Antimicrobial peptides from different plant sources: Isolation, characterisation, and purification

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ABSTRACT
Antimicrobial peptides (AMPs), the self-defence products of organisms, are extensively distributed in plants. They can be classified into several groups, including thionins, defensins, snakins, lipid transfer proteins, glycine-rich proteins, cyclotides and hevein-type proteins. AMPs can be extracted and isolated from different plants and plant organs such as stems, roots, seeds, flowers and leaves. They perform various physiological defensive mechanisms to eliminate viruses, bacteria, fungi and parasites, and so could be used as therapeutic and preservative agents. Research on AMPs has sought to obtain more detailed and reliable information regarding the selection of suitable plant sources and the use of appropriate isolation and purification techniques, as well as examining the mode of action of these peptides. Well-established AMP purification techniques currently used include salt precipitation methods, absorption-desorption, a combination of ion-exchange and reversed-phase C18 solid phase extraction, reversed-phase high-performance liquid chromatography (RP-HPLC), and the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) method. Beyond these traditional methods, this review aims to highlight new and different approaches to the selection, characterisation, isolation, purification, mode of action and bioactivity assessment of a range of AMPs collected from plant sources. The information gathered will be helpful in the search for novel AMPs distributed in the plant kingdom, as well as providing future directions for the further investigation of AMPs for possible use on humans.

1. Introduction

Antimicrobial peptides (AMPs), also known as host defense peptides (HDPs), constitute part of the innate immune system found in almost all classes of life including microorganisms, arthropods, plants and animals (Bulet et al., 1999; Carvalho and Gomes, 2009; Zasloff, 2002). These AMPs are potent, broad-spectrum antibiotics against pathogenic bacteria (Gram-negative and Gram-positive), fungi, enveloped viruses and other parasites (Reddy et al., 2004).

Plant AMPs are an abundant group of proteinaceous compounds produced in plants (Jenssen et al., 2006). The first reported plant AMP was purothionin from wheat (Triticum aestivum) (De Caleya et al., 1998). Several groups of plant AMPs with antimicrobial activity have since been identified, characterised and purified, including defensins, snakins, puroindolines, glycine-rich proteins, cyclotides, hevein-type proteins, and lipid transfer proteins (Pelegrini and Franco, 2005; Witkowska et al., 2007). Such AMPs have been isolated from different plants and plant organs such as the stem, root, seed, flower and leaf. They exhibit potent microbicidal activities against viruses, bacteria, fungi, parasites and protozoa (Nawrot et al., 2014). Plant AMPs have become important candidates for developing potential new techniques for controlling crop losses as well as novel antibiotics for treating various infections in humans (Pelegrini et al., 2008).

The adverse effects of chemical pesticides, the frequent emergence of drug-resistant bacteria and the failure of some traditional antibiotics have all led to an urgent search for new antimicrobial agents (Nordström and Malmsten, 2017). Although more than 5000 AMPs have been identified from different sources so far (Hu et al., 2016; Zhao et al., 2013), only just over 2400 AMPs have been deposited in the AMP Database (http://aps.unmc.edu/AMP/main.php), of which 343 are from...
Table 1
Main families of plant antimicrobial peptides with representative peptides and their sources.

<table>
<thead>
<tr>
<th>Family</th>
<th>Plant organ</th>
<th>Plant</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclotides:</td>
<td>Leaf &amp; flowers</td>
<td>Oldenlandia affinis</td>
<td>(Craik, 2001; Nawrot et al., 2014; Sellitrennikoff, 2001; Stec, 2006)</td>
</tr>
<tr>
<td>Impatients:</td>
<td>Seeds</td>
<td>Impatiens balsamina</td>
<td>(Stepek et al., 2005; Tailor et al., 1997)</td>
</tr>
<tr>
<td>β-AMPs</td>
<td>Seeds</td>
<td>Physioca americana, Mitisilis jalapa</td>
<td>(Marcus et al., 1999; Nawrot et al., 2014; Terras et al., 1995)</td>
</tr>
<tr>
<td>Lipid transfer proteins (LTPs): LTP1s and LTP2s</td>
<td>Seeds</td>
<td>Zea mays</td>
<td>(Nawrot et al., 2014; Pelegrini et al., 2007; Stec, 2006)</td>
</tr>
<tr>
<td>Defensins:</td>
<td>Seeds</td>
<td>Phaseolus vulgaris</td>
<td>(García-Olmedo et al., 1998; Mendez et al., 1990; Ramos et al., 2014)</td>
</tr>
<tr>
<td>Puroindolines:</td>
<td>Endosperm</td>
<td>Tricium aestrum</td>
<td>(Liu et al., 2000; Nawrot et al., 2014; Tailor et al., 1997)</td>
</tr>
<tr>
<td>PINA and PINB</td>
<td>Tubers</td>
<td>Solanum tuberosum</td>
<td>(Fujimura et al., 2003; Nawrot et al., 2014; Terras et al., 1995)</td>
</tr>
<tr>
<td>Thionesin:</td>
<td>Endosperm</td>
<td>Tricium aestrum</td>
<td>(De Caley et al., 1972; Nawrot et al., 2014)</td>
</tr>
<tr>
<td>ζ-1-purothionin</td>
<td>Seeds</td>
<td>Macadamia integrifolia</td>
<td>(Marcus et al., 1999)</td>
</tr>
<tr>
<td>Hevein-like AMPs:</td>
<td>Leaves</td>
<td>Brosssonetia papyrera syn. Morus papyrera</td>
<td>(Zhao et al., 2011)</td>
</tr>
<tr>
<td>PMAPI</td>
<td>Seeds</td>
<td>Cicer arietinum</td>
<td>(Ramos et al., 2014; Tailor et al., 1997; Ye et al., 2002)</td>
</tr>
<tr>
<td>Others:</td>
<td>Seeds</td>
<td>Cicer arietinum</td>
<td>(Rivillas-Acevedo and Soriano-García, 2007)</td>
</tr>
<tr>
<td>Ay-AMP</td>
<td>Seeds</td>
<td>Amaranthus hypochondriacus</td>
<td>(Rivillas-Acevedo and Soriano-García, 2007)</td>
</tr>
<tr>
<td>Cicerin</td>
<td>Seeds</td>
<td>Cicer arietinum</td>
<td>(Souza et al., 2011; Ye et al., 2002)</td>
</tr>
<tr>
<td>Shepherins</td>
<td>Roots</td>
<td>Capsella bursa-pastoria</td>
<td>(Marcus et al., 1999; Remuzgo et al., 2014)</td>
</tr>
<tr>
<td>Hispidulin</td>
<td>Seeds</td>
<td>Benincasa hispida</td>
<td>(Sharma et al., 2014)</td>
</tr>
<tr>
<td>Lunatustin</td>
<td>Seeds</td>
<td>Phaseolus lunatus</td>
<td>(Wong and Ng, 2005a)</td>
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<td>Peptidea</td>
<td>Seeds</td>
<td>Brassica napus</td>
<td>(Del Mar Yust et al., 2004)</td>
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<tr>
<td>Vulgarinian</td>
<td>Seeds</td>
<td>Phaseolus vulgaris</td>
<td>(Wong and Ng, 2005b)</td>
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plants (Liu et al., 2017). Many of these identified AMPs have not been tested in clinical trials, and a number of those that have reached the clinical phase have failed due to unacceptable toxicity or lack of efficacy.

There is thus a need to search for new AMPs to widen the range of AMPs potentially available for therapeutic and similar uses. However, variations in screening, identification and purification methods, as well as the wide variety of different plant organs and species involved, have made the discovery process complicated and time-consuming. A comprehensive study involving the isolation, characterisation and purification methods, as well as the wide variety of different plant organs and species involved, have made the discovery process complicated and time-consuming. A comprehensive study involving the isolation, characterisation and purification of AMPs together with an analysis of their antimicrobial activity and cytotoxicity should help to identify novel AMPs with improved antimicrobial activity and reduced cytotoxicity, thereby accelerating the application of new AMPs for clinical and agricultural uses (Zhao et al., 2013).

This study summarises and updates current information on the isolation, identification and purification strategies for AMPs from plant sources. In addition to reviewing existing methods, this study also looks at a wide range of possible options for obtaining novel AMPs from the plant kingdom. Above all, this review aims to provide insights to help guide researchers interested in the isolation, identification, evaluation and how to test antimicrobial activity of novel AMPs from plant sources.

2. Isolation of antimicrobial peptides from plants

2.1. Selection of plant materials

The immense biodiversity in plant species means that there is enormous scope to explore new prospective AMPs for drug development in human welfare and other potential applications in agriculture. However, that very diversity also means that enormous efforts are required to select, identify and characterise plant materials in the search for AMPs. The availability of morphological, physiological and molecular data on plant species has made them suitable targets for research into the collection of AMPs. Data derived from molecular techniques designed to characterise and identify genes responsible for AMP synthesis and regulation, including the in silico analysis of AMPs along with the gene products responsible for inhibiting pathogens, is required to validate the functional aspects of AMPs (Clara Pestana-Calsa et al., 2010). The increasing number of plant species being deposited in public databases, together with the rapid growth in information available on proteins, genomes, transcripts and other molecular data, has made plants attractive as potential sources of AMPs.

Plant species that exhibit a strict ecological relationship with a pathogen and produce AMPs in a symbiotic context might be particularly suitable for use as sustainable biological controlling agents for pests and pathogens (Údvary et al., 2011). The first identified plant-derived AMP was purothionin, which was found to be active against Xanthomonas phaseoli, Pseudomonas solanacearum, Erwinia amylovora, Corynebacterium flaccumfaciens, C. michiganense and C. fascians (De Caley et al., 1972). Several other plant AMPs have subsequently been discovered and characterised through a range of morphological, physiological and molecular analyses. The major AMPs collected from different plant species have been categorised into several groups, including defensins, cyclotides, lipid transfer proteins and thionins (Nawrot et al., 2014; Pelegrini et al., 2007; Stec, 2006; Údvary et al., 2011), as well as less common groups such as the impatins, puroindolines, vicilin-like, glycinine-rich, shepherins, snakins and heveins (Berrocal-Lobo et al., 2002; Fujimura et al., 2003; Liu et al., 2000; Marcus et al., 1999; Zottich et al., 2011).

AMPs have been isolated from whole plants, such as lunatins collected from Phaseolus lunatus L. (the Chinese lima bean), as well as from seeds such as vulgarinins from Phaseolus vulgaris (haricot beans), hispidulin from a medicinal plant (Benincasa hispida), and cicerin and arietin from Cicer arietinum (chickpea) (Rivillas-Acevedo and Soriano-García, 2007; Wong and Ng, 2005a; b). Other AMPs have been obtained from the thick cell walls of Spinacia oleracea cv. Matador (spinach leaves) (Segura et al., 1998). The names of known AMPs, plant species, and the related references are summarised in Table 1 below.
Plant AMPs have been isolated from different plants and plant organs, including the stems, roots, seeds, flowers and leaves from a wide variety of plant species (Montesinos, 2007). The number of AMPs isolated from plants is extremely high, with some plant species possessing hundreds of different AMPs or more. In sum, this wide range of plant organs and plant species represent a potentially huge source of AMPs which might be used as drugs or food preservatives.

2.2. Isolation and extraction of plant AMPs

The first step in obtaining AMPs is to perform a series of processes to extract and purify them from potential source plant materials. Although AMPs have been successfully isolated directly from crude plant extracts in several plant species (Kovaleva et al., 2009), fractionating compounds and isolating AMPs from plant materials generally require efficient and sophisticated techniques (Moreira et al., 2011). The extraction and purification of potential new AMPs, followed by sequencing and searching for similar AMPs in databases, is an effective way to identify novel AMPs, as described by Odintsova et al. (2009) (Fig. 1).

In fact, a number of AMPs have already been identified using the above techniques. For example, AMPs were isolated directly from crude plant extract followed by functional assays and characterisation from the plant in *Lippia sidoides* Cham. (rosemary pepper) flowers through Octyl-Sepharose hydrophobic column separation (Moreira et al., 2011). Similarly, an antiviral peptide (2 kDa) purified from sorghum (*Sorghum bicolor*) seeds by gel filtration, ion exchange and high-performance liquid chromatography showed potent inhibitory effects on the herpes simplex virus type 1 and bovine herpes virus (Camargo Filho et al., 2008). Two other AMPs with anti-yeast properties were isolated from the seeds of *Capsicum annuum* (pepper) and identified by amino acid sequencing (Ribeiro et al., 2011). Other AMPs isolated from *Sesamum indicum* (sesame) flour have been shown to exhibit anti-bacterial activity against *Klebsiella* sp. (Costa et al., 2007). The direct isolation of AMPs from plants has also been observed in *Crotalaria pallida* (the African legume), a widely dispersed weed in South America, producing a novel peptide structurally similar to defensin (Pelegrini et al., 2008). Some antibacterial and antifungal peptides (passiflin, Pg-AMP1, and 2S albumin-like) have been isolated from the seeds of *Psidium guajava* (guava) and *Passiflora edulis* (passion fruit) (Lam and Ng, 2009). Some studies have also reported the successful isolation of AMPs from common vegetables and spices, including *Pisum sativum* L. (garden pea).
Water is the most commonly used solvent to extract AMPs from dried plants such as teas, either by steeping the plants in hot water or boiling suspensions of the parts using steam. Dried plant parts can also be added to oils or petroleum jelly and poultices (concentrated teas or tinctures) (Brantner and Grein, 1994).

All of the above components extracted and identified from plants are active against microorganisms; most of them are aromatic or saturated organic compounds, often obtained through ethanol or methanol extraction (Zhang and Lewis, 1997). Different plant parts may contain active antimicrobial components, such as saponins in the root of Ginseng (Panax ginseng) plants, essential oils and tannins in Eucalyptus (Eucalyptus spp) leaves, and phenolic compounds in the bark, leaves and shoots of Balsam poplar (Populus balsamifera) (Cichewicz and Thorpe, 1996; Taylor et al., 1996).

In sum, research has shown that AMPs can be isolated and extracted from whole plants as well as from the seeds and flour of plant parts.

3. Purification and characterisation of antimicrobial peptides from plants

3.1. Concentrating the supernatant of AMPs

Following the extraction of AMP from plant materials as described in Section 2.2 above, it is necessary to purify and characterise the relevant antimicrobial peptide. Plant extract contains much lower levels of AMPs than other compounds (He et al., 2010), so it is essential to concentrate the AMP solution in order to recover the AMPs present. Many procedures have been developed to concentrate AMP containing supernatant, but two of the most common are ammonium sulphate precipitation and organic solvent extraction.

3.1.1. Ammonium sulphate precipitation

AMPs are short peptides, which can be concentrated through a salting-out method with ammonium sulphate as a common reagent. Solid ammonium sulphate or 75% ammonium sulphate is added to the extracted plant sample until the desired saturation level of ammonium sulphate is reached. For this, the hulled seeds or other relevant parts of a plant sample are homogenised with sodium acetate buffer and stirred overnight, then centrifuged. The supernatant is concentrated under reduced pressure and saturated with ammonium sulphate (Yokoyama et al., 2008). Sometimes AMPs are precipitated at lower ammonium sulphate concentrations (<70%). In such cases, it is essential to monitor and determine the right concentration of salt to isolate and concentrate the AMPs. The concentrated AMP suspension can then be desalted by dialysis against a phosphate buffer using benzoylated membranes or with dialysis cassettes (Pingitore et al., 2007; Yokoyama et al., 2008). Plant extract may also contain tannin, which needs to be eliminated before salt precipitation. To achieve this, plant extract is dissolved in water and stirred for 30 min, then centrifuged for 15 min at 3144 × g to remove insoluble polysaccharide excipients. The solution then needs to be dried down using a Genevac sample concentrator under reduced pressure at 30 °C, and re-suspended in 10 mg/mL in water and methanol (1:1 v/v). About 300 mg of polynonyl pyrrolidone is added, and the solution is stirred for 30 min and centrifuged. Finally, the supernatant is removed and dried down to yield de-tannized plant extracts (Miyasaki et al., 2013; Pingitore et al., 2007).

3.1.2. Organic solvent extraction

Organic solvent extraction from crude extract is a successful protocol for concentrating AMPs from free-cell supernatant that has been treated with polar solvents (acetone, methanol, etc.) stored at −30 °C overnight. The extracted AMPs are centrifuged to recover the peptide fraction in the pellet, and the suspension is subjected to ion exchange chromatography (Pingitore et al., 2007).

3.2. Purification and characterisation of AMPs

Ammonium sulphate precipitation and solvent extraction are generally used for the initial partial purification of plant AMPs. Such AMPs then need to be subjected to further purification using other methods, such as Ion exchange chromatography (IEXC), Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), MALDI-TOF MS/MS, 2D-gel electrophoresis, Reversed-phase (RP) HPLC and Mass Spectrometry (Fig. 1).

3.2.1. Ion exchange chromatography (IEXC)

Ion exchange chromatography (IEXC) can be used for both cation- and anion-charged AMPs that require separation by their electric charge at a defined pH. For this, the crude extract needs to be loaded onto a cellulose column, equilibrated with ammonium acetate, pH 5.2, and washed with buffer. The fractions are collected, re-suspended in acetic acid, lyophilized, and stored at −20 °C. Alternatively, crude extract can be dialysed into ammonium acetate, pH 5.2, using spectrophotometry tubing with exclusion and aliquots loaded onto a cation exchange column. In both processes, the plant extract is passed through the column and washed with phosphate buffer containing NaCl, and the eluted AMPs are collected (Duvick et al., 1992; Pingitore et al., 2007).

Ion exchange protein purification can be applied to purify plant AMPs because most plant AMPs contain non-zero net electrostatic charges at all pHs except the pH equal to pI (isoelectric point). When the pH is higher than its pI, an AMP becomes negatively charged (an anion), while at a pH lower than its pI the AMP becomes positively charged (a cation). Ion exchange chromatography works for two reasons: (i) the electrostatic attraction between buffer-dissolved charged AMPs; and (ii) the oppositely charged binding sites on a solid ion exchange adsorbent. In sum, the charge (both net and local), stability and conformation of the charged form of the buffer can all play an important role in the purification of plant AMPs by the ion exchange technique (Khan, 2012).

3.2.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Fractions containing AMPs can be analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), using 15% gel. After completing the electrophoresis, the gel is sensitised using a sodium thiosulphate (Na2S2O3) solution, followed by staining with silver nitrate at room temperature for 2 h. Sodium carbonate containing formalin in water can be used as the developer. Finally, the gel needs to be placed in a solution of acetic acid in water to stop the staining process (Schägger and von Jagow, 1987). An SDS-PAGE analysis helps to separate effective antibacterial substances which are peptides with a low molecular weight.

3.2.3. Reverse-phase (RP) HPLC

Reverse-phase high-performance liquid chromatography (RP-HPLC) is the most useful analytical technique for the purification of AMPs from plant and other sample sources. Plant AMPs are often resistant to various organic solvents used as mobile phases, making RP-HPLC a suitable option for the purification of plant AMPs (Dolezilova et al., 2015; Duvick et al., 1992). In this method, samples are lyophilized and dissolved in a MilliQ water solution of trifluoro-acetic acid (TFA), then separated using HPLC. HPLC is a frequently used semi-preparative technique. Elution of pure AMP fraction from a column can be carried out with a gradient of solvent or a solution with constant water-solvent composition. Previous studies have reported that the elution times of hydrophilic and hydrophobic fractions are 45 and 75 min respectively. The absorbance is monitored at 214 nm. The fractions can then be collected manually, reconstituted and lyophilized to obtain a final concentration in MilliQ water prior to analysis for antimicrobial activity (Dolezilova et al., 2015; Pingitore et al., 2007).

RP-HPLC is also useful for the separation and removal of plant
pigments before proceeding to purification. For example, an HPLC based on a reversed-phase C8 column and mobile phases containing pyridine was developed by Yacobi et al. (1996) for the simultaneous separation of plant pigments such as chlorophylls and carotenoids. The benefits of RP-HPLC in terms of speed and sensitivity as well as high resolution in separating plant pigments are particularly dependent on the type of column material used. Different reverse-phase columns, such as C8, C18, C18 monolithic, ρ-NAP and choleran, can be used for the separation of pigments at several specific states of the mobile phase as well as at different temperatures. Of the range of columns available, the choleran column is most suitable for separating not only hydrophobic pigments in a simple mobile phase but also pigments at a broad range of polarities (Shioi et al., 2015).

3.2.4. Circular dichroism spectral (CDS) analysis

Circular dichroism spectral (CDS) analysis is a useful way to predict the secondary structure of a purified protein. The purified AMP and buffer are subjected to both 250–350 nm (Near UV) and 190–250 nm (Far UV) light in order to obtain spectral scans. For the spectral measurement, a volume of 300 μl of Tris-HCl buffer and purified peptide is used, with Tris-HCl buffer as blank for the scan. Depending on the concentration of AMP, the antimicrobial protein can be diluted twof- or three-fold with Mili-Q water before measurement. CDS analysis can then be carried out using a CD Spectrolopearimeter to obtain the structural information of the purified AMP (Sakthivel and Palani, 2016).

3.2.5. 2-Dimensional gel electrophoretic analysis

2-D gel electrophoresis is another useful technique for analysing the secondary structure of purified protein. A native-PAGE electrophoresis is first carried out to obtain an initial separation of the protein. After that, the gel slice containing separated protein is incubated with SDS sample buffer, but without beta-mercaptoethanol, for 10–15mins. SDS-separated gel electrophoresis is then carried out for the second phase of protein separation. This analysis is particularly useful to verify whether the purified AMP is in a monomeric form or otherwise (Sakthivel and Palani, 2016).

3.2.6. Amino acid analysis

High performance liquid chromatography (HPLC) can also be used to carry out an amino acid analysis of purified AMPs. First, the purified peptide is subjected to chromatography separation with an HPLC column. Next, the amino acids eluted from the column are detected with a photodiode array detector at 280 nm. The amino acids are identified based on the retention times of their corresponding standards and the calibration curves obtained from with respective amino acid standards which have undergone the same process of derivatization as the AMP samples. The internal standard method is applied based on the areas of the peaks of the derivatives. Finally, the chromatograms for the amino acids from AMP samples, along with blank, are obtained. Amino acids sequence in AMP can also be further studied using a MALDI-TOF MS/MS analysis (Sakthivel and Palani, 2016) – described below.

3.2.7. MALDI-TOF MS/MS analysis

A matrix-assisted laser desorption-ionization time-of-flight tandem mass spectrometer (MALDI–TOF MS/MS) that uses a pulsed nitrogen laser is a useful tool to measure the molecular mass and peptide sequence of AMPs. A matrix solution containing saturated acid in acetonitrile is put on a sample plate, then air dried completely. Mass spectra are collected using a linear delayed extraction mode (DE mode) and positive ion mode (PI mode), with accelerated voltage, grid voltage, guide wire voltage, low mass gate, and laser intensity. The signals from the excitation pulses are accumulated and averaged to yield each recorded mass spectrum. The spectra are externally calibrated using matrix ion peaks as per international standards. The data obtained from the mass spectrometer are put through a database search (NCBI nonredundant/SwissProt) and analysed using software. A programme such as MASCOT (http://matrixscience.com) is useful for such analysis, as it allows up to one missed trypsin cleavage and a monoisotopic mass tolerance of 1.2 Da. The calibrated peptide masses are searched with 200 ppm mass accuracy. Blast2GO and UniProtKB software (http://uniprot.org) can then be used for functional classification. The similarity of mass spectra, tandem MS data and information on peptide sequences in the database are used in combination in order to detect AMPs with an appropriate molecular weight (Dos Santos et al., 2017; Sakthivel and Palani, 2016; Yokoyama et al., 2008).

3.3. Common properties of plant AMPs

Most plant AMPs are basic in nature, have a molecular weight between 2 and 10 kDa, contain 4 to 12 cysteine residues, and form disulphide bonds that give them structural and thermodynamic stability (García Olmedo et al., 2001). Plant AMPs are therefore generally classified based on their amino acid sequences and the number and position of cysteines forming disulphide bonds (de Souza Cândido et al., 2011; Marcus et al., 1997). These distinctive disulphide bond patterns determine their tertiary structure folding and thermal stability. Plant AMPs contain 2-6 intra-molecular disulphide bonds which bolster structural stability without affecting the general scaffold. The cross-bracing of multiple disulphide bonds in plant AMPs makes them structurally compact, with high thermal, chemical, and enzymatic stability. These properties of plant AMPs make them suitable for purification by warming, homogenizing, adjusting the pH, and separating the target protein/peptide from other components by chromatography, including affinity-based methods and salt precipitation (Naderi et al., 2015; Tam et al., 2015).

AMPs also display peptide promiscuity – meaning that a single peptide is able to exert multiple biological functions, often containing three domains (N- and C-terminal pro-domains and a mature AMP domain) (Tam et al., 2015). Plant AMPs also demonstrate antifungal and antibacterial activity; are active against oomycetes and herbivorous insects; exhibit enzyme inhibitory activities; have a heavy metal and abiotic stress tolerance; and display anticancer activity against different cancer cells (Allen et al., 2008; Koike et al., 2002; Kong et al., 2004). These unique characteristics and broad antimicrobial coverage make AMPs superior to synthetic or chemical antibiotics.

4. Antimicrobial activity of plant AMPs on targeted organisms

AMPs of plant origin can be tested against a variety of different microorganisms, including bacteria, fungi, viruses, protozoa and helminths. An initial assay for potential antimicrobial compounds derived from plants can be performed with either pure substances (Afolayan and Meyer, 1997) or crude extracts (Freiburghaus et al., 1996). Crude plant extracts demonstrating antimicrobial activities have been shown to be rich in plant AMPs. For example, crude Indian herbal extract (ethanolic extract) from Piper longum fruits has counteracted Entamoeba infections, while chloroform extract from the legume Milletia thonningii has been shown to prevent the establishment of Schistosoma mansoni infection on the skin of mice (Ghoshal et al., 1996; Sohni et al., 1995). Among other studies, various extracts of the bark of Pteleopsis suberosa against stomach ulcers caused by Helicobacter pylori, papaya latex against infections with the helminth Heligmosomoides polygyrus, and Santolina chamaecyparissus essential oil against candidal infections, have all been observed as promising (He et al., 2010; Suresh et al., 1997).

The procedures generally used by researchers for antiviral, antibacterial and antifungal tests on AMPs are described below.

4.1. Antiviral activity

Previous studies have reported that both RNA and DNA viruses can be controlled by antiviral AMPs (Horne et al., 2005). Antiviral AMPs
neutralise such viruses by incorporating themselves into the viral envelope or host cell membrane. This causes membrane instability and disruption. As a result, the viruses are unable to infect host cells (Robinson et al., 1998). AMPs such as defensins also can reduce the binding sites of viruses to their host cells; for example, by binding to viral glycoproteins and inhibiting the binding of herpes simplex viruses (HSV) to the surface of their host cells (Yasin et al., 2004). Some antiviral AMPs prevent the entry of the viral particles into their host cells by occupying specific receptors (Song et al., 2001). For example, defensins bind to viral glycoproteins, preventing HSV from binding to the surface of host cells and blocking virus-receptor interactions (Andersen et al., 2004; WuDunn and Spear, 1989). Some AMPs cannot compete with viral glycoproteins in binding to the heparan sulphate receptors on the cell surface, but can cross the cell membrane and localise in the cytoplasm. These types of AMPs can cause changes in the gene expression profile of the host cells and stimulate the host defence system to fight against viruses or modulate cellular pathways to block viral gene expression, thereby inhibiting viral replication. Both lunatustin and vulgarisin were shown to inhibit HIV-1 reverse transcriptase and HIV-1 gene expression in a cell-free rabbit reticulocyte lysate system, which suggests that antimicrobial activity might be linked to gene expression and protein synthesis (Wong and Ng, 2005a; b). In some cases, AMPs can also serve multiple roles to exert anti-viral effects. For instance, Malfromin A1, a cyclic penta-peptide containing one disulphide bond isolated from an endophytic fungus, has been found to strongly inhibit both infection and the replication of the Tobacco mosaic virus (TMV) in host plants (Tan et al., 2015).

Several methods can be used to evaluate the antiviral activity of plant AMPs. For viruses which form plaques in suitable cell systems, there are two basic approaches: plaque reduction assays and titer reduction assays. In plaque reduction assays, a constant number of viral particles are used together with a range of substance concentrations. The level of antiviral activity is determined by measuring the reduction in the size or number of plaques formed in a cell monolayer. Titer reduction assays, on the other hand, use a fixed amount of test substance but a varying number of viral particles. By serial dilution of the virus suspension to a concentration that renders a number of viral plaques small enough to be counted, an obvious lower virus titer (compared to the virus titer determined in the assays without the test substance) can be detected, showing antiviral activity (Abou-Karam and Shier, 1990).

For viruses that induce cytopathic effects but do not readily form plaques in cell cultures, inhibition assays of virus-induced cytopathic effect and virus yield reduction assays can be carried out. Antiviral activity is detected by determining a virus-induced cytopathic effect in cell monolayers cultured in liquid medium, infected with a limited dose of virus, and treated with a nontoxic dose of the test substance. By serial dilution of the viral suspension to a concentration that renders a number of viral plaques small enough to be counted, an obvious lower virus titer (compared to the virus titer determined in the assays without the test substance) can be detected, showing antiviral activity (Abou-Karam and Shier, 1990).

For viruses that do not induce cytopathic effects and do not form plaques in cell culture either, assays based on the measurement of specialised functions and viral products can be used. Antiviral activity is detected by determining virus specific parameters, such as the inhibition of cell transformation (Epstein-Barr virus), haemagglutination and hemadsorption tests (myxoviruses), and immunological tests detecting antiviral antigens in cell cultures (Epstein-Barr virus, HIV, HSV, cytomegalovirus). Antiviral activity can also be expressed as a reduction or inhibition of the synthesis of virus-specific product/poly-peptides in infected cell cultures, such as viral nucleic acids, determination of the uptake of radioactive isotope-labelled precursors, or viral genome copy numbers (Cowan, 1999; Lee et al., 1996; Paris et al., 1993; Vlietinck and Vanden Berge, 1991).

In sum, the common endpoints for antiviral activities are cytopathic effects, plaque formation, and transformation or proliferative effects on cell lines. Viral replication can be assayed by detecting viral products such as DNA, RNA or poly-peptides.

However, Vlietinck and Vanden Berge (1991) noted that the methods used for assaying antiviral substances in various laboratories were not standardised, and so the results from different such tests could not reliably be compared. They also pointed out that further research is needed to explain the differences between the merely toxic effects of agents on host cells and actual antiviral properties in plant AMPs (Esimone et al., 2005; Vlietinck and Vanden Berge, 1991). There is therefore a need to develop alternative techniques, such as in vitro pharmaco-dynamic screening. This type of method can contribute not only to the rapid screening of bioactive plant compounds, but also to the standardization and pharmacokinetic-pharmaco-dynamic profiling of bioactive components. Various reported gene-based antiviral screening assays have been shown to be flexible, rapid, unbiased and amendable to high throughput (Johansen et al., 2004; Kimpton and Emerman, 1992; Marschall et al., 2000; Profitt and Schindler, 1995). Esimone et al. (2005) reported using a vector-based assay technique for an in vitro pharmacodynamic evaluation of antiviral medicinal plants, while the cytotoxicity of the extracts was evaluated in parallel on target cell lines.

4.2. Antibacterial activity

Antibacterial AMPs are the most studied cationic AMPs. Many of them target the bacterial cell membranes and cause disintegration of the lipid bilayer structure (Shai, 2002). They are also amphipathic in nature, containing both hydrophilic and hydrophobic domains. Both domains provide the capability of binding to lipid (hydrophobic region) and phospholipid groups (hydrophilic region) (Brogden, 2005). Some AMPs can kill bacteria at low concentrations without affecting membrane integrity (Shai, 2002). They do so by inhibiting some important pathways inside the cell such as DNA replication and protein synthesis (Brodgen, 2005; Jensen et al., 2006; Le et al., 2017).

The most frequently used methods for screening AMPs to determine their antimicrobial susceptibility are the disc diffusion assay or agar well diffusion assay (Jenkins and Schuetz, 2012), and the agar and broth dilution assays (Citron and Goldstein, 2011; Jaskiewicz et al., 2016). For the disc diffusion as well as agar and broth dilution assays, standardised guidelines detailing microbial strains, culture media, incubation conditions, quality control procedures, and criteria for interpreting results have been published by organisations such as the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST).

The disc diffusion assay is the most common method used for antimicrobial susceptibility testing, largely due to its suitability for testing a wide range of aerobic and anaerobic microorganisms and anti-microbial compounds. It is also a simple experiment without the need for specialised equipment, and capable of testing a relatively large number of antimicrobial compounds at the same time. This method involves the inoculation of nutrient agar with a standardised suspension of the test organism, followed by the application of a filter paper disc containing the test compound on the surface of the agar plate. The agar plate is then incubated at the appropriate temperature and duration for the test organism. The antimicrobial agents diffuse through the agar to inhibit the growth of the bacteria, giving rise to a clear zone of inhibition for which there is no bacterial growth. The diameter of the zone of inhibition is then measured and reported in millimetres. It is generally recommended to perform quality control testing for each test.
organism using a disc containing a known antibiotic so as to ensure that the zone of inhibition falls within acceptable limits, before interpreting the results.

The agar well diffusion assay is a variant based on the disc diffusion assay. Wells of about 6–10 mm in diameter are formed on the inoculated nutrient agar plates using a sterile cork borer. The wells are then filled with a solution of the antimicrobial compound (100 μl). The plates are kept at room temperature for a short period of time to allow diffusion of the antimicrobial agents, before being incubated at suitable conditions for microbial growth.

The above two methods have been reported to produce generally consistent results with each other for certain bacterial species (Luangtongkum et al., 2007). Although agar diffusion methods are popular due to their low cost and simplicity, they do have some disadvantages, including difficulties in determining the minimum inhibitory concentrations (MICs) – defined as the minimum concentrations of antimicrobial agents required to inhibit the growth of the microorganism.

One good example of how to conduct antimicrobial tests using agar well diffusion tests was shown by Tadele et al. (2005). They carried out experiments to screen the antimicrobial activities of some traditional Ethiopian medicinal plants used in the treatment of various skin disorders through agar well diffusion assays at three concentration levels (100, 50 and 25 mg/mL) for crude extracts and at two concentration levels (25 and 5 mg/mL) for fractions. During this experiment, 80% methanol, 100% methanol, chloroform and distilled water were used as negative controls for bacteria and fungi. The results were the averages of triplicate tests, and the zones of inhibition included the diameters of the wells.

The agar and broth dilution tests (macrodilution or microdilution) are routinely used to determine the MICs of new antimicrobial agents (Wiegand et al., 2008). In the agar dilution assay, the solution containing the compound of interest is serially diluted at 10 × the intended test concentration and added to 9 parts of molten agar, before being poured into petri dishes to achieve an agar height of 3–4 mm. The standardised inoculum is then aliquoted onto the surface of the agar to give 10^4 CFU per 5–8 mm diameter spot using inoculation loops, micropipettes or inoculum replicators. Following incubation, the MIC is taken as the lowest concentration of antimicrobial agent that inhibits discernable microbial growth, often without taking into account the presence of a single remaining colony or a slight haze in the inoculated spot. A major advantage of this method is that it permits the simultaneous study of different bacterial strains on a single agar plate. In the broth dilution method, a series of dilutions (typically two-fold) are prepared. 1 mL is added into a test tube in the macrodilution method, while 0.1 mL is added into each well of a 96-well round bottom plate in the microdilution method. The standardised inoculum is added into the macrodilution test tubes or microdilution well plates, which are then incubated at approximately 35 °C for 16–20 h. The MIC is again regarded as the lowest concentration of antimicrobial agent that completely inhibits the growth of the test organism – determined either by viewing with the naked eye or by comparing spectrophotometric readings (O.D. 600 nm) before and after incubation.

Both the agar and broth dilution methods do have some limitations. These include the relatively laborious and expensive processes involved; also the fact that the end results can be greatly affected by experimental variables. As such, these experimental methods and the associated conditions must be tightly controlled to ensure that the results obtained are reproducible and can be interpreted accurately.

### 4.3. Antifungal activity

Antifungal AMPs can kill fungi by targeting the cell wall or intracellular components, while some antifungal AMPs can bind to chitin (major components of fungal cell walls) (Pushpanathan et al., 2012; Yokoyama et al., 2009). Such binding helps antifungal AMPs to target fungal cell walls and kill target cells by disrupting the integrity of fungal membranes (Terras et al., 1992; van der Weerden et al., 2010). Although antifungal AMPs have polar and neutral amino acids in their structures, there is no correlation between the structure of an antifungal AMP and the type of cells (Jiang et al., 2008; Lee et al., 2003).

As with antibacterial testing, agar diffusion and broth dilution are both commonly used methods for the evaluation of the antifungal activity of new compounds. The main difference lies in the type of culture medium or agar used as well as the incubation time. As a typical example, a fungal strain is removed from the stock and suspended in 2 mL of non-selective medium such as dextrose broth medium. This suspension needs to be uniformly spread on Petri plates containing dextrose agar media, using sterile swabs. Wells are formed by the same technique as for bacteria. The plates are incubated at 25 °C for 3 days in the case of Candida albicans and Aspergillus niger, and for 7 days in the case of Trichophyton mentagrophytes. The inhibition zones are measured and compared to a positive control (De Lucca et al., 1998; De Lucca and Walsh, 1999).

The broth macrodilution and microdilution methods have been used for the determination of MIC values in yeasts and moulds. Due to the consistency of results between the two methods and the fact that the microdilution method is easier to carry out than the macrodilution method, the former has been the preferred choice for many laboratories for use to determine antifungal susceptibilities.

For yeasts, the antifungal agent is prepared in water or a suitable solvent and serially diluted in a RPMI-1640 growth medium. One hundred microliters of each of the dilutions is then added in replicates to each well of a 96-well plate. The yeast strain is inoculated onto a nutritive agar medium such as Sabouraud dextrose agar or potato dextrose agar, and allowed to incubate at 35 °C. The inoculum is prepared by picking five representative colonies (≥ 1 mm) and suspending them in sterile saline or distilled water. The density of the suspension is then adjusted spectrophotometrically at 530 nm to match that of a 0.5 McFarland, giving an approximate concentration of 1–5 × 10^8 CFU/mL. An equal volume (0.1 mL) of the standardised inoculum (typically between 5 × 10^3 to 5 × 10^4 CFU/mL depending on the guideline being followed) is then added to each well. The microdilution plates are incubated at 35 °C without shaking, usually up to 72 h depending on the yeast strain. MIC determination is less clear cut than in the case of bacterial testing, being defined as the minimum concentration at which a certain degree of inhibition of growth occurs compared to the drug-free control. This is typically defined as ≥ 90% inhibition for amphotericin B and ≥ 50% inhibition for fluconazole, azoles, and echinocandins. A relevant control antifungal agent is therefore normally included to compare antifungal potency in the evaluation of new antifungal agents. The MIC can be determined either by viewing with the naked eye or spectrophotometrically, although the interpretation of results can be highly variable if only visual observations of turbidity are made.

To determine the MIC for moulds, the fungus is inoculated onto an appropriate agar medium and allowed to sporulate at 35 °C. Water supplemented with 0.1% Tween 20 is added to cover the colonies, and then a sterile swab is used to transfer the conidia to a sterile tube containing 5 mL of water with Tween 20. After vortexing to disperse clumps and removing a significant amount of any hyphae present by filtering through a 11 μm filter, the conidia is counted under a haemocytometer and diluted using sterile water to a working concentration of 2–5 × 10^5 conidia/mL. An equal volume of the conidia (0.1 mL) is then added to the serially diluted antifungal agent in the 96-well plate. The microdilution plates are incubated without agitation, typically for 24–72 h. The MIC is regarded as the lowest concentration at which no growth is observed by eye.

In addition to these assays, antifungal phytochemicals can be evaluated by a spore germination assay. This involves adding plant extracts to fungal spores collected from solid cultures, placing these on glass slides, and incubating them at an appropriate temperature (25 °C) for
24 h. The slides are fixed in lactophenol-cotton blue, and observed under the microscope for spore germination assessment (Rana et al., 1997).

4.4. Antiparasitic activity

Screening plant extracts against protozoa and helminths is more complicated than screening against bacteria, fungi or viruses, and the assays involved need to be specific to the microorganisms concerned (Freiburghaus et al., 1996). For example, two specific methods against Trypanosoma brucei were developed by Freiburghaus’s group to evaluate the trypanocidal effectiveness of compounds extracted from African medicinal plants (Freiburghaus et al., 1996). First, a 46-h cultured tissue of trypanosomes was exposed to various concentrations of extracts. Then the MICs to which the lowest concentration of extract capable of completely inhibiting the growth of the trypanosomes was determined with an inverted microscope. Second, a fluorescence assay was carried out to assess trypanosome viability in micro-titer wells. In this fluorescence assay, the lipophilic and methanolic plant extract as well as water extract was suspended in 10% dimethyl sulfoxide (DMSO), 10% Methanol, and filtered sterilised water respectively. Three-fold serial dilutions ranging from 500 to 0.07 μg/mL were performed on each extract, with complete culture medium in a 96-well microtiter plates (at least 2 times in duplicate). After 66 h of incubation at 37°C in a humidified incubator, the MIC of each extract was determined. For extracts that showed MIC values ≤ 56 μg/mL, a 100 μl of 4 μM BCECF-AM (2′,7′-bis(carboxyethyl)-5-(6-carboxyfluorescein-pacedoxy-methylester) was added to each well and the plate was further incubated for another 45 min before fluorescence units were read with a fluorescence plate reader at 485 nm and 530 nm wavelengths. The concentration of plant extracts that inhibited the growth of trypanosomes by 50% (IC50) was then calculated (Freiburghaus et al., 1996).

Plant AMPs which have anti-infective properties against the Plasmodium species are of interest to scientists. Parasitized erythrocytes are incubated both with and without test substances, and after the incubation period the numbers of Plasmodium organisms are quantified (François et al., 1996). In one study, ethanol extracts from nine different Bidens species were collected. Tests showed that seven of the nine extracts inhibited parasite growth in vitro by 65–91%. An HPLC analysis using a photo diode-array detector showed the presence of phenyl acetylene and flavonoids in the ethanol extract, while chloroform fractions caused 86% inhibition of parasite growth in vitro containing 1-phenyl-1,3-dimethyl-2-acetate (Brandão et al., 1997).

There are not many studies investigating the antiparasitic property of plant-derived AMPs. The first evidence for such antiparasitic (leishmanicidal) activity in plant-derived AMPs was presented by Berrocal-Lobo and co-researchers (2009). In their experiment, thionin, a barley lipid transfer protein derived from wheat and potato derived defensins, tested positive against Leishmania donovani (a human pathogen) at a low range of concentrations. Both AMPs managed to collapse ionic and pH gradients across this parasite plasma membrane. The mode of action of antiparasitic peptides is broadly the same as other AMPs: most kill cells directly by interacting with the cell membrane to form pores (Park et al., 2004).

Further proof of leishmanicidal activity in plant-derived AMPs has been published by a number of Brazilian researchers (Carvalho et al., 2006; Carvalho et al., 2001; dos Santos et al., 2010; Souza et al., 2013). They found evidence that a plant defensin (Vu-Defr), isolated from the seeds of Cowpea (Vigna unguiculata L. Walp), was able to negatively affect the growth of Leishmania amazonensis promastigotes. In addition to the cloning and overexpression of recombinant defensin from Cowpea seeds (Vu-Defr) in Escherichia coli, they also carried out various modifications and improvements to the expression and purification processes of Vu-Defr. They conducted experiments to compare the leishmanicidal activity of Vu-Defr to the natural defensin Vu-Defr and the results demonstrated that recombinant Vu-Defr was as biologically active as its counterpart, and retained its full level of biological activity after improved recombinant production and purification (Souza et al., 2013). These results are further evidence of the potential of plant-derived AMPs to be used as new antiparasitic agents.

Separately, in 2015, a group led by Gomes successfully isolated a plant AMP from the seeds of Perola (Phaseolus vulgaris), which they called PvD1, and which displayed inhibitory activity against protozoan L. amazonensis promastigotes (do Nascimento et al., 2015). In addition to evaluating the effect of PvD1 on the proliferation of this protozoan, they also characterised the antiparasitic mechanisms of this plant-derived AMP. Their results proved that the defensin PvD1 was able to cause membrane permeabilization, the formation of cytoplasmic vacuoles and cytoplasmic fragmentation, thus inhibiting the proliferation of L. amazonensis promastigotes cells. Interestingly, they also found the presence of this plant-derived AMP within L. amazonensis cells – which suggests that the toxicity of these peptides may not be restricted to the plasma membrane, but may also be acting on intracellular targets. This finding opens new perspectives on the antiparasitic mechanisms of plant-derived AMPs, offering new avenues for future research (do Nascimento et al., 2015).

5. Conclusions and prospects

Antimicrobial peptides (AMPs) of plant origin have a variety of amino acid compositions and structures, many of which exhibit potent broad spectrum antimicrobial activities and prove capable of killing microbes rapidly. Plant AMPs are thus a potentially valuable natural alternative to chemical antibiotics for use in both human healthcare and in agriculture to protect plants and animals from disease. Many transgenic plants have been developed to express AMPs that confer different degrees of protection against disease (Navrot et al., 2014). It has been shown that a number of transgenic plants containing AMPs can protect themselves from pathogen attack, such as the Mj-AMP1 (jalapa defensin) in tomato against Alternaria solani (Schaefer et al., 2005), Rs-AFP2 (radish defensin) in tobacco and tomato against Alternaria longipes (Terras et al., 1995), hevein Pn-AMP in tobacco against P. parasitica (Koo et al., 2002), and alfalfa defensin in potato for V. dahlieae (Gao et al., 2001). Similarly, thionins, cyclotides, protein transduction domains and cell-penetrating peptides are other important peptides for the development of transgenic crops. Genetic improvements in plants to increase their pathogenic resistance and reduce crop losses in agriculture could eventually lead to a reduction in pesticide use.

In sum, AMPs could play an important role in agriculture as plant protection products. However, there are problems to overcome. First of all, transgenic plants are not generally released as commercial cultivars due to regulatory limitations and social concerns. In addition, the intrinsic toxicity, low stability and high production costs of some AMPs are further obstacles to the commercial use of plant AMPs. There is thus a need to identify and develop commercial plant AMPs that are less toxic, more stable and can be produced at lower cost.

Several options for improving the quality, selectivity, durability and safety of AMPs have already been explored, including improving their functional and immunological properties by partial hydrolysis (Salas et al., 2015). AMPs in hydrolysate form can be used as a food additive for beverages and infant formulae, as a food texture enhancer, and as a pharmaceutical ingredient. They can also be computationally modelled, genetically manipulated, and expressed in different systems to serve various practical pharmacological and pesticidal purposes.

In conclusion, the progress made in developing peptide libraries, as well as the development of sophisticated proteomics, bioinformatics and modification strategies, have made plant AMPs promising antimicrobial agents. The further discovery and development of novel plant AMPs with potent biological properties should provide excellent opportunities to expand further their use in the treatment of human, animal and plant diseases.
Conflicts of interest

None.

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Frontiers in Life Sciences

Zakaria Hossain Prodhan has pursued his Ph.D. in genetics and molecular biology of the plant, and he is familiar with metabolomic analysis of natural compounds (extraction, identification, and quantification of volatile compounds from plants). Zakaria has obtained 1st class in both the M.Sc. (Thesis) and B.Sc. (Honors) examinations and his research theme was on the effect of plant extracts on salivary gland- and oocyte chromosomes of Musca domestica L. (Diptera: Muscidae). Dr. Zakaria is an author of twelve scientific publications, and his main research interests are in genetics, breeding and molecular biology of plants. He is the Editor in chief of the Scholastic Editing Service and is a reviewer for a prominent international journal (Rice Research journal).

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Dr Cheng-Foh Le is an Assistant Professor at the University of Nottingham Malaysia Campus. He obtained his PhD from the University of Malaya in 2013 for his research study on designing and development of antimicrobial peptides as alternative antimicrobials against Streptococcus pneumoniae. The significance of his works had been recognized in a number of conferences and symposia. In 2013, he received the Young Investigator Award in the 9th International Symposium on Antimicrobial Agents and Resistance (ISAAR-9) for his research on the design of a series of novel AMPs which successfully cured the mice from lethal pneumococcal infection. His major interest is grounded on antimicrobial drug discovery, particularly against the clinical pathogens.

Prof Dr. Shamala Devi Sekaran began her career centring in immunology which then expanded to the field of virology, bacteriology, diagnostic microbiology, anticancer as well as anti-microbial drug/peptide discovery and lately endothelium dysfunctions in infectious diseases. Dengue was her primary focus during her Ph.D. programme and has remained her stronghold particularly lie in the field of diagnostics. She and her team have also developed various molecular diagnostic kits and have been actively involved in evaluation projects of dengue diagnostic kits conducted by the WHO and collaborating partners around the world as well as with companies that are commercializing these kits. The lack of anti-dengue drugs and vaccines has prompted Prof Shamala to further venture into antiviral drug development. Her projects include (i) the application of siRNA to inhibit entry of dengue virus into target cells; (ii) use of natural products such as Phyllanthus plant species to inhibit dengue virus infections; and more recently (iii) using combinatorial bioinformatics to design antimicrobial peptides (AMPs) against dengue virus. Besides dengue, Prof Shamala and her team have begun to design and develop novel synthetic AMPs as standalone or combination antimicrobial agents against the bacteria Streptococcus pneumoniae, and the fungus Candida albicans. The research conducted in this area involve in vitro and in vivo antimicrobial and toxicity evaluation of the designed peptides which eventually will be investigated in appropriate animal models for therapeutic efficacy and clinical usefulness. In few such endeavours, her team had designed a series of hybrid peptides showing potent antipneumococcal activity with broad spectrum antibacterial activity against various gram-positive and gram-negative clinically important bacteria. Among the series, one of the peptides displayed significant therapeutic efficacy in murine lethal pneumococcal infection model and could potentially a promising candidature for further development.