biofilm biomass observed when treated at these combinations (Figure 5-13 to Figure 5-16). However, this result suggests that a combination of low concentrations of α-tocopherol and vancomycin; i.e. 4 µg/ml and 0.008 µg/ml, respectively, might be able to affect the viability of cells within *S. aureus* biofilm.

It is currently unknown why the combination of α-tocopherol and vancomycin at lower concentrations had affected cell viability. However, it was not due to a solvent effect. The solvent

control was not significantly different when compared with the negative control, indicating that the solvent used to dissolve α-tocopherol and vancomycin did not influence the observed reduction in viable count. More analyses would need to be conducted to explain the effect on cell viability. Furthermore, HPLC analysis on α-tocopherol and vancomycin alone and when in combination could be analysed to detect any possible interaction between the two compounds when in combination. Investigations on the combination of α-tocopherol with other antibiotics could also be done to verify whether similar effects on cell viability would occur.

5.5 Conclusion

In conclusion, investigation on the anti-biofilm activity of α-tocopherol demonstrated the presence of biofilm disruption activity of α-tocopherol at 0.01 – 0.5 mg/ml, and confirms its lack of biofilm inhibition activity. Currently, there has not been any study reported on the biofilm disruption effect of α-tocopherol. This will be the first study to report on the biofilm disruption activity of α-tocopherol against *S. aureus* biofilms or any other bacterial biofilms.

Further analysis on the biofilm disruption effect of α-tocopherol revealed that α-tocopherol does not affect the cells within the biofilms but instead affects the biofilm matrix in order to disrupt *S. aureus* biofilms. The time-course experiment showed that α-tocopherol exhibited a slow disruption effect, requiring minimum incubation time of 10 – 16 hours.

Besides *S. aureus*, α-tocopherol was also effective in disrupting *E. faecalis* (23% disruption) and *E. coli* (31% disruption) in monomicrobial biofilms, with both having MBDC of 0.01 mg/ml. α-tocopherol was also effective in disrupting the polymicrobial biofilms consisting of *S. aureus* and *E. faecalis* with it being effective against three out of four polymicrobial biofilms of *S. aureus* + *E. faecalis* tested (22-25% disruption).

The combination of α-tocopherol with vancomycin had showed indifference effect against three *S. aureus* strains while another showed antagonistic effect. The combination of α-tocopherol and vancomycin was indifferent to the presence of each other in reducing the biofilm biomass of *S. aureus* and would not cause a greater effect in disrupting biofilm as compared to using either α-tocopherol and vancomycin alone. Additionally, when α-tocopherol and vancomycin was tested against the cells within biofilm, the combination of α-tocopherol and vancomycin at low concentrations (4 μg/ml of α-tocopherol + 0.008 μg/ml of vancomycin) was shown to affect the viability of cells within *S. aureus* biofilms.

Chapter 6 Overall conclusion & future work

6.1 Overall conclusion

Dicranopteris linearis was determined to exhibit both antibacterial and anti-biofilm activities against *S. aureus*. The antibacterial activity of *D. linearis* was assessed against multiplying and non-multiplying cells of *S. aureus*. Through broth microdilution assay, the methanol crude extracts (MCE) (MCE(L) and MCE(R)) of *D. linearis* showed antibacterial activity against *S. aureus*. Through time-kill assays, MCE(L) exhibited bactericidal activity against the non-multiplying cells of *S. aureus,* when tested against growth arrested cultures caused by nutrient depletion and protein synthesis inhibition. It was also determined that MCE(L) was not effective at cold temperatures. It was determined that active protein synthesis may not be required in the mechanism of action against non-multiplying cells of *S. aureus*.

Among the fractions obtained from sequential solvent extraction (SSE) of *D. linearis*, only MeOH fraction showed antibacterial activity. When compared to MCE, the antibacterial activity of *D. linearis* did not improve after fractionation with SSE and therefore, further characterization of MeOH fraction and its antibacterial activity was not conducted.

For anti-biofilm activity of *D. linearis*, both biofilm inhibition and biofilm disruption activities were assessed. Among the MCE and SSE fractions tested, H_2O fraction was the most effective for biofilm inhibition activity while HEX fraction was the most effective for biofilm disruption activity, as they showed activity against all five *S. aureus* biofilm strains tested. Currently, there are no studies in literature on the anti-biofilm effect of *D. linearis*. This will be the first study to report on the anti-biofilm activity of *D. linearis*, for both biofilm inhibition and biofilm disruption activities.

H_2O fraction did not inhibit cell growth, thus the biofilm inhibition effect observed was only due to the biofilm structure itself or the genes that codes for the biofilm. H_2O fraction was able to inhibit *S. aureus* biofilm formation on various polymer materials commonly used in medical settings: polystyrene (85-93% inhibition), polyvinyl chloride (76-91% inhibition), polyethylene (68-90% inhibition); polypropylene (52-93% inhibition), silicone rubber (68-94% inhibition. H_2O fraction does not modify surface properties of polystyrene to prevent adhesion but may be involved in changing the cell surface hydrophobicity of *S. aureus* to inhibit biofilm formation on surfaces. The presence of various phytochemicals such as flavonoids, terpenoids, tannins, cardiac glycosides, phenols, quinones and saponins were identified in H_2O fraction. However, further purification and isolation of H_2O fraction was

not conducted due to difficulties in identifying the specific phytochemical responsible for the biofilm inhibition effect.

HEX fraction was able to disrupt about 42-75% of *S. aureus* biofilms. MBDC of HEX fraction ranges between 0.07-1.25 mg/ml against *S. aureus* biofilm. HEX fraction did not inhibit cell growth, thus the biofilm disruption effect observed was only due to the biofilm structure itself or the genes that codes for the biofilm. Through scanning electron microscopy, HEX fractions demonstrated destruction of the biofilm structure and reduced biofilms attached to the surface, with scant biofilms observed. Few phytochemicals were identified in HEX fraction, and thus, HEX fraction was selected for further purification and isolation process. Purification of HEX fraction using silica column chromatography, acetonitrile fractionation and HPLC analysis had yielded Fraction A. Based on NMR spectroscopy and LC-MS data, the compound from Fraction A was identified as α-tocopherol.

α-Tocopherol was tested for anti-biofilm activity and was found to exhibit biofilm disruption activity against *S. aureus* biofilms. Currently, there has not been any study reported on the biofilm disruption effect of α-tocopherol. Therefore, this will be the first study to report on the biofilm disruption activity of α-tocopherol against *S. aureus* biofilms or any other bacterial biofilms.

Further analysis on the biofilm disruption effect of α-tocopherol revealed its effect against the biofilm matrix and not against the cells within biofilms. Besides that, α-tocopherol exhibited a slow disruption effect, requiring minimum incubation time of 10 – 16 hours. Besides *S. aureus*, α-tocopherol was also effective in disrupting *E. faecalis* (23% disruption) and *E. coli* (31% disruption) in monomicrobial biofilms, and the polymicrobial biofilms consisting of *S. aureus* and *E. faecalis* (22-25% disruption). α-Tocopherol was also evaluated for its combination effect with antibiotic. The combination of α-tocopherol with vancomycin had mostly showed indifference effect towards the disruption of biofilm biomass. The combination of α-tocopherol and vancomycin was indifferent to the presence of each other in reducing the biofilm biomass of *S. aureus* and would not cause a greater effect in disrupting biofilm as compared to using either α-tocopherol and vancomycin alone. Additionally, the combination of α-tocopherol and vancomycin at low concentrations (4 µg/ml of α-tocopherol + 0.008 µg/ml of vancomycin) was shown to affect the viability of cells within *S. aureus* biofilms.

6.2 Future work

Several approaches can be conducted in the future as a continuation of this study.

Biofilm disruption activity of α-tocopherol

1. Biofilm disruption activity of various α-tocopherol derivatives

Several α-tocopherol derivatives have been synthesized for usage in supplements and cosmetics (Zingg, 2007). Tocophersolan is a polyethylene glycol derivative of α-tocopherol that is water soluble and used as a vitamin E supplement or to treat vitamin E deficiency in individuals who cannot absorb fats due to disease (Ash and Ash, 2004; Robin, 2015). Acetate and succinate esters of α-tocopherol such as α-tocopherol succinate (α-TOS) and α-tocopherol oxyacetic acid (α-TEA) are used as vitamin E sources in commercial supplements as they are more stable in the presence of air (oxygen) (Litwack, 2007; Zingg, 2007). The biofilm disruption activity of these α-tocopherol derivatives towards *S. aureus* biofilms could be investigated and their activity compared with the synthetic α-tocopherol used in this study.

2. Mechanism of action studies

Currently, the commonly known mechanisms in relation to disruption of biofilms are related to the solubilisation of the biofilm matrix components (DNA, protein, polysaccharides). Compounds such as DNases, dispersin B, and protease K have been reported to degrade DNA, polysaccharides and proteins matrix, respectively (Boles *et al.,* 2011). It is possible that α-tocopherol may act in a similar way like these compounds. Besides that, since previous studies have shown that alpha-tocopherol can have an effect on gene expression, there is also the possibility of α-tocopherol targeting the biofilm related genes controlling the production of matrix degrading enzymes, which can result to the disassembly of established biofilms. The only known molecular mechanism of staphylococci biofilm disassembly is controlled by a cyclic autoinducing peptide (AIP) (Boles *et al.,* 2011). Additionally, the effects of α-tocopherol against biofilm will also be elucidated with the aid of microscopy techniques such as scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM).

- ***Quantification of the biofilm matrix components***

  In order to identify the biofilm matrix components specifically targeted by α-tocopherol, quantifications using NanoQuant, Bradford assay and phenol sulfuric acid assay will be used to quantify DNA, proteins and polysaccharides, respectively (Chiba *et al.,* 2014; Jiao *et al.,* 2010). One

or more components reduced when treated with α-tocopherol would indicate the possibility of α-tocopherol solubilizing the particular matrix component(s).

- ***Quantitative PCR analysis of biofilm gene expression***

  In order to ascertain the possibility of a molecular mechanism of action, the expression levels of biofilm related genes after treatment with α-tocopherol are defined using quantitative PCR (qPCR) analysis (Atshan *et al.,* 2013; Resch *et al.,* 2005). If α-tocopherol has an effect on the gene expression of biofilm related genes, it may affect the expression of genes controlling the production of matrix degrading enzymes, resulting to the disassembly of established biofilms. Differential expression (upregulation or downregulation) of genes is expected to be observed if α-tocopherol affects the gene expression of biofilm related genes.

- ***Scanning electron microscopy (SEM)***

  Scanning electron microscopy has been widely used to visualise the structure of biofilms. SEM provides information about the morphology of biofilm, the thickness of biofilm and the presence of EPS (Kerekes *et al.*, 2013). It can be used to investigate the structural modifications of biofilms after treatment with α-tocopherol.

- ***Confocal laser scanning microscopy (CLSM)***

  SEM analysis of biofilms, though useful, often involves a dehydration and fixation step by necessity. The exopolysaccharide matrix in the biofilms will be reduced to a fraction of its original volume. This sometimes results in some loss of 3D information. Therefore, in order to further understand the mechanism of action of α-tocopherol, CLSM analysis can be performed. CLSM has been widely used to study the biofilm structure, composition and metabolism in several different microorganisms. CLSM allows in-depth analysis of the biofilm structures, viability and biomass changes when in contact with α-tocopherol. CLSM does not kill or damage the biological structure and does not dehydrate the EPS matrix unlike SEM analysis. Live/dead staining is included for indicator of cell viability (Cerca *et al.*, 2012).

3. Biofilm disruption effect of α-tocopherol on polymer surfaces

As mentioned previously, medical devices are susceptible to bacterial colonization, which pose an important public health concern. When these devices are implanted, they become a site for bacterial adhesion, colonization and infection. Medical devices made using polymer material were listed in Table 3-8. The biofilm disruption effect of α-tocopherol could be evaluated against pre-formed *S. aureus*

biofilms on these polymer materials: polystyrene, poly-vinyl chloride, polyethylene, polypropylene, and silicone rubber.

4. Biofilm disruption effect of α-tocopherol against *S. aureus* biofilm infections *in vivo*

It is standard practice in many institutions to remove catheters and begin antibiotic treatment as soon as a staphylococcal infection is suspected. While this treatment may be possible for many catheters, it more problematic for other indwelling devices such as artificial heart valves or prosthetic joints. Infections are treated with long-term antibiotics in an attempt to eradicate the infections and this might lead to the possibility of development of antibiotic resistance. Since α-tocopherol can disrupt *S. aureus* biofilms *in vitro*, it is possible that α-tocopherol may also be effective against *S. aureus* biofilm infections *in vivo.* Furthermore, if α-tocopherol would show effective biofilm disruption effect on the polymer materials used to make catheters, it would indicate the possibility of α-tocopherol eradicating established biofilm from indwelling medical devices. Therefore, the effectiveness of α-tocopherol for the *in vivo* clearance of *S. aureus* biofilm-associated catheter infections could be investigated by using catheterized mouse model (Kokai-Kun *et al.,* 2009).

Other studies – Future work for H_2O fraction

1. Purification and identification of active compound(s) in H_2O fraction & mechanism of action studies

As discussed in Chapter 3, the H_2O fraction showed effective biofilm inhibition activity. Therefore, purification and identification of the active compound(s) in H_2O fraction that was responsible for the biofilm inhibition activity observed against *S. aureus* would be conducted. Besides that, the mechanism of action involved in the biofilm inhibition effect by the active compound(s) in H_2O fraction would be explored. Assays such as aggregation assay and anti-quorum sensing assay would be conducted. Aggregation is the second stage of biofilm development and therefore, aggregation assay could be conducted to determine whether the active compound(s) in H_2O fraction affects aggregation of cells to prevent biofilm formation. Quorum sensing has significant role in biofilm formation and the production of virulence factors. Quorum-sensing systems are important targets to address the sensitivity of bacteria to anti-biofilm compounds. The anti-quorum sensing assay could be performed to identify whether the active compound(s) in H_2O fraction inhibits the quorum sensing systems.

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Appendices

Appendix I

Standard procedures for phytochemical screening

Test for flavonoids

- Quercetin and water were used as positive control and negative control, respectively.
- ***Shinoda test***: The extract was dissolved in 95% ethanol, added with magnesium and treated with a few drops of concentrated hydrochloric acid. Formation of orange, pink, red to purple colours indicates the presence of flavones, flavonols, the corresponding 2, 3-dihydro derivatives and/or xanthones. By using zinc instead of magnesium, only flavanonols give a deep red to magenta colour, while flavanones and flavonols will give weak pink to magenta colours, or no colour at all (Jones & Kinghorn, 2006).

Test for terpenoids

- Dihydrocholesterol and water were used as positive control and negative control, respectively.
- ***Salkowski test***: The extract was dissolved in 1 ml of chloroform and 0.5 ml of concentrated sulphuric acid was carefully added. A reddish brown precipitate at interface suggests the presence of terpenoids while the appearance of golden yellow colour indicates the presence of triterpenes (Neelam *et al.*, 2014; Ugochukwu *et al.*, 2013).
- ***Liebermann-Burchard test***: The extract was dissolved in chloroform and the added with 1 ml of anhydrous acetic acid and 2 ml of concentrated sulphuric acid. The appearance of a reddish colour at the interface and a bluish green colour in the acetic acid layer suggests the presence of triterpenes (Jones & Kinghorn, 2006).

Test for tannins

- Tannic acid and water were used as positive control and negative control, respectively.
- ***Ferric chloride test***: The extract was added with a few drops of 5% alcoholic ferric chloride and the formation of a blue, blue-black or blue-green solution suggests for the presence of tannins (Jones & Kinghorn, 2006).
- ***Gelatin test***: The extract was added with 1% gelatin solution containing 0.5% NaCl. The formation of a white precipitate suggests the presence of tannins (Jones & Kinghorn, 2006).

Test for alkaloids

- Caffeine anhydrous and water were used as positive control and negative control, respectively.
- ***Wagner's reagent test***: The extract was treated with 3 – 5 drops of Wagner's reagent (1.27 g of iodine and 2 g of potassium iodide in 100 ml of water) and observed for the formation of reddish brown precipitate/ colouration, which suggests the presence of alkaloids (Jones & Kinghorn, 2006).

Test for cardiac glycosides

- ***Keller Killiani test***: The extract was treated with 1 ml of glacial acetic acid and a drop of ferric chloride solution was added to it. One ml of concentrated sulphuric acid was then added and the appearance of a brown ring at interface indicates the presence of deoxysugar characteristic of cardenolides (Ugochukwu *et al.*, 2013).
- ***Kedde test***: The extract was treated with one drop of 2% of 3, 5-dinitrobenzoic acid in MeOH and one drop of 5.7% aqueous potassium hydroxide. A bluish to purple colour will appear within 5 minutes, which indicates the presence of compounds containing α,β-unsaturated lactone functional groups (Jones & Kinghorn, 2006).

Test for phenols

- 4- methylcathecol and water were used as positive control and negative control, respectively.
- ***Ferric chloride test***: The extract was added with a few drops of 5% alcoholic ferric chloride and the formation of a blue, blue-black or blue-green solution indicates the presence of polyphenols (Jones & Kinghorn, 2006).

Test for sterols

- ***Liebermann-Burchard test***: The extract was treated with a solution containing 1 ml of anhydrous acetic acid and 1 ml of chloroform that was cooled to 0°C and added with one drop of concentrated sulphuric acid. The change in colour to blue, green, red or orange indicates the presence of a sterol backbone. A blue-greenish colour suggests the presence of Δ^5 sterols, with maximum intensity at 30 minutes (Jones & Kinghorn, 2006).
- ***Salkowski test***: The extract was dissolved in 1 ml of chloroform and was carefully added with 1 ml of concentrated sulphuric acid. Two phases were formed, with red or yellow colour indicating the presence of sterols and methylated sterols (Jones & Kinghorn, 2006).

Test for quinones

- The extract was treated with concentrated hydrochloric acid (HCl) and observed for the formation of yellow precipitate/ colouration, which suggests the presence of quinones (Ugochukwu *et al.*, 2013).

Test for saponins

- Sodium dodecyl sulphate (1%) and water were used as positive control and negative control, respectively.
- ***Foam test***: The extract was dissolved in water, shaken vigorously for 10 seconds and left to stand for more than 15 minutes. The presence of saponins was indicated by the foam persisting after 15 minutes (Jones & Kinghorn, 2006).
- ***Identification of bio-surfactant properties***
  - *Emulsification capacity*: The extract was dissolved in water and added with petroleum in equal volumes. The mixture was shaken vigorously for 1 minutes and left to stand overnight at room temperature for formation of stable emulsion layer. The height of emulsion layer was compared with controls. Sodium dodecyl sulphate (1%) and water were used as positive control and negative control, respectively (Batista *et al.*, 2006).
  - *Drop collapse assay*: The extract was dissolved in water. Twenty microliter of extract was added with 1 µl of methylene blue, mixed and pipetted carefully on a parafilm. The methylene blue was added solely for visualization purposes and does not influence drop collapse activity. The mixture was allowed to stand for 5 minutes prior visually analysed. Sodium dodecyl sulphate (1%) was used as positive control and the expected outcome was to observe a collapsed droplet after 5 minutes. Water was used as negative control with the expected outcome of droplet remaining beaded (Berti *et al.*, 2007).

Appendix II

Lack of biofilm inhibition activity of HEX, DCM and EA fractions

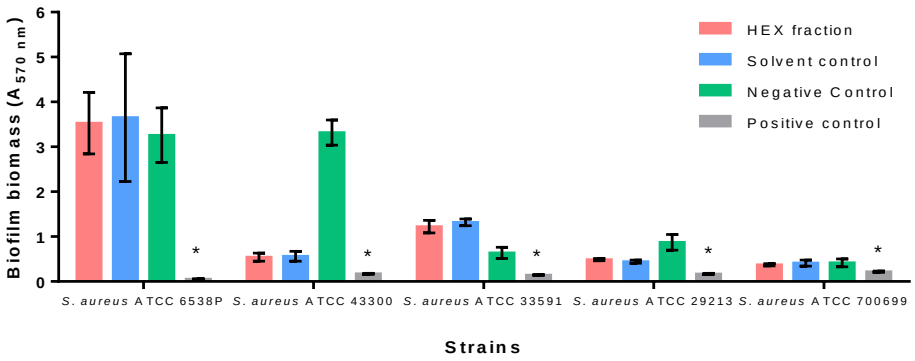

Figure A 1: Biofilm inhibition activity at sub-MIC of HEX fraction was not observed against all five strains of *S. aureus*. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against *S. aureus* strains. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm inhibition when compared with solvent control.

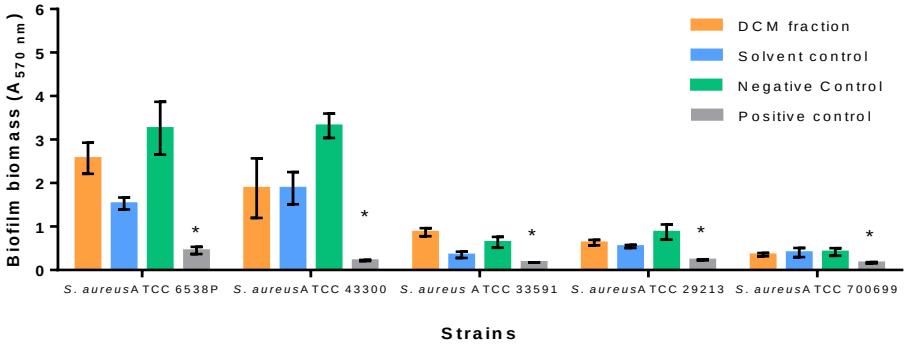

Figure A 2: Biofilm inhibition activity at sub-MIC of DCM fraction was not observed against all five strains of *S. aureus*. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against *S. aureus* strains. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm inhibition when compared with solvent control.

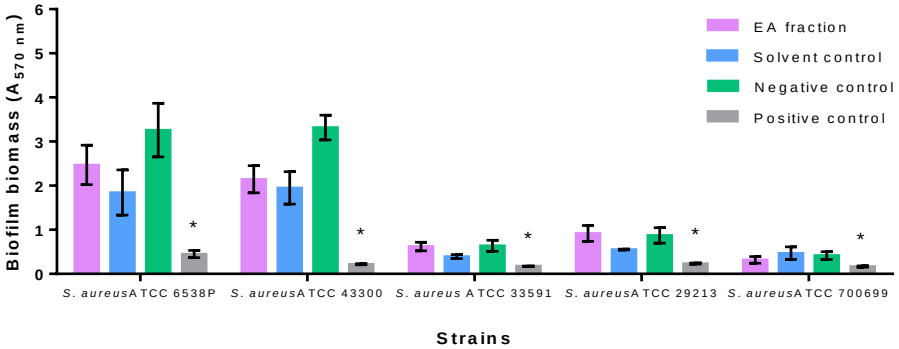

Figure A 3: Biofilm inhibition activity at sub-MIC of EA fraction was not observed against all five strains of *S. aureus*. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against *S. aureus* strains. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm inhibition when compared with solvent control.

Appendix III

Lack of biofilm disruption activity of MeOH and H_2O fractions

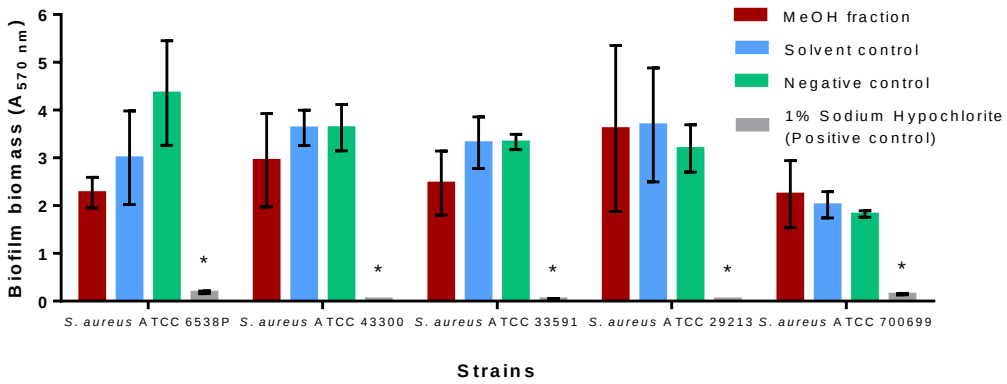

Figure A 4: Biofilm disruption activity at 5 mg/ml of MeOH fraction was not observed against all five strains of *S. aureus*. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against *S. aureus* strains. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm disruption when compared with solvent control.

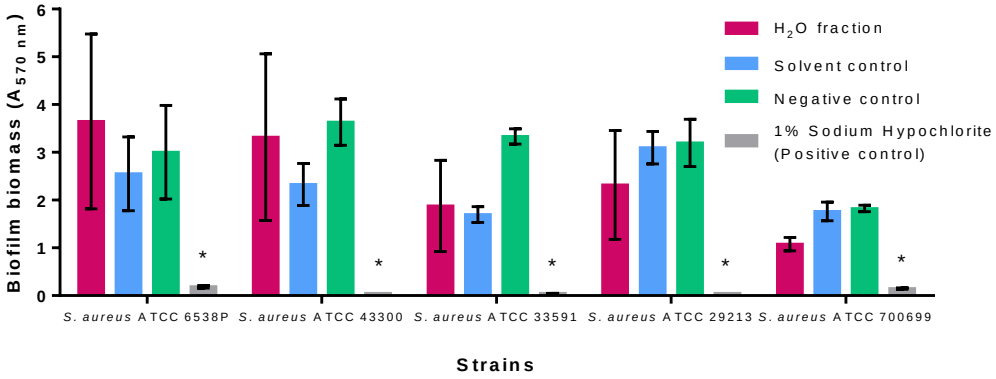

Figure A 5: Biofilm disruption activity at 5 mg/ml of H_2O fraction was not observed against all five strains of *S. aureus*. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against *S. aureus* strains. * denotes statistically significant difference at $p<0.05$ and exhibiting biofilm disruption when compared with solvent control.

Appendix IV

Growth curves of *S. aureus* strains when treated with H_2O fraction

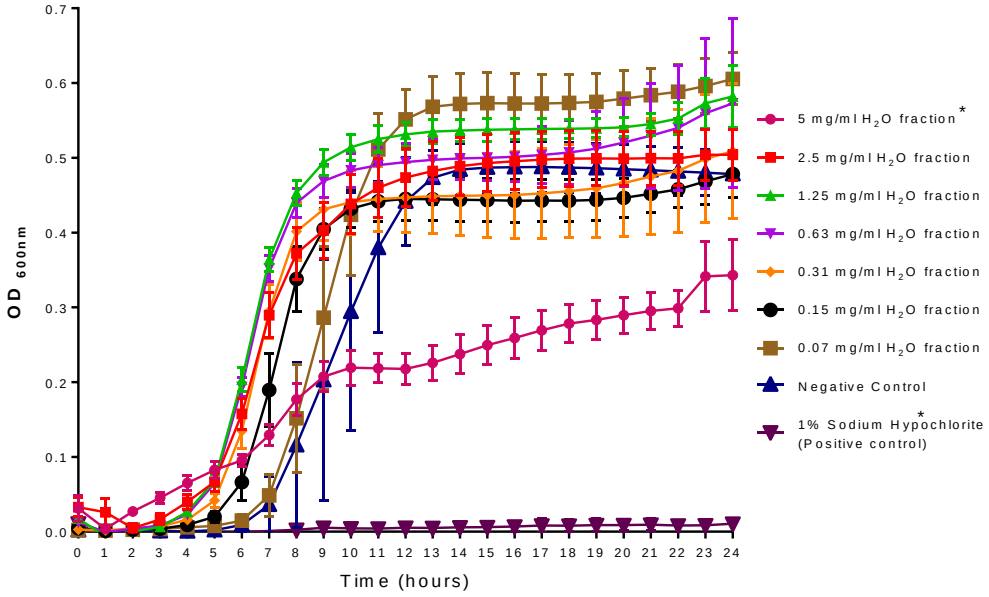

Figure A 6: The effect of H_2O fraction on the growth of *S. aureus* ATCC 6538P. Mean growth at OD_{600nm} ± SD plotted against time. * denotes statistically significant difference in terms of growth rate when compared with the negative control ($p<0.05$) (Table 3.6).

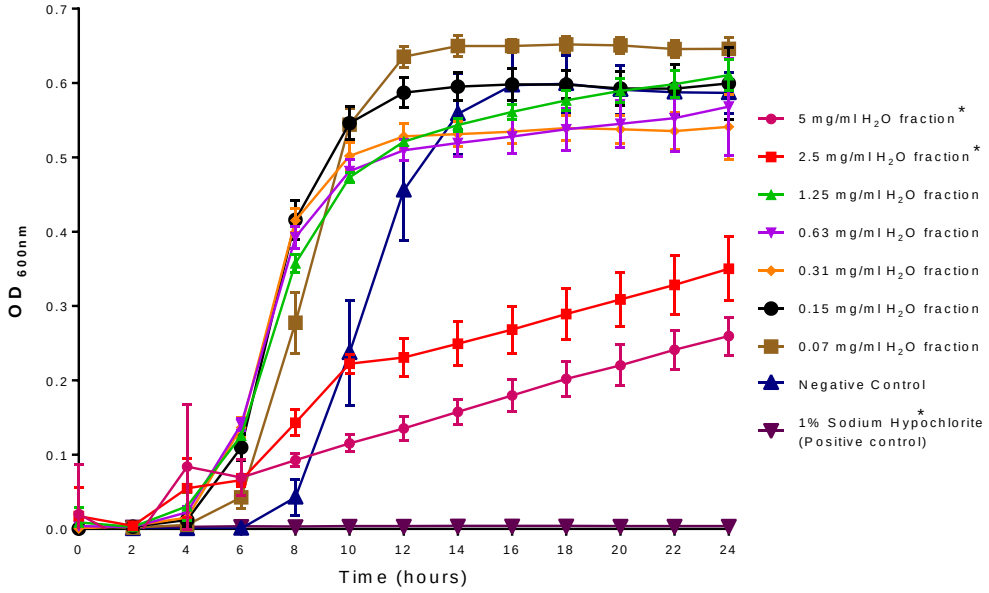

Figure A 7: The effect of H_2O fraction on the growth of *S. aureus* ATCC 43300. Mean growth at OD_{600nm} ± SD plotted against time. * denotes statistically significant difference in terms of growth rate when compared with the negative control ($p<0.05$) (Table 3.6).

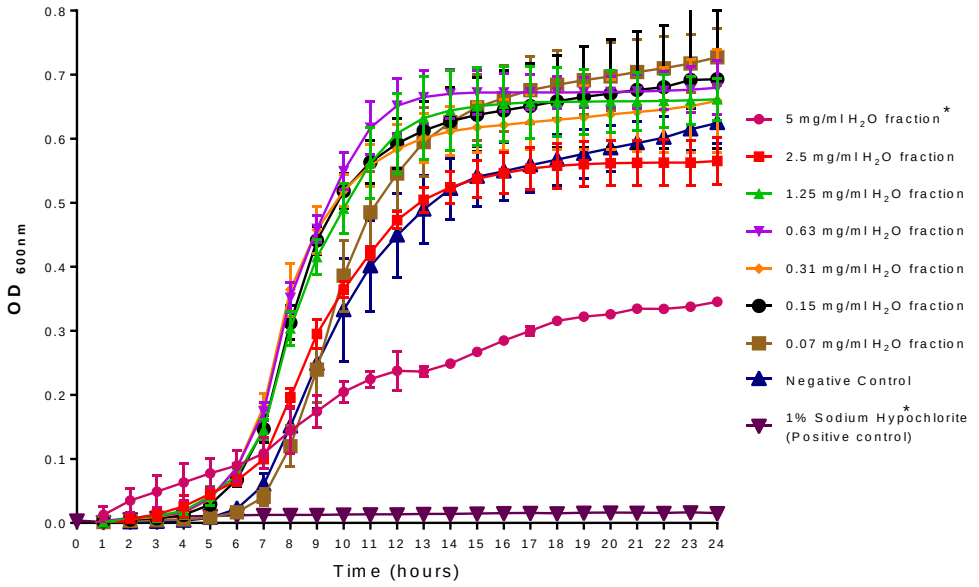

Figure A 8: The effect of H_2O fraction on the growth of *S. aureus* ATCC 33591. Mean growth at OD_{600nm} ± SD plotted against time. * denotes statistically significant difference in terms of growth rate when compared with the negative control ($p<0.05$) (Table 3.6).

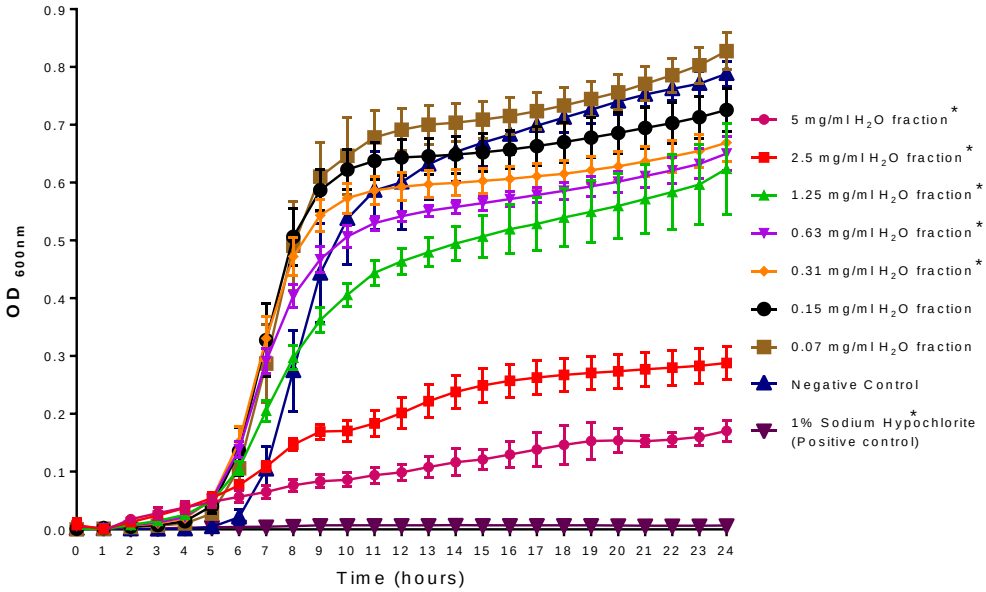

Figure A 9: The effect of H_2O fraction on the growth of *S. aureus* ATCC 29213. Mean growth at OD_{600nm} ± SD plotted against time. * denotes statistically significant difference in terms of growth rate when compared with the negative control ($p<0.05$) (Table 3.6).

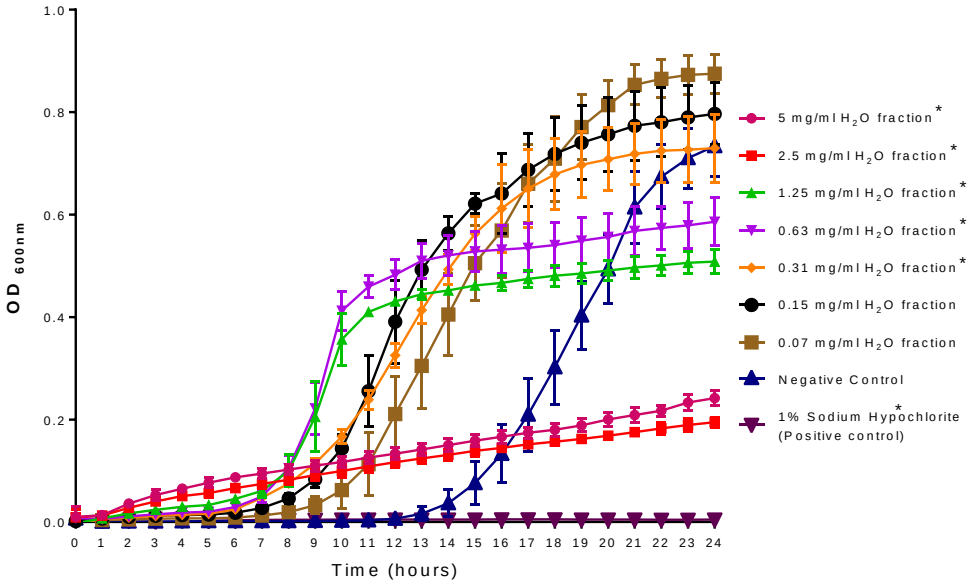

Figure A 10: The effect of H_2O fraction on the growth of *S. aureus* ATCC 700699. Mean growth at OD_{600nm} ± SD plotted against time. * denotes statistically significant difference in terms of growth rate when compared with the negative control ($p<0.05$) (Table 3.6).

Appendix V

List of chemicals

1,9-Dimethyl methylene blue zinc chloride double salt	Sigma-Aldrich, USA
Acetonitrile, HPLC grade	Merck KGaA, Germany
α–Tocopherol $\geq$95.5%	Sigma-Aldrich, USA
Chloramphenicol $\geq$98% (HPLC)	Sigma-Aldrich, USA
Crystal violet	Merck KGaA, Germany
D(+)-Glucose monohydrate	Merck KGaA, Germany
Daptomycin	Tocris Bioscience, UK
Dichloromethane, analytical grade	Merck KGaA, Germany
Erythromycin, *Streptomyces erythreus*	Merck KGaA, Germany
Ethanol, analytical grade	Merck KGaA, Germany
Ethyl acetate, analytical grade	Merck KGaA, Germany
Hexane, analytical grade	Merck KGaA, Germany
Methanol, analytical grade	Merck KGaA, Germany
Oxytetracycline hydrochloride $\geq$95% (HPLC), crystalline	Sigma-Aldrich, USA
Penicillin G, potassium salt	Merck KGaA, Germany
Phosphate buffer saline	Merck KGaA, Germany
Resazurin sodium salt	Sigma-Aldrich, USA
Silica gel 60 (0.015 - 0.040 mm)	Merck KGaA, Germany
Sodium chloride	Merck KGaA, Germany
Sodium hypochlorite	Clorox®, Malaysia
Tryptic soy agar	Merck KGaA, Germany
Tryptic soy broth	Merck KGaA, Germany
Vancomycin hydrochloride from *Streptomyces orientalis*	Sigma-Aldrich, USA

List of consumables and glassware

Column chromatography column	Favorit, Malaysia
COSMOSIL Guard Column 5C-18-MS-II (10ID x 20 mm)	Nacalai Tesque, Japan
Falcon tube (15 ml)	SPL Life Sciences Co., Ltd., Korea
Falcon tube (50 ml)	SPL Life Sciences Co., Ltd., Korea
HPLC syringe filters (PTFE, nylon)	Agilent Technologies, USA
HPLC vials (2 ml)	Agilent Technologies, USA
Micro centrifuge tube (1.5 ml)	SPL Life Sciences Co., Ltd., Korea
Nunc™ 96-well polystyrene microtiter plates	Thermo Fisher Scientific Inc., USA
Nunc™ 24-well polystyrene microtiter plates	Thermo Fisher Scientific Inc., USA
Petri dishes	Jatikhas, Malaysia
Pipette tips (1ml)	Extra Gene Inc., Taiwan

Pipette tips (200 µl)	SPL Life Sciences Co., Ltd., Korea
Pipette tips (10 µl)	Extra Gene Inc., Taiwan
Schott glass bottles	Schott AG, Germany
Syringe filters (25 mm)	Merck KGaA, Germany
Trident vials	Labchem, Malaysia
Universal bottles	Labchem, Malaysia

List of equipment

Acquity™ Waters UPLC – Synapt High Definition Mass spectrophotometer
Agilent 1200 Series preparative HPLC system
Bruker Ascend™ 700 NMR spectrometer
Elma ultrasonic bath LC130H
Eppendorf® 5418 R and 5810 R centrifuges
Eyela rotary evaporator N1110 V
Hirayama autoclave HVE-50
Hitachi S-3400N VP scanning electron microscope
Labconco™ Freezone™ bench top freeze dry systems, 4.5L
Memmert water bath
Memmert Incubator I
Nikon microscope YS100
Sartorius weighing balance CP124S
Secomam Prim Light spectrophotometer
Smith shaking incubator A3555
Tecan Infinite® 200 Pro microplate reader
Velp Scientifica vortex mixer