Jagannath University Journal of Life and Earth Sciences, **6**(1): 95-110 pISSN 2414-1402

JnUJL&ES

Review Article

PLANT ANTIMICROBIAL PEPTIDES IN BANGLADESH: SOURCES, EXTRACTION, PURIFICATION AND CHARACTERIZATION

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Received: 19 March 2021, Accepted: 14 July 2021

ABSTRACT

Antimicrobial peptides (AMPs) are naturally occurring short peptide molecules produced by every living organism and act against intruding microbes such as viruses, bacteria and fungi. Plants are promising sources of AMPs that exhibit defensive action against several invading pathogens. AMPs can be obtained from various plants and plant parts such as roots, seeds, flowers, stems, and leaves. Generally, chemical and solid phase extraction techniques have been utilized to isolate AMPs from plant parts. For purification and characterization of peptide molecules SDS-PAGE, IEXC, HPLC, MS, RT-PCR and CD have been extensively used. Plant-derived AMPs can be classified as thionins, defensin, lipid transfer protein, 2S albumin, snakins, hevein, knottin, etc. However, the structure and antimicrobial activity of AMPs contained in Bangladeshi plants have not been studied yet. In this review, we provide a list of plants containing AMPs in Bangladesh and summarize the extraction, purification and characterization processes of plant AMPs. This review would be helpful to isolate newer AMPs from Bangladeshi plants, which could be used as potential therapeutic antimicrobial agents.

Keywords: *Antimicrobial peptides, extraction, purification, characterization, source, Bangladesh*

Introduction

Antimicrobial peptides (AMPs) includes a large group of naturally occurring defense molecules produced by all multicellular organism having a wide range of activities against viruses, bacteria and fungi (Ting *et al.*, 2020). In 1939, antimicrobial peptide was firstly discovered by Dubos from bacteria (Dubos 1939a; 1939b). After that, Hotchikss and Dubos (1940) identified gramicidin from bacteria which was used as topical treatment for wounds and ulcer (Van Epps 2006). First plant antimicrobial peptide purothionin was discovered from Wheat (*Triticum aestivum*) in 1941

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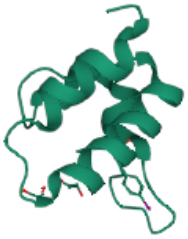
(Balls *et al.*, 1942) which was effective against fungi and some pathogenic bacteria (Ohtani *et al.*, 1977). In 1956, first animal antimicrobial peptide defensing was discovered from rabbit leukocytes (Hirsch 1956) and after few years, another animal antimicrobial peptide was isolated from lactoferrin of cow milk (Groves *et al.*, 1965). Antimicrobial peptides are available in both prokaryotic and eukaryotic cell (Radek and Gallo 2007; Leippe 1999). There are about 2,000 natural AMPs from different sources enlisted in the antimicrobial peptide database (APD) in which 293 from plant source (Wang 2013).

Plant AMPs can be isolated from varieties of plants and their organs such as the bark, root, flower, seed, rhizome and leaf. They show important activities against viruses, bacteria, fungi, parasites and other microorganisms and can be used as important candidates for developing potential new drugs to protect any living organisms. The plant AMPs have a wide variations in terms of extraction, purification and characterization methods. So, it is required to summarize these methods together for the ease of developing a unique lead compound (Tang *et al.*, 2018). Different plants of Bangladesh contain a notable amount of AMPs which can be used to design therapeutically active antimicrobials. So far our knowledge, there is no published work that summarizes the sources of AMPs from Bangladeshi plants.

Structural Features and Mechanism of Action of AMPs

Generally, AMPs are short peptide molecules having 10-50 amino acid residues. Most of them contain basic amino acids in their structure, therefore they are cationic in nature showing a net positive charge ranging from +3 to +11. Due to positive charges, most of the AMPs can bind electrostatically with negatively charged surface of the pathogen e.g. teichoic acid of gram-positive bacteria, lipopolysaccharide of gram-negative bacteria and cytoplasmic membrane of both gram-positive and gram-negative bacteria having negatively charged phospholipids (Hancock and Diamond 2000; Boman 2000). However, AMPs exhibit weak interactions with the mammalian cytoplasmic membrane as it contain mostly neutral phospholipids. As a result, they selectively and preferentially act against bacteria and other pathogens, while avoid binding with the mammalian cells (Matsuzaki 1999; Zasloff 2002). Due to this striking feature, AMPs have been considered as natural antibiotics as well as antimicrobial agents. Another important feature of AMPs is the presence of hydrophobic amino acid residues which control the hydrophobic binding and insertion behavior in the lipid membrane. In some AMPs, the cationic and hydrophobic residues are located in opposite direction, therefore, these peptides exhibit amphipathicity (Tossi *et al.*, 2000). This amphipathic arrangement enables them to bind with both the hydrophilic and the lipophilic surface of the target membranes. Depending on secondary structure, AMPs can be divided into α-helix, β-sheet, mixed or random-coil structure (Takahashi *et al.*, 2010; Nguyen *et al.*, 2011). The general secondary structures of plant AMPs are shown in Table 1. Several AMPs remain unstructured in aqueous solution but obtain a secondary structure or conformation (e.g., α-helix) in presence of lipid membranes. This conformational change sometimes plays significant role on the antimicrobial activity of AMPs (Pasupuleti *et al.*, 2012).

Table 1. Secondary structures of some significant plant AMPs.

Structural motif	Structure*	Example	References
α-helix	 Snakins-1	Lipid transfer proteins, Puroindolines, Snakins, 2S Albumin	Segura *et al.*, 1999; Berrocal-Lobo *et al.*, 2002
β-sheet	 Defensin	Defensin	Stotz *et al.*, 2009; Rogozhin *et al.*, 2011
Mix	 Thionin	Thionin	Hussain *et al.*, 2013

*Structures are obtained from https://www.rcsb.org/

The mechanism of action of AMPs differs greatly based on their target pathogens. For example, antiviral AMPs interfere with viral envelopes (Robinson *et al.*, 1998; Sitaram and Nagaraj 1999), antibacterial and antifungal AMPs causes membrane permeabilization (De Lucca *et al.*, 1999; Park *et al.*, 2004) and inhibit the protein synthesis (Brogden 2005). The mechanism of antibacterial activity of AMPs has been extensively studied and it can be classified into two groups- one is membrane permeabilization which leads to cell lysis and cell death (Chan *et al.*, 2006), another is blocking the synthesis of intracellular organelles e.g. enzymes or proteins (Otvos 2005; Brogden 2005; Pfalzgraff 2018).

Sources of Plant AMPs in Bangladesh

Plants are good sources of antimicrobial peptides (AMPs), which provide defensive action against a wide range of microbes (Nawrot *et al.*, 2014). Plant antimicrobial peptides can be classified as thionin, purothionin, defensin, lipid transfer protein, 2s albumin, napin, snakins, knottin-type, peptide so-d1, cycloteinases and glycine rich protein. These AMPs also could be isolated from Bangladeshi plants which are summarized in Table 2.

Table 2. List of plants containing antimicrobial peptide in Bangladesh.

Plant name	Local name	Scientific name	Name of peptide	Activity of antimicrobial peptide	References
Wheat	Gam	*Triticum aestivum*	Thionein: alpha-1-purothionin	Antibacterial	Hussain *et al.*, 2013
Oldenlandia	Oldenlandia	*Oldenlandia affinis*	Cyclotides: kalata B1 and B2	Antibacterial, Antifungal, insecticide	Pelegrini *et al*., 2007
Mallow	Napa shak	*Malva parviflora*	2S albumin	Antibacterial, allergen	Wang and Bunkers 2000
Radish	Mula	*Raphanus sativus*	2S albumin	Antibacterial, allergen	Terras *et al*., 1992
Corn	Bhutta	*Zea mays*	Lipid transfer proteins	Antibacterial	Hussain *et al.*, 2013
Mirabilis plant	Sondhya maloti	*Mirabilis jalapa*	knottin-type	Antibacterial	Pelegrini *et al*., 2007
Potato	Alu	*Solanum tuberosum*	Snakins	Antibacterial	Segura *et al.*, 1999; Berrocal-Lobo *et al*., 2002
Rubber tree	Rubber	*Hevea brasiliensis*	Heveins	Antifungal,antibacterial	Van Parijs *et al.*, 1991
Bean	Sim	*Phaseolus vulgaris*	Peptides	Antifungal, antibacterial	del Mar Yust*et al.*, 2004
Wax gourd	Kumra	*Benincasa hispida*	Hispidulin	Antibacterial,antifungal	Ye *et al.*, 2002
Chickpea	Chola	*Cicer arietinum*	Lipid Transfer Protein	Antifungal, antiviral	Bogdanov *et al*., 2016
Spinach pie	Palongshak	*Spinacia oleracea*	Peptide So-D1(Superoxide Dismutase Peptide)	Antifungal, antibacterial	Segura *et al.*,1998
Apple of Sodom	Makalfol	*Calotropis procera*	Proteins from latex	Antifungal	Ye *et al.*, 2002
Papaya	Papaya	*Carica papaya*	Proteinases	Antifungal	Ye *et al.*, 2002
Rapeseed	Shorisa	*Brassica napus*	Peptides	Antiviral	Pelegrini *et al.*, 2007
Coyote tobacco	Tamak	*Nicotiana attenuate*	PR-13 thionins	Antibacterial	Hussain *et al.* 2013

Plant name	Local name	Scientific name	Name of peptide	Activity of antimicrobial peptide	Reference
Dahlia	Dahlia	*Dahlia pinnata*	Defensin	Antifungal	Stotz*et al.*, 2009
Nigella	Kalojira	*Nigella sativa*	Defensin	Antifungal,antibacterial	Rogozhin *et al.*, 2011
Onion	Piyaj	*Allium cepa*	Lipid Transfer Protein	Antifungal,antibacterial	Cammue *et al.*, 1995
Momordica	Kakrol	*Momordica cochinensis*	Cyclotides	Trypsin inhibitor	Thongyoo *et al.*, 2009
Chili	Morich	*C. annuum*	Defensin	Antifungal, antibacterial, and insect amylase inhibitor activities	Maracahipes *et al.*, 2019
Jujuba tree	Jujuba	*Z. jujube*	Snakin	Antifungal,antibacterial	Daneshmand *et al*., 2013
Jambo tree	Jambo	*E. malaccensis*	Napin	Antibacterial	da Silva Dantas *et al.*, 2014
Water chestnut	Panifol	*Trapa natans*	Napin	Antibacterial	Byczynska and Barciszewsk 1999
Green coconut	Dab	*Cocos nucifera* L	Napin	Antibacterial	Mandal *et al.*, 2009
Guava	Peyara	*Psidium guajava*	glycine rich protein	Antifungal, antibacterial	Pelegrini *et al.*, 2008
Cucumber	Shosa	*Cucumis sativus*	Defensin	Antifungal, antibacterial	Al Akeel *et al.*, 2018

Variation of Plants AMPs Based on Sources

AMPs are found in the different species of plant, animal, insects and microorganisms. A single AMP can be found in various sources and a single source may contain multiple AMPs. For example, defensin is present in *Cucumis sativus, Nigella sativa, Dahlia pinnata* (Al Akeel *et al.*, 2018; Rogozhin *et al.*, 2011; Stotz *et al.*, 2009) whereas wheat (*Triticum aestivum*) contains several AMPs including purothionin, hevein-like peptides and defensin (Hussain *et al.*, 2013; Odintsova *et al.*, 2013). However, geographical variation may cause discrepancies in the structure, mode of action and activity of AMPs of the same species. *Staphylococcus hominis* (MBL AB63), a jute endophyte isolated from Bangladeshi jute seeds, exhibited significant antibacterial activity against *Staphylococcus aureus* SG511 (Uddin *et al.*, 2021). Therefore, it is important to extract and evaluate the activity of different AMPs present in the Bangladeshi plants.

Extraction of AMPs

Extraction process involves the isolation of soluble material from an insoluble residue using a suitable solvent system (organic and aqueous) on the basis of the physical nature of the compound

to be extracted. Generally solid phase extraction (SPE) and chemical extraction processes have been used to isolate different class of plant AMPs. Before going to extraction phase, the different plant parts are homogenized using grinding or milling. The overall process of extraction, purification and characterization is shown in Figure 1.

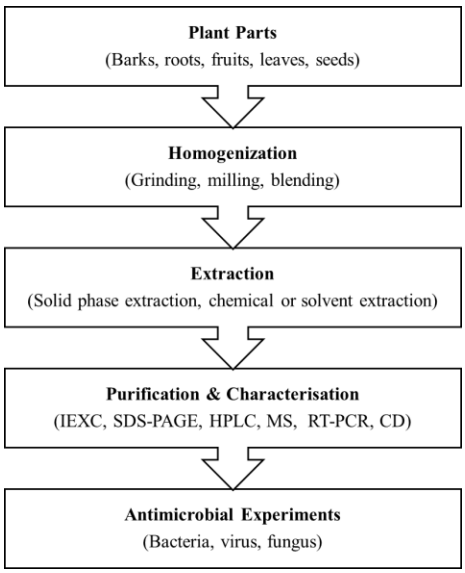


Fig. 1. Diagram for extraction, purification and characterization of the AMPs from the plant parts.

Solid phase extraction (SPE)

Solid phase extraction (SPE) is a very popular extraction method that involves partitioning between a liquid sample matrix and a solid phase that can be used for extraction, amount enrichment, derivatization and compound fractionation. The SPE process can analyze samples which are in solution, free of contaminating matrix compounds and concentrated enough for detection. Solid phase extraction is used to concentrate and purify the analytes from solution by sorption on a solid sorbent molecules and purification of extract after collection. The process can be used for extraction of molecules from different matrices which includes physiological fluid, water, beverages, soil, and living tissues (Żwir-Ferenc and Biziuk 2006). For substances which are in a solution, ion exchange SPE can be utilized. The electrostatic interaction of the charged functional group of the compound to the charged group bound to the silica surface is the compound's major retention strategy (Font *et al.*, 1993). However, pH in SPE is very crucial. Silica-based surfaces have a pH range of 2 to 7.5, and at pH levels above and below this range they are stable (Sabik *et al.*, 2000).

Chemical Extraction

Generally, two types of solvents are used to extract AMPs from plant sources. One is water and buffer solutions containing Na_2HPO_4 (Disodium phosphate), NaH_2PO_4 (Monosodium phosphate), KCl (Potassium chloride), EDTA (Ethylene diamine tetra-acetic acid). The other solvent system includes organic-based solutions such methanol, ethanol, acetonitrile, methylene chloride, chloroform etc. Some plants contain very low amount of AMPs, therefore it is necessary to

concentrate the AMPs solution. For this purpose, organic solvent extraction and ammonium sulfate precipitation method have been used. In case of ammonium sulfate precipitation method, the AMPs are concentrated by salting out approach. Then, concentrated AMPs are desalted by dialysis. After formation of the pellets of extractable materials, they are resuspended and finally dialyzed using a definite molecular weight cut-off dialysis tubing (Osborn *et al.*, 1995; Barber 1988). Moreover, several organic solvents are used to extract and concentrate specific group of AMPs including cyclotides (Craik *et al.*, 1999). However, these organic solvents also extract low molecular weight compounds other than peptides.

Purification and Characterization of AMPs

Plant AMPs are partially purified using the extraction methods discussed above. After that, these partially purified extracts have been subjected to several purification and characterization techniques for complete purification as well as structural identification.

Ion exchange chromatography (IEXC)

Ion exchange chromatography is a separation technique that can be used for both positively charged and negatively charged AMPs. The separation is done at a definite pH for their electric charge. In this process, the plant extract is passed through a cellulose column and washed out by phosphate buffer containing NaCl, and the eluted peptides are collected (Pingitore *et al.*, 2007). Ion exchange chromatography is used for the electrostatic attraction between buffer-dissolved charged AMPs and the oppositely charged binding sites on a solid ion exchange adsorbent (Khan 2012).

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is one of the most popular methods for size-based separation and analysis of peptides. Here, proteins are sorted exclusively on the basis of their polypeptide chain length (Nowakowski 2014). All types of the SDS-PAGE process are based on the ability of SDS to form complex with the proteins. SDS binds with non-polar regions of the peptide molecule by hydrophobic interactions (Hamdan and Righetti 2005). Not only proteins but also nucleic acids and peptide nucleic acids can be purified by this method. Higher nuclease resistance, increased binding affinity with target mRNAs, and lack of association with serum proteins are all advantages of uncharged nucleic acid analogues. The lack of charge prevents standard nucleic acids electrophoresis, the most widely used and convenient laboratory method for detecting and separating nucleic acids, from being used to examine these nucleic acids mimics (Bell 2009). This process provides an effective separation of antibacterial peptides having a low molecular weight.

High-performance liquid chromatography (HPLC)

High-performance/pressure liquid chromatography (HPLC) is one the leading analytical tools for qualitative and quantitative analysis of a large varieties of chemical compounds including proteins and polypeptides. On the basis of the side chains of amino acids, they are classified as hydrophobic and hydrophilic; uncharged polar, positively charged and negatively charged. Based on this classification, different modes of HPLC are used for isolation and purification of peptides-size exclusion HPLC (based on the size of the peptides), ion-exchange HPLC (based on the net charge of the peptide), reverse phase HPLC (based on the hydrophobicity of the peptides (Dorsey *et al.*, 1992; Mant and Hodges 1996).

RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR) involves a reverse transcription reaction with the help of reverse transcriptase enzyme to generate cDNA from mRNA. An RNA molecule is used as the template for the enzyme. The single-stranded DNA that produced is then used as a substrate for the process. Primers against established coding regions of mRNA are used for cloning. The process is very sensitive and requires a very small amount of sample (Bachman 2013). RT-PCR technique can be used in different sectors of clinical microbiology, oncology, single nucleotide polymorphism study and any type of gene expression study. It also can be utilized for nucleic acid multiplication and measurement. Protein analysis can benefit from the capacity to monitor fluorescence while manipulating temperature in numerous samples (Southard 2014).

Circular Dichroism (CD)

Circular dichroism (CD) is an important technique for quick determination of the secondary & tertiary structure, providing comparability of conformation, measuring thermal stability, detection of molten globule-like structure of proteins and biophysical analyses of cell-penetrating peptides (Ranjbar and Gill 2009). When the chromophores of the amino acids of the proteins are arranged in arrays, their optical directions are shifted or split into multiple transitions (Sreerama and Woody 2004).

Mass Spectrometry (MS)

Mass spectrometry is a widely used technique for the purpose of identification, characterization and quantification of different biomolecules. Simple mass spectrometry can be used for estimating the molecular mass of a protein. Advanced MS techniques can determine several structural features of the polypeptide which includes the amino acid sequence and type of posttranslational modifications (Domon and Aebersold 2006). There are two advanced features which has increased the capacities of the MS process:

(i) Electrospray ionization mass spectrometry (ESI-MS)

(ii) Matrix assisted laser desorption/ionization (MALDI)

(i) Electrospray ionization mass spectrometry (ESI-MS): Simple MS technique was restricted to small and thermostable compounds as there was lacking of process to softly ionize the molecules and transfer them to gaseous phase without excessive fragmentation. But development of the ESI technique has removed the barrier. ESI-MS has been used to study the single protein, multimeric protein, protein ligands and nucleic acid ligand complexes. For example, it can be used for analyzing the binding of vancomycin antibiotics to bacterial cell wall peptide analogues (Hofstadler and Sannes-Lowery 2006).

(ii) Matrix assisted laser desorption/ionization (MALDI): MALDI is another advanced ionization process for polypeptides and commonly used to complement results produced by ESI-MS. The sensitivity and tolerability of MALDI-MS greater than ESI during the presence of any excess materials such as salts or detergents. The MALDI technique has been used in combination with Time-of-flight (ToF) analyzers for determining the mass of a molecule. The ToF analyzers have improved performance in resolution and mass accuracy of the analytes (Jurinke *et al.*, 2004). The commonly used extraction, purification and characterization techniques of different plant AMPs are summarized in Table 3.

Table 3. Methods of extraction, purification and characterization of plant AMPs.

AMP	Source of AMPs	Extraction, purification and characterization techniques	References
Thionin	Mistletoe, wheat, barley seed	SPE, MALDI/ToF, MS/MS	Mak*et al*., 1976; Plattner *et al*., 2015; Rees and Lipscomb 1982; Herraiz 1997
Defensin	Wheat, barley, horse-chestnut, chili, cucumber	Chemical extraction, RP-HPLC	Carvalho and Gomes 2011; Maracahipes *et al*., 2019a; 2019b; Taveira *et al*., 2013
LTP	Onion, corn, radish, pea	Ultrafiltration, ion exchange chromatography, RP-HPLC, RT-PCR	Hussain *et al*. 2013; Cammue *et al*., 1995; Finkina *et al*., 2007; Rudresh *et al*., 2002
Puroindoline	Wheat	RT-PCR, SDS-PAGE	Dhatwalia *et al*., 2009; Bhave and Morris 2008
Snakin	Potato, jujube	Chemical extraction, ultrafiltration, MALDI/ToF	Van Parijs *et al*., 1991; Daneshmand *et al*., 2013; Asoodeh *et al*., 2012; Lehrer *et al*., 1991
2S albumin	Mallow, radish, lotus	Chemical extraction, ion exchange chromatography, SDS-PAGE, ESI-MS, CD	Wang and Bunkers, 2000; Terras *et al*., 1992; Khan *et al*., 2016
Hevein like protein	Rubber	Chemical extraction, SDS-PAGE, Gel filtration	Van Parijs*et al*., 1991; Van Damme*et al*., 1999

Therapeutic Potential of AMPs

AMPs exhibit broad-spectrum activities against several pathogenic microbes such as bacteria, fungi and viruses. Moreover, they can selectively act against microbial cells, while showing less toxicity to mammalian cells (i.e., low side effect) (Matsuzaki 1999; Zasloff 2002). Therefore, AMPs have been extensively studied to be used as potential antimicrobial drug candidates. Besides antimicrobial activities, AMPs exhibits cytotoxicity against a wide range of cancer cell lines such as breast, bladder, lungs and melanoma. Notably, some AMPs are highly selective to cancerous cell than normal cells, thus provides lower side effect (Guzmán-Rodríguez *et al.*, 2015), which is rarely observed in current anti-cancer therapy. Thus AMPs are also could be considered as potential anti-cancer drug. A large number of AMPs are in clinical trial phase and few AMPs are now registered for clinical use as anti-infectives e.g., gramicidins and polymyxins (Stevenson 2009). There are some obstacles of using AMPs as drug including proteolytic disintegration, immunogenicity problems, hemolytic activity, quick renal and liver clearance after ingestion, specificity and delivering issues, and expensive production costs (Kumar *et al.*, 2018; Wang *et al.*, 2019; Divyashree *et al.*, 2020). In order to increase the stability of AMPs, various ways have been investigated. Chemical modification is one of the most extensively used methods that includes addition of D-amino acids or non-natural amino acids, acetylation, cyclization, lipidation, glycosylation, and PEGylation (Erak *et al.*, 2018; Mahlapuu *et al.*, 2016; Moradi *et al.*, 2016; Kowalczyk *et al.*, 2017). In the coming days, extensive research can be conducted on the

therapeutic utility of AMPs as natural antibiotics against infectious microorganisms, various diseases, allergic reactions, and also to boost immunity for unpredictable interactions.

Conclusion

Many plants in Bangladesh contain medicinal compounds which have been extensively used as traditional medicine. Bangladeshi plant parts (seeds, barks, flower, leaves) also contain many AMPs, for example, thionins (from wheat, barley), defensin (from cucumber, chili), LTP (from pea, onion, radish), snakin (from jujube), 2S albumin (from lotus). However, the structure and antimicrobial activity of AMPs contained in Bangladeshi plants are rarely studied. This review will be helpful to identify new AMPs distributed in the plant kingdom that could be used as potential alternatives to conventional antimicrobials.

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