ORIGINAL ARTICLE

Antibacterial Efficacy *Oreochromis niloticus* (Nile Tilapia) Acid Solubilized Collagen towards *Staphylococcus aureus* and *Pseudomonas aeruginosa* Skin Infection in Mice

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ABSTRACT

Introduction: *Oreochromis niloticus* is one-third of the most aqua cultured fish around the world. Recent studies shows that *Oreochromis niloticus* extracted collagen from skin, scale, and bones shows promising quality for wound healing due to its biocompatibility. However, few studies are recorded regarding the antibacterial efficacies of the *Oreochromis niloticus* extracted collagen. The objective of the research is to evaluate the antibacterial efficacy of acid solubilized collagen extracted from *Oreochromis niloticus* scales toward *Staphylococcus aureus* and *Pseudomonas aeruginosa* skin infection in mice. **Materials and Methods:** 54 Balb-c mice weight around 25-30 g were divided equally under two bacteria groups, and further separated into three groups, which is positive, negative and tested group. The positive group were treated with acriflavine, tested group were treated with *Oreochromis niloticus* scales collagen, and the negative group were left untreated. The treatment were given everyday and the duration of treatment were performed for nine days. In every three days interval of treatment, swab sample on the skin infection of mice were taken to perform the Colony Formation Unit (CFU) counting. The data recorded were analysed using analysis of variance (ANOVA) test and student T-test. **Results:** There are no significance differences between the antibacterial efficacies between the treatments for all mice groups. However, acriflavine shows the highest bacterial efficacy towards *Staphylococcus aureus* and *Pseudomonas aeruginosa* skin infection on mice, followed by acid solubilized collagen treatment. **Conclusion:** *Oreochromis niloticus* scale extraction acid solubilized collagen shows low antibacterial efficacy towards *Staphylococcus aureus* and *Pseudomonas aeruginosa* skin infection.
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Keywords: acid solubilized collagen, antibacterial efficacy, *oreochromis niloticus*, *pseudomonas aeruginosa*, *staphylococcus aureus*

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INTRODUCTION

Wound infection persists to be an arduous complication and exhibiting significant burden toward healthcare sector (1). This issue is one of most frequent and can be most potentially dangerous at same time. The complication of the badly managed wound infection can consequently increase the medical expenses, invoke secondary complications, and even cause loss of extremity and fatality. Immediate identification of infection along with early, suitable, and functional intervention are more critical than ever in minimize its economic and health repercussion, particularly in the climate of growing crisis of antibiotics resistance (1). Bacteria have vital role-play in influencing the magnitude of inflammatory response in tissue repair still there are many others element can modulate magnitude of infection. The other factors can also cause various effect from development of wound colonization to the infectious bacterial count, the bacteria species present, host innate immune response, the number of other types of bacterial species present in area, the virulence factor of the microorganism and its symbiotic relationship with the organism (2).

Among all pathogen that present in wound, *Pseudomonas aeruginosa* pose high potential of developing antibiotic resistance, hence increasing its pathogenicity factor. The

antibiotic resistant strains of *pseudomonas aeruginosa* were categorized by many healthcare groups for it high risk toward public health sector. Accounting of *Pseudomonas aeruginosa* high mortality rate and limited treatments option causing this species under classify as high priority in research for establishing new revolutionary therapies (3).

Oreochromis niloticus, or commonly known as Nile Tilapia, is one-third of the most aqua cultured fish around the world. Nile Tilapia is one of the popular fishes that used for food due to its meat contain mild flavour and white coloured. Malaysia's neighbour country, Thailand have mass-produced Nile Tilapia around 140,000 tons, which is considered as the world's sixth tilapia production in the world. Few of the fish production are contribute to tilapia fillets. After processing, it is estimate that the scales generated as waste weight around 700 tons. Normally, the tilapia scales are discard as waste or used for fertilizers; however, its value can be added by converting the waste scale to collagen for multiple uses (4). In Malaysia, the production of the tilapia has increased consistently and becoming an important source of fish supply. The fish is classify as a long lasting, can grow rapidly compared to another fish species and highly resistance to disease (4).

Currently, there are few studies regarding the antibacterial efficacy of the Nile Tilapia collagen. In recent studies, it is proven that Nile Tilapia collagen have a good quality for soft and hard tissue regenerations. It is also worth mention that its quality, cost effective and the good response from in vivo and in vitro studies revolving around the fish making it possibly as a satisfactory source of collagen (4). Another studies also noted that pepsin-solubilized collagen that derived from the Nile Tilapia skin is applicable for the formation of vascular endothelial cells. It is also compatible in nature and possibly used as biomedical material (4). In wound healing, collagen are made from fibroblast. Collagen make up the most extensive type of protein present in human body, and it is vital in all stages of the tissue healing process by activating cellular activity, contributing to new tissue growth while encouraging autolytic debridement, angiogenesis, and reepithelialisation. The presence of collagen in damaged tissue is highly benefit toward promoting repair in hard-to-heal wounds. With the ability to stimulating cytokine of macrophages and fibroblasts to the damaged site, causing production the deposition of new collagen matrix (5). Nile Tilapia collagen also can be used as an alternative source of collagen, as the current main source of collagen, which is tendon or skin of bovine and porcine have religious restriction towards Muslim and Jews (4). Collagen extracted from Nile Tilapia collagen also proven to be a biocompatible type I collagen with potential use as biomedical material (4).

MATERIALS AND METHODS

Materials

List of drugs that were used in this experiment were acriflavine, xylazine and ketamine.

- 5 ml of acriflavine were used as topical treatment for *P. aeruginosa* infection in positive control group.
- 5mg/kg of xylazine and 80mg/kg ketamine were used for local anaesthesia to induce sleep and numbness. The calculation of dosage drug use in the study as followed.

Bacteria Culture Preparation

In this experiment, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were selected for antibacterial testing of Nile Tilapia collagen. The bacteria were collected from the UniKL MESTECH bacteria inventory, and will be undergo serial dilution following the McFarland standard 1.0 ($1.5x10^8$ CFU/ mL) to obtain fixed amount of bacteria for the experiment. To obtain the bacteria broth of McFarland standard 1.0, two test tubes were filled with 2mL of sterile normal saline 0.9% and labelled accordingly. With sterile micropipette, several drops of original bacteria broth were dropped into the labelled test tubes until it reached McFarland standard 1.0 turbidity by comparing the tubes with Wickerham Card.

Animal Preparation

All animal protocols used in the study were approved by the Universiti Kuala Lumpur Research Ethics Committee (UNIKL REC/2022/FRGS/CO/03). 54 male BALB/c mice weight around 25-30 g were used. The animals were housed for one-week prior to experimentation for acclimatization to the new environment. The mice were divided equally under two different bacteria categories, which is a total of 27 mice in the *Staphylococcus aureus* group and 27 mice in the *Pseudomonas aeruginosa* group. The mice were further divided equally into three groups, which are the positive control, negative control and the tested group. All mice were randomly assigned to the groups. Each cage contains three mice and the kept in a controlled environment, which include a 12-hour day/night cycle and a setting for room temperature. The animals had free access to food and water throughout the experiment period.

Preparation of Nile Tilapia Scale Collagen

0.5 kg/piece of fresh Nile Tilapia were obtained from the local fish market. Scales were washed, drained, and packed in a polyethylene bag, frozen and were kept below -18°C. The scale were stored 1 month before the extraction process of collagen. Before collagen extraction, running water was used for thawing the frozen scale until the temperature increase until 8°C reached. All the procedures of acid solubilized collagen extraction were carried out under 4°C with some adjustment following the ASC extraction procedure from (4) and (6). First, the scales were mixed and stirred

with 0.1 M NaCL at the ration of 1:10 (w/v) for 24 hours to remove the non-collagenous protein. Then, the fish scale undergo demineralization process by treating with 0.4 mol/L HCI solution with the ration of one part of dry scales to 15 parts of solution for 90 minutes and then were rinsed with distilled water around three times. After that, the extraction of collagen are followed the method by (7) with some adjustments. All procedure were done at 4°C. First, the scale were cut into smaller pieces and were put into a 0.5 M acetic acid solution for extraction on (1:1 ratio) for two days. After that, the extracts suspension were centrifuged at 4000 rpm for 30 minutes. Then, the supernatant were gathered, and the residues were re-extracted with the same solution for another one day and centrifuged under same condition. The supernatant of both extracts were combined and salt out by adding NaCL to a final concentration of 0.7 M. Then, the precipitated collagen were collected by centrifugation at 4000 rpm for 45 minutes and then re-dissolved in 0.5 M acetic acid to precipitate with NaCL again. The resulting precipitated was dialyzed against water and lyophilized.

Wound Incision and Bacteria Inoculation

In this study, wounds were inflicted on the dorsal area of mice. Prior to wound incision, mice were anesthesized using a combination of 2.6 mL ketamine (100 mg/kg) and 1.6 mL xylazine (10 mg/kg), diluted in 15.8 mL of sterile distilled water, administered via the intraperitoneal route. The dorsal area was then prepared by trimming the hair with an electric trimmer and swabbing with an alcohol swab for sterilization. Wound incision was performed using an 8 mm biopsy punch. Subsequently, the wounds were inoculated with 40 µL of *Staphylococcus aureus* and *Pseudomonas aeruginosa* ($3.0x10^8$ CFU/mL) according to the respective mice groups, followed by a three day growth period to ensure the bacteria have established a robust infection. Treatment were not given during the three day growth period. The negative control group received no treatment, while the positive control group was treated with acriflavine, and the tested group was treated with acid solubilized collagen (ASC) extraction. Biochemical tests and Gram staining were conducted to confirm the bacterial growth on the wound after three days growth period. For the biochemical test, catalase test and coagulase test were performed to *Staphylococcus aureus* as it has various defence mechanism and catalase enzyme is one of them. This enzyme enables the bacteria to neutralize hydrogen peroxide, which has bactericidal effects (8).

Animal treatment and monitoring

The experiment were done for 9 days. Extraction of ASC and acriflavine were applied at the wound site one time every day until day 9. The mice health condition and behaviour were monitored daily. If the mice shows severe weight loss, impaired mobility, and severe wound necrosis during the experiment, the mice will be sacrificed according to the animal ethics procedure.

Sample collection and Colony Formation Unit (CFU) count

The mice were sacrificed on day 3, 6 and 9 for sample collection. Before the mice were sacrificed, the wound were swabbed in a circular motion with the Q-swab that is moistened with sterile saline for sample collection. The wound was measured with digital calliper and the swab were used for CFU counting. The data were recorded.

Data Analysis

After the CFU count has been collected, statistical analysis were carried out using Microsoft excel 2211 (year 2022). The data will be comparing the mean differences and standard deviation of each group. Comparing between all three-group using the analysis of variance (ANOVA) and T-test to compare between the two groups. All data are quantitative data.

RESULTS

After three days of bacteria inoculation, symptoms of bacterial skin infection are present, which confirms that there are presence of bacteria on the bare mice skin. Three days after wound infliction and *Staphylococcus aureus* inoculation, accumulation of pus and abscess were observed on all the mice groups. Due to the wound infliction, the physical skin barrier were opened, leading to an increase of pH on the skin surfaces, leading to the growth of *Staphylococcus aureus* (9). This shows that *Staphylococcus aureus* are able to colonize the inflicted wound at the dorsal area of the mice. The *Pseudomonas aeruginosa* infection in mice skin wound have similar sign symptom as exhibit in human. It is known to cause skin wound infection, tissue damage, delayed remodelling, and systemic infection (10). On a normal condition, the bacteria does not able to colonize the skin, however when there is change in environment of the skin it may promotes the growth of commensal pathogens (11).

Colony Formation Unit (CFU) of *Staphylococcus aureus* and *Pseudomonas aeruginosa* between mice group on day 9 post treatment

There is a decreasing trend of CFU numbers of *Staphylococcus aureus* skin infected mice during treatment, which indicate that the treatment are able to reduce the bacteria amount. It is worth noticed that acriflavine treated group shows the lowest number of CFU among the other group for nine days for *Staphylococcus aureus* skin infected mice while for *Pseudomonas aeruginosa* skin infected mice, there are occurrence of irregular pattern of CFU data numbers. This may due to improper technique of swabbing or dilution technique during experimental days (Fig.1-3). Standardized swabbing technique should be implemented for future suggestion.

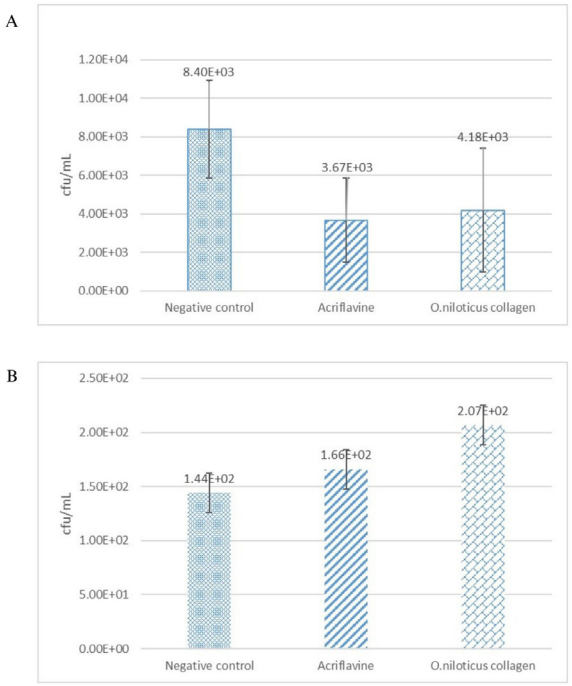

Fig. 1: Comparison of *Staphylococcus aureus and Pseudomonas aeruginosa* CFU mean between mice groups on day 3. The average of mean value for the CFU unit of *Staphylococcus aureus* in Acriflavine treatment is the lowest (3.67 x 10^3 cfu/ml) compared to collagen treatment (4.18 x 10^3 cfu/ml) and negative control (8.40 x 10^3 cfu/ml) (A). The average of mean value for the CFU unit of *Pseudomonas aeruginosa* in negative control is the lowest (1.44 x 10^2 cfu/ml) compared to Acriflavine treatment (1.66 x 10^2 cfu/ml and collagen treatment 9 2.07 x 10^2 cfu/ml) (B).

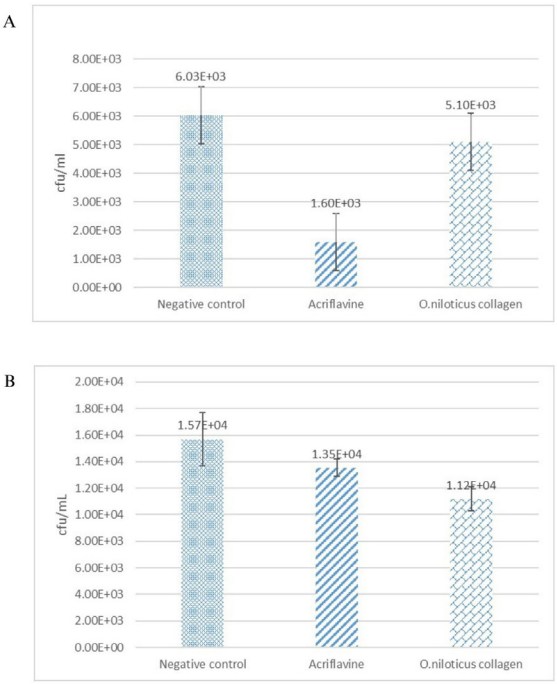

Fig. 2: Comparison of *Staphylococcus aureus and Pseudomonas aeruginosa* CFU mean between mice groups on day 6. The average of mean value for the CFU unit of *Staphylococcus aureus* in Acriflavine treatment is the lowest (1.60 x 10^3 cfu/ml) compared to collagen treatment (5.10 x 10^3 cfu/ml) and negative control (6.03 x 10^3 cfu/ml) (A). The average of mean value for the CFU unit of *Pseudomonas aeruginosa* in collagen treatment is the lowest (9.12 x 10^3 cfu/ml) compared to Acriflavine treatment (9.50 x 10^3 cfu/ml) and negative control (1.24 x 10^4 cfu/ml) (B).

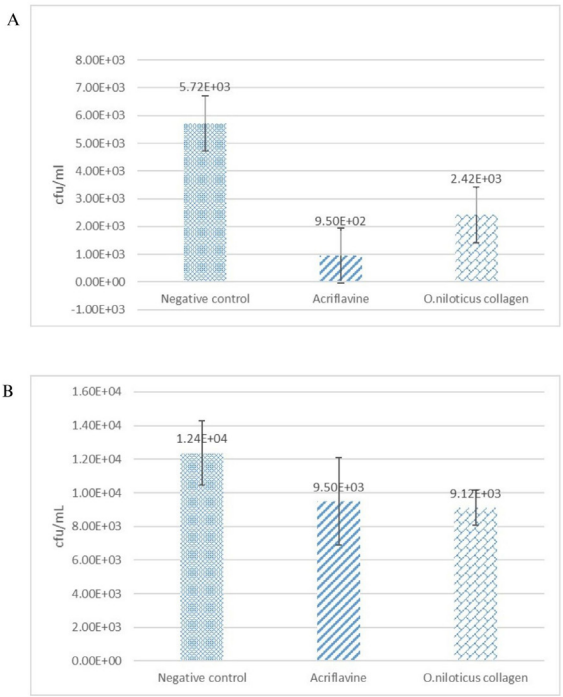

Fig. 3: Comparison of *Staphylococcus aureus and Pseudomonas aeruginosa* CFU mean between mice groups on day 9. The average of mean value for the CFU unit of *Staphylococcus aureus* in Acriflavine treatment is the lowest (9.50 x 10^2 cfu/ml) compared to collagen treatment (2.42 x 10^3 cfu/ml) and negative control (5.72 x 10^3 cfu/ml) (A). The average of mean value for the CFU unit of *Pseudomonas aeruginosa* in collagen treatment is the lowest (1.12 x 10^4 cfu/ml) compared to Acriflavine treatment (1.35 x 10^4 cfu/ml) and negative control (1.57 x 10^4 cfu/ml) (B).

Comparison mean value of *Staphylococcus aureus and Pseudomonas aeruginosa* CFU and mice group on day 9

On day nine of treatment for *Staphylococcus aureus* group, acriflavine showed the lowest amount of CFU ($9.50x10^2$), followed by ASC from *Oreochromis niloticus* ($2.42x10^3$). Based on the One Way ANOVA test, it is noted that the difference between every treatment is statistically not significant (Table I).

As for the *Pseudomonas aeruginosa* group, the group treated with ASC showed the lowest CFU count ($1.12x10^4$), followed by acriflavine treated ($1.35x10^4$). One Way ANOVA test showed that the difference between every treatment is statistically not significant (Table II).

Comparison of acriflavine treatment and acid solubilized collagen treatment against *Staphylococcus aureus* and *Pseudomonas aeruginosa.*

According to the Student T-Test analysis of both bacteria group, it is statistically not significant between both acriflavine and ASC treatment against *Staphylococcus aureus* (P=0.111) and *Pseudomonas aeruginosa* (P=0.064). However, the declining number of CFU of the bacteria indicates a limited antibacterial effect. (Table III-IV).

Table I: Comparison mean value of *Staphylococcus aureus* Colony Forming Unit (CFU) between mice group on day 9

Variables	Treatment	Mean ± SD	F	P-value
CFU	Negative Control	$5.72x10^3$	2.503753	0.115276
	Acriflavine	$9.50x10^2$		
	ASC	$2.42x10^3$		

One Way ANOVA test, Standard Deviation (SD)

** significant if $p < 0.05$

From the table above, it is shown that the mean value of CFU on day 9 for the mice that were treated with acriflavine ($9.50x10^2$) is the lowest compared to mice treated with ASC ($2.42x10^3$), and no treatment ($5.72x10^3$). At F = 2.504, alpha = 0.05, the p-value is 0.115, the One-Way ANOVA test analysis shows that the difference mean of CFU calculation between mice treatment on day 6 was statistically not significant (p = 0.115).

Table II: Comparison mean value of *Pseudomonas aeruginosa* Colony Forming Unit (CFU) between three groups of day 9

Variable	Treatment	Mean ± SD	F	P-value
CFU	Untreated	1.57E±04	2.843	0.089
	Acriflavine	1.35E±04		
	Collagen	1.12E±04		

One Way ANOVA Test, Standard Deviation (SD)

** significant if $p< 0.05$

From the table above, it is shown that the mean value of CFU on day 9 for the mice that were treated with ASC ($1.12x10^4$) is the lowest compared to mice treated with Acriflavine ($1.35x10^4$), and no treatment ($1.57x10^4$). At F = 2.843, alpha = 0.05, the p-value is 0.089, the One-Way ANOVA test analysis shows that the difference mean of CFU calculation between mice treatment on day 6 was statistically not significant (p = 0.089).

Table III: Comparison of acriflavine treatment and acid solubilized collagen treatment against *Staphylococcus aureus* on day 9

Variables	Treatment	Mean (SD)	Df	P-value	T
CFU	Acriflavine	$9.50x10^2$	10	0.11091	2.2281
	ASC	$2.42x10^3$			
	Student's T-Test, Standard Deviation (SD)				
	** significant if $p < 0.05$				

From the table above, it is displayed that the mean value of CFU for acid solubilized collagen treated mice ($2.42x10^3$) is higher compared to the positive control group which use the acriflavine ($9.50x10^2$). At dF = 10, alpha=0.05, the p-value for the calculated t = 2.228 is 0.111. The student t-test analysis shows that, the difference of the mean value of CFU between acid solubilized collagen and acriflavine treated mice on day 9 is statistically not significant (p = 0.111, alpha 0.05).

Table IV: Comparison of acriflavine treatment and acid solubilized collagen treatment against *Pseudomonas aeruginosa* on day 9

Variables	Treatment	Mean (SD)	Df	P-value	T
CFU	Acriflavine	$1.35x10^4$	10	0.06387	2.2281
	ASC	$1.12x10^4$			
	Student's T-Test, Standard Deviation (SD)				
	** significant if $p < 0.05$				

From the table above, it is displayed that the mean value of CFU for Acriflavine treated mice ($1.35x10^4$) is higher compared to the positive control group which use the ASC ($1.12x10^4$). At dF = 10, alpha=0.05, the p-value for the calculated t = 2.2281 is 0.06387. The student t-test analysis shows that, the difference of the mean value of CFU between acid solubilized collagen and acriflavine treated mice on day 9 is statistically not significant (p = 0.06387, alpha 0.05).

DISCUSSION

This study aims to evaluate the effectiveness of antimicrobial properties of collagen extracted from *Oreochromis niloticus* scale in mice skin tissue healing infected with *Pseudomonas aeruginosa* and *Staphylococcus aureus*. BALB/c mice models were used in experimental study including negative control group, positive control group and treated group. The effectiveness of *Oreochromis niloticus* collagen has been frequently studied due to its regenerative properties, high collagen content and great adherence mechanism in wound making it a popular biomaterial in regenerative medicine (12). However, there are few studies accessing its properties in antimicrobial efficiency on infected skin, so it is still remains unclear. It is well proven that bacteria presence in open wound can lead to infection and delayed tissue remodelling process. The microenvironment of wound is cradle for multiple species of microorganism and making it ideal habitat bioburden pathogen. One of the most common types of bacterial species can be identify in wound is the opportunistic gram-negative *Pseudomonas aeruginosa* (13).

Based on the trends of *Staphylococcus aureus* CFU mean between the positive control, negative control and tested group on day 3, 6, and 9, there are no significance difference ($p>0.05$) among the mice groups. This imply that each treatment that were given may have similar bacterial efficacy towards *Staphylococcus aureus* skin infection as all groups shows a decrease number of CFU mean at a consistent rate. However, on day 6, the mice that were treated with ASC shows an increase number of CFU mean compared to day 3, which may due to bacteria growth are more influential than ASC treatment, suggesting limited antibacterial effects. In addition, based on the student t-test statistical analysis between group treated with acriflavine and ASC, it is noted that there is no significant difference between the CFU mean of the two groups. However, the positive control showed the lowest number of CFU mean among the groups. These findings are supported with a study by (14), which stated that acriflavine shows a strong antibacterial efficacy against *Staphylococcus aureus*.

Based on result on day 3 CFU count there were no significant number of *Pseudomonas aeruginosa* in skin wound between all groups of mice. This suggest that all treatment have little to minimal effect on wound on early stage of infection. Furthermore the group that have been treated with collagen exhibit the highest count of CFU compared to another group. This finding similar with prior research done by Guo et al., (2010) (15) conclude that the presence of bacteria and its virulence factor can influence the bacteria thrive in this environment. He stated that bacteria such as *Pseudomonas aeruginosa* embedded in wound could develop biofilm created by the bacteria communities' clusters roots at the

extracellular polysaccharide matrix. This biofilm then will matured enveloping the colony thus protecting it microenvironment from outside threat consequently making the *Pseudomonas aeruginosa* colony establish resistant toward wound treatment.(15).

There also no significant count of CFU on day 6 and 9 however compared to day 3 the number of bacterial present in collagen treated group have drop to lowest compared to other group. This may suggest that the collagen treatment have minimal anti-microbial properties toward *Pseudomonas aeruginosa*, which is not significance for this study. The previous study by Prasad et al., (2020) (16) have documented that *Pseudomonas aeruginosa* wound infection can impede with process of wound healing by disturbing the formation of the collagen. In normal wound repair, it requires of multiple function from other mediator such as cell population, growth factor, cytokine and extracellular matrix in top optimize the healing process. This shows that the ASC does have low antibacterial efficacy towards *Staphylococcus aureus* and *Pseudomonas aeruginosa* skin infection, which supports the null hypothesis of the experiment.

CONCLUSION

This study aims to help to test the potential of new antimicrobial peptide and discover it properties in combating bacteria virulence factor. The results produced have prove that the *Oreochromis niloticus* scale extraction ASC have minor antibacterial efficacy towards the *Staphylococcus aureus* and *Pseudomonas aeruginosa* skin infection. Both acriflavine and ASC shows comparable antibacterial efficacy against both bacterial skin infections. However, ASC extracted from *Oreochromis niloticus* scale could be explored to be a potential value as a biomaterial for tissue regeneration. There are few methodological limitations in the study due to a few swabbing and dilution error occurred during the experiment. As for future work and recommendations, a standardized methodological procedure, higher doses of collagen, and combination of therapy might improve the efficacy of the treatment.

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