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Review on antimicrobial peptides from Malaysian amphibian resources: status, research approaches and ways forward

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Abstract: Antimicrobial resistance (AMR) is a significant threat to human health globally. Diseases caused by AMR are getting more challenging to cure due to the increasing rate of resistance of microbial pathogens. Drugs formulated with antimicrobial peptide (AMP) can be applied to treat these diseases. AMPs are found naturally within organisms, especially amphibians, and is related to the high adaptability of defence mechanisms against various pathogens and predators in the environment. This review focuses on the current status of research works and the different approaches applied to stimulate and collect amphibian secretions, extract and identify peptides, and conduct antimicrobial assays. The concerns of AMR include human health, animal health, economy, and agriculture. This section provides an overview of the potential uses of AMPs from Malaysian amphibians, other than their antibacterial and antifungal properties. The exploration of AMP on other amphibian species and the recommended steps for protein/AMP profiling via mass spectrometry are also included.

Key words: Anura, antimicrobial resistance, antiviral, secretion, foam nest, mass spectrometry, protein profiling

1. Introduction

A staggering 10 million mortalities are estimated to occur annually from antimicrobial resistance (AMR) by 2050 (World Health Organization, 2018a). The World Health Organisation (WHO) reported that at least 500,000 people had been infected by antibiotic-resistant bacteria across 22 countries (World Health Organization, 2018b). These common resistant bacteria include *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pneumonia* and *Salmonella* spp. AMR has been a severe issue in both human health and to the economic system since 1970. One of the earliest warnings regarding AMR was reported by Alexander Fleming in 1945 (Fleming, 1945). According to the WHO, the AMR-related deadly diseases are pneumonia, tuberculosis, gonorrhoea, and salmonellosis. These diseases are increasing in frequency and are becoming more challenging to treat. AMR occurs naturally, in the wild. Nevertheless, the rate of these resistance has significantly increased due to the misuse of antibiotics. AMR's concerns other than human health include animal health, the clinical economy, and the socioeconomy (Barriere, 2014; Malik and Bhattacharyya, 2019). Hence, there is an urgent need for novel drug/medicine/antibiotic discoveries to combat AMR.

Antimicrobial peptides (AMPs), also known as host defence peptides have broad-spectrum activity against microbes, including bacteria, fungi, parasites and some viruses (Bahar, 2013; Sang and Blecha, 2018; Conlon et al., 2019). These bioactive peptides represent a specific segment of proteins that have a positive outcome and function to our health (Rollins-Smith, 2001). Different species possess AMPs with different AMP properties as their defence mechanism. These organisms include plants, microorganisms, humans, and animals (König et al., 2015; Dahham et al., 2016; Chai et al., 2019; Conlon et al., 2019). The first AMPs isolated and fully revealed from an animal were cecropins isolated from the Native American moth (*Hyalophora cecropia*) (Steiner et al., 1981). Expression of these AMPs was due to duplication of an ancestral gene, and each corresponding gene (multigene family) originated from a common ancestor of the AMP family (Wang et al., 2008; Conlon and Sonnevend, 2011). AMPs are generally short molecules consisting of 10–100 amino acids that are positively charged ranging (+2 to +11), due to the presence of positively charged amino acids such as arginine, lysine and histidine. These amino acids are also amphiphilic and hydrophobic (generally 50%) (Yeaman and Yount, 2003; Hancock and Sahl, 2006; Niyonsaba et

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al., 2006; Pasupuleti et al., 2011). Most AMPs are effective against AMR microbes due to their cationic (positive charge) and hydrophobic features (Lu et al., 2008; Galdiero et al., 2013; Let et al., 2019), which permits them to bind selectively, penetrate and interact with the surface of negatively charged pathogen membranes to kill them (Lu et al., 2008; Galdiero et al., 2013; Let et al., 2019). The binding of these pathogens eventually lead to non-enzymatic disruption (Lu et al., 2008).

Amphibians (class: Amphibia) have among the highest diversity of vertebrates. AmphibiaWeb (2020)[1], reported 8160 species of amphibians as of May 2020; with the highest number in the order: Anura (frogs and toads), which represents 7204 species from 54 families. Anurans can be identified by certain physical characteristics such asshort and stocky body, lack of a tail, long, muscular hind legs, short forearms, large bulging eyes and a very wide mouth (O'Shea and Halliday, 2002; Inger et al., 2017). The diversity of amphibians is due to their high adaptability to different environments and habitats. Amphibians are dispersed worldwide, except for the North and South Poles and several small islands[1]. Amphibian species tend to congregate in the Neotropical, Panamanian, Oriental and Afro tropical areas, due to its consistently warm and humid climate all year round[1]. According to Adalberto et al. (2016), tropical regions occupy approximately 40% of the Earth's surface and are located within the latitudes of 23.5° N and 23.5° S. These optimal conditions give rise to the diversity of amphibian species.

There are 36 biodiversity hotspots, including Sundaland that comprise south-eastern Asia- Malay Peninsula, Borneo Island, Java Island, Sumatra Island, as well as smaller islands surrounding the region (Critical Ecosystem Partnership Fund, 2020)[2]. The Oriental/Asia region has the highest diversity of amphibians (1021) after the Neotropical area/South America (2231) and Panamanian/southern portions of North America (1098)[1] (Jenkins et al., 2013; Pimm et al., 2014). Thus, there is a higher probability for new AMPs to be discovered in the Asian region. With the rapid decline/extinction of these species, AMPs are being lost before being isolated and identified by researchers (Calderon et al., 2012).

Most AMP studies have been performed on amphibians because they have a higher exposure to microorganisms and pathogens from the environment (Clarke, 1997). According to Calderonet al. (2012), anuran AMPs can be correlated with each species' habitat and ecology because different species live in different habitats. Consequently, various feeding and behavioural adaptations and defences against pathogens, parasite, and predators of these species may contribute to the variations in the AMPs they produce (Nicolas and Mor, 1995; Clarke, 1997; Simmaco et al., 1998). Frogs' skin secretions are their first line of defence against penetrating infectious microorganisms (Hancock and Diamond, 2000; Schadich, 2009). Pressure from rapidly evolving pathogenic microorganisms has led to the diversity of AMPs in amphibians, as these pathogens have evolved to their changing environments (Wang et al., 2007). Each AMP differs in sequence, size, charge, hydrophobicity, tri-dimensional structure, mode and spectrum of action (Tossi, 2003; Pirtskhalava et al., 2016). According to the AMP database synthesised by Wang et al. (2016), 1092 active peptides have been identified from amphibians. These AMPs were mostly found in Ascaphidae, Bombinatoridae, Hylidae, Hyperoliidae, Leptodactylidae, Myobatrachidae, Pipidae and Ranidae families (Conlon, 2011).

Most AMPs are synthesised and stored within the secretory glands, commonly known as the poison glands in anurans' skin. However, AMPs can also be found within the stomach, gastric mucus, blood, internal organs, eggs, tadpoles and foam nests (Moore et al., 1991; Duellman and Trueb, 1994; Nicolas and Mor, 1995; Bullet et al., 2004; Conlon et al., 2004; Wells, 2007; Han et al., 2008; Shahrudin et al., 2017). Toxic secretions that contain AMPs and proactive substances are expelled onto the skin surface when the animal is injured, stressed or threatened (Simmaco et al., 1998). These bioactive substances include biogenic amines and neuroactive peptides, which act as hormones, neurotransmitters, and neuromodulators (Bevins and Zasloff, 1990; Erspamer, 1994). Skin secretions are often used to screen AMPs. The advantages of using skin secretions are: 1) the method is noninvasive; 2) can be collected regularly from the same individual; 3) their applicability during fieldwork; 4) minimal/no contamination and 5) the ability to generate cDNA libraries (Simmaco et al., 1998). The secretions can be obtained through both chemical and physical stimulations (Lai et al., 2002a). Chemical stimulation is the use of adrenergic agents such as ether, chloroform, epinephrine or norepinephrine which can be introduced to the animals by air exposure/direct exposure or immersion (Bevins and Zasloff, 1990; Simmaco et al., 1998; Conlon et al., 2008; Al-Ghaferi et al., 2010; Lai, 2010). Meanwhile, the physical intervention can be accomplished via low voltage electrical stimulation (Lai, 2010; Dahham et al., 2016).

The first AMP (caerulein) was isolated by Erspamer in 1968, followed by bombinin (a 24-residue peptide) in 1969, which possesses antimicrobial and haemolytic activity (Anastasi et al., 1968; Csordas and Michl, 1970). Marginins from *Xenopus laevis* (Pipidae), was the first AMPs to be completely characterised in 1987 and

[1] AmphibiaWeb (2020). University of California [online]. Website https://amphibiaweb.org [accessed 7 January 2020].

[2] Critical Ecosystem Partnership Fund (2020). Website https://www.cepf.net/ [accessed 6 Jun 2020].

reported to have cytotoxic activities against mammalian cells (Csordas and Michl, 1970; Giovannini et al., 1987; Zasloff, 1987). Since then, the identification and discovery of AMPs from other amphibian species have been widely explored by various researchers, particularly AMPs from Hylidae, Pipidae, and Ranidae families (König et al., 2015; Wang et al., 2016; Conlon et al., 2019). At least 15 well-studied AMP families, have been identified in Ranidae, luding brevinin-1, brevinin-2, esculentin-1, esculentin-2, japonicin-1, japonicin-2, nigrocin-2, palustrin-1, palustrin-2, palustrin-3, ranacyclin, ranalexin, ranatuerin-1, ranatuerin-2, and temporin (Zhou et al., 2006; Wang et al., 2009; Lai, 2010; Ma et al., 2010; Paddock, 2011; Conlon et al., 2014; Ciocan-Cartita et al., 2019). Protein profiling is an essential tool for biomarkers, drug discovery, vaccine development and the elucidation of evolutionary histories and phylogenetic relationships between species (Barra and Simaco, 1995; Yang and Huang, 2007). Amphibian AMPs have 9–50 positively charged amino acids, ranging from +1 to +6 and are amphipathic, hydrophobic molecules (Rinaldi et al., 2002; Powers and Hancock, 2003; Boland and Separovic, 2006; Xiao et al., 2011).

2. Studies of AMPs in Malaysian amphibian species

There are 266 species of amphibians in Malaysia up to March 2016 (Inger et al., 2017; Norhayati, 2017). However, the number of amphibian species recorded has increased to 289 (Frost, 2020)[3], as shown in Table 1. However, the exact number of amphibian species is uncertain, as new species are being discovered every year (Inger et al., 2017). These amphibians can be found everywhere from Mount Kinabalu's highlands (Sabah) to saltwater mangrove swamps. Malaysia holds a high diversity of amphibians; however, only a few studies have explored the potential antimicrobial activity within these species (Table 2). These studies included two toad species and several frog species from peninsular Malaysia, several more from East Malaysia (Sarawak), and three Malaysian frog species from other countries (extralimital areas). Examples of the samples studied among Malaysian amphibians are shown in Figure 1.

Table 1. The number of amphibian species in Malaysia (Norhayati, 2017; Frost, 2020)[3].

Family	Number of species
Bufonidae	48
Ceratobatrachidae	2
Dicroglossidae	36
Megophryidae	40
Microhylidae	48
Ranidae	41
Rhacophoridae	61
Ichthyopiidae	12
Total species of Anura	276
Total species of Amphibia	288

[3] Frost D (2020). Amphibia Species of the World 6.0, an Online Reference 2020 [online]. Website http://research.amnh.org/herpetology/amphibia/index [accessed 8 January 2020].

The skin secretions of *Phrynoidis asper*, the Asian Giant Toad, have been demonstrated to contain more than 50 types of proteins with a broad-spectrum antimicrobial effect against gram-positive and gram-negative bacteria, including *Staphylococcus aureus* and *Bacillus subtilis* (Dahham et al., 2016). Parotoid secretions of the common Sunda toad (*Duttaphrynus melanostictus*) have weak inhibitory activity towards microbes (Zahri et al., 2015). Conlon et al. (2008) revealed a structural characterisation of skin secretions of *Odorrana hosii* and *Hylarana picturata* from Malaysia that contained eight AMPs. These AMPs belong to the esculentin-1, esculentin-2, brevinin-1, brevinin-2, and nigrocin-2 families. A preliminary study from various frog species from peninsular Malaysian frog skin secretions by Rahman et al. (2016) demonstrated the antimicrobial effects against gram-negative and gram-positive bacteria; unfortunately, the frog species were not specified. Partially purified peptides from East Malaysian (Sarawak) frog species' skin secretions showed that some of the frog species contain AMPs and some are more potent than others (Sabri et al., 2018; Shahabuddin et al., 2018). Brevinin-2 has been identified from protein profiling of a foam nest from *Polypedates leucomystax* (Shahrudin et al., 2017)

At least five studies have been performed on extralimital frog species that can also be found in Malaysia (Table 2) (Lu et al., 2008; Song et al., 2009; Al-Ghaferi et al., 2010; Wang et al., 2012; Suhyana et al., 2015). Skin secretions of *Limnonectes kuhlii,* Kuhl's wart frog, from China revealed that the five novel AMPs thatwere purified and characterised showed strong antimicrobial effects against gram-positive and gram-negative bacteria and fungi (Wang et al., 2012a). The five novel AMPs were temporin-LK1, rugosin-LK1, rugosin-LK2, gaegurin-LK1, and gaegurin-LK2 (Wang et al., 2012a). According to Al-Ghaferi et al. (2010), structural characterisation of *Hylarana erythraea* skin secretions from Vietnam showed AMPs found from the brevinin-1, brevinin-2, esculentin-2, and temporin families. This species' skin secretions also revealed that they are generally active against gram-positive and gram-negative bacteria, particularly *S. aureus* and *E. coli* (Al-Ghaferi et al., 2010). Two studies

Table 2. List of AMP studies done on Malaysian amphibian species.

No.	Family	Species name	Locality	Source	Reference
1	Bufonidae	*Phrynoidis asper*	Peninsular Malaysia	skin secretion	Dahham et al. (2016)
2	Bufonidae	*Duttaphrynus melanostictus*	Peninsular Malaysia	paratoid secretion	Zahri et al. (2015)
3	Ranidae	*Odorrana hosii*	Peninsular Malaysia; East Malaysia	skin secretion; skin	Conlon et al. (2008); Sabri et al. (2018); Shahabuddin et al. (2018)
4	Ranidae	*Pulchrana picturata*	Peninsular Malaysia	skin secretion	Conlon et al. (2008)
5	Ranidae	*Pulchrana glandulosa*	East Malaysia	skin secretion	Sabri et al. (2018)
6	Ranidae	*Pulchrana signata*	East Malaysia	skin secretion	Sabri et al. (2018)
7	Ranidae	*Pulchrana baramica*	East Malaysia	skin secretion	Sabri et al. (2018)
8	Ranidae	*Chalcorana raniceps*	East Malaysia	skin secretion	Sabri et al., 2018); Shahabuddin et al. (2018)
9	Ranidae	*Meristogenys jerboa*	East Malaysia	skin secretion	Sabri et al. (2018); Shahabuddin et al. (2018)
10	Ranidae	*Staurois guttatus*	East Malaysia	skin	Shahabuddin et al. (2018)
11	Ranidae	*Hylarana erythraea*	Vietnam	skin secretion	Al-Ghaferi et al. (2010)
12	Dicroglossidae	*Limnonectes leporinus*	East Malaysia	skin	Shahabuddin et al. (2018)
13	Dicroglossidae	*Limnonectes kuhlii*	East Malaysia; China	skin secretion; skin	Shahabuddin et al. (2018); Wang et al. (2012)
14	Dicroglossidae	*Fejervarya cancrivora*	China	skin secretion	Lu et al. (2008); Song et al. (2009)
15	Dicroglossidae	*Fejervarya limnocharis*	Indonesia	skin secretion	Suhyana et al. (2015)
16	Rhacophoridae	*Polypedates leucomystax*	Peninsular Malaysia	foam nest	Shahrudin et al. (2017)

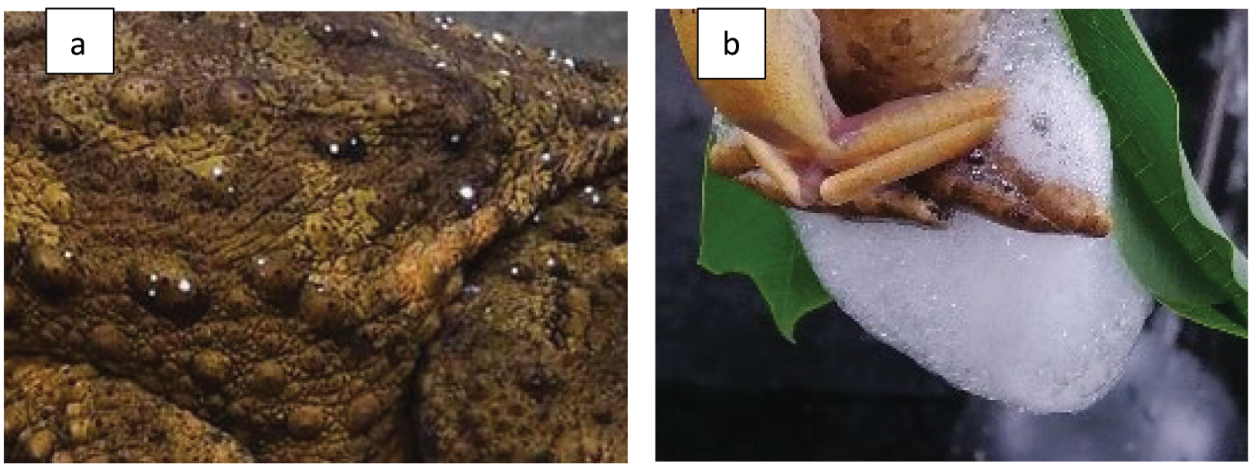

Figure 1. Examples of the type of samples studied among Malaysian amphibians; a) skin and skin secretion, and b) foam nest.

on AMPs of *Fejervarya cancrivora* from China disclosed that this species contains tigerinin-like peptides and cancrin (Lu et al., 2008; Song et al., 2009). Indonesian frog AMPs from *Fejervarya limnocharis* suppress the growth of *S. pneumoniae* multidrug-resistant strain SPN1307 (Suhyana et al, 2015).

3. Different approaches to discover the AMPs in Malaysian amphibian species

The methodologies to identify amphibian AMPs include stimulation and collection of secretions, extraction, identification of the peptides and an antimicrobial assay. Many approaches had been considered to study the AMPs from Malaysian amphibian species. A summary of the different approaches is presented in Figure 2.

3.1. Stimulating and collecting secretions

Hormonal stimulation is the most utilised method to collect skin secretions. The skin is an endocrine organ that exhibits hormonal activity. With the introduction of hormone or chemical stimulant, the skin produces positive feedback towards skin secretion production. This type of stimulation is animal-friendly and does not have a long term effect on the individual. Dahham et al. (2016) used a minimal electrical voltage to introduce stress in *P. asper* (Asian Giant Toad) to stimulate skin secretions. Amphibians produce skin secretions when they feel threatened/stressed (Hancock and Sahl, 2006).

Zahri et al. (2015) and Shahrudin et al. (2017) directly collected secretions without stimulation because parotoid secretions and foam nests do not need any stimulant for collection. These secretions were collected either by immersing the animals in a low concentration, salt solution (sodium chloride or sodium acetate) or directly rinsing them with deionised water. Centrifugation is essential to sort out these secretions from any contaminants (small organic particles). All of these secretions were frozen and lyophilised. The lyophilisation process is essential to remove water from the secretions for long-term storage and for the convenience of transporting the samples to another place (Shukla, 2011; Khairnar et al., 2013).

3.2. Peptide extraction

An extraction buffer was the most common method utilised to extract and purify peptides in previous studies. These extraction buffers can either be phosphate (Lu et al., 2008; Song et al., 2009; Wang et al., 2012a; Suhyana et al., 2015) or Tris-HCl (Dahham et al., 2016; Shahrudin et al., 2017). The extraction buffer acts as a lysis buffer that improves the peptides' stability, as they can easily be denatured, damaged or lost during the extraction process. The samples are fractionated either by gel permeation chromatography, solid-phase extraction or gel separation. The fractionation process refers to the isolation and separation process of samples into smaller fractions (quantities) from their original conformation. These smaller fractions increase the sensitivity of peptide identification. All samples are finally analysed by reversed-phase high-performance liquid chromatography (RP-HPLC) before identification of the peptide, except in Suhyana et al. (2015) and Shahriza et al. (2017). RP-HPLC is utilised to separate small fraction samples according to their polarity.

3.3. Peptide identification

Identification and structural characterisation of AMPs are essential to specify their properties. Only two studies have identified AMPs; one focused on AMPs of skin secretions and the other on AMPs from foam nests (Conlon et al., 2008; Shahrudin et al., 2017). A protein profiling analysis of *P. asper* skin secretions revealed more than 50 proteins, and some were undiscovered in the available database (Dahham et al., 2016). Thus, there is a high potential for discovering AMPs in other skin secretions and egg nests (foam and gel) of other amphibian species. However, in East Malaysia (Sarawak), only partially purified crude peptides have been extracted from frog specimens. These peptides have high potential as AMPs due to their similar molecular weight ranges with previous studies on amphibian AMPs. There is a high chance to uncover new AMPs of amphibian species in East Malaysia (Sabah and Sarawak) as there is a higher species diversity in East Malaysia (Sabah and Sarawak compared to Peninsular Malaysia, 266 vs 183, as of March 2016 (Inger et al., 2017; Norhayati, 2017).

According to previous studies, mass spectrometry-based protein profiling is more reliable because proteins are very complex biomolecules (Tyers and Mann, 2003; Kislinger and Emili, 2005). Mass spectrometry refers to an analytical technique that measures the mass to charge (m/z) ratio of ions from a particular compound/mixture/solution. Most studies that have identified proteins (AMPs) used matrix-assisted laser desorption/ionisation- time-of-flight- mass spectrometry (MALDI-TOF-MS) instead of liquid chromatography-tandem mass spectrometry (LC-MS/MS). This is arguablydue to the cost factor, which makes MALDI-TOF-MS more widely available. Furthermore, before the introduction of newer LC-MS/MS technologies, such as Orbitrap and SWATH, MALDI-TOF-MS was the instrument of choice for protein and peptide analyses. Nevertheless, the downside of an MS/MS system is its inability to distinguish between leucine and isoleucine residues. This problem can be solved by performing genetic comparisons (cDNA) of the host by Edman degradation, as previously done by Wang et al. (2012).

3.4. Antimicrobial assay

The general steps taken for antimicrobial assays are microbial selection, microbial growth/culture,

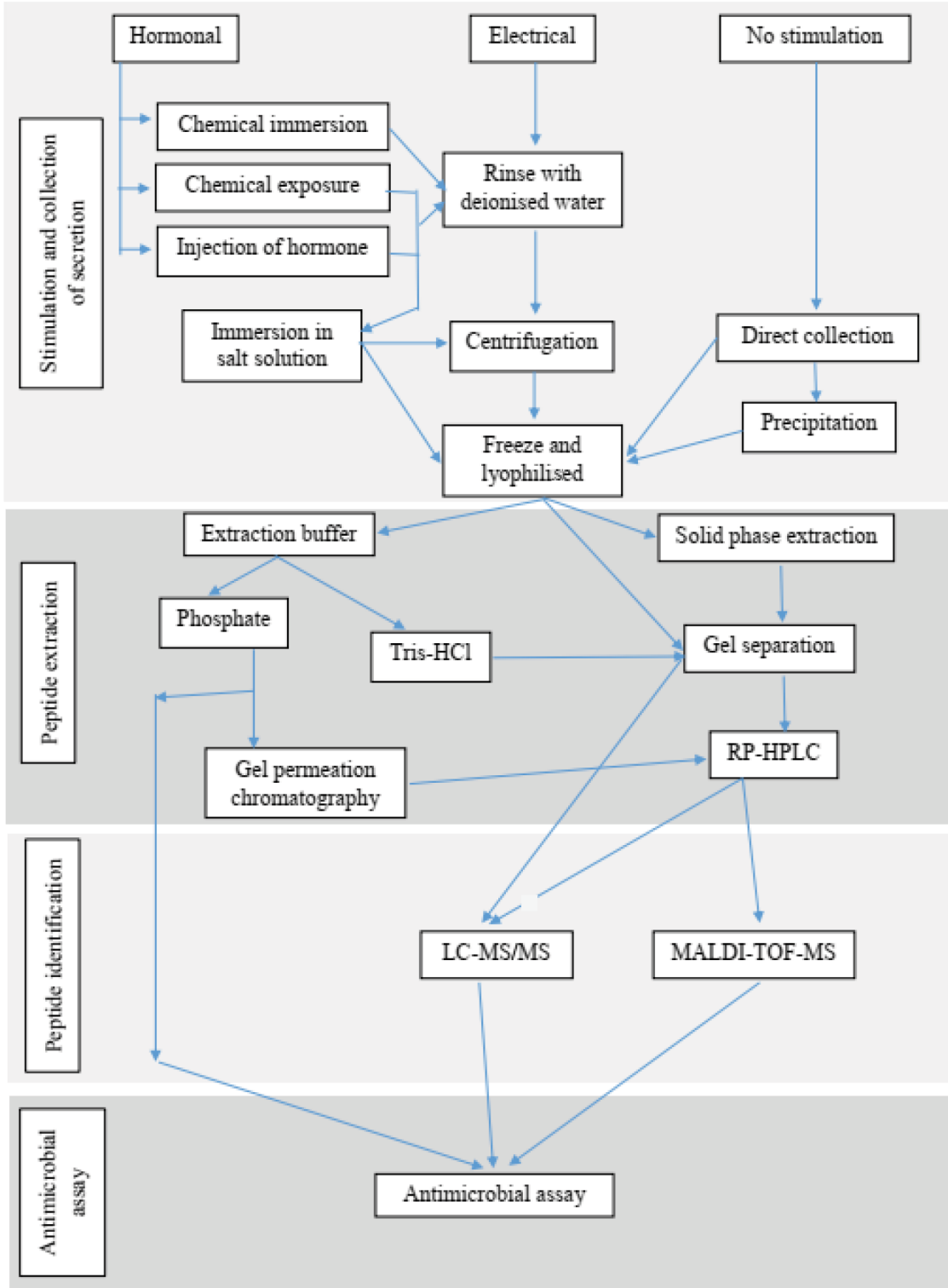

Figure 2. The workflow of various methods applied to stimulation and collection of secretions, extraction, identification and assay of antimicrobial peptides from Malaysian amphibian species. HCl, hydrochloric acid; RP-HPLC, reversed-phase high-performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption/ionisation -time-of-flight- mass spectrometry.

microdilution/series dilution of AMP solution, and AMP solution incubation into a microbial plate (depending on different concentrations), followed by antimicrobial evaluation tests. The antimicrobial assays in most studies were performed against common bacteria and fungus, which include *E. coli, S. aureus, Candida albicans* (yeast), Methicillin-resistant *Staphylococcus aureus* (MRSA), *Bacillus cereus, Bacillus subtilis, Shigella dysenteriae, Pseudomonas aeruginosa* and *Salmonella typhimurium* (Conlon et al., 2008; Wang et al., 2009; Al-Ghaferi et al., 2010; Dahham et al., 2016; Sabri et al., 2018; Shahabuddin et al., 2018).

These assays were evaluated by disc diffusion, minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC) tests. The MIC is the lowest concentration that an antimicrobial agent is bacteriostatic (Andrews, 2001). In comparison,MBC indicates the lowest concentration needed for an antimicrobial agent to completely inhibit (kill) a bacterium over a fixed time and under a specific set of conditions (Andrews, 2001). A range for both MIC and MBC determinations (mg/L) is needed to establish the antimicrobial agent's bactericidal effect. In this case, the antimicrobial agent refers to the antimicrobial peptide solution.

However, identification of the peptides in their samples indicated the presence of AMPs which have a high potential for inhibiting microbes, especially for resistant strains. Several antimicrobial studies have been performed on the peptides of Malaysian frog species found in other countries, namely *Hylarana erythraea* from Vietnam and *Limnonectes kuhlii* and *Fejervarya cancrivora* from China (Lu et al., 2008; Al-Ghaferi et al., 2010; Song et al., 2009). These studies indicated that the relationship between AMPs and their antimicrobial activities could be justified for further investigations.

4. Ways forward

The increase in cases and mortality from the coronavirus disease (COVID-19) pandemic and a few other viral diseases such as Ebola, dengue, influenza A, and other corona virus-respiratory diseases such as the Middle East respiratory syndrome coronavirus and severe acute respiratory syndrome (SARS), has put the world in an urgent state to discover new antiviral drugs. According to the WHO, the most frequent viral disease cases reported globally are influenza virus diseases, which include H5N1 (avian influenza), H1N1 (swine flu), H7N9 (Asian lineage avian influenza A), the common flu and the US seasonal flu Dengue virus disease was ranked first in Malaysia, with the most cases and fatalities among Malaysians in the past decade.

Several studies have revealed the peptides secreted by amphibians have antiviral properties. For example, Holthausen et al. (2016; 2017) discovered that the urumin antimicrobial peptide is virucidal for the H1 hemagglutinin-bearing human influenza A virus and may potentially contribute to a cure during an influenza outbreak. Other antiviral properties that have been found in amphibian AMPs include anti-Dengue (HS-1 and dermaseptin 01), anti-Herpes (magainins I and II, modified brevinin-1, temporin B, temporin-SHa (SHa) and its synthetic analogue [K^3] SHa, human cathelicidin LL-37) and anti-HIV (maximin 3, caerin 1.1, caerin 1.9, and maculatin 1.1) (Aboudy et al., 1994; Yasin et al., 2000; Lai et al., 2002b; Chinchar et al., 2004; Clara et al., 2004; Van Compernolle et al., 2005; Wang, 2012; de Souza Cardoso et al., 2013; Van Compernolle et al., 2015; Monteiro et al., 2018; Roy et al., 2019).

Pneumonia was the second highest (12.7%) cause of death within Malaysia in 2017, after coronary heart disease (13.9%) and is the leading cause of death (21.9%) within 64 districts in Malaysia making pneumonia the most potent killer among antimicrobial resistance-linked diseases (The Star Online, 2019)[4]. The AMR bacteria responsible for pneumonia include *A. baumannii, S. pneumonia* and *K. pneumonia*. According to the National Antibiotic Resistance Surveillance Report (NSAR) (2017), *S. pneumonia* showed increased resistance rates towards erythromycin from 33.9% in 2013 to 35.7% in 2014. Resistance decreased to 33.2% in 2015 due to antibiotics' potency (National Antibiotic Resistance Surveillance Report, 2017). Notwithstanding, in 2017, resistance fluctuated to 36% compared to 33.2% in 2015, because of their constant mutating ability. The resistance trend was also seen for penicillin G and cotrimoxazole (National Antibiotic Resistance Surveillance Report, 2017). The high resistance to erythromycin is not surprising as it is the most common and widely used antibiotic to treat bacterial infections.

A. baumanii had shown a higher resistance rate against all antibiotics except polymixin B in 2017 compared to 2016 (National Antibiotic Resistance Surveillance Report, 2017). Resistance towards carbapenem drugs (imipenem and meropenem) by *K. pneumoniae*, increased from 2.3% and 2.6% in 2016 to 2.7% and 2.9% in 2017, respectively. With the rapidly increasing rate of resistance, the urge to research, discover and develop new drugs had increased.

Some of the other important emerging AMR bacteria in human health include MRSA, vancomycin-resistant *Enterococcus*, carbapenem-resistant *Enterobacteriaceae* gut bacteria,and multidrug-resistant *Mycobacterium tuberculosis* (Department of

[4] The Star Online (2019, January 24). Do you know...about Malaysia's killer diseases? [online]. Website https://www.thestar.com.my/news/nation/2019/01/24/do-you-know-about-malaysia-a-killer-diseases [accessed 8 Jun 2020].

Health & Human Services, 2015)[5]. The challenge to overcome antibiotic resistance is becoming more difficult, as no new novel classes of antibiotics have been approved since 1987 (Roundtable, 2014)[6]. With the rapid emergence of new AMR microorganism strains, the need for new drugs to fight them intensifies. Moreover, AMP research should not be limited to common microorganisms that are vital for human health, but also to microorganisms that are vital for the well-being of livestock, agriculture and the environment. This will significantly affect the effort against AMR microorganism strains in every area of life, introducing the 'One Health' concept in research.

A lot has been achieved in the fight against AMR microbes (Mwangi et al., 2019). For examples, AMP from frogs, such as in *Phyllomedusa tarsius* and *Limnonectes fujianensis* inhibit the growth of MRSA by disrupting and destroying the membrane of this bacteria (Gao et al., 2017; Yuan et al., 2019). Among Malaysian anuran species, some AMP was effective against AMR microbes such as MRSA using MIC and MBC tests (Sabri et al., 2018; Shahabuddin et al., 2018). Furthermore, the works on the AMPs mechanism of actions have beenextensively described previously, as such by Raheem and Straus (2019).

Agriculture is an essential sector of the Malaysian economy, contributing 7.3% of the national gross domestic product in 2018. The importance of AMPs in this sector may be due to the concern of unprescribed antibiotic residues deposited in agricultural products. These antibiotic residues may cause an increase of AMR in pathogenic microorganisms, and impair human health. AMR in pets and livestock can affect humans primarily through zoonotic diseases, which can shift from animals to humans and vice versa. In Malaysia, some zoonoses include leptospirosis, rabies, influenza, Japanese encephalitis, toxoplasmosis, and monkey-pox.

Other properties of amphibian AMPs besides their antibacterial and antifungal properties include antiviral, antiinflammatory, antioxidant, antidiabetic, antileishmanial, antibiofilm and anticancer effects, which promote blood clotting during wound healing and suppresses the growth of chytrid fungus (frog killing disease) (Mangoni, 2006; Kumar et al., 2015; Demori et al., 2019). Studies of AMPs in Malaysian amphibians have only bactericidal and fungicidal aspects using common microorganisms. Consequently, there is a need to explore the research on each of the other aspects. There is a need for new drug discoveryto mitigate these new health problemsglobally. With the progress and technology of biomedicine, research on AMPs and AMR should be conducted efficiently. Discoveries of AMPs should also be made in other amphibian species, especially from Bufonidae, Ranidae, and Dicroglossidae families, as previous studies have shown a high level of AMPs, particularly in the common species. More studies need to be conducted on the secretions of egg nests (gel/foam), as these secretions' primary function is to protect the eggs.

Overall, for future work, we recommend a workflow involving hormonal stimulation, extraction in a suitable buffer, identification using LC-MS/MS and validation using antimicrobial assays. Hormonal stimulation is recommended to collect skin secretions. This stimulus acts as positive feedback within the frog's physiology, which produces more secretions with a minimal impact on the individual than other methods. The extraction buffer acts as a stabilising agent for the protein molecule within the secretion. Protein profiling data based on LC-MS/MS is more comprehensive as it is more sensitive and reliable than those collected by MALDI-TOF-MS (Kinslinger and Emili, 2005; Rathore et al., 2018; Ciocan-Cartita et al., 2019).

Moreover, the fractionation procedure before LC-MS/MS such as 2D-gel electrophoresis is arguably no longer necessary if robust LC is used (preferably nano-LC), thus reducing the processing steps and saves time. Finally, antimicrobial assays support and validate data that have been obtained from LC-MS/MS and in-silico analyses. Another essential method to identify AMPs is a comparison to the genetics of the host. This technique looks at the host's cDNAs from mRNAs released during glandular secretion (Wang et al., 2012b). It is hoped that more comprehensive and robust studies on Malaysian amphibians will produce reliable and multifunctional AMPs.

5. Conclusion

Malaysia is a diversity hotspot. Much work has been done to identify and characterise the AMPs from Malaysian amphibian species; however, it is far from complete. Of the 288 species, only 6% of Malaysian amphibian species had their secretions screened for AMPs. Most of the amphibians were from the family Ranidae, followed by Dicroglossidae. Hormonal/chemical stimulation is the best method to stimulate skin secretions from frogs, whereas LC-MS/MS is the most robust tool to identify these proteins and peptides.

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