



Faculty of Medicine and Health Sciences

**Purification and Characterization of Antimicrobial Peptides with
Insulinotropic Activity from the Skin Secretion of Bornean Frogs**

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Purification and Characterization of Antimicrobial Peptides with Insulinotropic Activity from the Skin Secretion of Bornean Frogs

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

The antimicrobial and insulintropic properties of skin secretions from two Bornean frog species in the Ranidae family—*Pulchrana baramica* and *Hylarana erythraea*—and *Limnonectes paramacrodon* from the Dicroglossidae family were investigated. A comprehensive approach utilizing various techniques was employed to study the characteristics of antimicrobial peptides (AMPs) isolated from these frogs. Initially, crude skin secretions were partially purified using Sep-Pak C-18 cartridges, followed by further purification through AKTA HPLC. SDS-PAGE analysis demonstrated that the AKTA HPLC method produced clearer, more distinct bands compared to Sep-Pak, confirming its superior effectiveness. In disc diffusion tests, the peptides exhibited significant inhibition zones against *Escherichia coli* and *Staphylococcus aureus*, indicating strong antibacterial activity. Specifically, *P. baramica* showed inhibition zones of 11.66 ± 3.44 and 11.33 ± 0.9 mm at 1000 $\mu\text{g/ml}$ and 500 $\mu\text{g/ml}$ concentrations, respectively, against *E. coli*, and 5.6 ± 0.6 mm against *S. aureus* at 1000 $\mu\text{g/ml}$. Meanwhile, *H. erythraea* displayed inhibition zones of 7.33 ± 0.5 and 4.3 ± 0.6 mm at 1000 $\mu\text{g/ml}$ and 500 $\mu\text{g/ml}$ concentrations, respectively, against *E. coli*, and 4.6 ± 0.6 mm against *S. aureus*. No antimicrobial activity was observed for *L. paramacrodon*. Minimum inhibitory concentration (MIC) tests revealed species-specific values, with AMPs from *P. baramica* demonstrating higher potency than those from *H. erythraea* (MIC: 250 $\mu\text{g/ml}$). Regarding cytotoxicity, AMPs purified from both Ranid frogs caused over 50% lysis of red blood cells, indicating potential impacts on mammalian erythrocyte membrane integrity, whereas *L. paramacrodon* peptides displayed lower toxicity with less than 50% cell lysis. Furthermore, the insulintropic effects of Bornean frog AMPs on HIT-T15 cells were examined. These AMPs were found to enhance insulin secretion from pancreatic cells, with statistical analysis indicating a significant effect ($p < 0.05$) for *P.*

baramica. In contrast, the effects of *H. erythraea* and *L. paramacrodon* were not significant ($p > 0.05$). These findings suggest that multifunctional peptides play a crucial role in protecting Bornean frogs against invading pathogens and hold potential as antidiabetic agents for managing glucose levels and diabetes.

Keywords: Antimicrobial peptides, Bornean amphibians, frog skin secretions, insulin-related properties.

Penulenan dan Pencirian Peptida Antimikrob yang mempunyai Aktiviti Insulinotropik daripada Rembesan Kulit Katak Borneo

ABSTRAK

Sifat antimikrob dan insulinotropik dari Rembesan kulit katak Borneo dari keluarga Ranidae, termasuk Pulchrana baramica dan Hylarana erythraea, serta Limnonectes paramacrodon dari keluarga dicroglassidea, telah disiasat. Pendekatan menyeluruh menggunakan pelbagai teknik telah digunakan untuk mengkaji ciri-ciri peptida antimikrob (AMP) yang diasingkan daripada katak-katak ini. Pada permulaan, rembesan kulit mentah telah melalui proses separa penulenan menggunakan katrij Sep Pak C-18, diikuti dengan penulenan lanjut melalui kaedah HPLC AKTA. Analisis ketulenan peptida melalui elektroforesis SDS-PAGE menunjukkan bahawa kaedah HPLC AKTA menghasilkan jalur yang lebih jelas dan bersih berbanding dengan Sep-Pak, menunjukkan keberkesanan yang lebih baik. Dalam ujian penyebaran cakera, zon-zon perencatan yang signifikan terhadap kedua-dua E.coli dan S.aureus telah diperhatikan, menunjukkan keberkesanan peptida dalam menghalang pertumbuhan bakteria. P. baramica menghasilkan zon perencatan sebanyak 11.66 ± 3.44 dan 11.33 ± 0.9 untuk kepekatan $1000 \mu\text{g/ml}$ dan $500 \mu\text{g/ml}$, masing-masing, terhadap E.coli, dan 5.6 ± 0.6 terhadap S.aureus pada $1000 \mu\text{g/ml}$, manakala H. erythraea menunjukkan zon-zon perencatan sebanyak 7.33 ± 0.5 dan 4.3 ± 0.6 untuk $1000 \mu\text{g/ml}$ dan $500 \mu\text{g/ml}$, masing-masing, terhadap E.coli dan zon perencatan 4.6 ± 0.6 terhadap S.aureus. L.paramacrodon tidak menunjukkan aktiviti antimikrob. Ujian kepekatan perencatan minimum (MIC) memberikan nilai-nilai spesifik untuk bakteria yang diuji, dengan AMP dari P.baramica menunjukkan keberkesanan yang lebih besar berbanding dengan H.erythraea, dengan MIC sebanyak $250 \mu\text{g/ml}$. Mengenai sitotoksik, AMP yang disucikan dari kedua-dua katak Ranid telah menyebabkan lebih daripada 50% lisis sel

darah, mencadangkan kesan potensi terhadap integriti membran eritrosit mamalia, berbeza dengan toksisiti yang lebih rendah yang dilihat dalam peptida L.paramacrodon. Selanjutnya, kesan insulinotropik AMP dari katak Borneo ke atas sel HIT-T15 telah disiasat. AMP ini didapati meningkatkan pengeluaran insulin dari sel pankreas, dengan analisis statistik menunjukkan perbezaan yang signifikan ($p < 0.05$) bagi P.baramica manakala kesan H.erythraea tidak signifikan ($p > 0.05$). Penemuan ini menunjukkan bahawa peptida multifungsional ini memainkan peranan penting dalam mempertahankan katak Borneo daripada patogen yang menyerang dan mempunyai aplikasi potensi sebagai ejen antidiabetik untuk menguruskan aras glukosa dan diabetes

Kata kunci: Peptida antimikrob, amfibia Borneo, sekresi kulit katak, sifat berkaitan insulin.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
%	Percentage
µg/mL	Microgram Per Milliliter
µL	Microliter
ACPs	Anticancer Peptides
AMP	Antimicrobial Peptides
APS	Ammonium Persulphate
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CFU/ml	Colony Forming Unit Per Milliliter
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
HC	Hemolytic Concentration
HCL	Hydrochloric Acid
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HSV-1	Herpes Simplex Virus Type-1
KDa	Kilo Dalton
LDH	Lactase Dehydrogenase
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin Resistant <i>Staphylococcus Aureus</i>

MWCO	Molecular Weight Cut Off
PGLa	Peptide Starting with a Glycine and Ending with a Leucine Amide
RBC	Red Blood Cell
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide
T2DM	Type-2 Diabetes Mellitus
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic Acid
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 Exploring Frog-Derived Antimicrobial Peptides (AMPs): A Promising Frontier in Understanding Insulin Secretion

This chapter introduces the research focus on exploring frog-derived antimicrobial peptides (AMPs) and their potential role in regulating insulin secretion. It provides background information on the significance of insulin in metabolic processes and highlights the issue of insulin deficiency, which underpins metabolic disorders like diabetes. The chapter further examines the unique bioactive peptides found in amphibian skin secretions, emphasizing their therapeutic potential. It specifically explores insulin-releasing peptides derived from frog skin, which have shown promise in stimulating insulin secretion and offering new therapeutic options for diabetes management. The chapter concludes with an overview of the study's objectives, focusing on isolating, purifying, and characterizing the insulinotropic properties of host defense peptides from Bornean frog species.

The insulin hormone, discovered nearly a century ago, plays a significant role in regulating metabolic processes within human physiology, as noted by White et al. (2021). As one of the body's principal hormonal messengers, a profound influence on the maintenance of blood glucose levels, energy utilization, and overall metabolic harmony is exerted by insulin. Its discovery revolutionized the understanding of diabetes and metabolism, remaining at the forefront of medical research and serving as a lifeline for millions of individuals with diabetes worldwide.

Produced and secreted by specialized cells within the pancreas known as beta cells, insulin, a peptide hormone, facilitates the uptake, utilization, and storage of glucose by cells

throughout the body, ensuring the vital energy necessary for their functions (Michael et al., 2006). Beyond its central role in glucose homeostasis, lipid and protein metabolism are also regulated by insulin (Qaid et al., 2016), making it a master conductor in the intricate symphony of metabolic processes.

However, the balance maintained by insulin can be disrupted, leading to insulin deficiency. This hormone, often called the 'master regulator' of glucose metabolism (Hatting et al., 2018), plays a crucial role in regulating blood sugar levels. When the mechanisms responsible for insulin production, secretion, or action are impaired, it can result in various metabolic disorders, with diabetes mellitus being a primary concern affecting millions globally.

A critical juncture in the intricate interplay of hormonal regulation is marked by insulin hormone deficiency, impacting the fundamental processes underpinning energy utilization and storage. This deficiency can manifest in different forms, each with its unique implications for metabolic health. Whether due to autoimmune destruction of insulin-producing beta cells in type 1 diabetes (Haller et al., 2005) or because of impaired insulin signalling and resistance in type 2 diabetes (Saini et al., 2010), the consequences of insulin deficiency reverberate throughout the body, affecting not only glucose metabolism but also lipid and protein homeostasis.

In conclusion, this exploration into insulin hormone deficiency underscores the critical importance of understanding and addressing metabolic disorders, particularly diabetes mellitus. As the complexities of insulin deficiency are navigated, traditional treatments such as insulin replacement therapies and glucose-lowering medications continue to play a crucial role in disease management (Tan et al., 2019). However, the ever-evolving

landscape of medical innovation has introduced novel alternatives, for example insulin-triggering peptides derived from frog skin secretion, promising new avenues for the development of new sustainable drugs in the treatment of diabetes mellitus.

1.2 Amphibian Skin Secretions

The amphibian skin, exposed directly to various environmental elements, plays a pivotal role in their interactions with microorganisms, parasites, predators, and physical surroundings (Varga et al., 2019). Its essential chemical diversity and versatile nature are crucial for the everyday survival of these amphibians, leading to considerable interest in amphibian skin secretions due to their distinct chemical properties, biosynthesis pathways, and potential clinical applications (Mans et al., 2020).

In recent decades, extensive research efforts have been directed towards uncovering the bioactive components within amphibian skin secretions, with a focus on biologically active peptides. These peptides, spanning diverse families such as myotropical peptides, opioid peptides, corticotropin-releasing peptides, angiotensins, protease inhibitor peptides, neuropeptides, antioxidant peptides, lectins, insulin-releasing peptides, mast cell degradation/histamine-releasing peptides, wound-healing peptides, immunomodulatory peptides, neuronal nitric oxide synthase inhibitors, antimicrobial peptides, antiviral peptides, antitumor peptides, anti-parasite peptides, pheromone peptides, granains, and other varieties, have been extensively studied (Indriani et al., 2023).

Specialized skin granular glands store these peptides, which can be released in significant concentrations into skin secretions, especially during times of stress or injury for the amphibian (Rollins-Smith et al., 2023). Moreover, the historical use of skin extracts from frogs and toads in traditional medicine highlights the enduring significance of these

compounds, with Chinese traditional medicine, for instance, employing them for centuries to treat inflammation and infections (Yacoub et al., 2020).

The exploration of the structures and biological functions of these active peptides derived from amphibian skin secretions holds great promise for the development of novel therapeutic agents. This not only illuminates the intricate world of amphibian biology but also provides numerous opportunities for advancing medical science and enhancing human health.

1.2.1 Biological Properties of Amphibian Skin Peptides

In general, peptides consist of a chain of amino acids linked by peptide bonds between the carbonyl and amine groups of adjacent amino acids, as shown in **Figure 1.1**, and divided into classes based on classification criteria such as net charge, secondary structure and solubility (Depta et al., 2022). Most AMPs are low molecular weight, contain 11 to 46 amino acid residues, and have a net cationic charge at physiological pH due to the high content of lysine and arginine in their peptide chains (Brown & Hancock, 2006). The net charges of AMPs have a key effect on their antimicrobial abilities. Increasing positive charge of AMP is a strategy to improve antimicrobial potency (Xiao et al., 2011).

In addition, AMPs with hydrophobic surface are highly amphiphilic (Reddy et al., 2004). Previous studies have shown that frog AMPs form helical structures in a simulated solution with a membrane called trifluoroethanol (TFE). In addition, most AMPs identified so far have positive charges ranging from +1 to +34 (Fillian et al., 2015). For example, the net charges of Temporin-L from *Rana temporaria* and temporin-CPa from *Lithobates* is 3+ (Mongoni et al., 2011). The net charge of phylloepin-3 isolated from *Phylomedusa sauvagi* is +1 (King et al., 2013).

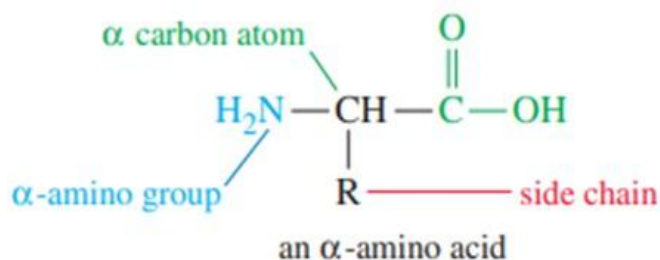


Figure 1.1: Schematic diagram of an amino acid structure. The central carbon of the amino acid is depicted in the diagram with its four bonds, which include the bonds with the carboxyl group, amino group, a hydrogen atom, and a side chain (Sandlers, 2019).

The skin secretions of all frog species take a unique approach to skin peptides. To date, no species with similar skin secretion characteristics have been identified (Conlon et al., 2007). However, some peptides were found to be dominant due to geographic area. For example, bomber-like peptides are found in the skin secretions of European and Asian frogs, whereas sirloin peptides are common in South American and African frogs (Roelants et al., 2013). Species of the different family and genus may possess the same peptide. For example, Brevinin-1 from *Hoplobatrachus rugulosus* and *Odorrana grahami* store phenylalanine, proline, alanine, cysteine, and lysine at the same positions in the amino acid sequence (Tian, et al 2023, Allen, 2011).

Peptide profiles can be obtained by examining crude secretions via chromatographic methods such as high-performance liquid chromatography (HPLC). Another method that can be used for protein analysis and identification is laser adsorption or matrix ionization (MALDI). Organic compounds called “matrix” are used to increase adsorption and ionization efficiency (Sato, 2018).

Frog skin, typically moist and exposed, creates an ideal environment for microorganisms to thrive. However, the evolutionary forces of natural selection have gradually shaped the frog's defence system, enabling it to protect itself from the various

threats present in its natural habitat (Longo et al., 2017), The composition of a frog's skin primarily consists of two layers, the epidermis and dermis, housing essential components such as mucous glands, poison glands, and pigment cells (Varga et al., 2019). Notably, mucous glands play a pivotal role in the frog's defence mechanism. They produce copious amounts of mucous containing glycoproteins and proteoglycans, forming a complex polypeptide mesh that acts as a natural barrier, preventing pathogens from invading the skin (Mauricio et al., 2021). In their adaptation to diverse habitats and ecological niches, amphibians have developed poison glands within the dermal layer. These glands produce an array of peptides, proteins, steroids, and alkaloids.

The utilization of frog secretions for medicinal purposes can be traced back to ancient Chinese history, where the secretions from the auricular and skin glands of *Bufo gargarizans* Cantor and *Bufo melanostictus* Schneider were collected and desiccated. This traditional Chinese medicine, known as "Venenum Bufonis," was employed for the treatment of conditions such as sunstroke, fainting, and acute gastrointestinal issues like abdominal pain, vomiting, and diarrhea (Wei et al., 2019). Furthermore, dried toad skins found their place in another Chinese medicinal tradition for the treatment of cancer (Xu et al., 2015).

In contemporary times, both the secretions and skins of toads have been scientifically proven to possess significant therapeutic effects, particularly in relieving conditions such as neglected tropical diseases (NTDs), arrhythmias, and other heart diseases (Lewies, et al., 2015). To date, a plethora of natural peptides extracted from frog skins have exhibited diverse biological activities, including but not limited to haemolytic activity, smooth muscle activity, antimicrobial activity, anticancer activity, and anti-diabetic activity, among others. These findings underscore the immense potential of frog skin-derived compounds in the

realm of biomedicine, opening doors to novel therapeutic avenues and furthering our understanding of nature's ingenious adaptations. Table 1.1 summarizes the diverse biological activities exhibited by peptides from frog skin.

Table 1.1: Diverse Biological Activities Exhibited by Peptides Extracted from Frog Skins, (Wang, 2020). (APD) Antimicrobial Peptide Database

Activity	APD Count	Example
Antibacterial	982	Magainin 2, Brevinin-1, Temporin-SHf
Anti G+	169	Temporin-10la, Aurein 5.2, Phylloseptin-S3
Anti G-	84	XT-2, Dermaseptin DA4, Leptoglycin
Anti MRSA	54	Ranalexin, Temporin-1GHd, Temporin-10La
Anti-fungal	490	Dermaseptin-S1, Temporin-1GHd, PGLa
Anti-virus	45	Brevinin-1, Urumin, Temporin B,
Anti-HIV	36	Brevinin-1, Urumin, Temporin B,
Antiparasitic	53	Dermaseptin-S1, Temporin L, Phylloseptin-S4
Anticancer	93	Magainin 2, Aurein 1.2, Dermaseptin
Anti diabetes	15	Esculentin-1, Amolopin, Dermaseptin-B4
Wound healing	4	Magainin 2, Temporin A, Brevinin-2Ta
Anti-oxidant	19	Temporin-TP1, Pleurain-A1, Brevinin-1TP3

1.2.2 Insulin-Releasing Peptides from Skin Secretion of Frogs

The study of amphibian skin secretions has emerged as a treasure trove of bioactive compounds, offering a rich source of molecules with diverse physiological activities. Among these, insulin-releasing peptides derived from frog skin secretions have garnered

considerable attention in recent scientific exploration. Frogs, with their unique skin anatomy and the presence of specialized glands, produce a plethora of bioactive peptides as a part of their innate defense mechanisms. Beyond their role in amphibian immunity, certain peptides have exhibited the remarkable ability to influence insulin release, presenting an intriguing avenue for potential therapeutic applications. The search for antidiabetic peptides from animal sources has gained momentum after the approval of exenatide, isolated from the venom of *Heloderma suspectum*, for the treatment of Type 2 diabetes mellitus (Ahren., 2019). The peptides found in skin secretions of frogs, which were initially characterised based on antimicrobial activity showed the ability to demonstrate insulin-releasing activity (Conlon et al., 2014).

The isolated frog skin peptides, as demonstrated in studies by Conlon et al. (2018) and Musale et al. (2019), exhibited insulin release both in vitro using rat clonal pancreatic beta cells (BRIN-BD11 cells), human-derived pancreatic beta cells (1.1B4), primary islet cells, and in vivo in lean and high-fat-fed mice. Additionally, these peptides were found to possess insulin-releasing activity when derived from frog skin secretions of various families, including Alytidae, Hylidae, Pipidae, and Ranidae. The isolated active compound demonstrated concentration-dependent insulin release, exhibited identity with brevinin-1, and temporin, the antimicrobial peptides found in the skin secretion of various *Rana* species. Subsequently, novel insulin releasing peptide (24 amino acid) was isolated from skin secretion of *Rana pipiens*. This peptide showed 100% homology to pipinin-1, which was initially characterised as an antimicrobial peptide (Musale et al., 2019). Similarly, four insulintropic peptides were isolated from skin secretions of *Rana saharicus* that were identical to antimicrobial esculentin-1, esculentin-1B brevinin-1E and brevinin-2EC (Ojo et al., 2013).

Antimicrobial peptides belonging to the temporin family have also demonstrated insulin-releasing activity. Temporin-A, Temporin -CBF, Temporin -DRb, and Temporin -TGb isolated from *Rana temporaria*, *Lithobates cetasbeianus*, *Rana draytonii* and *Rana tagoi* (Musale et al., 2018) stimulated dose-dependent insulin release from BRIN-BD11 cells without affecting cell viability.

In the literature, Temporin-Oe derived from *Rana ornativentris* was found to be highly potent, exhibiting a remarkable 2.6-fold increase in insulin release compared to the control group, as reported by Conlon et al. (2014). Additionally, peptides belonging to the Phylloseptin family displayed significant insulinotropic effects. Phylloseptin-L2, a 15 amino acid peptide isolated from *Hylomantis lemur*, was shown to stimulate insulin release from BRIN-BD11 cells through a K-ATP channel-independent pathway, as evidenced by Soltaninejad et al. (2021). This peptide was also observed to enhance glucose tolerance in healthy mice by elevating insulin concentrations. Moreover, skin secretions from frogs within the subfamily *Phyllomedusinae* were found to contain peptides with insulin-releasing properties. For instance, the RK-13 peptide from the skin secretions of *A. calcarifer* was able to stimulate insulin release from BRIN-BD11 cells in a dose-dependent manner without affecting cell viability. Early mechanistic studies indicated that the AMPs may trigger insulin release through the activation of the protein kinase A (PKA) pathway, as suggested by Musale et al. (2021).

The recent literature review delves into a study by Musale (Musale, 2019), emphasizing the insulinotropic effects of the Tigerinin-1R peptide (RVCSAIPICH.NH₂) found in the skin secretions of the frog *Hoplobatrachus rugulosus*, previously identified as *Rana rugulosa*. This unique peptide's insulinotropic effects were notably independent of

LDH enzyme release, indicating the preservation of plasma membrane integrity. Intriguingly, Tigerinin-1R exhibited a maximum response surpassing that of GLP-1 and GIP in identical experimental conditions. It shared similarities with KCl and alanine in depolarizing BRIN-BD11 beta cells and elevating intracellular Ca^{2+} levels. These insights suggest potential therapeutic applications of Tigerinin-1R in the realm of insulin regulation. Furthermore, the study underscores the essential role of C-terminal amidation in conferring robust insulintropic potency. The amine group at the C-terminus is critical for this amidation process, contributing to the peptide's stability and bioactivity. This functional group also plays a key role in receptor binding and the subsequent signaling cascade that enhances insulin secretion. Previous study on the findings of Conlon et al. (2023) reported dose-dependent insulin-releasing effects of Ocellatin-3N and [D4K] ocellatin-3N, isolated from the Caribbean frog *Leptodactylus nesiotus*, in both BRIN-BD11 and isolated mouse islets. Preliminary mechanistic studies hinted at these peptides stimulating insulin release through the PKA pathway. Additionally, in high-fat-fed mice, Hymenochirin-1B was observed to significantly improve glycemic control, accompanied by an increase in insulin release after acute administration (Amatya, et al., 2020). These collective findings contribute to the expanding understanding of peptides and their potential in diabetes management and related therapeutic avenues.

1.3 Frog Species Used in the Study

The selection of *Pulchrana baramica*, *Hylarana erythrae*, and *Limnonectes paramacrodon* frog species for this study was based on their unique and diverse peptide profiles, which provide valuable insights into amphibian-derived bioactive compounds. Their distinct geographic distribution and ecological adaptations also offer a broad spectrum of peptides for comparison, enhancing the scope of the study. These frogs, found in the

Borneo area, are considered a natural treasure that has not been fully investigated yet. Therefore, this study will contribute to the Sarawak frog database. By investigating these frogs, the research aims to explore the insulinotropic effects of their peptides and identify novel bioactive molecules that could inform future therapeutic strategies for metabolic disorders.

1.3.1 *Pulchrana baramica*

Pulchrana baramica, shown in **Figure 1.2**, is classified as a member of the "true frog" species within the Ranidae family. It is found across various regions in Asia, in accordance with its distribution pattern. This species was originally identified in the vicinity of the Baram River in Sarawak, Malaysia, which contributes to one of its commonly used names. Its native habitats primarily encompass tropical moist lowland forests and swamplands.



Figure 1.2: Genus *Pulcharana baramica* from the Family Ranidae

1.3.2 *Hylarana erythraea*

The common green frog (*Hylarana erythraea*), shown in **Figure 1.3**, is a member of the Ranidae family, and is commonly referred to as the "true frog," thrives in Southeast Asia. This frog species is recognized by various names, including the green paddy frog, red-eared frog, or leaf frog. While some sources still use the former name *Rana erythraea*, *Hylarana erythraea* is the currently accepted scientific nomenclature for this amphibian.



Figure 1.3: Genus *Hylarana erythraea* from the Family Ranidae

1.3.3 *Limnonectes paramacrodon*

Limnonectes paramacrodon **Figure (1.4)**, commonly recognized as the masked swamp frog, represents a frog species within the Dicroglossidae family. This amphibian species can be encountered on Balambangan Island in Sabah, as reported by Zainudin et al. in 2011. Additionally, it inhabits the Ulu Sebuyau National Park (USNP), situated along the lower Batang Lupar river, which encompasses regions primarily characterized by peat swamp forests within the Samarahan and Sri Aman Divisions of Sarawak, as documented by Silang in 2014. These frogs naturally inhabit lowland swamp forest areas with small rivers and streams. Unfortunately, due to habitat loss, they are gradually becoming scarce and have

consequently earned the classification of near threatened (NT) in the IUCN Red List for the year 2015.



Figure 1.4: Genus *Limnonectes paramacrodon* from the family Dicroglossidae.

1.4 Problem Statement

Antimicrobial resistance (AMR) is one of the most pressing health challenges of the 21st century, threatening the effectiveness of current antibiotics and increasing the burden of infectious diseases worldwide. The search for novel antimicrobial agents is critical to addressing this growing threat. Amphibians, including frogs, produce a diverse range of bioactive compounds in their skin secretions, notably antimicrobial peptides (AMPs), which serve as a defense mechanism against microbial pathogens. These AMPs have demonstrated broad-spectrum antimicrobial activity and may also possess additional biological properties, including insulintropic effects, which could make them valuable for treating both infections and metabolic disorders like diabetes.

While there has been increasing interest in amphibian-derived peptides, the Bornean frogs, particularly those from the Ranidae family, remain largely underexplored as a source of therapeutic peptides. Despite their potential, there is limited research focused on isolating,

characterizing, and evaluating the antimicrobial and insulintropic properties of these peptides. This research aims to fill this gap by isolating, purifying, and characterizing antimicrobial peptides from the skin secretions of three Bornean frog species. The study will further investigate the effects of these peptides on cell viability and their potential to stimulate insulin release in vitro, with the goal of identifying novel candidates for therapeutic applications in both infectious and metabolic diseases.

1.5 Objectives of Study

This research aims are to isolate and identify antimicrobial peptides from the skin secretions of three Bornean frogs belonging to the Ranidae family. The isolated peptides, which were subsequently purified to homogeneity, were subjected to an array of investigations to characterize their antimicrobial and insulintropic properties.

The specific objectives of this research include:

- i. To isolate, purify and characterize the antimicrobial peptides from Bornean frogs' skin secretions.
- ii. To determine the effect of the antimicrobial peptide on cell viability of HIT-T15 cells.
- iii. To determine the insulintropic activities of the identified antimicrobial peptides.

1.6 Hypothesis

- i. The antimicrobial peptides isolated from the skin secretions of Bornean frogs exhibit significant antimicrobial activity against a range of bacterial, suggesting their potential as novel antimicrobial agents.

- ii. The antimicrobial peptides isolated from Bornean frogs do not induce cytotoxic effects on HIT-T15 cells at concentrations required for antimicrobial activity, demonstrating their selectivity and safety for use in therapeutic applications.
- iii. The antimicrobial peptides isolated from Bornean frogs possess insulinotropic activity, stimulating insulin secretion from HIT-T15 cells, which could have potential applications in the treatment of diabetes.

1.7 Thesis Outline

The thesis comprises six chapters. Chapter 1 presents an introduction of the whole thesis; the research background and the research objectives. Chapter 2 presents the literature review. Chapter 3 contains the methodology of this research. It describes the research process, materials and methods, equipment and justifications based on contemporary work done. Chapter 4 presents the results with comparisons done in the characteristics of the three anuran families studied in this thesis. Chapter 5 presents the explanation and discussion of the results. Chapter 6 presents the conclusion of the whole research project.

CHAPTER 2

LITERATURE REVIEW

2.1 General Review of Distribution and Habitat of Anurans in the World

Anurans, a captivating and diverse assemblage of amphibians, traverse both aquatic and terrestrial realms, spanning across the continents of North and South America, Europe, Africa, Asia, and Australia. Notably, they remain conspicuously absent from the frigid landscapes of Antarctica. This expansive group encapsulates an astonishing tapestry of biodiversity, comprising a staggering 7,338 species globally, as meticulously documented by Frost (2021). Among these continents, South America emerges as a focal point, exhibiting the highest concentration of frog species, with approximately 1,119 meticulously documented by Guerra et al. (2020), and Brazil alone contributing substantially to this rich diversity. On the African front, Nigeria hosts a notable presence of 388 frog species, as illuminated by Onadeko et al. (2016). Simultaneously, Europe, North America, Australia, and Asia each offer diverse habitats for Anurans, accommodating variable numbers of frog species that range from as few as 10 to as many as 90, a spectrum that underscores the fascinating regional variations in Anuran diversity.

In Figure 2.1, sourced from (Zapata et al.,2022), an illustrative depiction unfolds, offering a visual synthesis of the worldwide distribution of anurans. These remarkable ectothermic vertebrates have displayed an exceptional capacity for adaptation, successfully inhabiting an extensive spectrum of environments, which include freshwater ecosystems, ponds, lakes, swiftly flowing streams, verdant rainforests, and even elevated vegetative landscapes. Notably, the distribution patterns of frogs on a global scale are intricately shaped by influential variables such as humidity levels, altitude, and the specific types of vegetation,

as expounded upon by Glime et al. 2017) in their study. Noteworthy is the observation that frog species richness tends to decline at higher elevations, a phenomenon intricately intertwined with various factors, encompassing physiological ecology and the environmental characteristics of particular region (Carvalho-Rocha et al., 2021).

Extensive research endeavors have been devoted to the examination of frog populations in various tropical regions across different continents. However, some areas have been insufficiently investigated, with Libya serving as a notable illustration, as highlighted by Bauer et al. (2017). Nonetheless, the predominant issue centers on the concerning decline in the populations of numerous frog species. This troubling trend is primarily ascribed to a constellation of factors, including infectious diseases, the impacts of global climate change, habitat degradation, and the ominous threat of extinction, as expounded upon by Rose et al. (2021).

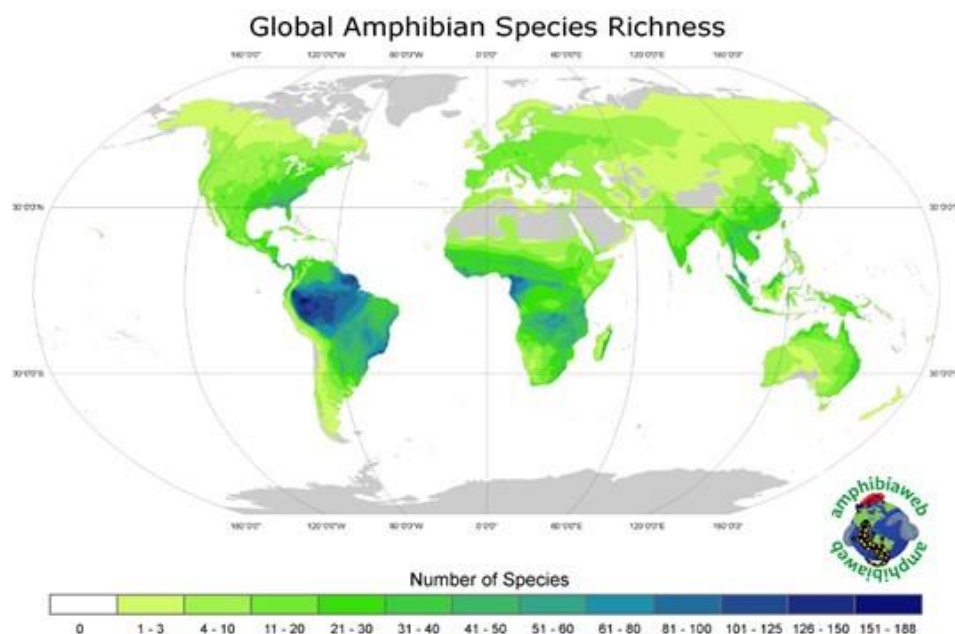


Figure 2.1: Distribution of Anurans in the World (Zapata et al.,2022)

2.2 Bornean Frogs

Bornean frogs, the amphibian inhabitants of Borneo, an island divided among Indonesia, Brunei, and East Malaysia (Sabah and Sarawak), constitute a diverse and intriguing subject of study. Inger and Stuebing (2005) documented approximately 150 frog species thriving in this region. These frogs exhibit a remarkable adaptability, occupying a wide array of habitats, including peat swamps, waterfalls, streams, high-altitude areas, and the forest floors of tropical rainforests.

Within Borneo, a mosaic of frog diversity is represented by seven distinct families: Bombinatoridae, Bufonidae, Ceralobatrachidae, Dicroglossidae, Microhylidae, Ranidae, and Rhacophoridae. Notably, some of these families, like Bombinatoridae, are comprised of a single species, exemplified by *Barbourula kalimantanensis*, as noted by Inger and Stuebing (2005). In contrast, other Bornean frog families thrive abundantly in the lush rainforests and national parks of Sabah and Sarawak, as attested by Das et al. (2014) in their findings.

Frogs have a wide range of habitat preferences, including rocky streams, marshy areas, and hilly terrains, as documented by Inger and Stuebing (2009). Interestingly, even among frog species from the same genus, there can be differences in where they are commonly found in a particular region. For instance, in Sarawak, Zainudin et al. (2017) studied three species from the *Limnonectus* genus. *Limnonectus finchi* and *Limnonectus ibanorum* are well adapted to hilly terrains and can be found throughout Borneo. On the other hand, *Limnonectus leporinus* is typically found at elevations below 600 meters above sea level, except in peat swamps.

Another interesting case is the frog species *Staurois latopalmatus*, part of the *Staurois* genus, which exclusively inhabits rocky streams with swift currents across all regions of

Borneo. However, it does not occur in vegetated or hilly terrains, as observed by Inger (2003). These variations in habitat preferences among closely related frog species highlight the complex ecological factors at play and offer valuable insights into the distribution and adaptation of frogs in the diverse landscapes of Borneo.

In a study carried out by Das et al. (2007), a total of 55 amphibian species were documented in Kubah National Park and Matang National Park, comprising 53 Anura (frogs) and 2 Gymnophiona (caecilians). These national parks are located within the Matang Range, situated approximately 22 kilometers northwest of Kuching in Sarawak. The sampled species represented various families, including Microhylidae, Bufonidae, and Ranidae. Most of these anurans were predominantly observed at elevations below 200 meters within the mixed dipterocarp forest. Additionally, frogs can also be found in plantation areas in west-central Sarawak and along the streams of the Kapit district. Figure 2.2 below summarizes the distribution of frogs throughout Borneo.

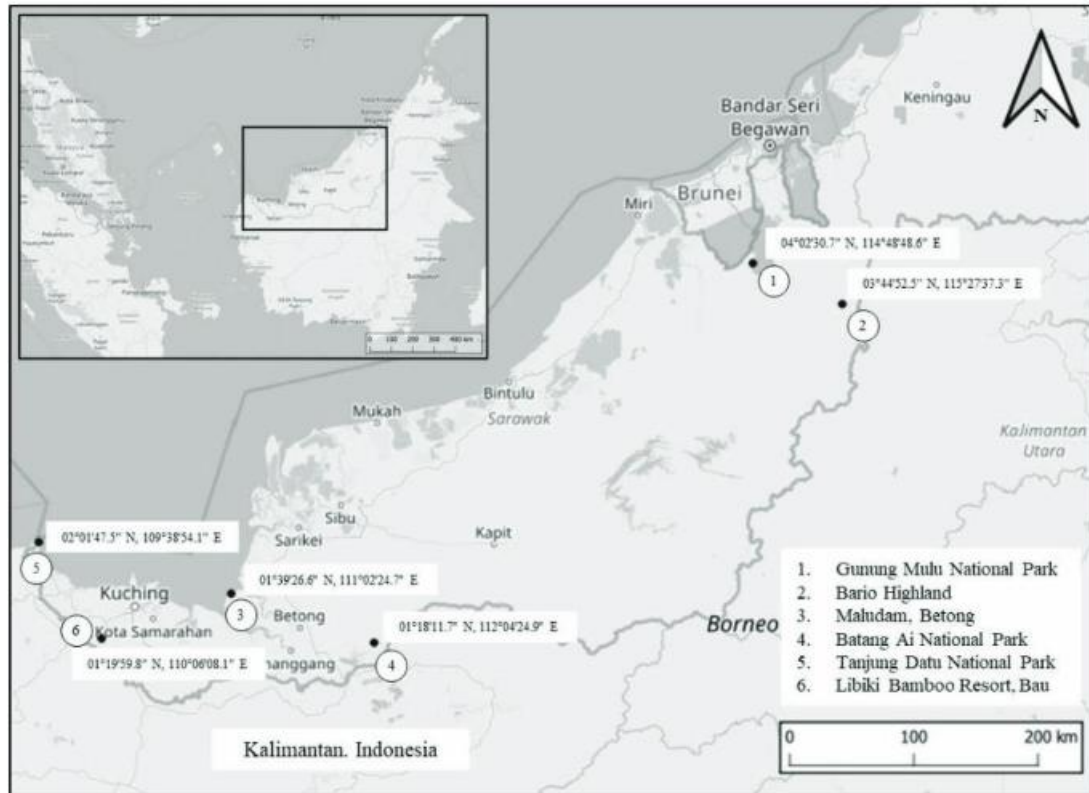


Figure 2.2: Distribution of frogs in Borneo . A map of the sampling localities in Sarawak that are populated with varieties of anurans species.(Zulkefliet al.,2022).

2.3 Frogs Belonging to Ranidae and Dicroglossidae Families

The current study focused on the Ranidae and Dicroglossidae families. Ranid frogs are known to have the AMPs as part of their defense system against the adverse microorganisms in their environments (Lin et al., 2021). However, only a few genera are found in Southeast Asia, including *Abavorana*, *Chalcorana*, *Huia*, *Hylarana*, *Meristogenys*, *Odorrana*, *Pulchrana*, and *Staurois* (see Table 2.1), while the Dicroglossidae distributed by the tropical and subtropical regions of Africa, Asia, and Papua New Guinea (Escoriza et al., 2019). The taxonomy of these anurans was recently updated in the Amphibian Web, as indicated in Table 2.1. This research specifically investigated skin secretions from the *Pulchrana baramica*, *Hylarana erythraea* and *Limnonectes paramacrodon* genera, which are native to the specific areas sampled in this study.

Table 2.1: Location and Count Details of Identified Family Ranidae and Dicroglossidae Species (Moen, et al 2013).

Frog Genus	Number	Location
Ranid Frog distribution		
Amnirana	12	South East Guinea
Amolops	51	China
Babina	10	China
Chalcorana	11	South East Asia
Clinotarsus	3	India
Glandirana	5	China
Huia	5	South East Asia
Humerana	3	North East India
Hydrophylax	4	Sri Lanka
Hylarana	12	South East Asia
Meristogenys	13	Western Sri Lanka
Odorrana	60	South East Asia
Papurana	16	South Asia
Pelophylax	26	Greece, Albania
Dicroglossidae frog distribution		
Fejervarya	17	Southeast Asia
Hoplobatrachus	6	Malay Peninsula, Myanmar
Limnonectus	17	Asia: China, Indochina
Occidozyga	19	Thailand, Malay, China

2.3.1 Bornean Ranids: Exploring Ranidae Genus in Borneo

Ranid frogs are locally known as true frogs. In Borneo, East of Malaysia, nine genera were reported by Inger and Stuebing (2005) as discussed earlier in Section 2.2. Each species under the said genus were tabulated in Table 2.1. Currently, the genus *Pulchrana* of Sarawak consists of *P. baramica*, *P. glandulosa*, *P. laterimaculata*, *P. picturata* and *P. signata* anuran species (Marly et al., 2017). While four Asiatic amphibian species belonging to the genus *Hylarana*, of the family Ranidae, namely *Hylarana erythraea*, *Hylarana macrodactyla*, *Hylarana taipehensis*, and *Hylarana tytleri*, have been known for over a century. However, three of these species (*H. erythraea*, *H. taipehensis*, and *H. tytleri*) continue to be confounded due to a similar green color pattern on their backs (Hasan et al., 2019). This confusion has led to numerous identification mistakes and a limited understanding of the species distribution ranges. Consequently, there is an urgent need to acquire more comprehensive insights into the distribution areas and to enhance our understanding of how these species interact with each other.

The remaining species are predominantly found in Sabah. This study specifically focused on analyzing secretions from *Pulchrana baramica* and *Hylarana erythraea*, commonly recognized as a torrent frog, inhabiting rocky streams within the lowland and hillside forests of western Sarawak. Recent study conducted on Bornean frogs belonging to this genus, which is *Pulchrana glandulosa* showed a wide range of activities with biomedical importance, such as antimicrobials, anti-tumour, anti-cancerous, anti-viral, wound healing, anti-inflammation, anti-ageing and maintaining homeostasis (Ong et al., 2023).

Currently, there are 6 species that are classified under this genus. The present study characterized the active peptides from three species; *Pulchrana baramica* and *Hylarana*

erythraea were found to inhabit sedimentary and peat swamp forests. *Pulcharana baramica* is identified by its mottled brown characteristic at the dorsal side and yellowish at the flanks. The skin texture is granular and warty. Besides that, it has a dark tympanum that lightens up in the center. *Hylarana erythraea*, which is also known locally as rough sided frog, lives in muddy primary or secondary forest areas, such as the peat swamps. The physical features include has cream colored dorso-lateral folds that are sometimes bordered with black. Limbs are yellowish with irregular spotting. This species has smooth skin, and long, free fingers that dilate into minute discs with grooves. It has long hindlimbs. The inner metatarsal tubercle is present, but the outer metatarsal tubercle is absent (Inger & Stuebing, 2005).

The only recent proteomic study on the Bornean species was conducted by Conlon et al. (2008). The researchers discovered Brevinin-1 and Brevinin-2, isolated from two Bornean frogs, *O. hosii* and *H. picturata* and tested the AMPs activities against *E. coli* (MIC<150 µg/ml) and *S. aureus* (MIC<125 µg/ml). However, these findings were only covered on small parts of the Bornean frog belonging to the family Ranidae. Therefore, the findings from the present study will fill the existing knowledge gap in the characteristics of AMPs from Bornean frog secretions and its broad-spectrum activities.

2.4 Innate Immune System of Frogs

Frogs inhabit diverse environments, relying on both terrestrial and aquatic habitats or a blend of these. This diverse habitat exposes them to various microorganisms, including pathogens, making their skin susceptible to parasitization and infections (Wake et al., 2018). To combat these challenges, amphibians have evolved intricate defense mechanisms. They possess specialized glands beneath their skin that produce an array of antimicrobial and toxic substances, fortifying their innate immune defenses. These adaptations underline the

importance of robust skin defenses against environmental factors. As a result, frogs have collectively developed unique skin adaptations to thrive in both aquatic and terrestrial environments (Varga et al., 2019).

The presence of both adaptive and innate immunity is observed in frogs, akin to higher vertebrates. Innate immunity serves as the primary defense mechanism, providing an immediate response against diverse pathogens. This defense includes antimicrobial peptides, phagocytic macrophages (Manning & Horton, 1982), and natural killer cells. Despite the extensive research on the crucial role of antimicrobial peptides in amphibian skin survival, relatively little attention has been given to the contribution of the frog epithelium in pathogen defense. Therefore, the understanding of interactions between frog skin, pathogens, and environmental factors is deemed essential. Unlike innate defenses, adaptive immunity operates at a slower pace due to its mechanism, which entails pathogen recognition through their antigens (Ramsey et al., 2010).

2.5 Histological Features of Frog Skin

The skin of a frog comprises an epidermis and dermis, each with specialized layers and structures essential for its physiology. The outer stratum corneum of the epidermis, made of thin corneal cells (Haslam et al., 2014), forms a protective seal on the skin's surface, while the basal layer, or bud layer, consists of cuboidal cells that renew the skin (Çömüden et al., 2023). The dermis contains chromatophores, or pigment cells, that contribute to skin coloration for camouflage or signaling, and two types of glands: mucous glands, which secrete mucus to keep the skin moist for cutaneous respiration, and granular (or poison) glands, which produce toxins for defense, often concentrated around the frog's head (Mauricio et al., 2021). The dermis also includes the stratum spongiosum, a layer of loose

connective tissue with glands and blood vessels that provide support, and the stratum compactum, a layer of densely packed collagen fibers that adds structural strength and stability. Together, these components enable frogs to maintain moisture, breathe through their skin, and protect themselves from predators.

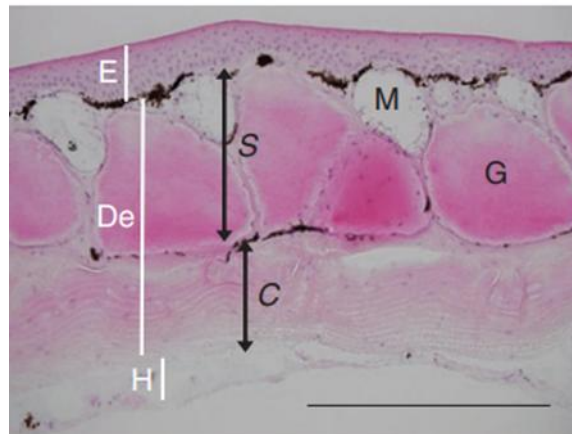


Figure 2.3: Morphology of adult *Xenopus tropicalis*, white lines demarcating epidermis (E), dermis (De) and hypodermis/subcutis (H). Black arrows indicate margins of the stratum spongiosum (S) and stratum compactum (C). M, small mucus gland; G, large granular/poison gland (Haslam et al., 2014).

When stress is applied, the myoepithelial cells are signaled by the sympathetic nerve to contract, leading to the expulsion of a sticky secretion from the granulation gland. This secretion, known to contain a peptide blocking the cross-section of the Australian toad (Rollins-Smith et al., 2011), is a mucous granular gland present in the dermis of green and golden bell frogs (*Litoria raniformis*), as noted by Sciani et al. (2013). The secretions, containing antibacterial peptides (AMPs), are crucial in combating pathogenic microorganisms. Amphibians possess abundant glands that secrete bioactive peptides to counteract pathogenic infections. Additionally, these secretions contain non-peptide compounds like amines, alkaloids, and polyphenols. Simultaneously, the mucus glands

generate mucus, facilitating moisture on the frog's skin and regulating the skin's pH, respiration, and hydration levels (Çömden et al., 2023).

The inducible expression of AMPs in frogs has been observed (Woodhams et al., 2020). Various effective methods have been utilized for collecting secretions from frog skin to date. Inducing granular secretion can be achieved through methods like electrical stimulation or exposure to adrenergic substances such as ether, chloroform, and norepinephrine (Lai, 2010). Ether exposure is commonly preferred due to its cost-effectiveness, non-invasiveness, and harmlessness to animals. When the animal is anesthetized, it begins secreting in response to the stimulus, manifesting as colorless or milky discharge bubbles. Furthermore, advancements in peptide isolation methods, particularly using high-performance reversed-phase liquid chromatography, have enhanced approaches to peptide processing.

2.6 Antimicrobial Peptides

In general, gene-encoded cationic and hydrophobic molecules ranging from 12 to 50 amino acids in length are known as antimicrobial peptides (AMPs) (Varga et al, 2019). A mixture of various peptides that are active against invasive pathogens has been shown to be contained within frog skin secretions, as demonstrated by several published studies (Conlon et al., 2019; Jiménez et al., 2022; Perez-Rodriguez et al., 2022). Their interaction with microbial cells leading to membrane disruption and death has been documented (Diniz et al., 2020).

AMPs are utilized by organisms to combat potentially hazardous microbial pathogens. It was reported that more than 1,200 types of peptides with antimicrobial activity have been isolated from various cells and tissues (Zhang et al., 2022). Broad-spectrum

effects against numerous bacteria, fungi, and viruses have been demonstrated in many studies.

The initial description of these bioactive molecules occurred in insects by Stephens (1962), followed by consecutive studies that led to the characterization of one of the largest groups of antimicrobial peptides from the American butterfly *Hyalophora cecropia*. This butterfly became the first animal to induce AMP isolation and comprehensive discovery, as documented by Steiner et al. (1981).

In comparison to conventional antibiotics, AMPs exhibit differences in the size and composition of the natural organic molecules they contain. Reports indicate that antimicrobial peptides (AMPs) have the capability to interact rapidly and efficiently, as outlined by Wimley (2019). Unlike antibiotics, which target specific intracellular proteins before inducing bacterial lysis, AMPs have the potential to directly act on the bacterial cell wall (Hommes et al., 2022). Additionally, it has been observed that AMPs are not secreted as a discrete group of molecules; rather, they are released as a complex mixture of peptides and defense molecules, as documented by Shen et al. (2016).

The preparation techniques for potential peptide drugs often involve the initial screening of active peptides within frog skin secretions. Active peptides have also been reported in other parts of frogs, such as blood, internal organs, stomach, eggs, and frogs themselves (Ong et al., 2021). However, the primary utilization of skin secretions stemmed from their acquisition method, which ensured that these 26 secretions were obtained without harm to the frogs. Subsequent procedures involve the purification and characterization of the peptide of interest using chromatographic and mass spectrometry techniques. Generally, mass spectrometry measures the mass and amino acid residues of peptides to construct

molecular structures with high precision. For instance, residues correspond to protein database fragments and generate a fingerprint of peptide mass, as exemplified by Walck and Clark (2018).

A general overview is provided regarding the utilization of host defense peptides from diverse organisms as substitutes for traditional antibiotics. The investigation was carried out to explore the additional biological activity of AMP, aiming to enhance the understanding of this cationic peptide as a potential therapeutic agent with a broad spectrum of activity. Numerous AMPs, derived from frog secretions, have been identified as anti-inflammatory (He et al., 2023; Li et al., 2023) and antidiabetic agents (Conlon et al., 2023) and have shown promise in viral vaccine development (Chianese et al., 2023; de Amaral et al., 2022).

2.6.1 The AMPs as Anticancer agents

Cancer can be characterized as a disease where an uncontrollably growing group of abnormal cells due to genetic or environmental factors. It is a serious disease affecting human mortality (Hausman, 2019). It is still the leading cause of death in economically developed countries and the second leading death in developing countries (Nagai et al., 2017). However, early cancer detection through accurate and timely treatment can extend the life expectancy of cancer patients by more than 5 years after diagnosis (American Society of Clinical Oncology, 2020). Understanding statistics used to guide prognosis and evaluate treatment. Chemotherapy, surgery, radiation therapy, and drugs are now common treatments for cancer (Debela et al., 2021). However, this treatment has unwanted side effects. Patients who received systemic chemotherapy generally experienced persistent fatigue regardless of tumor type (Strebkova., 2020).

Owing to the limited number of currently available treatments, only alternative anti-tumor agents with specific mechanisms of action affecting cancer cells have been investigated. Studies have shown that cancer cells have multiple targets that can be attacked. Interestingly, cancer cells consist of cell membranes with altered chemical composition compared to noncancerous wound cells, resulting in increased net negative charge (Oelkrug et al., 2015). Therefore, the modified membrane is an ideal target for cationic antimicrobial peptides (AMPs) with additional anti-cancer properties, also called anticancer peptides (ACPs). In vitro and in vivo studies using PCR were performed to find novel therapies for cancer cells in different types of cancer as shown in Table 2.2.

As reviewed in many literatures, potential anti-cancer agents derived from frogs consists of a series of short peptides composed of 2–50 amino acids (Wang et al., 2017) that have varied benefits over alternative chemical agents by having low molecular weight, low toxicity, high selectivity, simple structure, easy absorption, fewer adverse reactions, and low risk for causing multidrug resistance. (Jafari, et al. 2022; Orafaie, et al 2021).

The mechanism involves guiding the ion anticancer agent from frogs to direct the disruption of growth through the induction of apoptotic cell death., principally by caspases and involves intrinsic mitochondrial pathways and extrinsic death receptor transduction pathways (Ju et al., 2021).

Table 2.2: An Overview of AMPs that Possess Anti-Cancer Activities

Peptide	Species (Family)	Cancer cells	Reference
Ascaphin-8	<i>Aschapus truei</i> (Aschapidae)	Human- hepatocarcinoma Derived HepG2cell	Conlon et al. (2008)
Pentadactylin	<i>Leptodactylus</i> <i>labyrinthicus</i> (Leptodactylidae)	Murine melanoma cell, B16F10	Libério et al. (2011)
Esculentin-2Cha	<i>Lithobates</i> <i>chiricahuensis</i> (Ranidae)	Lung adenocarcinoma cell, A549	Attoub et al. (2013)
Hymenochirin- 1B	<i>Hymenochirus</i> <i>boettgeri</i> (Pipidae)	Lung and breast adenocarcinoma, HepG2 cell	Attoub et al. (2013)

2.6.2 The AMPs as Anti-Viral Agents

The potential antiviral properties of natural AMPs have garnered significant interest. Extensive research has focused on the impact of frog AMP on herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2). While Acyclovir and its derivatives are currently employed in treating herpes simplex virus 1, prolonged use of these drugs can induce resistance (Oka et

al., 2021). Hence, the significance of exploring new therapeutics for human viral diseases becomes pivotal, especially in addressing emerging infectious viruses and those developing resistance to existing drugs (Shartouny, 2019).

The discovery of 31 new effective herpes combinations signifies a promising breakthrough. For instance, *Xenopus laevis* mezzanine-1 and *Xenopus* PGLA-AM1 exhibit the ability to eliminate over 90% of extracellular HSV-1 strains within the initial five instances of direct contact (Conlon et al., 2014). Additionally, Temporin-SHa (SHa), previously recognized for its anti-HSV-1 properties, demonstrated a twofold inhibition of viral replication (52%) at the highest tested concentration of 10 μ M (Maëva Roy, 2019). Furthermore, *X. laevis* maginin-2 and PGLa significantly diminish the likelihood of catfish contamination. *Phyllomedusa sauvagei* dermaceptin-S4 exhibits high cytotoxicity in HSV-2 cells, with an LC concentration below 6.0 mM, serving as a potent HSV-2 inhibitor (Conlon et al., 2014).

The antiviral activity of AMPs against human immunodeficiency virus (HIV) has also been explored. Caerin 1.1, Caerin 1.9, and Maculatin 1.1 from the skin of *Litoria caerulea*, *Litoria chloris*, and *Litoria genimaculata* respectively, exhibit inhibitory effects on HIV at concentrations of 7.8 μ M, 1.2 μ M, and 11.3 μ M (Shartouny, 2019).

Studies by Conlon et al. (2014) have elucidated the mechanism of viral inactivation involving the use of AMPs as viral inhibitors during the cell cycle. Other literature indicates that AMPs might act promptly on the virus during its entry into cells, causing disruptions in the removal of the outer envelope.

2.6.3 The AMPs as Anti-Inflammatory Activity

AMPs have been noted not only for their antimicrobial properties but also for their potential as anti-inflammatory agents. Direct anti-inflammatory properties are possessed by some AMPs, whereby inflammatory signaling pathways are modulated, leading to the inhibition of the production of pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1 β), which are central mediators of inflammation (Luo & Song, 2021). The immune response can be modulated by AMPs, with immune cells such as macrophages, neutrophils, and dendritic cells influenced by them. The resolution of inflammation can be promoted by AMPs through the enhancement of the clearance of inflammatory mediators and the promotion of tissue repair processes, as aligned with findings by Duarte et al. (2023). AMPs such as defensins and cathelicidins exhibit anti-inflammatory activity alongside their antimicrobial properties. While their primary function is to combat microbial pathogens, they also play a role in modulating the immune response and reducing inflammation by regulating the production of pro-inflammatory cytokines and chemokines.

In a study by Lu et al. (2024), it was observed that some AMPs exhibit antioxidant properties, whereby they scavenge reactive oxygen species (ROS) and reduce oxidative stress-induced inflammation. By mitigating oxidative damage to cells and tissues, inflammation can be attenuated, and tissue homeostasis promoted. In the context of Parkinson's disease, peptides with antioxidant properties may potentially play a crucial role by scavenging excessive ROS, thereby mitigating oxidative stress associated with dopaminergic neuron damage and disease progression.

A study by He et al. (2019) demonstrated that immune cell recruitment and activation at inflammation sites can be regulated by AMPs. Otolin-wound healing-promoting peptide (Ot-WHP), derived from the Chinese concave-eared frog *Odorrana tormota*, promotes wound healing by accelerating closure in mouse models of full-thickness wounds. Ot-WHP enhances neutrophil and macrophage infiltration, triggers neutrophil activity and cytokine production in macrophages, and induces chemoattractant production, facilitating immune cell recruitment to wounds.

2.6.4 The AMPs as Antidiabetic Agent

Frogs' skin peptides have garnered attention for their potential antidiabetic activity. These peptides, derived from the skin of various frog species, exhibit bioactive properties that may contribute to managing diabetes. Research in this area suggests that certain peptides derived from frog skin possess the ability to modulate glucose metabolism, insulin sensitivity, and other factors associated with diabetes.

Studies have explored the isolation and characterization of these bioactive peptides with the aim of understanding their mechanisms of action. Some frog skin peptides have demonstrated the capability to enhance insulin secretion, improve glucose uptake, and regulate key enzymes involved in carbohydrate metabolism. These findings suggest a potential role for these peptides in mitigating hyperglycemia and insulin resistance, two critical aspects of diabetes management. Figure 2.4 elucidates a proposed mechanism for these peptides.

Insulin release is induced by the AMPs through different mechanisms depending on the peptide type. It was observed in bioinformatics analysis that an enhancement of the insulintropic effect of peptides can be achieved by an increase in positive net charge and a

reduction in hydrophobicity. Peptides exhibiting higher positive net charge and Boman index demonstrated elevated activity (Soltaninejad, et al., 2021).

Temporin peptides, including Temporin-DRa, Temporin-DRb, Temporin-Oe, Temporin-CBa, Temporin-Va, Temporin-Vb, Temporin-Vc, Temporin-CBf, and Temporin-TGb, sourced from different amphibians, have exhibited robust insulin release stimulation. These peptides operate through a K-ATP channel-independent pathway, showcasing diversity in their mechanisms and underscoring their potential versatility in antidiabetic effects. On the other hand, bombesin and bombesin-related peptides, derived from the toad *Bombina variegata*, demonstrate significant insulin release stimulation. Interestingly, these peptides operate through a shared mechanism involving a cAMP-dependent, G protein-insensitive pathway. This suggests a convergence in the antidiabetic effects of bombesin peptides, indicating a commonality in their action despite diverse amphibian sources. The comparison highlights the richness of mechanisms among frog skin peptides, offering a spectrum of pathways for potential therapeutic applications in diabetes management.

Ranatuerin peptides, namely Ranatuerin-1CBa, Ranatuerin-2CBc, and Ranatuerin-2CBd, isolated from bullfrog *Lithobates catesbeianus*, showcase insulin release stimulation. Among them, Ranatuerin-2CBd stands out for its pronounced potency in stimulating insulin release. Noteworthy is the observation that these peptides effectively maintain the integrity of the plasma membrane without adversely affecting lactate dehydrogenase levels. This indicates a dual benefit, where the peptides not only stimulate insulin release but also ensure the structural integrity of the cellular membrane (Mechkarska et al., 2011). Similarly, Xenopsin and Xenopsin-AM2, sourced from *Xenopus laevis* and *X. amieti* secretions, respectively, induce significant insulin release without impacting lactate dehydrogenase

levels. This commonality with the Ranatuerin peptides suggests a shared attribute among these amphibian skin peptides. (Zahid et al., 2011).

The ability to stimulate insulin release while preserving membrane integrity underscores the potential of these peptides for therapeutic applications in diabetes. This comparison highlights a convergence in the insulinotropic effects and membrane-preserving properties of Ranatuerin and Xenopsin peptides, adding to the diverse mechanisms observed across frog skin peptides with antidiabetic potential. A comparative analysis of Palustrin, Phylloseptin, Pseudin, and Dermaseptin peptides reveals a fascinating array of mechanisms contributing to their potential as antidiabetic agents. Palustrin peptides, derived from *Lithobates catesbeianus*, exhibit significant insulin release stimulation at low concentrations, showcasing their efficacy. In contrast, Phylloseptin-L2, a member of the phylloseptin family, stands out for its potent insulin release, operating independently of Ca^{2+} influx or ATP-sensitive K^{+} channels (Conlon et al., 2009). Pseudin-2, sourced from *Pseudis paradoxa*, adds another dimension with its insulin-inducing action through a Ca^{2+} -independent pathway (Abdel-Wahab et al., 2008.). The Dermaseptins, recognized as multifunctional AMPs, including Dermaseptin B4 and Dermaseptin-LI1, also contribute to antidiabetic activity, yet the exact mechanism remains elusive. This comparative exploration underscores the richness and diversity of mechanisms among frog skin peptides, offering a plethora of potential therapeutic avenues for addressing diabetes with multifaceted approaches.

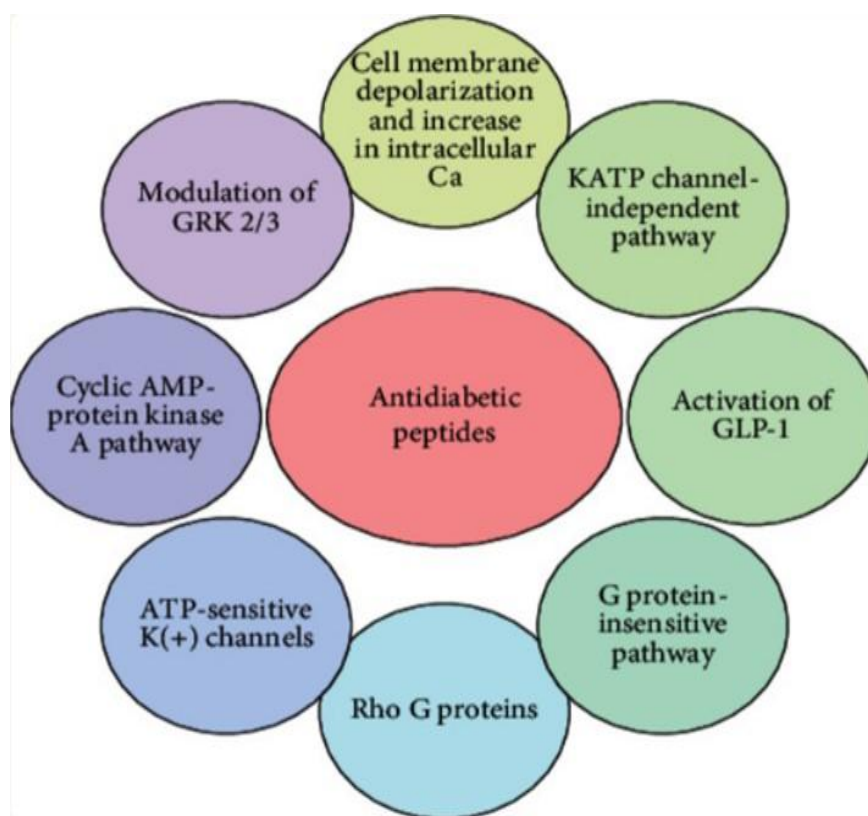


Figure 2.4: Proposed possible mechanisms of action for antidiabetic peptides (Soltaninejad, et al 2021).

Table 2.3 provides a comprehensive overview of peptides derived from various frog species, highlighting their net charge, which ranges from +2 to +6, and their hydrophobicity, which exceeds 48%. These peptides are typically composed of 8 to 46 amino acids, exhibiting significant diversity, even in the Boman index. The Boman Index is a measure used in peptide research to assess a peptide's binding potential to other proteins. It quantifies the sum of all potential binding interactions and is expressed as the estimated binding energy in kcal/mol per residue (Gómez, et al., 2017). A higher Boman Index indicates a stronger binding affinity, which can be beneficial in designing therapeutic peptides that interact effectively with target proteins. In contrast, a lower Boman Index may indicate reduced binding potential, which could help avoid unwanted interactions. This makes the Boman Index a valuable tool for peptide design and optimization, aiding in the development of novel peptide-based therapeutics with enhanced biological activity and stability.

Table 2.3: Antidiabetic peptides from amphibians

Name	Source	Sequence	Net charge	Hydrophobic residue (%)	Boman index
Brevinin-1CBb	<i>Lithobates catesbeianus</i>	FLPFIARL AAKVFP IICSVTKKC	+4	62	-0.36
Brevinin-1Pa	<i>Rana pipiens</i>	FLPIIAG VAAKVF PKIFCA ISKKC	+4	66	-1.11
Brevinin-1E	<i>Rana esculenta</i>	FLPAIFR MAAKVV PTIICSI TKKC	+4	62	-0.36
Brevinin-2GUb	<i>Hylarana guentheri</i>	GVIID TLKGAA KTVAA ELLRKAH CKLTNSC	+4	50	0.79

Table 2.3 continued

Brevinin-2EC	<i>Pelophylax esculentus</i>	GILLDK LKNFAK TAGKGV LQSLN TASCK LSGQC	+4	44	0.53
Esculentin-1	<i>Rana esculenta</i>	GIFSKL GRKKIK NLLISGL KNVGKE VGMDVV RTGIDIA GCKIKGEC	+6	41	0.98
Esculentin-1b	<i>Rana esculenta</i>	GIFSKL AGKKLK NLLISGL KNVGKE VGMDV VRTGIDI AGCKIKGEC	+5	43	0.62

Table 2.3 continued

GM-14	<i>Bombina variegata</i>	GKPFYPP PIYPEDM	-1	21	0.72
IN-21	<i>Bombina variegata</i>	IYNAICP CKHCNKC KPGLLAN	+3	47	0.57
Tigerinin-1R	<i>Hoplobatrachus rugulosus</i>	RVCSA IPLPICH	+1	58	-0.01
Caerulein-B1	<i>Xenopus borealis</i>	EQDY (SO3) GT GWMDF	-3	27	2.08
Magainin-AM2	<i>Xenopus amietii</i>	GVSKIL HSAGKF GKAFL GEINKS	+4	39	0.58
Hymenochirin-1B	<i>Hymenochirus boettgeri</i>	IKLSPE TKDNLK KVLKGA IKGAIA VAKMV	+6	48	0.47

The Boman Index for these peptides ranges from -0.01 to 0.9. Noteworthy contributions to antidiabetic peptides have come from the Asian frog *Hylarana guentheri* (among the *Rana* species) and four Hylidae species (*Phyllomedusa trinitatis*, *Agalychnis calcarifer*,

Agalychnis litodryas, and Hylomantis lemur). Additionally, six toad species within the Bombinatoridae family, particularly those in the genus Bombina, demonstrate insulin-releasing properties. However, the distribution of antidiabetic peptides in other species lacks a clear pattern.

Despite their promising potential, these peptides face challenges related to their short half-life, immune system stimulation, hemolysis, and toxicity, limiting their utility as antidiabetic agents. For instance, Ocellatin-L2, known for its high hemolytic activity, also exhibits intense stimulation of insulin release. Alterations in amino acid content have shown potential in enhancing peptide efficacy, with an increased net charge positively impacting insulin release potency. However, this modification does not consistently yield uniform results. Hydrophobicity, crucial for antimicrobial peptides, does not significantly affect the insulintropic effect but can reduce hemolytic activity. Peptides with lower hydrophobicity, such as Magainin-AM2, display decreased hemolytic activity. In summary, frog skin peptides exhibit diverse antidiabetic potential, with notable contributions from various species. Despite challenges like short half-life and toxicity, ongoing research explores amino acid modifications and structural enhancements. The Boman index correlates with antidiabetic activity, emphasizing binding interactions. Strategies such as L-to-D amino acid substitution and protective coatings aim to enhance stability. Overall, frog skin peptides represent a promising avenue for advancing antidiabetic therapeutics.

2.7 Characteristics of Antimicrobial Peptides

In general, the composition of peptides involves the linkage of amino acids forming a chain through peptide bonds between the carboxyl and amino groups of adjacent amino acids. Most AMPs are characterized by low molecular weight, comprising 11-46 amino acid

residues. These peptides exhibit a net cationic charge of 32 at physiological pH, attributed to the abundance of lysine and arginine within their peptide chains (Pirtskhalava et al., 2021). The first twenty amino acids were tabulated in Table 2.4. Most anurans secrete small molecular weight peptides, mostly within 1 to 5 kDa as depicted in Table 2.6 (Rash et al., 2011).

Table 2.4: Details of the twenty common amino acids occur in nature.

No	Name	Abbreviations	Symbol	Molecular weight
1	Alanine	Ala	A	89
2	Arginine	Arg	R	174
3	Aspartic acid v	Asp	D	133
4	Asparagine	Asn	N	132
5	Cysteine	Cys	C	121
6	Glutamic acid	Glu	E	147
7	Glutamine	Gla	Q	146
8	Glycine	Gly	G	75
9	Histidine	His	H	155
10	Isoleucine	Lle	I	131
11	Leucine	Leu	L	131
12	Lysine	Lys	K	146
13	Methionine	Met	M	149
14	Phenylalanine	Phe	F	165
15	Proline	Pro	P	115

Table 2.4 continued

16	Serine	Ser	S	105
17	Threonine	Thr	T	119
18	Tryptophan	Trp	T	104
19	Tyrosine	Tyr	T	181
20	Valine	Val	V	117

Table 2.5: Summary of reported AMPs from frog skins with their dominant secondary structures

Peptide	Species	Mr _a	Structure	References
Brevinin-1HSa	<i>Odorrana hosii</i> (Poisonous rock frog)	2571.4	Helix	Conlon et al. (2008)
Brevinin2HSa1	<i>Odorrana hosii</i> (Poisonous rock frog)	1962.0	Helix	Conlon et al. (2008)
Citropin 1.12	<i>Litoria citropa</i> (Australian blue mountain Tree frog)	1614.0	Helix	Wegener et al. (1999)
Caerin 1.12	<i>Litoria splendida</i> (Australian green tree frog)		Helix	Wong et al. (1997)
Defensin-TK3 ⁴	<i>Theloderma kwangsiensis</i> (Tree frog)	2582.0	β-sheet	Shen et al. (2016)

Table 2.5 continued

Distinctin	<i>Phyllomedusa distincta</i> (Tree frog)		β -sheet	Batista et al. (2001)
Dermaseptin-B24	<i>Phyllomedusa bicolor</i> (Tree frog)	4521.3	Helix	Fleury et al. (1998)
Esculenti ¹ -1	<i>Odrorrana hosii</i> (Poisonous rock frog)		Helix	Conlon et al. (2008)
Magainin-2 5	<i>Xenopous laevis</i> (African claw frog)	5400.0	Helix	Zasloff (1987)
Temporin-PTa	<i>Hylarana picturata</i> (Spotted stream frog)		Helix	Conlon et al. (2008)
Thaulin-1 ⁶	<i>Pleurodema thaul</i>	2780.4	Helix	Marani et al. (2017)

Furthermore, microbiocidal peptides with hydrophobic surfaces also exhibit a highly amphiphilic nature (Ye et al., 2020). Previous research has shown that amphibian antimicrobial peptides (AMPs), when exposed to a simulated membrane environment

containing trifluoroethanol (TFE), tend to adopt helical structures. Additionally, the positive net charges of most identified microbiocidal peptides range from +1 to +34 (Fillion et al., 2015). For example, Temporin-L from *Rana temporaria* and Temporin-CPA from *Lithobates* exhibit a net charge of +3, while Phylloseptin-3, isolated from *Phyllomedusa sauvagii*, has a net charge of +1 (Mongoni et al., 2011; King et al., 2013).

2.8 Isolation and purification of peptides from frog skin secretion

In addition to its diverse array of proteins, frog skin secretions have garnered significant interest for their potential therapeutic applications in various fields, including medicine. The intricate composition of these secretions, as highlighted by Indriani et al. (2023), encompasses proteins with potent biological activities that extend to defense against pathogens, promotion of wound healing, and potential therapeutic interventions. Building upon the findings of Ong et al. (2021), the high protein concentration within frog skin secretion underscores its importance as a reservoir of bioactive molecules. AMPs stand out as the predominant protein group, showcasing the crucial role of frog skin in defending against microbial invaders. Moreover, the presence of hormones, enzymes, and structural proteins hints at the multifunctionality of these secretions, with implications for diverse physiological processes such as antidiabetes agent and in tissue regeneration (Mans et al., 2021).

When it comes to the purification of membrane proteins versus secretory proteins, different strategies are employed due to their distinct characteristics. Membrane proteins are notoriously challenging to purify due to their hydrophobic nature and tendency to aggregate (Engel & Gaub 2008). Techniques such as detergent solubilization combined with chromatography, including affinity chromatography and size exclusion chromatography, are

commonly used for membrane protein purification (Bellotti et al 2021). On the other hand, secretory proteins, being more soluble and typically found in higher abundance in the extracellular environment (Wang, et al 2024), can often be purified using simpler techniques such as precipitation, ion exchange chromatography, or affinity purification. The choice of purification method depends on the specific properties of the target protein and the desired purity and yield.

In the context of protein loss in chromatography, optimization of elution conditions, as well as column design and packing, aims to ensure uniform flow dynamics and maximize protein binding capacity. Using very short columns offers benefits such as reduced analysis time, column pressure drops, and potentially improved solute recovery (Espindola et al., 2023).

However, the realization of the therapeutic potential inherent in frog skin secretions necessitates overcoming various challenges associated with protein purification. Kumar et al. (2021) emphasizes the critical role of purification processes in isolating proteins from the complex mixture of compounds present in the secretion. Yet, the inherent biochemical properties of certain proteins or inadequacies in extraction methods may result in their loss during purification.

Addressing these challenges requires the implementation of meticulous purification techniques tailored to the unique characteristics of frog skin proteins. Bocanegra et al. (2021) exemplify this through the utilization of Box-Behnken design and response surface methodology, a statistical approach that optimizes multiple variables at three levels (low, medium, and high) while minimizing experimental runs. This design enables systematic

optimization of extraction conditions to maximize protein yield. Such strategic refinement not only enhances the recovery of proteins but also augments their therapeutic efficacy.

In purification processes, absorbance measurements are commonly utilized to monitor the concentration of proteins. A decrease in absorbance is indicative of protein loss from the sample, thereby suggesting potential issues with the purification process, Mariano et al. (2018) present innovative approaches to minimize protein loss during purification, such as decreasing the intensity of chromatogram absorbance at 220 nm, which significantly improves chromatographic results compared to conventional methods. Absorbance at 214nm offers high sensitivity for detecting proteins, even at low concentrations (Dauly et al., 2006), thus enabling the detection and quantification of trace amounts of proteins and minimizing the risk of protein loss during analysis. Absorbance at 214nm is widely accepted and validated for protein analysis in HPLC methods, providing confidence in the accuracy and reproducibility of results (Emami, et al., 2022). This validation helps reduce the likelihood of errors and protein loss during analysis. These advancements underscore the importance of continuously refining purification strategies to unlock the full therapeutic potential of frog skin secretion proteins.

2.9 The AMPs Structure and Function Relationship

AMPs can generally be categorized based on their secondary structures, encompassing α -helical, β -sheet, loop, and extended peptides. The Antimicrobial Peptide database (<http://aps.unmc.edu/AP/main.php>) is composed of 682 high-resolution AMP structures, of which 18.7 percent are helical, 18.9 percent are β -strand, and 60.1 percent are mixed or coil, as reported by Lee et al. (2016). Valuable information on peptide sequences,

structures, and functions for any submitted amino acid sequence is provided by the Antimicrobial Peptide database, as emphasized by Wang et al. (2020).

The parameters that made the overall structure of an AMP includes its conformation (x), charge (Q), hydrophobicity (H), Amphipathicity (A) and polar angle (θ). It is important to take note that, these parameters are interdependent, whereby any alteration of any parameter will lead to compensatory alterations in others (Lee et al., 2016; Yeaman & Yount, 2023). The understanding of mode of actions of AMPs and the function of structural features thus, help to design a novel molecule that is potent against a wide array of microorganisms.

It has been strongly suggested by previous literature that the biological activity of a peptide is influenced by interdependent parameters (Giraldo-Lorza, et al, .2024). . One of the most studied peptides is Dermaseptin from *Phyllomedusa sauvagii*. The structures of Dermaseptin's analog were extensively modified to elucidate the structure and function relationships of the antimicrobial peptide.

2.9.1 Peptide's Structural Conformation

Conformation is a three-dimensional structure of a protein. There are four major conformational structures levels of protein, which are the primary structure, secondary structures, tertiary structures and quaternary structures (La Pelusa & Kaushik 2020). Primary structure is the unfolded state of peptides, appeared as a linear strand of amino acids, whereas a secondary structure is identified as a repeated of hydrogen-bonding patterns “turn” and “bridge”.

Generally, repeating turns are known as ‘helices’, repeating bridges are ‘ladders’, and subsequently connected ladders are ‘sheets. Tertiary structure is a native-like secondary

structure but lacking the tight packing and rigid structure and the quaternary structure is the native state in which the side chains are tightly interdigitated. Generally, AMPs can be classified into four major groups which includes α -helix and β -sheet secondary structure as shown in Figure 2.5 and Figure 2.6, extended peptides, and loop peptides (Sun et al., 2018). Amino acids are joined together by hydrogen bonds between the carbonyl and amino groups. It has been acknowledged that α -helical peptides become helical only upon interaction with amphipathic phospholipid membranes (Pantic et al., 2017). The amphipathicity of the peptides is segregated along the axis of the α -helix and the effect onto the antimicrobial activity will be discussed in detail in Section 2.9.2.

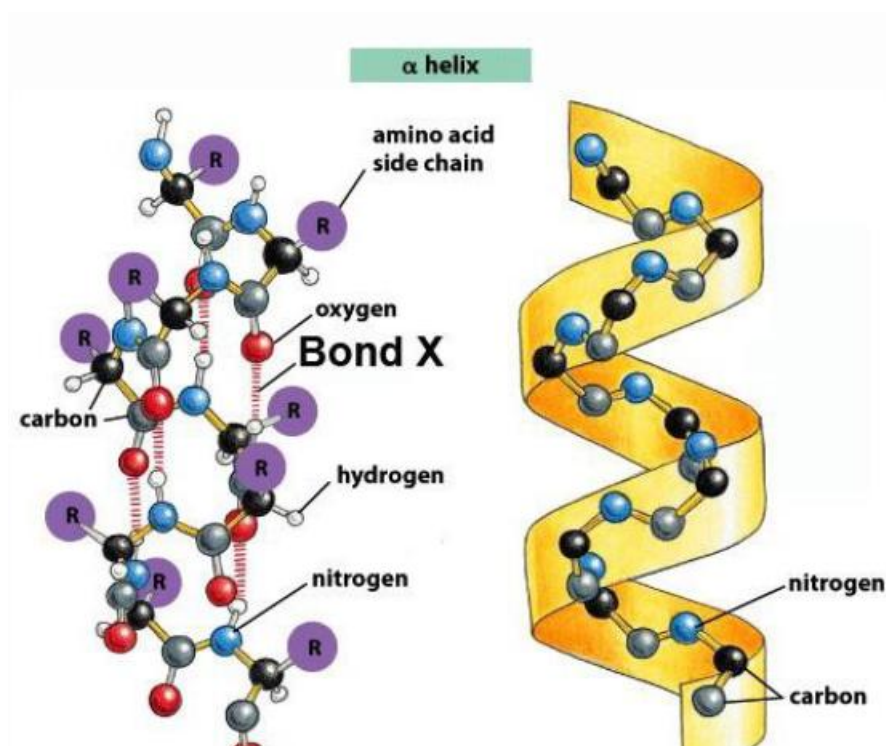


Figure 2.5: A representation of an α -helical amphipathic peptide showed amino acids joined together by hydrogen bonds between the carbonyl and amino groups shown by dashed line. Figure adapted from (Corrêa, et al., 2019).

Meanwhile, the β -sheet, characterized by antiparallel stabilization through a series of disulphide bridges, also possesses an amphipathic structure (Figure 2.6). Unlike the helix

structure, it is noted for being a highly diverse group of molecules at the level of primary structure (Zhou, X., et al. 2023). The β -sheet, as possessed by the Distinctin peptide, features a helix-bend-helix structure that favors pore formational mechanism attributed to the tilted orientation of shorter chains inside the bilayer (Becucci et al., 2013). The β -sheet conformations of these peptides are frequently stabilized by disulfide bridges between conserved Cys residues (Wang et al., 2023).

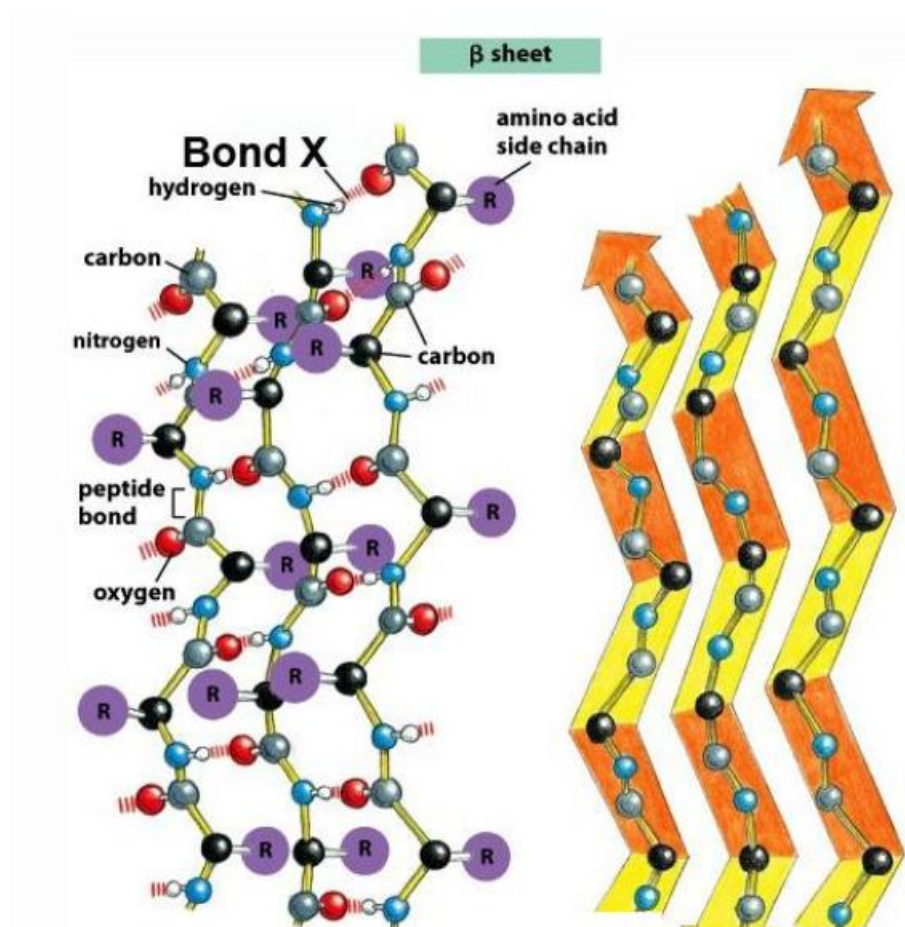


Figure 2.6: A representation of a β -sheet amphipathic peptide showed amino acids, antiparallel stabilised by a series of disulphide bridges. The center image is the notation used to indicate a beta sheet when protein structure is drawn schematically (Corrêa, et al., 2019)

The disruption of bacterial membranes by many of these peptides occurs through the formation of toroidal pores, as exemplified by the porcine peptide protegrin I. Oligomeric transmembrane barrels are formed by this peptide in anionic membranes, while it aggregates

into β -sheet structures on the surface of cholesterol-containing membranes (Nguyen et al., 2011). It is worth noting that most amphibian peptides isolated to date have not been structurally characterized. Therefore, it is likely that there may still be peptides either undiscovered or uncharacterized with other secondary structures. There are a few AMPs that do not fold into helical or sheet structures, such as human salivary histatins. However, histatins, rich in Arginine, Tryptophan, Proline, or Cystine residues, are known to exhibit potent activity against parasites and fungi, such as *Candida albicans* and *Cryptococcus neoformans* (Sarkar et al., 2021).

2.9.2 Amphipathicity of Peptides

The formation of an amphipathic structure upon interaction with target membranes is a common characteristic of AMPs, as highlighted by Pirtskhalava et al. (2021). Amphipathicity in this context pertains to the relative distribution of hydrophilic and hydrophobic domains within the peptide. One highly effective amphipathic structure involves the amphipathic α -helix, where the hydrophobic portion is situated on one side while the hydrophilic portion is located on the other side of the peptide. When AMPs bind to lipid membranes, the hydrophilic part faces outward, interacting with the phospholipid headgroups, while the hydrophobic side becomes embedded within the acyl tail core, as visually depicted in Figure 2.7. This structural feature plays a pivotal role in the functional interaction of AMPs with lipid membranes.

Most AMPs are isolated from anurans and have exhibited an inherent amphipathic α -helical structure. Notably, in the context of Malaysian frogs, specific examples include Brevinin-2 and Brevinin-1, which were derived from *Hylarana picturata* and *Odoranna hosii*, respectively. Conlon et al. (2008) observed that these AMPs consistently adopted a

stable α -helix conformation when placed in a solvent mimicking a membrane environment. This finding underscores the prevalence of amphipathic α -helical structures in AMPs derived from anuran sources and provides valuable insights into their potential antimicrobial mechanisms.

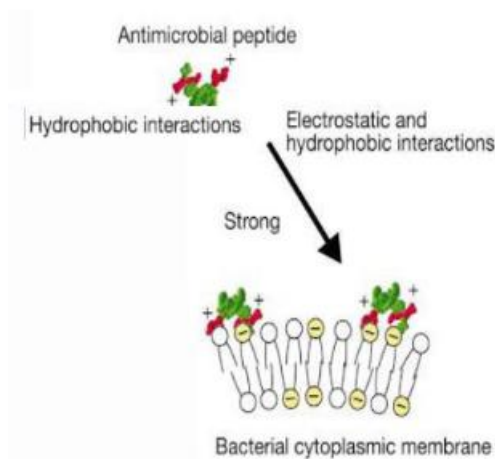


Figure 2.7: AMPs that showed amphipathic properties upon interaction with bacterial membrane. Red indicates hydrophilic region and green indicates hydrophobic region. Upon interaction with bacteria cytoplasmic membrane via electrostatic interactions, hydrophilic portion will face outward toward the phospholipid headgroup, whereas the hydrophobic side is embedded into the acyl tail core. Figure presented was adapted from (Yin, et al 2017).

2.9.3 The AMPs Net Charges

The net charges of AMPs often stemmed from the cumulative charge of their constituent amino acids (Aslam et al., 2022). Majority of these peptides tend to be cationic, although exceptions exist, and typically exhibit a net positive charge ranging from +1 to +9, as depicted in Figure 2.8, adapted from Hebaishi (2013). The antimicrobial peptide database has documented the overall charges and hydrophobicity of amphibian peptides.

Recognition has been gained for cationic AMPs as a crucial factor contributing to the efficacy of these peptides against invading pathogens. A strong correlation between cationicity and antimicrobial activity has been established in several studies, including those

conducted by Pantic et al. (2017). An illustration of the significance of cationicity in modulating the potency of AMPs against microbial threats is provided by the substitution of a Lysine residue with Glutamine at position 11 in Brevinin-1HSa (Pantic et al., 2017).

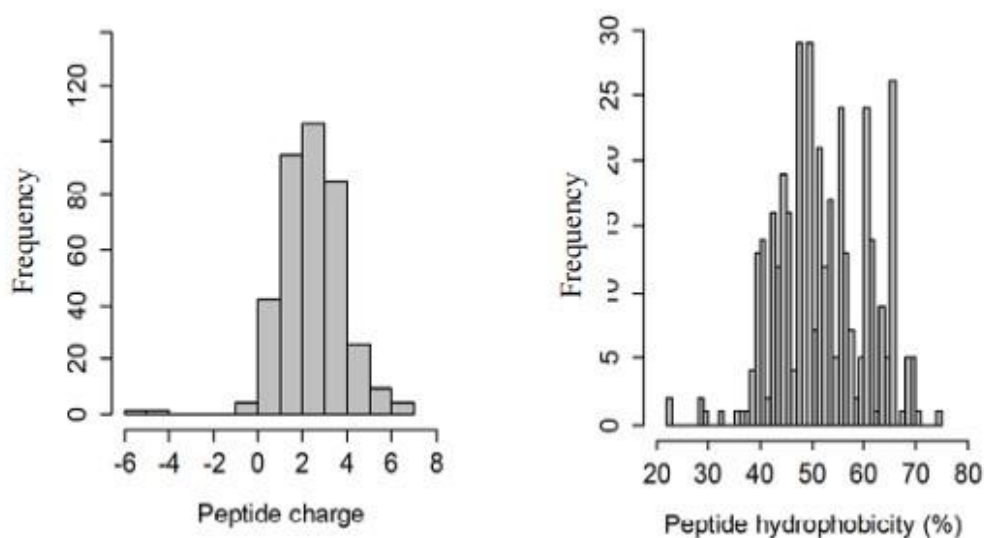


Figure 2.8: Overall charges and hydrophobicity of amphibian peptides statistics in the antimicrobial peptide database. The bar chart on the left shows notable feature where most frog's AMP have charges between +1 to +8. The bar chart on the right represent hydrophobicity of most frog's AMP that shows irregular pattern, ranging between 50 percent to 70 percent (Hebaishi, 2013).

An intriguing experiment involving Brevinin-1HSa showed that reducing its net charge from +2 to +1 had profound consequences. This modification led to a noticeable alteration in the peptide's potency against *E. coli*, with the Minimum Inhibitory Concentration (MIC) increasing from 62.5 $\mu\text{g/mL}$ to over 125 $\mu\text{g/mL}$, as reported by Conlon et al. in 2008. In another study, Kang, et al. (2018) demonstrated a direct relationship between the positive charge of Pseudin-2 and its toxicity towards bacterial cells. Increasing the positive charge of this peptide significantly heightened its efficacy in damaging bacterial cells.

The underlying principle governing these observations can be attributed to electrostatic interactions whereby positively charged AMPs target the negatively charged phospholipid membranes of bacterial cells. This electrostatic attraction facilitates the binding of AMPs to the bacterial cell membrane, ultimately culminating in cell lysis. These studies collectively highlight the pivotal role of charge modulation in shaping the mode of action and effectiveness of AMPs against microbial threats.

2.9.4 Hydrophobicity of Antimicrobial Peptides

The crucial role of hydrophobicity in the function and effectiveness of antimicrobial peptides (AMPs) is often defined as the proportion of hydrophobic residues within a peptide (Büyükkiraz et al., 2022). In Figure 2.6, it is observed that a hydrophobicity of at least 50% is exhibited by majority of the AMPs. The significance of this parameter lies in its influence on the peptide's capability to penetrate the lipid bilayer of cell membranes. The membrane permeabilization is enhanced with increased hydrophobicity; however, an excessive level of hydrophobicity has been associated with heightened cell toxicity and a reduction in antimicrobial activity, as demonstrated by Kumar et al. (2018).

In a study by Romero et al. (2020), an interesting finding was reported. They observed an increase in Temporin-A's toxicity against enterococcus bacteria after elevating its hydrophobicity. This highlights the complex relationship between hydrophobicity and peptide efficacy, with the potential for hydrophobicity to be a double-edged sword, boosting antimicrobial activity but also raising cytotoxicity levels.

Furthermore, when the therapeutic goal involves the selective elimination of specific cells, such as cancerous cells, an increase in cytotoxicity becomes advantageous. This concept was explored by Conlon et al. (2013). It was found that Alysterin-2A

(ILGKLLSTAAGLLSNL.NH₂) exhibited weak activity as an anticancer agent against cancer cells. However, by strategically increasing hydrophobicity through the substitution of Threonine at position 8, Alanine at position 9, and Alanine at position 10 with L-tryptophan, the peptide's toxicity against adenocarcinoma A549 cells was enhanced eleven-fold. The significance of hydrophobicity in tailoring the selectivity and efficacy of peptides for specific therapeutic applications is underscored by these findings (Conlon et al., 2013).

2.9.5 Polar Angle

The critical process of membrane binding involves the penetration of the lipid acyl chain region by the hydrophobic face of a peptide's helix, while the hydrophilic peptide groups interact with the polar lipid head groups and aqueous surroundings (Ciumac et al., 2019). The extent of membrane binding and perturbation is reliant on the relative size of the hydrophilic and hydrophobic helix cores, as highlighted by Pirtskhalava (2021). An important metric for characterizing the distribution of hydrophilic and hydrophobic residues within an amphipathic helical peptide is the polar angle, indicating the degree of hydrophobicity. This aspect was demonstrated in a study by Uematsu and Matsuzaki (2000), where PGLa (θ : 100°) exhibited higher membrane permeabilization, facilitated translocation, and induced a greater pore formation rate compared to Magainin-2 (θ : 180°). The pivotal roles played by the hydrophobic and hydrophilic structures of antimicrobial peptides (AMPs) in membrane disruption are underscored by these findings.

Furthermore, the balance in helical peptides is guided by the polar angle (Torres et al., 2017). Two peptides were designed with leucine substitutions to modify hydrophobic interactions and attain a balance favoring helical structures. The polar angle does not directly impact antimicrobial activity but contributes to conformational differences. Interestingly, a

crucial role in shaping the conformational and functional outcomes of these designed peptides is likely played by the polar angle.

2.10 AMPs Mechanism of Action

AMPs, or antimicrobial peptides, serve as nature's frontline defenders against invading pathogens. These small molecules, ubiquitous across various organisms, possess a fascinating mechanism of action that underscores their effectiveness in combating microbial threats. Operating through a multifaceted approach, AMPs disrupt microbial membranes, interfere with essential cellular processes, and even modulate immune responses. Their versatility and ability to target a broad spectrum of pathogens make them promising candidates for therapeutic development in the ongoing battle against antibiotic resistance. This introduction barely scratches the surface of the intricate interplay between AMPs and pathogens, offering a glimpse into the captivating world of antimicrobial peptides and their mechanisms of action.

2.10.1 Barrel Stave Model

In the barrel-stave model originally proposed by Sansom (1993), a complex is formed by a group of cationic AMP molecules with α -helical structures binding to the membrane surface. This complex is enabled to be inserted into the lipid bilayer membrane, as observed in the study by Lei et al. (2019). Upon insertion, a ring-like 'barrel' pore is formed by the AMP, serving as a conductance channel that disrupts transmembrane potential and ion gradients, resulting in leakage and ultimately leading to cell death. In this pore structure, the hydrophobic face of the peptide interacts with the acyl chains of phospholipids (located in the hydrophobic region of the bilayer). Simultaneously, the hydrophilic face of the peptide is oriented inward toward the aqueous pore, creating a hydrophilic channel that expands

along the membrane, as illustrated by Lee et al. (2016). Figure 2.9 depicts the mode of action by the AMPs on the membrane cell via the barrel stave model.

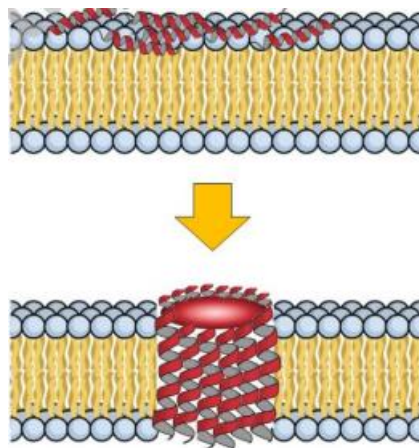


Figure 2.9: The barrel-stave mechanism, AMPs accumulate on the membrane surface and their successive insertions form an aqueous pore in the membrane (Corrêa, et al., 2019).

The carpet model, distinguished from other models, is characterized by the absence of involvement in pore formation and the presence of a "carpet"-type action mechanism (Seyfi et al., 2020). Furthermore, specific peptide-peptide interactions are not deemed essential for the carpet model. The functioning of a particular AMP may be influenced by more than one mechanism, with parameters such as peptide-to-lipid ratio playing a crucial role, as elucidated by Sandhu (2019).

The carpet model was originally proposed by Pouny et al. (1992) to describe the mode of action of Dermaseptin. This model has been observed to be employed by various AMPs as shown in Table 2.6, such as Aurein 1.2, as reported by Sitaram and Nagaraj (1999), Cecropin, as noted by Fernandez et al. (2012), and Indolicidin, as studied by Rozek et al. (2000).

Table 2.6: Proposed Models of AMPs Mechanism of Action and their Representative Antimicrobial Peptides

Antibacterial Mechanism	Representative AMPs
Non-permeabilizing	
Translocating via a transient pore	Buforin II
Translocating via sinking raft	δ -lysin
Permeabilizing	
Translocating via a transient pore	Almethicin
Translocating via sinking raft	Magainin 2
Huge toroidal pore	Lacticin Q
Disordered toroidal pore	Melittin
Aggregation model	Magainins
Interfacial activity model	Magainin2
Chaotic or non-stoichiometric model	Magainin2
Carpet mechanism	Aurein 1.2, Cecropin
Detergent model Membrane discrimination model	
Shai-Huang-Matsazuki (SHM) model	V13KL
Membrane thinning/thickening	LL-37

Similar to other models, the phospholipids in the outer layer of the membrane covering the bilayer are bound by cationic AMPs through electrostatic attractions, forming a "carpet," as previously demonstrated (López-Meza et al. 2011). Upon binding, conformational changes are undergone by the AMPs, and the hydrophobic region may or may not be inserted into the membrane core.

The peptides feature 47 hydrophilic surfaces facing the phospholipid group, while the hydrophobic residue is reoriented towards the hydrophobic core of the membrane, thereby forming a localized carpet (Cherry et al. 2014). As a specific threshold of peptide concentration is surpassed, the surface of the membrane is effectively covered by the peptides in a 'carpet-like' manner. In the final stage of the carpet mechanism, as described by Lee et al. (2016), the targeted membrane structure is ultimately destabilized, leading to its collapse into micelles.

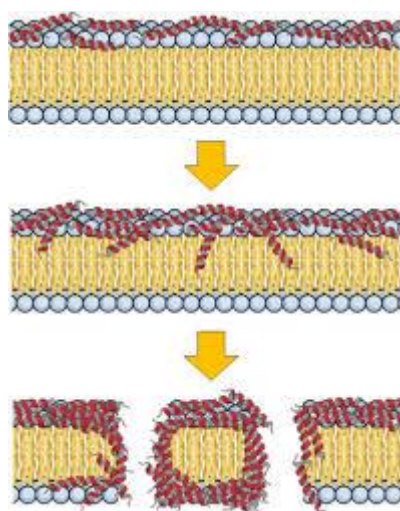


Figure 2.10: Schematic diagram of a carpet model. The total collapse of the membrane provoked by the carpet mechanism is the main consequence of the high density of AMPs accumulated on the surface. (Corrêa, et al.,2019).

2.10.2 Torroidal Pore

The understanding of cationic peptide action mechanisms has been greatly enhanced by the membrane model proposed herein. This model effectively elucidates the behavior of cationic peptides characterized by α -helical structures and those capable of forming disulfide bonds. It posits that peptide molecules initially adhere to the membrane's surface and subsequently "press" into the membrane, thereby inducing strain. As peptide concentration

increases, more molecules accumulate, exerting a cumulative "pushing" force that ultimately leads to the formation of a transmembrane pore, as visually depicted in Figure 2.11.

In this proposed model, peptide molecules exclusively interact with the hydrophilic heads of phospholipids within the lipid bilayer. In contrast, the hydrophobic tails of these phospholipids remain shielded within the membrane and are not exposed to the peptides. This mode of interaction results in the leakage of cellular contents and the ingress of water molecules, ultimately culminating in cell death. The efficacy of this peptide-driven mechanism is intricately linked to the diameter of the resulting pore, as it governs the rate at which molecules traverse the membrane, as supported by the research conducted by Sani et al. (2013).

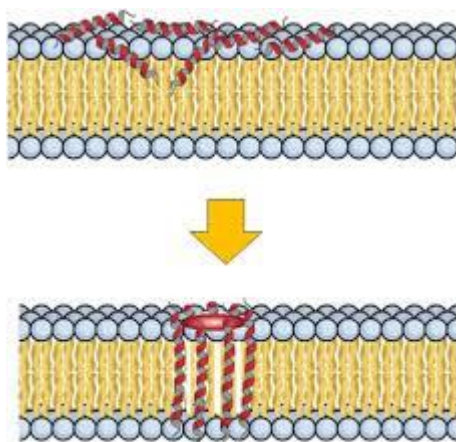


Figure 2.11: Schematic diagram showing toroidal models. The AMPs insert into the bilayer of the microbial cell membrane perpendicularly, pressing the membrane and forming aqueous pores which led to cell death (Corrêa, et al.,2019).

This model finds empirical support in the form of the toroidal pore mechanism, which has been substantiated in Maculatin-1.1 and Novicidin through advanced techniques such as electron microscopy and NMR spectroscopy. The observable swelling of the cell surface aligns with the proposed pore formation mechanism, as corroborated by studies conducted by Sani et al. (2013) and Bertelsen et al. (2012). The convergence of these findings

underscores the significance of this membrane model in elucidating the action mechanisms of cationic peptides and their consequences on cellular membranes.

CHAPTER 3

MATERIALS AND METHOD

3.1 Materials

3.1.1 Chemicals and Reagents

All reagents used were of analytical grade or better. All solutions were prepared using ultrafiltered water produced by the Millipore ultra-pure water system from Thermo Scientific. Acetonitrile (HPLC grade), water (HPLC grade), and petroleum ether were purchased from Fisher Scientific, USA. Methanol used was of HPLC grade, and trifluoroacetic acid for protein sequence analysis was purchased from Merck, USA. Tween-20 was purchased from Sigma-Aldrich, USA.

For the quantification of protein concentration, the Pierce BCA protein assay kit containing Bovine Serum Albumin (BSA), BCA Reagent A (comprising bicinchoninic acid, sodium carbonate, sodium bicarbonate, and sodium tartrate in 0.1M sodium hydroxide), and BCA Reagent B (comprising 4 percent cupric sulfate) were purchased from Thermo Fisher Scientific, USA. For gel electrophoresis, sodium carbonate powder was purchased from Bendosen, USA. Tricine powder was obtained from Sigma, USA. Sodium dodecyl sulfate was purchased from Bio-Rad, USA. Ammonium persulfate, glycerol, glutaraldehyde, and TEMED were purchased from Merck, USA. Tris (ultrapure grade) was purchased from Amresco, USA. The 30 percent (w/v) acrylamide/bis mixed solution was purchased from Nacalai Tesque. 2-Mercaptoethanol was obtained from Amresco, USA, and Coomassie Brilliant Blue R-250 was obtained from Bio-Rad, USA. The ultra-low-range molecular weight marker (1060-26600 kDa) was purchased from Sigma Aldrich, USA. For skin secretion extraction, diethyl ether was purchased from Fisher Scientific, USA.

3.1.2 Bacterial Cultures

For the preparation of culture media, Mueller Hinton agar powder was purchased from Himedia. Meanwhile, Luria Bertani broth and antibiotics such as Ampicillin were obtained from Fisher, USA. The two bacterial isolates used in this study were ATCC 25922 *Escherichia coli* and ATCC 25923 *Staphylococcus aureus*. All isolates were obtained from the Microbiology Lab strain collections, Faculty of Medicine and Health Sciences, UNIMAS.

3.1.3 HIT-T15 Cell Culture

The HIT-T15 cells (CRL-1777) were procured from the American Type Culture Collection (ATCC) in Innoniaga, Malaysia. F-12 medium was obtained from Nacalai Tesque. Horse serum, fetal bovine serum, and Penicillin-Streptomycin solution were purchased from Thermo Fisher Scientific and Gibco, respectively. The cytotoxicity assay kit was sourced from Sigma-Aldrich, and the Human/Canine/Porcine Insulin kit was acquired from R&D Systems.

3.2 Method

3.2.1 Solvents Preparation

Prior to purification of the samples, two HPLC solvents, namely HPLC Solvent A and HPLC Solvent B, were prepared for use as the mobile phase. The HPLC Solvent A was prepared by dissolving 1.2 mL of trifluoroacetic acid (TFA) in 1000 mL of ultrapure water, resulting in a final solution with a concentration of 0.1 % TFA in 99.9 % water. Subsequently, HPLC Solvent B was prepared by dissolving 1.0 mL of Trifluoroacetic acid in 700 mL of acetonitrile, and the volume was adjusted to 1000 mL by adding ultrapure water.

The setup of the mobile phase filtration apparatus involved clamping an inert container to a solvent reservoir connected to a vacuum pump. The HPLC solvent was then drawn through a 0.45 μm Whatman filter paper by applying a vacuum to the side arm of the solvent reservoir, with the pump maintaining flow rates of 10 mL/min. Finally, the solvent was transferred into a sterile Schott bottle for further use.



Figure 3.1: The Setup for Degassing Solvents A and B Using Vacuum Filtration Apparatus The assembly involves connecting a filter flask with a vacuum adapter to a vacuum pump via tubing. Filter paper is placed in the funnel, wetted with solvent, and the mixture to be filtered is poured into the funnel. Suction created by the vacuum pump pulls the liquid through the filter paper, and the filtrate is collected in the flask.

3.2.2 Collection of Skin Secretions

3.2.2.1 Collection of Frogs via Sampling

Frogs were collected at two study sites situated in Kota Samarahan. These sites encompassed the Real Living Lab and the surrounding river course areas within the Universiti Malaysia Sarawak. Frog surveys were carried out at nighttime. The frogs were manually captured using bare hands and were subsequently deposited into plastic bags.

Precise documentation was maintained regarding the time of collection and the specific location where each frog was found.

This study was approved by UNIMAS Animal Ethical Committee (AEC), approval references number: UNIMAS/AEC/T/F07/024

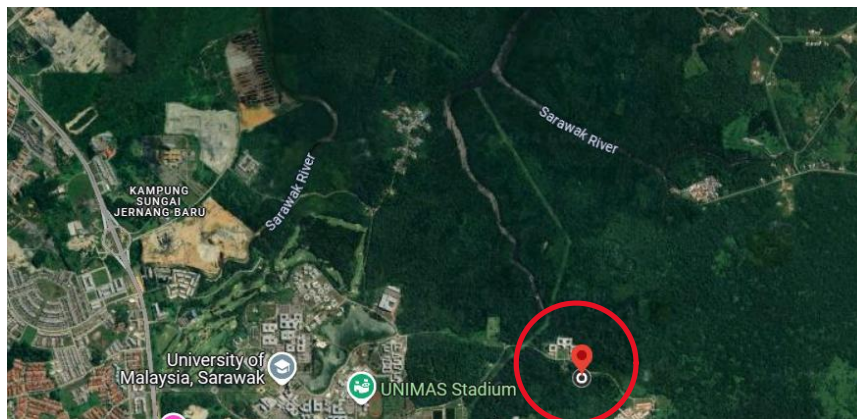


Figure 3.2: This figure shows the GPS coordinates of two study sites in Kota Samarahan, the Real Living Lab and the surrounding river course areas at Universiti Malaysia Sarawak

The identification of frog species was conducted utilizing the reference guide 'A Field Guide to the Frogs of Borneo,' authored by Robert F Inger et al, 2017.

3.2.2.2 Stimulated Extraction of Amphibian Skin Secretions using Ether

The method employed in this study was a slight modification of the one described by Lai et al. (2010). Adult specimens of selected Bornean frog species, encompassing both sexes (n=7 specimens per species), were collected from the surrounding river course areas within the Universiti Malaysia Sarawak vicinity.

The frogs were positioned inside glass beakers. Within each beaker, a piece of absorbent cotton soaked with diethyl ether (1 mL) was placed on top of the frogs. The

beakers were then sealed with aluminum foil for duration of 10 minutes to facilitate the permeation of volatilized anhydrous ether into the container. Skin secretions were obtained by washing the dorsal region of the frogs with distilled water, resulting in a total volume of 20 mL. Then 1.0% (v/v) trifluoroacetic acid was promptly introduced to prevent peptide degradation in the samples. Subsequently, the secretions underwent centrifugation at 3220 x g for 30 minutes at 4°C. Following centrifugation, the supernatant was meticulously transferred into sterile Falcon tubes and preserved at -80°C until the time of analysis to mitigate issues related to proteolysis. Alternatively, if the secretions were collected in the field without immediate access to a freezer, they were maintained at 0°C in an ice bath until freezer access became available (Conlon & Sonnevend, 2010).



Figure 3.3: Using an immersed cotton of diethyl ether, the skin secretions from amphibians are extracted by placing it inside the beaker.

3.2.3 Determination of Protein Concentration Content

The frogs were manually captured using bare hands and temporarily placed in plastic bags. combining Working Reagent A with Working Reagent B in a ratio of 50 parts A to 1-

part B, resulting in the formation of a clear green solution. This solution retained its stability for several days at room temperature.

For the determination of protein concentration, the Bicinchoninic Acid (BCA) Protein Assay Kit from Thermo Scientific was employed following the instructions provided by the manufacturer. Firstly, 50 μ L of the sample was mixed with 1.0 mL of Working Reagent (1:20 v/v) and the mixture was vortexed for 1 minute to ensure thorough mixing. Subsequently, the assay mixture was incubated at 37°C for 30 minutes, using a block heater. Following incubation, the absorbance was measured at 562 nm against a water blank, utilizing a spectrophotometer (Spectramax ID3/Molecular Devices).

Protein concentrations were determined using a standard curve that had been generated by incubating known concentrations of BSA (ranging from 0 to 1000 μ g/mL) with 1.0 mL of working reagent shown in **Appendix A**.

3.2.4 Partial Purifications of Crude Skin Secretions

3.2.4.1 Solid Phase Extractions using Sep Pak C-18® Plus C18 Cartridges

Impurities, such as acids and salts, which can strongly bind to reverse-phase high-performance chromatography, may be present in crude skin secretions (Conlon, 2007). Therefore, the crude extracts were subjected to concentration and partial purification by using Sep Pak C-18 cartridges (WATERS, USA), by the method outlined by Menegolloet al., (2019)

3.2.4.2 Frog skin secretion samples Preparation using Sep Pak Cartridges

The samples were thawed under running cold water and centrifuged at 5000 g for 30 minutes at 4°C to remove debris. The peptide-containing supernatant was decanted into a sterile beaker and kept on ice. The Sep Pak cartridge was activated with 12 mL of acetonitrile

(2 mL per cartridge) followed by 0.1% (v/v) Trifluoroacetic acid in water. Then, 5 mL of supernatant was loaded onto the cartridge at a flow rate of 4 mL/min, followed by a wash with 0.1% TFA in water. The peptides were eluted with 12 mL of 70% acetonitrile containing 0.1% TFA, collected in a glass beaker, and aliquoted into 1.5 mL microcentrifuge tubes. These samples were preserved at -80°C according to the protocol established by Sabri et al. (2018).

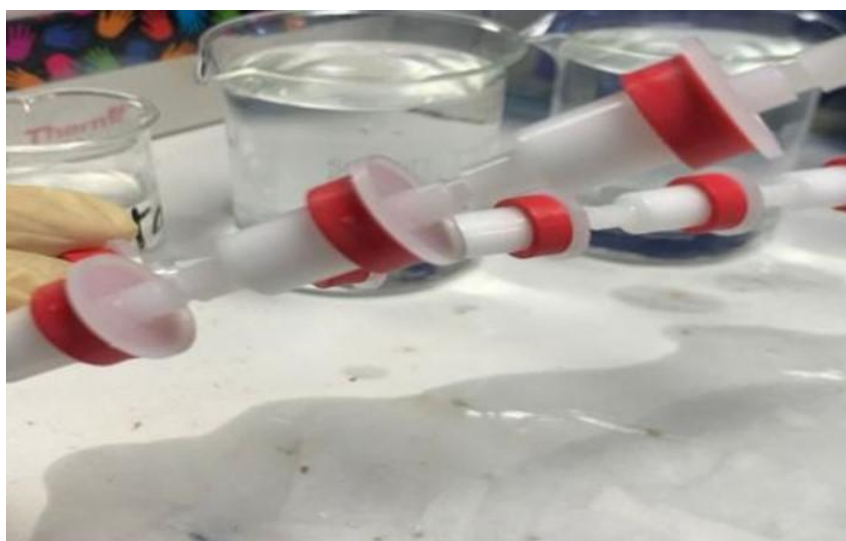


Figure 3.4: Sep Pak C-18® Plus C18 Cartridges (Waters) Connected in Series.

3.2.5 Concentration of Partially Purified Skin Secretions

3.2.5.1 Concentrating and Desalting of Samples

The partially purified samples were concentrated by using Amicon® Ultra-15 10 kDa Molecular Weight Cut Off (MWCO) Centrifugal Filter Devices (Merck). These devices served two purposes, which were concentrating on the diluted samples and desalting them. The weight of the filter device was recorded before being loaded with 5 mL of partially purified secretions. A tube filled exclusively with distilled water was included to balance the centrifuge rotor. The filter device was then centrifuged at 4°C at 3200 x g for 30 minutes

using an Eppendorf 5810R centrifuge. Then, 145 μ L of the supernatant was meticulously transferred into a microtube and stored at -20°C for subsequent HPLC analysis. accomplished by using Amicon® Ultra-15 10 kDa Molecular Weight Cut Off (MWCO) Centrifugal Filter Devices (Merck). These devices served the dual purpose of further concentrating the diluted samples and desalting them. Each device was loaded with 5 mL of partially purified secretions, and prior to utilization, the weight of the filter device was recorded. To balance the centrifuge rotor, a tube filled exclusively with distilled water was included. The device was then centrifuged at 4°C at 3200 x g for 30 minutes using an Eppendorf 5810R centrifuge. Approximately 150 μ L of the supernatant was meticulously transferred into a microtube and stored at -20°C for subsequent HPLC analysis.

3.2.5.2 Washing and Recycling of Ultramembrane Filter

The used filter device underwent a meticulous cleaning process in order to be used again. Initially, it was rinsed with 3 mL of distilled water for 2 minutes, followed by a subsequent 2-minute rinse with 3 mL of 100% ethanol, all while subjected to a centrifugation force of 3200 x g. This rigorous cleaning procedure ensured the removal of any contaminants and residues from the device.

Subsequently, the device was filled with 20% ethanol, serving as a preservative to maintain its sterility and integrity during storage. It was then carefully stored at a temperature of 4°C, preserving its readiness for future applications and minimizing the risk of contamination.

3.2.6 Peptide Profile Analysis

The purification of antimicrobial peptides (AMPs) from the skin secretions of *Pulcharana baramica*, *Haylarana erythraea*, and *Limnonectes paramacrodon* was carried

out using ÄKTA Pure 25 System (Chemopharm, Malaysia), (Figure 3.4). The column used was HPLC Siliachrom C18 mono column, with a column volume of 4 mL and a diameter of 4.6 mm. The column pressure was maintained at 7.9 MPa.

Before the commencement of the purification process, a 5-minute wash with 20% ethanol was administered to the ÄKTA machine pumps, with pump B being initiated first, followed by pump A. The buffer preparation encompassed two components: buffer A, comprising 0.1% TFA in 99% water, and buffer B, consisting of 70% acetonitrile and 0.1% TFA in water. The connection arrangement was as follows: inlet A was connected to buffer A, while inlet B was connected to buffer B.

Prior to injection into ÄKTA, the sample underwent filtration through a 0.22 μ m filter unit. The Peptide Silica C18 Mini Column was washed with buffer A using the Unicorn 7 system. Subsequently, a manual sample injection was executed through the injector loop, and the initiation of elution with buffer B was performed.

The data collection process was overseen by the Unicorn 7 software. Following this, the acquired fractions were collected using the ÄKTA fraction collector, concentrated, and subsequently subjected to freeze-drying for further analysis.

Achieving precise separations involved packing the column with a solution comprising 0.1% (v/v) trifluoroacetic acid in 99.9% water. During elution, a crucial stage in separating target compounds, a 70% acetonitrile solution containing 0.1% (v/v) trifluoroacetic acid was employed. This solution flowed at a consistent rate of 0.5 mL/min, capturing isolated peaks corresponding to distinct compounds with accuracy.

Monitoring the purity and integrity of isolated compounds involved using a UV detector at two different wavelengths, 240 nm. This careful monitoring helped accurately identify and collect all relevant peaks.



Figure 3.5: ÄKTA Pure™ Chromatography System integrates essential components for efficient chromatographic separations. This includes a pump module, injector valve loop, a UV detector and a fraction collector for collecting eluted fractions.

3.2.6.1 Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis

3.2.6.2 Gel preparation

A 0.05% bromophenol blue solution was prepared by dissolving 0.010 grams of in 20 mL of distilled water. The 3M Tris solution was prepared by dissolving 36.34 g of Tris dissolved in 100 mL of distilled water, and the pH was adjusted to 8.45 by the addition of hydrochloric acid. Additionally, a 0.5 M Tris solution was prepared by dissolving 18.17 g of Tris in 300 mL of distilled water, with subsequent pH adjustment to 6.8 using hydrochloric acid.

A freshly prepared 10% ammonium persulphate solution was created by dissolving 50 mg of APS in 500 μ L of distilled water. Emphasis was placed on not exceeding 55 mg of ammonium persulphate per 500 μ L of water to prevent excessive concentration, which

could lead to gel hardening and the distortion of bands. Additionally, the 1x Sample Buffer was formed by diluting the 2x Sample Buffer two-fold with water; specifically, 2 mL of Sample Buffer was mixed with an equal amount of distilled water and divided into three 1.5 mL micro-centrifuge tubes. The ultra-low range molecular weight marker (Sigma-Aldrich) underwent a 20-fold dilution with 1x Sample Buffer; this involved mixing 10 μ L of the molecular weight marker with 190 μ L of Sample Buffer. The resulting aliquot was stored at -20°C.

The gel preparation procedure followed the guidelines outlined in Table 3.1, which specified the proportions for the separating gel, spacing gel, and stacking gel. The procedure commenced with the pouring of the separating gel, which was allowed to polymerize for 30 minutes at room temperature. Subsequently, the spacing gel was added and likewise allowed to polymerize for 30 minutes.

Table 3.1: The Volume and the Chemical Reagents of Separating Gel, Spacer Gel and Stacking Gel

Chemical reagents	Separating gel	Spacing gel	Stacking gel
30 % Acrylamide Stock	2.8 ml	1.65 ml	195 μ l
0.5 M Tris. pH 6.8	-	-	375 μ l
10 % SDS	-	-	15 μ l
Gel Buffer	1.65 ml	1.65 ml	-
Glycerol	0.135 ml	-	-
10 % APS	15 μ l	15 μ l	7.5 μ l
TEMED	2 μ l	2 μ l	2 μ l
Water to final Vol	5 μ l	5 μ l	1.5 μ l

After this duration, the stacking gel was introduced, and the gel comb was carefully inserted. The stacking gel was then given 15 minutes to polymerize. In parallel, loading buffer, anode buffer, and cathode buffer were prepared to reach final volumes of 8 mL and 500 mL, respectively, as detailed in Table 3.2.

Table 3.2: The Parameters for Loading Buffer, Anode Buffer and Cathode Buffer

	Loading buffer	Anode buffer	Cathode buffer
0.5 M Tris. pH 6.8	1.0 ml	-	-
Glycerol	0.8 ML	-	-
10 % SDS	1.6 ML	-	5.0 ml
2-B mercaptoethanol	0.4 ml	-	-
0.05 % bromophenol blue	0.2 ml	-	-
Tris	-	12.1 g	6.06 g
Tricin	-	-	8.96 g
Adjust pH to	-	8.8	8.25
Water to final Vol	8.0 ml	500 ml	500 ml

3.2.6.3 Frog skin secretion samples Preparation

The samples were thawed by being subjected to running cold water. Subsequently, approximately 10 μ L of these samples were dissolved in 20 μ L of loading buffer and were heated at 85°C on a heating block for 5 minutes before being loaded onto gels. Additionally, a volume of 1 μ L of the molecular weight marker (Sigma) was combined with 19 μ L of sample buffer (a 20-fold dilution) and was heated at 65°C for 2 minutes before loading.

3.2.6.4 Analysis of Peptides Purity Using (SDS-PAGE)

Assessing differences in peptide sample purity involved collecting a 10 μ L aliquot both before and after partial purification using Sep-Pak® C18 Cartridges and after each peak (fractions obtained from HPLC). These aliquots underwent Tricine Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) following the method outlined by Schaagger (2006). For gel preparation, a separating gel was poured to a height of approximately 3.0 cm, followed by a spacer gel of 1.5 cm. Water was added to create a level surface, and the gel was allowed to polymerize for 30 minutes. Subsequently, excess water was removed using absorbent filter paper to achieve the driest possible surface. Following this, a stacking gel was poured to a height of approximately 1.0 cm on top of the already polymerized separating and spacer gels.

The comb was promptly inserted, and the gel was allowed to polymerize for 15 minutes. Following this, 6 μ L of an ultra-low range molecular weight marker was loaded into the initial well, followed by the samples into the subsequent wells. The gels were then subjected to electrophoresis at 200 V and 150 A for 60 minutes until the dye had migrated to the bottom of the gel. Photographs of the resulting bands were captured for subsequent analysis.

3.2.6.5 Gel Staining

A crucial role is played by gel staining in numerous laboratory procedures aimed at visualizing and scrutinizing biological molecules, such as proteins. As outlined by Lawrence A. et al. in 2009, the process is initiated with the disassembly of the gel cassette, followed by the careful placement of the gel inside a container for subsequent washing steps. Approximately 100 milliliters of distilled water were introduced into the container and heated in a microwave oven for 30 seconds, with meticulous attention to preventing boiling.

Subsequently, the container, housing the gel, was positioned on a shaker for a duration of 3-5 minutes. This washing sequence was repeated twice, using fresh water each time.

Following the washing steps, a sufficient volume of Coomassie blue staining solution was added to ensure complete coverage of the gel within the container. The container was then briefly subjected to 10 seconds of microwave heating, with careful monitoring to avoid boiling. Subsequently, the container with the gel was returned to the shaker to complete the staining process. Upon the conclusion of the staining process, the staining solution was drained off, and an additional 50-100 milliliters of distilled water were introduced into the container to further destain the light blue background of the gel, all while the container was kept on the shaker. Fresh water was used to replace the water for further destaining. Typically, protein bands usually become visible within 24 to 48 hours.

3.2.7 Antimicrobial Peptide Test

3.2.7.1 Preparation of Agar and Luria Bertani Broth

Mueller Hinton agar (MHA; Himedia) was prepared by dissolving 19 grams of agar in 1000 mL of distilled water. The solution underwent thorough mixing, and additional distilled water was added to achieve a final volume of 1000 mL for the agar solution. Following this, the agar solution was subjected to autoclaving at 121 psi/lb for 15 minutes and was then allowed to cool to approximately 50°C. Subsequently, the solution was poured into petri dishes, and it was left to solidify at room temperature. Once the agar plates had solidified, they were inverted and stored in a refrigerator at 4°C for preservation.

For the preparation of Luria Bertani broth, 21 grams of the broth were dissolved in 1000 mL of distilled water. The solution was mixed thoroughly, and additional distilled water was added to bring the final volume to 1000 mL for the broth solution. The broth

solution was then autoclaved at 121 psi/lb for 15 minutes and allowed to cool to room temperature. Subsequently, 3 mL of the broth solution was aliquoted into bijoux bottles and stored in a dry place. It is important to note that this entire procedure was conducted within a laminar hood to maintain sterility throughout the preparation process.

3.2.7.2 Preparation of Glycerol Bacterial Stock

Growing bacteria on a fresh plate from a frozen stock is essential for ensuring consistent results. This method is supported by Lakshman et al. (2018), where it was demonstrated that bacteria preserved in this manner maintain their cultural, biochemical, and immunological characteristics over an extended period.

To initiate the process, a single colony of *E.coli* and *S.auerus* strains was isolated using a sterile 10 µL inoculating loop and dissolved in 5 mL of Luria Bertani broth contained within a sterile screw-cap tube. The tube was then subjected to vortexing for 30 seconds and subsequently incubated overnight at 37°C. the bacterial suspension underwent another vertexing step and was aseptically pipetted into sterile 1.5 mL cryovial tubes, resulting in a final glycerol concentration of 20% (v/v). The bacterial suspension was thoroughly mixed by vertexing and was then stored in a -80°C freezer for future utilization and long-term storage.

3.2.7.3 Obtaining Pure Culture Isolates

The frozen glycerol bacterial stock was subjected to thawing under running tap water. Subsequently, the three-sector T streak assay was executed to acquire pure culture isolates of both gram-positive and gram-negative bacteria. To achieve this, a loopful of the bacterial stock, which had been thoroughly vortexed, was applied to one edge of the Mueller Hinton agar surface in a back-and-forth motion, covering one quarter of the agar surface. This initial streak constituted the first section of the T streak.



Figure 3.6: An illustration of the three sector T streak assay performed to obtain pure culture isolates of gram positive and gram negative bacteria. (American Society for Microbiology , 2016).

Following this, the plate was rotated by 60 degrees, and another streak, perpendicular to the original inoculation, was performed, with overlapping in some instances. Again, the plate was rotated by 60 degrees, and streaking was carried out on a new surface, perpendicular to the second section, thereby creating a sufficient three-way dilution of bacteria, as depicted in Figure 3.5. Subsequently, the lid of the plate was closed, and it was incubated overnight at a temperature of 37°C. the isolated colonies were employed for the preparation of the inoculum.

3.2.7.4 Frog Skin Secretion Samples Preparation

Initially, peptide samples at a protein concentration of 1000 µg/mL, following their passage through Sep Pak C18 cartridges, underwent further dilution to achieve final concentrations of 500 and 250 µg/mL. This dilution process was carried out by performing 1:1 dilution with 0.1% (v/v) TFA. Subsequently, the resulting samples were placed into separate vials and stored at -80°C for preservation.

In the case of broth microdilutions, the peptides were twofold diluted directly in Nunc 96-well microplates with round-bottomed wells, using LB Broth. These dilutions were

freshly prepared just before use to ensure their accuracy and consistency. The samples were stored in a -20°C freezer and, when needed, were thawed by placing them under running cold water before use. Additionally, 10 µg ampicillin disks were utilized as a positive control in the experiments.

3.2.7.5 Inoculum Preparation using Growth Method

The guidelines outlined in the 9th Edition of the Clinical and Laboratory Standards Institute were strictly adhered to in this method. To prepare the inoculum for both *E. coli* and *S. aureus*, three to five well-isolated colonies of the same morphological type were individually selected from agar plate cultures. The tip of each colony was touched with a 10 µL sterile inoculating loop, and the growth was transferred into a tube containing 3 mL of Luria Bertani broth. The broth culture was then incubated for 4 hours to achieve a turbidity equivalent to the 0.5 MacFarland standard (OD₆₀₀= 0.08-0.1), which corresponds to 1 x 10⁸ CFU/mL and 2 x 10⁸ CFU/mL.

Subsequently, for the disk diffusion assay and broth microdilution, 40 µL of the bacterial suspension was further diluted (a 20-fold dilution) into 800 µL of broth. This final inoculum of 5 x 10⁶ CFU/mL was meticulously prepared within 15 minutes before its intended use, ensuring the accuracy and consistency of the experimental setup.

The calculation of the dilution factor is shown in Equation 3.1

$$\frac{5 \times 10^6}{1 \times 10^8} \text{ CFU per milliliter} = \frac{1}{20} \quad \text{Equation 3.1}$$

3.2.7.6 Disk Diffusion Assay

The activity of peptide mixtures against both *E. coli* and *S. aureus* was assessed through a disk diffusion assay, following the guidelines outlined in the Clinical Laboratory Standards Institute M02-A11. This experiment was conducted within a Biosafety Cabinet.

For the disk diffusion method, individual isolates were prepared by selecting three to five well-isolated colonies of the same morphological type from agar plate cultures. The tip of each colony was touched with a sterile 10 μ L inoculating loop, and the growth was transferred into 3 mL of Luria Bertani broth. This broth culture was then incubated for 2 hours in an orbital incubator at 37°C. After incubation, the turbidity was adjusted to match the 0.5 MacFarland standard (OD₆₀₀= 0.08-0.1), equivalent to 1×10^8 CFU/mL, by adding sterile PBS. The MacFarland suspension was further diluted 1:20 with LB Broth to achieve a final bacterial concentration of 5×10^6 CFU/mL. Within 15 minutes, the bacterial suspension was evenly spread across the entire surface of a Mueller Hinton Agar (Himedia) plate using a sterile cotton-tipped swab and a petri dish rotator to create a uniform lawn. The plates were allowed to air dry for 5 minutes at room temperature. Sterile 6 mm paper disks were autoclaved at 121 psi for 15 minutes in a beaker sealed with aluminum foil before impregnating them with 20 μ L of samples, positive controls (conventional antibiotics). These disks were then placed on the surface of the MHA plate using sterile forceps. The plates were incubated aerobically at 37°C for 18 hours, and after overnight incubation, the diameter of the inhibition zone was measured using a ruler.

3.2.7.7 Broth Microdilution Assay

Antimicrobial testing to determine the Minimum Inhibitory Concentrations (MIC) of partially purified secretions against *E. coli* and *S. aureus* was conducted following the guidelines provided by the Clinical and Laboratory Standards Institute M07-A9. All

preparations of the microtiter plate were performed within a laminar flow biological safety cabinet. Approximately 50 μ L aliquots of LB broth (for *E. coli* and *S. aureus*) were dispensed into columns 2-8 of a 96-well microtiter plate. A volume of 100 μ L of the highest concentration of the peptide sample was dispensed into column 1. Subsequently, two-fold serial dilutions were executed by transferring 50 μ L of the peptide sample from the first column well to the second column and then to the next column. This process resulted in a final concentration range of 500, 250, 125, 62.5, 31.2, 15.6, and 7.8, μ g/mL. These dilutions were carried out with a steady circular motion to prevent the formation of air bubbles. A volume of 50 μ L aliquots of a bacterial suspension at a concentration of 5×10^6 CFU/mL was introduced into columns 1 to 8.

Controls were established by introducing 100 μ L of LB broth into column 10 as a sterility control, and a mixture of 50 μ L of LB broth and 50 μ L of bacterial inoculum was introduced into the last column as a growth control. The 96-well microtiter plate was sealed using parafilm and then incubated at 37°C for 18 hours. After overnight incubation, bacterial growth was assessed by measuring turbidity as the optical density at 620 nm (OD₆₂₀) using a Spectra max ID3, ELISA plate reader (APPENDIX B). The MIC (Minimum Inhibitory Concentration) of the peptide samples was determined as the lowest concentration where no viability was observed in the wells of the 96-well microtiter plate after 18 hours.

3.2.8 Determination of HC50 Value of Partially Purified Peptides by Hemolysis Assay

3.2.8.1 Frog Skin Secretion Samples Preparation

A series of microcentrifuge tubes were set up, each containing 50 μ L of Phosphate Buffer Saline (PBS) with a pH of 7.4. In the first tube, 100 μ L of the partially purified peptide sample was transferred. Then, 50 μ L of the solution from the first tube was carefully mixed

with 50 μ L of PBS in the second tube. This process was repeated, creating a total of 10 dilutions, and the final tube in the series was not used. A Negative control was prepared, consisting of 50 μ L of PBS, while positive control was made with 50 μ L of 1% Tween-20 dissolved in PBS.

3.2.8.2 Hemolysis Assay

The assessment of the potential toxicity of the AMPs on normal mammalian cells involved the evaluation of peptide-induced hemolysis using human erythrocytes. The methodology described by Ju et al. (2021) was followed. Initially, red blood cells were procured from the Faculty of Medicine and Health Science at UNIMAS. These cells underwent separation from plasma and other blood components through centrifugation at 1800 x g for 10 minutes, utilizing an Eppendorf 5810R centrifuge. Subsequently, the red blood cells were resuspended in a volume of PBS equivalent to that of a whole blood sample. The concentration of these washed blood cells was determined using a hemocytometer machine and was then diluted 100-fold in PBS to achieve a final concentration of approximately 5×10^7 cells. It was established that the lifespan of these washed RBCs, following the washing process, was 24 hours.

Next, 50 μ L of the washed erythrocytes were introduced into all tubes containing samples and controls. The contents were thoroughly mixed and then subjected to incubation at 37°C for 1 hour. Following incubation, all tubes underwent centrifugation at 1325 x g for 30 seconds using a Wisepin® CF-10 (Daihan) centrifuge to pellet the cells and eliminate any cellular debris. A volume of 90 μ L of the supernatant was subsequently transferred to a 96-well plate with a flat bottom (NUNC™), and the absorbance was measured at 450 nm. To determine the extent of hemolysis, the positive control measurement was set at 100%, while the negative control was established at 0%.

3.2.9 Aseptic Techniques and Preparation for HIT-T15 Pancreatic Beta Cells

3.2.9.1 Preparation of Cell Culture Work

Creating a sterile environment and preventing contamination between microorganisms and the sterile cell culture hood is facilitated by adhering to good aseptic techniques. The process began with the removal of the hood's cover and its activation. Subsequently, the hood's sash was adjusted to the correct position to maintain laminar airflow. To ensure sterility, all surfaces were then meticulously sanitized with 70% ethanol. All equipment and materials, including pipette tips, pipettes, racks, flasks, and reagents, underwent thorough preparation and sterilization before introduction into the hood. Sterilization was achieved through autoclaving or by spraying 70% ethanol to pipettes and racks.

3.2.9.2 Preparation of Cell Growth Medium

Preparing the Ham's F12K medium involved creating a mixture by combining 50 mL of horse serum, 12.5 mL of fetal bovine serum, and 5 mL of penicillin-streptomycin solution with 500 mL of medium. This formulation was meticulously blended to ensure homogeneity. Subsequently, the resulting medium was aseptically dispensed into individual 50-ml conical-bottom tubes, which were then securely sealed. To shield the contents from light, the tubes were covered with foil. Finally, the prepared medium was stored at a temperature of 4 °C, ensuring its preservation and readiness for use when required.

3.2.9.3 Preparation of 0.25% (w/v) Trypsin-0.53 mM EDTA Solution

The 0.25% (w/v) Trypsin-0.53 mM EDTA solution was prepared by dissolving 19.7 mg of EDTA and 250 mg of trypsin in 100 ml of autoclaved water, ensuring the complete dissolution of the components through continuous shaking. Subsequently, the solution underwent aseptic filtration through a 0.2- μ m filter unit into a sterilized 50 ml tube,

maintaining sterility throughout the entire procedure. The resulting solution, once labeled , filtered and carefully stored at a temperature of -20 °C.

3.2.10 Cell Culture: HIT-T15 Pancreatic Beta Cells Thawing procedure

The HIT-T15 cells (CRL-1777) cells were bought from the ATCC (American Type Culture Collection) from INNONIAGA Malaysia.

The procedure was initiated by retrieving the frozen cells from liquid nitrogen storage. The vial containing the cells was then thawed by gentle agitation in a 37°C water bath for 2 minutes. Upon complete thawing, the vial was removed from the water bath and subjected to decontamination by spraying it with 70% ethanol. From this point onward, all operations were performed under rigorous aseptic conditions. The contents of the vial were then transferred into a centrifuge tube containing 9.0 mL of complete culture medium. Subsequently, the mixture underwent centrifugation at approximately 125 x g for 7 minutes.

Following centrifugation, the cell pellet was resuspended in the complete medium and dispensed into a 25 cm² flask. Finally, the flask was positioned in a 37°C incubator with a 5% CO₂ atmosphere for incubation.

3.2.10.1 Changing Medium

Daily microscopic examinations were conducted on the HIT-T15 cells to ensure their health and monitor their growth, aiming to confirm their thriving as anticipated. Additionally, media changes were conducted every 2 days to facilitate optimal growth and confluence, allowing the cells to reach confluence before splitting.

The process included pre-warming fresh media in a water bath for a minimum of 30 minutes. Subsequently, the old media were discarded into a waste container, and an

equivalent volume of prewarmed new media was added to the wells. The culture flask was then returned to the incubator.

3.2.10.2 Subculture Procedure (Splitting Cells)

After 14 days, when the cells had reached 80% confluency (as shown in Figure 3.6), cell splitting, 1:2 split was performed. Fresh cell culture medium was prewarmed to 37°C in either a water bath or an incubator for a minimum of 30 minutes. The existing medium in the culture flask was carefully poured into a waste container containing approximately 100 mL of a 10% sodium hypochlorite solution with bleach, serving as the waste medium.

To cleanse the cells and eliminate any residual media and FBS (Fetal Bovine Serum), 5 mL of prewarmed PBS (Phosphate Buffered Saline) was pipetted into the flask. The flask was gently rocked to wash the cells with PBS, and the media were then poured into the waste container. This rinsing step was repeated three times to ensure the removal of any lingering FBS for effective trypsinization. After completing the washes, 1 mL of prewarmed trypsin EDTA solution was added to the flask. The flask was placed in a 37°C incubator for 5 minutes. While gently tapping the flask, the cells were monitored under a microscope to confirm detachment. Once the cells were detached, fresh culture medium was added to the flask. Eliminating any additional adherent cells involved gently pipetting the medium up and down the walls of the flask. The cells were counted, and a necessary volume of cells was pipetted into new 25 cm² flasks. These flasks were then filled with culture media to achieve a total volume of 10 mL. Each flask was labeled with pertinent information, including the passage number, cell line, split ratio, and date. The cells were left undisturbed overnight in a CO₂ incubator to recover and settle.

3.2.10.3 Cell Counting

Cell counting was carried out manually using a hemocytometer to assess the cell viability, determine cell density, and calculate the percentage of viable cells. The cells were trypsinised and transferred into an Eppendorf tube. Following this, they were centrifuged at 100 x g for 6 minutes, and the supernatant was carefully removed. Subsequently, 1 ml of culture medium was added to resuspend the cells. A 1:1 dilution of cells with trypan blue was performed in a microcentrifuge tube. Before loading the cell suspension, the coverslip and counting surface of the hemocytometer were cleaned with 70% alcohol and lens paper. Using a pipette, 10 µL of the prepared sample was gently expelled into a full grid of 1 mm squares on the hemocytometer. Cell counting was conducted with a 4x magnification, focusing on the middle, two right, and two left squares.

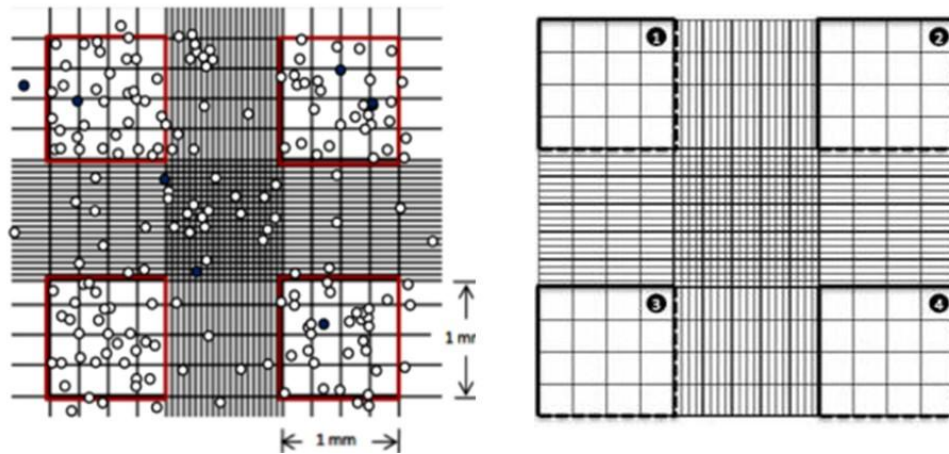


Figure 3.7: Hemocytometer Grid under a Microscope. Cells were counted within the four large corner squares of the central 1x1 mm area, with cells that touch the top or left borders of the grid square were included, while those touching the bottom or right borders are excluded. (<https://www.chegg.com/honorcode>)

The cell density was calculated using the following formula.

$$\text{TCPmL} = \frac{\text{TCC} \times \text{dilution factor} \times 10.000 \text{ cells/ml}}{\text{Number of squares counted}} \quad \text{Equation 3.2}$$

Where, TCPmL is the total cells per milliliter, and TCC is the total cells count. Cell seeding density: The cell seeding density was calculated using the following formula.

- i. The volume of cell suspension needed is the product of the total number of wells and the desired cell density per total cell per ml.
- ii. The total volume of medium needed is the product of the total number of cells and the volume per cell.
- iii. The volume of medium needed to pop-up is the volume of cell suspension needed, subtracted from the total volume of medium needed.

3.2.10.4 Freezing Cells

The freezing medium was prepared by adding 5% dimethyl sulfoxide to the complete growth medium, and it was stored at 2° to 8°C until needed. The cells were detached gently from the tissue culture vessel using trypsin, following the subculture procedure. The total cell count, and percent viability were determined using a hemocytometer, and based on this, the required volume of the freezing medium was calculated. Next, the cell suspension underwent centrifugation for 6 minutes at 100 g. The supernatant was aseptically decanted without disturbing the cell pellet. The cell pellet was then reconstituted in the freezing medium, and aliquots of the cell suspension were dispensed into cryogenic storage vials. Throughout this process, the cells were gently and regularly mixed to maintain a homogeneous cell suspension. Subsequently, the cells were placed in a controlled rate freezing apparatus, with the temperature gradually reduced at a rate of approximately 1°C per minute. Finally, the frozen cells were transferred to liquid nitrogen and stored in the gas phase above the liquid nitrogen.

3.2.11 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide (Cytotoxicity Assay)

To evaluate the impact of various antimicrobial peptides (AMPs) on cell viability in HIT-T15 cells, the widely recognized MTT assay kit from Sigma-Aldrich was employed. Different concentrations of AMPs, specifically 1 µg/ml, 5 µg/ml, 10 µg/ml, 25 µg/ml, and 50 µg/ml, were tested. HIT-T15 cells were initially seeded at a density of 2×10^4 cells per well within a 96-well plate and allowed to incubate for a 24-hour period. Subsequently, after this incubation period, the cells were exposed to the varying concentrations of the test samples for an additional 24 hours. Upon completion of the 24-hour treatment duration, the culture media were carefully removed. To determine cell viability, a 1 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added, and the cells were incubated at a temperature of 37°C for 2 hours, allowing for the conversion of MTT into formazan crystals within the viable cells. Following the 2-hour incubation with MTT, the MTT solution was aspirated, and 0.1 mL of a solubilisation solution was introduced to dissolve the formazan crystals. Finally, to quantitatively assess cell viability, the absorbance at a wavelength of 570 nm was measured using an ELISA plate reader. This comprehensive methodology offered precise insights into the cytotoxic effects of the AMPs on HIT-T15 cells across a range of concentrations.

3.2.12 Elisa Assay

3.2.12.1 Krebs-Ringer Bicarbonate Buffer Preparation

The Krebs buffer was prepared according to the manufacturer's instructions from Sigma-Aldrich, Malaysia. First, 90% of the required water volume was added, maintaining a temperature between 15 and 20°C. While the water was stirred, the powdered medium was added, and the package was rinsed to remove any remaining powder. The solution was stirred

until fully dissolved. Next, 1.26 grams of sodium bicarbonate were added and dissolved. The pH was adjusted to 0.3, anticipating a rise during filtration. The final volume was reached by adding additional water to bring the solution to 100 mL. Finally, the solution was sterilized and filtered through a 0.22-micron filter unit before being aseptically dispensed into sterile containers.

3.2.12.2 Supernatant Preparation

The HIT-T15 cells were cultured at a density of 5×10^4 cells per well in a 12-well plate for 48 hours, a critical period for establishing a stable cellular environment for subsequent testing. Following this, the cells were incubated for 30 minutes with 1 mL of Krebs-Ringer Bicarbonate Buffer (KRB buffer, Sigma-Aldrich), containing 1.8 g/L D-glucose, 0.00468 g/L $MgCl_2$, 7.0 g/L NaCl, 0.34 g/L KCl, 0.1 g/L Na_2HPO_4 , and 0.18 g/L NaH_2PO_4 . During this incubation, the cells were exposed to 5 μg concentrations of the test sample. Afterward, the conditioned media were collected, centrifuged at 12,000 rpm for 10 minutes, and the supernatant was isolated and quantified using the Human Insulin ELISA kit (R&D Systems).

3.2.12.3 Reagent Preparation

The kit contains several components: 21 mL of Human Insulin Conjugate (a monoclonal antibody specific for insulin conjugated to horseradish peroxidase with preservatives), Human Insulin Standard (recombinant human insulin in a buffered protein base with preservatives), 18 mL of Assay Diluent RD1-105 (a buffered protein base with preservatives), 21 mL of Calibrator Diluent RD5-62 (a buffered protein base with preservatives), and 2 vials (21 mL each) of Wash Buffer Concentrate (a 25-fold concentrated solution of buffered surfactant with preservatives). The reagents were allowed to reach room temperature before use. To prepare the wash buffer, 40 mL of concentrate was added to 960

mL of distilled water, resulting in 1000 mL of wash buffer. The kit also includes 12 mL of Color Reagent A (stabilized hydrogen peroxide), 12 mL of Color Reagent B (stabilized chromogen, tetramethylbenzidine), and 6 mL of Stop Solution (2 N sulfuric acid).

3.2.12.4 Substrate Solution

Equal volumes of colour reagent A and colour reagent B were mixed within 15 minutes of use. A 200 μ l aliquot of the resultant mixture is required per volume.

3.2.12.5 Human/Canine/Porcine Insulin Standard

The assay is based on a quantitative sandwich enzyme immunoassay technique. The insulin standard was reconstituted with distilled water, producing a stock solution of 5000 pmol/mol. The standard was thoroughly mixed to ensure complete reconstitution and left to set for a minimum of 15 minutes with gentle agitation prior to making dilutions. Next, 900 μ l of calibrator diluent RD5-62 was pipetted into the 500 pmol/L tube, and 500 μ l was pipetted into the remaining tubes. The stock solution was used to produce a dilution series, with each tube being mixed thoroughly before the next transfer (Figure 3.7). The 500 pmol/L standard served as the high standard, and the calibrator diluent RD5-62 served as the zero standard (0 pmol/L).

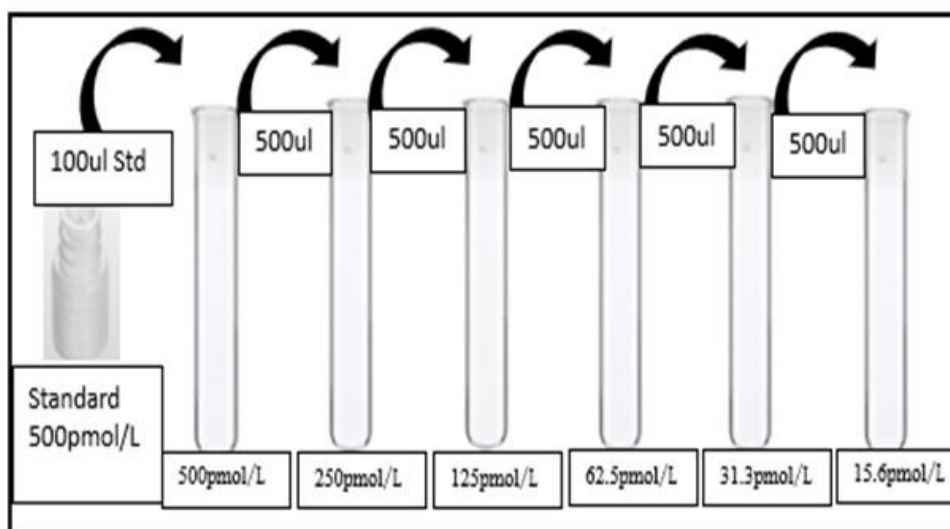


Figure 3.8: Reconstitution of Human/Canine/Porcine Insulin Standard for ELISA Assay, A dilution series was prepared from the stock solution, with each tube mixed before the next transfer. The 500 pmol/L standard served as the high standard, while RD5-62 acted as the zero standard (0 pmol/L).

3.2.12.6 Assay Protocol

The excess microplate strips were returned to the foil pouch with the desiccant pack. Each well was washed twice with wash buffer using an autowasher, ensuring complete removal of liquid for optimal performance. After the final wash, any remaining buffer was aspirated and the plate inverted, then blotted against a clean paper towel. Next, 150 µl of assay diluent RD1-105 was added to each well, followed by 50 µl of standard and sample solutions. The plate was covered with an adhesive strip and incubated for 2 hours at room temperature on a shaker at 500 ± 50 rpm. Afterward, the washing procedure was repeated, and 200 µl of Human Insulin conjugate was added to each well, with the plate incubated for another 2 hours. The washing step was repeated before 200 µl of substrate solution was added, and the plate was incubated for 30 minutes at room temperature, protected from light. Finally, 50 µl of stop solution was added to each well, resulting in a color change from blue

to yellow. The optical density of each well was promptly measured within a 30-minute timeframe using a microplate reader set at a wavelength of 450nm.

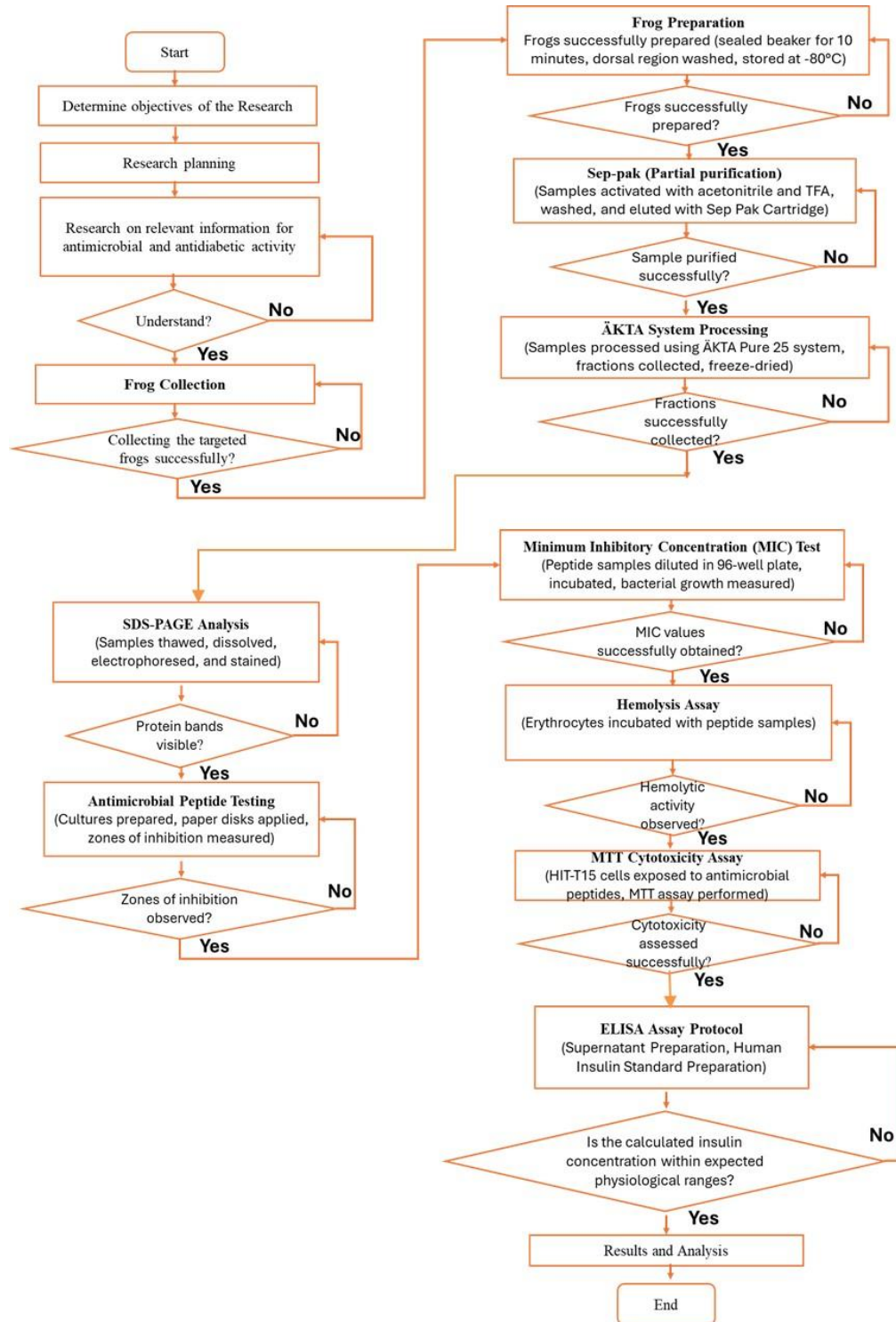


Figure 3.9: Flowchart illustrating the step-by-step methodology used in the study

CHAPTER 4

RESULTS

4.1 Study Locations

The present study focused on frog species within the families of Ranidae and Dicroglossidae, investigating multiple frog genera. Frogs belonging to the species *Pulcharana baramica*, *Hylarana erythraea*, and *Limnonectes paramacrodon* were collected from various locations, including the nearby forest, beat swamp forest, and the lake areas of east campus of Universiti Malaysia Sarawak, as shown in Figure 4.1.



Figure 4.1: The Frog Species Study Locations. The frogs were predominantly found distributed along water courses, leading to concentrated samplings along the nearby forest , beat swamp forest , and the lake areas of east campus of Universiti Malaysia Sarawak Unimas lake.

Table 4.1: Number of Specimens from Three Bornean Frog Species Collected from the Selected Sampling Sites

Genus	Specie	No. of specimens	Location
<i>Pulcharana</i>	<i>P.baramica</i>	8	East campus lake
<i>Hylarana</i>	<i>H. erythraea</i>	6	East campus lake and peat swamp forest
<i>Limnonectus</i>	<i>L.paramacrodon</i>	7	Zoology forest and peat swamp forest

The frog samplings, carried out during a three-day trip between September 2021 and August 2022, coincided with an annual temperature range of 24 °C - 30 °C. The frogs were found distributed along water courses, leading to concentrated samplings along the UNIMAS east campus lake areas. Throughout the sampling period, a total of three species were collected, comprising of 8 individuals from *P. baramica*, 6 individuals from *H. erythraea*, and 7 individuals from *L. Paramacrodon* (refer to Table 4.1). Identification of the frogs was conducted using the method outlined in "A Field Guide to the Frogs of Borneo. (Inger et al, 2017).

4.1.1 Bornean Frog Identification Keys

The identification of Bornean frog species relied on the comprehensive identification keys outlined by (Marly, et al 2017). These keys encompass various criteria, including snout-

vent length (SVL), coloration, size of the *tympanum* (a prominent external structure located on either side of the head behind the eye), the presence of a dorsolateral fold, and the extent of webbing on the toes. Three distinct frog species were studied, each revealing unique features, as shown in Figure 4.2. The identification keys of *P. baramica*, *H.erythraea* and *L.paramacrodon* are presented in Table 4.2. The *P. baramica* frog stands out with a mottled brown upper surface and lighter, sometimes yellowish, flanks. Interestingly, it exhibits a darker coloration during the day as compared to nighttime. Its skin texture is granular to slightly warty, and the *tympanum* appears dark but lightens up in the center.

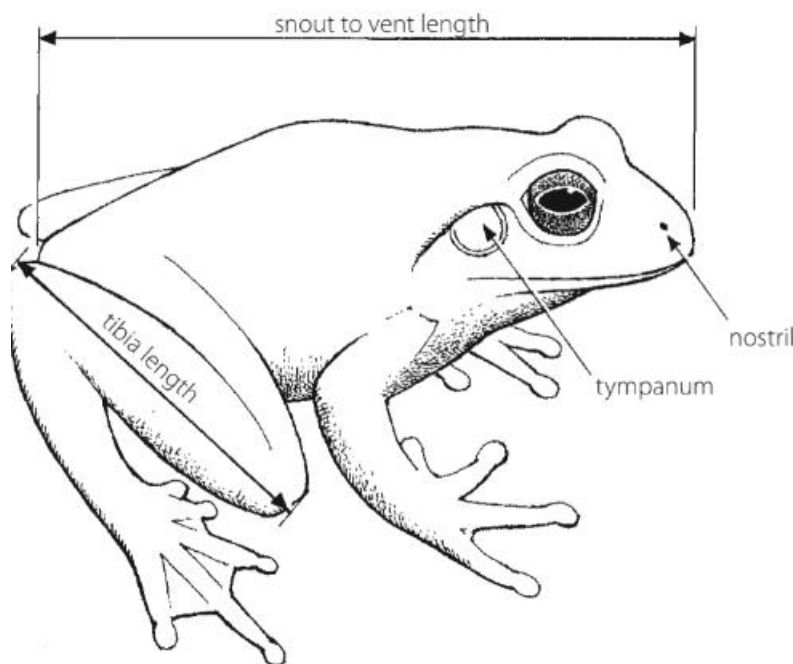


Figure 4.2: A Schematic Diagram of Frog's Identification Keys. The keys include the size and coloration of tympanum, nostril, tibia length, snout to vent length. (Knight et al 2020).

In contrast, *Hylarana erythraea*, or the Green Paddy Frog, is known as one of South Asia's most aesthetically pleasing frogs. Its bright to dark green color is accompanied by a broad dorsolateral fold adorned with a yellow stripe and a dark stripe along the sides of the head and body. The skin texture is notably smooth, and the *tympanum* is dark.

Table 4.2: The Identification Keys of *P. baramica*, *H. erythraea* and *L. paramacrodon* (Marly, et al 2017).

Specie	Identification key	Picture
<i>P. baramica</i>	The upper lips are marked with black bars separated by small white spots	
<i>H. erythraea</i>	Bright to dark green colour with broad dorsolateral fold covered with yellow stripe and broad dark stripe on sides of head and body	
<i>L. paramacrodon</i>	Black mask running through the eye, and the triangular shape of the black marking covering the tympanum (ear drum) and adjacent skin.	

As for *Limnonectes paramacrodon* or the Lesser Swamp Frog, distinguishes itself with fully webbed toes, except for the fourth toe, displaying a grayish to reddish-brown hue on the top and sides. They are of moderate size, with long hind legs, a rounded snout, and

visible eardrums. Its skin texture is granular to slightly warty, with some similarity to the *P. baramica*

Comparing the toe structure, all toes except the fourth toe of both the *P. baramica* and Lesser Swamp Frogs are fully webbed, contributing to their distinct characteristics. In contrast, the Green Paddy Frog lacks full webbing on its toes, adding another dimension to its unique features. The males of *Limnonectes paramacrodon* can grow between 60 mm and 75 mm in snout-vent length (SV), while females reach a range of 55 mm to 66 mm. For *H. erythraea*, males can reach a maximum SV of 48 mm, while adult females can attain a larger size of 78 mm.

4.2 Crude Protein Visualisations via Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

The process of crude protein visualization via Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was initiated by pooling skin secretions from specimens of the same species, followed by quantification of their concentration using the Bicinchoninic acid assay. Subsequently, the crude secretions were subjected to electrophoresis using SDS-PAGE, where 50 µg/µL of each sample was loaded into each well (wells 1-6). The molecular weight of peptides was determined by running the samples alongside a known protein standard, the Ultra Low Range Molecular Weight Marker from SIGMA (as depicted in Figure 4.3).

Subsequent to electrophoresis, the gel was visualized using a Coomassie blue staining protocol. During this procedure, proteins that had bound to SDS during electrophoresis transformed into rod-like structures, becoming visible through staining.

Both Ranidae and Dicroglassidae families exhibit efficient electrophoretic migration within an hour, suggesting optimal electrophoresis conditions. However, distinct protein profiles are evident, emphasizing species-specific differences in protein composition and structure within each family. In both families, Coomassie blue staining of crude and purified secretions reveals significant changes in protein composition during purification. Certain protein bands disappear or alter in the purified state, indicating dynamic alterations in protein profiles during purification.

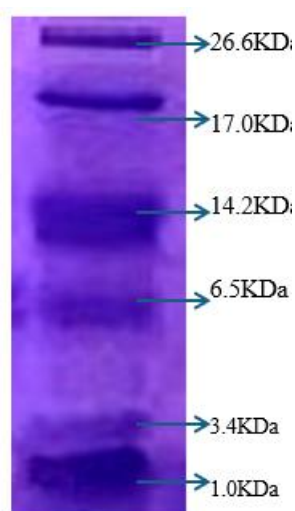


Figure 4.3: Protein ladder, Ultra Low Molecular Weight from SIGMA after stained using Coomassie blue staining method. Protein marker was heated for 2 mins at 65 °C prior to loading and was loaded. Upon visualization using coomassie stained protocol, the markers showed six visible protein bands arranged according to the relative mobility of the protein. Label on the right indicated the respective molecular weight of each proteins in kilo Dalton (kDa).

In Figure 4.4, the electrophoretic patterns of crude protein extracts from these species. Notable fractions from *P. baramica* and *H.erythraea* are discerned in bands 2, 4, and 5, showcasing variations in the size and location of protein bands. underscore the inherent diversity within the crude fractions of these two distinct species. These differences can be attributed to species variations in the composition and structure of proteins,

emphasizing the importance of these findings in elucidating the unique characteristics of each species.

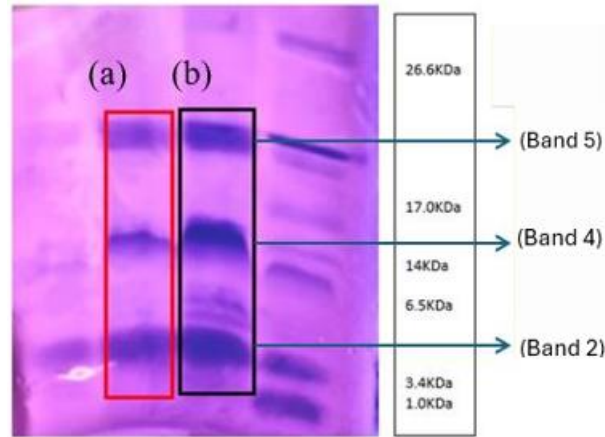


Figure 4.4: Coomassie Blue Tricine SDS-PAGE Staining Gel of Crude Secretions from Bornean Ranids (genera *Chalcorana* and *Pulchrana*) before passing through Sep-Pak cartridges. (a) represents *H. erythraea* and (b) *P. baramica* were loaded at 50 µg/uL for 60 mins at 150 V. Gel was visualised using Coomassie Blue protocol, carried out at room temperature.

Additionally, contrasting intensity levels of protein bands in *L. paramacrodon*'s electrophoresis pattern (Figure 4.5) suggest potential differences in protein abundance or types compared to other species. Notably, *L. paramacrodon* exhibited a slight band at 3.4 kDa and very little intensity band at 6.5 kDa, indicating distinct protein composition characteristics unique to this species.

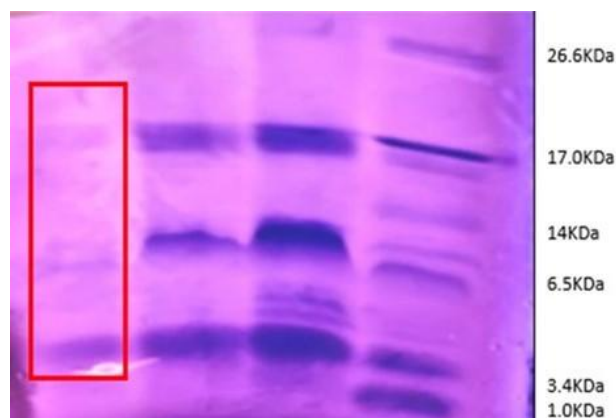


Figure 4.5: Coomassie Blue Tricine SDS-PAGE Staining Gel of Crude Secretions from Bornean Dicroglossidae (genera *L. paramacrodon*) before passing through Sep-Pak cartridges. The red box represents *L. paramacrodon* were loaded at 50 µg/uL for 60 mins at 150 V. Gel was visualised using Coomassie Blue protocol, carried out at room temperature.

The Sep Pak purification process has demonstrated its effectiveness in significantly altering the protein composition of frog skin secretions. As depicted in Figure 4.6, the transition from crude to partially purified secretions have revealed notable changes in protein profiles across different species. Specifically, while *P. baramica* (a) exhibited sharper and straighter protein bands at 6.5 kDa, indicative of various protein contents with larger molecular weights, *H. erythraea* (b) displayed more transparent bands at 3.4 kDa while *L. paramacrodon* (c) showed a diffused band indicating the presence of peptides smaller than 6.5 kDa and located in the area between 3.4 kDa and 1.0 kDa.

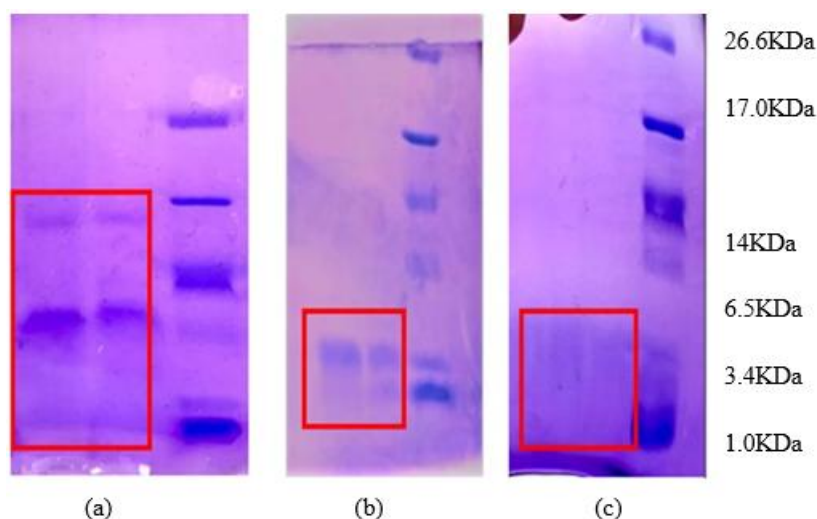


Figure 4.6: Coomassie Blue Tricine SDS-PAGE Staining Gel of partially purified secretions from Bornean ranids (genera *Haylarana* and *Pulchrana*) and Dicroglossidae (genera *L. paramacrodon*) after passing through Sep-Pak cartridges. (a): *P. baramica*, (b) *H. erythraea*, and (c): *L. paramacrodon*. were loaded at 50 µg/µL for 60 mins at 150 V. Gel was visualised using Coomassie Blue protocol, carried out at room temperature. The box in red indicates the presence of low molecular mass protein in all species tested.

Both Ranidae and Dicroglossidae families exhibit efficient electrophoretic migration within an hour, suggesting optimal electrophoresis conditions. However, distinct protein profiles are evident, emphasizing species-specific differences in protein composition and structure within each family. In both families, Coomassie blue staining of crude and purified secretions reveals significant changes in protein composition during purification process. Certain protein bands disappear or altered in the purified state, indicating dynamic alterations in protein profiles due to purification.

Comparing Coomassie blue staining of crude and partially purified secretions (Figure 4.6) reveals notable changes in protein composition during the Sep Pak purification process. For instance, purified samples show cleaner and more resolved protein bands compared to crude extracts, indicating successful purification. In *P. baramica* and *H. erythraea*, certain

protein bands disappear or alter in the purified state, indicating dynamic alterations in protein profiles during purification.

Notably, in both crude and purified *P. baramica* samples, protein bands corresponding to a molecular weight of 3.4 kDa are absent in the purified state, while new transparent protein bands emerge at band corresponding to a molecular weight of 6.5 kDa. Similarly, in both crude and purified *H. erythraea* samples, protein bands within the molecular weight range of 6.5-26.6 kDa are notably absent in the purified state. Furthermore, a distorted and skewed protein band diminishes in the gel containing purified samples.

In the analysis of the Dicroglossidae family (Figure 4.6(c)), a beamed band was apparent at 3.4 kDa, and clear bands were observed in the crude state at 3.4 kDa, indicating distinct protein profiles unique to this family.

4.3 ÄKTA Pure™ Chromatography System

B. baramica, *H. erythraea* and *L. paramacrodon* secretion peptides recovered from Sep-Pak C18 filtration were screened and further purified using an Akta-pure chromatography system. The aim of this analysis was to obtain pure peptides from skin secretion mixtures. Hence, HPLC chromatography was used to separate proteins from complex peptide mixtures. The purification process using the C18 column was based on the size exclusion principle. Subsequently eluted molecules correspond to a smaller particle size and thus separate smaller molecular weight protein bands when visualized via gel electrophoresis.

After injecting 500 µl through the sample injector and initiating protein elution, the system bypassed the C18 column, directing the flow to the fraction collector. When the

absorbance threshold was triggered, the system's flow was redirected from waste to outlet V9-Os, and the C18 column switched to online mode. Once a predetermined volume of eluate was loaded, the C18 column switched back to bypass mode, returning the system flow to the waste.

A peak fractionation command directed the system flow to drop 0.5 ml into the collector until an absorbance threshold was triggered, guiding the flow into tubes in the fraction collector as depicted in the chromatogram in Unicorn software in Figure 4.7. Collection ceased when the peak absorbance fell below the threshold, ensuring that each sample occupied different tubes, each filled with 0.5 ml in the fraction collector, and were promptly prepared for downstream processing.

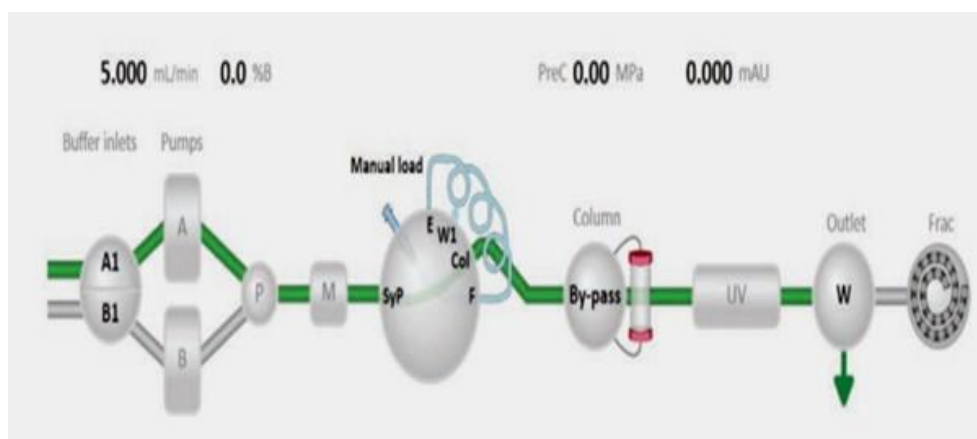


Figure 4.7: The UNICORN™ Process showing Active Flow Paths which includes the Inlet, Pre-column Filter, Injection Valve, Column Detector, Fraction Collector and Outlet. These components work together to facilitate the separation, purification, and analysis of biomolecules in a controlled and efficient manner within the UNICORN™ 7 system

Run conditions were optimized for binding flow rate, protein capacity, and sample load volume, identifying parameters that facilitated rapid sample processing times with minimal impact on protein quality.

Pure Akta HPLC chromatograms obtained at absorbance of 214 nm after passing through a C18 RP-HPLC column are shown in Figure 4.8, 4.9 and 4.10. Fractions were subsequently collected and concentrations were measured using a BCA protein assay. In the results, each fraction was indicated by the rinse time (Table 4.4).

Strikingly, skin secretory peptides extracted from the different species examined in this study showed distinct elution patterns on the chromatogram. Notably, diverse chromatographic elution profiles were observed during the analysis. These identified peptides have demonstrated activity against *Escherichia coli* and *Staphylococcus aureus* strains. Subsequently, the isolated peptide fraction underwent concentration through a centrifugal tube filter prior to being subjected to analysis via SDS-PAGE electrophoresis.

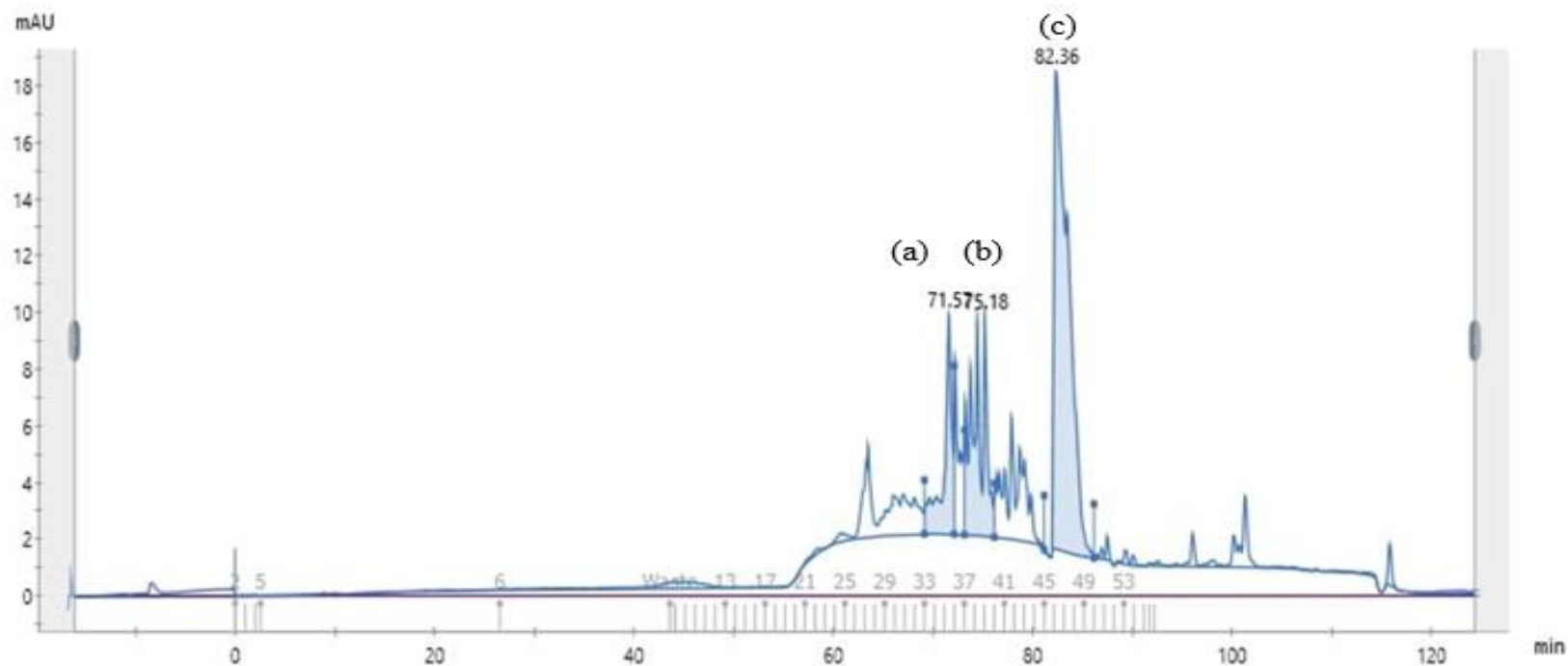


Figure 4.8: Akta pure chromatograms of *P. baramica* skin peptides peaks (A, B and C) using reverse phase HPLC. The peptide elution was conducted using 0.1% (v/v) TFA and 90% (v/v) acetonitrile gradient, under constant flow of 0.5mL/min. UV absorbance was monitored at 214nm. AMP is indicated as C at 82.36 minutes.

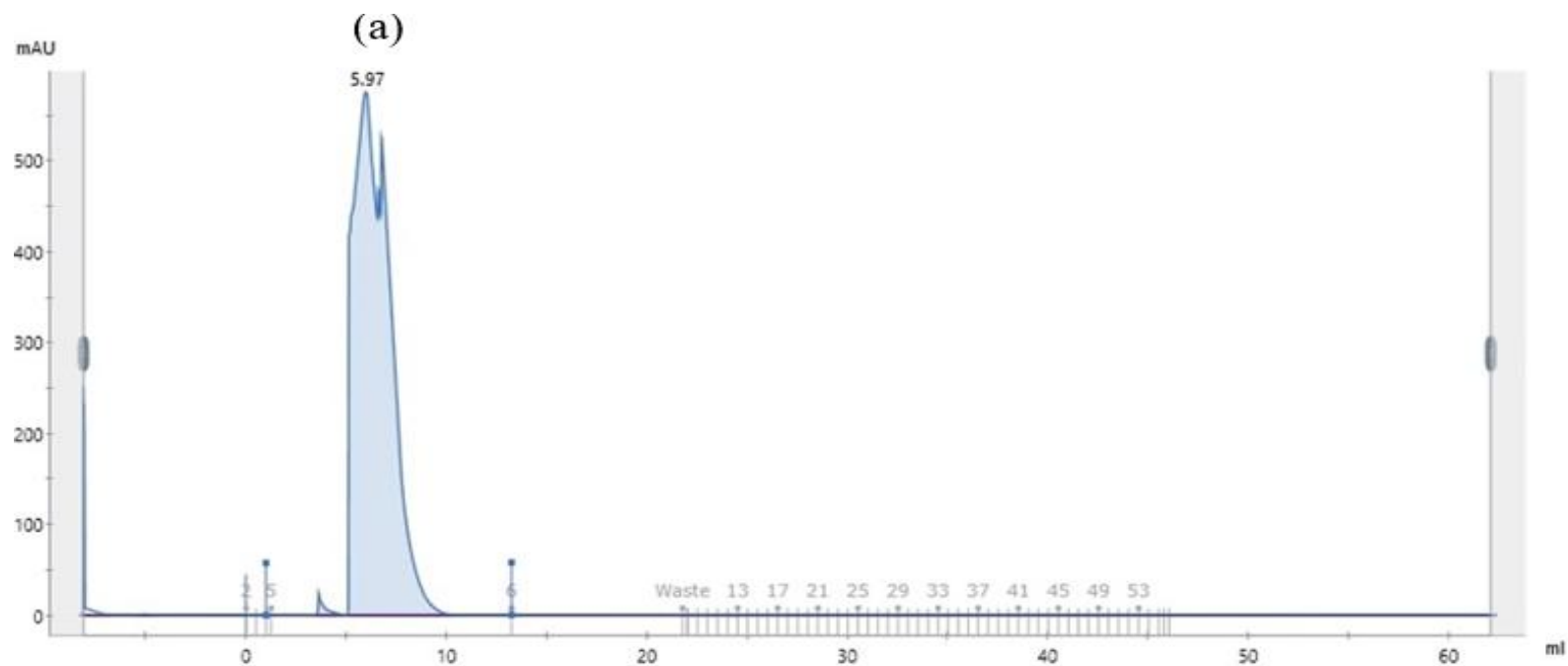


Figure 4.9: Akta pure chromatograms of *H.erythraea* skin peptides peak (a) using reverse phase HPLC. The peptide elution was conducted using 0.1% (v/v) TFA and 90% (v/v) acetonitrile gradient, under constant flow of 0.5mL/min. UV absorbance was monitored at 214nm. AMP is indicated as (a) at 5.97 minutes.

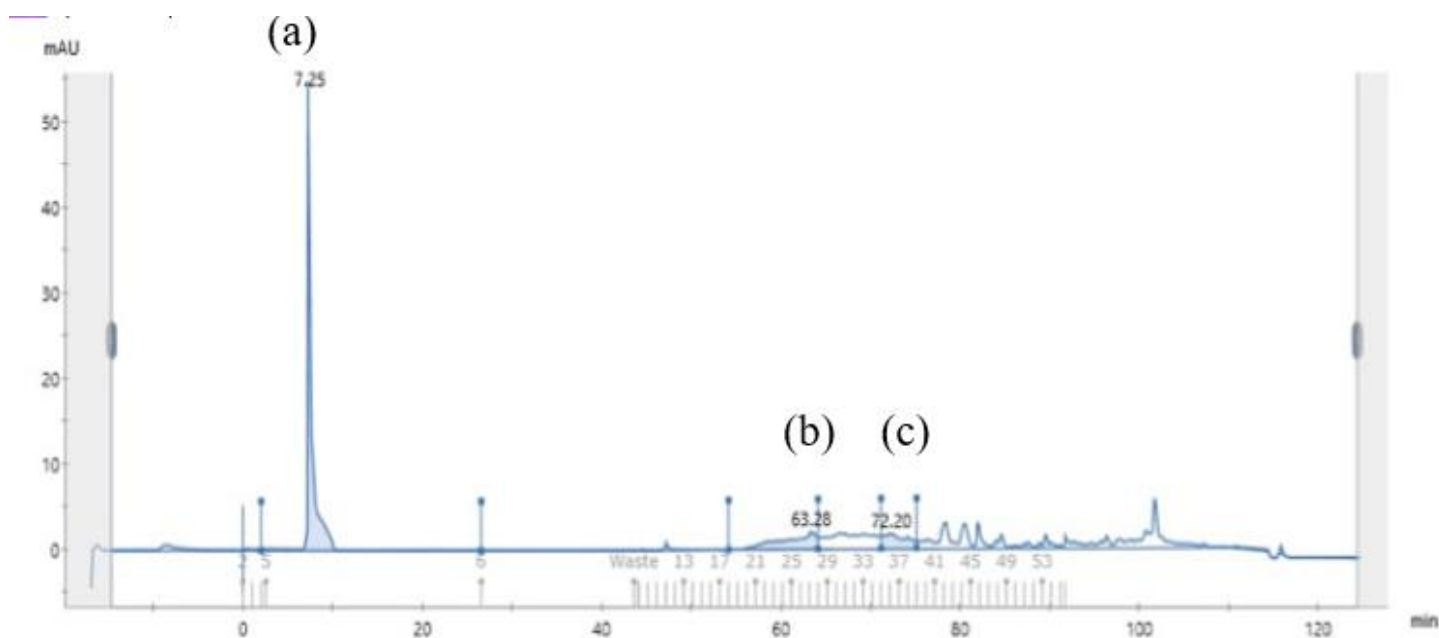


Figure 4.10: Akta pure chromatograms of *L.paramacrodon* skin peptides peaks (b) and (c) using reverse phase HPLC. The peptide elution was conducted using 0.1% (v/v) TFA and 90% (v/v) acetonitrile gradient, under constant flow of 0.5mL/min. UV absorbance was monitored at 214nm. AMP is indicated as (a) at 7.25 minutes.

Table 4.3: The Time of Elution and the Number of Proteins Observed from Gel

Specie	The numbers of the new peptide bands observed	Peak HPLC	Time of elution (min)
<i>P.baramica</i>	1	C	41.135
<i>H. erythraea</i>	1	A	7.459
<i>L.paramacrodn</i>	2	A	33.590

The introduction of supplementary purification steps using the ÄKTA system played a pivotal role in significantly improving the clarity of the molecular weight profiles of the peptides isolated from the Bornean frogs under investigation. Subsequently, these purified fractions underwent a second round of analysis through SDS-PAGE, as visually represented in Figure 4.11 and Figure 4.12. In this subsequent analysis, notably distinctive low-molecular-weight protein bands were discerned. Specifically, peak C from the AKTA chromatogram for *P. baramica* underwent a secondary round of SDS-PAGE. In this round, the bands were concentrated and prominently manifested at band 1, showcasing molecular weights measuring 1.0 and 3.4 kDa, complemented by transparent bands at 6.5 kDa. Conversely, in the case of *H. erythraea*, peak A was subjected to SDS-PAGE, where the observed bands were notably transparent, exhibiting molecular weights recorded at 1 kDa and 3.4 kDa, respectively as in Figure 4.11.

Upon comparing the *P. baramica* bands of partially purified secretions, notable distinctions emerged. Two new concentrated bands appeared at 1.0 and 3.4 kDa, while the 6.5 kDa bands disappeared, indicating the efficiency of the C18 column and AKTA system in purifying peptides of small molecular weight. In contrast, *H. erythraea* showed minimal difference pre and post AKTA treatment. Gel bands in partially purified secretions exhibited

transparent bands at 3.4 kDa, whereas post-AKTA treatment revealed a new transparent band at 1.0 kDa.

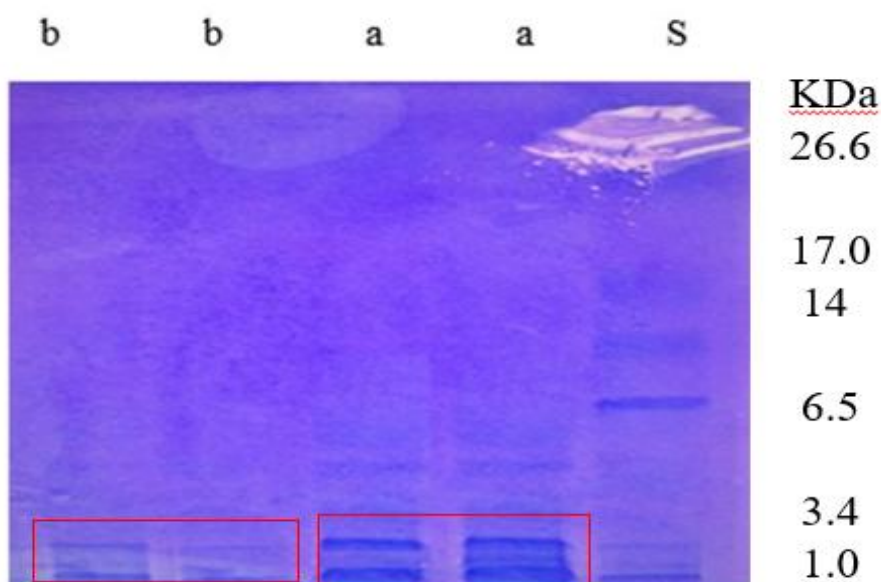


Figure 4.11: Coomassie Blue Tricine SDS-PAGE Staining Gel of Purified Secretions from Bornean Ranids after using AKTA Pure System Purification. Bornean Ranid (*P.baramica* and *H.erythraea* labeled as (a) and (b) respectively) were loaded at 50 $\mu\text{g}/\mu\text{L}$ for 60 mins at 150 V. Gel was visualised using Coomassie Blue protocol, carried out at room temperature. The boxes in red indicates the presence of low molecular mass protein in all species tested

In Figure 4.12, the fractions from *L. paramacrodone* revealed an interesting finding, the emergence of entirely new bands at 6.5 kDa, alongside distinctly clearer bands at 3.4 kDa. Interestingly, unlike Ranid frogs' secretions, *L. paramacrodone* did not exhibit any bands at 1.0 kDa. This disparity underscores the variation in peptide compositions across different frog families.

Upon meticulous comparison with the bands observed in partially purified secretions, it became evident that the purification process yielded significant enhancements. The once indistinct bands now appeared markedly clearer in the purification state. Notably, even with

the utilization of a sample concentrator before SDS running, no apparent bands were discernible.

In summary, this study highlights how effective the purification process is in improving the clarity and visibility of protein bands, especially as seen in the case of *L. paramacrodone*. These results underscore the significance of recognizing and considering the diverse peptide compositions found in various frog species.

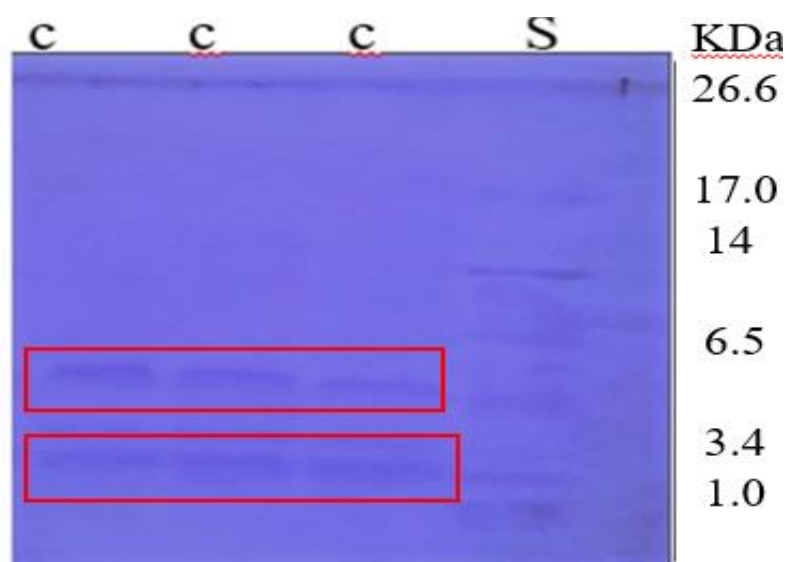


Figure 4.12: Coomassie Blue Tricine SDS-PAGE Staining Gel of Purified Secretions from Dicroglossidae after using AKTA Pure System Purification. M.W standard labelled as (S) and the *L. paramacrodon* fractions labelled as (c), were loaded at 50 µg/uL for 60 mins at 150 V. Gel was visualised using Coomassie Blue protocol, carried out at room temperature. The boxes in red indicates the presence of low molecular mass protein in all species tested.

4.4 Concentration Yield in Protein Purification (Protein Loss and Recovery)

Protein concentrations within biological samples can exhibit significant variability, influenced by factors such as experimental conditions and the organism's size. For example, the body condition of the frogs, expressed as the ratio between weight and length, was directly related to the level of plasmatic proteins (Brenes-Soto, et al 2017) In this study, it was observed that larger frogs generally yield more concentrated protein solutions compared to smaller ones. Such variations pose challenges in accurately assessing protein function and activity, necessitating purification to obtain homogeneous protein samples for reliable analysis. Moreover, contaminants present in crude samples can interfere with downstream applications, potentially leading to erroneous conclusions.

Following purification, the target protein's concentration ideally increases relative to contaminants, resulting in a higher purity sample suitable for subsequent assays and experiments. However, in the case of the crude secretions from three species, the protein concentration was initially high due to being in a concentrated phase. Through purification using Sep-Pak cartridges, the concentration significantly decreased due to high dilution with buffer during the purification steps listed in Table 4.5. Even after concentration using an Amicon filter device, the resulting concentration did not match that of the crude sample, albeit some improvements were observed. Percent recovery after this purification steps was calculated using the formula below:

$$\text{Percent yield \%} = \frac{\text{Amount of Protein X in creude extract}}{\text{Amount of Protein X obtained}} \times 100 \quad \text{Equation 4.1}$$

Table 4.4: Comparison Between the Concentration of Crude and Purified States of *P. baramica* Individuals

<i>P.baramica</i> individuals	Crude phase (µg/mL)	Partial purified - post sep-pak (µg/mL)	% Recovery	Partial purified- concentrated phase (µg/mL)	% of protein recovery
1	1520	400	26 %	1100	72.3 %
2	879.3	394	44 %	764	86.8 %
3	1020	312	30 %	742	72.7 %
4	1115	228	20.4 %	933	83.6 %
5	984	234	23.7 %	652	66.2 %
6	931	289	31 %	590	63.3 %

The protein concentration from the table above demonstrates a considerable decrease in protein yield following purification from the crude phase to the partially purified. In all instances, there was a marked reduction in protein concentration post-purification, with percent recovery ranging from 20.4% to 44%, depicted in Figure 4.13. It is important to note that while the sample concentration increased to over 50% after utilizing the Amicon filter, it remained significantly lower compared to the crude concentration. This reduction in concentration correlates with the loss that occurred during the purification process.

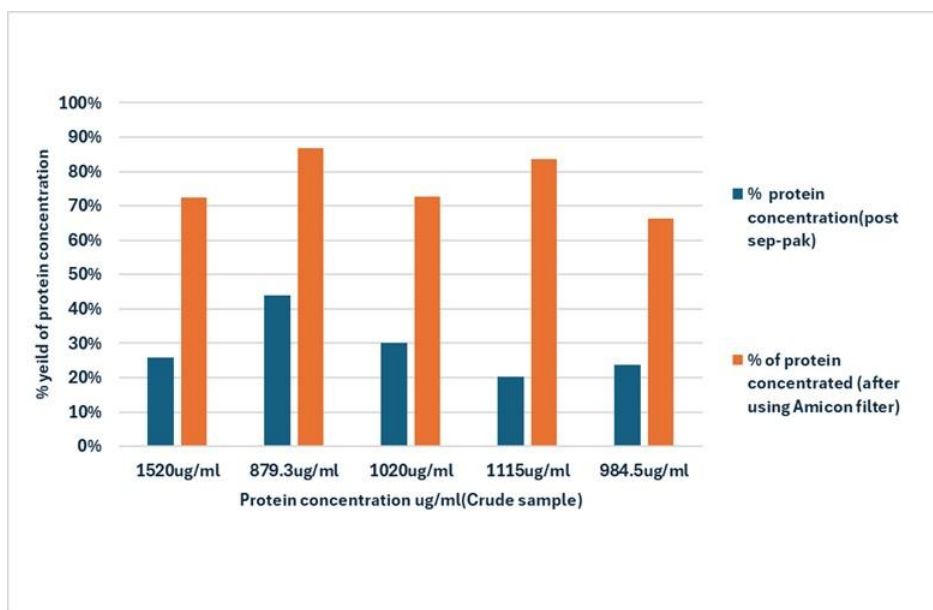


Figure 4.13: Percentage of Protein Recovery Following Partial Purification of *P. bramica* Skin Secretion. The blue column represents the protein concentration after using a Sep-Pak C18 column purification, while the orange columns shows the increase in concentration after using the Amicon filter concentrator.

Similarly, the protein concentration of *H. erythraea* in the crude state was initially relatively high before using the 6-Sep Pak cartridge. It decreased to less than 50% following purification from the crude phase to the partially purified. In each case, there was a notable decrease in protein concentration post-purification, with percent recovery ranging from 17% to 48%. Furthermore, after concentration with the Amicon filter, the percent increased up to 50%, indicating partial recovery but still lower than the initial crude concentration.

Table 4.5: Comparison Between the Concentration of Crude and Purified States of *H. Erythraea* Individuals

<i>H.Erythraea</i> individuals	Crude phase (µg/mL)	Partial purified - post sep-pak (µg/mL)	% Recovery	Partial purified-concentrated phase (µg/mL)	% of protein recovery
1	995.4	250	25 %	640	64 %
2	1432	689	48 %	1210	84.4 %
3	789	140	17 %	520	65 %
4	1221	532	43 %	976	79 %

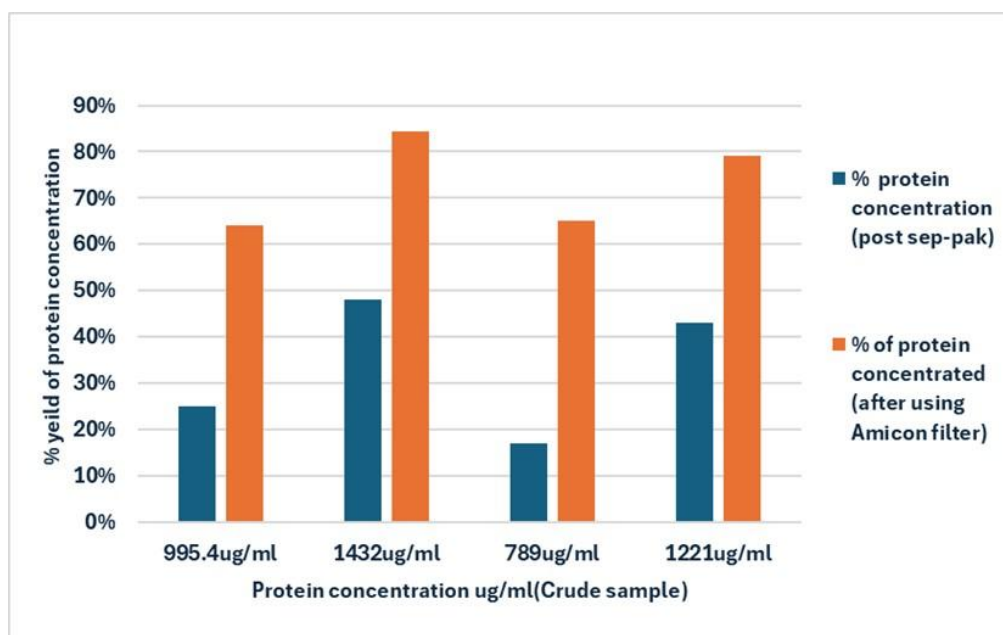


Figure 4.14: Percentage of Protein Recovery Following Partial Purification of *H.erythraea* Skin Secretion. The blue column represents the protein concentration after using a Sep-Pak C18 column purification, while the orange column shows the increase in concentration after using the Amicon filter concentrator.

The protein concentration of *L. paramacrodon* frog skin secretion showed a significant decrease following purification from the crude phase to both the partially purified (diluted phase) and the partially purified (concentrated phase). In each case, there was a notable decrease in protein concentration post-purification, with percent yields ranging from 18% to 31% in the diluted phase and from 44.6% to 49% in the concentrated phase **Figure (4.15)**. Despite the reduction in yield, the protein concentration increased in the concentrated phase compared to the diluted phase. However, achieving the required concentration for experimental procedures necessitated mixing many samples of *L. paramacrodon* in one tube. This suggests a partial recovery of protein concentration during the purification process, albeit with the need for additional sample consolidation to meet experimental needs.

Table 4.6: Comparison Between the Concentration of Crude and Purified States of *L.paramacrodon* Individuals

<i>L.paramacrodon</i> Individuals	Crude phase (µg/mL)	Partial purified -post sep-pak (µg/mL)	% Recovery	Partial purified- concentrated phase (µg/mL)	% of protein recovery
1	988	189	19 %	486	49 %
2	799	144	18 %	357	44.4 %
3	1321	421	31 %	629	47 %
4	1200	340	28 %	577	48 %

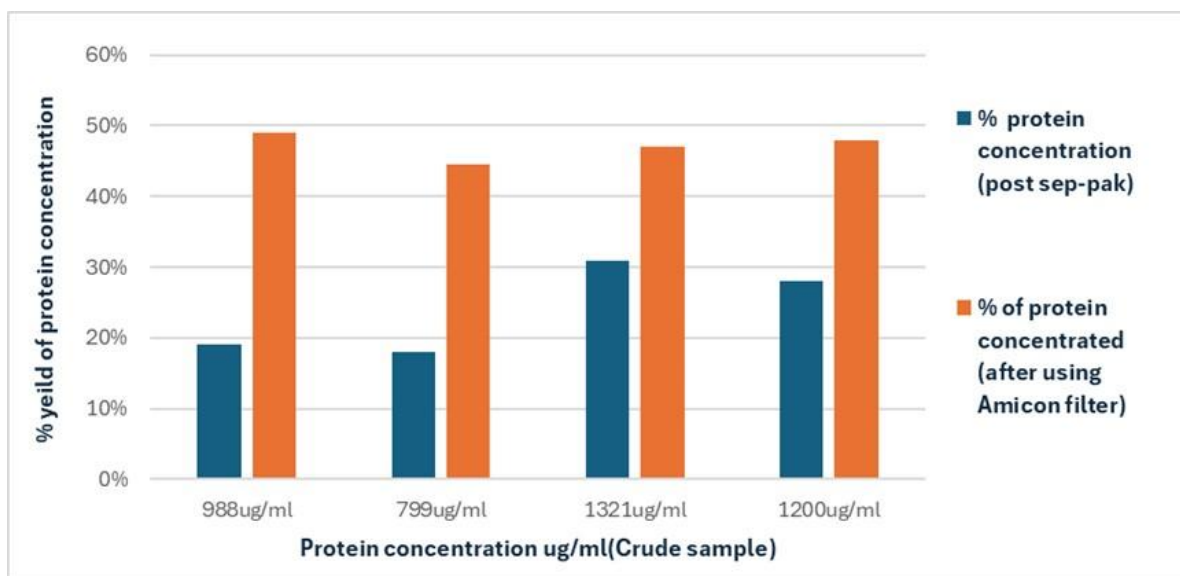
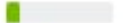
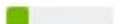


Figure 4.15: Percentage of Protein Recovery Following Partial Purification of *L.paramacrodon* Skin Secretion. The blue column represents the protein concentration after using a Sep-Pak C18 column purification, while the orange column shows the increase in concentration after using the Amicon filter concentrator.

Protein purification is a fundamental process aimed at isolating specific proteins for analysis. The AKTA system, a robust tool utilized for this purpose, operates in tandem with UNICORN software, which serves as the controlling interface. Through this integration, proteins are purified with precision and efficiency, guided by the automation and management capabilities of UNICORN software. The combined utilization of the AKTA system and UNICORN software streamlines the purification process. Upon purification via the AKTA Pure system, the concentrated partially purified secretions yielded satisfactory protein concentrations. Specifically for *P. baramica*, 789ug/ml was injected through the injector loop. When the absorbance threshold was triggered, the fraction collector dropped 0.5ml/min in each tube. According to the chromatogram Table 4.8, it showed 3 Peaks A, B, and C, each with different percent area indicating different protein concentrations. Peak C

exhibited the highest concentration at 410ug/ml, representing 62.03% of the peak area, corresponding to the protein concentration percentage.

Table 4.7: Unicorn 7 Software Chromatograms Illustrating the Analysis of Skin Peptides from *P.baramica* Secretion at 214UV Absorbance

Peak	Retention min	Area min* μ AU	Area %	Ext coeff. $\text{mg ml}^{-1} \text{cm}^{-1}$	Fraction(s)
Peak A	71.565	6.914	 15.18		33 - 35
Peak B	75.175	10.13	 22.23		37 - 39
Peak C	82.362	28.52	 62.6		45 - 49

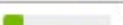
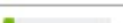
Upon purification of *H. erythraea* through Akta, peak A showed 100% of the area, with 500ug/ml injected through the injector loop as shown in Table 4.9.

Table 4.8: Unicorn 7 Software Chromatograms Illustrating the Analysis of Skin Peptides from *H.erythraea* Secretion at 214UV Absorbance

Peak	Retention min	Area min* μ AU	Area %	Ext coeff. $\text{mg ml}^{-1} \text{cm}^{-1}$	Fraction(s)
Peak A	11.993	2470	 100		Waste - 5

Additionally, the purification of *L. paramacrodon* revealed three different peaks: A, B, and C (Table 4.10). Peak A showed the highest protein content with an area percent of 65.44%, Peak B exhibited 21.2% of the area, while peak C showed 13.35% of the area percent.

Table 4.9: Unicorn 7 Software Chromatograms Illustrating the Analysis of Skin Peptides from *L.paramacrodon* Secretion at 214UV Absorbance

Peak	Retention min	Area min* μ AU	Area %	Ext coeff. $\text{mg ml}^{-1} \text{cm}^{-1}$	Fraction(s)
Peak A	7.248	24.33	 65.52		Waste - 5
Peak B	63.282	7.852	 21.14		18 - 27
Peak C	72.202	4.953	 13.34		35 - 38

4.5 Functional Characterization of Antimicrobial Peptides

4.5.1 Antimicrobial Activities of Partially Purified Peptides by Disk Diffusion Assay

Several investigations have demonstrated the potency of AMPs from frog secretions as antibacterial agents. (Conlon, 2011; Kumar et al., 2015; McLaughlin et al., 2016). In this exploratory investigation, the disc diffusion assay, a qualitative technique, was used to assess the antibacterial activity of frog skin secretions from a partly isolated peptide.

In this study, A suspension of *E. coli* and *S. aureus* strains, responsive to prescribed antibiotics, was evenly injected into the agar gel. Nutrient-rich Mueller-Hinton agar gel was used to promote the development and reproduction of microorganisms. During incubation, organisms started to multiply and continued until a critical concentration of an antibiotic or tested peptide solution prevented it. Bacteria continued to multiply. As a result, the medium became turbid, exposing a distinct zone of inhibition in contrast to the turbidity during the 24-hour incubation period at 37°C. The location of the zone boundary was influenced by the concentration of the tested solution or antibiotics that are diffusing, as indicated by Hewitt (1977).

In order to explore antibacterial activity, the maximum peptide concentrations were evaluated for the preliminary study, at 1000 and 500 g/mL, respectively. A sterile disc was loaded with 20 µL of each sample, including the positive control, and the assay was carried out twice, in triplicate. As a positive control, ampicillin was employed at a working concentration of 10 g/mL. The findings of the disc assay are displayed in Figure 4.16, 4.17, 4.18, 4.19 and Table 4.5 provides a summary of the diameter of the inhibition zone.



Figure 4.16: Antimicrobial Activity of Partially Purified Samples after Concentration Using Amicon Filter (MWCO 10 kDa) Against *E. coli* Strain. 'Ba' refers to *P. bramica*, 'Hy' refers to *H. erythraea*, and 'Pa' refers to *L. paramacrodon*



Figure 4.17: Antimicrobial Activity of Partially Purified Samples after Concentration using Amicon Filter MWCO 10 kDa against *E. coli* Strain. 'Ba' refers to *P. baramica*, 'Hy' refers to *H. erythraea*, and 'Pa' refers to *L. paramacrodon*

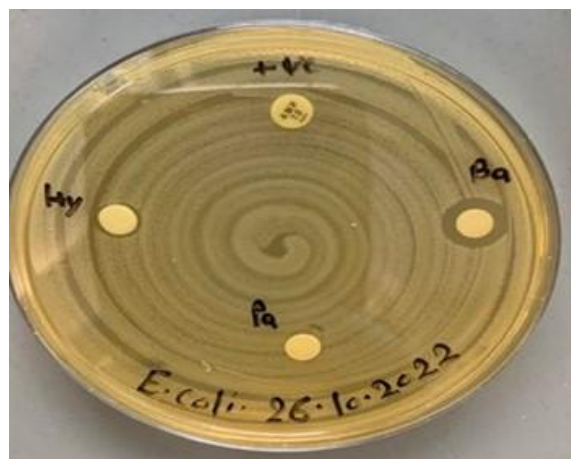


Figure 4.18: Antimicrobial Activity of Partially Purified Samples after Concentration using Amicon Filter MWCO 10 kDa against *S.auerus*. 'Ba' refers to *P. baramica*, 'Hy' refers to *H. erythraea*, and 'Pa' refers to *L. paramacrodon*.

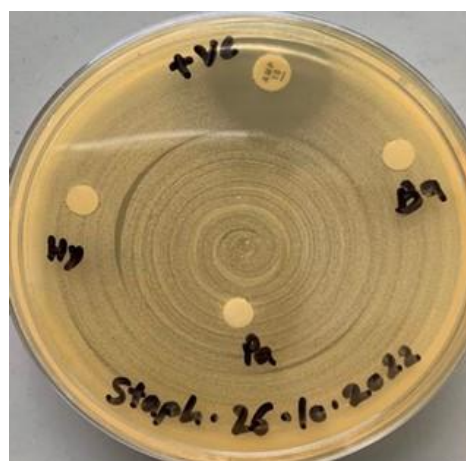


Figure 4.19: Antimicrobial Activity of *L.paramacrodon* against *S.aureus* Strain, 'Ba' refers to *P. baramica*, 'Hy' refers to *H. erythraea*, and 'Pa' refers to *L. paramacrodon*

Not all frog secretions from the investigated species exhibited antibacterial properties, as indicated by the disc diffusion results. Frog secretions from the *P. baramica* and *H. erythraea* species demonstrated a stronger inhibitory action against *E. coli* and *S. aureus* strains, as illustrated by Figures 4.17, 4.18, and 4.19. In contrast, *L. paramacrodon* did not demonstrate antibacterial activity against either strain.

Several observations can be made when comparing the plate assays. Notably, a larger zone of inhibition was generated by a higher peptide concentration. For further investigation, secretions that displayed a distinct inhibition zone between 1000 µg/mL and 500 µg/mL were selected.

Table 4.10: Diameter of Inhibition Zone of Frog Peptides against Two Strains of Bacteria, *E. coli*, and *S. aureus* over a Period of 24 Hours

Bacteria strains	<i>E. coli</i>		<i>S.aureus</i>
Concentration µg/ml	1000 µg/ml	500 µg/ml	1000 µg/ml
<i>P.baramica</i>	11.66±3.44	11.33±0.9	5.6±0.6
<i>H. erythraea</i>	7.33±0.5	4.3±0.6	4.6± 0.6
Positive control	17.6±0.46	16.3±0.6	15.3±0.6

4.5.2 Minimum Inhibitory Concentration of Frog Peptides using Broth Microdilution Method

The presence of AMPs in the frog skin peptide combinations from each analyzed sample was strongly suggested by the outcomes of the disc diffusion testing. Subsequently, a quantitative assessment of AMP's effectiveness in inhibiting bacterial growth, commonly referred to as the minimum inhibitory concentration (MIC), was undertaken. The procedure for obtaining bacterial isolates for the MIC measurement was executed like that employed in the disc diffusion experiment.

Nevertheless, to attain a bacterial suspension with a density of 5×10^5 CFU/mL for the broth microdilution procedure, a 0.5 McFarland suspension was diluted by a factor of 100, resulting in a density of 10^6 CFU/mL (achieved by combining 9.9 mL of broth with 0.1

mL of the 0.5 McFarland suspension). Following this dilution step, the resulting mixture was pipetted into wells that contained the necessary peptide concentrations in the broth, along with the addition of 50 μ L of bacterial inoculum and 50 μ L of liquid medium containing the peptides.

The antimicrobial efficacy of the AMPs was evaluated against both *E. coli* and *S. aureus* strains. After 24 hours of incubation, the growth of *E. coli* was observed under favorable conditions, specifically in Luria Bertani Broth. This was evidenced by the appearance of a turbid yellowish broth, with variations in color intensity observed among the three different species of frogs. *H. erythraea* and *L. paramacrodon* displayed a darker yellowish and higher turbidity compared to *P. baramica*, which exhibited a lighter yellowish color with less turbidity.

Notably, the highest concentration of AMPs displayed significant antibacterial activity against the *E. coli* strains, suggesting resistance to these strains. This phenomenon is further elucidated in Figure 4.20.

Meanwhile, a distinct yellow turbidity was observed surrounding the wells for *S. aureus* depicted in Figure 4.21. It appeared as a yellowish 'button-like' shape with a noticeable white circular ring at the center, particularly evident in the 500ug/ml concentration in *H. erythraea*. Interestingly, as the turbidity increased, the white circular feature tended to disappear. This phenomenon was especially notable in the 500ug/ml concentration in *H. erythraea* and 250 and 125 ug/ml *L. paramacrodon*.

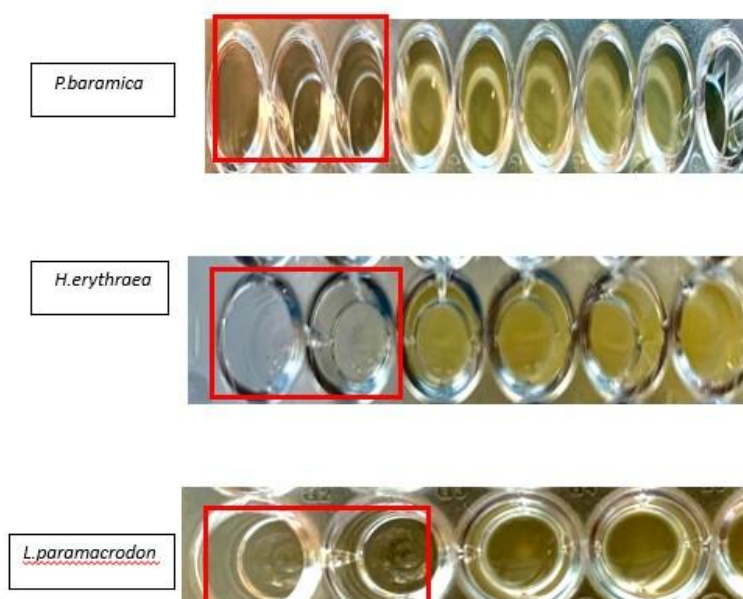


Figure 4.20: Comparison of Antibacterial Activity by Three Species of Bornean frogs; Frog secretions versus *E.coli*. Red box indicates the Minimum Inhibition Concentration (MIC) for each frog secretions.

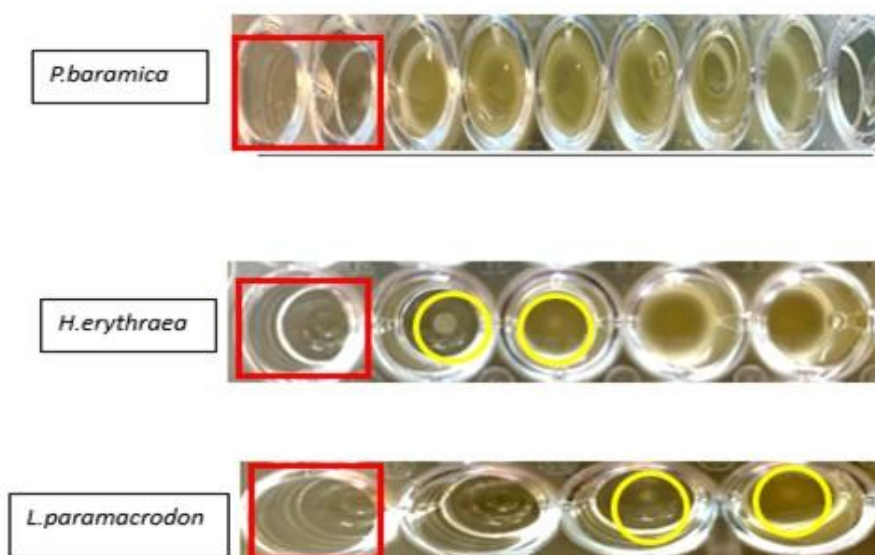


Figure 4.21: Comparison of Antibacterial Activity by Three Species of Bornean Frogs. Frog Secretions versus *S.aureus*. The red box showed a clear, non-turbid solution with the Minimum Inhibition Concentration, while the yellow circle appeared as a yellowish 'button-like' shape with a noticeable white circular ring at the center, indicating bacterial growth.

Like the *E. coli* wells, there was a notable divergence in turbidity intensity among the wells of the three frog species, suggesting a variance in the antibacterial potency of the AMPs sourced from these species. The turbidity of the broth solution was influenced by bacterial growth within the 3cm depth microplate well, facilitating the assessment of bacterial cell counts via optical density. The calculations were conducted utilizing Equation 4.2.

$$\frac{(\text{OD of well} - \text{OD negative control})}{\text{OD Positive control}} \times 100 \quad \text{Equation 4.2}$$

The negative control wells were associated with wells containing only the broth medium, thereby indicating a zero value. Conversely, positive wells were associated with wells containing a mixture of the broth medium and bacterial isolates. The first clear well observed after turbidity was recorded as the MIC. The skin secretions from various species of Bornean frogs against *E. coli* and *S. aureus* were compared in Table 4.12.

Table 4.11: Minimum Inhibitory Concentrations of Skin Peptides from Three Species of Bornean Ranids

Species	<i>E. coli</i>	<i>S.aueus</i>
<i>P.baramica</i>	250 µg	500 µg
<i>H. erythraea</i>	500 µg	1000 µg
<i>L.paramacrodon</i>	500 µg	1000 µg

The information presented above offers initial insights into the antibacterial properties of skin secretions concerning varying concentrations of skin peptides. Notably, the standard deviation was consistent across most strains, except for *P. baramica*, which

exhibited a deviation of approximately ± 0.45 . This divergence suggests stable minimum inhibitory concentrations (MICs) throughout triplicate experiments, consistent across all identified strains.

These findings underscore the potential of *P. baramica* skin secretions to effectively hinder the growth of *E. coli* in comparison to *S. aureus*. In summary, all secretions displayed antibacterial activity against both *E. coli* and *S. aureus* strains within the concentration range of 500 to 1000 $\mu\text{g/mL}$. This implies that the antimicrobial peptides (AMPs) found in the examined frogs' secretions do provide a degree of protection, though the bacterial strains exhibit relative resilience. Bacterial growth in this study was assessed following inoculation and an 18-hour incubation period on blood agar.

4.5.2.1 Comparing the Efficacy of Antimicrobial Peptides (AMPs) among Genera in the Ranidae and Dicroglossidae Families.

Genus *Pulchrana*: The growth of both *E. coli* and *S. aureus* in the microtitre plate wells is visualized in Figures 4.22, 4.23, and 4.24. The partially purified peptide from *P. baramica* exhibited greater potency (MIC: 250 $\mu\text{g/mL}$) against *E. coli* compared to other species within the same genus, specifically *H. erythrae* (MIC: 500 $\mu\text{g/mL}$). In Figure 4.22(A), the red box highlights clear wells with peptide concentrations ranging from 1000 $\mu\text{g/mL}$ to 250 $\mu\text{g/mL}$ for *P. baramica*. At concentrations lower than 250 $\mu\text{g/mL}$, adjacent wells of *P. baramica* showed a 'button-like' shape of *E. coli* isolates, indicating uninterrupted bacterial growth.

Figure 4.22 (B) illustrates the antibacterial activity of skin secretion peptides against *S. aureus* isolates. *Staphylococcus aureus* isolates were found to be susceptible to AMPs from *H. erythrae* only at the concentrations used (500 $\mu\text{g/mL}$). However, *P. baramica*

exhibited moderate antibacterial activity, with MICs of 250 $\mu\text{g/mL}$, 500 $\mu\text{g/mL}$, and 1000 $\mu\text{g/mL}$, as represented by the red box in the Figure.

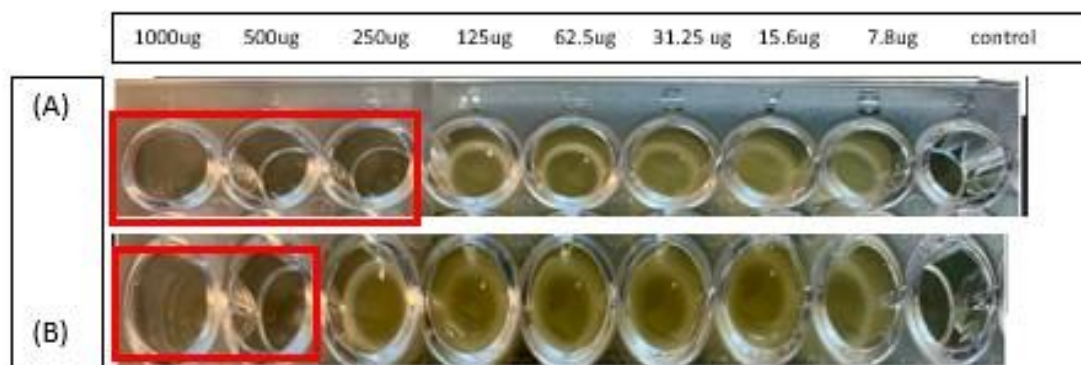


Figure 4.22: Comparison of Antibacterial Activity Demonstrated by Bornean Frogs under Genus *P.baramica* against (A) *E.coli*, (B) *S.aueurus*, the red boxes indicates the Minimum Inhibitory Concentration of partially purified frog secretion.

Genus *H.erythrae*: In Figure 4.23(A), skin secretions partially purified from *H. erythrae* exhibited activity against *E. coli* with a minimum inhibitory concentration (MIC) of 500 $\mu\text{g/mL}$, as indicated by the presence of a red box, signifying bacterial lysis. Notably, the only clear wells were those containing skin peptide concentrations of 1000 $\mu\text{g/mL}$ and 500 $\mu\text{g/mL}$.

Conversely, Figure 4.23(B) displayed distinct white "dot"-shaped *S. aureus* isolates at the center of the well containing a 500 $\mu\text{g/mL}$ skin peptide concentration, marked by a blue box, indicating the absence of bacterial lysis in the surrounding wells. *Staphylococcus aureus* isolates were primarily observed at the highest concentration tested (MIC = 1000 $\mu\text{g/mL}$). Nevertheless, turbid wells revealed the presence of tested *S. aureus* strains at concentrations lower than 500 $\mu\text{g/mL}$.

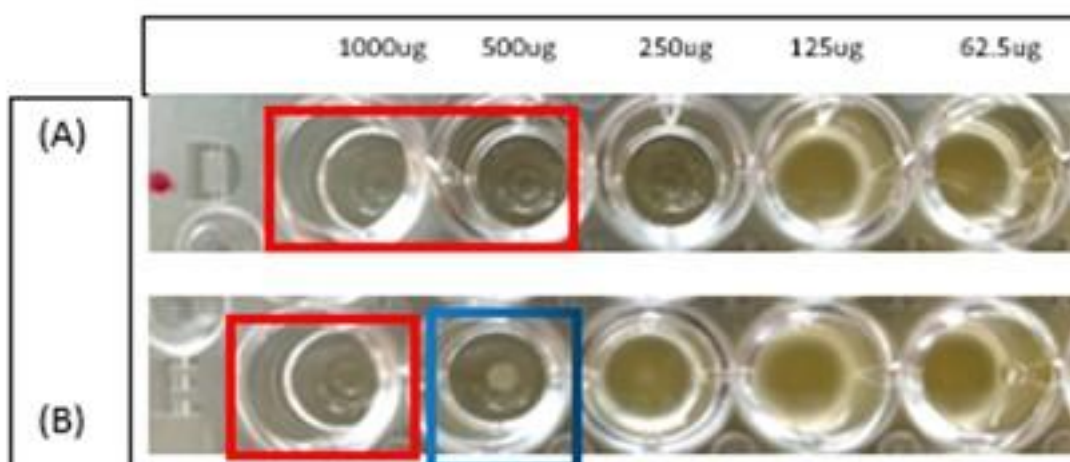


Figure 4.23: Comparison of Antibacterial Activity Demonstrated by Bornean Frogs under Genus *H.erythrae* versus *E.coli* (A) and *S.aureus* (B). Red boxes indicates the minimum inhibitory concentration of partially purified frog secretion. And the blue box highlight the white dot shape in the turbid well.

Genus *L.Paramacrodon*: In Figure 4.24 (A), only the wells containing 1000 $\mu\text{g/mL}$ and 500 $\mu\text{g/mL}$ of skin peptides exhibited a clear appearance after 24 hours of incubation with *E. coli* strains, as highlighted by the red box, indicating bacterial lysis (MIC = 500 $\mu\text{g/mL}$). Conversely, the turbid wells demonstrated the proliferation of the tested *E. coli* strains at concentrations lower than 500 $\mu\text{g/mL}$.

Moving to Figure 4.24 (B), the red box highlights wells containing 1000 $\mu\text{g/mL}$ and 500 $\mu\text{g/mL}$ of peptides, which were also devoid of *S. aureus* isolates. However, upon inoculation on blood agar to assess bacterial growth, a well with a 500 $\mu\text{g/mL}$ peptide concentration exhibited bacterial growth despite appearing clear, as indicated by the yellow circles in Figure 4.24 (B). Consequently, no bacterial lysis occurred in the neighboring wells, corresponding to 500 $\mu\text{g/mL}$.

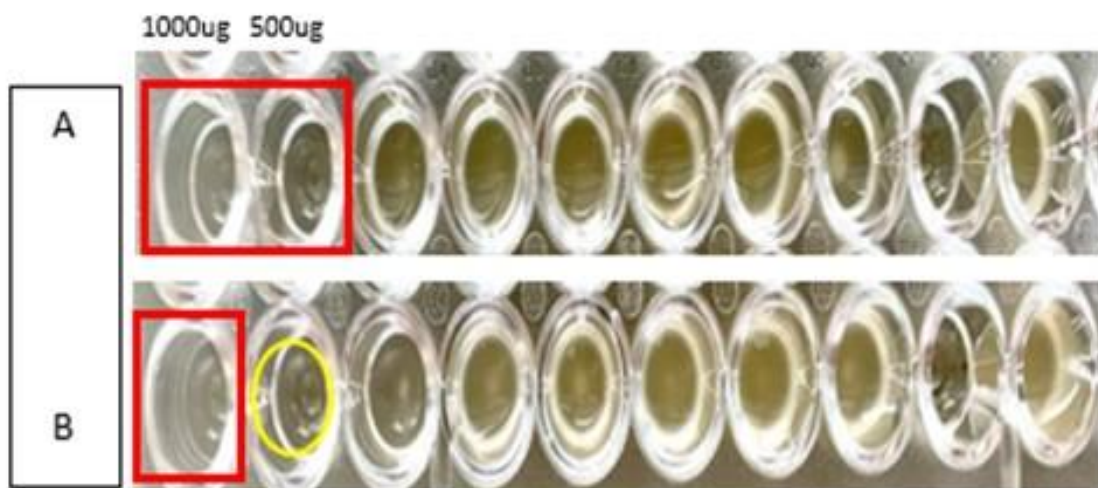


Figure 4.24: Comparison of the antibacterial activity demonstrated by Bornean frogs of the genus *L. paramacrodon* against (A) *E. coli* and (B) *S. aureus*. The red boxes indicate the minimum inhibitory concentration of partially purified frog secretion, and the yellow circle indicates bacterial growth, even though it appears as a clear solution

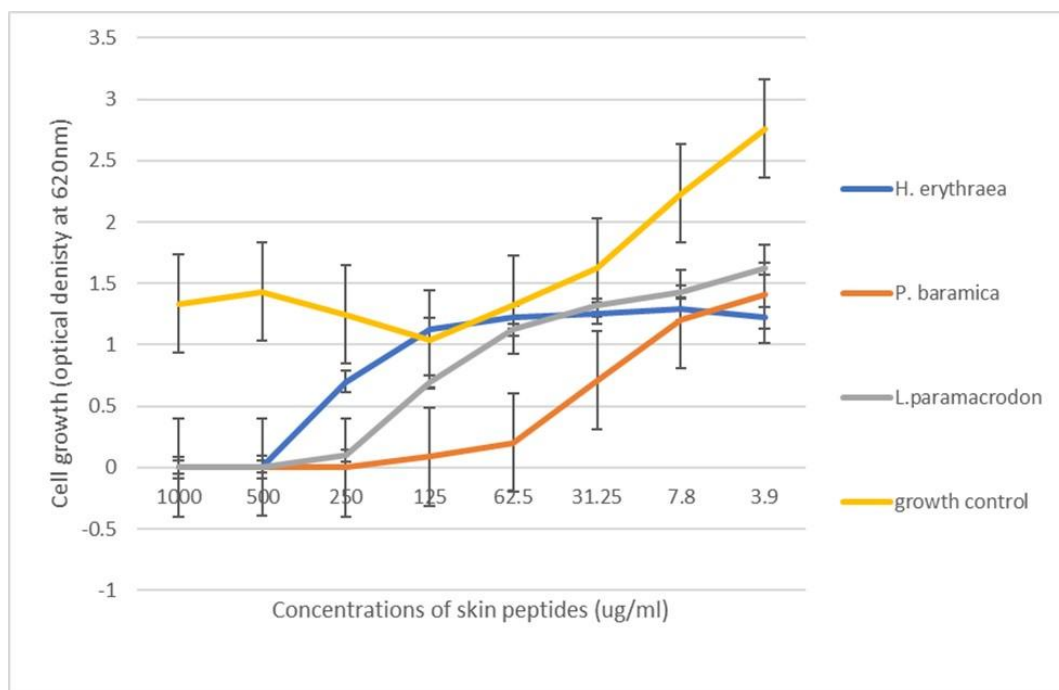


Figure 4.25: Growth Inhibition of *E. coli* by Partially purified peptides of *P. baramica*, *H. erythraea* and *L. paramacrodon*. at concentrations ranging from 500 $\mu\text{g/mL}$ to 3.9 $\mu\text{g/mL}$

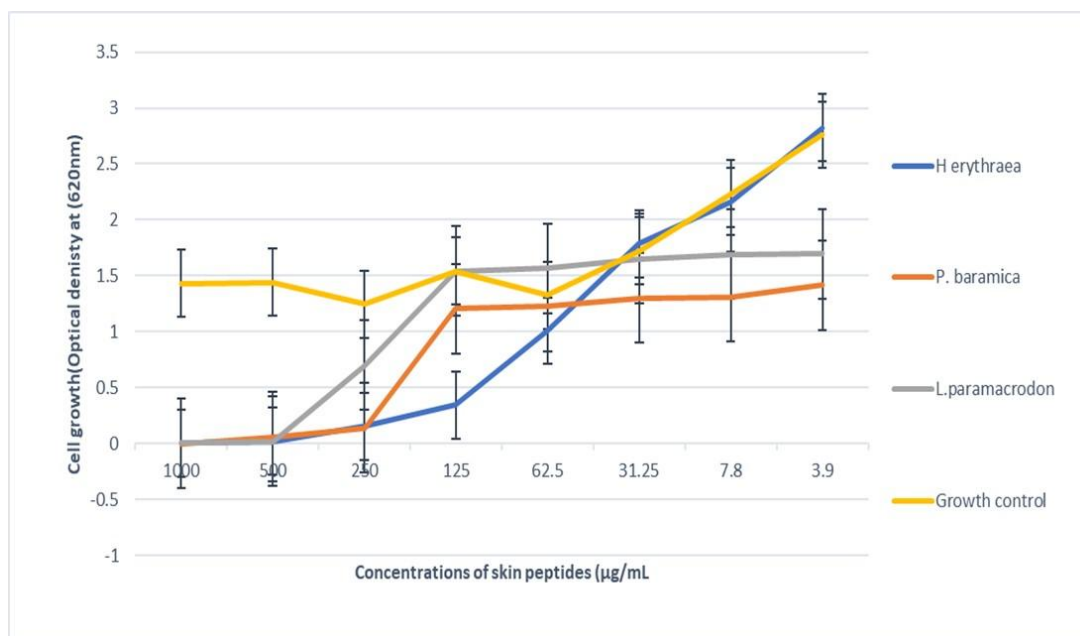


Figure 4.26: Growth Inhibition of *S.auerus* by Partially purified peptides of *P. baramica*, *H.erythraea* and *L. paramacrodon*. at concentrations ranging from 500 µg/mL to 3.9 µg/mL

4.6 Evaluation of Hemolytic Activity and Toxicity of Frog Skin Peptides on Human Red Blood Cells

This study aimed to assess the hemolytic activity and toxicity of frog skin peptides, specifically antimicrobial peptides (AMPs), on human red blood cells (RBC). The investigation involved incubating RBCs with varying concentrations of frog skin secretions for one hour. To determine the toxicity of AMPs, concentrations of 1.56, 3.125, 12.5, 25, 50, and 100 µg/mL were examined. The percentage of hemolysis was calculated based on the mean absorbance of triplicate experiments following the formula.

$$\frac{\text{OD}_{450\text{nm sample}} - \text{OD}_{450\text{nm PBS}}}{\text{OD}_{450\text{nm 1\% Tween}} - 20} \times 100 \quad \text{Equation 4.3}$$

The results in Figure 4.27 demonstrated that skin secretion concentrations less than 12.5 µg/mL had no significant influence on the viability of RBC in any of the tested species, as indicated by less than 25% hemolysis. Specifically, *P. baramica*, *H. erythrae*, and *L. paramacrodon* exhibited moderate hemolytic activity, with less than 50% hemolysis observed at concentrations lower than 100 µg/mL. Notably, *P. baramica* exhibited significantly higher hemolytic activity than *H. erythrae*, causing more than 70% RBC lysis at a concentration of 100 µg/mL. Conversely, *L. paramacrodon*, among the tested species, displayed the least hemolytic activity, with only 36% hemolysis at a concentration of 100 µg/mL.

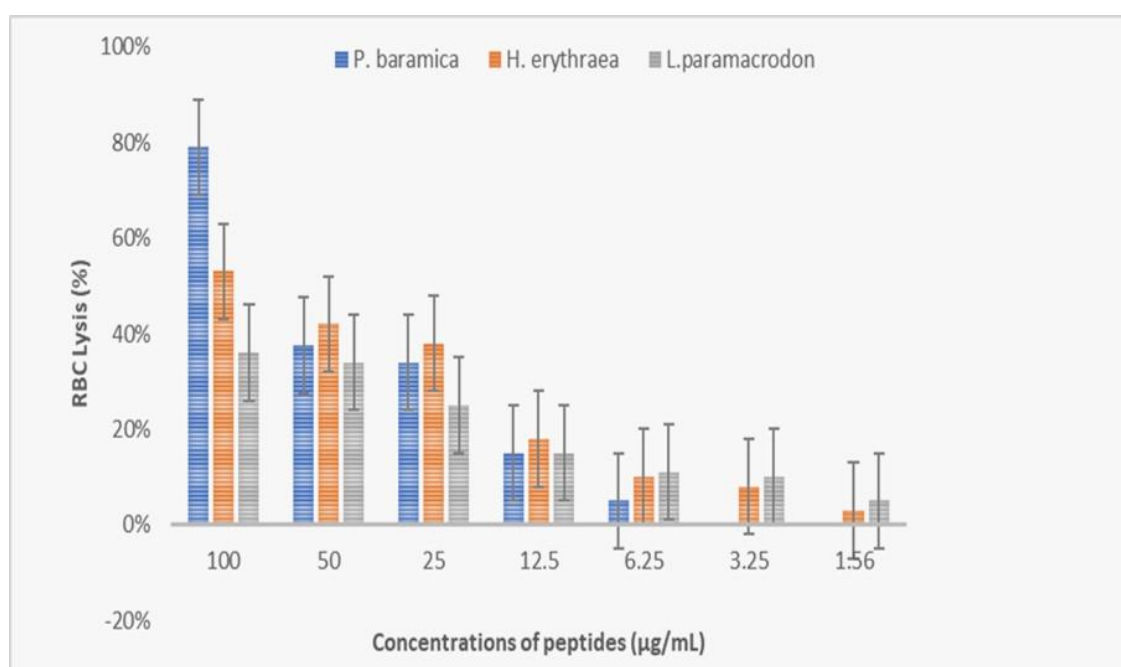


Figure 4.27: Percentage of cell lysis induced by partially purified skin secretions on human red blood cell after 1 hour of incubation. Bar charts showed the percentage of red blood cell lysis, expressed as mean $\pm$ SD. The negative control, 0% hemolysis was a sample of red blood cell treated with Phosphate Buffer Saline and positive control, 100 % hemolysis was red blood cell treated with 1 % Tween 20. Values were taken as mean absorbance at 450 nm.

4.7 Cell Culture Maintenance: Optimizing Growth Conditions and Observing Replication Dynamics

HIT-T15 cells were originally derived from a transplantable insulinoma tumor in a Syrian hamster. They exhibit characteristics of pancreatic beta cells, including the ability to produce and secrete insulin, making them a valuable model for studying insulin secretion and glucose metabolism. The cells were maintained in a controlled environment set to 37°C with a humidity level of 95% and 5% CO₂. The culture medium was changed every 48 hours to sustain optimal conditions for cell growth and function.

Throughout the culturing process, cells were incubated under controlled conditions to mimic physiological settings. Careful attention was given during the medium changing process to minimize stress and preserve cell viability. Specifically, for HIT-T15 cells, the F12k medium was refreshed every 2 days, resulting in 80% confluency after 14 days of culturing.

Figure 4.28 illustrates the initial phase of cell growth, where the cells are observed to be at less than 20% confluency. At this stage, the cells are in the early stages of proliferation, with a limited number of cells adhering to the culture substrate and beginning to replicate. The low confluency indicates that the cells are still in the early phases of growth, and the culture is not yet densely populated with cells.

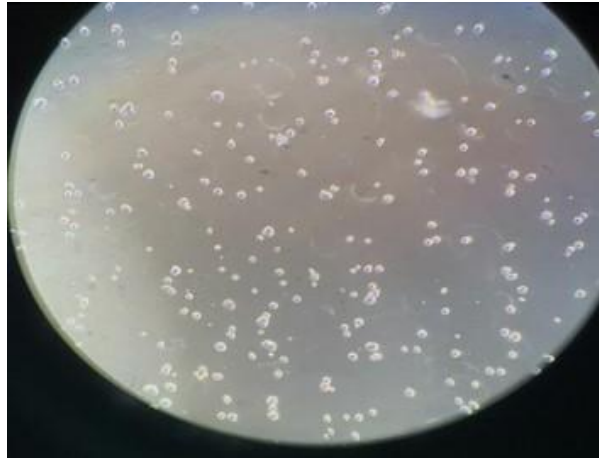


Figure 4.28: The Initial Phase of HIT-T15 Cell Growth after 48 Hours

In Figure 4.29, the cell culture demonstrates an intermediate stage of growth, with the cells reaching approximately 50% confluency. At this stage, the rate of cell proliferation has increased, resulting in a higher number of cells occupying the culture surface compared to Figure 4.28. The increased confluency suggests that the cells have continued to replicate and proliferate over time, leading to a more densely populated culture.

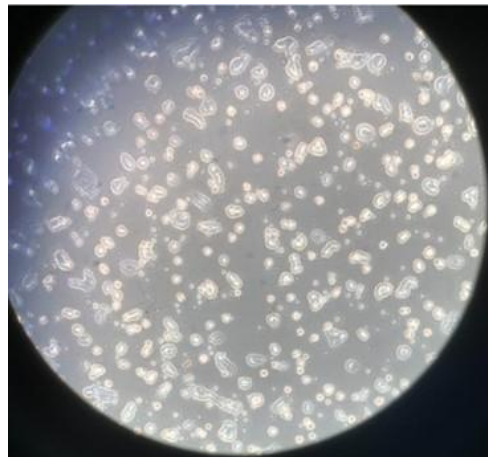


Figure 4.29: HIT-T15 Cell Growth after 7 Days

Finally, in Figure 4.30, the cell culture has reached approximately 80% confluency, indicating that the cells are nearing confluence. At this stage, the culture surface is densely populated with cells, and cell proliferation has slowed down compared to earlier stages. The higher confluency observed in Figure 4.30 suggests that the cells have undergone multiple rounds of replication and division, resulting in the increased cell density observed in the culture.

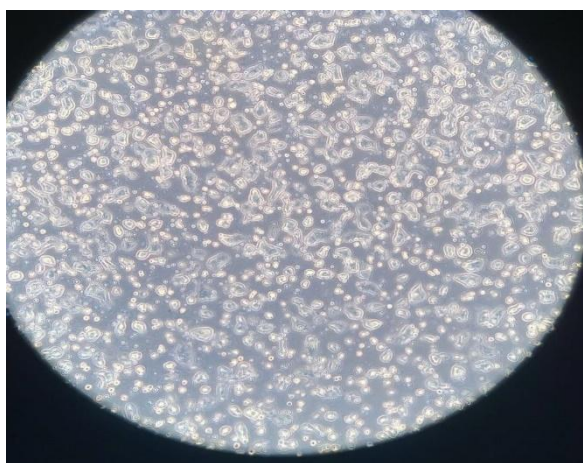


Figure 4.30: HIT-T15 Cell Growth after 14 Days

Overall, the progression from Figure 4.28 to Figure 4.30 demonstrates the dynamic nature of cell growth and proliferation in culture. Over the 14-day period, the cells replicate and proliferate, gradually increasing in number and leading to higher confluency levels in the culture. This process is driven by the cells' ability to divide and multiply, ultimately resulting in the establishment of a densely populated cell culture reaching 80% confluency.

4.8 Cell Assessment of Cytotoxicity Induced by Antimicrobial Peptides (AMPs) on HIT-T15 Cells Using the MTT Assay

Following the successful preparation and attainment of desired confluency in cell cultures, the subsequent step in many experimental procedures involves assessing cell

viability. One widely used method for this purpose is the MTT assay, which provides a quantitative measure of cell viability based on the conversion of a tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into formazan crystals by metabolically active cells. This assay is valuable for evaluating the effects of Antimicrobial Peptides (AMPs) on cell viability and proliferation. Intriguing variations in cell viability were revealed when these cells were exposed to different concentrations of skin secretions from *P. baramica* and *H. erythraea*.

After seeding HIT-T15 cells at a density of 2×10^4 cells per well in a 96-well plate, incubation took place for a 24-hour period. Figure (4.31) illustrates the cells within the wells on the first day, while Figure (4.32) depicts the cell confluency on the second day before treatment with different concentrations of AMPs for an additional 24 hours. Subsequently, the culture media were carefully removed. Cell viability was assessed by adding a 1 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), as outlined in the methodology.

After adding a 1 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The wells color in 96 well plate changed start from 25ug concentration to 1 ug concentration in different color intensity indicating formation of formazin crystal and viability of the cells in Figure 4.33 while the color of 50ug concentration wells doesn't changed indicating there's no formazen crystal has formed in Figure 4.34.

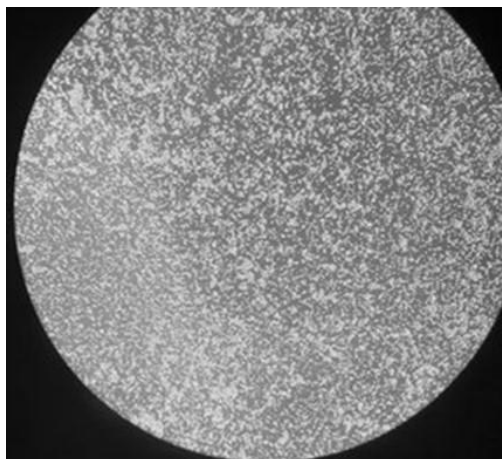


Figure 4.31: 1st Day Seeding of HIT-T15 Cells within 96 Well Plate

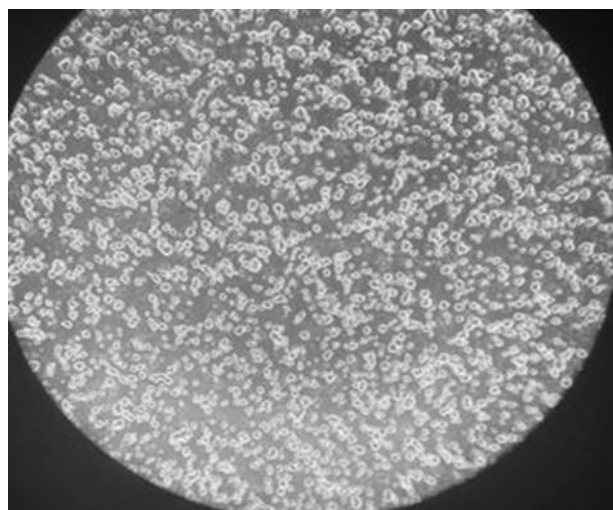
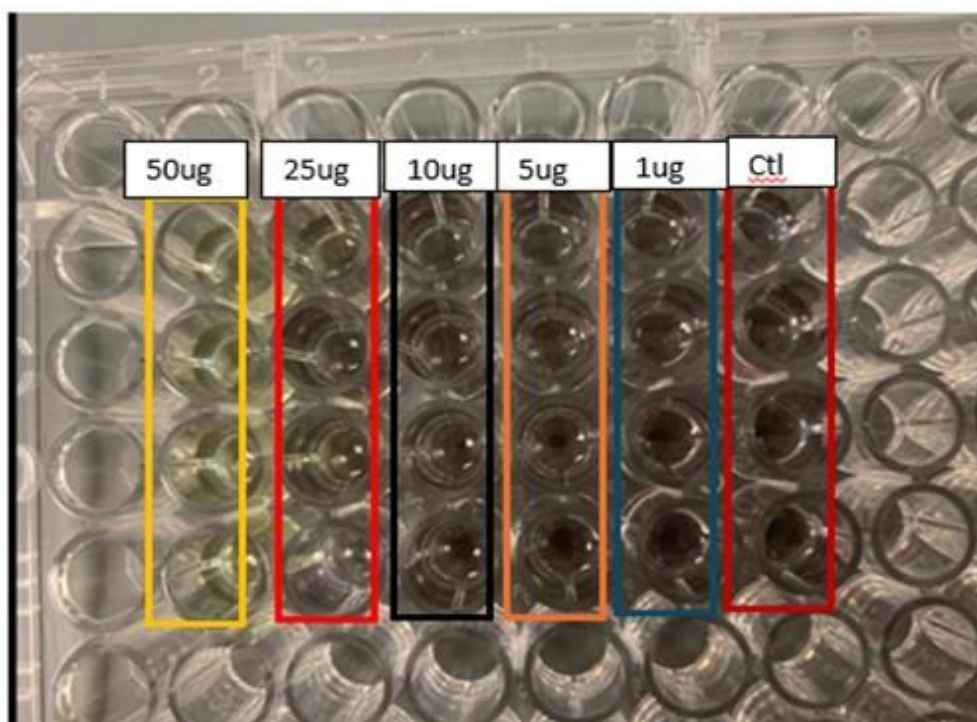
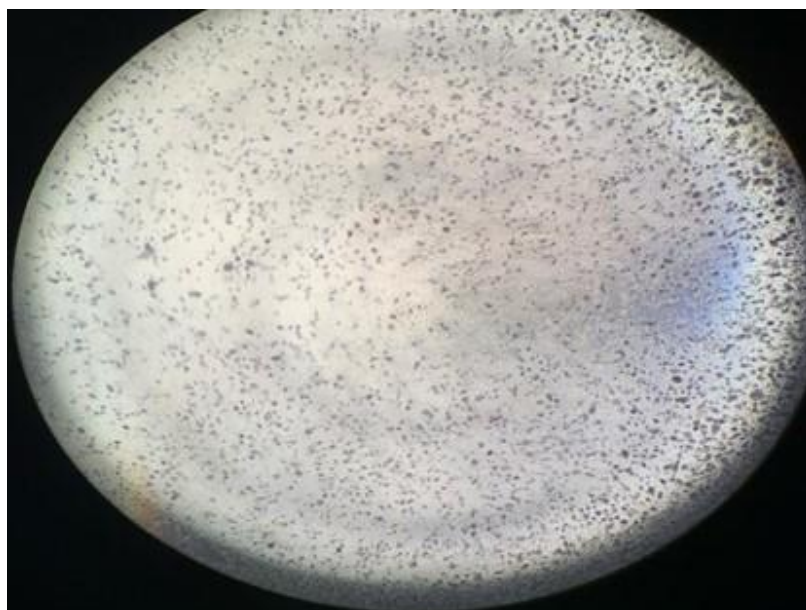


Figure 4.32: 2nd Day Seeding of HIT-T15 Cells within 96 Well Plate



The skin secretions from three distinct frog species—*P. baramica*, *H. erythraea*, and *L. paramacrodon*—were shown to reveal concentration-dependent responses. It was observed that for the *P. baramica* frog, cell viability was exhibited as a concentration-dependent pattern, with 76% viability at a lower concentration of 25 µg, compared to 27% at the higher concentration of 50 µg. Similarly, for *H. erythraea*, cell viability was indicated as 60% at 25 µg and 40% at 50 µg, showing a concentration-dependent decrease. In contrast, *L. paramacrodon* was shown to exhibit higher cell viability, with over 80% viability at 25 µg and over 60% at 50 µg. At lower concentrations of 1 µg and 5 µg, exceptional cell viability was demonstrated by all three species with 100% viability. When the concentration was increased slightly to 10 µg, a slight reduction in viability to 97% was shown by *P. baramica*, while 100% viability was maintained by both *H. erythraea* and *L. paramacrodon*.

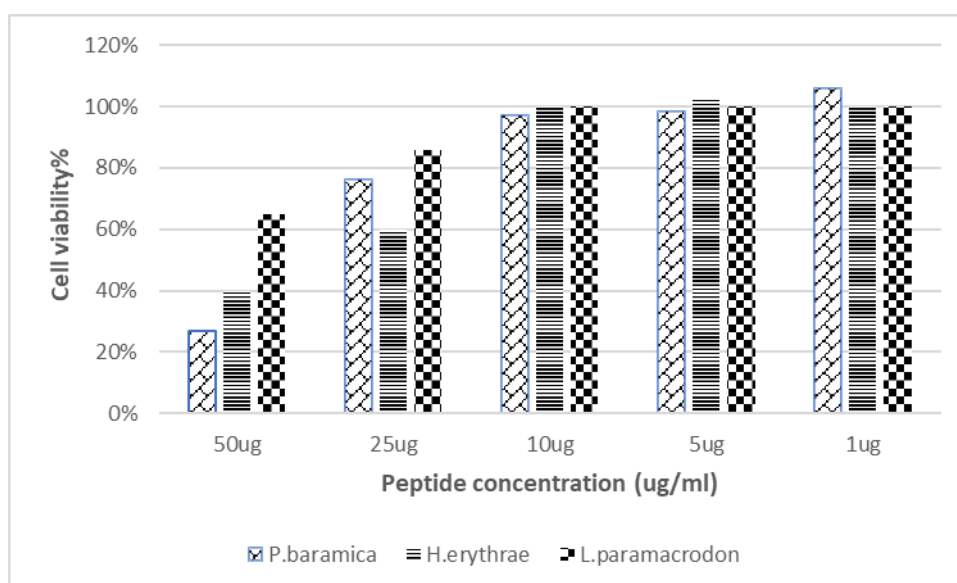


Figure 4.35: The effect of different concentrations of antimicrobial peptides (AMPs) from frog skin secretion of *P. baramica*, *H. erythraea*, and *L. paramacrodon* on HIT-T15 cells, measured using the MTT assay

4.9 Evaluation of Insulin Secretion in HIT-T15 Cells

In this study, the impact of frog skin secretions on insulin release was investigated using HIT-T15 cells. Supernatants from different frogs, specifically *P. baramica*, *H. erythraea* and *L. paramacrodon* were prepared in 12-well plates. The experiment, conducted in triplicate, involved treating the control group with Krebs bicarbonate buffer, while Krebs buffer containing 5 μ g of AMPs was administered to the other wells. Cell morphology was depicted in Figure 4.36. Furthermore, quantification of the supernatant was performed using the Human Insulin ELISA kit from R&D Systems.

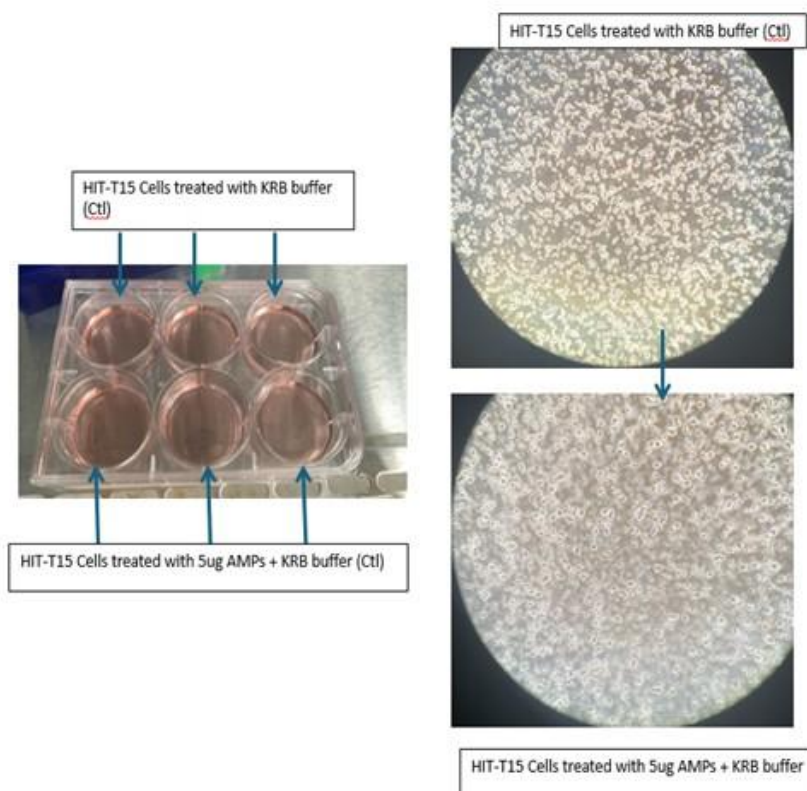


Figure 4.36: Supernatant Preparation in a 12-Well Plate: The supernatant was prepared in triplicate. (A) shows the cells treated with KRB buffer (control), and (B) shows the cells treated with 5 μ g AMPs and KRB buffer. Cell morphology changes were observed before and after treatment with AMPs

In the Elisa assay procedure, the autowasher plays a crucial role by ensuring consistent and thorough washing of the microplate wells. It automates the process of adding and removing wash buffers, which helps to minimize variability between wells and ensures accurate and reliable results. By precisely controlling the washing process, the autowasher helps to remove any unbound substances and reduce background noise, thereby enhancing the sensitivity and specificity of the assay.

After adding the stop solution, a color change occurs in the wells, transitioning from blue to yellow Figure 4.37. This color change is a key indicator of the reaction occurring within the wells. In many assays, including the one described here, the color change corresponds to the completion of a chemical reaction. In this case, the stop solution halts the enzymatic reaction initiated by the substrate solution, leading to the formation of a colored product. The change in color intensity correlates with the concentration of the target analyte in the sample. By measuring the optical density of the yellow-colored solution using a

The basal insulin release rate under normal glucose conditions (1.8 g/L) was quantified as 138.2 ± 0.01 ng/ 10^5 cells/30 min. In the first experiment, exposure to 5 μ g of AMPs from *P. baramica* led to a significant, dose-dependent increase in insulin release ($P < 0.05$). The insulin release rate increased to 145.7 ± 0.002 ng/ 10^5 cells/30 min, as shown in Figure 4.38. In the second experiment, AMPs from *H. erythraea* induced a concentration-dependent stimulation of insulin secretion at 5 μ g, although this increase was not statistically significant ($P > 0.05$) compared to the basal insulin release rate, as depicted in Figure 4.39. In the third experiment, AMPs from *L. paramacrodon* also caused a concentration-dependent stimulation of insulin secretion. The basal insulin release rate under normal glucose conditions was 19.57 ± 0.028 ng/ 10^5 cells/30 min. At 5 μ g, the insulin release rate

increased to 21.3 ± 0.0051 ng/ 10^5 cells/30 min, as shown in Figure 4.40, although this increase was not statistically significant ($P > 0.05$) when compared to the basal insulin release rate.

microplate reader.

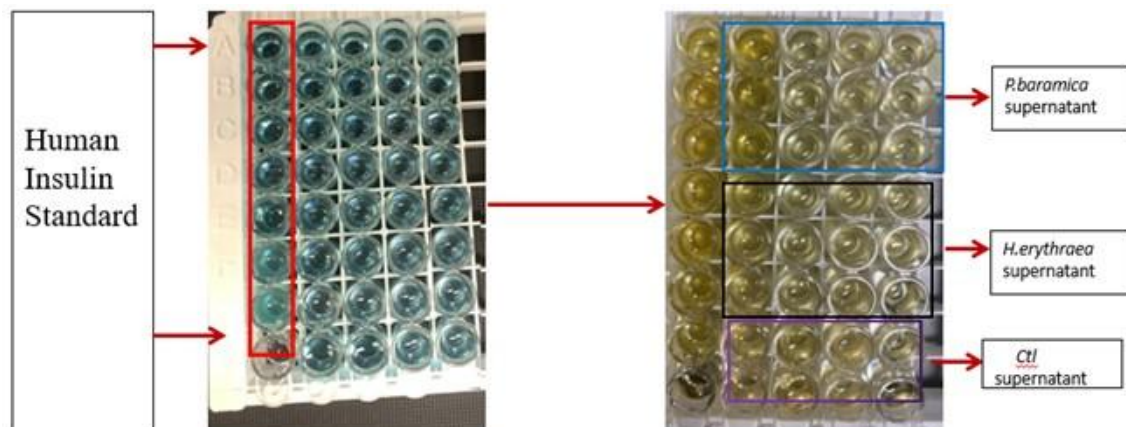


Figure 4.37: The Enzymatic Reaction for Human Insulin ELISA, wells were coated with a monoclonal antibody specific to human insulin. The washing buffer was added to remove unbound substances. The supernatant were added, binding to the immobilized antibody. A secondary antibody conjugated to HRP was added, binding to captured insulin. Subsequently, TMB substrate was added, and the conversion to a blue-colored product was catalyzed by HRP. The reaction was stopped with sulfuric acid, changing the color from blue to yellow.

Collectively, the research findings suggest that frog skin secretions from both *P. baramica*, *H. erythraea* and *L.paramacrodon* have the potential to influence insulin release in HIT-T15 cells. However, the magnitude and statistical significance of these effects varied between the three types of secretions, highlighting the variability in their impact on insulin secretion and opening avenues for further investigation, with potential therapeutic implications in diabetes research.

Table 4.12: AMPs-Induced Insulin Release in HIT-T15 Cells

Concentration $\mu\text{g/mL}$	Control (1.8g/L) glucose	5 $\mu\text{g/mL}$ (AMPs)
<i>P.baramica</i>	138.2 $\pm$ 0.001	145.7 $\pm$ 0.002
<i>H. erythraea</i>	122.2 $\pm$ 0.48	122.7 $\pm$ 0.46
<i>L.paramacrodon</i>	19.57 $\pm$ 0.0028	21.3 $\pm$ 0.0051

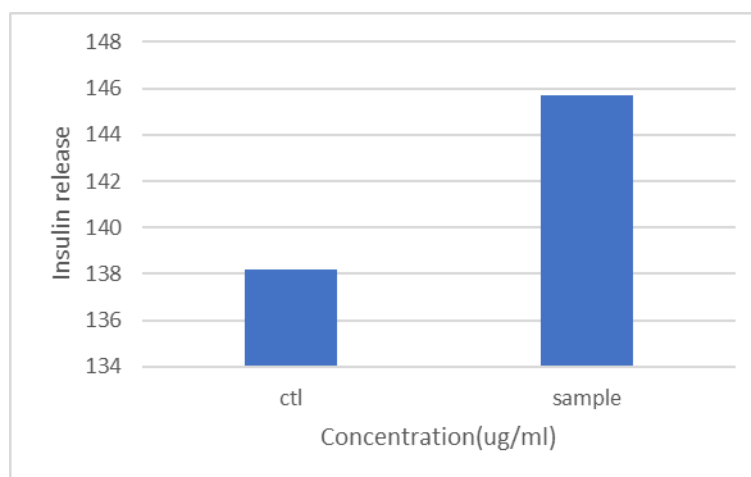


Figure 4.38: Effects of 5ug AMPs *P.baramica* on the Rate of Release of Insulin from HIT-T15 Clonal β -cells Compared with Control

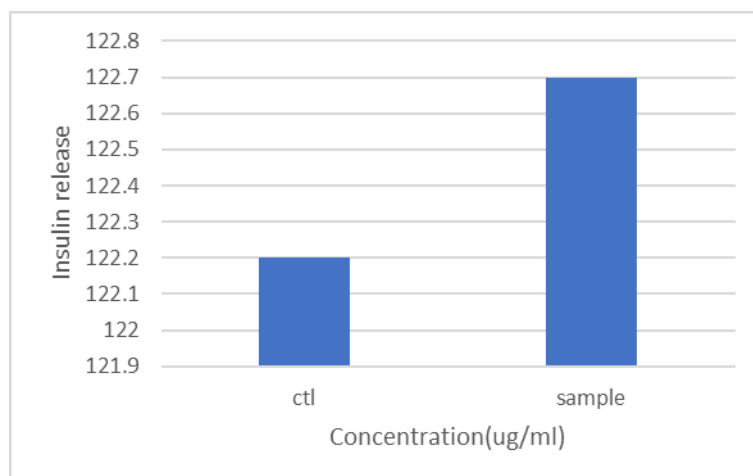


Figure 4.39: Effects of 5ug AMPs *H.erythraea* on the Rate of Release of Insulin from HIT-T15 Clonal β -cells Compared with Control

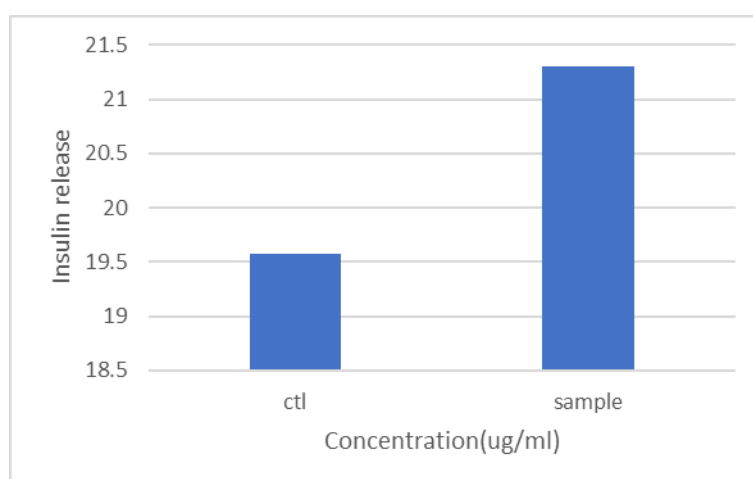


Figure 4.40: Effects of 5ug AMPs *L.paramacrodon* on the Rate of Release of Insulin from HIT-T15 Clonal β -cells Compared with Control.

CHAPTER 5

DISCUSSION

5.1 Collection of Skin Secretions using Chemical Stimulation

Amphibian skin secretion is renowned for its high concentration of bioactive compounds (Chenet al., 2022). Chemical stimulation methods for extracting frog skin secretion typically involve applying chemical agents or solutions to the frog's skin to induce secretion. One common method involves inducing frogs to anhydrous ether in a closed container, which has been found to be not only practical but also ethical, efficient, and reliable. However, ongoing debates persist regarding the effectiveness of this chemical stimulation method compared to alternative techniques, such as electrical stimulation and norepinephrine injection, as discussed by Melzer et al. (2013) and Kakehashi et al. (2022). An alternative approach, studied by Govender et al. (2012), utilizes electrical stimulation, resulting in the complete emptying of granular glands. Each of these methods can be tailored to the specific needs of the research or extraction process, depending on factors such as the type of compounds being extracted, the species of frog, and ethical considerations.

Electric stimulation a low-voltage electric current was applied to the skin of the Rain Frog to induce secretion of skin compounds. This allowed the subsequent detection of skin peptides using RP-HPLC. However, it's important to mention that while most methods (though not all) have successfully isolated known antimicrobial peptides (AMPs) using anhydrous ether, it's unclear whether peptides were completely expelled from granular glands in the current study. This uncertainty arises because the skin wasn't dissected, and the morphology of the skin before and after stimulation wasn't examined. Future research should consider these measures to ensure that AMPs are not retained within granular glands, thus

aiding in the efficient isolation and characterization of biologically active peptides from frog skin secretions.

5.2 Monitoring Purification via SDS Page Electrophoresis

The SDS-PAGE analysis and purification strategies detailed in the study align closely with the objectives of isolating, purifying, and characterizing host defense peptides from Bornean frog skin secretions, as well as supporting their functional evaluation. Chemical stimulation enabled the collection of crude secretions, which exhibited complex protein band patterns that were refined through purification using Sep-Pak C18 cartridges and AKTA systems. These methods successfully removed impurities, salts, and larger proteins (>17 kDa), enriching peptides of interest, as confirmed by changes in band intensity and migration patterns observed in Figures 4.4, 4.5, and 4.6. The use of Tricine SDS-PAGE with Coomassie blue staining improved the visualization of smaller peptides, facilitating their characterization (Duarte et al., 2018). The study emphasized the need to monitor peptide loss and protein concentrations during purification, noting the sensitivity of BCA assays to low-molecular-weight peptides (Brandoch Cook, 2018) and dilution effects during Sep-Pak processing. Additionally, the emergence of high-intensity bands in partially purified samples validated the enrichment of target proteins, with AKTA purification yielding bands corresponding to expected molecular weights, such as the viral E protein (Pato et al., 2019). The purification process, which selectively retained peptides below 11 kDa using washing buffers containing trifluoroacetic acid (Tang et al., 2024), ensured that the peptides were suitable for downstream functional assays. Furthermore, structural changes, such as protein denaturation and disulfide bond formation during heating, were considered (Khalid et al., 2023). These findings confirm the effectiveness of the methods in isolating peptides for

evaluating their effects on HIT-T15 cell viability and insulinotropic activities, fulfilling the study's objectives.

5.3 Purifications of Peptides using Akta-pure Chromatography Purification System

The attainment of nearly uniform purity in peptide purification is deemed crucial for ensuring accurate and reliable research outcomes. In this study, the Akta-Pure chromatography purification system was employed, and the Unicorn 7 software was incorporated for the analysis of peaks and calculation of retention times. This method is widely recognized for its capacity to achieve high purity in peptide samples.

The role of the C18 column, specifically sourced from Siliachrom, is considered pivotal in the peptide purification process. It is essential to note that the column selection represents a fundamental aspect of the methodology. However, variations in the literature are observed concerning the specific type of C18 column utilized for peptide purification. In an extensive literature review, a preference for the utilization of a C18 column was identified among most researchers, although not uniformly. In a study conducted by Emma Renard in 2019, peptide 2as was successfully purified using a similar analytical column from Siliachrom. Nevertheless, the preference for different column types, as also demonstrated in studies by Conlon et al. (2018) and Gusmao et al. (2017), may contribute to the disparities noted in peptide elution times when compared to the findings of other research studies.

Within the scope of this study, the efficient elution of all peptides was consistently achieved within a relatively brief timeframe, typically not exceeding forty minutes. In contrast, a duration of fifty minutes to sixty minutes was extended by Yang & Li (2023), employing a C18 column. It is significant to note that variations in the retention times of identical amino acids within distinct peptides were recurrently observed, a phenomenon

previously documented by Skiadopoulou et al. (2023). These differences in elution times are generally considered to have a minimal impact on the bioactivity of the peptides. Nevertheless, the confidence associated with peptide identifications may be marginally affected, an aspect of concern in the realm of peptide research.

Furthermore, it is essential to highlight that the absence of an in-house RP-HPLC system for Akta-Pure software necessitated specific adjustments to the Unicorn software, aligning with the manufacturer's recommended protocol. This serves to underscore the adaptability and flexibility of chromatographic methods in the realm of peptide purification. The diverse methodologies and column selections, while resulting in variations in elution times, collectively contribute to the depth and diversity of research within the domain of peptide purification.

The manual adjustment of column pressure in the Unicorn software is a critical aspect of chromatographic optimization, particularly in C18 column chromatography. By manually adjusting the column pressure to 7 units and the flow rate of the mobile phase to 0.5 mL passing through the column, chromatographic parameters were fine-tuned.

The pressure within the column is directly influenced by the flow rate of the mobile phase (Thermo Fisher Scientific, 2024). As the flow rate increases, the pressure within the column also increases due to the resistance encountered by the mobile phase, but it varies slightly with composition-programmed gradient elution because of the viscosity differences of mixed solvent (Fekete et al., 2022). As shown in Figure 5.1, there is a slight drop in pressure once the elution begins, which might be due to the mixing of the solvent and samples, resulting in a change in viscosity. Variations in peak intensity or resolution may be observed in the chromatogram obtained during the analysis, particularly at higher flow rates.

This phenomenon may appear as a slight decrease in peak intensity at the highest peak. Such variations underscore the delicate balance between flow rate, pressure, and chromatographic performance.

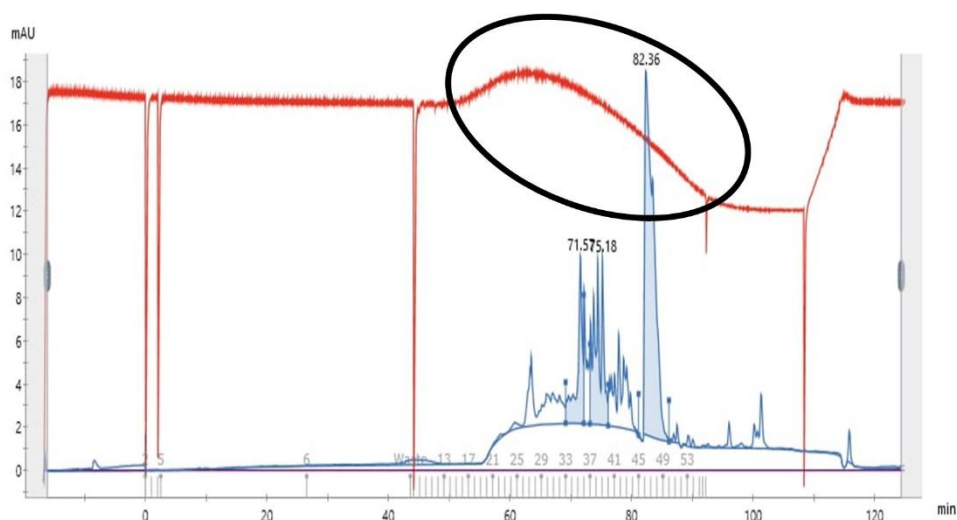


Figure 5.1: Chromatograms Illustrating the Analysis of Column Pressure during the Elution Process of Skin Peptides from *P. baramica* Frog

Peptides were eluted at a 214nm wavelength, enhancing sensitivity and ensuring the detection of a wider variety of proteins in the secretion. Various interfering components, such as peptides, enzymes, and other biomolecules, may be present in frog skin secretion. AKTA systems are equipped with detectors and filters optimized for various wavelengths, including 214 nm, making it a practical choice for protein purification from frog skin secretion or similar samples based on instrumentation compatibility and specific purification protocol requirements.

The use of the 214 nm wavelength in AKTA systems for purifying proteins from frog skin secretion or other sources is primarily determined by the specific properties of the proteins in the sample and the characteristics of the purification process. Proteins in frog skin

secretion, like those from other biological sources, contain aromatic residues such as tryptophan, tyrosine, and phenylalanine, which absorb UV light at specific wavelengths. Tryptophan exhibits the highest molar absorptivity at 280nm commonly used for protein detection due to its absorbance peak. At 214 nm, the amide peptide bond has a strong absorbance, allowing for improved sensitivity in analyzing limited or low-concentration proteins (Peptides Guide), At 214 nm, the amide peptide bond has a strong absorbance. Improved sensitivity allows for analysis of sample limited or low concentration proteins (Hong et al., 2012).

5.4 Protein Concentration (Protein Loss and Recovery)

Protein recovery refers to the percentage of the target protein obtained compared to the initial amount present in the crude sample. High yield is desirable to maximize the production of the target protein. The variation in the crude protein content of frog skin secretion can be influenced by factors such as the species of frog, environmental conditions, and the physiological state of the frog (Varga et al., 2019). It is known that frog skin secretion contains a diverse array of proteins, peptides, and other bioactive molecules (Ong et al., 2021). The quantification of the crude protein content of frog skin secretion typically entails the extraction of proteins from the secretion, followed by the analysis of the total protein concentration using biochemical assays. Downstream applications may be disrupted by contaminants in crude samples, potentially leading to incorrect conclusions that necessitate the purification technique to remove the undesirable molecules.

This study revealed an acceptable level recovery in the concentration of protein for three species of frog protein after purification with Sep Pak columns. as mentioned before in Figures 4.13, 4.14 and 4.15. the protein concentration in the *P.baramica* had protein

recovery 20.4% to 44%% after using amicon filter. Similarly, the protien recovery for *H.erythrae* was 17% to 48% and *L.paramacrodon* was 18% to 31% %. This enhancement in protein concentration after purified by SEP-PAK following concentrated with amicon filter indicates the effectiveness of Sep Pak columns in concentrating the target proteins while removing interfering substances.

The enhancement in protein concentration after Sep Pak purification can be attributed to several factors. Sep Pak columns selectively retain target biomolecules while washing away impurities, leading to a higher concentration of the desired protein in the eluted fraction (Smith & Nguyen 2023). Additionally, the removal of contaminants such as salts, nucleic acids, and other cellular debris during the purification process contributes to the overall increase in protein concentration (Tang et al.,2024).

On the other hand, significant protein recovery was achieved after purification using an AKTA system, emphasizing the importance of purity and quality. A study by Yoo et al. (2014) demonstrated that the recovery of protein is affected by the flow rate. Different flow rates and sample loads were tested to observe their impact on protein recovery. When a higher flow rate was utilized, better recovery of the protein was generally observed, with over 90% recovery achieved at a flow rate of at least 2 mL/min. Moreover, higher recovery of protein could be achieved by increasing the injection volume of the sample in the AKTA-pure system (Franke et al., 2020).

In a recent study (Winters et al., 2015), the effectiveness of the modified ÄKTA system in purifying proteins through a two-column process was demonstrated. Different levels of dilution were tested to observe their effect on protein recovery, with both levels yielding similar results. Optimization of purification conditions, such as solvent and elution

buffer choice (e.g., TFA and acetonitrile), can further improve yield and purity depend on factors such as the sample type, chemical or biological modifications of the peptides (Bugyi,2023). These findings underscore the importance of considering various parameters in protein purification processes to achieve optimal recovery and quality.

5.5 Antimicrobial Activity of AMPs from Bornean Ranids

5.5.1 Variations in Minimum Inhibitory Concentration (MIC)

The research presented in this study has unveiled a notable dose-dependent relationship between increasing protein concentrations and the demonstrated antibacterial activity against the tested strains. This finding aligns with previous studies, providing further support for the reliability of the observed trends.

However, it's worth highlighting that slight variations in MIC values were observed in this study when compared to other studies. For instance, the MIC was either one-fold greater or one-fold reduced for the same bacterial specimen. These differences can be attributed to several factors, with the type of bacterial strains used in the assay being a significant consideration. In our study, *E. coli* ATCC 25243 and *S. aureus* ATCC 23433 were employed. These bacterial strains exhibit variations in their lipid compositions and the percentage of anionic lipids on their membrane leaflets, which can lead to different levels of susceptibility to antimicrobial agents.

Additionally, it is essential to note that variations in the dilution technique, may also contribute to differences in MIC values. This underscores the importance of meticulous and standardized procedures in MIC determination. These variations in MIC values across studies add depth and complexity to our understanding of antibacterial activity and

underscore the need for careful consideration of the specific experimental conditions and bacterial strains when interpreting MIC data.

5.5.2 Effectiveness Amongst Species: Environmental Factors and Life History

AMPs from *P. baramica* demonstrated higher activity against both *E. coli* and *S. aureus* compared to the other tested species. Consequently, it could be considered a promising candidate for future therapeutic development.

In this study, it was also noted that AMPs from *P. baramica* displayed the highest protein concentration when assessed using the BCA assay. Moreover, they exhibited the most potent antibacterial activity when compared to the *H.erythraea*. This observation might be attributed to environmental factors, as *P. baramica* is known to thrive in peat swamp areas, which typically have a high-acidic environment, unlike secondary forest habitats. These unique environmental conditions could contribute to the distinctive properties of AMPs from *P. baramica*, potentially enhancing their antibacterial efficacy.

However, a significant discovery was made by Gammill in 2012, indicating that the concentrations of AMPs are directly proportional to the range of stimuli received by frogs. This relationship was exemplified in *Xenopus laevis*, where the levels of AMPs notably increased when the quantity of peptides collected following one injection at 80 nmol norepinephrine/gbw was 20-fold higher than the amount released due to natural norepinephrine signaling during a simulated predator attack. These findings underscore the profound influence of environmental factors on the expression of antimicrobial peptides in frogs.

This dynamic response highlights the adaptive nature of AMP production, allowing frogs to adjust their defenses in response to the microorganisms and environmental challenges they encounter. The ability of frogs to modulate their AMP levels based on external stimuli provides valuable insight into the intricate interplay between these amphibians and their surrounding ecosystems, ultimately contributing to our understanding of host-pathogen interactions and immune defense mechanisms in amphibians.

In terms of the effectiveness of these AMPs against bacterial cells, the frog skin secretions (AMPs) from *P. baramica* and *H. erythrae* exhibited higher activity against the gram-negative *E. coli* when compared to the impact observed on the gram-positive *S. aureus*. This disparity can be attributed to the distinctions in the composition of the cytoplasmic membranes of gram-negative and gram-positive bacteria. These findings emphasize the multifaceted interactions between AMPs and different bacterial cell types, shedding light on the complexities of peptide-mediated antimicrobial activity.

Although both bacterial strains feature negatively charged surface membranes, a marked distinction is observed in the thickness of the peptidoglycan mesh within the cell wall. It is well-documented by Malanovic & Lohner (2016). This structural disparity within the cell wall serves as a plausible explanation for the differing responses exhibited by the two bacterial types when exposed to antimicrobial peptides. These insights provide a deeper understanding of the intricate interplay between peptides and bacterial membranes, particularly how structural variations in bacterial cell walls influence the peptides' antimicrobial activities.

5.6 The Purified Peptides Decreased Human RBC Viability

In this study, the reduction in red blood cell (RBC) viability was directly influenced by the peptide concentration, as illustrated in Figure 4.26. Significant hemolytic activity was observed in the skin peptides extracted from the purified samples, notably not occurring around the Minimum Inhibitory Concentration (MIC) values. This observation runs counter to prior research findings, as demonstrated in studies by Kumar et al. (2015), where hemolysis was shown to occur around the MIC concentration value. While the specific mechanism behind RBC lysis upon interaction with the peptides was not investigated in this study, it is widely acknowledged that antimicrobial peptides (AMPs) derived from frog secretions tend to exhibit toxicity towards mammalian cells. This heightened toxicity has been linked to their elevated hydrophobicity. This observation aligns with the findings of Jiang et al. (2014), who demonstrated that the alteration of the positions of positively charged residues on the hydrophobic surface of peptides, such as piscidin-1 and dermaseptin-S4, resulted in a reduction in their hemolytic activity and an enhancement of their antimicrobial activity. This concept has been termed ‘Specific Determinant’.

Furthermore, the contribution of their cationic nature to hemolytic activity has been extensively investigated and verified through research conducted by Pedron et al. (2017). In their study, it was observed that heightened hemolytic activity correlates with an increase in the net charge of peptides. This observation highlights the crucial role played by the positive charge present on peptide molecules in their capacity to disrupt red blood cells.

In line with these findings, Ruiz et al. (2014) proposed a classification system for antimicrobial peptides (AMPs) based on their toxicity levels. It consists of three categories: low toxicity (LTP) for peptides inducing cell lysis below 20 μ M, moderate toxicity for those

within 20 μM to 100 μM , and highly toxic peptides (HTP) causing cell lysis at concentrations over 100 μM . This categorization aids in understanding the hemolytic activity of AMPs and their interactions with biological membranes.

It is worth noting that these factors are not necessarily isolated but can be involved in complex interactions contributing to cell lysis. Previous literature has described intricate interactions involving hydrophobicity, cationicity, α -helicity, and amphipathicity of the peptides (Santana, et al 2020). This multifaceted interplay underscores the intricate nature of AMP interactions with RBCs and their overall hemolytic activity.

5.7 Cell Culture and Viability Assessment in HIT-T15 Cells

Under controlled conditions, the HIT-T15 cells are cultured in a 37°C incubator with a 5% CO₂ atmosphere to maintain their viability and functionality. The cells are routinely passaged (split, sub-cultured) in 25 flasks, and the F-12K medium is changed every two days. When they reach 80% confluency, they are split at a 1:2 ratio using trypsin-EDTA solution and Phosphate-buffered saline (PBS) for various cell culture procedures, including washing cells.

These solutions cause the dissociation of cells from the culture surfaces, enabling their transfer to new culture vials with fresh medium, thus maintaining optimal cell growth and health. However, when adherent cells are partly transferred to new culture vials containing fresh culture medium, they may experience a disadvantage, as they are prevented from being exposed to excessively tight cell-cell contacts, resulting in a reduction of their cell division rate due to cell contact inhibition (Roth, 2022). The phosphate-buffered saline is used for diluting cells for counting and maintaining cells during experiments. It helps maintain the pH and osmolarity of the cell culture environment, providing a stable buffer

solution that is compatible with cell physiology (Tyagi & Mani, 2023). The cells were counted by trypan blue method, the Trypan blue selectively stains dead cells, leaving live cells unstained. The process involves harvesting cells from a culture flask, mixing them with trypan blue, and loading the stained cell suspension into a hemocytometer. After counting cells under a microscope, the concentration is calculated based on the counted cells and chamber volume. Viability is assessed by determining the proportion of live cells to dead cells, expressed as a percentage. A disadvantage of the trypan blue method is that it cannot differentiate between apoptotic and necrotic cells, as both types of dead cells are stained blue (Banfalvi, 2017). Conversely, trypan blue staining can be performed relatively rapidly, allowing for efficient cell counting and viability assessment (Kamiloglu et al., 2020).

5.8 Effect of purified AMPs on HIT-T15 Cells Viability

It is important to note that the MTT assay measures metabolic activity rather than cell number directly (Rai et al., 2018), with the absorbance reflecting the number of viable cells (Rai et al., 2018). It is often used in high-throughput setups, like 96-well plates, to assess cell viability across multiple conditions (Riss et al., 2016). One benefit of this method is that it allows testing of various concentrations of AMPs while minimizing reagent use.

However, the MTT assay has limitations. Since it measures metabolic activity, factors like mitochondrial function and overall cell metabolism can affect the results (Ghasemi, 2021). For instance, exposure to AMPs might alter metabolic activity, which can change absorbance readings without reflecting a real change in cell number. Prolonged exposure to certain compounds, such as EPZ005687 enzyme, can also lead to cell death, which would be detected by the MTT assay (Knutson et al., 2012).

In addition, factors like peptide nature, concentration, and exposure time can influence how AMPs affect cell viability. Peptides that regulate key cellular processes, such as p21, play an important role in this process (Yosef et al., 2017). AMPs can alter the cell cycle and even cause cell death in some cases. Understanding how these peptides impact cell survival and stress responses is critical (Sandoval et al., 2018).

5.8.1 Exploring the Role of Apoptosis: Insights from Frog Studies and Cell Cultures

In this study, three frog species were involved, and it was demonstrated that cell viability exceeding 50% was observed at a concentration of 25 µg/ml, while at 50 µg/ml, cellular shrinkage indicative of apoptosis was observed under the microscope (Olympus), as shown in Figure 5.3. The influence of proteins involved in the regulation of apoptosis on B-cell viability is highlighted, with apoptosis induced by AMPs being emphasized as a novel mechanism (Wang et al, 2022).

The significance of apoptosis in diverse contexts is underscored by various studies, with apoptosis emerging as the predominant cause of cell death in industrially relevant cell lines. For instance, apoptosis contributes to 80% of cell deaths in Chinese Hamster Ovary (CHO) cultures and up to 90% in TB/C3 hybridoma cell cultures (Grilo et al., 2019; Xiao et al., 2023). Dysregulation of apoptosis-related proteins, such as those belonging to the Bcl-2 family and Inhibitors of Apoptosis Proteins (IAPs), may lead to either increased or decreased B-cell survival, depending on the specific protein and context (Shahar et al., 2020). Moreover, the activation of endonucleases leading to DNA breaks is a primary mechanism of apoptosis, as elucidated in the research by Arya et al. (2014).

Regarding apoptosis mechanisms in pancreatic beta cells, Ghorbani et al. (2019) elucidated several factors contributing to apoptosis, including cytokines, glucotoxicity, and

lipotoxicity. However, further investigation is warranted to confirm the specific mechanism of apoptosis in HIT-T15 cells and elucidate its intricacies in this cellular context.

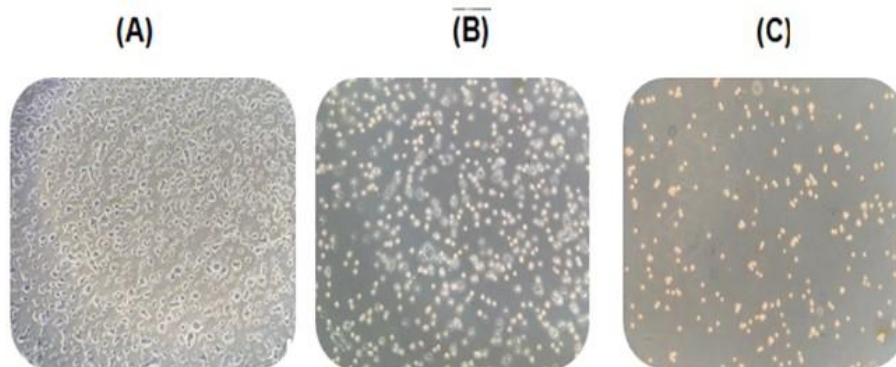


Figure 5.2: Impact of Varied AMP Concentrations from *P. baramica* on HIT-T15 Cell Viability as observed under an Olympic microscope. (A) Depicts 97% viability at a concentration of 50 $\mu\text{g/ml}$. (B) Illustrates 67% viability at a concentration of 25 $\mu\text{g/ml}$. (C) Demonstrates 27% viability at a concentration of 10 $\mu\text{g/ml}$.

5.9 Evaluation of Insulin Secretion in HIT-T15 Cells

The 12-well plate was seeded with HIT-T15 cells at a specified concentration. A concentration of 5 μg of AMPs and Krebs-Ringer bicarbonate buffer were applied to the cells to maintain pH (Bjarnsholt, et al 2019). Similarly, the control supernatant was obtained from cells treated with bicarbonate buffer alone, with the Krebs buffer containing 1.8 glucose to distinguish the effects of the experimental treatment from baseline cellular activity.

The human insulin ELISA is based on the principle of antigen-antibody binding. In a human insulin ELISA, insulin in the sample is bound to specific antibodies immobilized on a solid surface (e.g., a microplate). After washing away unbound substances, a secondary antibody conjugated with an enzyme is added, which binds to the insulin-antibody complex. A detectable signal is produced by the enzyme in the presence of a substrate, allowing quantification of insulin levels.

The ELISA assay is performed by adding the samples to the microplate wells coated with insulin-specific antibodies. After incubation and washing steps to remove unbound substances, the enzyme-conjugated secondary antibody is added. Following another incubation and washing step, a substrate solution is added, leading to the development of a colorimetric or fluorescent signal proportional to the amount of insulin present.

The absorbance or fluorescence of each well is measured at wavelength 450nm using a microplate reader. The signal generated is compared to a standard curve constructed using known concentrations of insulin standards as shown in Figure 5.4, allowing the quantification of insulin levels in the samples.

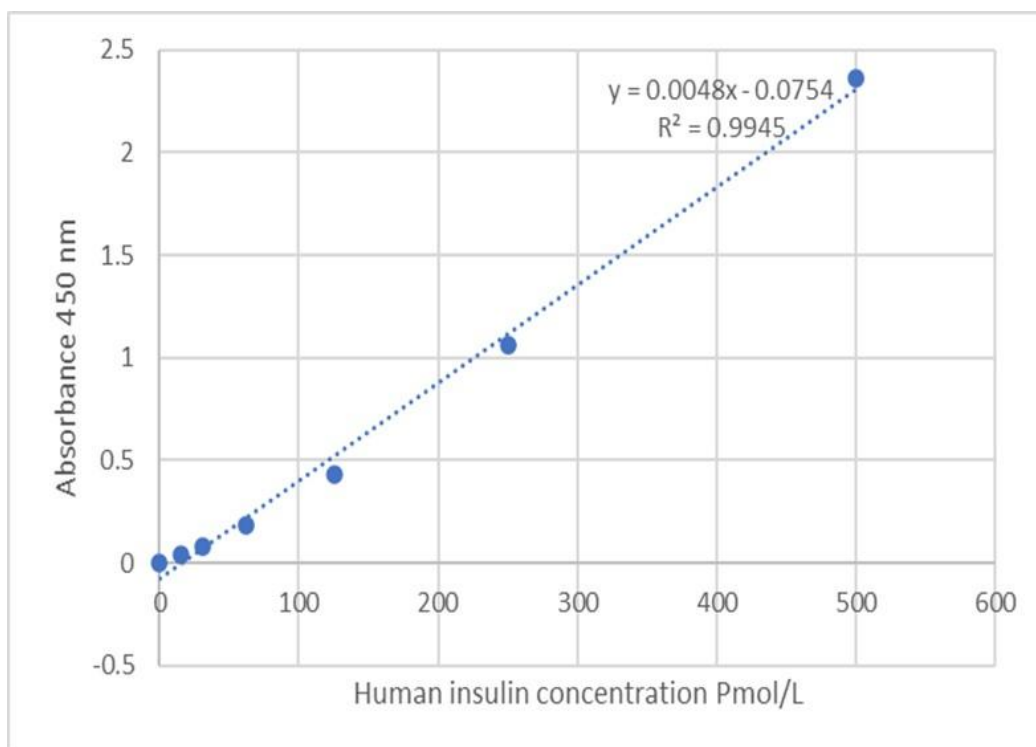


Figure 5.3: A Standard Curve of Human Insulin ELISA

The variability observed in the insulinotropic effects of frog skin secretions from *P. baramica*, and *H. erythraea*, underscores the complexity of their impact on insulin release.

This variability could stem from several factors. Firstly, different cell types may exhibit varying sensitivities and responses to insulintropic peptides due to differences in their receptor expression profiles, signaling pathways, and physiological characteristics. For example, HIT-T15 cells, which are insulinoma-derived pancreatic beta cell lines, may respond differently to insulintropic peptides compared to other cell lines or primary beta cells.

Moreover, the structure of the insulintropic peptides themselves may play a significant role in their variability of action. Peptides with diverse amino acid sequences, charge distributions, and secondary structures may interact differently with cellular receptors or membrane components, leading to distinct signaling cascades and insulin release patterns. Additionally, post-translational modifications or variations in peptide conformation could further influence their activity and efficacy in stimulating insulin secretion. Previous studies, such as those conducted by (Gagat et al., 2024), have demonstrated the importance of specific structural features, such as cationicity and amphipathicity, in enhancing the potency and effectiveness of peptides in influencing insulin release.

These findings suggest that modifications to peptide structures, such as amino acid substitutions, can alter their insulintropic properties. In addition, experimental conditions such as peptide concentration, incubation time, and culture media composition can significantly impact cellular responses to insulintropic peptides (Holst et al., 2020, Chu et al., 2015). Variations in these parameters across different studies could contribute to inconsistencies in the observed effects on insulin release. Additionally, factors such as cell culture passage number, and cell density, may also influence cellular physiology and peptide responsiveness.

Insulin secretion from pancreatic beta cells involves a complex process that integrates and interacts with multiple external and internal stimuli. This process includes steps such as GLUT-2 mediated transport into beta cells, metabolism of ATP, closure of KATP channels, and Ca^{2+} influx, ultimately leading to exocytosis (Ashcroft, 2023). Temporin and esculentin-1 Ranid frog peptides have been shown to exert insulintropic effects in various cell models, including BRIN-BD11 cells, human clonal β -cells, and isolated mouse islets (Musale et al., 2019).

The differential effects of temporin and esculentin-1 peptides on membrane potential and intracellular calcium concentration in BRIN-BD11 cells suggest distinct mechanisms of action. Esculentin-1 peptides appear to modulate insulin release through the KATP channel-dependent pathway (Ashcroft, 2023). whereas temporin peptides may act through a KATP channel-independent mechanism (Zhang et al 2021). These observations highlight the complexity of peptide-mediated regulation of insulin secretion and warrant further investigation into their specific molecular targets and signaling pathways.

the collective findings discussed in this study provide valuable insights into the potential of frog skin secretions to modulate insulin release. The variability observed among different frog species and their secretions underscores the need for further exploration to fully understand their insulintropic effects and therapeutic implications in diabetes management. This research sets the stage for future investigations into the molecular mechanisms underlying the insulin-regulating properties of frog skin peptides and their potential application as novel therapeutic agents.

CHAPTER 6

CONCLUSION

6.1 General Conclusion

Analysis of the skin secretions from three species of Bornean frogs, *P. baramica*, and *H. erythraea*, belonging to the family Ranidae, showed promising antimicrobial activities and insulintropic activity. The most potent peptides in this current study were secretions isolated from *P. baramica* frogs, with MIC = 250 µg/mL against *E. coli* and MIC = 500 µg/mL against *S. aureus*, along with insulin-releasing activity of 11.1 ± 5.58 . The expressions of antimicrobial and insulintropic activities from frog skin secretions have been demonstrated in many studies, and the current findings are therefore consistent with the activities reported. The antimicrobial and insulintropic results obtained prove that the peptides from Bornean frogs may have the potential to be developed as therapeutic agents to overcome antibiotic resistance and diabetic crises.

One aspect of this relationship between the antimicrobial and insulintropic activity lies in the structural and functional similarities between certain antimicrobial peptides (AMPs) and peptides involved in glucose metabolism. Some frog peptides exhibit both antimicrobial and antidiabetic properties due to their ability to interact with microbial membranes (Guryanova et al., 2022). and cellular receptors involved in insulin signaling pathways (Musale et al., 2021). For example, peptides with amphipathic structures, commonly found in frog skin secretions, can disrupt bacterial membranes while also modulating insulin secretion or enhancing insulin sensitivity in target cells.

Moreover, the peptides delineated in this investigation exhibited molecular weights spanning from 1.0 to 3 kDa. The AKTA Pure purification system demonstrated its efficacy in purifying these peptides, yielding clearer bands on SDS gel analysis. It is widely recognized that peptides with low molecular weights hold promise for the development of novel antimicrobial agents. However, the correlation between antibacterial activity and peptide structure, particularly in terms of hydrophobicity, remained unexplored in this study. Hence, there is a pressing need to expand the scope of this research to furnish additional data and insights into the structure-function relationship among the peptides.

At concentrations exceeding 50 µg/mL, nearly all peptides displayed a reduction in red blood cell viability, indicating that although some antimicrobial peptides (AMPs) exhibit toxicity, many possess moderate levels of toxicity at concentrations effective against bacteria. This presents a significant hurdle in harnessing frog AMPs for therapeutic purposes.

6.2 Challenges in Developing Frog AMP as Therapeutic Potential

Despite the effectiveness of these AMPs as therapeutic peptides, there is a significant drawback for their usage in the clinical applications. Most, although not all, AMPs are reported to cause cell toxicity at a dose effective to kill bacteria. The low toxicity AMPs include Pseudin-2 (Kong et al., 2024), Temporin-A (Simmons, et al., 2023). and Esculentin-1 (Albuquerque et al., 2023). that demonstrated low haemolytic activity towards mammalian cells. Hence, it is not safe to be administered into the human body to treat diseases but the topical applications of the AMPs were considered on the other hand, pseudin-2 and temporin-A and Esculentin-1 exhibit insulin releasing activities and showed low B cell cytotoxicity which is finding application in Type 2 diabetes treatment (Musale et al., 2018). For example, peptide antibiotics such as Polymyxin-B and Gramicidin are safe and effective when applied

topically but are too toxic at clinically used doses to be utilized systemically as anti-bactericidal agents (Marr et al., 2006). Conlon and Sonnevand (2011) agreed that peptides applied to infected skin or skin lesions in the form of spray or ointments can penetrate the stratum corneum to effectively kill microorganisms. This suggests that future therapeutic applications using peptide-based agents are more likely to involve topical rather than systemic applications.

Pexiganan, an analog of Magainin-2, emerged as the sole peptide derived from anurans to undergo clinical studies in 1999 (Gordon & Romanonski, 2015). The application of topical Pexiganan acetate and Floxacin at 800 mg per day in 835 diabetic patients with infected ulcers demonstrated significant improvement, with 90 percent of the participants showing positive outcomes by the end of the therapy. However, FDA approval was later denied due to the lack of substantial advantages over existing conventional antibiotics. Consequently, there have been no planned trials to further develop amphibian-based peptides since then.

However, the activity of magainin-AM2 suggests the potential for exploiting this peptide in the treatment of type 2 diabetes. It has been shown to improve glucose tolerance, enhance insulin sensitivity, and augment islet beta cell secretory responsiveness in mice with obesity-diabetes (Ojo et al., 2015). This indicates a promising avenue for research into the therapeutic potential of amphibian peptides in addressing metabolic disorders such as diabetes. Furthermore, the cost of production of AMPs are expensive for daily use and this was not convenience in underdeveloped countries. For instance, the cost for currently known AMPs were ranging from 50 to 400 dollar per gram (Marr et al., 2006).

Another arguable reasons to use AMP as therapeutic agent is the shelf-life of a peptide. Peptide is known to be vulnerable to enzyme A peptide needs to effectively inhibit the growth of pathogen before enzymatic breakdown occurred in plasma. Strategies such as substitution of peptide fragments susceptible to proteolytic degradation and modifications of N-terminus may be employed to increase the lifespan of peptide in plasma (Tao et al, 2023).. To sum up, AMP must possess superior properties and profound activities to be clinically used. Such properties include the ability to inhibit bacteria at lower concentrations than the conventional antibiotics, considering the cost of production of AMP is higher.

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APPENDICES

Appendix A: Equipment Used

ÄKTA pure™ CHROMATOGRAPHY SYSTEMS

ÄKTA pure™ is a flexible and intuitive chromatography system (Fig 1) for fast purification of proteins, peptides, and nucleic acids from microgram levels to tens of grams of target product. ÄKTA pure chromatography system is a reliable system where hardware and UNICORN™ software are designed to work together with columns and chromatography resins to meet purification challenges.

We offer two versions of ÄKTA pure chromatography system: ÄKTA pure 25, designed for a broad range of research applications and purification tasks in a multiuser environment; and ÄKTA pure 150, created for optimizing resource utilization and productivity in routine large-scale preparative purification. The system supports a wide range of chromatography techniques and meets automation requirements needed to deliver high purity. You can add on to the system at any time with a wide range of options to further increase its capabilities depending on your purification needs.

ÄKTA pure chromatography system is the product of over 50 years of expertise in protein research and three decades of experience in the development of ÄKTA™ purification systems.

ÄKTA pure chromatography system provides you with the following benefits:

- Modular system design with a large range of options to allow flexibility in purification of proteins and peptides
- Intuitive and flexible method creation, system control, and evaluation with UNICORN software
- Practical size, for easy placement on laboratory bench or in cold cabinet
- Reliable system with components and integrated features based on the proven design of ÄKTA protein purification systems
- Predefined method settings for all our laboratory-scale chromatography columns

System overview

ÄKTA pure chromatography system is a highly versatile, modular system with a number of design features to facilitate reliable purification.

The system consists of the ÄKTA pure instrument and UNICORN software. The system is modular in design with all valves, monitors, and columns mounted on the forward-facing



Fig 1. ÄKTA pure is a flexible chromatography system for the reliable purification of proteins, peptides, and nucleic acids at laboratory scale.

wet side of the system. The design allows easy interaction with the instrument modules (Fig 2). Additional components such as valves, monitors, and sensors from the wide range of optional modules can easily be added to the available positions. Multiple rails for attachment of column holders and equipment are located at the front and on the side of the instrument. A buffer tray on the top of the instrument provides a large storage area for vessels and bottles. The instrument control panel shows the system state and allows the possibility to interact with the run (pause/continue) at the touch of a button.

The system weighs 48 kg in basic configuration and 53 kg when fully equipped. The relatively low weight enables easier placement in the laboratory. The system dimensions allow it to fit conveniently into a standard cold cabinet for work with labile samples.

Regardless of configuration, ÄKTA pure chromatography system always comes with two high-performance system pumps, system pressure monitor for column protection, mixer, injection valve, and UV monitor. ÄKTA pure chromatography system has a wide range of optional modules to allow a large number of possibilities. The system flow path is designed to minimize band-broadening effects, and all wetted materials used in the flow path are biocompatible and resistant to commonly used solvents. The instrument front is designed with empty module positions where optional valves and monitors can be mounted to enable a flexible configuration of the flow path. Examples of two ÄKTA pure system configurations are shown in Figure 2.

SpectraMax iD3 Multi-Mode Microplate Reader

Unparalleled performance on a personalized platform

KEY FEATURES

- Use the touchscreen to easily set up methods, run experiments or view tutorial videos
- Personalize workflows with one-tap near-field communication (NFC) functionality
- Push data to workstations, eliminating the need to retrieve data directly from the instrument
- Validate your instrument and software with our extensive suite of tools

The SpectraMax® iD3 Multi-Mode Microplate Reader is the cornerstone of a complete laboratory solution to help you expand the boundaries of your research capabilities. With optimized reagents and the industry-leading data acquisition and analysis tool, SoftMax® Pro 7 Software, the SpectraMax iD3 allows you to customize your workflow to perfectly match your needs.

A microplate reader as personalized as your research

Built-in near-field communication (NFC) functionality in the SpectraMax iD3 reader enables you to pull up your custom protocols with a single tap, saving you precious time better spent on your research.

It features a large, high-resolution touchscreen interface with an embedded software package allowing you to set up custom protocols, take advantage of pre-loaded protocols, and run your experiment without the need for a dedicated computer workstation.

A complete solution to answer all your research needs

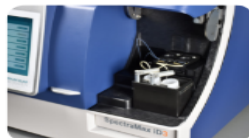
The SpectraMax iD3 reader measures absorbance, fluorescence, and luminescence. The superior optical system includes a xenon flash lamp and features an ultra-cooled photomultiplier tube (PMT) that reduces background noise for excellent sensitivity and a wide dynamic range.

Featuring temperature control up to 65°C, orbital and infinity shaking, a four-monochromator optical pathway with high efficiency gratings, well scanning up to a 20x20 read matrix, spectral scanning and detection of plate formats from 6- to 384-wells, the SpectraMax iD3 reader is the complete solution for all your research needs.



Capture flash assays with ease

Expand your lab's capabilities to include flash applications such as dual luciferase and ATP assays with the SpectraMax iD3 injector system with SmartInject™ Technology ensuring equal mixing across the plate for high-precision experiments. Engineered for high performance, the dual injector system can be used with any read mode and includes safeguard features such as overflow protection to save precious reagents and maximize performance for those critical experiments in your lab.



Quick initialization time allows you to start your experiments in seconds.



NFC allows you to personalize workflows with one tap.



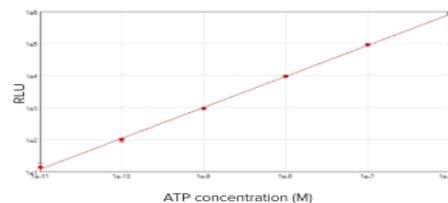
Easy-to-use touchscreen interface allows you to easily set up your experiments, use preconfigured analysis methods, or view tutorial videos.



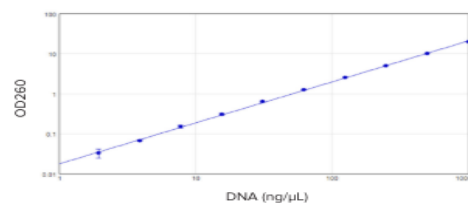
Network connectivity allows you to run multiple instruments from one workstation. Data is automatically delivered to workstations, eliminating the need to retrieve data directly from the instrument.

Reliable performance with a proven track record

For nearly 30 years, Molecular Devices has provided scientists with tools to expand the boundaries of their research. Our microplate readers are the industry's most cited instruments and have empowered life science researchers to advance protein and cell biology, breaking the barriers to novel, landmark discoveries. The SpectraMax iD3 reader is built on the same foundation that has made our entire SpectraMax microplate reader product line among the most trusted in the industry.



An ATP standard curve spanning five decades was run using the ATPlite 1step Luminescence Assay System (PerkinElmer) on the SpectraMax iD3 reader. Standard concentrations ranged from 1x10⁻¹¹ M to 1x10⁻⁶ M. A wide linear dynamic range ensures accurate assay results across a broad span of sample types. Standards were plotted using a log-log curve fit in SoftMax Pro Software.



The SpectraMax iD3 reader is fully compatible with the SpectraDrop™ Micro-Volume Microplate, enabling quantitation of precious low-volume samples. 4-μL DNA standards from 2 ng/μL to 1000 ng/μL were read in absorbance detection mode with a preconfigured protocol in SoftMax Pro Software. Performance matching the 2 ng/μL sensitivity specification is demonstrated here.

The data you need anywhere you want it

Reduce the amount of time you spend collecting data and increase your productivity. The SpectraMax iD3 reader allows you to interact with your data any way you want from anywhere you want. View your data quickly using the large touchscreen interface, export your data to a USB drive for analysis in the program of your choice, or analyze your data using the industry's leading data acquisition

and analysis tool, SoftMax Pro 7 Software. The SpectraMax iD3 also features network connectivity that allows you to walk away from the instrument to focus on additional research. Data is automatically delivered to any workstation on the same network, eliminating the need to physically retrieve data from the instrument.

View data on the large touchscreen for quick reading of results



Export data to a USB drive for analysis in the program of your choice



Automatically send data to any workstation on the same network



Use SoftMax Pro 7 Software for advanced data acquisition and analysis



SoftMax Pro GxP Compliance Software

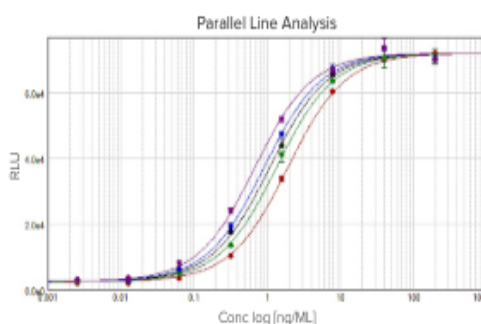
SoftMax Pro GxP Compliance Software extends Molecular Devices leading data acquisition and analysis solution into regulated laboratories working under GMP, GLP, 21 CFR Part 11, and other similar guidelines for secure electronic records.

Secure, traceable electronic recordkeeping

- Controlled user access through a granular permission structure and unique logins
- Electronic signature support for verification, authorization, and approval
- Audit trails to document the history of user actions for each data file
- Local and remote administration of user accounts for straightforward deployment

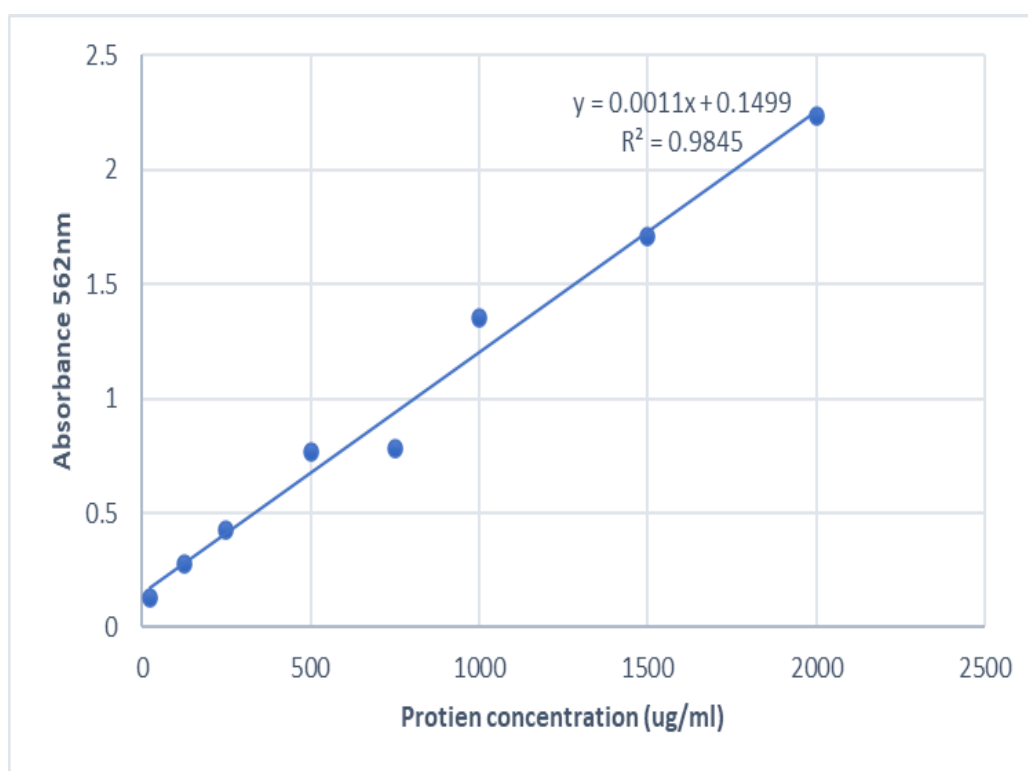
Save time and reduce cost

- Extensive suite of tools available for validation can reduce the cost and time of validation by 50% as compared to using multiple platforms to collect and analyze data
- Provides end-to-end chain of custody from capture through analysis to validation of data
- Validation tools for PLA, 4-P and 5-P curve fits
- Ready-to-use data for OQ confirmation tests
- Printable IQ/OQ documents for GLP/GMP paper trail



Appendix B: Supplementary Results

Standard curve for determination of protein concentration using BCA assay. The thin line represent a linear gradient and absorbance was read at 562nm against the concentrations of bovine serum albumin (BSA) ranging from 0-2500 µg/mL .



Appendix C: Conference and ExPo

Winning the gold award in the Intex competition for this research study(i) ,Research study poster for the Intex competition



Winning the 1st prize winner in the HMHS open day research (Laboratory based research category)



Anurans Treasure in Combating Diabetes Mellitus

Problem Statement:
Diabetes: "A Growing Global Challenge"

- Diabetes is rapidly becoming more prevalent, posing a significant threat to individuals and societies globally.
- Health Impact: Diabetes poses a significant health challenge, leading to elevated blood sugar levels that can result in various complications.
- Healthcare Strain: The prevalence of diabetes strains healthcare systems due to increased medical visits, medications, and treatments.
- Economic Consequences: Diabetes exerts a financial burden on economies, stemming from healthcare costs and reduced productivity.

How to Solve the Problem:
Frog Peptides: "Transforming Diabetes Treatment"

Natural Blood Sugar Regulation: Frog peptides offer a natural way to help regulate blood sugar levels in individuals with diabetes, potentially reducing the reliance on synthetic medications.

Enhanced Insulin Sensitivity: These peptides have shown the ability to increase insulin sensitivity, improving the body's response to insulin and promoting better glucose utilization.

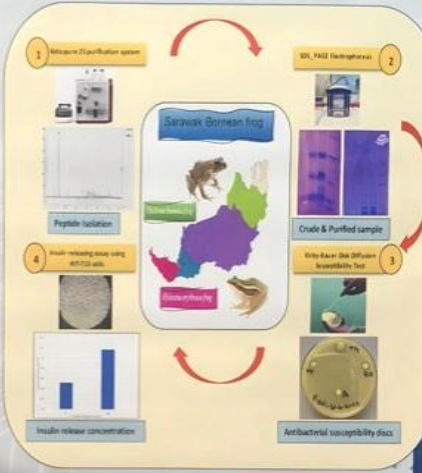
Novelty:
"Sarawak's Exclusive Frog Wonders"
The novelty of this research resides in the dual potential of these exceptional amphibians:

- Capability of antibacterial prowess.
- Ability to trigger insulin production.

Research Grant:
Special Top Down Grant (TOS/SP1DG/1769/2018)
Universiti Malaysia Sarawak

Intellectual Property:
IP application accepted by UNIMAS RIEC.
Application ID:82922 Pending MyIPO

Team Members
(Faculty of Medicine and Health Sciences)
AP,Dr.Muna BI Sabri
Amara Abdullah
AP,Dr.Zunika binti Amit
Prof,Dr.Ramlah binti Zainudin



Commercial Potential:

- Frog skin secretion-derived peptides offer potential for new antidiabetic drugs, diversifying treatment options.
- Introducing innovative antidiabetic products could tap into new market segments seeking alternative diabetes management.

Selected Publication:

SABRI, MUNA, ELIZABETH JEGA, JENGQUT, RAMLIAN ZAINUDIN, and ZUNIKA AMT. "Antimicrobial activity of partially purified peptides isolated from the skin secretions of Bornean frogs in the family of Ranidae." *Malaysian Applied Biology*, 47 (2) (2018).

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Community-Driven University For A Sustainable World