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ORIGINAL ARTICLE

Identification of α-tocopherol as a bioactive component of *Dicranopteris linearis* with disrupting property against preformed biofilm of *Staphylococcus aureus*

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Keywords
α-tocopherol, biofilm, *Dicranopteris linearis*, disruption, *Staphylococcus aureus*.

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Abstract

Aims: The potential of *Dicranopteris linearis* leaves' extract and its bioactive components were investigated for the first time for its disrupting ability against *Staphylococcus aureus* biofilms.

Methods and Results: The leaves of *D. linearis* were subjected to sonication-assisted extraction using hexane (HEX), dichloromethane, ethyl acetate and methanol (MeOH). It was found that only the MeOH fraction exhibited antimicrobial activity using broth microdilution assay; while all four fractions do not exhibit biofilm inhibition activity against *S. aureus* ATCC 6538P, *S. aureus* ATCC 43300, *S. aureus* ATCC 33591 and *S. aureus* ATCC 29213 using crystal violet assay. Among the four fractions tested, only the HEX fraction showed biofilm disrupting ability, with 60–90% disruption activity at 5 mg ml^{-1} against all four *S. aureus* strains tested. Bioassay-guided purification of the active fraction has led to the isolation of α-tocopherol. α-Tocopherol does not affect the cells within the biofilms but instead affects the biofilm matrix in order to disrupt *S. aureus* biofilms.

Conclusions: α-Tocopherol was identified to be the bioactive component of *D. linearis* with disruption activity against *S. aureus* biofilm matrix.

Significance and Impact of the Study: The use of α-tocopherol as a biofilm disruptive agent might potentially be useful to treat biofilm-associated infections in the future.

Introduction

Biofilms are defined as bacterial cells that are irreversibly attached to a surface and embedded in an extracellular polymeric substance (EPS) matrix. Biofilms can grow on living tissues, indwelling medical devices and implants, leading to infections that can threaten human health (Wu *et al.* 2015). 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 (Lebeaux *et al.* 2013). 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. Treatment of biofilms with antibacterial agents often results in incomplete killing, allowing unaffected bacteria to cause recurrent infection following the withdrawal of antibacterial treatments (Wu *et al.* 2015). Furthermore, this might allow bacterial cells to develop resistance towards the antibacterial agents, rendering them ineffective.

Staphylococcus aureus is one of the most frequent causes of biofilm-associated infections. 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 (Mohamed *et al.* 2017). *S. aureus* biofilms have been associated with chronic wound infections such as diabetic foot ulcers, venous ulcers and pressure sores (Archer *et al.* 2011). Infection treatments are further complicated with the increase in the emergence and prevalence of antibiotic-resistant *S. aureus* strains. *S. aureus* is also known for evolving and spreading antibiotic resistance mechanisms, consequently causing further challenges in biofilm treatments (Wu *et al.* 2015).

Due to the various challenges in biofilm treatments, there is a need to search for effective compounds for biofilm treatments. Plants are a good source for discovering antibiofilm compounds because of their therapeutic values in traditional medicine (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 a sophisticated immune response and therefore, rely on the cellular and biochemical defence systems for protection against biofilm infections (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 biofilm infections.

Dicranopteris linearis, belongs to the Gleicheniaceae family. 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. It was previously reported to possess antimicrobial, antinociceptive, anti-inflammatory, antipyretic, antioxidant and wound-healing potential (Zakaria *et al.* 2007, 2008, 2011; Lai *et al.* 2009; Ponnusamy *et al.* 2015). Thus far, the antibiofilm properties of *D. linearis* against *S. aureus* have not been reported. Hence, the objectives of this study are to evaluate the biofilm disruption ability of *D. linearis* against *S. aureus* biofilms, to purify and identify the compound(s) that are responsible for the activity.

Materials and methods

Bacterial strains

The strains that were used in this study were *S. aureus* ATCC 6538P (methicillin susceptible, strong biofilm former), *S. aureus* ATCC 43300 (methicillin susceptible, moderate–strong biofilm former), *S. aureus* ATCC 33591 (methicillin susceptible, weak–moderate biofilm former) and *S. aureus* ATCC 29213 (methicillin susceptible, weak–moderate biofilm former). The biofilm-forming ability of the *S. aureus* strains were classified according to Darabpour *et al.* (2017): strong biofilm producer (OD_{570} >4), moderate biofilm producer ($2 \leq OD_{570} \leq 4$) and weak biofilm producer (OD_{570} <2). All strains were cultured in tryptic soy broth (TSB) (Merck, Darmstadt, Germany) at 37°C for 20 h and maintained at −80°C in 20% (v/v) glycerol (Merck) for long-term preservation.

Plant collection and extraction

Fresh leaves of *D. linearis* were collected from its natural habitat in Genting Highland, Selangor (GPS coordinate: N03·40357°, E101·78545°) and identified by a botanist from Forest Research Institute Malaysia (FRIM). 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. The freeze-dried leaves were then subjected to sonication-assisted extraction using hexane (HEX), dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH), sequentially for three times. All solvents were purchased from Merck. The mixtures were filtered and concentrated under reduced pressure. The freeze-dried extracts were stored at −20°C prior to analysis (Lai *et al.* 2009).

Broth microdilution assay

The minimum inhibitory concentration (MIC) values were determined using broth microdilution assay according to Clinical and Laboratory Standards Institute standard method. The MIC is defined as the lowest concentration of an extract that inhibits the visible growth of a micro-organism after 16–20 h of incubation. Briefly, the test micro-organisms were grown in TSB at 37°C for 20 h and adjusted to 0·5 McFarland standard (OD_{625} 0·08–0·11), corresponding to $1{\cdot}5 \times 10^8$ colony-forming unit (CFU) per ml. The adjusted cultures were then diluted 100× in TSB and used as inocula. The extracts were twofold serially diluted (0·07–5 mg ml^{-1}) using sterile TSB in a 96-well flat-bottomed microtitre plate (Nunc, Rochester, New York, USA). One hundred microlitres of the adjusted *S. aureus* culture was added to each well. The negative control contained TSB with *S. aureus* culture while the blank control consisted only of TSB. The microtitre plate was then incubated aerobically for 16–20 h at 37°C. The MIC was determined in triplicates by the concentration of extract (mg ml^{-1}) where no visible growth was observed.

Preparation of inoculum suspension for antibiofilm assay

Overnight cultures of *S. aureus* grown in TSB at 37°C for 20 h were adjusted to 0·5 McFarland standard (0·08–0·1 at OD_{625}), corresponding to approximately 10^8 CFU per ml. The suspension was further diluted 1 : 100 in TSB supplemented with 1% (v/v) glucose (Merck), resulting in a final inoculum suspension of ~10^6 CFU per ml.

Determination of the minimum biofilm inhibition concentration of the extracts

Biofilm inhibition assay was performed to determine the effect of the extracts in inhibiting the formation of *S. aureus* biofilms. The minimum biofilm inhibitory concentration was defined as the lowest concentration of extract in a serial twofold dilution series (0·07–5 mg ml^{-1}) that inhibits biofilm formation as compared with the solvent control. Briefly, a twofold dilution series of various concentrations of extract was added to 100 μl of the adjusted inoculum suspensions in a 96-well microtitre plate (Nunc). Solvent control refers to treatment with solvents used (0·125% Tween 80 in 2·5% acetonitrile) to dissolve the extracts while treatment with 1% (v/v) sodium hypochlorite in TSB served as the positive control. Negative control contained TSB with the *S. aureus* culture. The plates were incubated at 37°C for 24 h. Inhibition of biofilm formation was quantified using crystal violet staining.

Determination of the minimum biofilm disruption concentration of the extracts

Biofilm disruption assay was performed to determine the effectiveness of extract in disrupting preformed biofilms of *S. aureus*. Two hundred microlitres of inoculum suspension was added to the wells of microtitre plates (Nunc) and incubated for 24 h to allow the formation of biofilms. After 24 h, the planktonic cells were decanted and the wells were washed three times with sterile water. Twofold dilution series (0·07–5 mg ml^{-1}) of various concentrations of the extracts were applied to the wells. Solvent control refers to treatment with solvent used (0·125% Tween 80 in 2·5% acetonitrile) to dissolve extract while treatment with 1% (v/v) 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 h. Disruption of preformed biofilms was quantified using crystal violet staining. The experiment was performed in triplicates.

Crystal violet staining

Crystal violet staining was performed to quantify the biofilm biomass after treatment with and without extract. Crystal violet stains both the bacterial cells (living and dead cells) and the extracellular matrix in the wells. After treatment for 24 h, liquid cultures were removed and the wells were rinsed three times with sterile distilled water to remove unattached cells. The plates containing the attached bacteria with extracellular matrix were heat fixed at 60°C for 1 h. The wells were stained with 0·1% (w/v) crystal violet (Merck) for 15 min. Excess stain was removed by washing the wells three times with sterile distilled water. The plates were air-dried. The bound dye was eluted from attached cells with MeOH. The amount of crystal violet bound in each well was measured at OD_{570} nm using a Tecan Infinite® 200 Pro microplate reader (Tecan, Männedorf, Switzerland) (Kwasny and Opperman 2010; Xu *et al.* 2016).

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to investigate the structural modifications of biofilms after treatment with extract at minimum biofilm disruption concentration (MBDC). The method was performed according to Kerekes *et al.* (2013), with modifications. Sterile polystyrene discs were placed in the wells of a 24-well plate containing the adjusted inoculum suspension and served as the attaching surface for *S. aureus* cells. The plate was incubated for 24 h at 37°C to allow the formation of biofilms on the polystyrene discs. The polystyrene discs were carefully washed with phosphate-buffered saline (PBS) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) 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 (0·125% Tween 80 in 2·5% acetonitrile) and positive control (1% sodium hypochlorite) were included. Then, the plate was incubated for 24 h 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% (v/v) glutaraldehyde (Sigma-Aldrich, St. Louis, Missouri, USA) in PBS for 4 h at room temperature, soaking in PBS for 10 min, and then followed by ethanol dehydration of concentrations: 20, 40, 60, 70, 80, 90, 95 and 100% (v/v) in PBS. Each ethanol treatment lasted for 10 min at room temperature. The discs were then placed in a desiccator overnight. The discs were sputter coated with gold and examined with a Hitachi S-3400N VP SEM (Hitachi, Tokyo, Japan).

Purification of bioactive compound(s)

Bioassay-guided fractionation method was employed in the purification of the active compound(s) from the

active fraction(s). Each fraction was evaluated for its biofilm disruption activity using the biofilm disruption assay followed by crystal violet assay against all *S. aureus* strains. The active fraction(s) was purified on an open silica column with HEX–EtOAc at 7 : 3 (v/v) as the mobile phase, followed by further purification using a reversed-phase preparative HPLC equipped with a COSMOSIL Guard column 5C-18-MS-II (10ID × 20 mm) (Nacalai Tesque, Kyoto, Japan). The mobile phase consisted of 5% water (solvent A) and 95% acetonitrile (solvent B) at isocratic conditions for 10 min. The flow rate was at 20 ml min^{-1}. Detection was performed at 210 and 450 nm.

Identification of purified active compound(s)

Identification of the purified active compound(s) was carried out with nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography-mass spectrometry. One-dimensional (1D) NMR experiment of the compound(s) dissolved in chloroform-d were performed on a Bruker Ascend™ 700 NMR spectrometer (Bruker, Billerica, Massachusetts, USA) with ^{1}H NMR (700 MHz) and ^{13}C NMR (176 MHz). The LC chromatogram was obtained using Acquity™ Waters Ultra Performance Liquid Chromatography (UPLC) with the ACQUITY UPLC BEH C18 (1·7 μm, 2·1 × 50 mm) column (Waters, Milford, Massachusetts, USA). The solvent system consisted of 5% of water + 0·1% formic acid (solvent A) and 95% of acetonitrile + 0·1% formic acid (solvent B) at isocratic conditions for 15 min. ESI-MS(+) was obtained using a Synapt High Definition Mass Spectrophotometer quadrupole orthogonal acceleration, time-of-flight detector.

Characterization of the biofilm disruption effect of α-tocopherol

Dimethyl methylene blue assay

Dimethyl methylene blue (DMMB) assay was performed to evaluate the effects of α-tocopherol on the biofilm matrix of *S. aureus*, as according to Darabpour *et al.* (2017). 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 twofold dilution series of α-tocopherol from 0·01–0·5 mg ml^{-1} for treatment. After 24 h of incubation, the plates were rinsed with water. DMMB complexation solution was added and the plates were incubated in dark for 30 min. The plates were then centrifuged, washed to remove unbound DMMB and added with the decomplexation solution before further incubation for 30 min. Absorbance was measured at 650 nm. The experiment was performed in triplicates.

Resazurin assay

Resazurin assay was performed to evaluate the effects of α-tocopherol on the viability of cells within biofilms, as according to den Driessche *et al.* (2014) and Peeters *et al.* (2008). The nonfluorescent 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 twofold dilution series of α-tocopherol from 0·01–0·5 mg ml^{-1} for treatment and incubated for 24 h. The microtitre plates were rinsed with water and 5 μg ml^{-1} of resazurin in sterile distilled water was added into each well and further incubated for 2 h at 37°C. The fluorescence intensity (λ_{ex}: 560 nm and λ_{em}: 590 nm) was then measured. The experiment was performed in triplicates.

Statistical analysis

Statistical analysis was conducted using the one-way analysis of variance test for comparing mean scores of more than two groups, with significance at $P < 0·05$. Independent sample *t*-test was performed to compare the mean scores of two groups, with significance at $P < 0·05$ using IBM SPSS Statistics 20 software All graphs were generated using the GraphPad Prism 6 software.

Results

Bioactivity of *D. linearis* crude extracts

Among the four fractionated fractions, only the MeoH fraction showed antimicrobial activity at 5 mg ml^{-1} while the other fractions showed MIC values >5 mg ml^{-1}. Compounds that can be extracted by MeOH such as tannins and flavonoids, had been reported to exhibit antibacterial activity and these compounds had been identified to be present in *D. linearis* by other studies (Raja *et al.* 1995; Jaishee and Chakraborty 2015). It was also found that the four fractions do not inhibit the biofilm formation by the four *S. aureus* strains tested. However, the HEX fraction was able to disrupt the preformed biofilm of all four *S. aureus* strains when compared to the other fractions, which include DCM, EtOAc and MeOH fractions (Table 1). The MBDC of the HEX fraction showed significant biofilm disruption activity at 1·25 mg ml^{-1} (38·4 ± 5·5% disruption) for *S. aureus* ATCC 6538P, at 0·63 mg ml^{-1} (55·0 ± 6·9% disruption) for *S. aureus* ATCC 43300, at 0·31 mg ml^{-1} (55·8 ± 11·3% disruption) for *S. aureus* ATCC 33591 and at 0·070 mg ml^{-1} (55·7 ± 8·8% disruption) for *S. aureus* ATCC 29213 (Fig. 1). At a higher concentration, a

Table 1 Minimum biofilm disruption concentration (MBDC) of each fraction against four different *S. aureus* strains

Strains	Biofilm production	MBDC (mg ml^{-1})			
		HEX	DCM	EtOAc	MeOH
S. aureus ATCC 6538P	Strong	1·25	>5	>5	>5
S. aureus ATCC 43300	Moderate to strong	0·63	5	>5	>5
S. aureus ATCC 33591	Weak to moderate	0·31	5	>5	>5
S. aureus ATCC 29213	Weak to moderate	0·07	5	5	>5

HEX, hexane; DCM, dichloromethane; EtOAc, ethyl acetate; MeOH, methanol.

significantly higher percentage of biofilm disruption was observed for two *S. aureus* strains, indicating a concentration-dependent antibiofilm activity. At 5 mg ml^{-1}, the percentage of disruption was 72·7 ± 5·9% for *S. aureus* ATCC 33591 and 81·5 ± 1·9% for *S. aureus* ATCC 29213. Additionally, the MBDC determined also showed a strain variation effect. The two stronger biofilm producers had higher MBDC values, indicating that these preformed 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^{-1}. Thus, the difference in biofilm production among these four strains

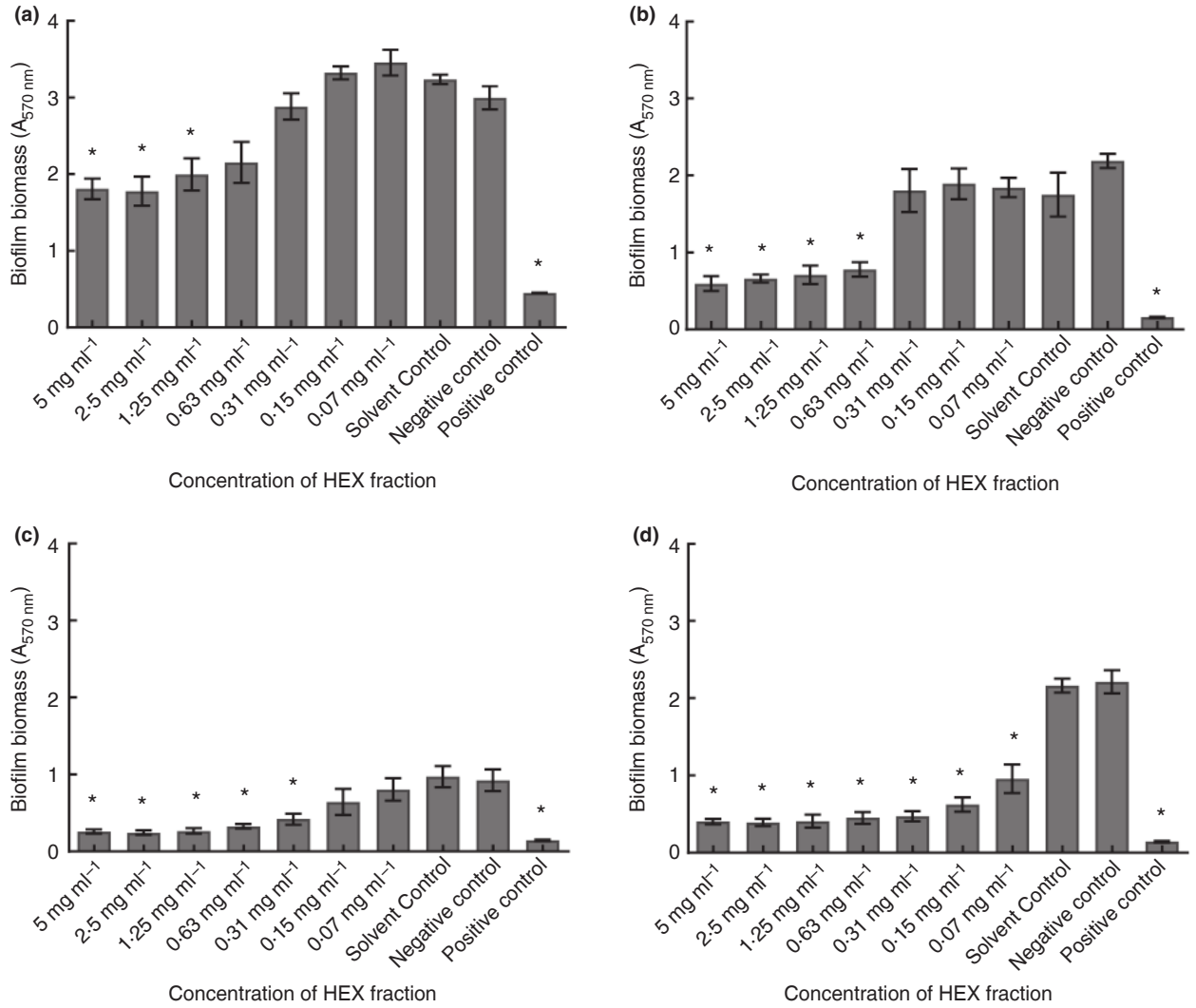

Figure 1 Biofilm disruption activity of hexane (HEX) fraction at various concentrations (0·07–5 mg ml^{-1}) against (a) *S. aureus* ATCC 6538P; (b) *S. aureus* ATCC 43300; (c) *S. aureus* ATCC 33591 and (d) *S. aureus* ATCC 29213. Mean biofilm biomass ($A_{570\ nm}$) ± SD plotted against various concentrations of HEX fraction. *denotes statistically significant difference at $P < 0·05$ and exhibiting biofilm disruption activity when compared with the solvent control. The negative control used was water while the solvent control was 0·125% of Tween 80 in 2·5% acetonitrile. Positive control was 1% (v/v) sodium hypochlorite.

might have influenced the disruption effect by HEX fraction.

Investigation of HEX fraction on the biofilm structure

In order to investigate the structural modifications of biofilms after treatment with HEX fraction, SEM was performed. SEM has been widely used to visualize the structure of biofilms. Through SEM, the morphology of bacteria adhered on a material surface can be observed, providing 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 antibiofilm compounds on biofilm structure and morphology (Khan and Ahmad 2012; Kerekes *et al.* 2013; Neto *et al.* 2014). It was shown that the solvent used to dissolve HEX fraction does not affect cell morphology and biofilm structure of *S. aureus* (Fig. 2b), as there was no difference in cell morphology and biofilm structure when compared with the negative control (Fig. 2a). Treatment with 1·25 mg ml^{-1} HEX fraction demonstrated destruction of the biofilm structure and reduced biofilms attached to the surface (Fig. 2c). Scant biofilms were observed, with only few bacterial cells. The SEM images prove that the HEX fraction had an effect on preformed biofilms and confirm the results obtained with crystal violet assay, which demonstrated reduction in biofilm biomass of *S. aureus* after treatment. As this study has preliminarily shown that the HEX fraction can uniquely disrupt preformed biofilm, but not exhibiting biofilm inhibition or bacterial inhibitory properties, the HEX fraction was chosen for further purification.

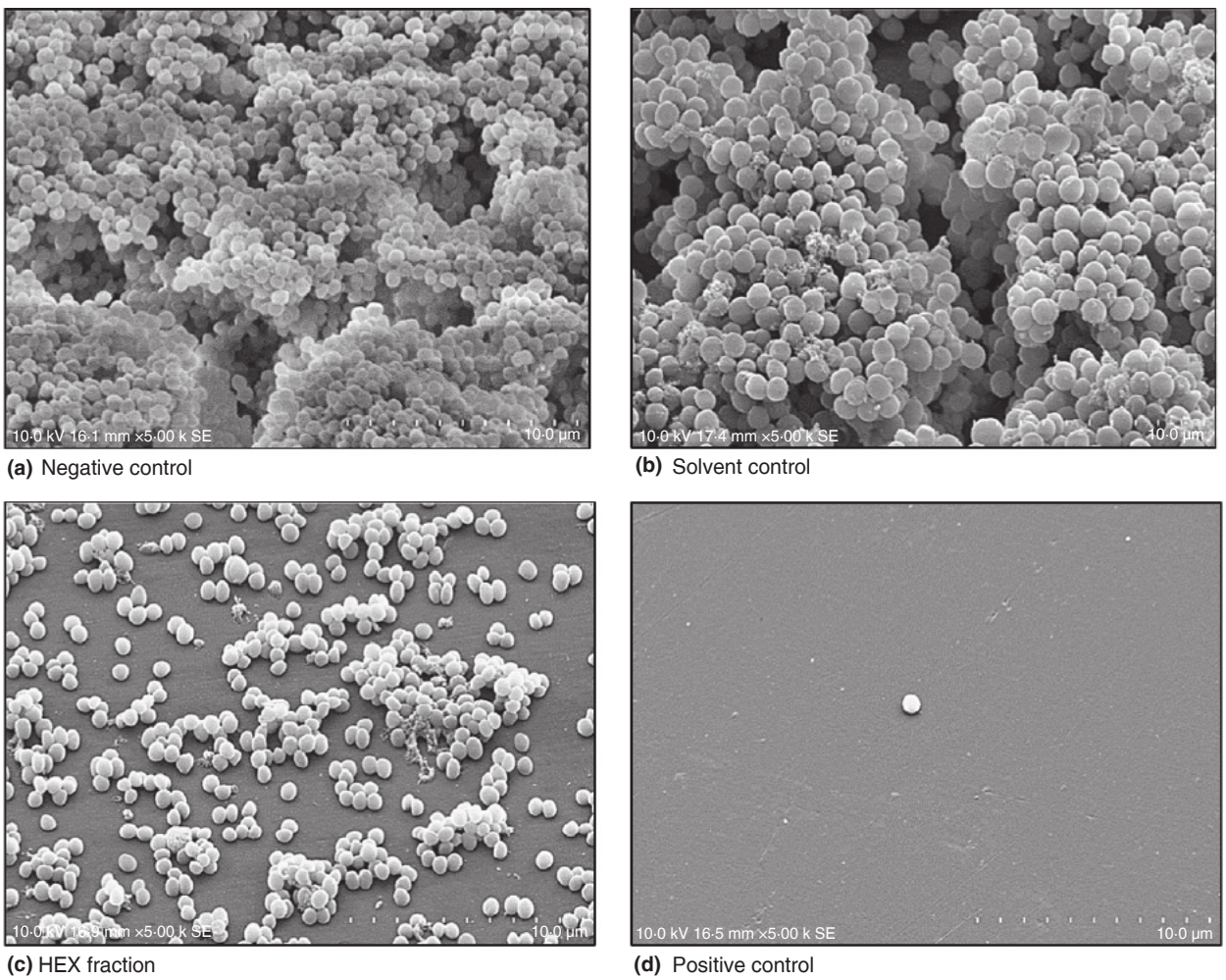

Figure 2 SEM images of *Staphylococcus aureus* ATCC 6538P: (a) Negative control (water), (b) solvent control (0·125% Tween 80 in 2·5% acetonitrile), (c) treatment with HEX fraction was at 1·25 mg ml^{-1} and (d) positive control (1% sodium hypochlorite). Images were taken under 5000× magnification at 10·0 kV.

Identification of the purified active compound(s)

Bioassay-guided purification of the HEX fraction has led to the isolation of one bioactive compound with biofilm disruption property. The compound was identified to be (2R)-2,5,7,8-Tetramethyl-2-[(4R,8R)-4,8,12-trimethyltridecyl]-3,4-dihydro chromen-6-ol, also known as α-tocopherol, as the ^{1}H (Table 2) and ^{13}C NMR (Table 3) chemical shifts were in good agreement with literature (Matsuo and Urano 1976; Baker and Myers 1991). Following NMR analyses, the identity of α-tocopherol was further confirmed using mass spectrometry data (Fig. 3) with a molecular formula of $C_{29}H_{50}O_2$. It has been reported in literature that the principle ions of α-tocopherol were of *m*/*z* 165, 205, 429, 430 and 431 (Matsuo and Urano 1976; De Leenheer *et al.* 1978). The peaks corresponding to these ions were observed in the mass spectrum (Fig. 3), further supporting its identity as α-tocopherol. The structure of α-tocopherol is shown in Fig. 4.

Characterization of the biofilm disruption activity of α-tocopherol

The extracted α-tocopherol demonstrated significant biofilm disruption effect at the concentrations of 0·01–0·5 mg ml^{-1}, with the MBDC value of 0·01 mg ml^{-1} for all four *S. aureus* biofilm strains. At 0·01 mg ml^{-1}, α-tocopherol was able to disrupt 32·8 ± 6·2% of *S. aureus* ATCC 6538P, 41·9 ± 10·3% of *S. aureus* ATCC 43300, 56·6 ± 4·5 of *S. aureus* ATCC 33591 and 62·7 ± 4·0% of *S. aureus* ATCC 29213 biofilms. 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 (Fig. 5). 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.

In the resazurin assay, the nonfluorescent 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

Table 2 Comparison of ^{1}H NMR chemical shift assignments of the extracted α-tocopherol with literature (Baker and Myers 1991)

	Extracted α-tocopherol	α-Tocopherol
Position		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

Signal multiplicity: s = singlet; t = triplet; m = multiplet.

Table 3 Comparison of ^{13}C NMR chemical shift assignments of the extracted α-tocopherol with literature (Matsuo and Urano 1976; Baker and Myers 1991)

		α-Tocopherol	
Position	Extracted α-tocopherol	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

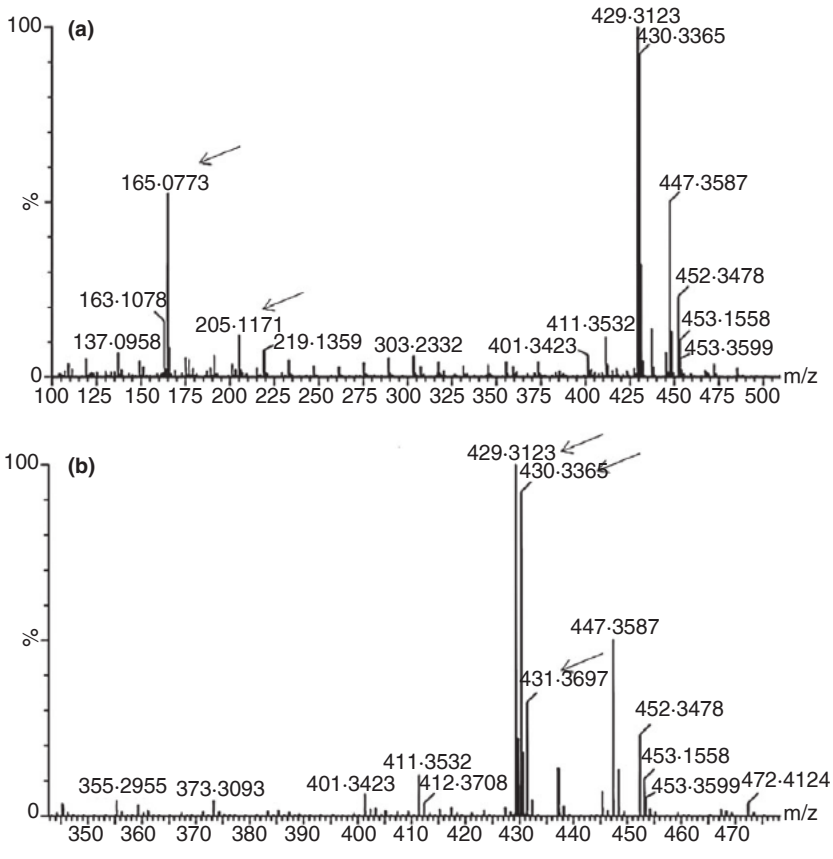

Figure 3 (a) ESI-MS(+) spectrum of extracted α-tocopherol. (b) Zoomed in image of ESI-MS (+) spectrum of extracted α-tocopherol. The arrows indicate the peaks corresponding to *m/z* 165, 205, 429, 430 and 431, which are the main fragmentation and molecular ions of α-tocopherol.

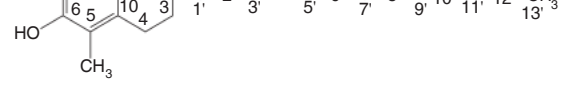

Figure 4 Structure of α-tocopherol with numbering system.

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. 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 (Fig. 6).

Discussion

Biofilms are known to be difficult to disrupt due to the their EPS, which are impermeable to many antibacterial agents (Kaali *et al.* 2011). The biofilm EPS matrix blocks the transport of antibacterial agents by interacting with the antibacterial agents and inactivating them. 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 (Stewart and Costerton 2001). Therefore, it is important to look for new biofilm-disrupting agents to effectively eradicate biofilms, allowing the antibacterial agents to function once again.

In the present study, the HEX fraction of *D. linearis* leaves demonstrated biofilm-disrupting activity against four *S. aureus* biofilm strains without affecting their growth. These results were further substantiated by SEM analysis on the effect of HEX fraction on the preformed biofilms, in which the biofilms were dispersed without affecting the cell morphology of the *S. aureus* culture. Further purification on the HEX fraction had led to the isolation of α-tocopherol.

The extracted α-tocopherol was able to disrupt *S. aureus* biofilms. Thus far, the mechanism of action of α-tocopherol as a biofilm-disrupting agent is not well

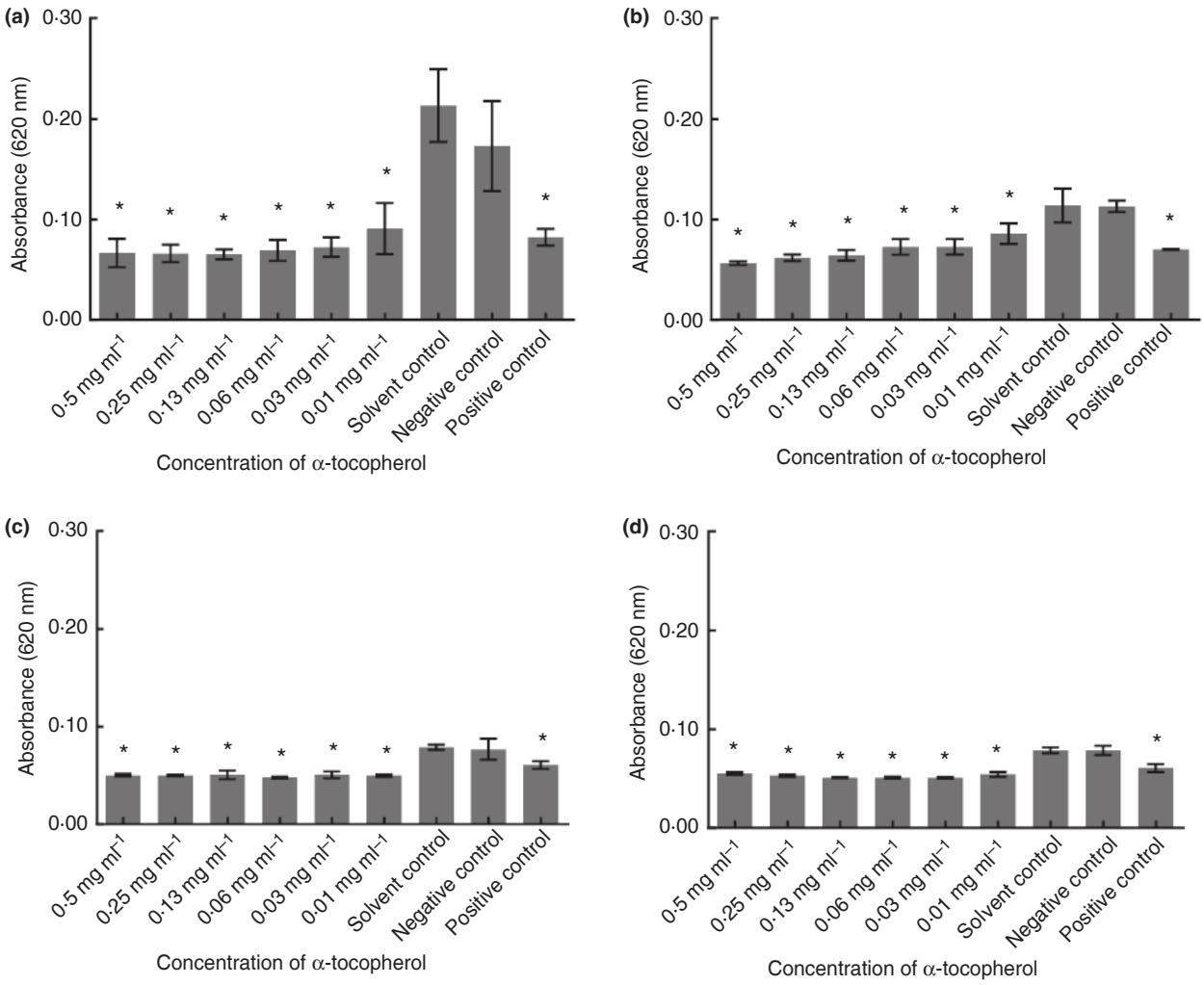

Figure 5 Quantification of the biofilm matrix after treatment with α-tocopherol at various concentrations (0·01–0·5 mg ml^{-1}) against (a) *S. aureus* ATCC 6538P; (b) *S. aureus* ATCC 43300; (c) *S. aureus* ATCC 33591 and (d) *S. aureus* ATCC 29213. Mean absorbance (620 nm) ± SD plotted against various 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.

characterized. Currently, the commonly known mechanisms in relation to disruption of biofilms are related to the solubilisation of the biofilm matrix components such as polysaccharides, and it is possible that α-tocopherol may act in a similar way (Boles and Horswill 2011). Hence, to evaluate the mechanism of α-tocopherol as a biofilm-disrupting agent, the biofilm polysaccharide was quantified with DMMB after being treated with α-tocopherol.

The 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*. 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 (Fig. 5). 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. Furthermore, it can also be assumed that α-tocopherol affects a specific component

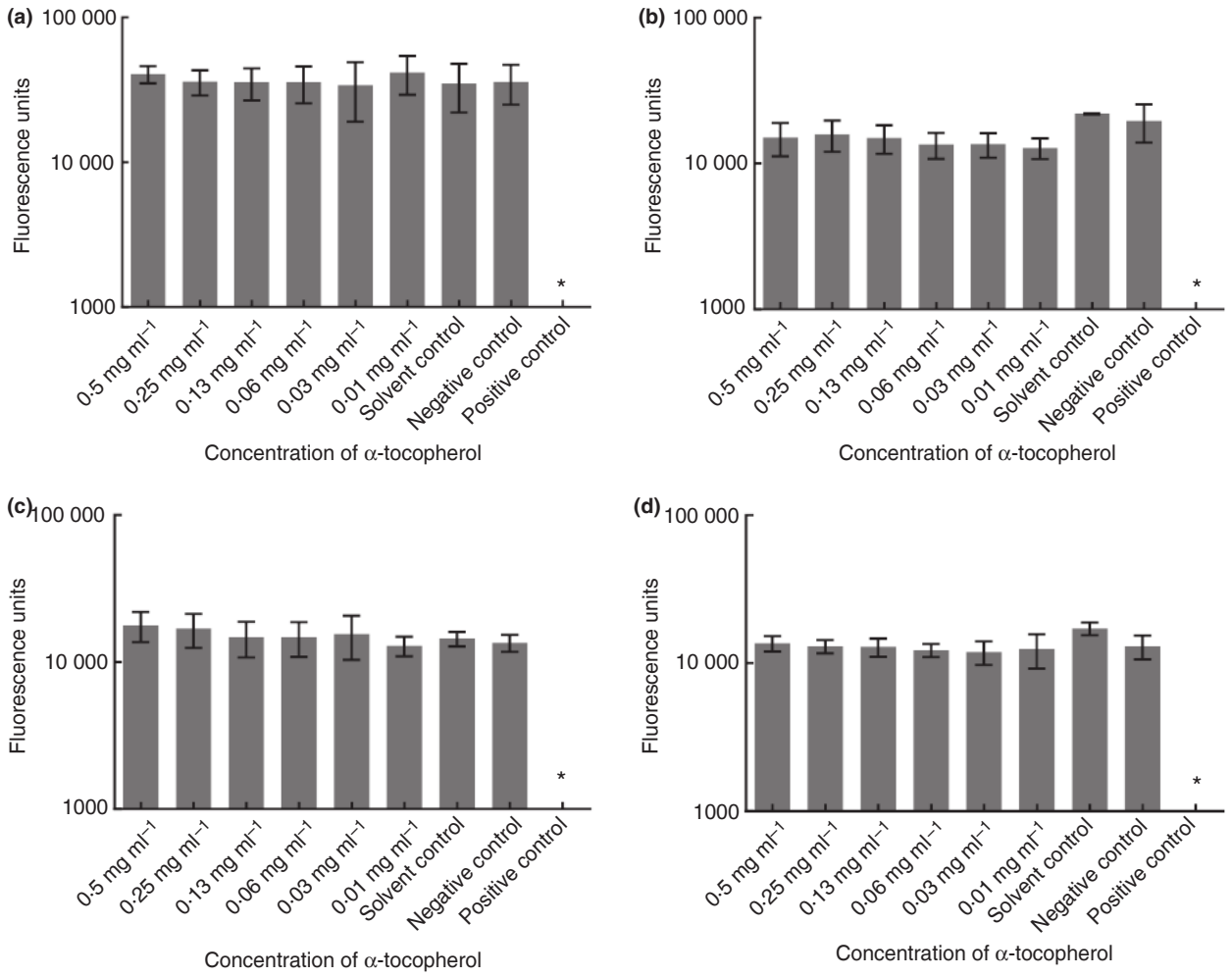

Figure 6 Quantification of viable cells within biofilm after treatment with α-tocopherol at various concentrations (0·01–0·5 mg ml^{-1}) against (a) *S. aureus* ATCC 6538P; (b) *S. aureus* ATCC 43300; (c) *S. aureus* ATCC 33591 and (d) *S. aureus* ATCC 29213. Mean fluorescence units ± SD plotted against 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.

of the biofilm matrix, which is the polysaccharides, as DMMB reflects the amount of polysaccharides present in the matrix. 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 and Horswill 2011). Analyses are needed to verify the possible effect of α-tocopherol on these other biofilm matrix components.

Since α-tocopherol is an antioxidant, there is a possibility that the antioxidant property of α-tocopherol might be involved directly or indirectly in affecting the biofilm matrix. An example of an antioxidant that disrupts biofilms through DNA intercalation is glutathione. In *Pseudomonas aeruginosa* biofilms, pyocyanin intercalates directly with extracellular DNA, to confer structural integrity to the biofilm. The glutathione reacts with pyocyanin, directly interfering with pyocyanin's ability to intercalate with extracellular DNA and thus, resulting in the disruption of biofilms (Klare *et al.* 2016). Besides that, a potent thiol-containing antioxidant, N-acetyl cysteine (NAC) have been reported to decrease biofilm formation of *Enterococcus faecalis, Escherichia coli* and *Klebsiella pneumoniae,* and may reduce the production of extracellular polysaccharide matrix while promoting the disruption of mature biofilm (Silveira *et al.* 2013; Dinicola *et al.* 2014). It was suggested that NAC may be directly disrupting disulphide bonds in enzymes involved in extracellular polysaccharide production or excretion, causing the molecules to be less active, or NAC competitively inhibiting cysteine utilization. Moreover, it was also

suggested that NAC may interfere with control or signalling systems involved in extracellular polysaccharide production (Dinicola *et al.* 2014).

The resazurin assay showed that the cells within the biofilms of all four *S. aureus* strains remained viable after treatment with various concentrations of α-tocopherol. This is advantageous for α-tocopherol as an antibiofilm 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 antibiofilm agents is low. Overall, it can be concluded that α-tocopherol might be a potent candidate to disrupt bacterial biofilm infections, allowing antibiotics to effectively eradicate the persistent cells underlying the biofilms. Nevertheless, further work such as *in vitro* testing of α-tocopherol against polymicrobial biofilms and synergistic studies with antibiotics could be performed in the near future.

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Conflict of Interest

The authors have declared no conflict of interest.

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