# Screening and Characterization of antimicrobial peptides from fish mucus in three major lakes of Uganda

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## DECLARATION

The work presented in this thesis is the result of my original research work. Where I have used the works of other persons, due acknowledgements are clearly stated. No portion of this work has been submitted in support of an application for a degree or qualification to any other university or institute of higher learning. I present it without any reservations for external examination.

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The research work culminating in this thesis was conducted under my guidance and supervision.

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## DEDICATION

This thesis is dedicated to my children, *Elisha*, *Elijah* and *Elizabeth*.

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## LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| ACAP: | African Catfish Antimicrobial Peptides |
| ALP: | Alkaline Phosphatase |
| AMP: | Antimicrobial Peptide |
| AMR: | Antimicrobial resistance |
| ANOVA: | Analysis of Variance |
| APD: | Antimicrobial peptide Database |
| ATCC: | American Type Culture Collection |
| CFU: | Colony Forming Units |
| ESBL: | Extended-spectrum ß-lactamase-producing bacteria |
| FDA: | Food and Drug Authority |
| FRC: | Faculty Research Committee |
| GPS: | Global Positioning System |
| HLP: | Hemolytic Peptide |
| IC: | Inhibitory Concentration |
| I-TASSR: | Iterative Threading Assembly Refinement |
| kDa: | Kilo Dalton |
| LC-MS/MS: | Liquid chromatography Mass spectrometry mass spectrometry |
| LOMETS: | Local Meta Thereading Server |
| MALDI-TOF: | Matrix Assisted Laser Desorption Ionization Time-of-flight |
| MBC: | Minimum Bactericidal Concentration |
| MIC: | Minimum Inhibitory Concentration |
| MWCO: | Molecular Weight Cut Off |
| NMR: | Nuclear Magnetic Resonance |
| PDB: | Protein Data Bank |
| PRIDE: | Proteomics Identification Database |
| ProSA: | Protein Structure Analysis |
| REC: | Research Ethics Committee |
| RP-HPLC: | Reverse phase high performance liquid chromatography |
| SDS-PAGE: | Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis |

| | |
|---|---|
| SEM: | Standard Error of Mean |
| SPE: | Solid Phase Extraction |
| TI: | Therapeutic Index |
| TOR: | Term of Reference |
| UNCST: | Uganda National Council for Science and Technology |
| WHO: | World Health Organizations |

## ABSTRACT

**Background**: Antimicrobial peptides (AMPs) constitute a broad range of bioactive compounds in diverse organisms, including fish. Currently, several peptide antibiotics have been approved for clinical use. Still, skin mucosal AMPs of African catfish and African lungfish are largely unexplored despite their potential in contributing novel drug candidates. Accordingly, the African Catfish Antimicrobial peptides (ACAPs) were investigated.

**Methods:** Native peptides were extracted from fish mucus scrapings in 10 % acetic acid (v/v) and ultra-filtered using a 5 kDa molecular weight cut-off membrane. The ultrafiltrate were purified using $C_{18}$ Solid-Phase Extraction cartridges. Later, their antibacterial activity was determined using the Agar Well Diffusion and broth-dilution methods utilizing *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922). Sephadex G-25 gel filtration was further utilized in bio-guided isolation of the most active fractions prior to peptide identification using Orbitrap Fusion Lumos Tribrid Mass Spectrometry. Discriminant Analysis Machine and Deep learning algorithms were utilized in predicting antimicrobial scores of each peptide. Toxicity of the AMPs was assessed using rabbit blood cells alongside *HLPpred-Fuse* and *ToxinPred* servers. The AMPs possible mode of action was assessed using SevenGo Duo SG-23 digital conductivity meter, *QuantiChrom* Alkaline phosphatase kit, and molecular docking engines prior to their chemical synthesis.

**Results:** The skin mucus extracts from fish in three major lakes of Uganda exhibited antimicrobial activity on *E. coli* and *S. aureus*. Lake Albert's African catfish demonstrated the highest antimicrobial activity with the lowest MIC of 2.84 and 0.71 µg/mL on *S. aureus* and *E. coli*, respectively. Sephadex G-25 peak I mass spectrometry revealed seven short peptides (11–16 amino acid residues) of high antimicrobial scores (0.561–0.905 units). In addition, these peptides had a low molecular weight (1005.57–1622.05 Da) and had a percentage hydrophobicity above 54%. Partially purified extracts were non-hemotoxic ($p=0.447$). Peptide ACAP-V demonstrated the highest binding and global energies against Monomeric sarcosine oxidase (PDB Id: 2gb0; -9.40 kcal/mol) and NADH peroxidase (PDB Id: 1nhp; BE: -88.84 kcal/mol) respectively. Synthesized ACAP-IV and ACAP-V had the MIC of 520.7 and 625.0 µg/mL on *E. coli*.

**Conclusion and Recommendation:** Collectively, the 7 African catfish antimicrobial peptides were identified as potential novel drug candidates. Future research is recommended to validate their activity *in vivo* on a wider range of bacteria.



**Key words:** African catfish, African lungfish, Aquatic, Antimicrobial peptides, Cell lysis, Electrolytes, Hemolytic, Drug-leads, *In silico*, MIC, Molecular docking, Skin mucus, Uganda

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background

Antimicrobial resistance (AMR) is one of the most critical global public health threat (Lomazzi et al., 2019). In 2019, more than 1.27 million lives were lost to antibiotic resistance (Murray et al., 2022). This resistance has largely been accelerated by; misuse as well as overuse of available antibiotics (Lomazzi et al., 2019) and lack of effective new antibiotics on the market (Ayukekbong et al., 2017). While, rational use of antimicrobials, regulation of over-the-counter availability, improving hand hygiene as well as infection control and prevention have been employed as key interventions, the situation continues to worsen (Uchil et al., 2014). This is attributed to the to discovery void, given that the last successfully introduced antibiotic class was discovered in 1987 (Silver, 2011). Accordingly, identification of new entities as potential antimicrobial drug candidates is in great demand (Uchil et al., 2014; Annunziato, 2019).

Antimicrobial peptides (AMPs) have recently attracted research attention as potential source of novel drug candidates (Mahlapuu et al., 2016; Cipolari et al., 2020). They are quick-acting (Raheem and Straus, 2019), short in length, and the majority are Generally Recognized As Safe (GRAS) (Hansen et al., 2020). Interestingly, AMPs have the potential to target both the intracellular and membrane (lytic) protein receptors (Hancock, 2001; Wilcox, 2004; Brogden, 2005). Moreso, AMPs have been reported to have a low risk of pathogen resistance. Accordingly, peptides and proteins are now gradually gaining ground over small molecule-based drug searches (Otvos and Wade, 2014). For example, up to 239 peptides and proteins have been approved by US

Food and Drug Authority (FDA) for clinical use (Usmani et al., 2017), accounting for 3.5 % of the FDA-approved drugs (Wishart et al., 2018).

Aquatic habitat being one of the homes of novel bioactive compounds (Giordano, 2020; Pooja et al., 2020), presents a crucial opportunity. This is due to their rich biodiversity (Tadesse, 2018). Beside, aquatic inhabitants like fish, effectively utilize innate immune response to thrive in such pathogen-dense medium (Masso-silva and Diamond, 2014). Their skin mucus is the principal component of innate immune response; as the first barrier to combat infection by trapping and sloughing off all potential pathogens (Corrales et al., 2010). This form of immune response is more pronounced in scaleless fish species (such as the African catfish and African lungfish), whose skin is richer mucus than scaled fish (Dash et al., 2018). The African catfish, *Clarias gariepinus* is a benthic dweller in the family *Clariidae* (Aliyu and Solomon, 2012). African lungfish, *Protopterus annectens* on the other hand, is an obligate air breather and a demersal in the family *Lepidosirenidae* (Omeji et al., 2017).

Being poikilothermic, fish adaptive immune response is slow, and hence they rely more on above mentioned skin mucus enhanced innate immunity (Gomez et al., 2013). Removal of such layers have been reported to increase susceptibility of common carp, *Cyprinus carpio* to bacterial infections (Lemaitre et al., 1996) and even death of Ayu, *Plecoglossus altivelis*; Turbot, *Scophthalmus maximus* (Fouz et al., 1990). Secreted by goblet cells (Shephard, 1993), fish skin mucus is largely composed of antimicrobial peptides, mucin (glycoproteins), flavozymes, clotting factors, hemolysin, immunoglobulin, C-reactive proteins, lysozymes and, complement proteins that are responsible for inhibiting microbial activity as well as immune-

modulation (Rakers et al., 2013). Since 1930s, several peptide antibiotics like Gramicidin, Bacitracin, Polymyxin, and Tyrothricin, among others have been in clinical use (Lei et al., 2019). They display rapid action (Raheem and Straus, 2019) targeting both intracellular (Hancock, 2001; Wilcox, 2004) and membrane receptors (Brogden, 2005). In addition, they are short and amphipathic for easy synthesis (Hansen et al., 2020) and cell membrane penetration (Fjell et al., 2012) respectively. Given their effectiveness on a wide range of bacteria, including those that resist chemical antibiotics (Fuochi et al., 2017), discovery of novel antimicrobial peptides is a promising strategy for development of effective antibiotics against AMR. In fact, antimicrobial peptides have been dubbed as the most promising strategy to inhibit antibiotic resistant bacteria (Reverter et al., 2018).

Regrettably, only a few fish AMPs (127) have been identified (Wang et al., 2016) as of 19th May 2019 <https://aps.unmc.edu/>. Yet, fish are by far the most successful vertebrates on earth comprising of 33,932 recorded species worldwide (Froese and Pauly, 2020). Moreover, the number of AMPs is even lower in the context of fish mucosal AMPs. The few notable examples of the mucosal AMPs are Pelteobagrin from yellow catfish, *Pelteobagrus fulvidraco* (Su, 2011); Myxinidin from hagfish, *Myxine glutinosa* (Subramanian et al., 2009); L40 peptide from Atlantic cod, *Gadus morhua* (Bergsson et al., 2005); SAMP-H1 from Atlantic salmon, *Salmo salar* (Luders et al., 2005); Oncorhyncin II from rainbow trout, *Oncorhynchus mykiss* (Fernandes et al., 2004); Hipposin from Atlantic alibut, *Hippoglossus hippoglossus* (Birkemo et al., 2003); Pleurocidin from winter flounder, *Pleuronectes americanus* (Cole et al., 1997); Pardaxin from Moses sole fish, *Pardachirus marmoratus* (Oren and Shai, 1996) among others. The majority of the currently identified AMPs are from marine fish. Emphasis on freshwater fish skin mucus in the African

region remains insufficient and there is no record of AMPs class that has been identified by primary structure in the skin mucus of the African Catfish, *Clarias gariepinus*. In addition, the safety profiles and possible mode of action of AMPs such peptides are unknown. Therefore, in an effort to develop novel alternative drug leads for potential drug discovery this study for the first time, explored the fish-derived AMPs from the three major lakes of Uganda.

### 1.2 Statement of the problem

Antimicrobial resistance remains one of the biggest threats to human health. The situation is even made worse by lack of new class of antibiotics in market (Spellberg et al., 2004). Still, antimicrobial peptides (AMPs) are seen as possible source of new drug candidates. Whereas, previous studies revealed that the fish skin mucus of African catfish, *Clarias gariepinus* is antimicrobial in nature (Wang et al., 2012; Nwabueze, 2014; Akunne et al., 2016; Jameel et al., 2019), the antimicrobial potentials of skin mucus of African catfish, *Clarias gariepinus* and African Lungfish, *Protopterus annectens* inhabiting the three major lakes of Uganda were unexplored. The identity and safety profiles of potent AMPs in such skin mucus remained unknown. More so, their hemo-toxicity profiles, possible mode of action were unrevealed. Understanding the fish skin mucus antimicrobial activity, identity, safety profiles and possible mode of action of these AMPs in the most active fractions of skin mucus extracts avails novel antimicrobial leads for pharamaceutical applications.

## 1.3 Objective

### 1.3.1 Overall objective

The study was set to screen and characterize the antimicrobial peptides from skin mucus of African catfish and African lungfish inhabiting Lake Albert, Lake Victoria, and Lake Kyoga.

### 1.3.2 Specific objectives

i. To determine the antimicrobial activity of skin mucus from African catfish and African lungfish obtained from three major lakes of Uganda
ii. To identify antimicrobial peptides purified from the potent skin mucus extract
iii. To evaluate the toxicity of the most potent antimicrobial peptides
iv. To determine the mechanism of action of the most potent antimicrobial peptides

## 1.4 Research questions

i. Does the skin mucus of African catfish and African lungfish in three major lakes of Uganda have antimicrobial activity?
ii. What are the antimicrobial peptides in the potent skin mucus extracts?
iii. How safe are the potent antimicrobial peptides from skin mucus?
iv. What is the mechanism of action of these potent antimicrobial peptides?

## 1.5 Scope of the study

This study focused on the extraction, isolation and characterisation of AMPs from the skin mucus of two freshwater fish species *viz.* African catfish (*Clarias griepinus*) and African lungfish (*Protopterus annectens*). The fish were sourced from three major lakes of Uganda, and they

include Lake Kyoga, Lake Albert and Lake Victoria. It was field-based and *in silico* non-clinical *in vitro* investigation designed to output potential novel native antimicrobial drug candidates. Basing on the antimicrobial scores, selected antimicrobial peptides were subjected to toxicity profiling and mode of action study. The the purposive selection of the two fish species (African catfish and African lungfish) was guided by available literature that reported higher antimicrobial activity of their skin mucus compared to other fish species (Jameel et al., 2019). More so, the fact that scale-less fish species produces more skin mucus compared to the sacaled, for an effective innate immune response further justified the choices (Dash et al., 2018).

### 1.6 Significance of the study

In the present study, novel antimicrobial peptides in the skin mucus of African catfish, *Clarias griepinus* were identified and dataset added to *ProteomeXchange* Consortium via the Proteomics Identification Database (*PRIDE*) with dataset identifier PXD029193 and 10.6019/PXD029193. This will not only contribute knowledge but most importantly avail new alternative animal-derived antimicrobial candidates from which potential drug development in this era of antimicrobial drug resistance can be built upon, with aquatic inhabitants at heart.

Furthermore, the study optimized the pipelines to screen, isolate, characterize and optimize fish-derived antimicrobial peptides. Such established pipelines can now be customised to build the capacity in peptide research and natural product development so as to enhance their indemnity, applicability and acceptance by local communities, food and pharmaceutical industries. This is justified by the fact that the biggest obstacles to natural products development is the technical barriers associated with their screening, isolation, characterization, and optimization.

The structure-based approach to Computer Aided Drug Discovery (CAAD) especially the molecular docking techniques and toxicity profiling exceuted by the present study can be applied to identify other potentially safe and most promising novel antimicrobial peptides, from large libraries of ligands. With such potentials to sieve and be reference points prior to animal studies, such approaches can be employed to reduce financial constraints; labor intensiveness and time-consuming procedures processes associated with basic research.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Antimicrobial resistance

Antimicrobial Resistance (AMR) is the ability of microorganism (such as bacteria, viruses, fungi and parasites) to stop an antimicrobial (such as antibiotic, antiviral and antimalarial) from working on it (WHO, 2021). This renders standard treatments ineffective and as well promotes microbial persistence and spread. As a result, more than 700,000 lives are lost annually, with a projected rise to 10 millions by 2050 (Figure 2.1) (O'Neill, 2014). At the beginning of 2017, the WHO made a desperate global call for new class of antibiotics (Willyard, 2017). In the same year, WHO confirms 'the world is running out of antibiotics' and declared a global health emergency that called for an urgent need for more investment in research and development for antibiotic-resistant infections especially for the ranked 'priority pathogens' that if not watched may within this century, return the world back to the "pre-antibiotic era" (World Health Organization (WHO), 2019).

Accordingly, the identification of new potential antimicrobial targets and/or the identification of new chemical entities and biologics as potential antimicrobial drugs are in great demand (Aguilar et al., 2002; Uchil et al., 2014; Annunziato, 2019), with aquatic habitat being tagged an unexplored residence to potential novel bioactive compounds (Giordano, 2020; Pooja et al., 2020). This due to their rich biodiversity (Tadesse, 2018). Also, the rapid advancement in processing technologies such as recombinant technology, solvent extraction, enzymatic treatment, solid-phase synthesis have enhanced the search for natural bioactive compounds from such habitats (Rai et al., 2016).

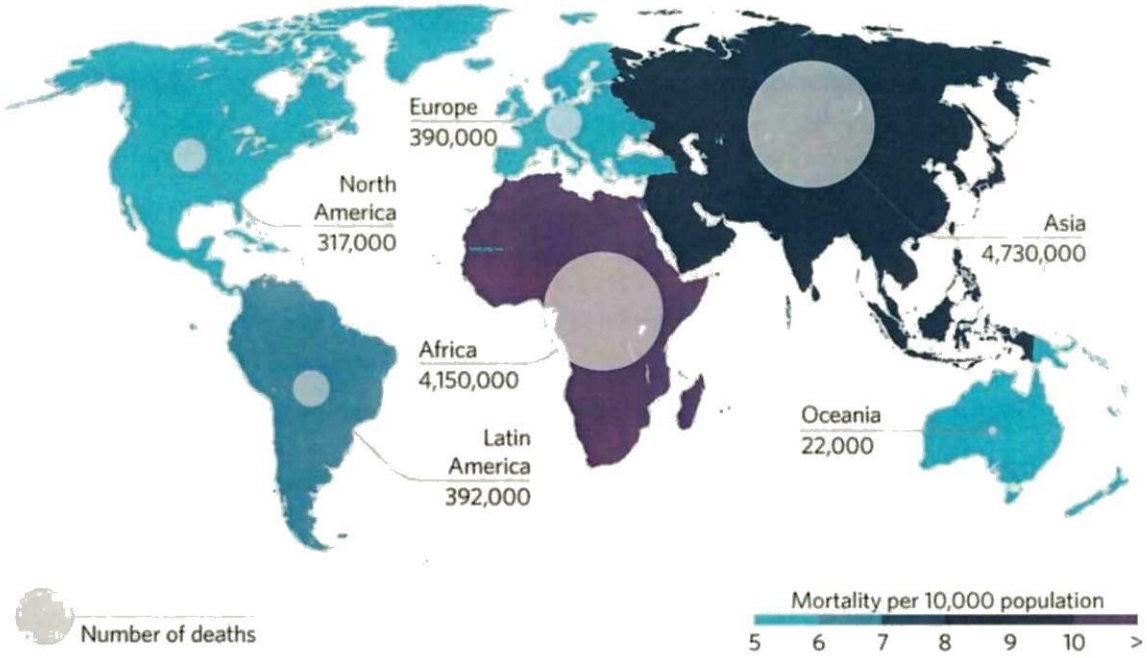



**Figure 2.1: Deaths attributable to AMR every year by 2050.** The Figure was adapted from O'Neil (2014).

## 2.2 Antimicrobial Peptides

Antimicrobial peptides (AMPs) are pivotal components of the innate immune system, widely distributed across all forms of life (Wang et al., 2019b). They are believed to have evolved over 2.6 billion years ago to combat microbial invasion challenges (Gordon and Eric, 2005). The first report on AMPs is dated back to 1939, when gramicidin D, a 15 amino acid residue antimicrobial agent was isolated from a prokaryote, *Bacillus brevis* (Dubos, 1939). Gramicidin D is an isoform of 80% gramicidin A, 6 % gramicidin B as well as 14 % gramicidin C and is active on both Gram-negative and Gram-positive bacteria. Thus, were the first AMPs to be produced on commercial scale as peptide antibiotics (Ageitos et al., 2017). Later on, gramicidin was combined with tyrocidines to form Tyrothricin peptide antibiotic, that has been in clinical use for over six decades without any record of being resisted against (Atiye et al., 2014).

Just like in prokaryotes, in 1942, a peptide substance (Purothionin) of broad antimicrobial activity on phyto-pathogens were isolated from wheat endosperm, *Triticum astivum* (Fernandez de Caleya et al., 1972). Purothionin, 45 amino acid residue antibiotics was effective on both bacteria and fungi. However, the 1943 discovery of penicillin and streptomycin, unwrapped the 'Golden age of Antibiotics' that in turn rendered antimicrobial peptide exploratation an unworthy venture (Zaffiri et al., 2012). It was not until the appearance of antibiotic-resistant microbes in the early 1960s when research on AMPs re-gained thrust (Davies, 2006; Katz et al., 2006) and since then, up to 3240 AMPs from diverse forms of life have been reported in the antimicrobial peptide database (APD3), as of 19th May 2019 https://aps.unmc.edu/ (Wang et al., 2016). Up to 127 of these AMPs were from fish, in which two most active motifs were designed using machine learning algorithms (Okella et al., 2020).

To date, AMPs have been reported effective against a wide range of bacterial species, including bacteria that resist traditional antibiotics (Fuochi et al., 2017; Välimaa et al., 2019; Tiralongo et al., 2020). They are known for rapid action (Raheem and Straus, 2019), being short, they can easily be synthesized (Hansen et al., 2020), together with reduced chances of resistance given their potentials to exhibit both the intracellular targeting (Hancock, 2001; Wilcox, 2004) and membrane (lytic) mode of action (Brogden, 2005). As earlieron stated, peptides and proteins are now gradually gaining grounds over small molecule-based drug search (Otvos and Wade, 2014). Up to 239 peptides and proteins have been approved by US Food and Drug Authority (FDA) for clinical use (Usmani et al., 2017), contributing 3.5 % of the FDA-approved drugs (Wishart et al., 2018).

However, high extraction cost (Dutta and Das, 2015; Pfalzgraff et al., 2018), toxicity against mammalian cells (Shilpakala et al., 2013; Conlon et al., 2014), plus susceptibility of linear peptides to peptidase degradation (Fosgerau and Hoffmann, 2015; Marqus et al., 2017) have been reported as the chief drawbacks to new peptide antimicrobial candidates. One approach to address such drawbacks is a bio-guided exploration of novel native (natural) active peptide fractions from which optimizable antimicrobial peptide templates can be obtained. The templates can then, be utilized in peptide synthesis (Sun et al., 2019), structural modifications such as dimerization as well as methylation (Lorenzon et al., 2019) and encapsulation (Molhoek et al., 2011; Obuobi et al., 2019) so as to reduce the high extraction cost, toxicity and peptidase susceptibility of novel antimicrobial peptides. The above approaches have seen, several AMPs into clinical use as peptide antibiotics as exemplified by Gramicidin D (DrugBank Id: DB00027), Salmon calcitonin (DrugBank Id: DB00017), Polymyxin B (DrugBank Id: DB00781), Colistin (DrugBank Id: DB00803), Bacitracin (DrugBank Id: DB00626), Tyrothrycin (DrugBank Id: DB13503) among others (Wishart et al., 2018). Such peptide antibiotics have stood the test of time as far as the battle against drug resistant pathogens is concerned.

### 2.3 Fish as a fountain of antimicrobial agents

Fish inhabits aquatic ecosystem full of pathogenic bacteria, fungi, viruses and protozoa (Magnadottir, 2010). These habitats predispose fish, especially scale-less species to higher infection risks compared to terrestrial vertebrates (Austin and McIntosh, 1988). Being poikilothermic organisms with less developed adaptive immunity, fish rely mostly on innate immunity (Sahoo et al., 2005; Kumar et al., 2018) comprising of three main components: physical barrier, phagocytic cells and chemical mediators (Dalmo et al., 1997; Smith et al., 2019). These

immune components are most abundant in the fish slime (mucus) layer (Rottmann and Durborow, 1992). Over decades, it has been demonstrated that the fish mucus layer on the skin, gills, nose and gut is the principal first-line physical and chemical barrier against infections (Dash et al., 2018; Cipolari et al., 2020). Here, the mucus components inhibits pathogens and as well as immune-modulation (Alvarez-Pellitero, 2008; Rakers et al., 2013; Guardiola et al., 2014).

With over 33,932 species recorded worldwide (Froese and Pauly, 2020), fish are by far the most abundant vertebrates on earth. The antibacterial potentials of their skin mucus have been demonstrated against several human and fish pathogenic bacteria (Table 2.1). Majority (60.00%) of the encountered principal studies focused on marine fish species at the expense of freshwater species, indicating less attention to the freshwater fish species. In addition, mild acidic extraction solvents utilizing Acetic acid dominated the studies (40.00%) with only two studies (0.08%), utilising aqueous solvents. This has been attributed to the fact that maximum solubilisation of the native peptides is enhanced in slightly acidic solvents (Rao et al., 2015).

Furthermore, the potentials of skin mucus extracts reviewed; varied across fish species, due to the fact that the mucus secreting cells differ among fish species (Shephard, 1993; Roberts and Powell, 2005). Therefore, exploring the unexamined freshwater fish species is a worthy venture; if novel antimicrobial agents against human and animal infections is to be unvailed (Thomas, 2008). Moreover, none of the available studies compared the effect of habitat on the antimicrobial activity of fish skin mucus. This inspired habitat expedition focusing on the skin mucus of two freshwater fish species *viz*. African catfish and African lungfish from the three major lakes of Uganda (Lake Victoria, Lake Albert, and Lake Kyoga).

**Table 2.1: List of principal studies on antimicrobial activity of fish skin mucus against human and fish pathogens**

| ies | Common names | Habitat | Pathogens sensitive to fish skin mucus extracts | Extraction solvent | References |
|---|---|---|---|---|---|
| *triatus* | Snakehead fish | Freshwater | *K. pneumonia, P. aeruginosa, B. subtilis, A. hydrophila* | Crude; Distilled water; 3% Acetic acid | (Wei et al., 20 |
| *asii* | Caspian kutum | Marine | *S. iniae, Y. ruckeri, S. aureus, L. monocytogenes, P. aeruginosa, E. coli, Saprolegnia sp., F. solani, C. albicans, A. flavus* | Physiological saline | (Adel et al., 20 |
| *mis niloticus* | Tilapia | Freshwater | *V. harveyi* | Ethanol | (Wibowo and Fadjar, 2015) |
| *sephen; Himantura* | Marine stingrays | Marine | *S. typhi, K. pneumoniae, S. aureus, E. coli, V. cholerae, S.typhi, A. niger, Penicillium sp, T. mentagrophytes, A. alternaria.* | Phosphate buffer; 10 % Acetic acid; Ethanol & Dichloromethane | (Vennila et al., 2011) |
| *eustis fossilis; Clarias us; Channa punctatus* | Stinging catfish; Green snakehead ; African catfish | Freshwater | *E. faecalis, S. aureus, S. pneumonia, E. coli, S. typhi, K. pneumoniae, V. inaba, P. aeruginosa* | Crude; 10 % Acetic acid; Ethanol & Dichloromethane | (Jameel et al., 2019) |
| *pastinaca* | Blue stingray | Marine | *K. pneumoniae ( the most sensitive strain), E. coli, Paeruginosa, C. albicans, C. glabrata, C. tropicalis* | Physiological saline | (Fuochi et al., 2017) |
| *mbalus armatus* | Spiny eel | Freshwater | *Y. ruckeri, A. hydrophila, P. aeruginosa, A. formican, A. liquefaciens, A. niger, A. flavus, C. albicans, C. neoformans, Mucor sp.* | Phosphate buffer; 10 % Acetic acid; Ethanol & Dichloromethane | (Uthayakumar al., 2012) |
| *arhua; Labrus bergylta; ya flesus; Pollachius virens; lmus rhombus; Solea solea* | Atlantic cod; Ballan wrasse; European flounder; Saithe; Brill; Dover sole | Marine | *B. subtilis, B. cereus, B. megaterium, Streptococcus sp., S. aureus, E. coli, K. pneumoniae, S. marcescens, P. vulgaris, P. aeruginosa, C. bruset, C. albicans, C. tropicalis, S. cerevisiae, I. orientalis* | Ethanol & Dichloromethane | (Hellio et al., 2002) |
| *Anguilla* | Eel fish | Marine | *E. coli, K. oxytoca, K. pneumonia, L. vulgaris, P. microbilus, P. aeruginosa, S. paratyphi, S. typhi, S. aerius, V. parahemolyticus.* | Crude | (Bragadeeswar and Thangaraj 2011) |
| *gariepinus* | African catfish | Freshwater | *E.coli, A.hydrophila, E. tarda* | PBS buffer | (Wang et al., 2012) |
| *ila; Cirrhinus mrigala; Anguilla* | South Asian carp; Carp fish; Eel fish | Marine | *A. awamori, C. falcatum, F. oxysporum* | 10 % Acetic acid | (Pethkar and Lokhande, 201 |
| *aaus arel; Nemapteryx* | Largescale tonguesole; Thickspined catfish | Marine | *S. aureus; S. aureus, V. cholera, V. parahemolyticus* | Physiological saline | (Bragadeeswar et al., 2011) |
| *thalmodon schlosseri* | Giant Mudskipper | Marine | *B. anthracis, E. coli, K. pneumoniae, P. mirabilis, P. aeruginosa, S. typhi, S. aureus, V. cholerae, A. flavus, C. albicans, Mucor sp., T. longibrachtin* | Physiological saline; Ethanol & Dichloromethane | (Mahadevan et 2019) |
| *negalensis* | Senegalese sole | Marine | *P. damselae, V. Harveyi, V. anguillarum* | Tris-buffered saline | (Guardiola et a 2017) |
| *rchus labrax; Anguilla ; Pagellus bogaraveo* | European eel: Eel fish; Blackspot Seabream | Marine | *V. alginolyticus; P. damselae* | Physiological saline | (Gabriella et a 2014) |
| *gariepinus* | African catfish | Freshwater | *S. aureus, B.subtilis, E. coli, P. aeruginosa, S. typhi, K. pneumonia, C. albicans, A. niger* | Absolute acetone | (Akunne et al., 2016) |
| *rion clarkia* | Yellowtail clownfish | Marine | *A.hydrphila, V. Harveyi, V. alginolitiens, V. parahaemolyticus, P. fluorescens, C. irritans* | Ammonium bicarbonate; 10 % Acetic acid; Ethanol | (Wang et al., 2019a) |
| *elus tauvina* | Greasy grouper | Marine | *E. coli, S. typhi, K pneumoniae, P. mirabilis, S. aureus, V. alginolytiens, V. parahaemolyticus, A. hydrophila, P. fluorescens, V. harveyi* | Ammonium bicarbonate; 10 % Acetic acid; Ethanol | (Manikantan e 2016) |
| *us alpinus; S. fontinalis; s carpio; Morone saxatilis; gramnus aeglefinu; Myxine a* | Arctic char; Brook trout; Koi carp; Striped bass; Haddock; Hagfish | Marine | *E. coli, S. enterica, S. epidermis, P. aeruginosa, C. Albicans* | Ammonium bicarbonate; 10 % Acetic acid; Ethanol | (Subramanian al., 2008) |
| *schlegelii* | Korean rockfish | Marine | *A.Salmonicida, P. damselae, V. parahaemolytus* | Aqueous | (Kitani et al., 2008) |
| *gariepinus* | African catfish | Freshwater | *Bacillus sp, E. coli, S. aureus, Streptococcus sp* | Physiological saline | (Nwabueze, 2( |
| *s mrigala* | Indian major carp | Freshwater | *S. paratyphi, P. aeruginosa, . S. typhi S. aureus, V. cholerae* | Triple-distilled water; 3% acetic Acid; 0.1% Trifluoroacetic acid | (Nigam et al., 2015) |
| *aculatus* | Marine catfish | Marine | *E. coli, P. aueruginosa* | Physiological saline | (Manivasagan al., 2009) |
| *batrachus* | Walking catfish | Freshwater | *P. aeruginosa, A. hydrophila, V. anguillarum, V. fischeri, E. coli* | Physiological saline | (Loganathan e 2014) |
| *batrachus;Tilapia mossambicus* | Walking catfish; Mozambique mouth-breeder | Freshwater | *E. coli, P. aeruginosa, A. hydrophila, K. pneumonia, P. vulgaris, B. coagulans S. aureus* | Ammonium bicarbonate | (Elavarasi et a 2013b) |

As the first-line physical and chemical defence, the skin mucus is rich in antimicrobial peptides, hydrolytic enzymes, glycoproteins, hemolysin, immunoglobulin, C-reactive proteins, and complement proteins (Shephard, 1993). However, antimicrobial peptides have been recognised as the most vital component and a promising strategy to inhibit bacteria, including those that resist traditional antibiotics (Rakers et al., 2013; Reverter et al., 2018). Table 2.2 presents key mucosal antimicrobial peptides isolated from different fish species. Again, majority of the identified antimicrobial peptides (76.19 %) were from fish skin mucus of marine fishes. Besides, most encountered studies (61.90 %) were basically from Europe, followed by Asia (23.81 %). The least number of studies (14.28 %) were from North America with none that explored the fish-derived mucosal antimicrobial peptides from the African freshwater bodies. This *status quo*, therefore, positioned the present study as the first in the continent to identify fish skin mucus antimicrobial peptides. Here, the peptides were extracted from the skin mucus of two freshwater fish species: African catfish, *Claris griepinus* and African lungfish, *Protopterus annectens*.

**Table 2.2: The key antimicrobial peptides from skin mucus of fish**

| crobial ides | AMPs Sequence | Source organism | Common name | Habitat | Continent | MIC/MBC/ $IC_{50}$ | Referenc |
|---|---|---|---|---|---|---|---|
| cidin | IFHHIFKGIVHVGKTIHRLG | *Siniperca chuatsi* | Mandarin fish | Freshwater | Asia | 5-160 µM | (Sun et al., 20 |
| n | RQRVEELSKKGAAARRRK | *Misgurnus anguillicaudatus* | Pond Loach | Freshwater | Asia | 4-16 µg/mL | (Park et al., 19 |
| I | KGRGKQGGKVRAKAKTRSS | *Parasilurus asotus* | Japanese Catfish | Freshwater | Asia | 1-4 µg/mL | (Park et al., 19 |
| n | GFFALIPKIISSPLFKTLLSAVGSALSSSGGQE | *Pardachirus marmoratus* | Red Sea Moses sole | Marine | Europe | ND | (Shai et al., 19 |
| din | GIHDILKYGKPS | *Myxine glutinosa* | Hagfish | Marine | N. America | 1-10 µg/mL | (Subramanian al., 2009) |
| agrin | GKLNLFLSRLEILKLFVGAL | *Pelteobagrus fulvidraco* | Yellow catfish | Freshwater | Asia | 2-64 µg/mL | (Su, 2011) |
| idin | GWGSFFKKAAHVGKHVGKAALTHYL | *Pleuronectes americanus* | Winter flounder | Marine | N. America | 2.2-35 µM | (Cole et al., 19 |
| | FIGGIISFFKRLF | *Grammistes sexlineatus* | Goldenstriped soapfish | Marine | Asia | 3.13-50 µg/mL | (Sugiyama et 2005) |
| | AERVGAGAPVYL (partial) | *Oncorhynchus mykiss* | Rainbow trout | Marine | Europe | 0.15-1.2 µM | (Fernandes et 2002) |
| a 1 | FFHHIFRGIVHVGKTIHKLVTG | *Morone saxatilis* | Hybrid striped bass | Marine | Europe | ND | (Lauth et al., 2002) |
| | PDPAKTAPKKGSKKAVTKXA | *Ictalurus punctatus* | Channel catfish | Freshwater | N. America | ND | (Robinette et 1998) |
| n 2 | FFHHIFRGIVHVGKTIHKLVTG | *Morone saxatilis* | Hybrid striped bass | Marine | Europe | 1.25-20 µM | (Lauth et al., 2002) |
| in | GRGKTGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGNYAHRVGAGAPVYL | *Hippoglossus hippoglossus* | Atlantic halibut | Marine | Europe | 0.4-40 µM | (Birkemo et al 2003) |
| ycin III | PKRKSATKGDEPA | *Oncorhynchus mykiss* | Rainbow trout | Marine | Europe | 0.2-0.8 µM | (Fernandes et 2004) |
| h3 | RRSRSGRGSGKGGRGGSRGSSGSRGSKGPSGSRGSSGSRGSKGSSGSRGSKGSRGGRSGRGSTIAGNGNRNNGGTR | *Gadus morhua* | Atlantic cod | Marine | Europe | ND | (Maier et al., 2008) |
| ycin II | KAVAAKKSPKKAKKPAT | *Oncorhynchus mykiss* | Rainbow trout | Marine | Europe | 0.2-0.8 µM | (Fernandes et 2004) |
| idin-α | FIHHIIGGLFHVGKSIHDLIR(-CONH2) | *Pterois volitans* | Lionfish | Marine | Europe | 1-50 µM | (Houyvet et al 2018) |
| -H1 | AEVAPAPAAAAPAKAPKKKAAAKPKKAGPS | *Salmo salar* | Atlantic salmon | Marine | Europe | 1-4 µM | (Luders et al., 2005) |
| | IIEPSLRMLAQKYNCDKMIXRXXYARLHPR (partial) | *Gadus morhua* | Atlantic cod | Marine | Europe | 0.1-10 µM | (Bergsson et a 2005) |
| lin | GCRFCCNCCPNMSGCGVCCRF | *Morone saxatilis* | Hybrid striped bass | Marine | Europe | ND | (Shike et al., 2002) |
| efensins ) | ASFPWSCPSLSGVCRKVCLPTELFFGPLGCGKGFLCGVSHFL | *Sparus aurata* | Gilthead seabream | Marine | Europe | ND | (Cuesta et al., 2011) |

MIC, Minimum Inhibitory Concentration; IC, Inhibitory Concentration; MBC, Minimum Bacterial Concentration; AMPs, Antimicrobial peptides; ND, Not detected

## 2.4 Diversity and distribution of fish species in Ugandan lakes

The diversity, distribution and abundance of fish species vary with physicochemical parameters of the aquatic habitat (Karthika et al., 2017; Rappe, 2013; Koenig et al., 1976). Notable examples of these parameters are water temperature, dissolved oxygen, pH, electrical conductivity, total dissolved solids, water transparency, turbidity, daily water level fluctuations, among others. The

aforementioned physicochemical parameters are inturn influenced by the climate variability (Nsubuga et al., 2017; Ogutu-Ohwayo et al., 2016), anthropogenic activities (Ongom et al., 2017) and regional geomorphological changes of the basin that holds the lake (Omide, 1971). These have, therefore, greatly impacted on the distribution, abundance, and diversity of aquatic species especially in the upper nile basin and lake Kyoga basin (Bastawesy et al., 2013; Ojara et al., 2020).

It's worth noting that, physicochemical parameters too, affects physiological processes in fish (Mbalassa et al., 2014). For instance, Nigam et al. (2012) reported that, the amount and the composition of the fish-secreted mucus were greatly affected by water, temperature, dissolved oxygen and pH. This was not any different from the earlier reports that environmental disturbances like temperature, dissolved oxygen, pH, salinity, turbidity among others hugely affected the amount and quality of fish mucus produced (Lebedeva, 1999; Subramanian et al., 2008). Therefore, varying the habitat of study for the respective fish species under investigation is essential as far as bioprospecting is concerned. Regrettably, none of such attempts on fish inhabiting Uganda's water bodies were encountered, paving way for the present study.

With more than 15 % of her total area being made up of lakes and swamps, Uganda is commonly referred a 'country of lakes'. The country has 28 lakes, four of which are the east African great lakes (Lake Victoria, Lake Kyoga, Lake Albert, and Lake Edward). More so, of the four ancient lakes in Africa (Lake Turkana, Lake Tanganyika, Lake Victoria and Lake Albert), Uganda boast of up to two such lakes (Omide, 1971). Ancient lakes are those that have consistently carried water for more than one million years due to plate tectonics in the active rift zone. Due to their prolonged

life, ancient lakes, serve as models for isolated evolutionary traits and novel biologics given their enhanced speciation (Balian et al., 2008; Hirano et al., 2019).

In regards to species, up to 273 fish species from 19 families have been reported in Uganda (Froese and Pauly, 2020). Lake Victoria harbours the highest percentage of Nile tilapia, *Oreochromis niloticus*; Silver fish, *Rasterineobola argentea* and Nile perch, *Lates niloticus* (Namisi, 2005). Lake Kyoga also known as 'Lake Kyoga Complex' on the other hand, is endowed with mud-loving benthic natives like African catfish, *Clarias gariepinus* (Figure 2.2); Walking catfish, *Clarias batrachus*; African lungfish, *Protopterus annectens,* Nile tilapia, *Oreochromis niloticus* (Figur 2.4) and spiny eel, *Macrognathus aculeatus* (Mbabazi et al., 2004; Nsubuga et al., 2017). The lake is famous for its extensive network of shallow open water areas fringed by papyrus swamps that makes it an ideal home for demersal fish species such as African lungfish.

Located towards the tip of the western arm of the African Great Rift Valley, Lake Albert supports the most diverse commercial fisheries in Uganda with at least 55 species that vary markedly in size with the commonest being African lungfish, *Protopterus annectens* (Figure 2.3); Nile perch, *Lates niloticus*; Tiger fish, *Hydrocynus vittatus*; Barbines, *Berbus plebejus* and Pebbly fish or Silversides, *Alestes baremose* among others (Wandera et al., 2010). The vast species diversity has been attributed to the geomorphological changes of the Albertine rift valley (Mbalassa et al., 2014), just like in other western rift valley lakes like Lake Edward and Lake George. But again, the reception of the Nile water by Lake Albert further endows the lake, more than any other lake in the western arm of the African Great Rift Valley (Ogutu-Ohwayo et al., 2016).

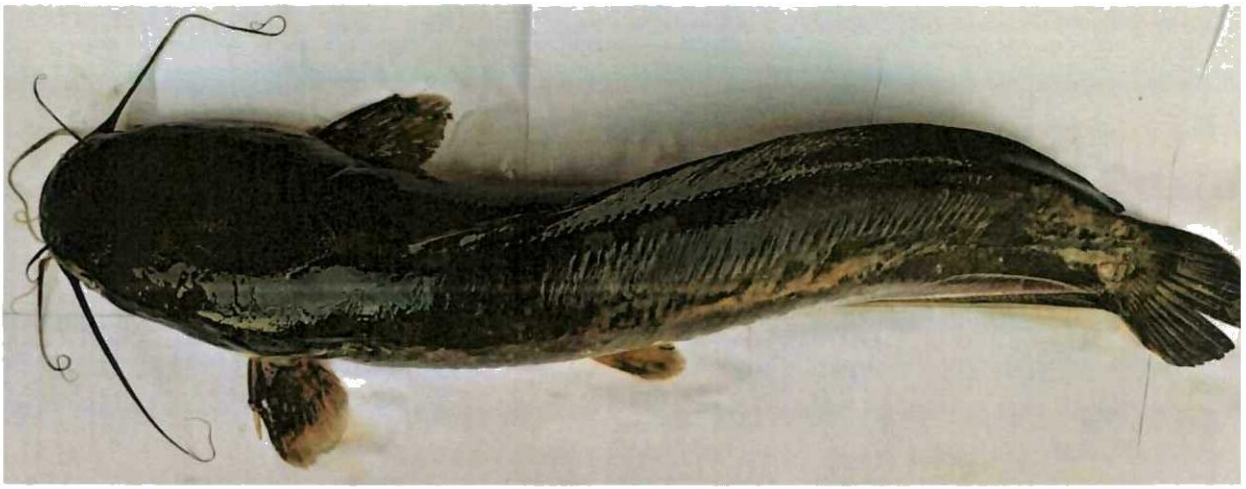


**Figure 2.2: *Clarias gariepinus* (Burchell, 1822).** Common name, African Catfish; Lango, *Rec Lango/Twang*; Luganda, *Semutundu*; Alur, *Nyaii*; Kiswahili, *Kambali*. Benthopelagic freshwater scales-less fish, with four pairs of slender, whiskerlike sensory organs (barbel) near the mouth. Photo by Hedmon Okella.

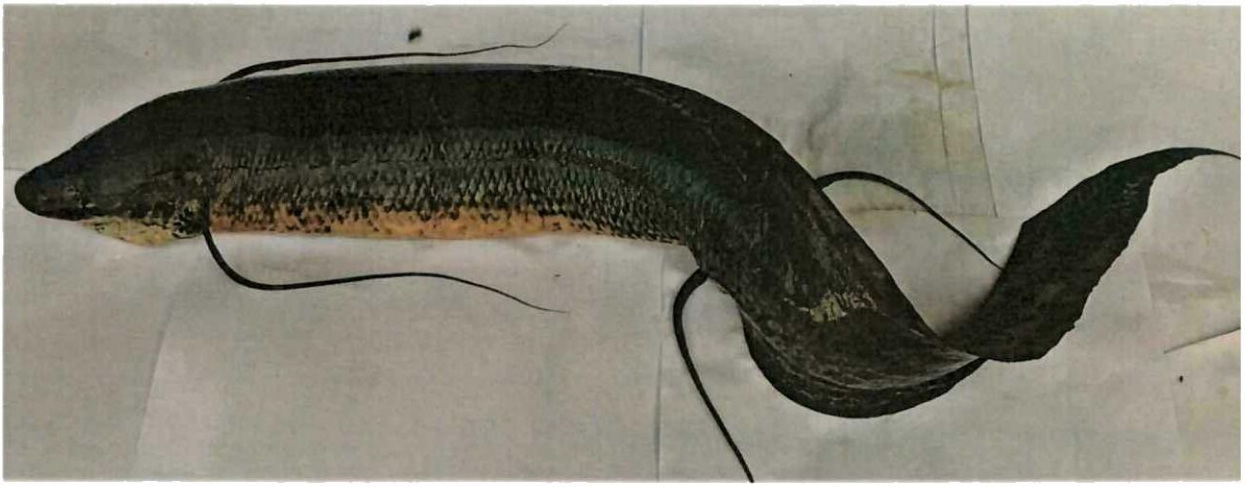


**Figure 2.3: *Protopterus annecten* (Owen, 1839).** Common name, African Lungfish; Lango, *Lut*; Luganda, *Emamba*; Kiswahili, *kambaremamba*. A dimersal lung breather with two pairs of long filamentous fins. Long and eel-like freshwater dweller with a prominent snout and small eyes. Photo by Hedmon Okella.

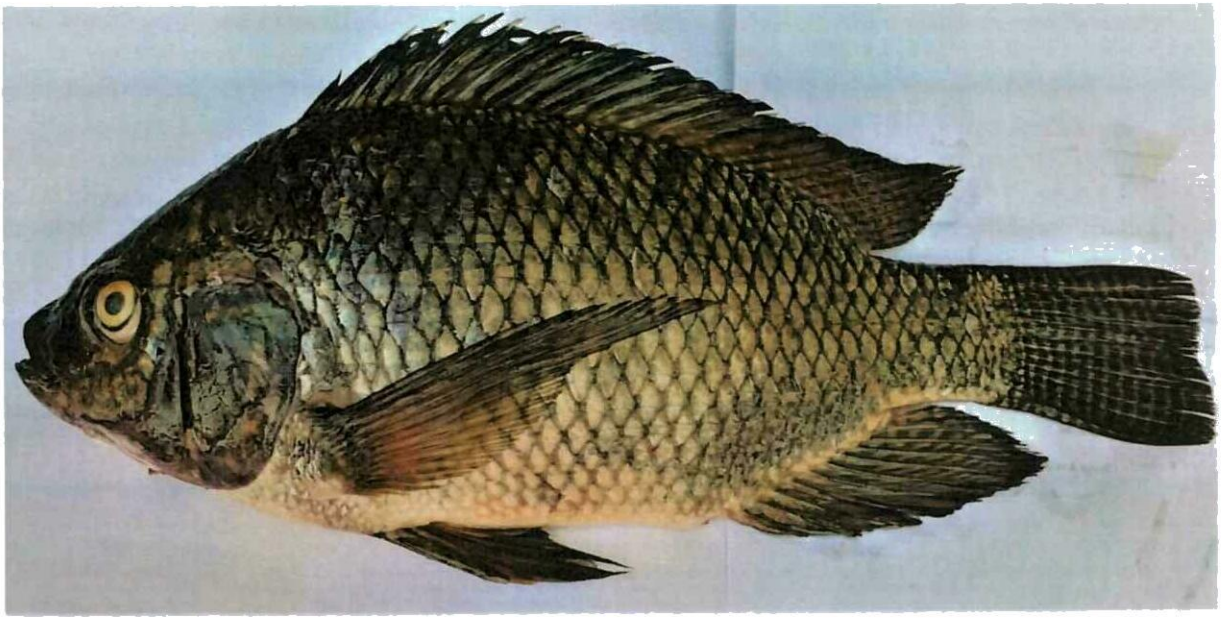


**Figure 2.4: *Oreochromis niloticus* (Linnaeus, 1758).** Common name, Nile tilapia; Lango, *Apok*; Luganda, *Engege*; Kiswahili, *Ngege*. A deep-bodied and compressed shape freshwater dweller having a truncated caudal fin that has many thin black bands and a pinkish-red posterior margin. Photo by Hedmon Okella.

### 2.5 Extraction and downstream processing of peptides

Three major extraction approaches are utilized to release bioactive peptides from the source: enzymatic hydrolysis, fermentation, and solvent extraction (Välimaa et al., 2019). Here, the choice of the extraction method is guided by the parameters like desired purity, peptide nativity, bioactivity, yield, safety profile and production cost (Wang et al., 2019b). The solvent systems in the downstreaming process can be modified to yield antimicrobial peptides of desired hydrophobicity, charge, size and amphipathicity (Najafian and Babji, 2012; Välimaa et al., 2019).

### 2.5.1 Extraction of peptides

Of the aforementioned extraction approaches, enzymatic hydrolysis is the most commonly used. The method doesn't leave residual organic solvents or toxic chemicals on its product therewith enhancing peptide purity and applicability to pharmaceutical and food industries (Kim and Wijesekara, 2010), as the yield, bioactivity and purity of the released peptides are retained (Huang et al., 2015). Besides, the approach avails a specific and controlled liberation of peptides from the sample. However, the nativity of such peptides is lost due to the peptidase degradation and this may compromise the bioactivity and safety of the isolated peptides (Abuine et al., 2019).

During enzymatic hydrolysis, the substrate is prepared, right enzymes chosen, reaction parameters (pH, temperatures and processing time) are set at optimal, hydrolysis degree measured and hydrolysis terminated by either adjusting the pH or heating prior to downstream processing (Bernardini et al., 2011). This method has successfully been employed to extract several peptides including; a seven amino acid residues peptides; DLVKVEA, from the black eelpout fish, *Lycodes diapterus* (Lee and Byun, 2019), three novel ACE inhibitory peptides; AVFPSIVGR, PPVLVFV, and PGKVHIT (Yang et al., 2019) and aquatic weed novel antimicrobial peptides; TPDSEAL (Jiao et al., 2019).

Fermentation too, has been employed as a more natural method of protein hydrolysis. Notable examples are; the isolation of ACE inhibitory peptides from fermented fish sauce from Salmon fish, *Salmo salar* (Okamoto et al., 1995) and European anchovy, *Engraulis encrasicolus* (Ichimura et al., 2003). Also, Najafian and Babji (2018), identified two antioxidant peptides; LDDPVFIH and VAAGRTDAGVH from fermented anchovy fish, *Ilisha melastoma* sauce. Furthermore,

*Lactobacillus* strains have as well, been reported to yield bioactive peptides of improved therapeutic potentials as antioxidants (Padghan et al., 2018) and antimicrobial leads candidates (Hayes et al., 2006; Rana et al., 2018). In addition, Casein-derived antimicrobial peptides of strong antibacterial potency against *Enterobacter sakazakii* ATCC 12868 and *Escherichia coli* DPC5063 were produced through *Lactobacillus acidophilus* DPC6026 fermentation of sodium caseinate (Hayes et al., 2006). During the fermentation process, proteases and other hydrolytic enzymes produced by microorganism together with those in substrate hydrolyses and transforms protein substrate into soluble peptides and amino acids (Lopetcharat et al., 2001). The fermentation process enhances the taste, nutraceutical values, bio-preservative and antimicrobial qualities. However, the process is time consuming and more predisposing as far as peptide nativity loss and contamination are concerned (Satyanarayana, 2016).

Therefore, if one is to recover natively existing innate bioactive peptides such as antimicrobial peptides from any source, solvent extraction is inevitable (Välimaa et al., 2019). This approach was utilized by Su (2011), to extract a novel antimicrobial peptide, pelteobagrin; GKLNLFLSRLEILKLFVGAL from the skin mucus of Yellow catfish, *Pelteobagrus fulvidraco*. Likewise, antimicrobial peptides from skin mucus of *Parasilurus asotus* (Park et al., 1998), *Myxine glutinosa* (Subramanian et al., 2009), and *Puntius sophore* (Patel et al., 2020) among others were successfully isolated using this method. However, the extractive yield of this method is usually low. Hence, the method can only be employed to isolate, identify, and characterize fish bioactive peptides; after which production of the identified peptides can be up scaled through peptide synthesis and recombinant protein technology. Interestingly, aquaculture has of late been explored to produce raw materials in sufficiently large quantities for purification of bioactive molecules

from aquatic animals (Välimaa et al., 2019). Since the focus of the present study was to isolate and characterize native antimicrobial peptides from the fish skin mucus, this approach to extraction was fully adopted. Here, mild acidic solvent (10 % Acetic Acid) was utilized in the solubulisation of novel antimicrobial peptides that were identified prior to their up scaling using chemical peptide synthesis (Okella et al., 2021).

### 2.5.2 Downstream processing of antimicrobial peptides

The major physiochemical parameters that determine the activity of antimicrobial peptides are: size, charge, hydrophobicity and amphipathicity (Wang et al., 2019b). The downstream procedures should, therefore, take into consideration such physiochemical parameters. The influence of low molecular size in enhancing antimicrobial activity have been reported in a 4.7 kDa LEAP-2 from Topmouth culter, *Erythroculter ilishaeformis* (Chen et al., 2020b); 2.2 kDa Pelteobagrin, from yellow catfish, *Pelteobagrus fulvidraco* (Su, 2011); 2.8 kDa SAMP-H1 from Atlantic salmon, *Salmo salar* (Luders et al., 2005) and 2.7 kDa Pleurocidin, from winter flounder, *Pleuronectes americanus* (Cole et al., 1997). This enhanced activity is due to the fact that, low molecular weight peptides (usually below 5 kDa) exhibit high mobility and therefore, easily penetrate the bacterial cell membrane to cause an inhibitory activity (Enan et al., 1996). Methods like membrane ultrafiltration and size exclusion chromatography (Figure 2.5), can be employed to exclude high molecular weight protein components during downstream processing (Satyanarayana, 2016). Centrifugation on the other hand, can be utilized to sediment high molecular weight contaminats (Majekodunmi, 2015), from which the soluble supernatant can be precipitated and lyophilized.

Lyophilization involves; freezing of the liquid sample, sublimation of frozen sample under vacuum, evaporation of moisture from the amorphous matrix followed by desorption of chemisorbed water resident in the apparently dried cake (Rajeevani et al., 2015). During the process all the solvents like acetic acid, acetonitrile among others are sublimed below their eutectic points, leaving the samples in the dried cake (Harcum, 2008; Katsuki et al., 2017).

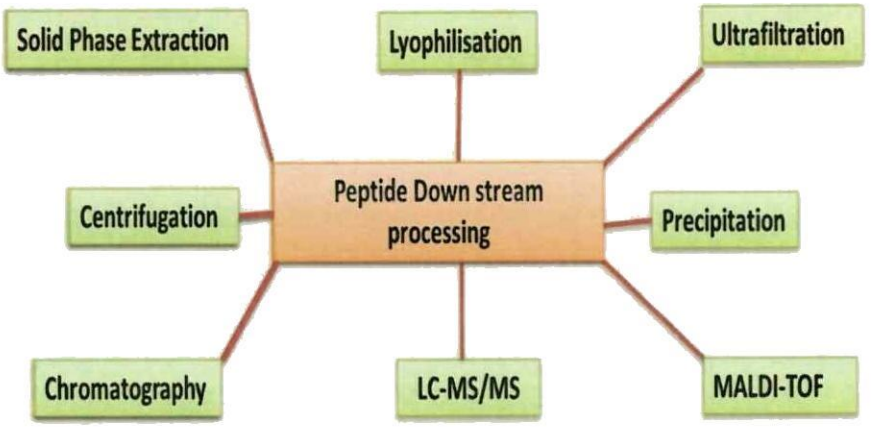



**Figure 2.5: Schematic illustration of different techniques for peptide downstream processing**

Ion exchange chromatography on the other hand can be used to fractionate peptides basing on their net charge. Peptides rich in positively charged amino acids like Arginine (Arg) and Lysine (Lys) can easily be separated using a cationic exchanger. Net charge is an essential physiochemical parameter as, positively charge peptides display enhanced affinity for electrostatic interactions with the negatively charged phospholipid head groups on the outer surface of the microbial cell membrane. This further justifies, introduction of cations like $Zn^{2+}$ co-factors to anionic antimicrobial peptides (Dashper et al., 2005). In addition, positive charge is an asset to membrane penetration as bacteria will always find it hard to elude their negatively charged membrane to

evade such interactions (Brogden, 2005). However, for any cationic peptide, charge should not exceed +9, as Therapeutic Index (TI), rapidly decreases beyond this threshold (Jiang et al., 2008).

Reverse phase High Performance Liquid Chromatography (RP-HPLC) permits rapid separation and detection of hydrophobic as well as hydrophilic characteristics of the peptides (Pratap et al., 2016). The HPLC is usually utilized in union with extra analysing equipment like Ultra violet detector or mass spectrometer (Najafian and Babji, 2012). However, in low-resourced settings, pre-packed disposable reverse phase cartridges can as well be utilised to effectively isolate hydrophobic peptides through solid phase extraction. The cartridge's solid-phase hydrophobic matrix optimally captures the hydrophobic peptides that are later recovered through organic solvent systems (Rana et al., 2018). The isolated peptides can then be identified through peptide sequencing. Solid Phase Extraction has been used to isolate peptides like Myxinidin (Subramanian et al., 2009), Hipposin (Birkemo et al., 2003), Pardaxin (Shai et al., 1994) among others. This informed the present study as far as downstream processing is concerned.

Hydrophobic peptides are rich in hydrophobic amino acids like Valine (Val), Glycine (Gly), Leucine (Leu), Isoleucine (Ile), Alanine (Ala), Phenylalanine (Phe), Tryptophan (Trp), and Proline (Pro). Such peptides, easily interact with cellular hydrophobic lipid bilayer, penetrate and cross the membrane (Bahar and Ren, 2013; Burton et al., 2013). Most hydrophobic peptides show α-helix structure conformation. Such conformation enhances amphipathicity of a peptide. The optimal AMPs polarily is exhibited by the polar nature of the amino acids forming it as determined by their lateral (R) chains (Cooper and Hausman, 2013). Here, amino acids like His, Lys, and Arg

›n the R group and hence serves in the formatting polar cationic AMPs

nical property has been reported to be most important physiological
›bial peptides (Wang et al., 2019b). The polar region of the amphipathic
tion with the microbial membrane through electrostatic interaction with
l head groups (Sato and Feix, 2006), while the non-polar region forms
els through hydrophobic interactions with the non-polar region of the phospholipid bilayer, resulting into increased permeability and loss of barrier function of the bacterial cells (Sato and Feix, 2006; Mihajlovic and Lazaridis, 2012).

To reveal the identity of the peptides by primary structure, liquid chromatography accompanied by mass spectrometry detection (LC-MS/MS) is most commonly used approach (Perkins et al., 1999). Also, Matrix Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) mass spectroscopy is as well useful in peptide profiling of partially purified fractions. But MALDI-TOF is not effective at detecting those peptides that are unable to ionise and 'fly' with the matrix molecules. Moreover, peptides with molecular masses below 0.5 kDa are hardly detected since, the α-cyano-4-hydroxycinnamic acid matrix molecules employed in energy absorption during the analysis also appear in this region of the chromatogram (Garbis et al., 2005). As such, the technique is mostly recommended for peptide molecular mass determination rather than sequencing.

On the other hand, a traditional method of sequence determination, Edman degradation can as well be utilised for short polypeptide sequencing (Smith, 2001). However, given its high cost of

operation and low efficacy on non-linear peptides Edman degradation method is now taken as obsolete. This leaves LC-MS/MS as highly recommended peptide sequencing platform and was therefore, adopted in the present study. The LC-MS/MS sequencing process involves for major stages like ionization, acceleration, deflection, and detection (Grebe and Singh, 2011). Here, vapourised samples from the nano-HPLC are first bombarded by stream of electrons prior to their acceleration by repelling positively charged chumber. Thereafter the magnetic fild is subjected to deflect the components based on the charge/mass ratio where the charged and lighter ions are defceected onto the various mass analyzers where they are detected and analyzed (Pitt, 2009; Grebe and Singh, 2011).

**2.6 Toxicity of Fish antimicrobial peptides**

Toxicity against mammalian cells is the chief drawback that impedes most peptides from penetrating the pharmaceutical market (Shilpakala et al., 2013; Conlon et al., 2014). To this effect, even some of the FDA approved peptide drugs like Dolastain from sea hare, *Dolabella auricularia* (Sone et al., 1993), and Cematodin from marine cyanobacteria, *Aureococcus anophagefferens* (Kang et al., 2018) have been withdrawn from clinical use. Toxic AMPs damage the cell membrane of eukaryotes to cause haemolytic side effects (Huan et al., 2020). Therefore, preliminary assessment of any potential lead biologic or their sources for hemo-compatibility is a crucial step in the drug discovery process.

Fortunately, majority of the fish-derived AMPs have been reported to hold a Generally Recognised As Safe (GRAS) legacy with exception to biologics from Soapfish, *Pogonoperca punctate* (Kaji et al., 2006), Spotted sea catfish, *Arius maculatus* (Manivasagan et al., 2009); Stonefish, *Synanceia*

*verrucosa* (Maillaud et al., 2020); Lionfish, *Pterois volitans* (Cohen and Olek, 1989; Robertson et al., 2014); Scorpionfish, *Scorpaena plumieri* (Andrich et al., 2010) and Pufferfish, *Akifugu rubripes* (Go et al., 2019). The biologics in such organisms just like in most reptiles and amphibians possess extreme hydrophobicity. Such hydrophobicity enables them penetrate the deeper hydrophobic core of the mammalian cell membrane to cause hemolysis (Wood et al., 2014).

The notable examples of non-toxic fish-derived AMPs include: Pelteobagrin, a 20 amino acids AMPs isolated from Yellow catfish was found to have no detectable haemolytic activity (Su, 2011); Misgurin (Park et al., 1997); Oncorhyncin II (Fernandes et al., 2004); Moronecidin (Lauth et al., 2002); Myxinidin (Subramanian et al., 2009); Paradaxin (Shai et al., 1994); Parasin I (Park et al., 1998). Such peptides have no record of haemolytic activity on eukaryotic red blood cells. However, no documented evidence was encountered on safety of AMPs from African lungfish, *Protopterus annectens* and African catfish, *Clarias gariepinus* on mammalian red blood cells, a task the present study investigated.

## 2.7 Mechanism of action of antimicrobial peptides

Antimicrobial peptides display two major modes of action: Membrane (lytic) and non-membrane (intracellular) targeting mechanism.

### 2.7.1 Membrane (lytic) mechanism

Classically, the antimicrobial peptides are known for targeting the cell membrane (Epand and Vogel, 1999), under two different models described as; Carpet and Pole models. In both models, the peptides electrostatically interact with negatively charged microbial cell membrane, prior to

membrane destruction. However, in carpet model, the antimicrobial peptides align parallel to the cell membrane like a carpet where it destroys the cell membrane in a 'detergent-like' manner (Oren and Shai, 1998), unlike in pole model where, the antibacterial peptides vertically penetrate the bilayer of the cell membrane. During the penetration, the peptides either accumulate then bend to form a ring hole/pore of 1-2 nm diameter as described in Toroidal pore/Wormhole model (Matsuzaki et al., 1995) or aggregates into channel forming multimers as described in Barrel-Stave model (Lohner and Prossnigg, 2009). Distruction of the cell membrane results in cytoplasmic outflow, cell collapse and death (Hollmann et al., 2018; Zhu et al., 2021).

Lytic mechanism presents a greatly reduced likelihood of bacterial resistance, since pathogens will always find it hard to recognize cationic peptides and be able to elude their negatively charged cell membrane composition so as, to evade the electrostatic interactions (Brogden, 2005). Therefore, to gain insight into the lytic mode of action of peptides, detecting the content of the extracellular alkaline phosphatase and electrolytes are commonly used. Here, an increase in the alkaline phosphatase activity signifies cell wall distruction (Robert et al., 2014). Alkaline phosphatase is localized in the periplasm between the cell membranes and cell walls of the bacteria (Sebastian and Ammerman, 2009) and any disruption of the cell wall penetrability, leads to its discharge out of the bacteria. This method has been employed to study bacterial cell wall distruction by *Porphyra yezoensis* peptides (Jiao et al., 2019) and Ornidazole (ORZ) (Chen et al., 2020a).

Furthermore, cell wall distruction increases the cell membrane permeability resulting to cytoplasmic outflow and hence, a rise in extracellular electrolytes (Green et al., 1929; Wu et al., 2016). And, by monitoring the electrical conductivity of the extracellular medium, the bacterial

cell membrane disruption can be studied (Molina-Guijarro et al., 2011) as exemplified bacterial membrane destruction by OVTp12 peptides on *E.coli* and *S. aureus* (Ma et al., 2020); *Porphyra yezoensis* peptides on *S. aureus* (Jiao et al., 2019); Essential oils on meat-borne *E.coli* (Zhang et al., 2017). Much as, cytoplasmic fluid outflow promotes cell collapse and death, it's an asset to freight peptides into the cell for intracellular target interactions (Schmidtchen et al., 2014).

It is also worth noting that, machine learning approaches like molecular docking can as well be employed to demonstrate the lytic mode of action of antimicrobial peptides. Through molecular docking, the lytic mode of action of cruzioseptin peptides (Cuesta et al., 2021) and flocculating polypeptides (Garcia et al., 2019) on bacterial cell membrane protein (PDB Id: 2dw6), have been demonstrated. This is substansiated by the fact that Bacterial cell membrane enzyme (PDB Id: 2dw6) is vital in the synthesis of unsaturated fatty acids that forms the the phospholipid bilayer (Kopp et al., 2020). Inhibition of such enzyme thus, impedes the cell membrane biosynthesis process (Leesong et al., 1996).

### 2.7.2 Non-membrane (intracellular) targeting mechanism

The non-membrane targeting mechanism involves entry of the antimicrobial peptides into the bacterial cell where they locate and bind on to the binding sites of intracellular target proteins (Hancock, 2001; Wilcox, 2004). Depending on the target, AMPs can inhibit protein biosynthesis, cell division, nucleic acid biosynthesis, protease activity, bacterial tolerance mechanism, among others. Well-known examples are; Bac7 1–35 peptide inhibits protein translation in bacteria by targeting ribosomes (Mardirossian et al., 2014), Indolicidin crosslinks DNA and as well inhibits

DNA Topoisomerase I (Subbalakshmi and Sitaram, 1998), MciZ peptide effectively inhibits Z-ring formation and localization (Cruz et al., 2020), among others.

Again, to establish such binding sites on the target protein and later, their binding affinities of the ligand with the target protein, molecular docking is the most widely utilized technique (Shukla et al., 2020). The technique permits simultaneous investigation of thousands of molecules and later incorporated the ligand-target interactions to rank such molecules based on their binding affinities (Lionta et al., 2014; Kumar et al., 2020). Such a virtual screening approach can therefore aid, the identification of potentially safe and most active novel molecules from among large libraries in a shorter time. Thus, can sieve and be a good reference prior to cell and animal studies and has so far delivered good results in the recent past at within a significantly shorter screening time (Lionta et al., 2014).

Notably, through virtual screening several drugs like Captopril, Dorzolamide, Saquinavir, Zanamivir, Oseltamivir, Aliskiren, Boceprevir, Nolatrexed, TMI-005, LY-517717, Rupintrivir and NVP-AUY922 were led into clinical trial and subsequent FDA-approval for therapeutic use, courtesy of Computer aided drug discovery (Talele et al., 2010). Similarily, novel putative drugs against Human papilloma virus protein E6 and E7 responsible for cervical cancer were discovered from natural products through virtual screening (Kotadiya and John, 2015). The biological activities of napin and cruciferin against bacteria were too established through the same approach (Rahman et al., 2020). Several docking engines like AutoDock, PatchDock, Discovery studio, GOLD, Schrodinger Maestro, PyMol, FireDock among others do exist, with their usability influenced by licencing. However, docking techniques is majorly limited by the approximated

scoring functions which in most cases present results that rarely correlate with the experimental binding affinities. Also, the docking pose prediction often hamper both the ligand and receptor sampling conformations thereby, necessitating experimental validations (Elokely and Doerksen, 2013; Pantsar and Poso, 2018; Okella et al., 2022a).

## 2.8 Summary and perspective

The majority of the currently identified mucosal AMPs were mostly reported from Europe followed by Asian and North America. No such records exist in entire African continent. In addition, most studies were on marine fish species with none conducted on freshwater species like African catfish, *Clarias gariepinus* (Burchell, 1822) and African lungfish, *Protopterus annectens* (Owen, 1839). The safety profiles and the possible mode of action of antimicrobial peptides in the skin mucus of such species remain unknown, thereby depriving foundational bio-prospecting efforts for novel antimicrobial leads in the era of novel antimicrobial search. These impelled, present study that screened and characterised the AMPs from selected freshwater fish species inhabiting the three major lakes of Uganda (Lake Albert, Lake Kyoga, and Lake Victoria).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Media, Reagents, Materials, Facilities and Approvals

Sephadex G-25 resin, SPE $C_{18}$ stationary phase columns, Acetonitrile, Ethanol, Phosphate Buffer Tablets, Sodium deodecyl sulphate, Acrylamide, 5 kDa Molecular Weight Cut-Off (MWCO) membrane, Coomassie R250, Methanol, Glacial acetic acid, Mercapto-ethanol, Coomassie blue G-250 and Triton X-100 were obtained from Sigma-Aldrich (St. Louis, USA). *QuantiChrom* Alkaline phosphatase assay kit was purchased from BioAssay Systems (Hayward, USA). Resazurin salt was supplied by Glentham (Wiltshire, UK). SDS-PAGE standard markers were purchased from Thermo Fisher Scientific (Waltham, USA).

Nutrient Agar (NA), Mueller-Hinton agar (MHA) and Brain Heart Infusion Broth (BHIB) were obtained from Himedia (Mumbai, India). Ciprofloxacin was sourced from Medicon (Vadodara, India). *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC25923 were were purchased from Thermo Fisher Scientific (Newport, UK). The present study utilized the archived bacteria at the Microbiology and Parasitology Laboratory, College of Veterinary Medicine, Animal Resources and Biosecurity (CoVAB), Makerere University (Kampala, Uganda). These were already imported by Makerere University following the recommended guidelines for importation of biological materials/pathogens. The two bacterial strains were the representatives of Gram positive (*S. aureus*) and Gram negative (*E. coli*), in the antimicrobial screening process. The choice was guided by the WHO Global Priority pathogen listing (Willyard, 2017). Here, a Gram-negative human pathogen in the critical priority tire (*E. coli*) (Shrivastava et al., 2018) and a Gram positive pathogen of high priority tire (*S. aureus*) (Asokan et al., 2019) were utilized.

Fish acclimatization, mucus harvesting, peptide extraction and purification were done at the Molecular Biology Laboratory, College of Veterinary Medicine, Animal Resources and Biosecurity (CoVAB), Makerere University (Kampala, Uganda). Susceptibility studies, safety profiling as well as bacterial cell envelope lysis assays were too carried out at Microbiology and Parasitology Laboratory, Makerere University (Kampala, Uganda).

Peptide quality control check and sequencing using Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fisher, USA) was performed at the *de Duve* Institute Proteomics platform, UCLouvain (Brussels, Belgium). Still at the same facility, database search for peptide identification was executed. Here, the Term of Reference (TOR) was basically a collaboration between the host laboratory and the study which culminated into a journal co-authorship. The antimicrobial peptides were characterised, their 3-D structures modeled, targets fished-out and later docked at Mbarara University of Science and Technology (Mbarara, Uganda). Later, the details of ligand-target interactions and the binding affinities were as well revealed. Peptides where chemically synthesized by Biomatik (Wilmington, USA) and *in vitro* experimental validation performed at the University of California, U.S.A.

Ethical approval and permission to conduct this study was obtained from the Faculty Research Committee (Appendix VI) and later Mbarara University of Science and Technology Research Ethics Committee (Appendix VII). In addition, the study was registered with Uganda National Council for Science and Technology (Appendix VIII). Furthermore, the permit to conduct study

on fish was granted by the Office of the Commissioner, Department of Fisheries Control, Regulation and Quality Assurance, Entebbe, Uganda (Appendix IX).

Trainings on Research Ethics, Methods and Bio-statistical analysis were obtained from the National Institute of Cancer Prevention and Research (NICPR), Noida, India. Additionally, a specialized skill on Molecular Biology and Biotechnology was acquired via Vrije Universiteit Brussel-Makerere University (VUB-Mak) fellowship. This was preceeded by a deep exposure to drug discovery research at the Center of Excellence in Drug Discovery, National Facility of Drug Discovery (NFFD), Rajkot, India.

### 3.2 Study design

The present study adopted both cross-sectional and *in silico* designs were adopted.

### 3.3 Study site

The samples were purposively collected from three different locations of Bweyale, Ggaba and Walukuba landing sites on Lake Kyoga (GPS: 1.5876, 33.0494), Victoria (GPS: 0.2578, 32.6375) and Lake Albert (GPS: 1.8481, 31.3819) respectively (Figure 3.1). These three lakes are the major lakes of Uganda that were purposively selected guided by their richness in the two fish species (Wandera et al., 2010; Nsubuga et al., 2017). In addition, priority was given to the ancient lakes of Uganda. Ancient lakes are those that have consistently carried water for more than one million years due to plate tectonics in the active rift zone. Due to their prolonged life, ancient lakes, serve as models for isolated evolutionary traits and novel biologics given their enhanced speciation (Balian et al., 2008; Hirano et al., 2019). Accordingly, the two ancient lakes of Uganda (Lake

Albert and Lake Victoria) were granted priority as far as study site selection for the present study was concerned. The busiest landing sites across the selected lakes were purposively considered to increase the chances of obtaining qualifying fish samples.

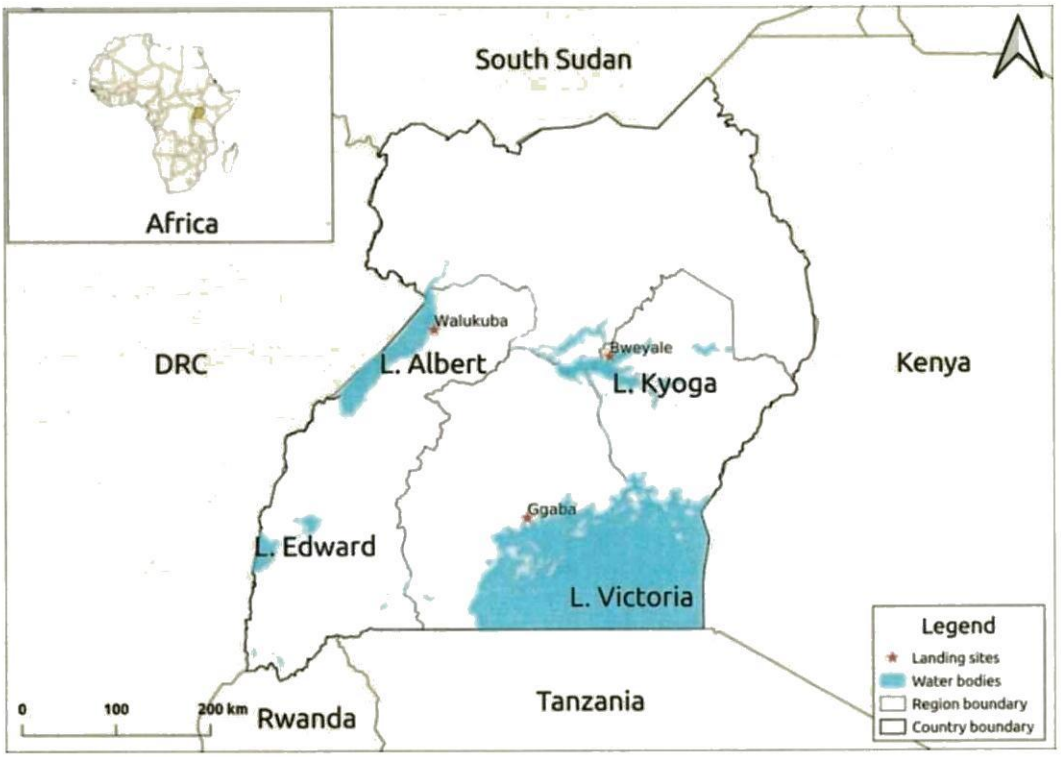



**Figure 3.1: Map of Uganda showing the study sites**. The samples were collected from the landing sites on Lake Victoria, Lake Kyoga and Lake Albert. The Map was drawn in *Quantum GIS V. 3.10* using the GPS coordinates taken from the landing sites during sample collection. The points were mapped on the Uganda shape files retrieved from *Humanitarian Data Exchange v1.43.6.*

### 3.4 Study sample and size

A total of twenty-four live mature African catfish, *Clarias gariepinus* (Burchell, 1822) in the family *Clariidae* (mean weight 300.50±5.98 g, mean length 30.60±2.11 cm) and another twenty-four live mature African lungfish *Protopterus annectens* (Owen, 1839) in the family *Lepidosirenidae* (mean weight 262.50±7.79 g, mean length 35.90±4.24 cm), all in the second growth phase, were purposively sourced from each of the selected three major freshwater lakes (Kyoga, Victoria and Albert). The second growth phase of fish is during which fish matures. The weight range for most fish species at this stage is 100-1500 g and fish in this stage of life produces substantial quantity of skin mucus even at high stocking density (Nieuwegiessen et al., 2009). The fish species were selected on the basis of available literature as scale-less produces more skin mucus compared to the scaled fish, for an effective innate immune response (Dash et al., 2018). Here, the sample size was empirically adopted based on the previous study in which Pelteobagrin, a 20 amino acids (GKLNLFLSRLEILKLFVGAL) novel native antimicrobial peptide was successfully identified and characterized from 8 Chinese Yellow catfish, *Pelteobagrus fulvidraco* by Su (2011).

### 3.5 Sample preparation and preliminary processing

The live fish were separately transported from the landing sites to the College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University (Kampala, Uganda) in 125 L freshwater tanks and later acclimatized and fed *ad labitum* for seven days on commercial 8 mm Catfish pellets (Kaffiika, Kampala, Uganda). Acclimatization was done in three separate 1000-litre plastic tanks, under contineous freshwater circulation. To optimize the harvest, the fish were starved for 24 hours prior to mucus collection. Skin mucus (45 mL) of all the eight fish representing

each lake was dorso–laterally scrapped using a sterile plastic spatula and pooled into 50 mL centrifuge tubes (Corning, New York, USA). To avoid contamination from anal and intestinal excreta, scrapping was avoided from the ventral part of the fish. The harvested skin mucus was first deep frozen at - 80 °C, then lyophilized using *FreezeZone* Labconco lyophilizer (Labconco, Kansas, USA) at -104 °C and 0.013 milibars to remove excess water and concentrate the samples. The lyophilized samples were later stored at 4 °C for one week given its stability between 4-25 °C and reduced chances of degradation at lower temperature. The guidelines from the Department of Fisheries Control, Regulation and Quality Assurance, Ministry of Agriculture, Animal Industry and Fisheries, Entebbe, Uganda alongside that of American Fisheries Society guidelines for the use of fish in research (Jenkins et al., 2014), were strictly followed.

### 3.6 Peptide extraction

Extraction of peptides was carried out using a method adopted from Subramanian et al. (2009), with minor modifications. Briefly, lyophilized skin mucus (0.4 g) was reconstituted in 15 mL of 10 % (v/v) acetic acid and heated for 5 min in a boiling water bath at 95 °C. The acid mucus mixtures were then homogenized for 1 minute using a polytron homogenizer (Kinematica, Malters, Switzerland). Insoluble compounds were then removed by centrifuging at 10,000 x *g* at 4 °C for 1 hour (Hermle, Wehingen, Germany). The supernatant (14 ml) was then collected into Vivaspin tubes containing a 5 kDa Molecular Weight Cut-off membrane (Sartorius, Gloucestershire, UK) and centrifuged at 6000 x *g* at 4 °C for 10 hours to exclude the high molecular weight proteins such as hydrolytic enzymes that could potentially demonstrate antimicrobial activity were left in the residues as peptides of interest proceed into the ultra-filtrate (Huiping et al., 2015). The ultra-

filtered peptides with Molecular weight below 5 kDa were collected for Solid Phase Extraction (SPE).

### 3.7 Solid Phase Extraction and yield calculation

As described by Rana et al. (2018), low molecular weight contaminants were removed from ultra-filtrate by Solid Phase Extraction. The mucus extracts were applied on disposable SPE $C_{18}$ stationary phase cartridges (50 µm particle size, 60 Å pore diameter, Column Volume 15 mL) containing 5 g sorbent (ThermoScientific, Bellefonte, U.S.A). Columns were conditioned with 30% (v/v) acetonitrile, and subsequently, the ultra-filtered sample was loaded at a flow rate of 1 mL/min. During this process, only the peptides can bind transiently to hydrophobic matrix, leaving contaminants that may confound the antimicrobial activity of the peptides to flow through. Bound peptides were mildly washed with deionized water (pH 7.01), for their extra cleaning. Finally, cleaned and concentrated peptides were eluted in 5 mL volume with 70 % (v/v) acetonitrile and lyophilized at -104 °C and 0.013 millibars. Lyophilization below the Acetonitrile eutectic point; -45.0 °C and 1.67 milibars (Harcum, 2008) and Acetic acid; -26.3 °C (Katsuki et al., 2017), eliminate all the solvents that could possibly confound the antimicrobial activity of the peptides. Prior to antimicrobial susceptibility assays, the extractive yield was then calculated using the formula:

$$Extractive\ yield\ (100\%) = \frac{Mass\ of\ extracted\ peptides}{Mass\ of\ mucus}\ x\ 100$$

### 3.8 Sub study I: Antimicrobial activity of fish skin mucus extracts

#### 3.8.1 Agar well diffusion assay

The agar well diffusion method described by Oliveira et al. (1998) was adopted. In this method, *S. aureus* ATCC25923 and *E. coli* ATCC25922 were spread-plated on the solid Mueller-Hinton agar (MHA) medium with a concentration of $10^6$ CFUs/mL. Pluralities of small wells with a diameter of 6 mm were punched out with a cork borer, and 50 µl of 50 µg/mL laboratory-prepared antibacterial peptides in 0.01 M PBS were dispensed into the wells in triplicates. Ciprofloxacin (50 µg/mL) in dionized water was, dispensed in separate wells as the control drug, while 0.01 M Phosphate Buffer Saline (50 µL) and 70 % Acetonitrile (50 µL), were used as negative controls. Besides, complete evaporation of acetonitrile in the SPE dry peptide powder required reconstitution in a non-antimicrobial solvent (0.01 M PBS) that preserves peptide integrity (Chen et al., 2017). After 24 hours of incubation at 37°C, the antibacterial activities of the extracted peptides were determined by measuring the diameter of the zone of inhibition (mm).

#### 3.8.2 Determination of Minimum Inhibitory Concentration (MIC)

Using the broth dilution method, as previously described by Teh et al. (2017), in a 96-well U-shaped cell culture plate (Greiner, Kremsmünster, Austria), 50 µL of Brain Heart Infusion Broth was introduced into nine vertical columns. To the first six wells of the plate columnwise, 50 µL of 500 µg/mL extracts of *C. gariepinus* from Lake Kyoga, Lake Victoria, and Lake Albert were added followed by extracts of *Protopterus annectens* from Lake Kyoga, Lake Victoria, and Lake Albert. The three subsequent columns contained ciprofloxacin (50 µg/mL) as a positive control, 0.01 M PBS as a negative control, and sterile broth. Subsequently, a two-fold serial dilution was performed after which 50 µL of diluted bacterial suspension ($1.5 \times 10^6$ cell/mL) was added into all wells

(except the broth sterility control column) and mixed thoroughly using a pipette. Micro-dilution was performed in triplicates for each bacterial species. After overnight incubation at 37°C, 5.0 µL of 6.75 mg/mL Resazurin (Glentham, Wiltshire, UK) was added to all wells and incubated at 37°C for another 4 hours. Color changes were observed and recorded, with microbial growth implied by an irreversible change in the color from the blue of resazurin to pink resorufin. The lowest concentration prior to the color change was considered as the Minimum Inhibitory Concentration (MIC). Skin mucus extract with the lowest MIC across indicator strains was later selected for further purification.

### 3.9 Sub study II: Antimicrobial peptides purification and identification

#### 3.9.1 Sephadex G-25 gel filtration purification of antimicrobial peptides

Lake Albert's *C. gariepinus* extract (SPE lowest MIC across bacterial strains) was further purified by a Sephadex G-25 gel filtration column [(1.2 × 55 cm; Cytiva, Uppsala, Sweden) (Neumann et al., 1996)]. Briefly, 5 mL of the peptides in 0.01 M PBS with a 10 mg/mL concentration were loaded into the column and eluted by deionized water (pH 7.01) at a flow rate of 0.3 mL/min. Deionized water was utilized as eluent of no antimicrobial effect. The fractions were collected at 9-minutes intervals with a fraction collector and absorbance was monitored at 280 nm using a *NanoDrop* Spectrophotometer (Thermo Scientific, New York, USA). A wavelength of 280 nm was used since presence of amino acids especially those with aromatic rings implies maximum absorbance peak at 280 nm (Schmid, 2001). The antibacterial activity of each of the peak fractions on *E. coli* and *S. aureus* was evaluated using the broth-dilution method as previously described. The peak fraction with the highest antibacterial activity was lyophilized and shipped to Proteomics

platform, *de Duve* Institute, UCLouvain (Brussels, Belgium) for peptide sequencing by High-Resolution, Accurate-Mass (HR/AM) mass spectrometry.

### 3.9.2 Analysis of purified antimicrobial peptidesby SDS-PAGE

Prior to peptide sequencing, presence and profiles of Peak I peptides was confirmed using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) as described by Laemmli (1970). A 15% separating gel and 4% stacking gel was used. Here, 0.01 g of lyophilized Peak I fractions and 0.01 g of lyophilized $C_{18}$ SPE elute were dissolved in 50 µL deionized water, respectively. Thereafter, 40 µL of samples and 40 µL of sample loading buffer [12% SDS (w/v), 6% mercaptoethanol (v/v), 30% glycerol (w/v), 0.05% Coomassie blue G-250, 150 mM Tris/HCl, pH 7.0] were mixed, boiled for 5 minutes at 100 °C to denature the proteins utilizing heating block (Biobase, Shangdon, China). Later, 10 µL of the samples were then loaded into each well of the gel. Later, 8 µL of pre-stained SDS-PAGE standard markers (Thermo Fisher Scientific, Waltham, USA) were included to estimate the molecular mass of the proteins. Electrophoresis was run at a constant voltage of 150 V for 55 minutes, until the dye front migrated to 2 cm from the bottom of the gel (Bio-Rad, Hercules, USA). Thereafter, the protein bands on the gel was directly stained in 100 mL staining solution (1 g Coomassie R250, 30 mL, methanol, 65 mL deionized water, 50 mL acetic acid) for 4 hours. Peptides were then directly visualised in distaining solution I (10 mL methanol, 10 mL Glacial acetic acid, and 80 mL deionized water) at 55 rpm rotation at room temperature; 25 °C (Edmund Buhler GmbH, Bodelshausen, Germany), until clear bands were observed.

### 3.9.3 Peptide sequencing by Mass Spectrometry

The molecular mass and the amino acid sequence of peptides in the most active fraction from gel filtration chromatography (Peak I) were determined using the Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fisher, Waltham, USA) at the *de Duve* Institute Proteomics platform, UCLouvain, Brussels, Belgium. Freeze-dried sample was reconstituted at 1 mg/mL in distilled water prior to 10-fold dilution in 3% Acetonitrile (ACN)/0.1% Trifluoroacetic acid (TFA). Later, 2 µL of the peptides were directly loaded onto reversed-phase pre-column (Acclaim PepMap 100, Thermo Scientific, Waltham, USA) and eluted in back-flush mode. Peptide separation was performed using a reversed-phase analytical column equilibrated in 3.5% ACN/0.1% Formic Acid (FA) in water (Acclaim PepMap RSLC, 0.075 x 250 mm, Thermo Scientific, Waltham, USA) with a linear gradient of 4%-27.5% solvent B (0.1% FA in 80% ACN) for 100 minutes, 27.5%-40% solvent B for 10 minutes, 40%-95% solvent B for 1 minute and holding at 95% for the last 6 minutes at a constant flow rate of 300 nL/min on an Ultimate 3000 RSLC nanoHPLC system (Thermo Fisher Scientific, Waltham, USA). The peptides were analyzed in positive mode by an Orbitrap Fusion Lumos tribrid mass spectrometer (ThermoFisher Scientific, Waltham, USA). The peptides were subjected to Nanospray Ionization (NSI) source followed by tandem mass spectrometry (MS/MS) in Fusion Lumos coupled online to the Ultra Performance Liquid Chromatography (UPLC). Intact peptides were detected in the Orbitrap at a resolution of 120,000 within a mass-charge (*m/z*) range from 350 to 1500. A charge state decision tree was applied for all precursors' ions prior to MS2 fragmentation. Peptides with charge states +2 and +3 were selected for MS/MS using Higher-energy C-trap dissociation (HCD) setting at 30; ion fragments were detected in the Orbitrap at a resolution of 30,000 units. For precursors with charge states from +3 up to +8, MS/MS was obtained by Electron-Transfer/Higher-Energy Collision

Dissociation (EThcD) fragmentation with supplemental energy set at 30, ion fragments were detected in the Orbitrap at a resolution of 30,000 units. A data-dependent procedure of MS/MS scans was applied for the top precursor ions above a threshold ion count of 2.0E4 in the MS survey scan with 60.0s dynamic exclusion. The total cycle time was set to 4 seconds. The electrospray voltage applied was 2.1 kV. The MS1 spectra were obtained with an Automatic Gain Control (AGC) target of 4E5 ions, and MS2 spectra were acquired with an AGC target of 5E4 ions, maximum injection time was set to auto for both type of scans. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the Proteomics Identification Databases (PRIDE) (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD029193 and 10.6019/PXD029193.

### 3.9.4 Peptide identification and database search

The resulting MS/MS data were processed using *Sequest HT* search engine within *Proteome Discoverer 2.4* against a custom database containing 102688 sequences from catfish species (compiled from Uniprot proteins entries with taxonomy ID: 175774 *Bagarius yarrelli*; 310915 *Pangasianodon hypophtalmus*; 7998 *Ictalurus punctatus*; 35657 *Clarias microcephalus*; 219545 *Ameiurus melas*). No enzyme was specified as cleavage enzyme allowing a maximum peptide length of 30 residues, four modifications per peptide *b*- and *y*- ions for HCD fragmentation, *b*- *c*- *z*- and *y*- ions for EThcD fragmentation. The mass error was set to 10 ppm for precursor ions and 0.1 Da fragment ions. Oxidation on methionine (Met) was considered as variable modification. Peptide Spectrum Matches (PSM) were filtered using the *q*-value and Posterior Error Probability (PEP) calculated by the Percolator algorithm ensuring an estimated False Discovery Rate (FDR) below 5%. The filtered *Sequest HT* output files for each peptide were grouped according to the protein from which they were derived. Thereafter, all the identified peptides in *FASTA* file format

were subjected to a Discriminant Analysis (DA) machine learning algorithm at the Collection of Anti-Microbial Peptides ($CAMP_{R3}$) server (http://www.camp.bicnirrh.res.in/)_(Waghu et al., 2016), to reveal their antimicrobial probability. The server gives a score on 0 to 1 scale; with a score of above 0.5 classified as Antimicrobial Peptide (AMP) and those below 0.5 are Non-Antimicrobial Peptide (NAMP). Subsequently, the *FASTA* file sequences of AMPs with the highest score (Antimicrobial score >0.9), were then inputted to a more rigorous and accurate the Deep Learning algorithm cross-validation using *Deep-AmPEP30* server at https://cbbio.online/AxPEP/ (Yan et al., 2020). The server's default antimicrobial peptide classification cut-off of 0.5 was maintained. Its positive sequence (Product Probability >0.5) is scored as 1, negative sequence (Product Probability <0.5) is 0, with an invalid sequence of -1. Peptides with the highest antimicrobial activity were obtained for sequence characterization.

### 3.9.5 Sequence Characterisation

Using the raw sequence files, molecular weights, net charge at pH 7 and percentage hydrophobicity were determined using the European Bioinformatics Institute (EBI) tool; *EMBOSS PepStats* at (https://www.ebi.ac.uk/Tools/seqstats/emboss_pepstats/). Thereafter, the same African Catfish Antimicrobial Peptides (ACAPs) sequences were then utilized in secondary structure classifications using Profile network prediction HeiDelberg (PHD) software at https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_phd.html (Combet et al., 2000).

### 3.10 Sub study III: Antimicrobial Peptides toxicity profiles

#### 3.10.1 The *in vitro* Hemolytic activity of Peak I antimicrobial peptides

Peak I antimicrobial peptide fraction was assayed utilizing a modified Rabbit blood cells method described by Lin, Hui, Chen, & Wu (2013). Briefly, fresh Rabbit red blood cells were obtained by centrifuging whole blood from live rabbit in EDTA-coated Vacutainer (Becton & Dickinson, New Jersey, USA) in a refrigerated microfuge (Eppendorf, Dubai, UAE) at 211 x *g* for 5 min at room temperature (25 °C). Red blood cells were washed three times with PBS, and then diluted with PBS to a blood cell concentration of approximately 10 % (v/v). A portion of the Rabbit blood cell suspension (500 µL) was transferred to six micro-centrifuge tubes (CellTreat, Massachusetts, USA), and mixed with 500 µL of the peak I antimicrobial peptide extract solution in 0.01 M PBS at the desired concentrations (0, 20, 40, 60, 80 and 100 µg/mL). The tubes were then incubated at 37 °C (Heraeus, Hanau, Germany) to induce hemolysis. After 30 minutes of incubation, non-hemolyzed blood cells were separated by centrifugation at 211 x *g* for 5 minutes at room temperature. Aliquots (100 µL) of the supernatant were transferred to a 96-well plate (Corning, New York, USA), and hemoglobin release was monitored by measuring the absorbance of the supernatant at 540 nm using microtiter plate reader (Biochrom, Cambridge, England). A blood cell solution treated with 1 % Triton X-100 (positive control), to induce 100 % lysis through blood cell deeper to hydrophobic core penetration. The untreated blood cell suspension in 0.01 M PBS alone was on the otherhand used as a negative control. Each assay was performed in triplicate for three independent experiments, and data were expressed as the mean and SEM. The percentage of hemolysis was calculated using the following formula (Gao et al., 2015):

$$Hemolysis\ (\%) = \frac{(Ae - An)}{(Ap - An)}\ x\ 100$$

Where *Ae* is Absorbance of the extracts, *Ap* is the Absorbance of the positive control and *An* is the absorbance of the Negative control.

### 3.10.2 *In silico* hemo-toxicity profile

Given the fact that, clinical trials failure of potential therapeutic peptides are mostly attributable to their toxicity profiles like hemolytic activity (Conlon et al., 2014), an accurate double-staged foretelling of hemolytic peptides (HLPs) at the earliest possible stage of drug discovery process is essential. The present study employed an online server at *HLPpred-Fuse* at http://thegleelab.org/HLPpred-Fuse/, to predict the haemolytic activity of individual antimicrobial peptides from the most active peak. The *HLPpred-Fuse* is a two-layer prediction framework, which can accurately and automatically predict either hemolytic peptides (HLPs) or non-HLPs. For the HLPs it can further predict the potential degree of hemolysis caused by the peptide. Established on 54 probabilistic features derived by integrating nine different sequence-based encodings and six different machine learning classifiers, *HLPpred-Fuse* is treated as the most accurate tool in the hemolytic activity prediction (Hasan et al., 2020). Here, the *FASTA* file sequence of individual African catfish antimicrobial peptide (ACAP) were inputted and run to predict profile of the identified peptides. Peptides with HLP probability above 0.5 was classified as haemolytic while, those with the scores below 0.5 were classified as non-hemolytic.

For additional cross-validation of the toxicity profiles of antimicrobial peptides, *ToxinPred* an online server at https://webs.iiitd.edu.in/raghava/toxinpred/design.php (Gupta et al., 2013), was run. Here, raw peptide sequence files were inputted, and a Support Vector Machines algorithm

(SVM-SwissProt based) was run at a stringent 0.0001 *e*-value cut-off. Toxicity prediction status and SVM scores were considered prime outputs.

### 3.11 Sub study IV: Mode of action of African Catfish Antimicrobial Peptides

#### 3.11.1 Ligand preparation

Ligands were prepared using a method previously described by Okella et al. (2020). Briefly, antimicrobial peptide *FASTA* file sequences were inputted to predict the three-dimensional (3-D) structure of the AMPs utilizing Iterative Threading Assembly Refinement (*I-TASSER*) server (https://zhanglab.dcmb.med.umich.edu/I-TASSER/) (Yang and Zhang, 2015). The server utilizes protein templates identified by Local Meta-Threading Server (LOMETS) from the Protein Data Bank (PDB) library (https://www.rcsb.org/) (Berman et al., 2002). The LOMETS employs multiple threading approaches to align the query protein amino acid sequence against the PDB based on fold recognition. Template proteins with the highest sequence identity and *z*-score were used in the modeling exercise. The best models were identified using a -5 to 2 confidence score (*c*-score) scale, with a higher *c*-score value signifying models with high confidence and vice versa. The *c*-score is calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations (decoys).

To cross-validate the predicted peptide 3-D structures, *PEP-FOLD* v3.5, a web-based *de novo* peptide structure prediction tool at https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/ was run (Thévenet et al., 2012). Here, *FASTA* files were inputted, 100 simulations run and later, best output models ranked based on the coarse-grained optimized potential for efficient structure prediction (sOPEP) energies and Apollo predicted melting temperature ($t_m$). Models that release highest *sOPEP* energies at highest melting temperature were ranked as best predicted structures.

Thereafter, a two phased quality check was performed on the best modeled structures (*I-TASSER* and *PEP-FOLD* v3.5 modeled); Initially, a web-based Protein Structure Analysis (*ProSA*) tool at https://prosa.services.came.sbg.ac.at/prosa.php (Wiederstein and Sippl, 2007) was used. The ProSA tool outputs the query protein *z*-score, residual energy and thereafter plots local model quality. The ProSA *z*-score equates the query protein *z*-score against those of experimentally validated proteins at the Protein Data Bank (PDB) Library (https://www.rcsb.org/) (Berman et al., 2002). A higher *z*-score value indicates greater similarity.

Later, the stereo-chemical properties of the modelled antimicrobial peptides in *pdb* file format were estimated using *PROCHECK* tool (https://saves.mbi.ucla.edu/) (Laskowski et al., 1993). Here, the residues bonding patterns and their effects on the distribution of backbone dihedral angles were revealed in a Ramachandran Plot. Models with majority of their non-glycine and non-proline residues in the most favoured region (>83 %) and, fewest residues (<3 %) in the disallowed region were qualified as best quality models (Hollingsworth and Karplus, 2010).

### 3.11.2 Target Fishing and Preparation

Target proteins were selected based on previous reports on their vital roles in inhibiting and killing of bacteria. The selection utilized *DrugProt*, an EMBL-EBI web server at https://www.ebi.ac.uk/thornton-srv/databases/drugport/. The *DrugPort* is based on the latest (December 2021) version of *DrugBank* database, and it contains 1492 approved drug targets and 1664 unique protein targets. To fish-out potential targets; sequences of non-hemolytic AMPs in *FASTA* file format were submitted as input files. The target name, PDB identity, percentage target identity, *z*-score, and source organism's name were retrieved and used to qualify protein targets in

bacteria. Only targets with experimentally determined structures at the PDB database and known physiological roles were considered. Thereafter, the 3-D structures and class of the receptor proteins were retrieved from the PDB library based on X-ray crystallography (XRC) and Nuclear Magnetic Resonance (NMR) data. All the retrieved 3-D protein structures were visually inspected in *PyMOL* software. Thereafter, *BOVIA Discovery Studio* v2021 client was used to clean the protein receptors and define the attributes of the binding sites. During cleaning, water molecules, ligand groups and heteroatoms were dispelled from the receptor protein prior to the docking exercise. This was to ensure energy minimization and hence more factual docking energy outputs. Later, the binding site attributes were defined using the receptor cavity method at *BOVIA Discovery Studio* v2021 client. Here, hydrogen atoms were first added prior to binding site localization and docking box dimension (length, width, height) establisment.

### 3.11.3 Molecular docking studies against intracellular protein targets

In order to gain additional insight into the possible mechanism of action, *AutoDock VINA* (Trott and Olson, 2019) at DINC 2.0 Web server (http://dinc.kavrakilab.org/) (Antunes et al., 2017) was utilized to perform several molecular docking calculations. *AutoDock VINA* is ranked, the best tool to execute a fixed docking exercise where binding sites is not known (blind docking) (Hetényi and van der Spoel, 2002). Here, the *pdb* file format for both ligand and receptor proteins were submitted as input files with the receptor protein centered in the previously defined docking box. The binding energies (kcal/mol) and Root Mean Square Deviation (RMSD, Å) were outputted. Thereafter, *PatchDock*, an online docking server (https://bioinfo3-D.cs.tau.ac.il/PatchDock/php.php) was as well utilised in a cross-validation exercise. PatchDock's molecular docking algorithm is based on Shape Complementarity Principles (Duhovny et al.,

2002; Schneidman-Duhovny et al., 2005). Both the ligand and receptor *pdb* files were uploaded to the server and tool was later run at a default complex type and maximum Root Mean Square Deviation (RMSD) of 4.0 A°. The output transformation files (best 1000 transformations) were then, refined and rescored for a flexible (induced-fit) docking using Fast Interactive Refinement in molecular Docking (*FireDock*) tool at https://bioinfo3-D.cs.tau.ac.il/FireDock/php.php to generate global energies of the docked complexes. This global energies is generated by comparing the low-energy minima with the experimental values (Mashiach et al., 2008). Flavin adenine dinucleotide (DrugBank Id: DB03147), was used as an FDA-approved control drug for the above protein targets to compare the most promising peptide ligand. All ligand-receptor complexes were rendered in *BOVIA Discovery Studio* v2021 software and *Chimera* v1.15 (Pettersen et al., 2004).

### 3.11.4 Membrane mode of action

To gain insight into the lytic mode of action of ACAPs, non-hemolytic ACAPs were docked against bacterial cell membrane protein (PDB Id: 2w6d) using AutoDock, FireDock and PatchDock engines as previously described. The ligand-receptor complexes were rendered in *Chimera 1.15* software to uncover the degree of penetration of the ACAPs into the bacterial cell membrane.

### 3.11.5 Alkaline Phosphatase activity of antimicrobial peptides

To cross-valideate bacterial cell wall destruction potential of the AMPs, Alkaline Phosphatase (ALP) assay was conducted. Alkaline phosphatase assay kit (*QuantiChrom* BioAssay Systems, Hayward, USA) with *p*-nitrophenylphosphate (*p*NPP) as a substrate was utilized (Zhao et al., 2019). Briefly, 12-hour cultured *E. coli* strains were dissolved in fresh sterile medium to $10^6$ CFUs/mL. About, 0.5 mg/mL of laboratory prepared peak I antimicrobial peptide in 0.01 M

Phosphate Buffer Solution (PBS) was added to the medium and cultured at 37 °C (Heraeus, Hanau, Germany) for 30 minutes. The reaction mixture (1 mL) was then collected every hour, centrifuged at 5000 x *g* (Eppendorf, Dubai, UAE) for the next 20 hours. Thereafter, 150 µL of disodium *p*-nitrophenyl phosphate substrate buffer was added in the 50 µL supernatant and the mixture incubated in a 40 °C water bath (Grants, Cambridgeshire, UK) for 4 minutes. Absorbance was then measured at 405 nm using Microtiter plate reader (Biochrom, Cambridge, England) while 0.01 M PBS was used as a negative control. The experiment was carried out in triplicate. Thereafter, the Mean and Standard Error of Mean (SEM) were calculated.

### 3.11.6 Electrical conductivity detection

Electrical conductivity of cytoplasmic fluid was determined as previously described by Lee, Choi, & Cho (1998). Here, a 12-hour cultured *E. coli* was dissolved in fresh sterile medium to $10^6$ CFUs/mL. Thereafter, 0.5 mg/mL Peak I antimicrobial peptide 0.01 M PBS was added and cultured at 37 °C (Heraeus, Hanau, Germany) for 20 hours. The culture mixture (1 mL) was then collected every hour, centrifuged at 5000 x *g* (Eppendorf, Dubai, UAE) for the next 20 hours. On an hour interval, the electrical conductivity was detected with SevenGo Duo SG-23 digital conductivity meter (Mettler-Toledo, Columbus, USA). The experiment was carried out in triplicate before the Mean and Standard Error of Mean (SEM) was calculated, taking 0.01 M PBS as a negative control.

### 3.11.7 Peptide synthesis

Of the seven, two promising African catfish antimicrobial peptides (ACAP-IV and ACAP-V) were chemically synthesized by Biomatik Cooperation (Wilmington, USA). The standard solid-phase synthesis process involved a condensation reaction between the carboxylic terminus and amino

terminus to join the amino acid residues through peptide bonds (Marglin and Merrifield, 1970). Unlike in protein biosynthesis, in which the amino terminus is coupled to the carboxylic terminus, here the synthesis is achieved by coupling of the carboxylic group of the incoming amino acid to the amino terminus. The synthesized peptides were then purified by preparative High-Performance Liquid Chromatography (HPLC) using a YMC-Triart $C_{18}$ analytic column (4.6x25mmx5um). The linear gradient used was from solvent A (0.1% Trifluoroaceticacid in 100% Water) to solvent B (0.1% Trifluoroacetic in 100% Acetonitrile). A flow rate of 1.0 ml/min and run-time of 30 minutes. The peptides were detected at 214 nm using UV spectrophotometry, with a purity of 99.01% and 96.48 % for ACAP-IV and ACAP-V, respectively. The molecular masses were confirmed by mass spectrometry. The synthesized peptides were stored at -20 °C and resuspended in distilled, deionized water following the manufacturer's recommendation. The antimicrobial activity of individual peptides was assayed as earlier on described in sub-section 3.8.2.

### 3.12 Data management and analysis

#### 3.12.1 Proteomics data analysis

The *Proteome Discoverer 2.4* software utilizing *Sequest HT* search engine was employed to process the MS/MS data. A machine learning algorithm (Discriminant Analysis) at http://www.camp3.bicnirrh.res.in/ and Deep Learning algorithm, Deep-AmPEP30 server at https://cbbio.online/AxPEP/ were used to determine the antimicrobial scores of individual peptides. Later, Toxicity of antimicrobial peptides was established using the *HLPpred-Fuse* web server at http://thegleelab.org/HLPpred-Fuse/ and the *ToxinPred* tool at https://webs.iiitd.edu.in/raghava/toxinpred/design.php. Thereafter, non-hemolytic antimicrobial peptides were prepared using the protein 3-D structure prediction tools, *PEP-FOLD* v3.5 and the

*I-TASSER* at https://zhanglab.dcmb.med.umich.edu/I-TASSER/. The quality of the predicted structures was evaluated using *ProSA-web* (https://prosa.services.came.sbg.ac.at/prosa.php) and PROCHECK (https://saves.mbi.ucla.edu/). Theraftre, the binding energies and interactions between the antimicrobial peptides and target proteins were determined using AutoDock Vina (http://dinc.kavrakilab.org/), PatchDock (https://bioinfo3-D.cs.tau.ac.il/PatchDock/php.php) and FireDock (https://bioinfo3-D.cs.tau.ac.il/FireDock/php.php) engines. *PyMOL* v1.7.4, *Chimera* v1.15, *BOVIA Discovery Studio* v2021 client softwares were respectively utilized in the inspection, visualization, and exploration of the peptide-target interactions.

### 3.12.2 Statistical data analysis

*Tableau* Software *v2019.4* was used to present data. *GraphPad 5.0* statistical package was used to describe the data. Statistical software, *SPSS v16.0* was used to compare the means, in which a one-sample *t*-test was performed to determine the significance of hemo-toxicity of Peak I antimicrobial peptides on fresh rabbit blood cells. The effect of lake, fish species and their interactions on the antimicrobial activity and yield of the peptides was analysed using two-way ANOVA, followed by Tukey's Multiple Comparison Test set at $p<0.05$.

# CHAPTER FOUR

# RESULTS

## 4.0 Summary of results

The extractive yield was influenced by the source and fish species. The skin mucus extract of African catfish demonstrated a higher antimicrobial activity than that of African lungfish and hence was considered for downstream analysis. From Lake Albert's African catfish skin mucus extract, 7 novel potential African Catfish Antimicrobial Peptides (ACAPs) were identified using LC-MS/MS, five of which were predicted non-hemolytic. Furthermore, these five ACAPs demonstrated both membrane and non-membrane mode of action.

## 4.1 Solid Phase Extraction and extractive yield

By removing the low molecular weight contaminants, solid-phase extraction remains an essential technique for purifying and concentrating samples. Results indicate that extractive yield was influenced by Lake ($F_{2,18}$=119.23, $p<0.05$) and fish species ($F_{1,18}$=16.41, $p<0.05$). Upon, comparing the extractive yield in the same lake among fish species, a significantly higher yield in *P. annectens* (15.67±0.37 %, $p<0.05$) was observed as compared to the *C. griepinus* (13.57±0.37 %). Comparison of the yield of peptides in the same species across lakes revealed a higher yield from Lake Kyoga (20.00±0.45 %) as compared to those from Lake Albert (13.56±0.45) and Lake Victoria (10.33±0.45) (Figure 4.1).

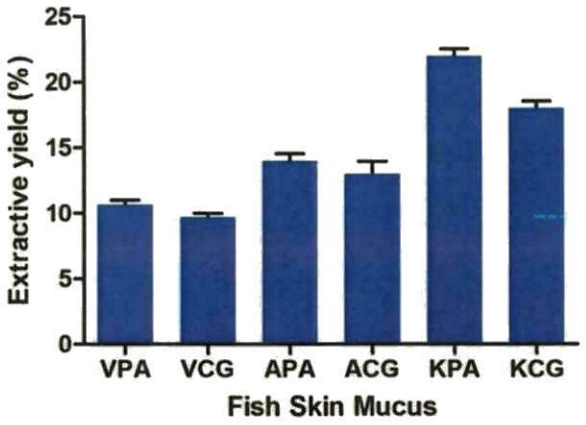



**Figure 4.1: Extractive yield of *P. annectens* and *C. griepinus* from three major lakes of Uganda.** VPA, Victoria *P. annectens*; VCG, Victoria *C. griepinus*; APA, Albert *P. annectens*; ACG-Albert *C. griepinus*; KPA, Kyoga *P. annectens*; KCG, Kyoga *C. griepinus*. Lake Kyoga's *P. annectens* has the highest extractive yield (22.00±0.58 %) while Victoria *C. griepinus* showed to have the least yield (9.67±0.33 %) among all species across the lakes. The image was rendered in *GraphPad 5.0* statistical package.

### 4.2 Objective I: Antimicrobial activity of peptides extracted from skin mucus

#### 4.2.1 Agar well Diffusion assay

The antimicrobial activity of the skin mucus extracted peptides on *E. coli* and *S. aureus* varied by fish species ($F_{2,18}$=7.53, $p<0.05$) and the interaction between the lake and fish species ($F_{2,18}$=14.67, $p<0.05$). Lake had no significant influence on the antimicrobial activity of peptides considering both *E. coli* ($p>0.05$) and *S. aureus* ($p>0.05$). Upon, comparing the antimicrobial activity of peptides in the same lake among fish species, a significantly higher activity was observed in peptides extracted from *C. griepinus* for both *E. coli* (ZOI, 12.0±0.46 mm, $p<0.05$) and *S. aureus* (ZOI, 11.0±0.29 mm, $p<0.05$) as compared to those from *P. annectens* on *E. coli* (ZOI, 9.22±0.29 mm) and *S. aureus* (ZOI, 8.72±0.49 mm) respectively. Here, the largest zone of inhibition

(15.00±0.58 mm) was demonstrated by the extracts from *C. griepinus* (Table 4.1). Both PBS and Acetonitrile did not show any antimicrobial activity (Figure 4.2).

### 4.2.2 Minimum Inhibitory Concentration (MIC) assessment

When subjected for the Minimum Inhibitory Concentration (MIC) assay (Figure 4.3), *C. griepinus* still showed a higher antimicrobial activity (lower MIC) (2.59±0.98 µg/mL, $p<0.05$) compared to *P. annectens* (26.40±14.19 µg/mL). The highest MIC (92.81±0.43 µg/mL) was registered on *S. aureus* by the peptide extracts from the skin mucus of *P. annectens* sourced from L. Albert (Table 4.2) yet extracts of *C. griepinus* demonstrated the lowest MIC (highest antimicrobial activity) across the studied bacterial strains (MIC: 0.71±0.33 µg/mL and 2.84±0.11 µg/mL on *E. coli* and *S. aureus* respectively) and, therefore, selected for the downstream analysis.

**Table 4.1: Zone of inhibition of SPE peptides from two fish species of different lakes**

| Lake | Fish species | Zone of Inhibition (mm) | |
|---|---|---|---|
| | | ***E. coli (p=0.007)*** | ***S. aureus (p=0.438)*** |
| Kyoga | *Clarias griepinus* | $9.00\pm0.58^{c}$ | $11.67\pm0.67^{a}$ |
| | *Protopterus annectens* | $12.00\pm0.58^{a,b}$ | $11.33\pm0.33^{a}$ |
| Victoria | *Clarias griepinus* | $13.00\pm1.53^{c}$ | $10.67\pm0.67^{a}$ |
| | *Protopterus annectens* | $10.0\pm0.58^{b,c}$ | $8.33\pm0.33^{a}$ |
| Albert | *Clarias griepinus* | $15.00\pm0.58^{a}$ | $8.67\pm0.33^{a}$ |
| | *Protopterus annectens* | $9.67\pm0.33^{c}$ | $9.00\pm0.58^{a}$ |

Data are expressed as Mean ± Standard Error of Mean; Means that do not share a letter are significantly different

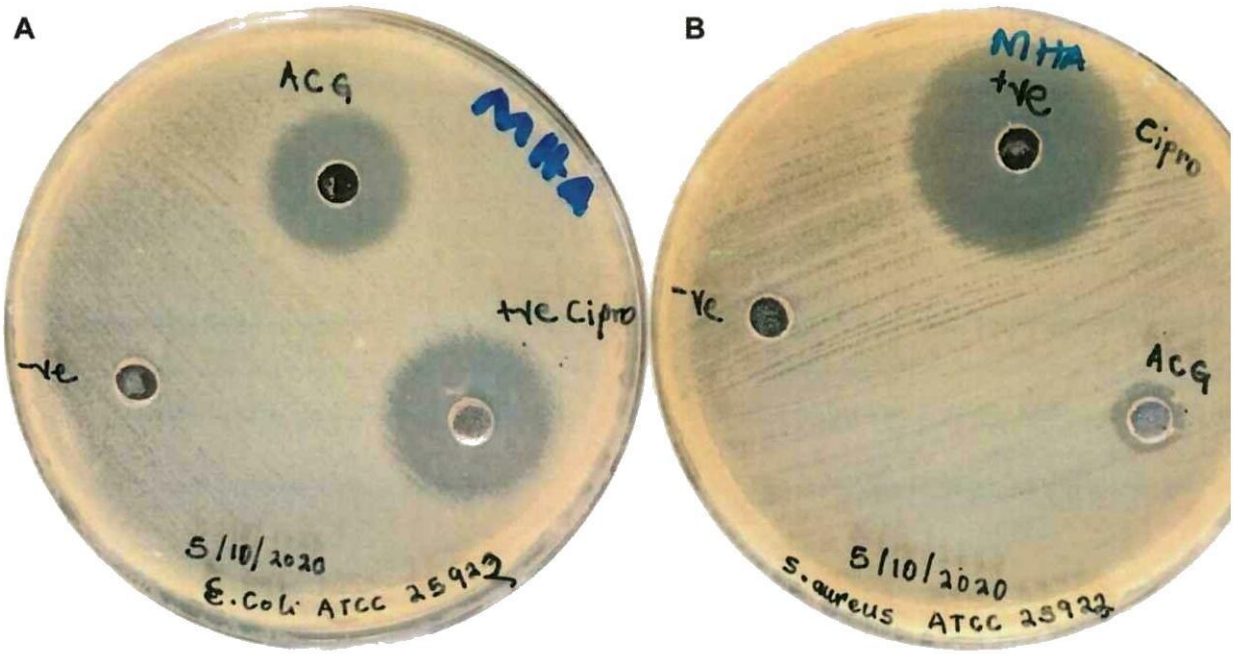



**Figure 4.2: Selected zone of Inhibition for skin mucus SPE peptide of *C. griepinus* from Lake Albert. (A)** *E.coli* zone of inhibition. **(B)** *S. aureus* zone of inhibition. **ACG,** Albert *C. griepinus; +ve cipro-* positive control containing Ciprofloxacin (Medicon, Vadodara, India)**; -ve** negative control containing 0.01 M PBS (Plate A) and 70 % Acetonitrile (Plate B). *E. coli* was more susceptible to Albert *C. gripinus* peptides (ZOI; 15.00±0.58) compared to *S. aureus* (ZOI; 8.67±0.33). Bacterial strains were cultured on Mueller-Hinton Agar (MHA).

**Table 4.2: Minimum Inhibitory Concentration on *E. coli* and *S. aureus***

| Lake | Fish species | MIC (µg/mL) | |
|---|---|---|---|
| | | ***E. coli (p<0.001)*** | ***S. aureus (p<0.001)*** |
| Kyoga | *Clarias griepinus* | $1.46\pm0.08^{c}$ | $2.92\pm0.04^{d}$ |
| | *Protopterus annectens* | $19.57\pm0.03^{a}$ | $19.57\pm0.03^{c}$ |
| Victoria | *Clarias griepinus* | $0.55\pm0.02^{c}$ | $7.04\pm0.13^{d}$ |
| | *Protopterus annectens* | $8.40\pm0.01^{b}$ | $33.79\pm0.33^{b}$ |
| Albert | *Clarias griepinus* | $0.71\pm0.33^{c}$ | $2.84\pm0.11^{d}$ |
| | *Protopterus annectens* | $1.45\pm0.07^{c}$ | $92.81\pm0.43^{a}$ |

Data are expressed as Mean ± Standard Error of Mean; Means that do not share a letter are significantly different

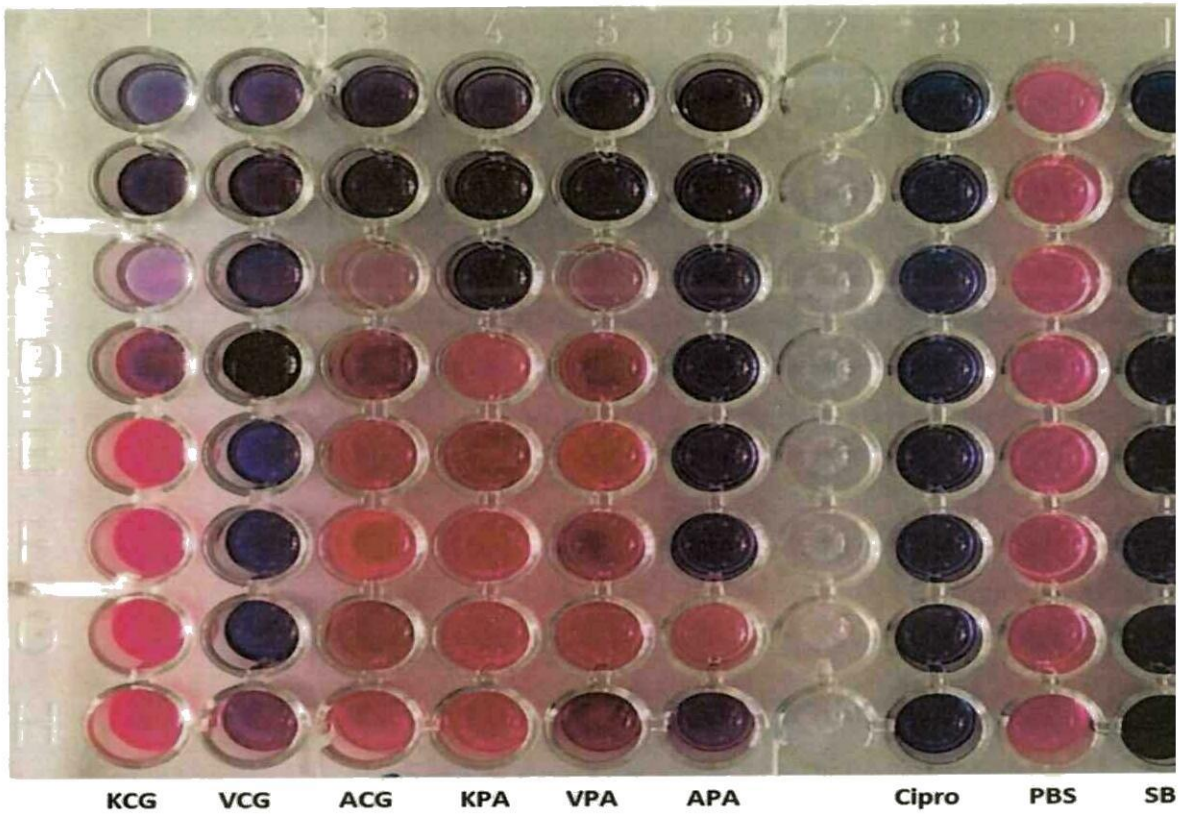



**Figure 4.3: MIC plate of skin mucus SPE peptides against *E. coli*.** VPA, Victoria *P. annectens*; VCG, Victoria *C. griepinus*; APA, Albert *P. annectens*; ACG-Albert *C. griepinus*; KPA, Kyoga *P. annectens*; KCG, Kyoga *C. griepinus; cipro-* positive control containing Ciprofloxacin (Medicon, Vadodara, India); PBS, negative control containing 0.01 M PBS. VCG demonstrated the lowest MIC aganist *E. coli*. Bacterial strains were cultured on Brain Heart Infusion Broth (BHIB).

### 4.3 Objective II: Antimicrobial peptides identification and characterizaion

#### 4.3.1 Sephadex G-25 gel filtration purification of antimicrobial peptides

Lake Albert's SPE isolated peptides of *C. griepinus* were further purified using Sephadex G-25 gel filtration chromatography to produce two peaks (Peak I and Peak II) (Figure 4.4). Up to 40 fractions (3 mL) were collected into fraction Pyrex fraction collectors (Figure 4.5). Across all fractions, maximum absorbance was observed at 280 nm further confirming the presence of the

extracted peptides. The purest fraction (Purity ratio: 0.68) was observed in fraction number 13, with maximum absorbance at 280 nm (Figure 4.6). The antimicrobial activity assessment revealed that, Peak I fraction demonstrated high antimicrobial activity with a MIC of 0.31±0.16 µg/mL and 1.99±0.13 µg/mL on *E. coli* and *S. aureus* respectively, whereas no antimicrobial activity was detected in peak II (Table 4.3). Peak I was broad in nature as attributed to presence of many peptide components.

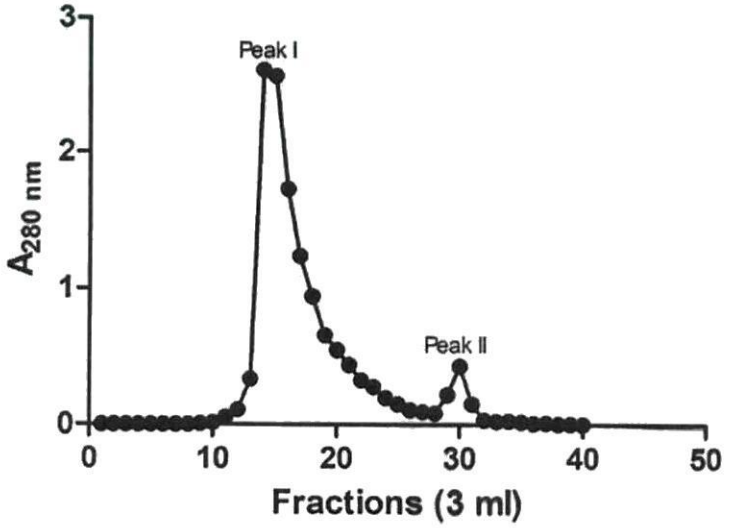



**Figure 4.4: Chromatogram for fractions of gel filtration chromatography.** Sephadex G-25 chromatographic resin was used, and two prominent peaks (peaks I & II) were observed. Absorbance was measured at 280 nm. The image was rendered in *GraphPad 5.0* statistical package.

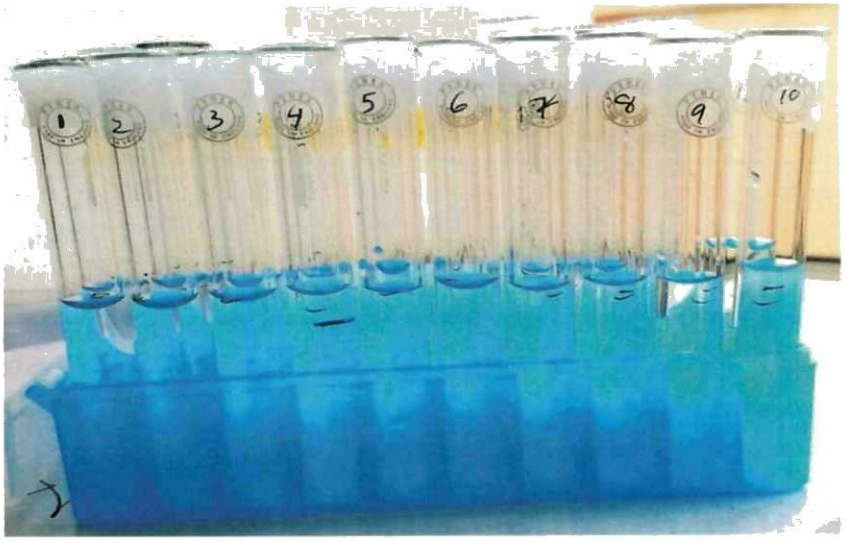



**Figure 4.5: Sephadex G-25 fractions of from manual column during size exclusion chromatography.** The peptides were eluted using deionized water, pH 7.01 at 9 minutes intervals. *NanoDrop* spectrophotometer was used to measure absorbance at 280 nm.

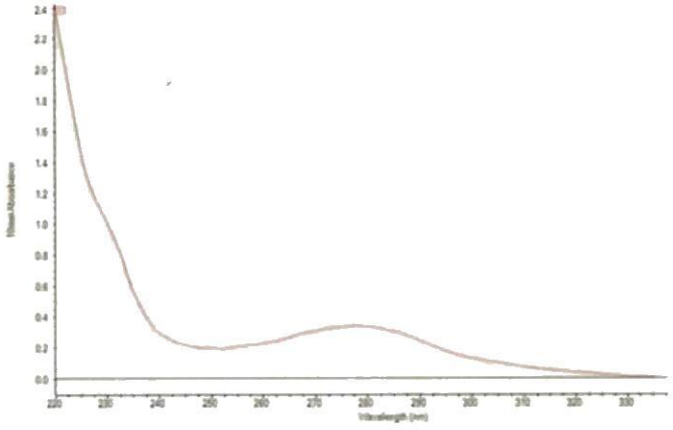


**Figure 4.6: Fraction number 13 Absorption spectrum from manual column of size exclusion chromatography.** Being the purest peptide fraction, maximum absorbance was observed at 280 nm. Purity ratio (A260/A280) was 0.68. Maximum absorption of nuclic acids occurs at 260 nm, and lower A260/A280 ratio implies least or no nucleic acid contaminants in the fractons. Sephadex G-25 was used as the chromatographic resin.

**Table 4.3: Minimum Inhibitory Concentration of Sephadex G-25 peaks on *E. coli* and *S. aureus***

| Run | MIC (µg/mL) | |
|---|---|---|
| | ***E. coli*** | ***S. aureus*** |
| Peak I | 0.31±0.16 | 1.99±0.13 |
| Peak II | ND | ND |
| Ciprofloaxacin | 0.50±0.02 | 0.61±0.31 |

Data are expressed as Mean ± Standard Error of Mean; ND-Not detected

### 4.3.2 Analysis of purified antimicrobial peptides

Purity of the eluted AMPs were analysed by SDS-PAGE. The SDS-PAGE peptide profiles of the peak I obtained from Sephadex G-25 purified peptides from the skin mucus of African catfish sourced from Lake Albert are shown in Figure 4.7. Low molecular weight peptides (below 5 kDa) were observed as a clear band for the Sephadex G-25 purified peptides extract unlike in the case of the $C_{18}$ SPE peptides.

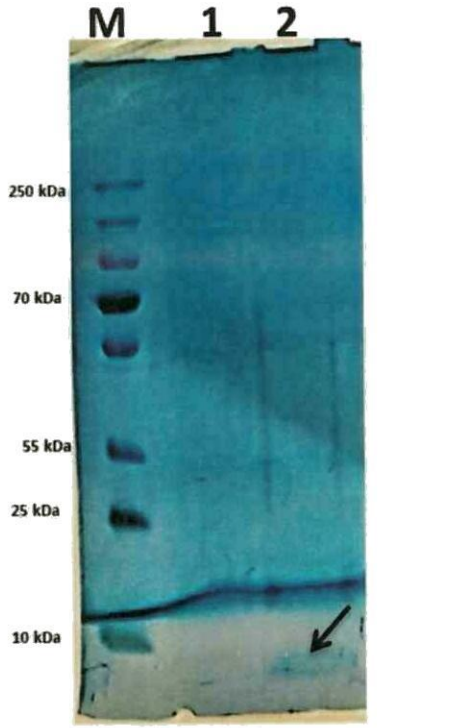



**Figure 4.7: SDS-PAGE (15%) of peptides extracted from African catfish, *C. gariepinus*.** SDS-PAGE molecular marker lane (M); $C_{18}$ SPE partially purified peptides (1); Sephadex G-25 peak I peptides (2). The purified peptides were below 5 kDa.

### 4.3.3 LC-MS/MS peptide identification

The Peak I fraction from gel filtration chromatography was subjected to LC-MS/MS at positive mode to uncover the molecular mass and amino acid sequences. A correlation of tandem mass spectral data of peptides with amino acid sequences in the compiled Catfish database using *Sequest HT*, positively identified 214 peptides in 126 proteins. Of the 214 peptides, some were confirmed using *in silico* tools as antimicrobial while the majority were non-antimicrobial. First, a

Discriminant Analysis machine learning revealed up to 40 of these peptides had antimicrobial probability score >0.5 (Table 4.4), and hence were classified as antimicrobial peptides (AMPs), leaving 174 peptides predicted as non-antimicrobial. The average antimicrobial score for the 40 AMPs was 0.797 with 12 peptides having higher antimicrobial scores (>0.9). Upon cross-validating, the 12 peptides using a more recently tuned rigorous Deep Learning approach, 7 peptides stood out with an average antimicrobial score 0.749 (Table 4.5). Their antimicrobial score ranged from 0.561 to 0.905, with ACAP-I (amino acid residues: AALKKALTAGGY), from H15 domain-containing protein (Figure 4.8), demonstrating the highest antimicrobial score of 0.905 units.

**Table 4.4: LC-MS/MS identified Peak I Antimicrobial peptides utilizing Discriminant Analysis machine learning algorithm**

| S/No | Seq. Processor. | Sequences | Antimicrobial score |
|---|---|---|---|
| 1 | A0A7J5ZUT8_2 | GAGGGFGAGGGFGSGGGFGGGFG | 0.997 |
| 2 | A0A7J5ZUT8_1 | GAGGGFGAGGGFGSGGGFG | 0.992 |
| 3 | A0A5N5LC08 | KVSKVLHKAIL | 0.975 |
| 4 | A0A7J6ATJ1_1 | AALKKALAAGGY | 0.963 |
| 5 | A0A7J6ALK6_3 | FGGAGVGKTVL | 0.954 |
| 6 | A0A2D0RRS1 | AGGGAGGGGGFGFGGGF | 0.951 |
| 7 | A0A7J6AHG0 | IAIIPSKKLRNKIAG | 0.936 |
| 8 | A0A7J6AA08_1 | VEDFKKKYEDEINKRAAAENE | 0.929 |
| 9 | A0A556UXS0 | VVLGSGGVGKSAL | 0.920 |
| 10 | A0A5N5KSD9 | GVASAPASGTGGFSFG | 0.917 |
| 11 | A0A7J6BGG9 | AALKKALTAGGY | 0.911 |
| 12 | A0A5N5PKX8 | INLRIEHEVAGGIAGLRAAMEALVV | 0.908 |
| 13 | A0A2D0RAQ3 | GAGGAGLALGGGGGAGAL | 0.889 |
| 14 | A0A5N5JEQ1_1 | VVINPYKNLPIY | 0.884 |
| 15 | A0A556TWZ9 | LGSGGLGLGLGSGGL | 0.875 |
| 16 | A0A5N5NK88 | IKQIPRILGPGLN | 0.832 |
| 17 | S4V1J6_1 | IINMKPPAISQ | 0.826 |
| 18 | A0A7J6AA08_3 | VEDFKKKYEDEINKRAAAENEF | 0.824 |
| 19 | A0A2D0S7F3_2 | ILDNGANGKIFV | 0.814 |
| 20 | A0A7J5ZJV7 | LAAGGIYHPRL | 0.796 |
| 21 | A0A5N5PZE3 | AGGGAGGGGGGGGGGGGGGGGGGL | 0.778 |
| 22 | A0A556U3B9_1 | FTNHNGTGGKSIYGNKF | 0.772 |
| 23 | A0A556V9V8 | VVINQKGIDPFSL | 0.761 |
| 24 | A0A5N5PXY6 | GGVGGGGGGGGGGGGGGSGGGTI | 0.759 |
| 25 | A0A556V121 | AAIEPSRGRPAGLGDALVPAAAGA | 0.754 |
| 26 | A0A2D0RPR7 | LAGRSGGAGTAGGGGAI | 0.752 |
| 27 | A0A5N5NJW0_2 | IFHSFGGGTGSGF | 0.750 |
| 28 | A0A7J5ZSW9_2 | IFHSFGGGTGSGF | 0.750 |
| 29 | W5UM08 | AILGGAKVKDKIQL | 0.702 |
| 30 | A0A556UYJ6_4 | IKVDKGVVPLAGTNG | 0.691 |
| 31 | Q90YT2_1 | LKVSKDKRALKF | 0.684 |
| 32 | A0A2D0T815 | VGGGGGGGGSGGGGFPGNGGGS | 0.682 |
| 33 | W5UKQ5 | LPGSSSSGFAGSGF | 0.671 |
| 34 | A0A2D0S7F3_1 | ILDNGANGKIF | 0.668 |
| 35 | A0A2D0SXG7 | KSVGGGGGGGVGGGGGGGGA | 0.656 |
| 36 | A0A2D0QLM1_1 | VYKVLKQVHPDTGISSKA | 0.621 |
| 37 | A0A5N5LUK7_1 | VFHSFGGGTGSGF | 0.618 |
| 38 | A0A5N5KZN5 | GKTAGWNIPIGTL | 0.591 |
| 39 | A0A5N5PRH7 | FKTQFGGGKTTGF | 0.577 |
| 40 | A0A5N5LBM3 | IGGISGGGATSRL | 0.543 |

S/No, Serial number; Seq., Sequence

**Table 4.5: Physiochemical properties of African Catfish Antimicrobial Peptides (ACAPs)**

| Seq. ID | Sequences | L | C | MW | α-helix (%) | H (%) | AS | Processor Protein |
|---|---|---|---|---|---|---|---|---|
| ACAP-I | AALKKALTAGGY | 12 | 2.0 | 1163.68 | 58.30 | 75.00 | 0.905 | H15 domain-containing protei |
| ACAP-II | AALKKALAAGGY | 12 | 2.0 | 1133.67 | 66.67 | 83.33 | 0.898 | H15 domain-containing protei |
| ACAP-III | GVASAPASGTGGFSFG | 16 | 0.0 | 1369.64 | 0.00 | 75.00 | 0.768 | Uncharacterized |
| ACAP-IV | KVSKVLHKAIL | 11 | 3.5 | 1235.82 | 45.45 | 54.54 | 0.754 | SERPIN domain-containing p |
| ACAP-V | VVLGSGGVGKSAL | 13 | 1.0 | 1143.67 | 0.00 | 76.92 | 0.684 | Small monomeric GTPase |
| ACAP-VI | FGGAGVGKTVL | 11 | 1.0 | 1005.57 | 0.00 | 81.81 | 0.677 | ATP synthase subunit beta |
| ACAP-VII | IAIIPSKKLRNKIAG | 15 | 4.0 | 1622.05 | 46.67 | 60.00 | 0.561 | 40S ribosomal protein |

L, peptide length; C, Peptide Charge; MW, molecular weight; H, hydrophobicity; and AS, antimicrobial score

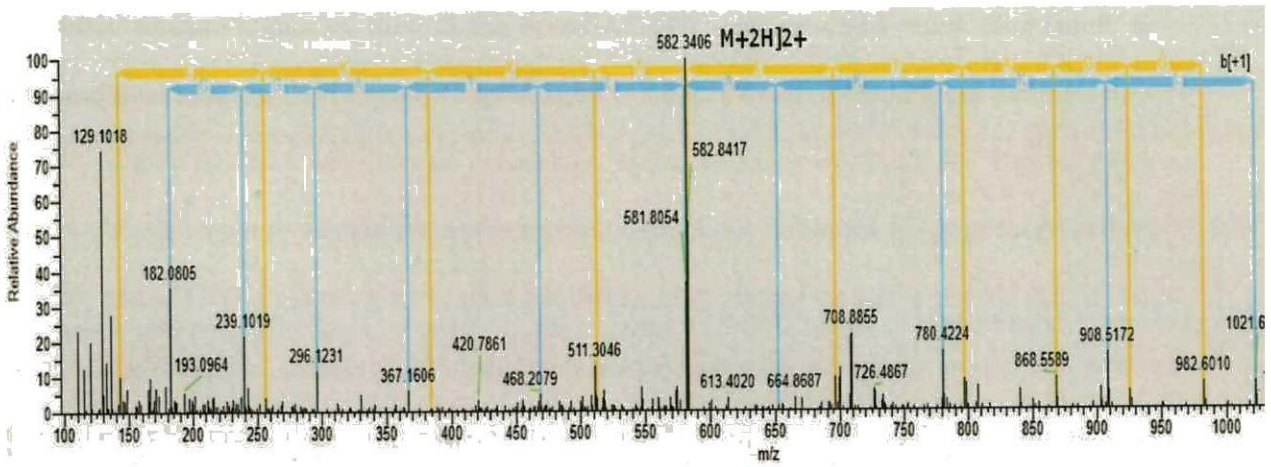



**Figure 4.8: Mass Spectrum (MS/MS) of the antimicrobial peptide (ACAP-I), from *Clarias gariepinus*.** Representative $MS^2$ data was obtained from a +2 parent ion with *m/z* 582.340 by HCD fragmentation and Orbitrap detection at 30.000 resolutions. The *y*- and *b*- series of ions allows identification of the peptide AALKKALTAGGY from protein with accession number A0A7J6BGG9.

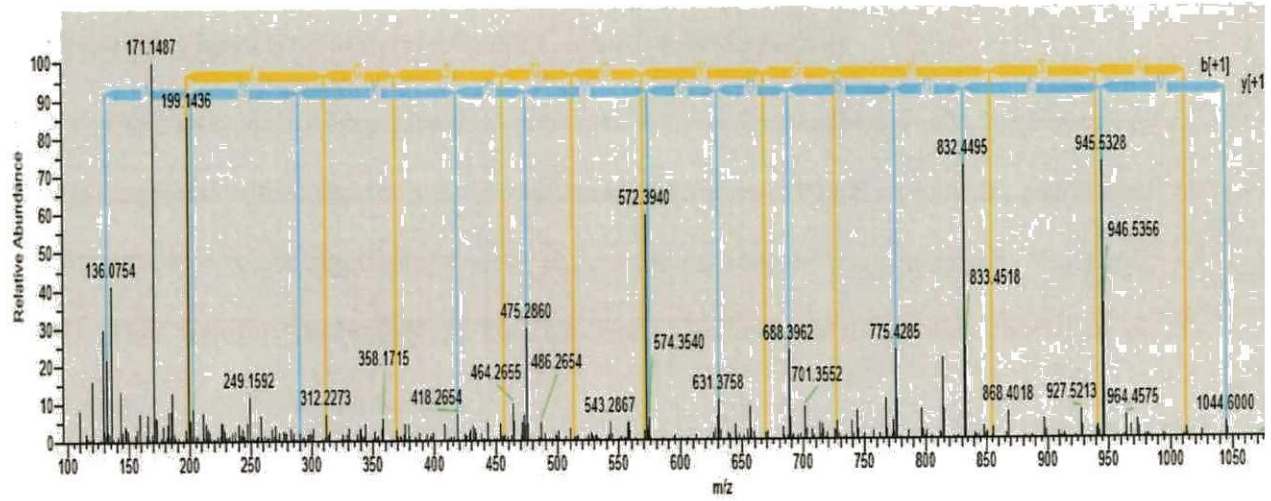



**Figure 4.9: Mass Spectrum (MS/MS) of the antimicrobial peptide (ACAP-V), from *Clarias gariepinus*.** Representative $MS^2$ data obtained from a +2 parent ion with m/z 572.338 by HCD fragmentation and Orbitrap detection at 30.000 resolutions. The *y*- and *b*- series of ions allows identification of the peptide VVLGSGGVGKSAL from protein with accession number A0A556UXS0.

### 4.3.4 Peptide sequence characterization

An *in silico* analysis indicated that all the seven ACAPs were generally short. The length as determined by the LC-MS/MS ranged 11 to 16 amino acid residues. All the peptides demonstrated high hydrophobicity; with an average percentage hydrophobicity of 72.37 %. The ACAP-II demonstrated the highest percentage hydrophobicity (83.33%) followed by peptide ACAP-VI (81.81%), with ACAP-IV having the lowest (54.54%). With exception to the neutral ACAP-III, the rest of the identified antimicrobial peptides were cationic with charge in the range of 1 to 4 (Table 4.5). Up to four of these antimicrobial peptides (ACAP-I, ACAP-II, ACAP-IV and ACAP-VII), demonstrated α-helix structure conformation. This implies they can possibly be amphipathic. But ACAP-V (Figure 4.9) and ACAP-VI did not show any α-helix structure conformation. The molecular weights of the identified antimicrobial peptides were as well low (1005.57-1622.05 Da).

## 4.4 Objective III: Toxicity profiles of antimicrobial peptides

### 4.4.1 The *in vitro* hemolytic activity of peak I antimicrobial peptides

When the peak I antimicrobial peptides were incubated with fresh rabbit blood cells, the percentage hemolysis increased with increase in their concentration (Figure 4.10). However, the increase in hemolytic activity was not significant even at the maximum assayed concentration (100 µg/mL, $p=0.447$). This signifies the safety of the fish-derived antimicrobial peptides (up to tested concentrations) on the Rabbit blood cells.

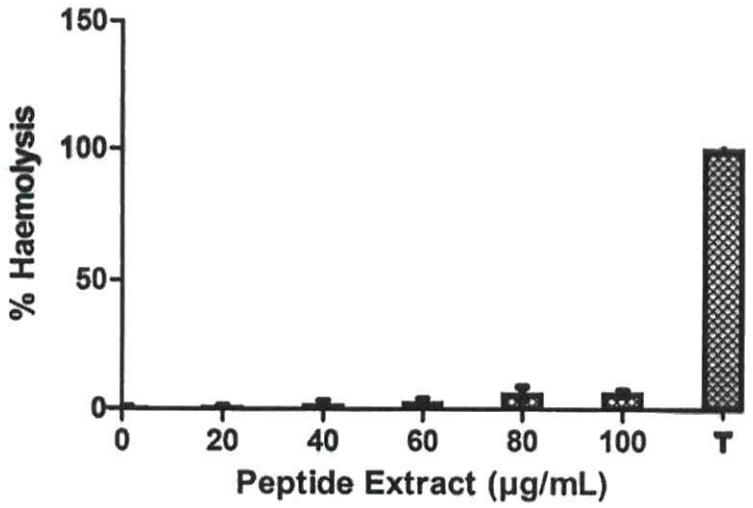



**Figure 4.10: Percentage hemolysis of Rabbit blood cells by Sephadex G-25 peak I peptides.** T-1% Triton X-100. For the concentration of 1-100 µg/mL, there was no significant increase in the percentage hemolysis of the Rabbit blood cells (*p=0.447*). The data are expressed as Mean ± SEM. The image was rendered in *GraphPad 5.0* statistical package.

### 4.4.2 The *in silico* hemolytic profiles

In the present study, toxicity of individual ACAPs as the chief cause of drug discovery failure (Conlon et al., 2014), was further cross-evaluated using *HLPpred-Fuse* tools. With exception to ACAP-II and ACAP-IV, the rest of the ACAPs had HLP Probability less than 0.5, and hence categorized non-hemolytic (Table 4.6). Of the two hemolytic antimicrobial peptides, ACAP-II had a higher Hemolytic activity level (High Hemolytic activity Probability = 0.5125) compared to ACAP-IV (High Hemolytic activity Probability = 0.3521). Upon, subjecting the five non-hemolytic ACAPs to a cross-validating Toxicity prediction tool; *ToxinPred* under stringent Machine Learning algorithm *viz.* 0.0000 SVM-SwissProt thresholds and 0.0001 error-value cut-

off and the five ACAPs were re-suggested as non-toxic (Table 4.7). Overall ACAP-III demonstrated the least SVM score (-1.52), followed by ACAP-V (-1.17).

**Table 4.6: The *HLPpred-Fuse* predicted hemolytic profiles of the ACAPs**

| Peptide Id | HLP Probability | HLP status | High Hemolytic activity Probability | Hemolytic activity level |
|---|---|---|---|---|
| ACAP-III | 0.0201 | Non-HLP | - | - |
| ACAP-I | 0.1839 | Non-HLP | - | - |
| ACAP-V | 0.2151 | Non-HLP | - | - |
| ACAP-VI | 0.4813 | Non-HLP | - | - |
| ACAP-VII | 0.4818 | Non-HLP | - | - |
| ACAP-II | 0.5576 | HLP | 0.5125 | High |
| ACAP-IV | 0.9349 | HLP | 0.3521 | Low |

ACAP, African Catfish Antimicrobial Peptides; HLP, Hemolytic Peptide; Id, Identity

**Table 4.7: The *ToxinPred* determined toxin profiles of the ACAPs**

| Peptide Id | SVM score | Toxin status |
|---|---|---|
| ACAP-III | -1.52 | Non-Toxin |
| ACAP-V | -1.17 | Non-Toxin |
| ACAP-VI | -1.15 | Non-Toxin |
| ACAP-I | -1.10 | Non-Toxin |
| ACAP-VII | -0.87 | Non-Toxin |

ACAP, African Catfish Antimicrobial Peptides; Id, Identity; SVM, Support Vector Machines algorithm

## 4.5 Objective IV: Mode of action of African Catfish Antimicrobial Peptides

### 4.5.1 Ligand preparation

The I-TASSER predictions produced between 1 to 5 models across different antimicrobial peptides: with ACAP-I and ACAP-VII yielding only one and two models, respectively. The rest of the antimicrobial peptides (ACAP-III, ACAP-V and ACAP-VI) yielded all the five models. Guided by the *c*-scores, models were selected for downstream analysis, with all top scoring models across the five non-hemolytic antimicrobial peptides having the highest *c*-score ranking and were thus, considered the best I-TASSER models. On the other hand, maximum numbers of models (five) were observed across all the PEP-FOLD modeled 3-D structures, with exceptions to ACAP-

I which had only two model outputs (Table 4.8). But just like in I-TASSER predictions, all the PEP-FOLD top scoring model across the seven peptides yielded lowest sOPEP energy with high Apollo predicted melting temperature ($t_m$), and were, therefore, considered the best models for downstream analysis.

Upon subjecting the I-TASSER and PEP-FOLD best models to structure quality checks, PEP-FOLD models demonstrated a better quality over I-TASSER's and hence, were considered for downstream analysis. Here, a Ramachandran plot demonstrated that; higher percentage of the residues in PEP-FOLD modeled structures was in the most favoured region. Even those with residues in additional allowed regions (ACAP-III and ACAP-VII), had fewer residues in this region (10.0 and 16.7 % respectively), when compared to I-TASSER modeled (50.0 and 33.3 %). Unlike in the I-TASSER modeled ACAP-III (Figure 4.11), with 10% of its residues in the disallowed region and I-TASSER modeled ACAP-VI (Figure 4.12), with 20% of its residues in the generously allowed region, none of the PEP-FOLD models had residues in the disallowed nor generously allowed region (Table 4.9). On the other hand, ProSA demonstrated analogous scores for I-TASSER and PEP-FOLD models, leaving PROCHECK as a better structure evaluation tool with, PEP-FOLD modeled ACAP-I (Figure 4.13), emerging as the 3-D structure of best quality.

**Table 4.8: Top output models with respective scores as predicted by I-TASSER and PEP-FOLD**

| Peptide Id | I-TASSER Output model *c*-score | | | | | PEP-FOLD Output model sOPEP and tm scores | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Model1 | Model2 | Model3 | Model4 | Model5 | Model1 | | Model2 | | Model3 | | Model4 | | Model5 | |
| | | | | | | sOPEP | tm | sOPEP | tm | sOPEP | tm | sOPEP | tm | sOPEP | tm |
| ACAP-I | -0.53 | - | - | - | - | -17.69 | 0.879 | -17.08 | 0.821 | - | - | - | - | - | - |
| ACAP-III | -1.34 | -1.38 | -1.92 | -3.33 | -5.00 | -11.53 | 0.228 | -9.31 | 0.286 | -9.10 | 0.268 | -9.00 | 0.298 | -8.52 | 0.291 |
| ACAP-V | -0.96 | -3.33 | -1.37 | -2.64 | -3.04 | -8.29 | 0.379 | -8.24 | 0.323 | -7.99 | 0.353 | -7.98 | 0.289 | -7.94 | 0.359 |
| ACAP-VI | -0.86 | -2.77 | -1.66 | -1.46 | -2.85 | -7.98 | 0.400 | -7.02 | 0.391 | -6.81 | 0.414 | -6.75 | 0.373 | -6.71 | 0.411 |
| ACAP-VII | -0.07 | -2.61 | - | - | - | -17.30 | 0.522 | -17.09 | 0.556 | -17.01 | 0.585 | -16.90 | 0.580 | -16.74 | 0.631 |

ACAP, African Catfish Antimicrobial Peptides; tm, Apollo melting temperature; *c*-score, confidence score

**Table 4.9: Evaluation scores for the predicted 3-D structures of African catfish antimicrobial peptides**

| | Parameters | Models | ACAP-I | ACAP-III | ACAP-V | ACAP-VI | ACAP |
|---|---|---|---|---|---|---|---|
| HECK | Residues in most favoured regions (%) | I-TASSER | 100.0 | 40.0 | 71.5 | 40.0 | 66.7 |
| | | PEP-FOLD | 100.0 | 90.0* | 100.0 | 40 | 83.3* |
| | Residues in additional allowed regions (%) | I-TASSER | 0.0 | 50.0 | 28.5 | 40.0 | 33.3 |
| | | PEP-FOLD | 0.0 | 10.0^ | 0.0 | 60.0 | 16.7^ |
| | Residues in generously allowed regions (%) | I-TASSER | 0.0 | 0.0 | 0.0 | 20.0 | 0.0 |
| | | PEP-FOLD | 0.0 | 0.0 | 0.0 | 0.0[a] | 0.0 |
| | Residues in disallowed regions (%) | I-TASSER | 0.0 | 10.0 | 0.0 | 0.0 | 0.0 |
| | | PEP-FOLD | 0.0[a] | 0.0[a] | 0.0 | 0.0[a] | 0.0[a] |
| | z-score | I-TASSER | -1.87 | 0.27 | 0.59 | 1.71 | -0.54 |
| | | PEP-FOLD | -1.85 | -0.67 | 0.28 | 0.86 | -0.13 |

ACAP, African Catfish Antimicrobial Peptides; *Higher percentage of residues; ^Lower percentage of residues; [a]None of the residues

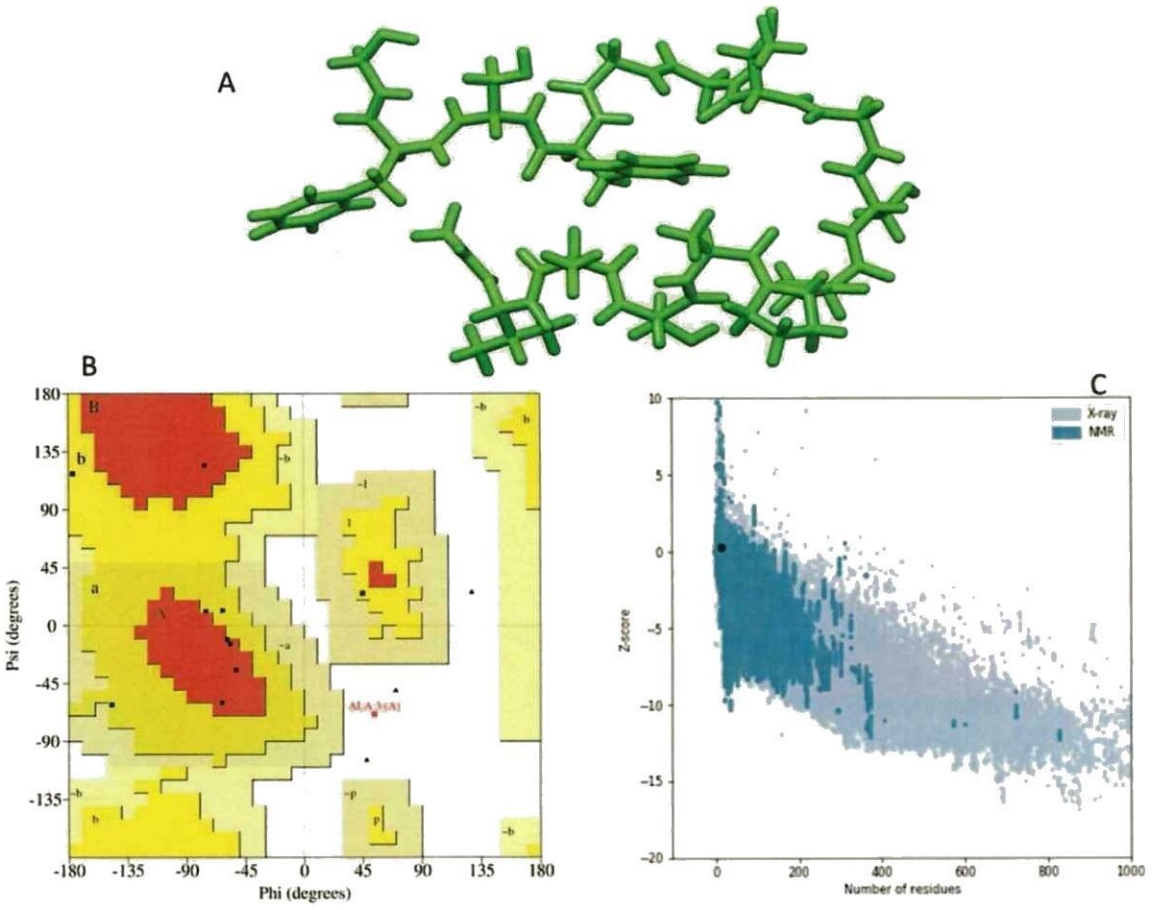



**Figure 4.11: I-TASSER-modelled peptide 3-D structures of ACAP-III together with respective Ramchndran and ProSA validation plots. (A)** I-TASSER-modelled ACAP-III stick format. **(B)** I-TASSER-modelled ACAP-III Ramachandran plot. **(C)** I-TASSER-modelled ACAP-III ProSA *z*-score. The I-TASSER-modelled ACAP-III peptide had 40.0% of non-Gly and non-Pro residues in the most favoured region, 50% in additional allowed region and 10% (Ala A:3) in the disallowed region. None of the ACAP-III residues were in the generously allowed region. The produced *z*-score of ACAP-III is 0.27.

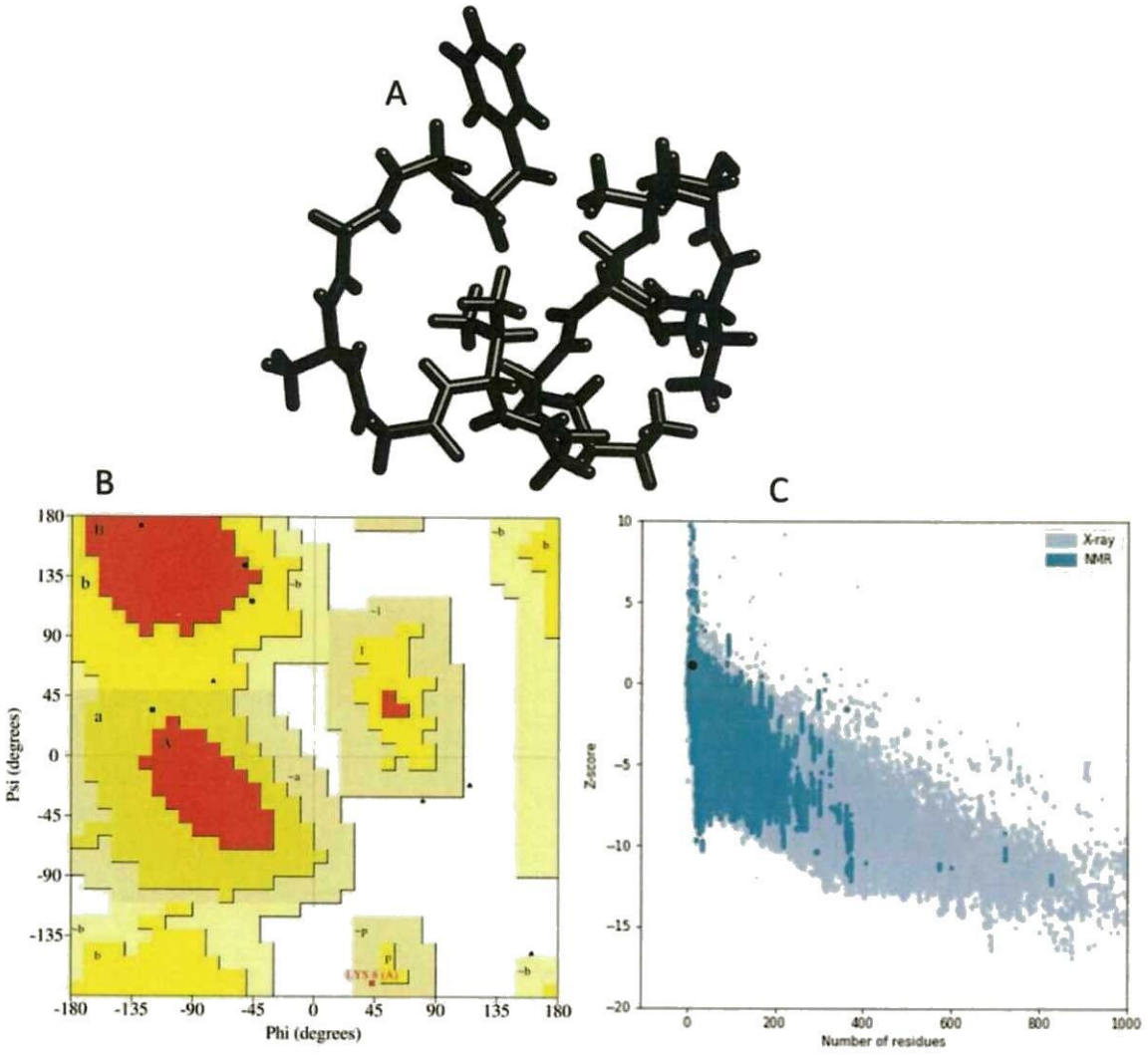



**Figure 4.12: I-TASSER-modelled peptide 3-D structures of ACAP-VI together with respective Ramachandran and ProSA validation plots.** (**A**) Stick format of I-TASSER-modelled ACAP-VI. (**B**) I-TASSER-modelled ACAP-VI Ramachandran plot. (**C**) I-TASSER-modelled ACAP-VI ProSA *z*-score. The I-TASSER-modelled ACAP-VI peptide had 40.0 % of residues in the most favoured region, 40 % in additional allowed region and 20 % in the generously allowed region. The produced *z*-score of I-TASSER modelled ACAP-VI was 1.71.

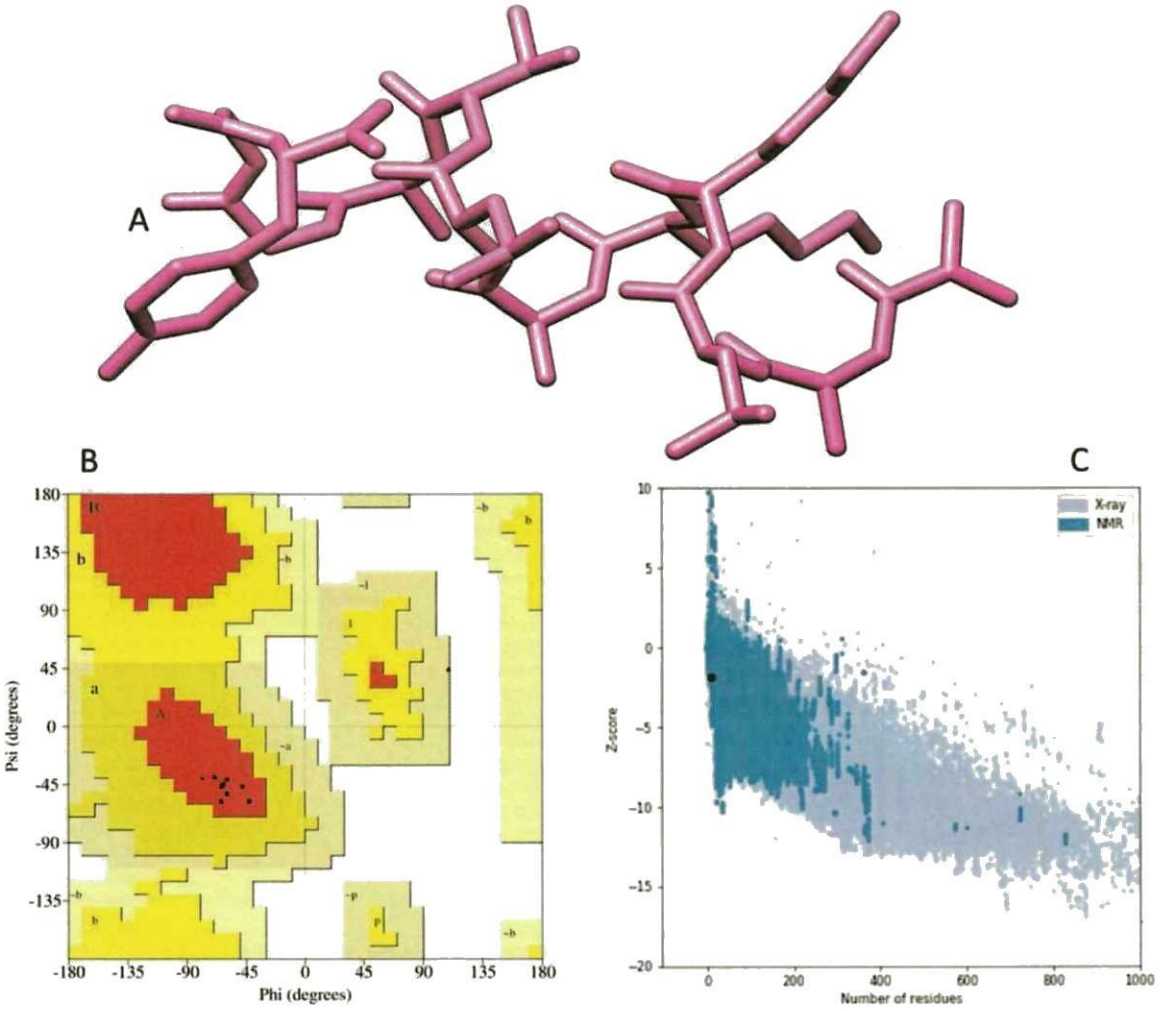



**Figure 4.13: PEP-FOLD modelled peptide 3-D structures of ACAP-I together with respective Ramchndran and ProSA validation plots.** **(A)** PEP-FOLD modelled ACAP-I stick format. **(B)** PEP-FOLD-modelled ACAP-I Ramachandran plot. **(C)** PEP-FOLD-modelled ACAP-I ProSA *z*-score. PEP-FOLD-modelled ACAP-I peptide had all (100%) amino acid residues in the most favoured region with a z-scores of -1.85.

### 4.5.2 Target Selection and Preparation

A total of 20 drug targets were fished from the DrugBank database (Table 4.10). Cholesterol oxidase (PDB Id: 2gew), demonstrated the highest target *z*-score (95.0%), followed by 94.6 % for Neocarzinostatin (PDB Id: 1noa). Majority of the receptor proteins (25.0 %) were of *Escherichia coli* origin followed by *Mycobacterium tuberculosis* (20.0%) and *Bacillus subtilis* (15.0 %). All the retrieved 3-D structures were previously elucidated by X-Ray crystallography.

**Table 4.10: Target proteins utilized in the docking exercise**

| Ligands | Target Protein name | Classification | PDB Id | Identity (%) | z-score | Organism |
|---|---|---|---|---|---|---|
| ACAP-I | Cholesterol oxidase | Oxidoreductase | 2gew | 58.3 | 95.0 | *Streptomyces sp.* |
| | Urease subunit alpha | Hydrolase | 1kra | 63.6 | 88.4 | *Klebsiella aerogenes* |
| | Catalase-peroxidase | Oxidoreductase | 1sj2 | 50.0 | 82.8 | *Mycobacterium tuberculosis* |
| | Pyridoxine/pyridoxamine 5'-phosphate oxidase | Oxidoreductase | 1jnw | 45.5 | 82.1 | *Escherichia coli* |
| | Renalase | Oxidoreductase | 3kkj | 62.5 | 78.2 | *Pseudomonas syringae* |
| ACAP-III | Isopentenyl-diphosphate delta-isomerase | Isomerase | 1p0k | 40.0 | 80.0 | *Bacillus subtilis* |
| | Enoyl-[acyl-carrier-protein] reductase | Oxidoreductase | 4fs3 | 38.5 | 78.7 | *Staphylococcus aureus* |
| ACAP-V | Flavohemoprotein | Lipid binding protein | 3ozw | 53.8 | 89.1 | *Cupriavidus necator* |
| | Thioredoxin reductase | Oxidoreductase | 1f6m | 50.0 | 84.4 | *Escherichia coli* |
| | NADH peroxidase | Oxidoreductase | 1nhp | 58.3 | 84.1 | *Enterococcus faecalis* |
| | Fumarate reductase flavoprotein | Oxidoreductase | 1kf6 | 50.0 | 79.5 | *Escherichia coli* |
| | Monomeric sarcosine oxidase | Oxidoreductase | 2gb0 | 41.7 | 79.0 | *Bacillus sp.* |
| | Dihydrolipoyl dehydrogenase | Oxidoreductase | 1ojt | 66.7 | 77.5 | *Neisseria meningitidis* |
| ACAP-VI | Morphinone reductase | Oxidoreductase | 1gwj | 66.7 | 83.7 | *Pseudomonas putida* |
| | Pyruvate oxidase | Oxidoreductase | 1pow | 63.6 | 79.7 | *Lactobacillus plantarum* |
| | 30S ribosomal protein | rRNA binding proteins | 3jzv | 100.0 | 78.5 | *Escherichia coli* |
| | Catalase-peroxidase | Oxidoreductase | 1sj2 | 54.5 | 78.0 | *Mycobacterium tuberculosis* |
| ACAP-VII | Neocarzinostatin | Antibacterial protein | 1noa | 33.3 | 94.6 | *Streptomyces carzinostaticu* |
| | Mersacidin decarboxylase | Oxidoreductase | 1p3y | 46.7 | 78.7 | *Bacillus sp* |
| | Beta-lactamase | Hydrolyse | 6t5y | 55.6 | 78.1 | *Escherichia coli* |

ACAP, African Catfish Antimicrobial Peptides; PDB Id, Protein DataBank Identity

### 4.5.3 Molecular docking against intracellular protein targets

To explore the structural-functional mechanism between the protein targets and antimicrobial peptides, molecular docking as a widely adopted and reliable modeling approach (Sulaiman et al., 2019), was performed using AutoDock, PatchDock and FireDock docking engines. The average binding energy (BE) and global energy (GE) were -7.60±0.23 kcal/mol and -60.07±2.68 kcal/mol respectively (Table 4.11). The ACAP-V peptide demonstrated the highest average binding energy (-8.47±0.32 kcal/mol), followed by ACAP-III (-7.87±0.15 kcal/mol) and ACAP-VI (-7.60±0.38

kcal/mol) against different protein targets. This was not any different when PatchDock and FireDock were used, as ACAP-V peptide demonstrated the highest average global energy (-70.78 kcal/mol), followed by ACAP-III (-69.20±0.29 kcal/mol) and ACAP-VI (-60.00±2.65 kcal/mol).

The highest binding energies (BE) of peptide ACAP-V were observed against Monomeric sarcosine oxidase (PDB Id: 2gb0; -9.40 kcal/mol) whereas its highest global energy was against NADH peroxidase (PDB Id: 1nhp; BE: -88.84 kcal/mol). Molecular docking of ACAP-V within the binding pockets of NADH peroxidase revealed four hydrogen bond interactions and up to eight hydrophobic bond interactions (Figure 4.14). Monomeric sarcosine oxidase, on the other hand, revealed four hydrogen bonds, five hydrophobic bonds and a covalent bond interaction with ACAP-V (Figure 4.15). Most interestingly, the binding of ACAP-V to the above target proteins was found to be like that of the FDA-approved drug (Flavin adenine dinucleotide; DrugBank Id: DB03147) for the respective binding sites (Figure 4.16). The lowest binding energy was observed in the interaction between ACAP-I peptide and Mersacidin decarboxylase (PDB Id: 1p3y; -5.80 kcal/mol).

## Table 4.11: Docking energies of the antimicrobial peptides

| Ligand Id | Biological Targets | PDB Id | Organism | PatchDock Solution # | Global energy (kcal/mol) | Binding Energ (kcal/mol) |
|---|---|---|---|---|---|---|
| ACAP-I | Cholesterol oxidase | 2gew | *Streptomyces sp.* | 487 | -45.32 | -7.30 |
| | Urease subunit alpha | 1kra | *Klebsiella aerogenes* | 236 | -60.38 | -7.60 |
| | Catalase-peroxidase | 1sj2 | *Mycobacterium tuberculosis* | 795 | -55.95 | -7.60 |
| | Pyridoxine/pyridoxamine 5'-phosphate oxidase | 1jnw | *Escherichia coli* | 476 | -43.23 | -6.90 |
| | Renalase | 3kkj | *Pseudomonas syringae* | 194 | -49.35 | -6.00 |
| ACAP-III | Isopentenyl-diphosphate delta-isomerase | 1p0k | *Bacillus subtilis* | 39 | -69.49 | -6.70 |
| | Enoyl-[acyl-carrier-protein] reductase | 4fs3 | *Staphylococcus aureus* | 225 | -68.92 | -8.90 |
| ACAP-V | Flavohemoprotein | 3ozw | *Cupriavidus necator* | 215 | -57.19 | -9.20 |
| | Thioredoxin reductase | 1f6m | *Escherichia coli* | 536 | -51.51 | -8.50 |
| | NADH peroxidase | 1nhp | *Enterococcus faecalis* | 60 | -88.84 | -8.40 |
| | Fumarate reductase flavoprotein | 1kf6 | *Escherichia coli* | 376 | -80.64 | -7.00 |
| | Monomeric sarcosine oxidase | 2gb0 | *Bacillus sp.* | 104 | -65.45 | -9.40 |
| | Dihydrolipoyl dehydrogenase | 1ojt | *Neisseria meningitidis* | 91 | -81.04 | -8.20 |
| ACAP-VI | Morphinone reductase | 1gwj | *Pseudomonas putida* | 477 | -62.22 | -8.60 |
| | Pyruvate oxidase | 1pow | *Lactobacillus plantarum* | 274 | -64.72 | -8.70 |
| | 30S ribosomal protein | 3jzv | *Escherichia coli* | 169 | -55.95 | -7.10 |
| | Catalase-peroxidase | 1sj2 | *Mycobacterium tuberculosis* | 155 | -68.59 | -8.50 |
| ACAP-VII | Neocarzinostatin | 1noa | *Streptomyces carzinostaticus* | 1 | -48.51 | -6.20 |
| | Mersacidin decarboxylase | 1p3y | *Bacillus sp* | 4 | -43.63 | -5.80 |
| | Beta-lactamase | 6t5y | *Escherichia coli* | 30 | -47.54 | -6.50 |
| FAD | Monomeric sarcosine oxidase | 2gb0 | *Bacillus sp.* | 78 | -59.47 | -7.20 |
| | NADH peroxidase | 1nhp | *Enterococcus faecalis* | 91 | -53.59 | -6.90 |

ACAP, African Catfish Antimicrobial Peptides; #, Number; PDB Id, Protein DataBank Identity

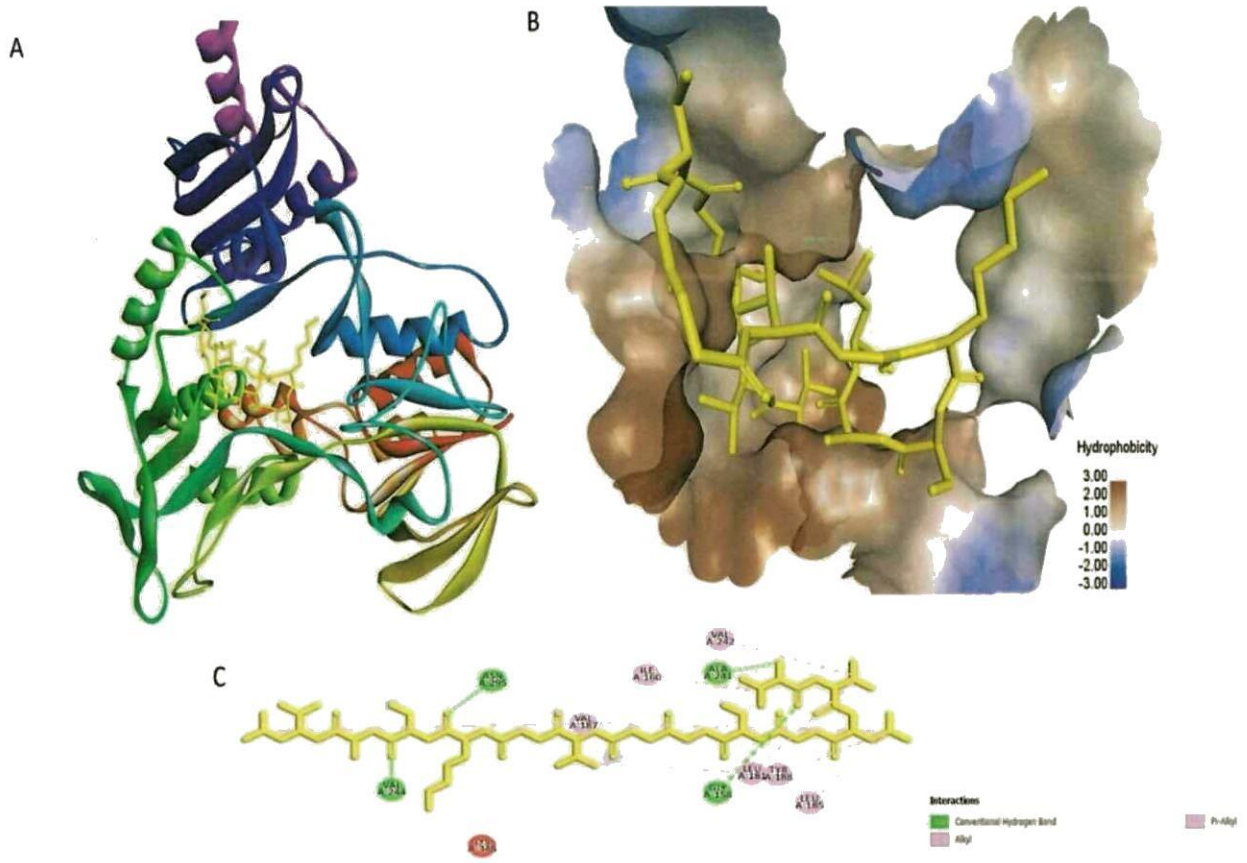



**Figure 4.14: The docking results and the interaction of ACAP-V with NADH peroxidase (PDB Id: 1nhp).** **(A)** 3-D structure of NADH peroxidase-ACAP-V complex. **(B)** The 3-D hydrophobicity surface plot at the binding site and **(C)** 2D diagram of the NADH peroxidase-ACAP-V molecular interactions. The interactions with residues are shown in different colors. ACAP-V formed four polar and eight non-polar interactions with NADH peroxidase (PDB Id: 1nhp). Val244, Asn295, Gly158 and Ala241 of NADH peroxidase bound ACAP-V by hydrogen bonding whereas Val187, Ile160, Val242, Leu181, Try188 Val187, Leu181 and Leu185 bound with ACAP-V by hydrophobic (Alkyl and Pi-alkyl) interactions.

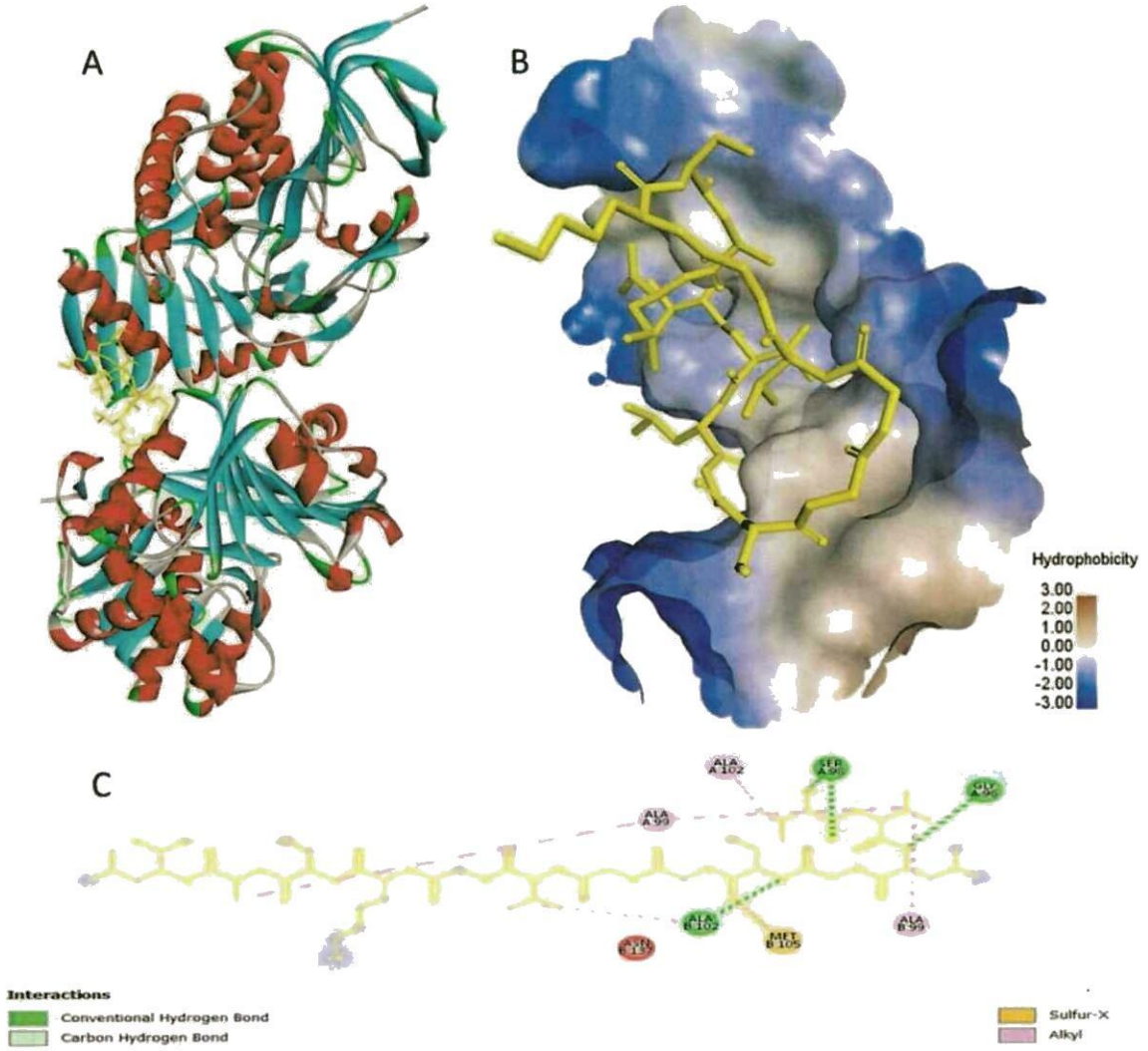
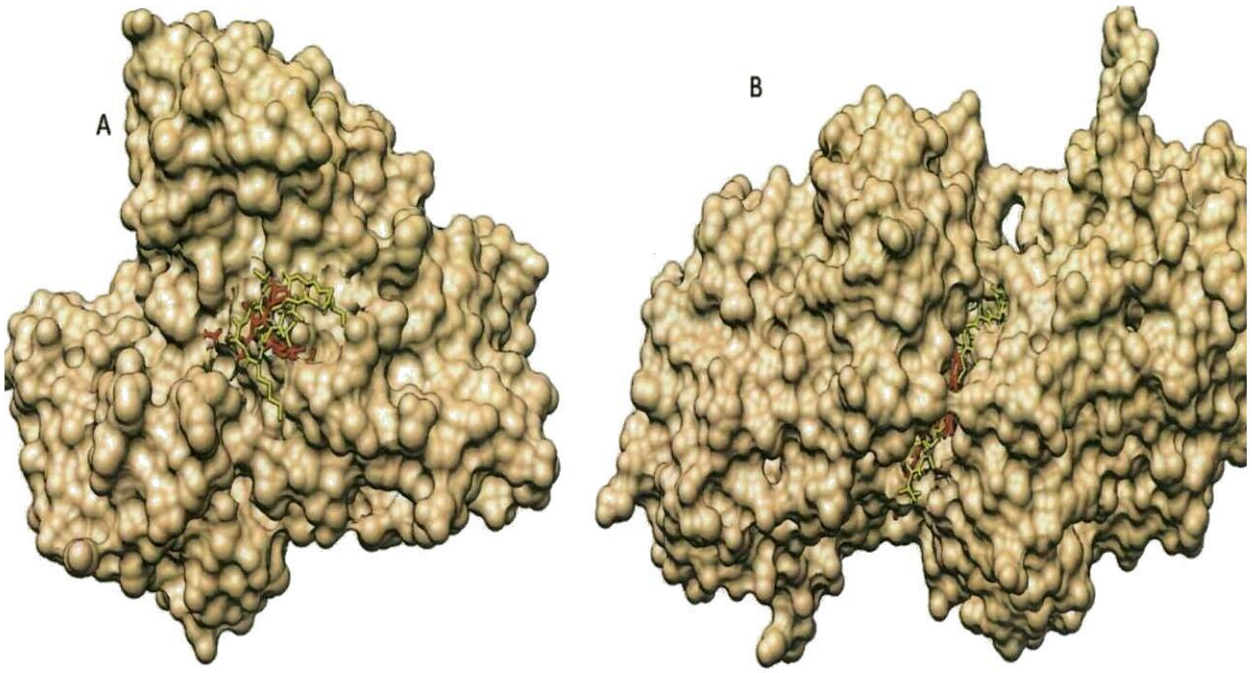



**Figure 4.15: The docking results and the interaction of ACAP-V with Monomeric sarcosine oxidase (PDB Id: 2gb0). (A)** 3-D structure of Monomeric sarcosine oxidase-ACAP-V complex. **(B)** The 3-D hydrophobicity surface plot at the binding site. **(C)** 2D diagram of the Monomeric sarcosine oxidase-ACAP-V molecular interactions. The interactions with residues are shown in different colors. ACAP-V peptide formed four with Ser98, Gly96, Ser98 and Ala102 as well as five alkyl bonds with Ala A:99, Ala A:102, Ala B:99, Ala B:102 and Ala A:99 and a sulfer-x bond with Met B:105 of Monomeric sarcosine oxidase.




**'igure 4.16: Binding of ACAP-V and Flavin adenine dinucleotide (FAD) to respective 'eceptor binding sites.** **(A)** Docking of NADH peroxidase (PDB Id: 1nhp) with ACAP-V (yellow) ınd FAD (Red). **(B)** Docking of Monomeric sarcosine oxidase (PDB Id: 2gb0) with ACAP-V yellow) and FAD (Red). In each of the target protein receptors, FAD binds to the similar binding ite with ACAP-V.

### ł.5.4 Bacterial cell membrane interaction study

Γhe action mechanism involving cell lysis was as well studied. Here, a molecular docking :alculation was performed between the five non-hemolytic ACAPs and bacterial cell membrane ›rotein (PDB Id: 2dw6). The top score pose demonstrated that all the five ACAPs were penetrating he bacterial cell membrane (Figure 4.17). The average global energy of -74.78 kcal/mol and ›inding energy -5.20 kcal/mol were observed in the studied ACAPs (Table 4.12). ACAP-V ›ersistently yielded the highest global energy (-87.35 kcal/mol) while the highest binding energy

(-5.6 kcal/mol) was from the interaction of ACAP-III with the bacterial cell membrane. Lowest global energy (-52.76 kcal/mol) and lowest binding energy (-4.30 kcal/mol) were observed in the interaction between bacterial cell membrane and ACAP-VII and ACAP-VI respectively.

**Table 4.12: Docking energies of the antimicrobial peptides against bacterial cell membrane (PDB Id: 2w6d)**

| Ligand Id | PatchDock Solution Number | Global Energy (kcal/mol) | Binding Energy (kcal/mol) |
|---|---|---|---|
| ACAP-I | 102 | -80.41 | -5.30 |
| ACAP-III | 778 | -73.81 | -5.60 |
| ACAP-V | 536 | -87.35 | -5.00 |
| ACAP-VI | 695 | -79.59 | -4.30 |
| ACAP-VII | 134 | -52.76 | -5.20 |

ACAP, African Catfish Antimicrobial Peptides

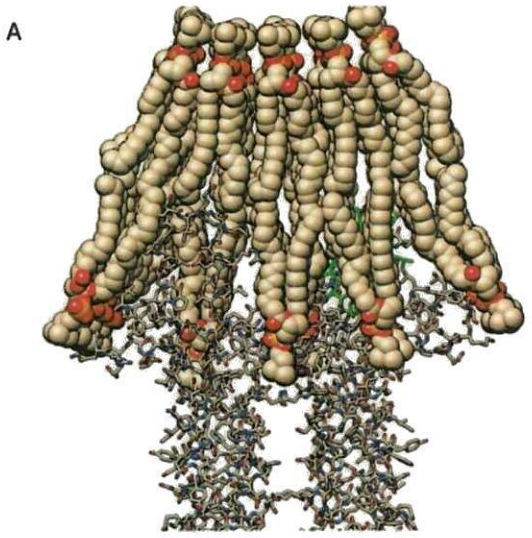



**Figure 4.17A: Interaction of the ACAP-I (green) with bacterial cell membrane.** ACAP-I was able to bind to the phospholipid bilayer especially on chain A of the intrinsic protein. The image was rendered on the *Chimera 1.15*

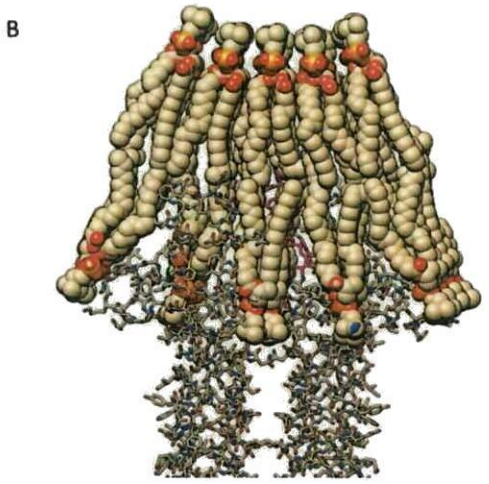



**Figure 4.17B: Interaction of the ACAP-III (pink) with bacterial cell membrane.** ACAP-III was able to bind to the phospholipid bilayer on both Chain A and chain B of the intrinsic protein. The image was rendered on the *Chimera 1.15*

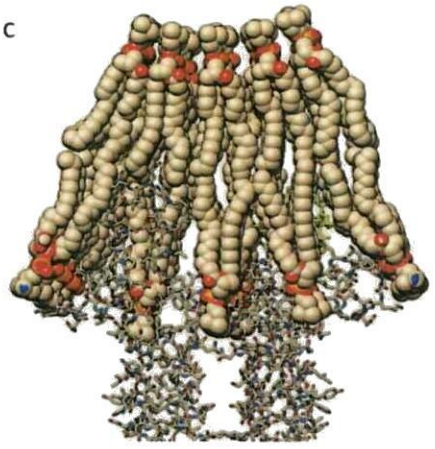



**Figure 4.17C: Interaction of the ACAP-V (yellow) with bacterial cell membrane.** ACAP-V was able to interact with the phospholipid bilayer through chain A of the intrinsic protein. The image was rendered on the *Chimera 1.15*

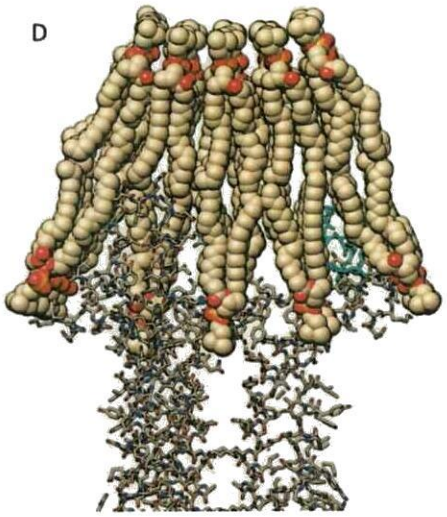



**Figure 4.17D: Interaction of the ACAP-VI (cyan) with bacterial cell membrane.** ACAP-VI was able to interact with the phospholipid bilayer through chain A of the intrinsic protein. The image was rendered on the *Chimera 1.15*

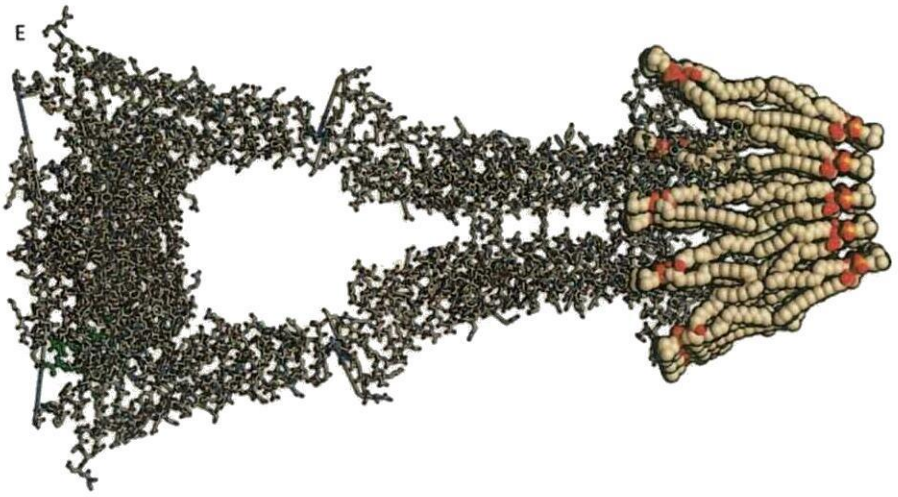



**Figure 4.17E: Interaction of the ACAP-VII (forest green) with bacterial cell membrane.** ACAP-VII interacts with the innermost end of the intrinsic protein. The image was rendered on the *Chimera 1.15*.

### 4.5.5 Alkaline phosphatase activity and electrolytic conductivity of antimicrobial peptides

This was a bacterial membrane lysis/permeability assay to reinforce the docking prediction of potential mechanism of action of the ACAPs. Throughout the culture process, the alkaline phosphatase activity in the control group (0.01 M PBS), remained at a low level (below 5 IU/L), indicating the bacterial cell wall was intact (Figure 4.18A). However, in the peak I antimicrobial peptide treated group, the alkaline phosphatase activity increased exponentially after an hour of treatment, as more alkaline phosphatase enzyme continues to leak out of the periplasm. Similarly, as indicated in Figure 4.18B, the conductivity of the control group (0.01 M PBS) remained relatively stable at low level throughout the culture process. On the other hand, just after an hour of treatment with the peak I antimicrobial peptides, the electrical conductivity was elevated significantly when compared to the control group, as the large volume of electrolyte continues to leak out of the cytoplasm during the culturing process.

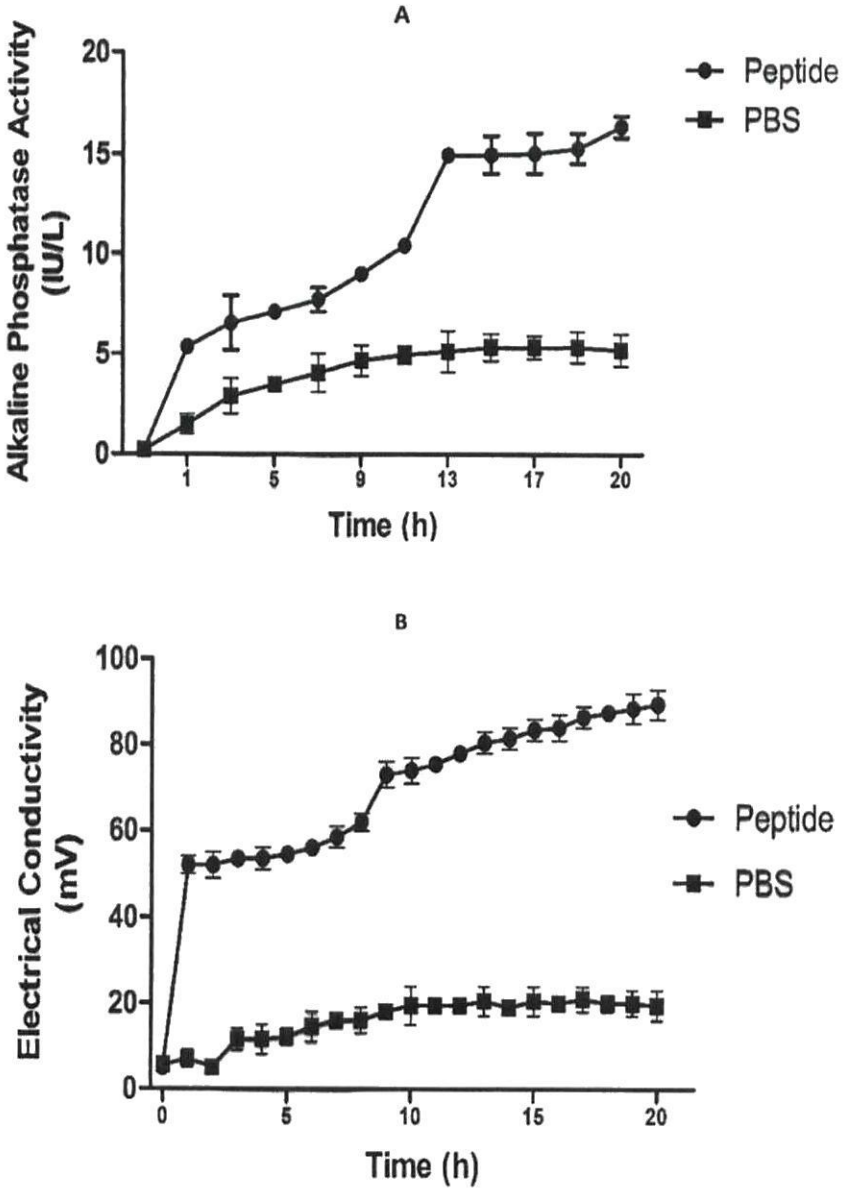



**Figure 4.18: Effect of antimicrobial peptide treatment on cell wall and cell membrane integrity. (A)** Alkaline phosphatase activity in the culture medium. **(B)** Electrical conductivity in culture medium. **PBS**-0.01 M Phosphate Buffer Solution. Both the Alkaline phosphatase activity and Electrical conductivity were measured each hour for 20 hours. The data are expressed as Mean ± SEM.

### 4.5.6: Peptide synthesis and in vitro validation

The purity of ACAP-IV and ACAP-V were 99.01% and 96.48 % (Figure 4.19), with molecular weight as confirmed by mass spectrometry was 1235.70 and 1143.34, respectively (Figure 4.20). The antibacterial activity of the synthesized ACAP-IV and ACAP-V was evaluated. Contrary to the results of the docking exercises, ACAP-IV demonstrated a higher antibacterial activity compared to ACAP-V with the MIC of 520.7±104.3 µg/mL and 1666.7±416.7 µg/mL against *E. coli* and *S. aureus*, respectively (Table 4.13).

**Table 4.13: Minimum Inhibitory Concentration ACAP-IV and ACAP-V on *E. coli* and *S. aureus***

| Run | MIC (µg/mL) | |
|---|---|---|
| | ***E. coli*** | ***S. aureus*** |
| ACAP-IV | 520.7±104.3 | 1666. 7±416.7 |
| ACAP-V | 625.0±00 | 2083. 3±416.7 |

Data are expressed as Mean ± Standard Error of Mean; MIC, Minimum Inhibitory Concentration

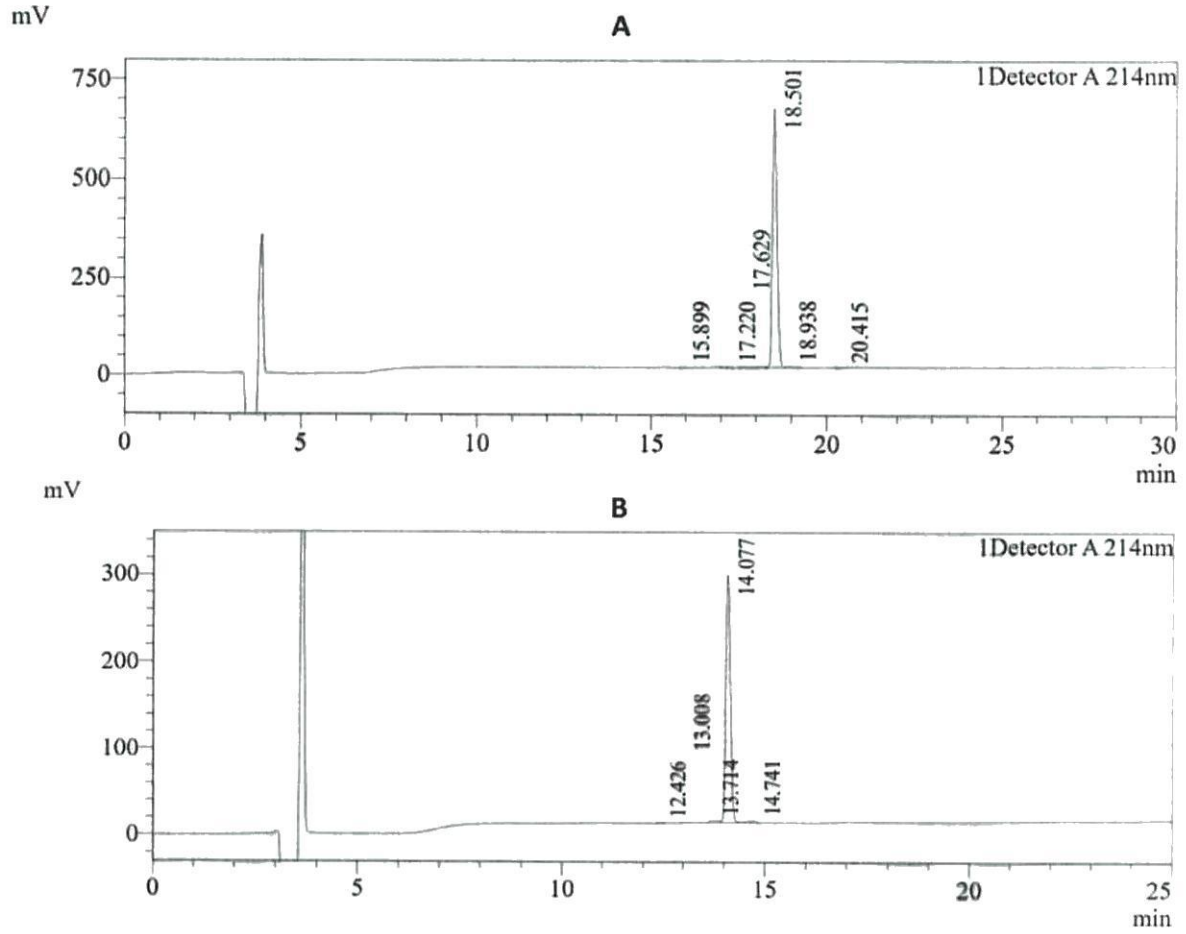



**Figure 4.19: Chromatograms of the purified African catfish antimicrobial peptides.** The synthesized peptides were loaded on a YMC-Triart $C_{18}$ (4.6×250mm×5μm) reversed-phase high performance liquid chromatography (RP-HPLC) column equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA)/water. Gradient elution was carried out with 0.1% (v/v) acetonitrile/TFA at a flow rate of 1.0 mL/min. (A) Chromatogram for ACAP-IV in the 30 minutes run at 214 nm. (B) Chromatogram for ACAP-V in the 25 minutes run at 214 nm. In both cases, 25 μL of samples were injected utilizing 100 % water dissolution method.

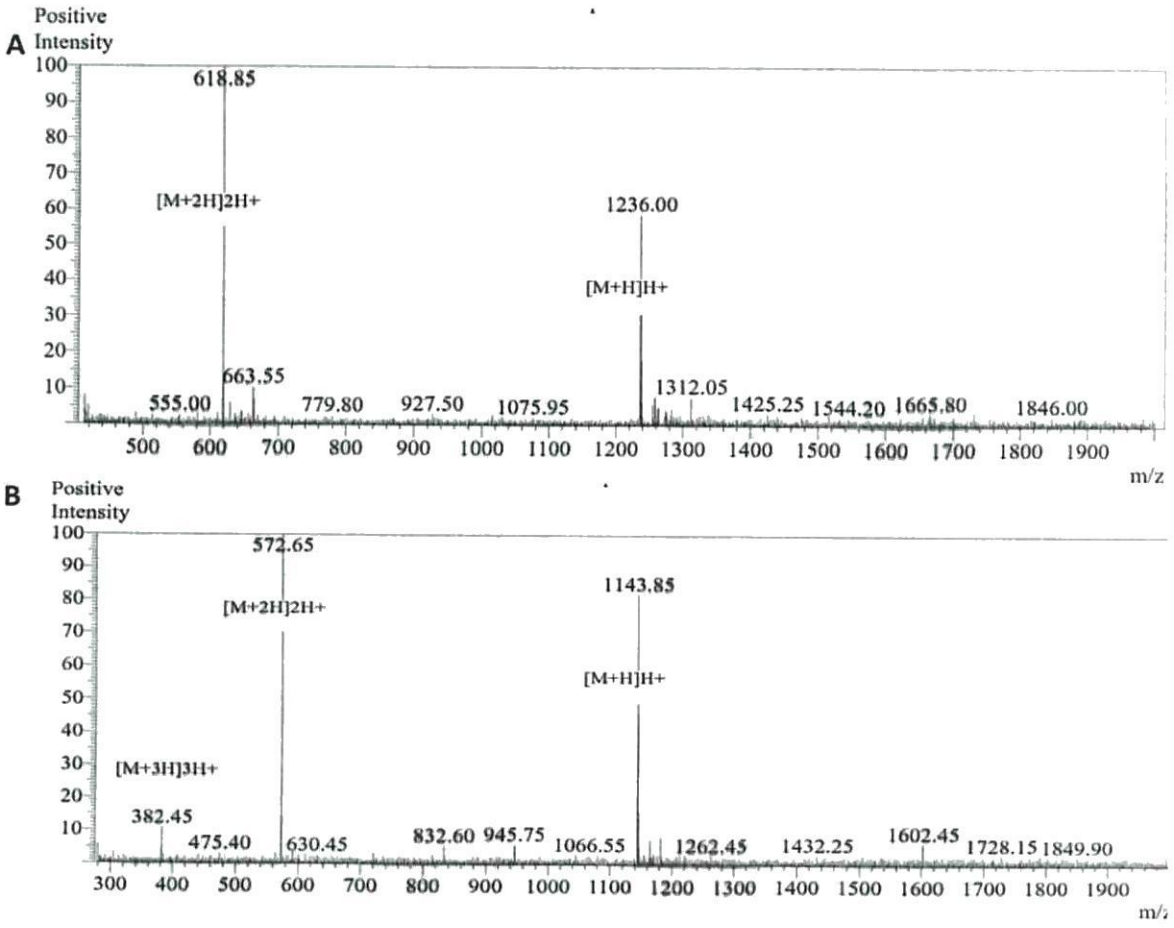



**Figure 4.20: MS spectra for the African catfish antimicrobial peptides.** 15 % ACN plus 85% water dissolution method was used prior to 0.2 µL injection. Electronspray Ionization (ESI) interface was used with the detector set at -2.4 kV. (A) MS spectrum of peptide ACAP-IV (KVSKVLHKAIL) with a theoretical mass of 1235.57 and an observed mass of 1235.70. (B) MS spectrum of peptide ACAP-V (VVLGSGGVGKSAL) with a theoretical mass of 1143.34 and an observed mass of 1143.30

# CHAPTER FIVE

# DISCUSSIONS

## 5.1 Antimicrobial activity of fish skin mucus extracts

The present study demonstrated that peptides extracted from the skin mucus of *P. annectens* and *C. griepinus* sourced from three major freshwater lakes of Uganda (Lake Albert, Lake Victoria and Lake Kyoga) are active against the two human priority pathogens: *E. coli* and *S. aureus*. The antibacterial activity varied according to fish species, with mucus extracts of *C. griepinus* presenting the best antimicrobial activity across the bacterial strains. When compared with the results obtained from previous studies, the current study reaffirmed the fact that the antibacterial activity of fish skin mucus extract is influenced by species, even on the same bacterial strain, with *C. griepinus* demonstrating the highest antimicrobial activity. In this regard, Adel, Safari, Soltanian, Zorriehzahra, & Esteban (2018) reported a high antimicrobial activity of Black Sea roach, *Rutilus frisii kutum* skin mucus extracts against different bacterial strains. Their study indicated MIC for *E. coli* (125 µg/mL) and *S. aureus* (500 µg/mL) which are much higher than the MIC in the present study [*E. coli* (0.71±0.33 µg/mL) and *S. aureus* (2.84±0.11 µg/mL)].

Additionally, the antibacterial activity of the marine stingrays, *Dasyatis sephen* and *Himantura gerrardi* (Vennila et al., 2011) was found to be lower when compared to that of *C. griepinus*. Here, *C. griepinus* demonstrated a lower MIC of 0.71±0.33 µg/mL (*E. coli*) and 2.84±0.11 µg/mL (*S. aureus*); values much lower compared with 32.34 ±1.89 µg/mL (*E. coli*) and 40.07±2.41 µg/mL (*S. aureus*) for *Dasyatis sephen* as well as 16.04±1.08 µg/mL (*E. coli*) and 32.27±1.84 µg/mL for *Himantura gerrardi* (Vennila et al., 2011). Furthermore, variation in the skin mucus antimicrobial activity across fish species have as well, been evidenced in Walking catfish, *Clarias batrachus*

and Mozambique mouth-breeder, *Tilapia mossambicus* (Elavarasi et al., 2013a); European eel, *Dicentrarchus labrax*, Eel fish, *Anguilla anguilla* and Blackspot fish, *Pagellus bogaraveo* (Gabriella et al., 2014); Largescale tonguesole, *Cynoglossus arel* and Thickspined catfish, *Nemapteryx caelata* (Bragadeeswaran and Thangaraj, 2011) among others. This differences in antimicrobial activity have been attributed to the variation in mucus secreting cells across fish species (Shephard, 1993).

Apart from the species of fish, the extraction solvents could have probably influenced the variation in the antimicrobial activity of fish mucus. In the present study, 10 % Acetic acid was utilized to extract the native peptides in the skin mucus. The activity of peptides associated with antibacterial properties has been reported to be greatly enhanced by mild acidic solvents. This is probably due to the fact that mild acidic solvents enhances the solubulization of native peptides (Okella et al., 2018; Välimaa et al., 2019). In their study on antimicrobial activity of different Malaysian freshwater fish species across solvents, Rao et al. (2015) reported that, only acidic extract was active against *E. coli* whereas aqueous, organic as well as crude extract showed no activity. A similar finding was reported for the skin mucus *Oreochromis niloticus*, *Clarias batrachus*, and *Channa striata* in acidic extraction medium (Lirio et al., 2019).More so, acidic extracts of three freshwater fishes Stinging catfish, *Heteropneustis fossilis*; Green snakehead, *Channa punctatus*; African catfish; *Clarias gariepinus* were reported to demonstrate the highest antimicrobial activity compared to organic and crude extracts (Jameel et al., 2019).

Looking at the antimicrobial peptide yield; fish species and lake greatly influenced the yield. This variation is thought to be due to diverse composition of the mucus as influenced by mucus secreting cells known to differ from one species to another (Shephard, 1993). Moreover, several reports (Lebedeva, 1999; Subramanian et al., 2008; Kumari et al., 2011; Nigam et al., 2012) revealed that environmental disturbances like temperature, dissolved oxygen, pH, salinity, ecological niches influence the amount and quality of the mucus produced. This lakes' physicochemical variations (Mbalassa et al., 2014) as well a the genetic make up of the fish (Abbas et al., 2020) greatly influences the variation.

**5.2 Identities of fish mucus-derived antimicrobial peptides**

In the present study, peak I antimicrobial peptides extracted from the skin mucus of freshwater African catfish, *Clarias gariepinus* demonstrated a high antimicrobial activity on both Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria (MIC: 0.31±0.16 and 1.99±0.13 µg/mL respectively). This is invariable from previous reports on mucosal AMPs like a 4.7 kDa LEAP-2 from Topmouth culter, *Erythroculter ilishaeformis* (Chen et al., 2020); 2.2 kDa Pelteobagrin, from yellow catfish, *Pelteobagrus fulvidraco* (Su, 2011); 2.8 kDa SAMP-H1 from Atlantic salmon, *Salmo salar* (Luders et al., 2005) and 2.7 kDa Pleurocidin, from winter flounder, *Pleuronectes americanus* (Cole et al., 1997). The molecular weight of the studied ACAPs determined by LC-MS/MS ranged between 1.005 to 1.622 kDa. It is, therefore, not surprising those ACAPs demonstrated high antimicrobial scores (0.561-0.905 units).

In addition, just like most AMPs like OVTp12 (Ma et al., 2020), Pelteobagrin (Su, 2011), Myxinidin (Subramanian et al., 2009), all the ACAPs except ACAP-III were predicted to have a net positive charge of 1 to 4. This cationic property is due to the abundance of positively charged

amino acid Arginine (Arg) and Lysine (Lys) residues in ACAPs. Several studies have indicated that net positive charge is one of the essential physiochemical parameters that determine the antimicrobial activity of any peptide (Bruston et al., 2007; Kim et al., 2018). Net positive charge on the peptides increases their affinity for electrostatic interactions with the negatively charged phospholipid head groups on the outer surface of the microbial cell membrane; an asset to membrane penetration (Brogden, 2005). However, this charge should not exceed +9, as Therapeutic Index (TI), rapidly decreases beyond this value (Jiang et al., 2008). The charges of identified ACAPs in the present study were far below this threshold value rendering them as more promising.

Simultaneously, ACAPs were found to be rich in hydrophobic amino acids like Valine (Val), Glycine (Gly), Leucine (Leu), Isoleucine (Ile), Alanine (Ala), Phenylalanine (Phe), Tryptophan (Trp), and Proline (Pro). Hydrophobic peptides easily interact with cellular hydrophobic lipid bilayer, penetrate and cross the membrane (Bahar and Ren, 2013; Burton et al., 2013). In the present study, SPE cartridges enhanced the recovery of such peptides. It's, therefore, not surprising that all the ACAPs demonstrated high hydrophobicity above 54%, a value much higher than those in the previously reported peptides like Cruzioseptin (Cuesta et al., 2021), A15_B (Okella et al., 2020), OVTp12 (Ma et al., 2020) among others.

Interestingly, four identified antimicrobial peptides (ACAP-I, ACAP-II, ACAP-IV and ACAP-VII) out of the seven identified ACAPs demonstrated α-helix structure conformation. Amphipathic physiochemical property has been reported as the most important for antimicrobial peptides (Wang et al., 2019). The polar region of the amphipathic peptide enhances association with the membrane

through electrostatic interaction with the phospholipid charged head groups (Sato and Feix, 2006), as the non-polar region forms transient pores or channels through hydrophobic interactions with the hydrophobic phospholipid bilayer zone, resulting into increased permeability and loss of barrier function of the bacterial cells (Sato and Feix, 2006; Mihajlovic and Lazaridis, 2012).

### 5.3 Toxicity of Antimicrobial Peptides

Toxicity, especially hemotoxicity remains one of the key drawbacks that have limited the number of approved AMPs in clinical use (Boto, De La Lastra, & González, 2018; Cuesta et al., 2019). In the present study, preliminary toxicity assessment of *C. gariepinus* peak I antimicrobial peptide revealed their non-hemolytic nature even at the highest assayed concentration (100 µg/mL). Non-hemotoxic findings have as well been demonstrated in the skin mucus extract of Striped Dwarf Catfish, *Mystus vittatus* (Jothi et al., 2013); Winter flounder, *Pleuronectes americanus* peptide extracts (Cole et al., 1997); skin mucus extract yellow catfish, *Pelteobagrus fulvidraco* (Su, 2011) and Marine Catfish, *Tachysurus Dussumieri* (Raja et al., 2020).

Contrary to findings in the present study, the mucus extracts of Spotted sea catfish, *Arius maculatus* (Manivasagan et al., 2009); and venomous fish such as Stonefish, *Synanceia verrucosa* (Maillaud et al., 2020); Lionfish, *Pterois volitans* (Cohen and Olek, 1989; Robertson et al., 2014); Scorpionfish, *Scorpaena plumieri* (Andrich et al., 2010); Pufferfish, *Akifugu rubripes* (Go et al., 2019) have been reported as hemotoxic and lethal. Such peptides penetrates the deeper hydrophobic core of the mammalian cell membrane, due to extreme hydrophobicity beyond the optimal threshold, thereby lysing the red blood cells (Wood et al., 2014) however, such fish are still edible.

Much as, the peak I antimicrobial peptides showed non-hemolytic property, the findings were not conlusive since only a maximum concentration of 100 µg/mL was assayed by this study. Besides, peak I antimicrobial peptides contained several peptides that could possibly interact to alter the hemolytic potentials, hence the need to explore individual peptides for hemo-compatibility. Nevertheless, *in silico* predictions were employed to shade light on the hemolytic activity of individual peptides rather than just peptide cocktail of Sephadex G-25 purified peptides.

Interestingly, when individual antimicrobial peptides were subjected to *in silico* toxicity profiling, revealed two antimicrobial peptides (ACAP-II and ACAP-IV) were predicted to be hemolytic (Table 4.6). These two peptides were dropped off at this screening stage. Conversely, of the non-hemolytic peptides (ACAP-I, ACAP-III, ACAP-V, ACAP-VI and ACAP-VII), peptide ACAP-III followed by ACAP-V stood out as the most promising with the least SVM scores of -1.52 and -1.17 respectively. Similar findings of non-toxicity have been reported in a novel angiotensin-converting enzyme (ACE) inhibitory peptides from *Oncorhynchus mykiss* nebulin (Yu et al., 2018); 15 amino acid residues peptide, LZ1 (Zhang et al., 2013); Moronecidin-like peptide from *Hippocampus comes* (Mohammadi et al., 2018) and lately, novel antimicrobial cruzioseptin peptides extracted from the splendid leaf frog, *Cruziohyla calcarifer* (Cuesta et al., 2021). Such peptides remain on the surface of the mammalian cell membrane without penetrating it (Cuesta et al., 2021).

### 5.4 Mode of action of African Catfish Antimicrobial Peptides

The choice was guided by the fact that cell envelope damage is the commonest killing mechanism of peptides with antimicrobial or host defense potentials (Hollmann et al., 2018). In the present study, a rapid increase in the alkaline phosphatase activity in the *E. coli* culture medium within an

hour of peptide treatment was observed, this increased activity persisted till 13$^{th}$ hour of treatment. Similar increase in ALP activity, have previously reported for *Porphyra yezoensis* peptides (Jiao et al., 2019), Dihydromyricetin against food-borne bacteria (Xiao et al., 2019) and *AIEgen* peptide conjugate (Zhang et al., 2020), signifying cell wall destruction (Robert et al., 2014). Alkaline phosphatase is localized between the cell membranes and cell walls of the bacteria (Sebastian and Ammerman, 2009) and any disruption of the cell wall penetrability, leads to its discharge out of the bacteria. Therefore, peak I antimicrobial peptides of *C. gariepinus* skin mucus peptides could destroy the cell wall.

Furthermore, cell wall distruction increases the cell membrane permeability resulting to cytoplasmic outflow and hence, a rise in extracellular electrolytes (Green et al., 1929; Wu et al., 2016). And, by monitoring the electrical conductivity of the extracellular medium, the bacterial cell membrane disruption can be measured (Molina-Guijarro et al., 2011). In this study, within an hour of peptide treatment, the conductivity of *E. coli* culture medium significantly increased (Figure 4.17B), suggesting the cell wall and cell membrane breakage leading to cytoplasmic fluid outflow, cell collapse and death. Later, the gradual increase in conductivity after an hour of treatment, is possibly since majority of the cytoplasmic electrolytes are lost within the first hour of membrane shock. This is in line with studies on OVTp12 peptide on *E.coli* and *S. aureus* (Ma et al., 2020) and *Porphyra yezoensis* peptides on *S. aureus* (Jiao et al., 2019). Bacterial cell membrane damage as well, ignites transient pore formation and freight of peptides into the cell for intracellular target interactions (Schmidtchen et al., 2014), that can be explored through molecular docking.

Accordingly, five non-hemolytic antimicrobial peptides were docked against respective target proteins. The lytic mode of action study further reaffirmed that; all the five antimicrobial peptides were able to interact with chain A of intrinsic bacterial cell membrane enzyme (PDB Id: 2dw6). Similar findings have been reported for cruzioseptin peptides (Cuesta et al., 2021) and flocculating polypeptides (Garcia et al., 2019). Bacterial cell membrane enzyme (PDB Id: 2dw6) is vital in the synthesis of unsaturated fatty acids, a key component of the phospholipid bilayer (Kopp et al., 2020), through a series of dehydration and isomerization reactions. Inhibition of the enzyme thus, impedes the cell membrane biosynthesis process (Leesong et al., 1996). Moreover, such interactions between the ACAPs and bacterial cell membrane also aids both cell membrane penetration (Lohner and Prossnigg, 2009) and 'detergent-like' distruction of the bacterial cell membrane (Oren and Shai, 1998), that can potentially lead to cell death (Okella et al., 2022b). During the penetration, the peptides either accumulate then bend to form a ring hole/pore of 1-2 nm diameter as described in Toroidal pore/Wormhole model (Matsuzaki et al., 1995) or aggregates into channel forming multimers as described in Barrel-Stave model (Lohner and Prossnigg, 2009).

Conversely, an in-depth focus on the non-membrane mode of action revealed that, each of the five non-hemolytic antimicrobial peptides interacted with the respective protein target to possibly cause an antimicrobial activity. Here, ACAP-V demonstrated the strongest binding affinities with a binding energy of -9.40 kcal/mol against NADH peroxidase (PDB Id: 1nhp). The best docking pose revealed that ACAP-V formed four polar and eight hydrophobic interactions with NADH peroxidase (PDB Id: 1nhp). Val244, Asn295, Gly158 and Ala241 of NADH peroxidase bound

ACAP-V by hydrogen bonding. Furthermore, Val187, Ile160, Val242, Leu181, Try188 Val187, Leu181 and Leu185 bound with ACAP-V by hydrophobic (Alkyl and Pi-alkyl) interactions.

Such interactions depend on the structure of the ligands and are crucial for the binding of the drug to the target, physiological changes during the binding (bioactivity), as well as drug elimination. Aromantic rings are for example vital in fostering both the π-π and hydrogen bond interactions. Steric bulk too, enhances hydrogen bond interactions. The amines and amides functional groups on the other hand, are responsible for dipole-dipole as well as additional hydrogen interactions between the ligands and the target protein (Ertl et al., 2020).

Although hydrophobic interactions are much weaker, they allow momentary interactions and unlike the covalent bonds that are usually hard to break, they easily break to release the ligands after their interactions with the target protein to enhance drug excretion (Varma et al., 2010). The hydrogen bonds on the other hand, enhances optimal interactions that allows timely dislodgment of the drug molecule from the binding site and hence, excretion after effecting action (Varma et al., 2010; Chen et al., 2016). Therefore, the four hydrogen bonds in the present study were the most essential given that, NADH peroxidase is a critical enzyme in bacterial tolerance mechanism. In this mechanism, NADH peroxidase catalyzes the degradation/inactivation of hydrogen peroxide produced within the cell by metabolic pathways, such as glycerol metabolism and dismutation of superoxide, before it damages essential cellular components (Miyoshi et al., 2003; Naraki et al., 2020). Inhibition of such enzymes by ACAP-V can potentially lead to the accumulation of cell-generated hydrogen peroxide leading to bacterial cell distraction and death.

On the other hand, the highest global energy of -88.84 kcal/mol was recorded still between the interaction of ACAP-V and monomeric sarcosine oxidase (PDB Id: 2gb0). Monomeric sarcosine oxidase catalyzes the oxidative demethylation of sarcosine (N-methlyglycine) to form glycine and formaldehyde. Molecular docking revealed that, ACAP-V peptide formed four hydrogen bonds with Ser98, Gly96, Ser98 and Ala102; five alkyl bonds with Ala A:99, Ala A:102, Ala B:99, Ala B:102 and Ala A:99 and a sulfer-x bond with Met B:105 of Monomeric sarcosine oxidase. These interactions inhibit the demethylation of sarcosine, thereby depriving energy production in such organisms. This can potentially lead to death of microbes whose sole source of energy is sarcosine (Hassan-Abdallah et al., 2006; Zhao et al., 2008). It's, therefore, not surprising that ACAP-V possessed a wide bioactivity spectrum against pathogens just like Polymixin B, used in the treatment of multidrug-resistant pathogens (Zavascki et al., 2007) and Colistin; the last line antibiotic (Falagas and Kasiakou, 2005).

Interestingly, the binding pocket of ACAP-V was not any different from that of FDA-approved Flavin adenine dinucleotide (DrugBank Id: DB03147). Just like Flavin adenine dinucleotide, ACAP-V was capable of binding onto the binding site of Monomeric sarcosine oxidase (PDB Id: 2gb0) and NADH peroxidase (PDB Id: 1nhp) in a similar way, thereby shadding the potential applicability of the ACAPs.

The synthesized ACAP-IV (KVSKVLHKAIL) demonstrated a higher *in vitro* antimicrobial activity than ACAP-V (VVLGSGGVGKSAL). However, its MIC of 520.7±104.3 and 1666.7±416.7 on *E. coli* and *S. aureus* respectively indicate a lower antibacterial activity when compared to Pelteobagrin (GKLNLFLSRLEILKLFVGAL) from yellow catfish, *Pelteobagrus*

*fulvidraco* (MIC, 2-64 μg/mL) (Su, 2011); Gs D (FIGGIISFFKRLF) from Goldenstriped soapfish, *Grammistes sexlineatus* (MIC, 13.3-50 μg/mL) (Sugiyama et al., 2005); Misgurin (RQRVEELSKKGAAARRRK) from Pond Loach, *Misgurnus anguillicaudatus* (MIC, 4-16 μg/mL) (Park et al., 1997).

During a solid-phase peptide synthesis, a condensation reaction between the carboxyl-terminus and amino-terminus occurs to link amino acids through amide/peptide bonds (Marglin and Merrifield, 1970). Variation in reaction conditions as well as various side chain protecting strategies during the chemical synthesis process impact the bioactivity of the synthesized products (Marglin and Merrifield, 1970; Abu-Baker M. Abdel-Aal, Richard Raz, George Papageorgiou, 2020) . Further, Kęska and Stadnik (2017) maintain that amino acid composition, structure, and physicochemical properties account for the wide variations in the antimicrobial activity of different peptides. These could explain the variation. However, the wide variations can still be reduced by ligand optimization.

# CHAPTER SIX

# CONCLUSIONS, LIMITATIONS AND RECOMMENDATIONS

## 6.1 Conclusions

- African catfish, *Clarias griepinus* skin mucus extracts demonstrated higher antimicrobial activity than African lungfish, *Protopterus annectens* against *E. coli* and *S. aureus*.
- Seven novel antimicrobial peptides of 11-16 amino acid residues, and molecular weight between 1.0-1.6 kDa were identified from the skin mucus extracts of African catfish.
- African catfish skin peptide fractions were generally non-hemolytic, but individual peptide level, two antimicrobial peptides (ACAP-II and ACAP-IV) were predicted hemolytic.
- The identified antimicrobial peptides exhibited membrane and non-membrane modes of action.

Hence, for the first time in the African continent, knowledge on the identity, toxicity profiles and the possible mode of action of novel antimicrobial peptides from the skin mucus of African catfish, *Clarias griepinus* from the three major lakes of Uganda have been added.

## 6.2 Limitations

- Due to facility constraints, only the most active extract (Peak I from Size Exclusion Chromatography) was shipped for peptide sequencing at the *de Duve* Institute Proteomics platform, UCLouvain (Brussels, Belgium).
- Low funding hindered lead optimization as well their upscaling hence, only two identified African catfish antimicrobial peptides were chemically synthesized.

- Similarly, the support extended to this investigation could only permit testing on few bacterial strains. Accordingly, only a representative of Gram positive (*S. aureus*) and Gram negative (*E. coli*), were utilized for the screening purpose.

### 6.3 Recommendations

- ✓ For any drug candidate to be qualified, the pharmacokinetic and drug-likeness properties should be within the acceptable range. Research and Development units of pharmaceutical companies may further explore profile identified antimicrobial peptides for pharmacokinetic and the drug-likeness profiles for potential uptake.
- ✓ The *in vitro* and *in silico* efficacies are not sufficient to ascertain the drug candidature. It will be necessary to further explore their *in vivo* efficacies of the peptides identified in the present study for natural product development. Such experimental validations are crucial for a drug lead progression along the drug discovery path.
- ✓ The present study revealed that ACAPs classically destroy the bacterial cell envelope. Future efforts towards elucidation of cell membrane at a molecular level is most welcomed.
- ✓ Given that the present study explored skin mucus extracts of only two fish species (African catfish and African lungfish), exploration of antimicrobial peptides from other fish species encouraged.

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## LIST OF APPENDICES

**Appendix I: Manuscript 1 Published in frontiers in Bioengineering and Biotechnolog**

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frontiers in Bioengineering and Biotechnology

ORIGINAL RESEARCH
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# New Putative Antimicrobial Candidates: *In silico* Design of Fish-Derived Antibacterial Peptide-Motifs

*Hedmon Okella[1]*, John J. Georrge[2]*, Sylvester Ochwo[3], Christian Ndekezi[3], Kevin Tindo Koffi[4], Jacqueline Aber[1], Clement Olusoji Ajayi[1], Fatoumata Gnine Fofana[5], Hilda Ikiriza[1], Andrew G. Mtewa[1,6], Joseph Nkamwesiga[3,7], Christian Bernard Bakwo Bassogog[8], Charles Drago Kato[3] and Patrick Engeu Ogwang[1]*

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Antimicrobial resistance remains a great threat to global health. In response to the World Health Organizations' global call for action, nature has been explored for novel and safe antimicrobial candidates. To date, fish have gained recognition as potential source of safe, broad spectrum and effective antimicrobial therapeutics. The use of computational methods to design antimicrobial candidates of industrial application has however, been lagging behind. To fill the gap and contribute to the current fish-derived antimicrobial peptide repertoire, this study used Support Vector Machines algorithm to fish out fish-antimicrobial peptide-motif candidates encrypted in 127 peptides submitted at the Antimicrobial Peptide Database (APD3), steered by their physico-chemical characteristics (i.e., positive net charge, hydrophobicity, stability, molecular weight and sequence length). The best two novel antimicrobial peptide-motifs (A15_B, A15_E) with the lowest instability index (−28.25, −22.49, respectively) and highest isoelectric point (pI) index (10.48 for each) were selected for further analysis. Their 3D structures were predicted using I-TASSER and PEP-FOLD servers while ProSA, PROCHECK, and ANOLEA were used to validate them. The models predicted by I-TASSER were found to be better than those predicted by PEP-FOLD upon validation. Two I-TASSER models with the lowest c-score of −0.10 and −0.30 for A15_B and A15_E peptide-motifs, respectively, were selected for docking against known bacterial-antimicrobial target-proteins retrieved from protein databank (PDB). Carbapenam-3-carboxylate synthase (PDB ID; 4oj8) yielded the lowest docking energy (−8.80 and −7.80 Kcal/mol) against motif A15_B and A15_E, respectively, using AutoDock VINA. Further, in addition to Carbapenam-3-carboxylate synthase, these peptides (A15_B and A15_E) were found to as well bind to membrane protein (PDB ID: 1by3) and Carbapenem synthetase (PDB: 1q15) when ClusPro and HPEPDOCK tools were used. The membrane protein yielded docking energy scores (DES): −290.094, −270.751; coefficient weight (CW):

**Appendix II: Manuscript 2 Published in frontiers in Microbiology (2021), doi: 10.3389/fmicb.2021.794631**

frontiers in Microbiology

ORIGINAL RESEAR
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# Identification of Antimicrobial Peptides Isolated From the Skin Mucus of African Catfish, *Clarias gariepinus* (Burchell, 1822)

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Antimicrobial peptides (AMPs) constitute a broad range of bioactive compoun
diverse organisms, including fish. They are effector molecules for the innate imr
response, against pathogens, tissue damage and infections. Still, AMPs from Af
Catfish, *Clarias gariepinus*, skin mucus are largely unexplored despite their pos
therapeutic role in combating antimicrobial resistance. In this study, African Ca
Antimicrobial peptides (ACAPs) were identified from the skin mucus of African Ca
*C. gariepinus*. Native peptides were extracted from fish mucus scrapings in
acetic acid (v/v) and ultra-filtered using 5 kDa molecular weight cut-off memb
The extract was purified using $C_{18}$ Solid-Phase Extraction. The antibacterial ac
was determined using the Agar Well Diffusion method and broth-dilution me
utilizing *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25
Thereafter, Sephadex G-25 gel filtration was further utilized in bio-guided iso
of the most active fractions prior to peptide identification using Orbitrap Fusion L
Tribrid Mass Spectrometry. The skin mucus extracted from African Catfish fr
the three major lakes of Uganda exhibited antimicrobial activity on *E. co*
*S. aureus*. Lake Albert's *C. gariepinus* demonstrated the best activity with the l
MIC of 2.84 and 0.71 μg/ml on *S. aureus* and *E. coli*, respectively. Sephadex
peak I mass spectrometry analysis (Data are available *via* ProteomeXchang
identifier PXD029193) alongside *in silico* analysis revealed seven short pe
(11–16 amino acid residues) of high antimicrobial scores (0.561–0.905 un
addition, these peptides had a low molecular weight (1005.57–1622.05 Da) ar
percentage hydrophobicity above 54%. Up to four of these AMPs demons
α-helix structure conformation, rendering them amphipathic. The findings

**Appendix III: Manuscript 3 Published in Asian Journal of Pharmaceutical Research and Development (2018), DOI: http://dx.doi.org/10.22270/ajprd.v6.i4.389**



Asian Journal of Pharmaceutical Research and Development
(An International Peer-Reviewed Journal of Pharmaceutical Research and Development)
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Open Access Available online at http://ajprd.com/index.php Review Article

# FISH MUCUS: A NEGLECTED RESERVOIR FOR ANTIMICROBIAL PEPTIDES

**Okella Hedmon[1]*, Aber Jacqueline[1], Kevin Tindo Koffi[3], Kato Charles Drago[2] and Ogwang Patrick Engeu[1]**

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**ABSTRACT:-**

Antimicrobial resistance has posed a great global burden, with the fear that by 2050 it would have killed more people than cancer if nothing much is done about it. Alongside several attempts in place, zoo-therapy is becoming one of important remedies in the modern society, with hope for solution believed to be hidden in nature. In this study, the authors present a review of journal articles and reports obtained through key word search of several literature databases on recent developments in the battle against the antimicrobial resistance using fish derived antimicrobial peptides. The findings indicate despite some limitations of these antimicrobial peptides, their very broad spectrum activity against pathogens keeps them among promising antibiotics as far as the battle against multidrug resistance is concerned. Much as various methods to study antimicrobial peptides do exist, fish mucus remains less explored. The study recommends aquatic habitat exploration in search for novel bacterial antimicrobial peptides.

*Key words: mucus, peptides, antimicrobial, fish, drug*

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## INTRODUCTION

The contemporary increase in mortality due to bacterial infections has solely been attributed to antimicrobial resistance [1]. The potentials of antibiotics to combat bacterial infections have greatly decreased due to the ever increasing multidrug resistance[2,3], that claims over 700,000 lives every year[4], with a projected rise to 10 million by 2050 if alternative solutions are not found[5]. Mortality due to bacterial infections will thus be far worse those caused by malaria which claimed 445,000 lives in 2016 [6], HIV which claims about a million lives annually [7] and cancers which accounted for 8.8 million deaths in 2015. [8]At the beginning of 2017, the World Health Organization (WHO) made a desperate global call for new class of antibiotics[4]. Seven month later (September, 2017), WHO confirms the world is running out of antibiotics and declared a global health emergency that calls for an urgent need for more investment in research and development for antibiotic-resistant infections especially for the ranked 'priority pathogens' that if not watched may within this century, return the world back to the "pre-antibiotic era"[9]. This point out the fact that, the search for novel antimicrobials is cardinal as far as combating the antimicrobial resistance is concerned. In light of the above, four new strategies of overcoming resistance by microbes, are being explored: modifying old antibiotics into entirely new classes, combining antibiotics, supplementing antibiotics with adjuvants or searching nature for novel antibiotics [10]. This is in line with the 2010 Infectious Diseases Society of America

**Appendix IV: Manuscript 4 published in East Africa Science Journal,**

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ORIGINAL ARTICLE

# Bacterial Cell Envelope Lysis and Hemotoxicity of Peptide Previously isolated From African Catfish, Clarias gariepinus

Hedmon Okella*[a], Clement Olusoji Ajayi[a], Hilda Ikiriza[a], Andrew Glory Mtewa Bruhan Kaggwa[a,c], Jacqueline Aber[a,d], Charles Drago Kato[b], Patrick Ogwang Enge

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## ABSTRACT

**Background:** The skin mucus layer of fish is endowed with biologics including, Antimicrobial peptides (AMPs) that off a first line of defence against pathogens. Such peptides can either inhibit bacterial growth or completely kill the bacter and hence are regarded as a viable alternative to traditional antibiotics, in addressing the ever-increasing incidenc of antimicrobial resistance. However, one of the major hurdles to AMPs use is their poor haemolytic profile. As a resu a thorough evaluation of prospective AMPs' bacterial cell membrane disruption and hemolytic potentials in the ea phases of drug discovery is critical. The current study presented cell membrane destruction as well as hemo-compatibil of antimicrobial peptides previously isolated from skin mucus of African catfish, ***Clarias gariepinus***.

**Methods:** A previously isolated antimicrobial peptide in the skin mucus of African catfish, *C. gariepinus* were profile using 15% Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The electrical conductivity ar alkaline phosphatase assays were utilised to measure bacterial cell envelope lysis activity as a classical mode of actic of the antimicrobial peptides. Afterwards, fresh Rabbit blood cells were then utilised for in vitro hemolytic assay.

**Results:** The peptides were found to be about 5 kDa molecular weight with, ability to damage the bacterial c envelope causing significant leakage in periplasmic alkaline phosphatase enzyme and cytoplasmic electrolytes. Even

**Appendix V: Manuscript 5 published in Frontiers in Molecular Biosciences,**

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# ADMET profiling and molecula docking of potential antimicrobial peptides previou isolated from African catfish, *Clarias gariepinus*

Hedmon Okella[1,2*], Emmanuel Okello[1,3], Andrew Glory Mte Hilda Ikiriza[2], Bruhan Kaggwa[2,5], Jacqueline Aber[2,6], Christian Ndekezi[7], Joseph Nkamwesiga[8,9], Clement Olusoji Ajayi[2], Ivan Mulongo Mugeni[10], Geofrey Ssentamu[11], Sylvester Ochwo[12], Steven Odongo[11] Casim Umba Tolo[2], Charles Drago Kato[11] and Patrick Ogwang Engeu[2]

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## Appendix VI: Mbarara University of Science and Technology Faculty Research Committee approval

**MBARARA UNIVERSITY OF SCIENCE AND TECHNOLOGY**
**FACULTY OF MEDICINE RESEARCH COMMITTEE**

Ref: DMS 6

October 11, 2018

Mr Okella Hedmon
2017/PHD/093/PS
Department of Pharmacy
MUST

Dear Mr Okello

Submitted Proposal **"Screening and Characterization of antimicrobial peptides from fish mucus in three major lakes of Uganda".**

Reference is made to the above protocol you re-submitted to the Faculty Research Committee (FRC) for review.

The Committee reviewed and approved your protocol.

You are now advised to proceed to produce enough copies of the **Protocol Form** and **Research Proposal** for eventual submission to the Institutional Research Ethics Committee. The Secretary to that Committee will advise you on the number of copies you are to submit.

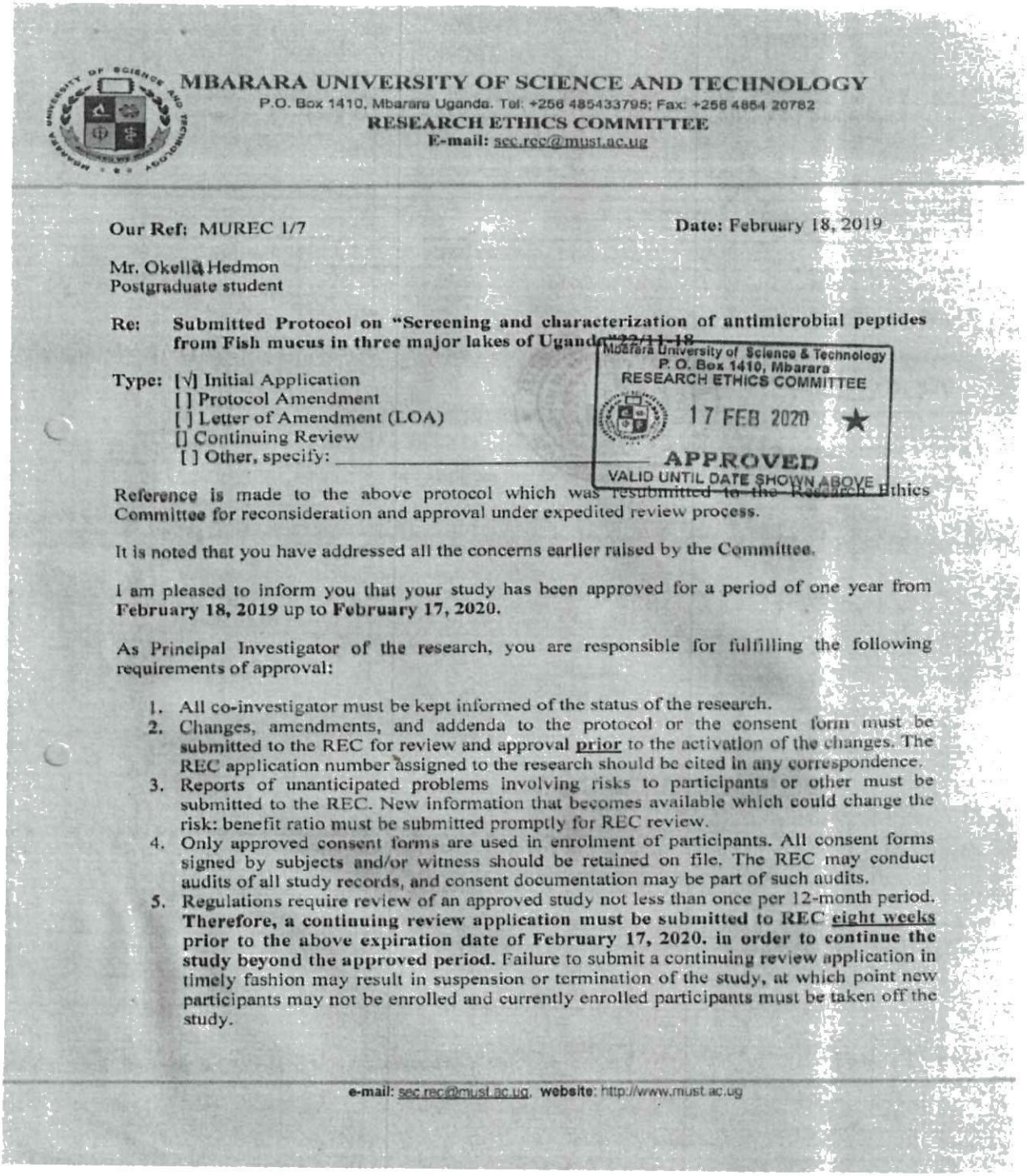
Please remember to attach a letter from the Head of Department or your supervisor when submitting.

Sincerely

Associate Professor Paul Alele
**Chairperson, Faculty Research Committee**

*Copy*: Drs Ogwang Patrick Engeu and Kato Charles Drago, Supervisors

**Appendix VII: Mbarara University of Science and Technology Research Ethics Committee approval**



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Our Ref: MUREC 1/7 Date: February 18, 2019

Mr. Okello Hedmon
Postgraduate student

Re: **Submitted Protocol on "Screening and characterization of antimicrobial peptides from Fish mucus in three major lakes of Uganda" 22/11-18**

Mbarara University of Science & Technology
P. O. Box 1410, Mbarara
RESEARCH ETHICS COMMITTEE
17 FEB 2020
APPROVED
VALID UNTIL DATE SHOWN ABOVE

Type: [√] Initial Application
[ ] Protocol Amendment
[ ] Letter of Amendment (LOA)
[] Continuing Review
[ ] Other, specify: _____

Reference is made to the above protocol which was resubmitted to the Research Ethics Committee for reconsideration and approval under expedited review process.

It is noted that you have addressed all the concerns earlier raised by the Committee.

I am pleased to inform you that your study has been approved for a period of one year from **February 18, 2019** up to **February 17, 2020.**

As Principal Investigator of the research, you are responsible for fulfilling the following requirements of approval:

1. All co-investigator must be kept informed of the status of the research.
2. Changes, amendments, and addenda to the protocol or the consent form must be submitted to the REC for review and approval **prior** to the activation of the changes. The REC application number assigned to the research should be cited in any correspondence.
3. Reports of unanticipated problems involving risks to participants or other must be submitted to the REC. New information that becomes available which could change the risk: benefit ratio must be submitted promptly for REC review.
4. Only approved consent forms are used in enrolment of participants. All consent forms signed by subjects and/or witness should be retained on file. The REC may conduct audits of all study records, and consent documentation may be part of such audits.
5. Regulations require review of an approved study not less than once per 12-month period. **Therefore, a continuing review application must be submitted to REC eight weeks prior to the above expiration date of February 17, 2020. in order to continue the study beyond the approved period.** Failure to submit a continuing review application in timely fashion may result in suspension or termination of the study, at which point new participants may not be enrolled and currently enrolled participants must be taken off the study.

e-mail: sec.rec@must.ac.ug, website: http://www.must.ac.ug

6. You are required to register the research protocol with the Uganda National Council for Science and Technology (UNCST) for final clearance to undertake the study in Uganda.

The following is the list of documents approved in the application:

| Document | Language | Version |
|---|---|---|
| Proposal | English | Version 2 |
| Protocol form | English | Version 2 |
| Data Collection tools | English | February 2019 |

I wish you all the best.

Dr. Francis Bajunirwe
CHAIR,
MUST RESEARCH ETHICS COMMITTEE

RESEARCH ETHICS COMMITTEE
MBARARA UNIVERSITY OF SCIENCE AND TECHNOLOGY
P. O. BOX 1410, MBARARA

**Appendix VIII: Uganda National Council for Science and Technology (UNCST) approval**

UNCST

**Uganda National Council for Science and Technology**

*(Established by Act of Parliament of the Republic of Uganda)*

**Our Ref: HS449ES** **17th October 2019**

Mr. Hedmon Okella
Principal Investigator
Mbarara University of Science and Technology
**Mbarara**

I am pleased to inform you that on **08/10/2019**, the Uganda National Council for Science and Technology (UNCST) approved your study titled, **Screening and Characterization of Antimicrobial Peptides from Fish Mucus in Three Major Lakes of Uganda.** Approval is valid for the period of **08/10/2019** to **08/10/2022.**

Your study reference number is **HS449ES.** Please, cite this number in all your future correspondences with UNCST in respect of the above study.

Please, note that as Principal Investigator, you are responsible for:

1. Keeping all co-investigators informed about the status of the study.
2. Submitting any changes, amendments, and addenda to the study protocol or the consent form, where applicable, to the designated local Research Ethics Committee (REC) or Lead Agency, where applicable, for re-review and approval prior to the activation of the changes.
3. Notifying UNCST about the REC or lead agency approved changes, where applicable, within five working days.
4. For clinical trials, reporting all serious adverse events promptly to the designated local REC for review with copies to the National Drug Authority.
5. Promptly reporting any unanticipated problems involving risks to study subjects/participants to the UNCST.
6. Providing any new information which could change the risk/benefit ratio of the study to the UNCST for final registration and clearance.
7. Submitting annual progress reports electronically to UNCST. Failure to do so may result in termination of the research project.

Please, note that this approval includes all study related tools submitted as part of the application.

Yours sincerely,

Hellen Opolot
For: Executive Secretary
UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

*LOCATION/CORRESPONDENCE*

*Plot 6 Kimera Road, Ntinda*
*P. O. Box 6884*
*KAMPALA, UGANDA*

*COMMUNICATION*

TEL: (256) 414 705500
FAX: (256) 414-234579
EMAIL: info@uncst.go.ug
WEBSITE: http://www.uncst.go.ug

**Appendix IX: Commissioner Fisheries control, Regulation and Quality control assuranc**

**approval**

TELEPHONE: 256-041-320578/320563/322026
FAX: 256-041-320496
E-mail: fishery_department@yahoo.com
wkc@ifinfo.com

In any correspondence on
this subject please quote No.

OFFICE OF THE COMMISSIONER
DEPARTMENT OF FISHERIES
CONTROL, REGULATION & QUALITY
ASSURANCE.
P O BOX 4,
ENTEBBE, UGANDA

THE REPUBLIC OF UGANDA

**The Executive Secretary,**

Uganda National Council for Science and Technology,
P.O Box 66884
Kampala, Uganda

**RE: RESEARCH PROPOSAL CLEARANCE**

Following a submission by Mr. Okella Hedmon (2017/PhD/093/PS), a PhD student of Mbarara University of Science and Technology who intends to conduct a study entitled **"Screening and Characterization of antimicrobial peptides from fish mucus in three major lakes of Uganda"**, the directorate, do here by gives a go ahead for the study.

His proposal involving purchase of 16 fish for research purpose from landing sites of each of the three lakes viz. L. Kyoga, L. Victoria and L. Albert does not in any way contravene ethics nor regulations of the Directorate of Fisheries Resources, Ministry of Agriculture, Animal Resources and Fisheries (MAAIF).

I therefore recommend his novel efforts to your council to enable him progress with the study.
Thank you for your cooperation.

COMMISSIONER FISHERIES
CONTROL, REGULATION AND
QUALITY ASSURANCE
SEP 2019
P. O. BOX
ENTEBBE - UGANDA

Mukasa Tom Bukenya
**Ag. Commissioner Fisheries Control, Regulation and Quality Control.**
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