International Journal of Engineering & Technology, 7 (4.14) (2018) 14-19

International Journal of Engineering & Technology

Website: www.sciencepubco.com/index.php/IJET

Research paper

Antibiofilm Activities in Skin Secretions of Malaysian Frogs

A Abdul-Aziz[1]*, M S Razali[2], W M S Wan Azmi[3], Z A Rahman[4], M F F Abdullah[5],

School of Biology, Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia
**Corresponding author E-mail: aziyah960@salam.uitm.edu.my*

Abstract

Biofilms are extracellular structures formed by many species of bacteria that attached on various surfaces. The formation of biofilms is responsible for many health problems as they are very difficult to treat effectively due to enhanced resistant to antibiotics. Frog skin secretions contain bioactive compounds that may exhibit antibiofilms properties against biofilms infection. This study aims to screen the presence of antibiofilm activities from lyophilized skin secretions collected from Malaysian frogs. In this study, lyophilized skin secretions from four species of Malaysian frogs were tested for biofilm inhibition activities against the bacteria *Staphylococcus epidermidis*, ATCC 35984. Antibiofilm activity was tested at three different stages of biofilm formation which were biofilm maturation, biofilm attachment and biofilm dispersion. Biofilm treated with skin secretion from *Fejervarya multistriata* showed the presence of antibiofilm activities of 45 % which was able to reduce biofilm formation to 55 % at the attachment stage. Similarly, the biofilm formation at the maturation stage was reduced to 65.8 % only. No antibiofilm activities of *Fejervarya multistriata* was detected at the dispersion stage. The sample showing positive results was fractionated using column chromatography and further purified by C18 reverse phase high-performance liquid chromatography column (HPLC). Mass spectroscopy revealed the presence of a single peptide. In conclusion, frog skin secretion of *F. multistriata* contains peptide that inhibits biofilm at both attachment and maturation stages.

Keywords: *Antibiofilm, Peptide, Biofilm formation, Biofilm inhibition assay, Frog skin secretions.*

1. Introduction

Bacteria often grow in the form of a biofilm [1] and are protected by this extracellular matrix [1, 2]. Biofilms are also formed by many other bacteria on various biotic and abiotic surfaces [1, 3]. Biofilms are thought to be responsible for at least 65% of all human infections especially in device-related infections, on body surfaces and in chronic infections [3, 4].

Formation of biofilm commonly proceeds in four different stages. The first stage is bacterial attachment to a surface, either abiotic or biotic surface [5]. The attachment of bacterial cells on the surface is due to van der Waal forces, steric interactions, and electrostatic interaction known as the DLVO (Derjaguin, Verwey, Landau, and Overbeek) forces [6]. The second stage is the sessile growth and extracellular polymeric protective matrix film production. The matrix production enhances the bacteria attachment to the matrix or directly to previous colonists [7]. The third stage is biofilm maturation which starts once biofilm grew in optimal growth conditions. The final stage is the detachment of bacteria via physical detachment or signaling events to colonize new areas [2, 3, 8, 9, and 10].

There are about 106 frog species that had been described in West Peninsular Malaysia meanwhile East Malaysia has their own frog population. According to the report, five species of frogs from the genus Hyalarana (*Hylarana baramica, Hylarana glandulosa, Hylarana signata, Hylarana picturata* and *Hylarana luctuosa*) have been found in Sarawak [11]. A frog's skins have mucus and granular glands that located mostly at the dorsal part of the frog's skin. These glands can produce bioactive peptide with inhibitory activities towards microbial growth [12]

Frog skin secretions have been reported to contain various bioactive compounds, such as antimicrobial peptides (AMPs), analgesic peptides, algesic peptides, protease inhibitors, lectins, antioxidant peptides and neurotoxins that important in their survival [13]. AMPs are bioactive peptides that demonstrated multifunctional activities against a great range of microorganisms such as Gram-negative and Gram-positive bacteria, fungi, protozoa and even viruses [14, 15, and 16]. The interactions between cationic AMPs and anionic phospholipids in the cell membrane by electrostatic forces will lead to cell death due to the destruction of cell membranes via hydrophobic interactions through several different mechanisms [17, 18]. Therefore, AMPs with these properties may represent a powerful weapon against resistant pathogenic microorganisms [19]. Some AMPs can inhibit biofilm formation as well as eradicating mature biofilm [20], which makes bacteria more resistant to conventional antimicrobial agents which could be up to 100-times more as compared to their planktonic counterparts [21].

Frog skins have been reported as the source of more than 300 different AMPs [22, 23]. Lys-a1 that isolated from frog species, *Hypsiboas albopunctatus* inhibit the biofilm growth of different strains of oral streptococci [24]. Meanwhile, L-K6 peptide that isolated from *Rana chensinesis* skin secretion has significantly reduced cell viability within *S. mutans* biofilms [25]. Esculentin(1-21), amphibian skin membrane-active peptide of *Rana* genus can kill both planktonic and biofilms forms of *Pseudomonas aeruginosa* strains. [26]. A recent study showed the

inhibition ability of peptide B1CTcu5 can be enhanced with the removal of four N-terminal amino acids [27].
In this current study, five lyophilized frog's skin secretions were tested for antibiofilm activity against *Staphylococcus epidermidis* (ATCC 35984). Antibiofilm activities were carried out at three stages of biofilm formation i.e. attachment, maturation and dispersion. Biofilm formation of *Staphylococcus epidermidis* in microtiter plate indicates the presence of antibiofilm activities. The aim of this study is to identify peptide with antibiofilm activities from frog species.

2. Method

Bacterial Culture
Brain Heart Infusion (BHI) (Difco) and Tryptic Soy (TS) (Difco) media were used for the culture of *S. epidermidis* ATCC 39584 and *S. epidermidis* ATCC 12228. Pure cultures of the *S. epidermidis* strains were grown on BHI agar for 18 – 24 hours at 37°C before incubation in fresh BHIB at 37°C with shaking for 24 hours.

Sample preparation
Five lyophilized frogs skin secretions from three different species were obtained from previous study, Zainon *et al*., 2015 [28]. They are *Fejervarya multistriata* (A), *Rana catesbiana* (B1 and B2, collected from different locations) and *Fejervarya limnocharis* (C1 and C2, collected from different locations). The samples were diluted in 0.1 M phosphate buffer, pH 6.0 (PBS) at 0.05 g/ml and stored at -20°C until further used.

Biofilm Formation Assay
Biofilm formation was determined using the microplate assay as described by Stepanović et al., 2007 [30]. An overnight culture of *S. epidermidis* ATCC 35984 (test bacteria and positive control) and *S. epidermidis* ATCC 12228 (negative control) were diluted in fresh Tryptase Soy Broth (TSB) supplemented with 1% glucose (TSBglu) at 1:100 ratio and incubated until the cell density reached ~10^{-6} cells/ml. Then, 100 µL of the test bacteria were transferred into 96-well flat bottom polystyrene microtitre plates and incubated at 37°C for 24 hour. The plate were gently washed three times with 300 µL of PBS, pH 7.2. The adherent biofilm layer formed were fixed with 150 µL of methanol for 20 minute and with 1 % crystal violet for 15 minutes at room temperature. The excess stain were removed by gentle washing under tap water. The wells were air-dried and the absorbed crystal violet stain was resolubilized with 150 µL of ethanol per well for 30 minutes. The plate was observed with microtiter plate reader at optical density value of 570 nm (OD_{570}).

Inhibition of Biofilm formation assay
For the biofilm inhibition assay, the above procedure for biofilm formation was carried out, except that after 2 hours of incubation, 100 µL of skin secretion samples were added to the wells followed by incubation for another 22 hours. All sample secretion was screened at 3 different stages of biofilm formation which are attachment, maturation and dispersion stages. All assays were performed in triplicate and repeated for 3 times. The average OD values of tested and control bacteria were calculated (Table 1,2 and 3). The positive control and negative control represented as 100% and 0 % biofilm formation respectively. The percentage of biofilm formation is calculated as

$$100 - [[(S - N) / (P - N)] \times 100\ \%]$$

where S = biofilm formation treated with sample
N = biofilm formation in the negative control
P = biofilm formation in the untreated positive control

Inhibition of biofilm attachment assay
The assay for inhibition of biofilm attachment was performed in a similar manner as biofilm formation assay. For the inhibition of biofilm attachment assay, the wells were pre-incubated with 100 µL of the samples before the bacteria culture were added and incubated for 24 hours. The biofilms were then fixed, stained, washed and the resolubilised as described in the biofilm formation assay. OD readings were taken and the percentage of biofilm formation was calculated.

Biofilm dispersion assay
For the biofilm dispersion assay, the test and control strains were allowed to form biofilm by incubation at 37°C for 24 hour before addition of the skin secretion samples followed by a further incubation of 2 hours. The biofilms were fixed, stained, washed and resolubilised as described in the biofilm formation assay. OD readings were taken and the percentage of biofilm formation was calculated.

Peptide Purification
Column chromatography was carried out according to He *et al*., 2012 [31] with modifications. Approximately 0.5 g of lyophilized crude skin secretion sample was dissolved in 10 ml of PBS and filter-sterilized with 0.45 µm (cellulose acetate or nylon) followed by centrifugation at 5000 g for 10 min**.** Gel chromatography was prepared by suspending Sephadex G-50 (Superfine, Amersham Biosciences) in 0.1 M phosphate buffer, pH 6.0 (PBS) in a column (diameter 2.6 cm, length 100 cm). The sample was then introduced and eluted with PBS and fractions of 3 ml were collected and monitored at absorbance of 280 nm.
All fractions were then lyophilized and diluted with 2 ml of PBS (0.1 M, pH 6). A small volume of diluted samples were transferred to 1 ml of PBS with 1:10 ratio before tested with biofilm inhibition assay for confirmation test. Positive fractions were pooled, lyophilized and re-suspended in 1 ml of PBS. Then, it was applied to a C18 reverse phase high-performance liquid chromatography (RP-HPLC, AGILENT 770995-902 300Extend-C18, 4.6x250mm, 5 micron) column.

Peptide sequencing
Mass spectroscopy of the sample was performed by the Australian Proteome Analysis Facility Ltd (Macquarie University, Sydney, Australia) on a Triple TOF 5600 (AB Sciex).

3. Results

Biofilm Inhibition Assay
Table 1 shows the classification of biofilm formation activity. Biofilm formation in *S. epidermidis* ATCC 12228 is weak at an OD of less than 0.3000, and this is considered as the negative control. Biofilm formation in *S. epidermidis* ATCC 39584, without treatment, is classified as very strong biofilm activity at OD readings of 2.000 – 3.000.

Table 1: Classification of biofilm formation activity

Optical Density (OD_{570})	Indicator
OD ≤ OD-ve	Non-biofilm activity
OD-ve< OD ≤ 2×OD-ve	Weak biofilm activity
2×OD-ve< OD ≤ 4×OD-ve	Moderate biofilm activity
4×OD-ve< OD ≤ OD+ve	Strong biofilm activity
OD >OD+ve	Very strong biofilm activity

F. multistriata (A), *R. catesbiana* (B1) and *F. limnocharis* (C1) were able to demonstrate inhibition of biofilm formation of *S. epidermidis* (ATCC 35984) at the attachment stage (Figure 1, Table 2). After treatment with the skin secretion of the *F. multistriata* (A), *R. catesbiana* (B1) and *F. limnocharis* (C1), the mean OD ± SEM of the biofilm formation were 1.42 ± 0.20, 1.17 ± 0.18 and 1.72 ± 0.18 respectively which was less than to the

positive control of *S. epidermidis* (ATCC 35984) at 2.21 ± 0.04. The biofilm formations were reduced to 55 % (*F. multistriata* (A)), 40.7 % (*R. catesbiana* (B1)) and 71.7 % (*F. limnocharis* (C1)). This indicates that skin secretion of *F. multistriata* (A), *R. catesbiana* (B1) and *F. limnocharis* (C1) contain bioactive compounds with antibiofilm activities which can inhibit almost 45 %, 28.3 % and 59.3 % of biofilm formation of *S. epidermidis* (ATCC 35984).

However, no reduction of biofilm was observed in the skin secretion of the remaining three samples.

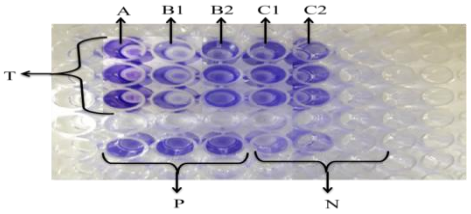

Figure 1: Inhibition of biofilm attachment. All frog skin secretion samples and *S. epidermidis* were incubate for 4 hour. From the left, Sample A, Sample B1, Sample B2, Sample C1 and Sample C2. Positive control (P) and Negative control (N) were placed at the bottom of samples. T is representing as triplicate of samples.

Table 2: Inhibition of biofilm attachment

Sample	Mean OD ± SEM	%biofilm formation	*t* Test (*P* < 0.05)
A	1.42 ± 0.20	55.0	0.00136
B1	1.17 ± 0.18	40.7	0.00003
B2	2.45 ± 0.14	113.3	0.11419
C1	1.72 ± 0.18	71.7	0.01493
C2	2.34 ± 0.07	106.9	0.14831
Positive	2.21 ± 0.04	100.0	-
Negative	0.46 ± 0.01	0	-

Data shown represent mean of the optical density (OD) with standard error of mean (SEM). Each sample was compared to the positive control using Student's *t* test.

F. multistriata (A) and *R. catesbiana* (B1) were capable to reduce *S. epidermidis* (ATCC 35984) biofilm formation at maturation stage (Figure 2, Table 3). The percentage of biofilm formation after the treatment with the skin secretion of the *F. multistriata* (A) and *R. catesbiana* (B1) were reduce to 65.8 % and 75.5 % respectively. The mean OD ± SEM for both *F. multistriata* (A) and *R. catesbiana* (B1) samples were 2.09 ± 0.19 and 2.32 ± 0.24 respectively which was less than to the positive control of *S. epidermidis* (ATCC 35984) at 2.92 ± 0.17. The biofilm formations were decreased by 34.2 % (*F. multistriata* (A)) and 24.5 % (*R. catesbiana* (B1)). This indicates that bioactive compounds in skin secretion of *F. multistriata* (A) and *R. catesbiana* (B1) can inhibit the formation of biofilm in *S. epidermidis* (ATCC 35984) at maturation stage.

Unlike at the attachment stage, *F. limnocharis* (C1) was unable to reduce biofilm formation at maturation stage. The mean OD ± SEM of *F. limnocharis* (C1) was 2.96 ± 0.18 which was more than positive control. However, still no reduction of biofilm was observed in the skin secretion of the two remaining samples.

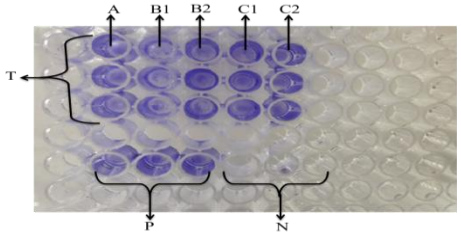

Figure 2: Inhibition of biofilm maturation. All frog skin secretion samples and *S. epidermidis* were incubate for 24 hour. From the left, Sample A, Sample B1, Sample B2, Sample C1 and Sample C2. Positive control (P) and Negative control (N) were placed at the bottom of samples. T is representing as triplicate of samples.

Table 3: Inhibition of biofilm formation (maturation)

Sample	Mean OD ± SEM	% biofilm formation	T Test (P < 0.05)
A	2.09 ± 0.19	65.8	0.00528
B1	2.32 ± 0.24	75.5	0.06171
B2	2.90 ± 0.17	99.2	0.93435
C1	2.96 ± 0.18	101.6	0.87742
C2	2.83 ± 0.17	96.5	0.72780
Positive	2.92 ± 0.17	100	-
Negative	0.48 ± 0.03	0	-

Data shown represent mean of the optical density (OD) with standard error of mean (SEM). Each sample was compared to the positive control using Student's *t* test.

All samples of the frog skin secretions except *R. catesbiana* (B2) were unable to remove matured biofilm of *S. epidermidis* (ATCC 35984) at the dispersion stage (Figure 3, Table 4). The mean OD ± SEM for all sample were very close to the positive control of *S. epidermidis* (ATCC 35984) at 3.35 ± 0.12 compare to *R. catesbiana* (B2) that was 2.96 ± 0.10. The frog skin secretion of *R. catesbiana* (B2) was able to reduce the biofilm formation to 86.1 %. This indicates that *R. catesbiana* (B2) skin secretion still inhibit 13.9 % of biofilm formation while no biofilm activities were observed in the skin secretion of the four remaining samples.

Table 4: Biofilm dispersion assay

Sample	Mean OD ± SEM	% biofilm formation	T Test (P < 0.05)
A	3.22 ± 0.15	95.4	0.50819
B1	3.07 ± 0.19	89.9	0.23117
B2	2.96 ± 0.10	86.1	0.02197
C1	3.37 ± 0.14	100.7	0.91583
C2	3.12 ± 0.07	91.7	0.11341
Positive	3.35 ± 0.12	100.0	-
Negative	0.58 ± 0.04	0	-

Data shown represent mean of the optical density (OD) with standard error of mean (SEM). Each sample was compared to the positive control using Student's *t* test.

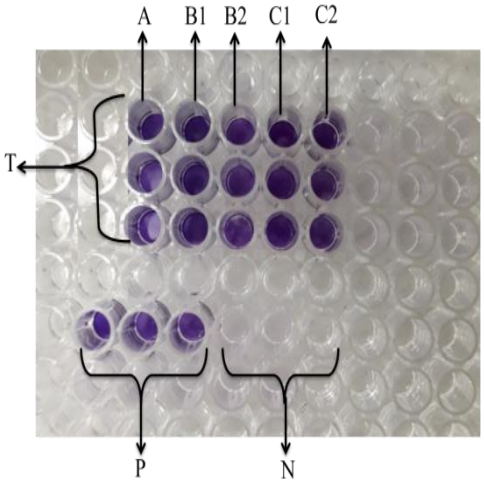

Figure 3: Inhibition of biofilm dispersion. *S. epidermidis* strain was incubating for 24 hour to allow biofilm to form. All frog skin secretion samples and *S. epidermidis* were incubated for 24 hour. From the left, Sample A, Sample B1, Sample B2, Sample C1 and Sample C2. Positive control (P) and Negative control (N) were placed at the bottom of samples. T is representing as triplicate of samples.

Purification of antibiofilm peptides

The skin secretion of *F. multistriata* was separated into 200 collection tubes by Sephadex G-50 gel filtration (Figure 4). Samples that suspected to have antibiofilm activity were selected and divided into 4 fractions. Fraction 3 was confirmed to have antibiofilm activity against *S. epidermidis* biofilm compare to other fractions when tested with biofilm inhibition assay at attachment stage.

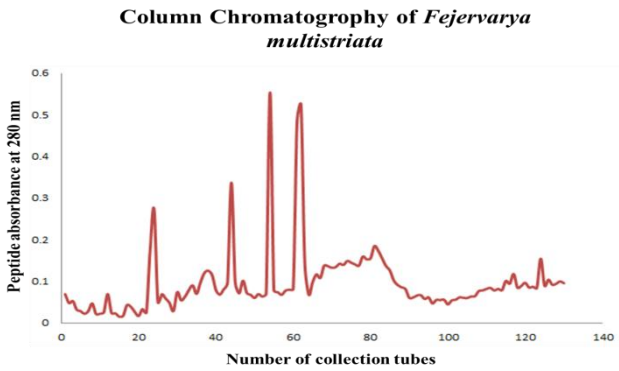

Figure 4: Separation of lyophilized skin secretion of *F. multistriata*. Lyophilized sample was applied to Sephadex G-50 gel filtration (flow rate of elution at 0.3ml/min.

Table 5: Inhibition of biofilm attachment (Confirmation test)

Fraction	Average OD	% biofilm formation
1	0.97	36.3
2	1.46	79.6
3	0.66	8.8
4	1.16	53.1
Positive	1.69	100
Negative	0.56	0

Fraction 3 were further purified by C_{18} reverse phase high-performance liquid chromatography (RP-HPLC) (Figure 5) and a peak (retention time = 4.116 min) was eluted. Purified peptide was further analyzed for peptide identification.

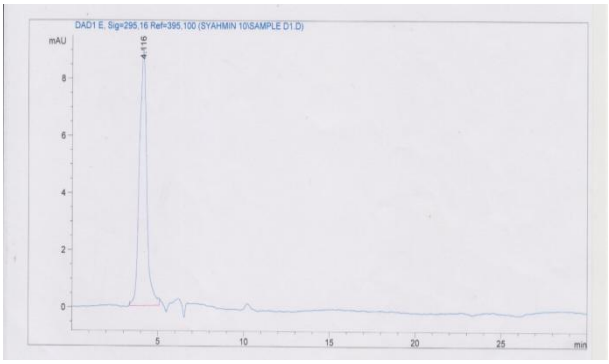

Figure 5: Purification of antibiofilm peptide. Fraction X was subjected onto C_{18}RP-HPLC column. Elution was performed with 65 % of acetonitrile and 35% of trifluoroacetic acid (TFA) at flow rate of 1.0 ml/min.

Structural characterization

The purified peptide was subjected to trypsin digestion and peptide mass fingerprinting. One peptide was identified with the sequence LCQSLPFGGVK. The peptide sequence is similar to peptide XP_010940924.1 which is predicted to be part of the aldehyde dehydrogenase enzyme.

4. Discussion

Staphylococcus epidermidis is a Gram-positive bacteria and a coagulase-negative staphylococcus [32] that commonly found from skin and mucous membranes of humans and other mammals [33]. *S. epidermidis* represents the major causative organism of infections related to any type of indwelling medical devices, such as peripheral or central intravenous catheters (CVCs) [34, 35]. Many *S. epidermidis* strains produce sessile microbial community known as biofilm. *S. epidermidis* produce extracellular polymeric matrix known as poly-N-acetyl glucosamine (PNAG) that resist to antimicrobial agent [36, 34].

Antimicrobial peptides (AMPs) are the alternative way to against bacterial biofilms and antibiotic resistant strains more effectively [37]. A number of peptides exhibit antibiofilm activities that cause inhibition or eradication of the biofilm and provide a first line of defense of virtually all organisms [47, 48]. Most frogs secrete AMPs through their skin glands [12]. As the source of more than 300 different AMPs, many of frog skin secretion have been used for inhibition of bacteria biofilm [22, 23]. Report shows that magainin 2 has antibiofilm activity against E. coli [38], *S. aureus* [39], and *P. aeruginosa* (at higher concentrations) [38, 39].

F. multistriata (belonging to the subfamily Dicroglossinae of the family Dicroglossidae) is known as Rice Paddy Frogs that found in in regions with rice agriculture [40]. Previous report shows that Tigerinin peptides from *Hoplobatrachus tigerinus* [41] and *F. cancrivora* [42] can display broad-spectrum antimicrobial activity despite inability to adopt a α-helical conformation [41, 42]. Another report has revealed that skin secretion of *Phyllomedusa hypochondrialis* contains distinctin-like-peptide-PH (DLP-PH) that displayed antibiofilm activities against *Escherichia coli* [46].

In this study, peptide from *F. multistriata* (A), *R. catesbiana* (B1) and *F. limnocharis* (C1) have shown antibiofilm activities by inhibiting *S. epidermidis* biofilm more than 20 % but less than 60 % at attachment stage. However, antibiofilm activities became less effective at other stages due to percentage of biofilm inhibition by each peptide samples from *F. multistriata* (A), *R. catesbiana* (B1) and *F. limnocharis* (C1) was less at maturation stage compare to attachment stage. However, no antibiofilm activity of peptide samples from *F. multistriata* (A), *R. catesbiana* (B1) and *F. limnocharis* (C1) were observed at dispersion stage. *S. epidermidis* strain (ATCC 35984) known as one of the strong biofilm producer [43]. Report shows that antimicrobial effect of F2, 5,12W was more effective against weak biofilm-producing *S. epidermidis* strain (BM185) compare to the strong biofilm-forming strain (BM492) [44]. This is due to the presence of polysaccharide intracellular adhesin (PIA) of staphylococcal biofilm matrix that increase the protection from AMPs penetration into the biofilm structure [45]. This might be reason for the reduction of antibiofilm activities of peptides at maturation and dispersion stages compare to attachment stages.

In this study, peptide from *F. multistriata* (A) and *R. catesbiana* (B1) have shown the best result to inhibit the biofilm formation of *S. epidermidis* strain (ATCC 35984) compare to *F. limnocharis* (C1) and *R. catesbiana* (B2) that only show antibiofilm activity at single stage. However, *F. multistriata* (A) has been chosen for further analyzed due to have better result compare to other samples at the maturation stage. MS fingerprinting of the peptide reveal a sequence that is not matched to known peptides from frogs. Rather the peptide sequence has similarities to a part of the plant aldehyde dehydrogenase enzyme.

5. Conclusion

The antibiofilm inhibition assays revealed that frog skin secretion of F. multistriata contain peptides with the ability to inhibit the bacteria biofilm but limited to different stages. Once the mature biofilm is formed, the peptide has no effects. However, further studies are needed to confirm the ability of this peptide to inhibit the bacteria with weak and moderate biofilm activities. This study will contribute to other biofilm studies for searching new antimicrobial peptides (AMPs) profiles from frogs that could be used as novel antibiofilm agents.

Acknowledgement

This study was supported by a Fundamental Research Grant Scheme (FRGS/2/2014/SG03/UITM/03/1) from the Ministry of Higher Education (MOHE), Malaysia. Aspects of this research have been facilitated by access to the Australian Proteome Analysis Facility supported under the Australian Government's National Collaborative Research Infrastructure Strategy (NCRIS).

References

[1] Costerton JW, Lewandowski Z., Caldwell DE, Korber DR & Lappin-Scott HM (1995), Microbial biofilms. Annu Rev Microbiol 49, 711–745.

[2] Donlan RM & Costerton JW (2002), Biofilms: survival mechanisms of clinically relevant microorganisms. ClinMicrobiol Rev 15, 167–193.

[3] Costerton JW, Stewart PS & Greenberg EP (1999), Bacterial biofilms: a common cause of persistent infections. Science 284, (5418), 1318-1322.

[4] de la Fuente-Nu´n˜ez C, Reffuveille F, Ferna´ndez L, Hancock REW (2013), Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. Curr Opin Microbiol 16, 580–9.

[5] O'Neill E, Pozzi C, Houston P, Humphreys H, Robinson DA, Loughman DA, Foster TJ & O'Gara J (2008), A novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. J Bacteriol 190, 3835–3850.

[6] Garrett TG, Bhakoo M & Zhang Z (2008), Bacterial adhesion and biofilms on surfaces. Prog Nat Sci 18, 1049–1056.

[7] Francois P, Schrenzel J, Stoerman-Chopard C, Favre H, Hermann M., Foster TJ, Lew DP & Vaudaux P (2000), Identification of plasma proteins adsorbed on hemodialysis tubing that promote *Staphylococcus aureus* adhesion. J Lab Clin Med 135, 32–42.

[8] Fey PD (2010), Modality of bacterial growth presents unique targets: how do we treat biofilm mediated infections?. Curr Opin Microbiol 13, 610–615.

[9] Hoiby N, Bjarnsholt T, Givskov M, Molin S & Ciofu O (2010), Antibiotic resistance of bacterial biofilms. Int J Antimicrob Ag 35, 322–332.

[10] Boles BR & Horswill AR (2011), Staphylococcal biofilms disassembly. Trends Microbiol 19, 449–455.

[11] Ramlah Zainudin M &Rahman Badr MM Za (2010), Mating Calls Description of Five Species of Frogs from the Genus *Hylarana tschudi* 1838 (Amphibia, Anura, Ranidae) from Sarawak, Malaysia. Sains Malaysian 39(3), 363-369.

[12] Leonardo de Azevedo Calderon, Alexandre de Almeid E Silva, Pietro Ciancaglini & Rodrigo Guerino Sta´beli (2010), Antimicrobial Peptides from Phyllomedusa frogs: from Biomolecular Diversity to Potential Nanotechnologic Medical Applications. Amino Acids DOI 10.1007/s 00726 - 010-0622-3.

[13] Guilherme DB, Raimunda CS, Luisa Mayumi Arake, Valdelânia GS, Leiz MCV, Vladimir C, Carlos Henrique NC, Selma SK, José Guilherme Alexandre, Maria JF & José Roberto SAL (2013), The Skin Secretion of the Amphibian *Phyllomedusa nordestina*: A Source of Antimicrobial and Antiprotozoal Peptides. Moleculaes 18, 2058-2070.

[14] Pukala TL, Bowie JH, Maselli VM, Musgrave IF & Tyler MJ (2006), Host-defence peptides from the glandular secretions of amphibians: structure and activity. Nat. Prod. Rep 23, 368–393.

[15] Kückelhaus SA, Leite JRS, Muniz-Junqueira MI, Sampaio RN, Bloch C & Tosta CE (2009), Antiplasmodial and antileishmanial activities of phylloseptin-1, an antimicrobial peptide from the skin secretion of *Phyllomedusa azurea* (Amphibia). Exp. Parasitol 123, 11–16.

[16] Bowie JH, Separovic F & Tyler MJ (2012), Host-defense peptides of Australian anurans. Part 2.Structure, activity, mechanism of action, and evolutionary significance. Peptides 37, 174–188.

[17] Yeaman MR &Yount NY (2003), Mechanisms of antimicrobial peptide action and resistance. Pharmacol. Rev 55, 27–55.

[18] Nguyen LT, Haney EF & Vogel HJ (2011), The expanding scope of antimicrobial peptide structures and their modes of action. Trends Biotechnol 29, 464–472.

[19] Liu J, Wu Q, Li L, Xi X, Wu D, Zhou M, Chen T, Shaw C & Wang, L (2017), Discovery of phylloseptins that defense against gram-positive bacteria and inhibit the proliferation of the non-small cell lung cancer cell line, from the skin secretions of Phyllomedusa frogs. Molecues 22, 1428.

[20] Grassi L, Maisetta G, Maccari G, Esin S & Batoni G (2017), Analogs of the frog-skin antimicrobial peptide temporin 1tb exhibit a wider spectrum of activity and a stronger antibiofilm potential as compared to the parental peptide. Front. Chem. 5.

[21] Davies D (2003), Understanding biofilm resistance to antibacterial agents. Nat Rev Drug Discov 2(2), 114-122.

[22] Conlon JM & Sonnevend A (2010), Antimicrobial peptides in frog skin secretions. Methods Mol. Biol. 618, 3–14.

[23] Ma YF, Liu CB, Liu XH, Wu J, Yang HL, Wang YP, Li JX, Yu HN & Lai R (2010), Peptidomics and genomics analysis of novel antimicrobial peptides from the frog, *Rana nigrovittata*. Genomics 95, 66–71

[24] da Silva BR, de Freitas VA, Carneiro VA, Arruda FV, Lorenzón EN, de Aguiar AS, Cilli EM, Cavada BS & Teixeira EH (2013), Antimicrobial activity of the synthetic peptide Lys-a1 against oral streptococci. Peptides 42, 78–83.

[25] Shang D, Liang H, Wei S, Yan X, Yang Q, & Sun Y (2014), Effects of antimicrobial peptide L-K6, a temporin-1CEb analog on oral pathogen growth, *Streptococcus mutans* biofilm formation, and anti-inflammatory activity. *Appl Microbiol Biotechnol* 98, 8685–8695.

[26] Luca V, Stringaro A, Colone M, Pini A & Mangoni ML (2013) Esculentin (1–21), an amphibian skin membrane-active peptide with potent activity on both planktonic and biofilm cells of the bacterial pathogen *Pseudomonas aeruginosa*. Cell Mol Life Sci 70, 2773–2786.

[27] Abraham P, Sundaram A, Asha R, George S & Kumar KS (2015), Structure activity relationship and mode of action of a frog secreted antibacterial peptide B1CTcu5 using Synthetically and Modularly Modified or Deleted (SMMD) peptides. PLoS One 10, e0124210

[28] Rahman ZA, Abdullah MFF, Wan Azmi WMS & Abdul-Aziz A (2016), Preliminary study of antimicrobial activity of the skin secretions of Malaysian frogs. Jurnal Teknologi 78(6-5), 65-69.

[29] Wu X, Pan J, Wu Y, Xi X, Ma C, Wang L, Zhou M & Chen T (2017), PSN-PC: A Novel Antimicrobial and Anti-Biofilm Peptide from the Skin Secretion of *Phyllomedusa-camba* with Cytotoxicity on Human Lung Cancer Cell. Molecules 22(11),1896.

[30] Stepanović S, Vuković D, Hola V, Di Bonaventura G, Djukić S, Cirković I & Ruzicka F (2007), Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS 115(8), 891–899.

[31] He X, Yang S, Wei L, Liu R, Lai R & Rong M (2013), Antimicrobial peptide diversity in the skin of the torrent frog, *Amolops jingdongensis*. Amino Acids 44(2), 481–487. Published online 2012 Jul 25. doi: 10.1007/s00726-012-1358-z

[32] Vuong C & Otto M (2002), *Staphylococcus epidermidis* infections. Microbes Infect 4, 481–489.

[33] Kloos W & Schleifer KH, Bergey's Manual of Systematic Bacteriology. PHA, S.; S, M.; ME, S.; JG, H., editors, Baltimore: Williams & Wilkins, (1986).

[34] McCann MT, Gilmore BF & Gorman SP (2008), *Staphylococcus epidermidis* device-related infections: pathogenesis and clinical management. J Pharm Pharmacol 60, 1551– 1571.

[35] Rogers KL, Fey PD & Rupp ME (2009), Coagulase-negative staphylococcal infections. Infect Dis Clin North Am 23, 73–98.

[36] Whitfield GB, Marmont LS & Howell PL (2015), Enzymatic modifications of exopolysaccharides enhance bacterial persistence. Front Microbiol 6, 471

[37] Hassan M, Kjos M, Nes IF, Diep DB & Lotfipour F (2012), Natural antimicrobial peptides from bacteria: characteristics and potential applications to fight against antibiotic resistance. J ApplMicrobiol 113, 723–736.

[38] Zasloff M., Martin B & Chen HC (1988), Antimicrobial activity of synthetic magainin peptides and several analogues. Proceedings of the National Academy of Sciences of the United States of America 85(3), 910–913.

[39] Giacometti A, Cirioni O, Barchiesi F & Scalise G (2000), In-vitro activity and killing effect of polycationic peptides on methicillin-resistant *Staphylococcus aureus* and interactions with clinically used antibiotics. DiagnMicrobiol Infect Dis 38, 115-118.

[40] Kaiser H, Carvalho V, Ceballos J, Freed P, Heacox S, Lester B, Richards S, Trainor C, Sanchez C & O'Shea M (2011), The herpetofauna of Timor-Leste: a first report. ZooKeys 109, 19–86

[41] Sai KP, Jagannadham MV, Vairamani M, Raju NP, Devi AS, Nagaraj R &Sitaram N (2001), Tigerinins: novel antimicrobial peptides from the Indian frog *Rana tigerina*. J BiolChem 276, 2701- 07.

[42] Yuzhu S, Yi L, Lijun W, Hailong Y, Keyun Z &Ren L (2009), Purification, characterization and cloning of two novel tigerinin-like peptides from skin secretions of *Fejervarya cancrivora*. Peptides 7, 1228-32.

[43] Christensen GD, Simpson WA, Bisno AL & Beachey EH (1982), Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infect Immun 37(1), 318–326.

[44] Molhoek EM, van Dijk A, Veldhuizen EJ, Haagsman HP & Bikker FJ (2010), A cathelicidin-2-derived peptide effectively impairs *Staphylococcus epidermidis* biofilms. International Journal of Antimicrobial Agents Volume 37, Issue 5, 476 – 479

[45] Vuong C, Voyich JM, Fisher ER, Braughton KR, Whitney AR, DeLeo, FR & Otto M (2004), Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. Cell. Microbiol. 6, 269–275

[46] Wu D, Gao Y, Tan Y, Liu Y, Wang L, Zhou M, Xi X, Ma C, Bininda-Emonds ORP, Chen T & Shaw C (2018), Discovery of Distinctin-Like-Peptide-PH (DLP-PH) From the Skin Secretion of *Phyllomedusa hypochondrialis*, a Prototype of a Novel Family of Antimicrobial Peptide. Frontiers in Microbiology. 9, 541.

[47] Haney EF, Mansour SC & Hancock RE (2017), Antimicrobial Peptides: An Introduction. Methods Mol Biol. 1548, 3-22.

[48] Andrea A, Molchanova N & Jenssen H (2018), Antibiofilm Peptides and Peptidomimetics with Focus on Surface Immobilization. Biomolecules. 8(2), 27.

[49] Jacobsen AS & Jenssen H (2012), Human cathelicidin LL-37 prevents bacterial biofilm formation. Future Med Chem. 4(12), 1587-99.

[50] Overhage J, Campisano A, Bains M, Torfs EC, Rehm BH, Hancock RE (2008), Human host defense peptide LL-37 prevents bacterial biofilm formation. Infect Immun. 76(9), 4176-82.