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Isolation of Antimicrobial Peptides (AMPs) From Different Sources: A Review

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Abstract

Antimicrobial peptides (AMPs) are small peptide molecules produced by a wide range of species, from bacteria to higher vertebrates, as a part of the innate immune system. These naturally produced peptides fight against intrusive microbes like viruses, bacteria, and fungi. AMPs are produced in various body parts by different organisms, such as skin secretion of amphibians; leaf, stem, fruits, or whole parts of plants; glandular secretion of mammals; the venom of reptiles and arthropods, etc. Due to this source variation, there is no universal method for the isolation, i.e., extraction and purification of the AMPs. Therefore, several techniques have been established to extract and purify these natural peptides from various sources. Generally, solid-phase extraction and chemical extraction techniques are the most common methods used for the extraction of AMPs. For purification and identification purposes, chromatography, gel electrophoresis, filtration, Edman degradation, circular dichroism, mass spectrometry, etc. are mainly adopted. In this review, we provide a brief discussion on the isolation process of AMPs based on their sources. This study would be helpful in isolating newer AMPs that could be used as potential antimicrobial agents.

Key words: Antimicrobial peptides, extraction, purification, source, characterization.

Introduction

Antimicrobial peptides (AMPs), also name as host defense peptides, are low molecular weight polypeptides that are naturally produced in almost every living organism. These peptides have a wide range of antimicrobial and immunomodulatory activities against bacteria, viruses, fungi and other pathogens (Zasloff, 2002). In 1922, the first antimicrobial protein, Lysozyme was isolated from human tissue and secretion by Alexander Fleming (Fleming, 1922). Purothionin and Phagocytin are the first antimicrobial peptides discovered in plants and animals, respectively (Balls *et al.*, 1942; Hirsch, 1956). After that, many AMPs were discovered in both eukaryotic and prokaryotic organisms (Radek and Gallo, 2007).

AMPs are found in various species of living organisms throughout their whole body or organs. For example, Discodermin A found in the whole body of *Discodeermia kiiensis* (Matsunaga *et al.*, 1985), Mytilins in blood of *Mytilus edulis* (Charlet *et al.*, 1996), Arasin 1 in the hemolymph of *Hyasaraneus* (Stensvåg *et al.*, 2008), Hadrurin in the venom of *Hadrurus aztecus* (Torres-Larios *et al.*, 2000), LEAP-1 in liver of human (Krause *et al.*, 2000), Magainin in skin secretion of *Xenopus laevis* (Zasloff, 1987), So-D1-7 in leaves of *Spinacia oleracea* (Segura *et al*., 1998), Snakin-1 in tuber of *Solanum tuberosum* (Segura *et al*., 1999), Arietin and Cicerin in seeds of *Cicer arietinum* (Ye *et al*., 2002), Napin in green coconut water (Mandal *et al.*, 2009) and so on. Based on their amino acid composition,

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the AMPs are classified as proline-rich AMPs, tryptophan- and arginine-rich AMPs, histidine-rich AMPs, and glycine-rich AMPs (Huan *et al*., 2020). The secondary structure of these AMPs is also diverse that includes linear α-helix, β-sheet, linear extension, and both α-helix and β-sheet (Lei *et al*., 2019; Hasan *et al*., 2022). There are the source and chemical composition variations in these AMPs, so there is no common method for isolating these peptides. Therefore, several extraction and purification techniques have been adopted for isolating AMPs. According to our knowledge, no study summarizes the isolation process of AMPs from various sources. In this review, we briefly described the extensively used isolation (extraction, purification, and characterization) techniques of AMPs from their available sources.

Isolation of AMPs: Extraction

Extraction is the separation process of soluble material from an insoluble residue using an appropriate solvent system (organic or aqueous) based on the physical characteristics of the substance to be extracted. The various animal and plant parts or portions are homogenized using grinding or milling before moving on to the extraction step. Generally, they were extracted through chemical extraction processes. Water acetonitrile-trifluoracetic acid was a common buffer solution to extract them. The other solvent system was nonpolar organic solvents like methanol, ethanol, chloroform, etc. Sometimes, they were extracted by $(NH_4)_2SO_4$ precipitation method (Hultmark *et al*., 1982). A common method for isolating and separating analytes from a liquid matrix is called Solid Phase Extraction (SPE). Commercially available SPE columns include tiny amounts of different chromatographic sorbents. For normal-phase separations, SPE columns may include polar sorbents such as silica, Florisil, or alumina, and for reversed-phase separations, nonpolar bonded silica phases or polymers. After passing the samples (containing AMPs) through these columns, these are adsorbed on the column that can be eluted from the column using a small amount of solvent (Lehotay and Schenck, 2000).

Isolation of AMPs: Purification and characterization

AMPs are partially purified in the extraction period. Following the extraction procedures, these substantially purified extracts were subjected to various purification and characterization methods to achieve maximum purification and structural identification. The following techniques are commonly used for purification and characterization purposes.

High-performance liquid chromatography (HPLC): Nowadays, HPLC (high-performance liquid chromatography) is universally recognized as the most effective method for analyzing and purifying a wide range of compounds. HPLC, in particular, has become a crucial method in the characterization of peptides and proteins. Instead, it has played a significant role in the biomedical and pharmaceutical sciences with rapid advancements over the decades. Amino acids are generally classified according to their polarity. Again, there are significant differences in the size, form, and characteristics of the side chains within each category. As a result, hydrophilicity/hydrophobicity and the quantity of charged groups present, play a vital role in peptide separation.

Reversed-phase HPLC (RP-HPLC) is a common separation technique used to separate antimicrobial AMPs, especially amphibian AMPs (He *et al*., 2012; Conlon *et al*., 2003). The maximum number of researchers have been inclined to perform HPLC at pH level 3, thus they could be benefited due to acidic evaporative mobile phase (particularly a linear gradient MeCN and TFA used as the mobile phase).

Ion-Exchange HPLC has proven to be extremely useful for peptide purification because HPLC packings can retain cationic and anionic-charged peptides. Human β-defensin 3 has been purified by cation-exchange HPLC (Xin *et al*., 2014).

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is one of the most abundantly used methods for purification of polypeptides according to their size variation (Gallagher, 2012). A porous acrylamide gel matrix has been used to bind denatured anionic polypeptide portions that segregates peptides with magnificent objectification based on their molecular mass (Nowakowski *et al.*, 2014). LL-37, a human cathelicidin, has been purified through the SDS-PAGE process (Dürr *et al*., 2006).

Ultrafiltration: Ultrafiltration is a process of separating solute from solvent by applying a force of hydraulic pressure gradient through a suitable membrane (Michaels *et al.*, 1971). It is generally used to purify AMPs from their solution because the molecular mass of AMPs is greater than the solvent. During the purification of a large amount of human β-defensin 6, AMPs were concentrated by ultrafiltration with an Amicon Ultra 3K device (Xin *et al.*, 2014).

Mass spectrometry: Mass spectrometry is a widely used process for identifying, characterizing, and quantifying various biomolecules. Simple mass spectrometry can be used to estimate the molecular weight of polypeptides. There are two sophisticated features which have enhanced the functionality of MS processes: Electrospray ionization mass-spectrometry (ESI-MS) and, Matrix-assisted laser desorption/ionization (MALDI); it also has been used in combination with Time-of-Flight (TOF) analyzers (Hofstadler and Sannes-Lowery, 2006).

Edman degradation: A variant of Edman digestion is described, in which peptides are sequentially cleaved on a solid support using a solid-phase peptide sequencer machine. Reagents are pumped through the column containing the peptide bound to the resin. the amino acid thiazolinone released after each digestion cycle is collected in a fraction collector for subsequent analysis (Laursen, 1971). It is widely used to analyze the primary structure and N-terminal sequencing of AMPs.

Circular dichroism (CD): Circular dichroism (CD) is a critical approach for the brief dedication of the secondary & tertiary shape, presenting a comparison of conformation, measuring thermal stability, detection of the molten globule-like shape of proteins and biophysical analyses of cell-penetrating peptides (Ranjbar and Gill, 2009). Peptide chromophores are responsible for shifting or electrical transition of optical directions, ensuring the structure of AMPs (Woody, 1985).

The overall process of extraction, purification, and characterization is shown in Figure 1.

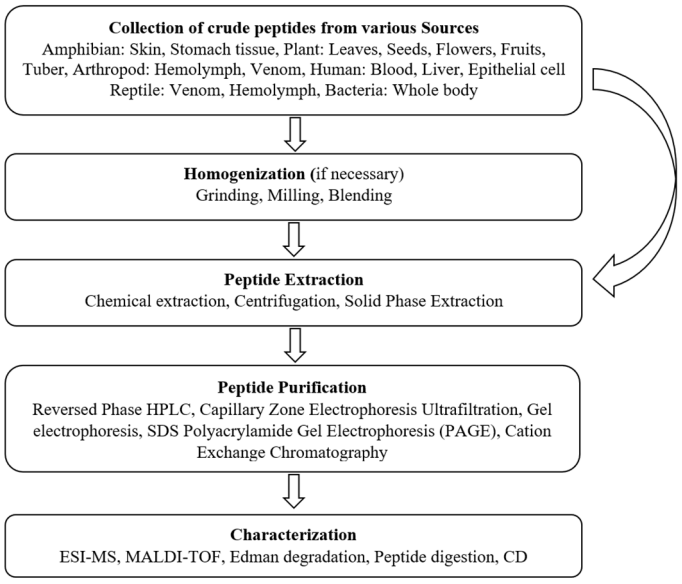

Figure 1. A general approach for the extraction, purification and characterization of the AMPs from various sources.

Isolation of AMPs from various sources

Both plants and animals are crucial sources of AMPs and other microorganisms that provide host defense activity against a wide range of pathogens. Animal AMPs could be further classified based on their source family names, e.g., amphibian AMPs, reptile AMPs, mammal AMPs, human AMPs, insect AMPs, etc. (Antimicrobial Peptide Database, 2021).

Amphibian AMPs: Almost 1093 AMPs have been discovered and partially synthesized from different frogs and toads (Wang, 2020). Most of them are found in their glandular secretion from the skin. AMPs were collected from the skin in both invasive and non-invasive ways. An invasive way, they were euthanized, and then their skins were immediately kept in dry ice. Then the small pieces of frozen skin tissues were homogenized at 0^{o}C temperature in an ethanol-HCl mixture. After the homogenization, the suspension was stirred and centrifuged to collect the supernatant. After another centrifugation of ethanol-free supernatant, it was eluted with a MeCN : TFA : water solvent mixture. The final extract was then kept freeze-dried for purification. Kassinatuerin-1, a host defense peptide, was isolated from the Hyperoliid frog, *Kassina senegalensis* according to this process (Wang *et al*., 2020). Several amphibian AMPs, including Bombinin-related peptide from *Bombina variegate* (Simmaco *et al*., 1991); Brevinin-1SY from *Rana sylvatica* (Matutte *et al*., 2000); Buforin I &II from *Bufo bufo gargarizans* (Park *et al*., 1996); Cancrin from *Rana cancrivora* (Lu *et al*., 2008); Dermaseptin b from *Phyllomedusa sauvagii* (Mor *et al*., 1994); Esculentin-2-ALa and ALb; temporin-ALd to -Alf from *Amolops lolensis* (Wang *et al*., 2010); Japonicin-2Cha to d from *Rana chaochiaoensis* (Conlon *et al*., 2006); Magainin-1 & 2 from *Xenopus laevis* (Zasloff, 1987); Maximins 1, 2, 3, 4 and 5 from *Bombina maxima* (Lai *et al*., 2002) are extracted in this invasive way.

Amphibian skin secretions were collected from noninvasive processes by chemical or electrical stimulation. *Amolops jingdongensis*, an Asian torrent frog, was stimulated with evaporated anhydrous ether (He *et al*., 2012), and their skin secretion was washed and collected in 0.1M NaCl solution (Wang *et al*., 2010). *Hymenochirus boettgeri*, an African dwarf-clawed frog, was injected with norepinephrine HCl through its dorsal lymph. On the other hand, *Leptodactylus syphax*, an American whistling foam frog, was stimulated by mild battery current discharge on its dorsal skin area (Dourado *et al*., 2007).

Using the same procedure, Brevinin-1PLa to c, Esculentin-1Pla & b, 2Pla to f, Plasturin-1a to d, -2a to c & -3a & 3b, Ranaturein-2Pla to f and Temporin-1pla from *Rana palustris* (Basir *et al*., 2000); Caerin-1 from *Litoria chloris* (Steinborner *et al*., 1998); Distinctin from *Phyllomedusa distincta* (Batista *et al*., 2001); PGLa from *Xenopus laevis* (Andreu *et al*., 1985) are extracted. After that, they were washed with distilled, deionized ultrapure water and kept frozen (Tyler *et al*., 1992). The isolation process of Magainin 2 from African clawed frog *Xenopus laevis* skin is given in Figure 2 (Zasloff, 1987; Gesell *et al*., 1997).

Plant AMPs: AMPs can be collected from various parts of a plant, including roots, tubes, leaves, flowers, fruits, seeds, bulbs, or the overall plant body (Hasan *et al*., 2021). At the homogenization stage, they need to be undergone mechanical destruction such as grinding, milling or blending. This process is selected according to the physical properties of the part of the plant. For example, Arietin and Cicerin are two peptides isolated from seeds of *Cicer arietinum* by the chemical extraction process. At first, the seeds are homogenized in distilled water, followed by centrifugation. The supernatant is dialyzed against distilled water. After that, Tris–HCl buffer is added to the dialyzed supernatant. This solution is subjected to affinity chromatography to isolate the target proteins (Ye *et al*., 2002). Almost similar approaches are used for the extraction of Hevein from the latex of *Hevea brasiliensis* (Van Parijs *et al*., 1991); Napin from *Cocos nucifera* (Mandal *et al*., 2009); Snakin-1 from *Solanum tuberosum* (Segura *et al*., 1999); So-D1-7 from *Spinacia oleracea* (Segura *et al*., 1998). However, the chemical composition of the dialysate,

homogenizer and buffer medium was different for these AMPs.

Arthropodal AMPs: Arthropods produce different classes of AMPs, including Defensins. Generally, these AMPs have been isolated at their larvae or pupae stages. The larvae were immunized and their hemolymph were collected to extract defensin-like peptide 4 (DLP4) from black soldier fly (*Hermetia illucens*). After that**,** the hemolymph was centrifuged to remove hemocytes and cell debris. The supernatant was diluted with an equal volume of ice-cold aqueous trifluoroacetic acid (TFA) followed by further centrifugation. The resulting supernatant undergoes several fractional analyses to collect the target AMPs (Park *et al.*, 2015).

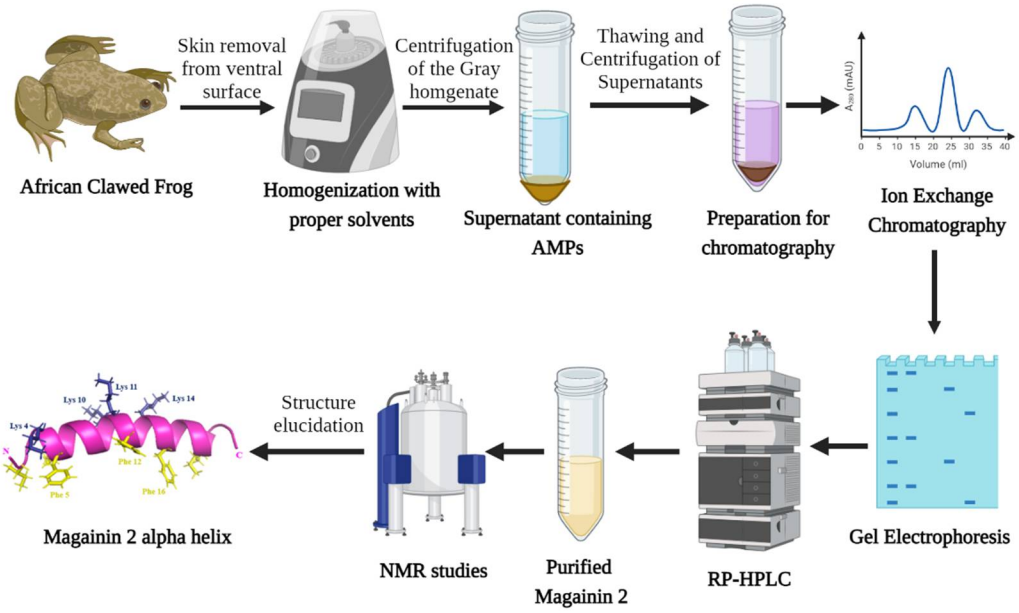

Figure 2. Isolation process of Magainin 2 from African clawed frog *Xenopus laevis.*

In general, chemical extraction with some solvents is the common method for extracting AMPs from arthropods. However, centrifugation, lyophilization, and homogenization are also used in several cases. Acaloleptins from the hemolymph of *Acalolepta luxuriosa* larvae (Imamura, 1999), Anoplin from the venom of *Anoplius samariensis* (Konno *et al*., 2001), Cupiennin 1 from *Cupiennius salei* venom (Kuhn-Nentwig *et al*., 2002), Diptericin A from larvae of *Phorrnia terranovae* (Dimarcq *et al.*, 1988), Hadrurin from the venom of *Hadrurus aztecus* (Torres-Larios *et al.*, 2000), House Fly Defensin from *Musca domestica* pupae (Dang *et al.*, 2010), Lacrain from *Scolopendra viridicornis* (Chaparro and da Silva, 2016), Ixocin from the salivary gland of *Ixodes sinensis* (Yu *et al*., 2006), QAK from *Antheraea mylitta* hemolymph (Chowdhury *et al.*, 2020), Scolopin 1 and 2 from *Scolopendra subspinipes mutilans* venom (Peng *et al*., 2010) are extracted following similar techniques.

However, Gomesin from *Acanthoscurria gomesiana* was synthesized using solid-phase methodology (Rodrigues *et al*., 2008).

Human AMPs: Human AMPs naturally occur in the human body and act as the first and second lines of the order of innate immunity. Alexander Fleming also isolated one of the first antimicrobial protein, Lysozyme, from human saliva (Fleming, 1922). Nowadays, they have been discovered in various parts of the body like skin, ocular cavity, blood, lungs, breast milk, urinary tract, gastrointestinal tract

(Wang, 2014) and, even in various wounded tissue and by-product of diseases (Dürr *et al*., 2006).

For example, LEAP has been isolated from blood ultra-filtrate fractions. For this purpose, the freeze-dried fraction is dissolved in 4-methyl morpholine and dithiothreitol added. After incubation for a definite period, iodoacetamide is added. Finally, the samples are desalted by solid-phase extraction, and proteins are eluted with an acetonitrile-trifluoroacetic acid mixture (Krause *et al*., 2000). Hepcidin (Park *et al*., 2001), Human β-defensin 3 (Harder *et al*., 2001), and Human platelet antimicrobial peptides (HPAPs) (Tang *et al*., 2002) are isolated from different organs of the human body by some mechanical and chemical extraction processes. Some of them have been isolated via their cDNA gene expression. After synthesis of the target AMP, they are subjected to chemical extraction processes. LL-37 (Dürr *et al*., 2006) and human β-defensin 6 (Xin *et al*., 2014) are isolated using this process.

Reptile AMPs: Reptiles are cold-blooded animals found in almost every environmental condition and have extensive susceptibility to adapt to the extreme environment. Several AMPs have been found in the venom of different snake species. Pelovaterin, a β-defensin-like peptide, has been isolated from the eggshell matrix of a Chinese soft-shelled turtle (Lakshminarayanan *et al*., 2008). A number of genome sequences were found in various reptiles, which suggests that several common and novel AMPs can be found and isolated from them (van Hoek, 2014).

TBD-1 is extracted from the blood samples of *Emys orbicularis.* From the incubated blood sample, the leukocyte layer was collected and homogenized using 10% (v/v) aqueous acetic acid, followed by centrifugation. The resulting supernatant is collected and ultrafiltered (Stegemann *et al*., 2009). Crocosin (Preecharram *et al*., 2010) and Leucrocin (Pata *et al*., 2011) from Crocodile (*Crocodylus siamensis*) blood are also extracted following the same technique. Omwarpin has been isolated from the venom of *Oxyuranus microlepidotus* using filtration and centrifugation techniques (Nair *et al*., 2007). Crotamine from Brazilian snake (*Crotalus durissus terrificus*) (Coronado *et al*., 2012) venom was collected using centrifugation and ion exchange chromatography.

Bacterial AMPs: Some bacterial cells are also responsibly synthesizing AMPs in their cell bodies, which have shown broad-spectrum antimicrobial activity. Generally, these AMPs are known as *bacteriocins*, and most of which are found in lactic acid-generating bacteria (Parada *et al*., 2007). A common method of extraction of these AMPs is the ammonium sulphate treatment [$(NH_4)_2SO_4$] of the bacteria culture. For example, Acidocin D20079-producing bacteria *Lactobacillus acidophilus* DSM 20079 was propagated in MRS broth resulting in the production of the AMP. After cultivation of *L. acidophilus* DSM 20079, cells were removed by centrifugation. The bacteriocin present in the supernatant fraction was concentrated by ammonium sulphate precipitation. The precipitate was dissolved in sodium acetate buffer (pH 5.0) and dialyzed using a 1 kDa cut-off membrane (Deraz *et al*., 2005). Almost similar approaches are used for the extraction of Enterocin CRL 35 (Farías *et al*., 1996), Pediocin AcH (Yang *et al*., 1992), Leuconocin Lcml (Yang *et al*., 1992), Sakacin A (Yang *et al*., 1992), Nisin (Yang *et al*., 1992), Mesentericin Y105 (Hechard *et al*., 1992) and Lactobin A (Contreras *et al*., 1997) from their source bacteria with some changes in the growth media and composition of the chemical used for extraction. The SDS-PAGE, HPLC, MALDI-TOF mass spectrometry, and cation exchange chromatography techniques were used for further purification. The overall isolation process of homicorcin from a jute endophyte *Staphylococcus hominis* strain MBL_AB63 is illustrated in Figure 3 (Aftab Uddin *et al*., 2021).

Marine AMPs: Almost 71% of the earth's surface is covered by the vast marine area. Therefore, it is a rich source of different types of animals, including sponges, corals, mussels, crustacean arthropods, and so on. They are exposed to pathogens ranging from 10^6 bacteria/ml to 10^9 viruses/ml of seawater (Mitta *et*

al., 1999). Thus, they inherently produce AMPs in their body to defend themselves. Hemolymph and hemocytes of marine crabs are good sources of AMPs. A novel AMP, Aurelin, was extracted from the mesoglea of *Aurelia aurita* (Ovchinnikova *et al*., 2006). AMPs from sponges were collected from the sliced specimens of the whole body. Then, generally, they were extracted through a solvent extraction process (Matsunaga *et al*., 1985), (Hansen *et al*., 2020). Arenicin-1 and Arenicin-2 are obtained from the coelomic fluid of the *Arenicola marina*. For this purpose, plasma is separated by centrifugation and the coelomocytes are resuspended in 10% acetic acid, homogenized and stirred overnight. Centrifugation is used to clarify the supernatant, which is used for further analysis (Ovchinnikova *et al*., 2004). Callinectin from haemocytes of *Callinectes sapidus* (Khoo *et al*., 1999), CgPep33 from whole flesh of *Crassostrea gigas* (Liu *et al*., 2008), Dolabellanin B2 from *Dolabella auricularia* body (Iijima *et al*., 2003), Mytimacin-AF from mucus of *Achatina fulica* (Zhong *et al*., 2013), Tachyplesin II, and Polyphemusins I and II from hemocytes of *Tachypleua tridentatus* (Miyata *et al*., 1989) are extracted by similar centrifugation, and chemical extraction methods. However, Turgencin A & B extraction from the whole body of *Synoicum turgens* includes solvent extraction and solid phase extraction (SPE) methods (Hansen *et al*., 2020).

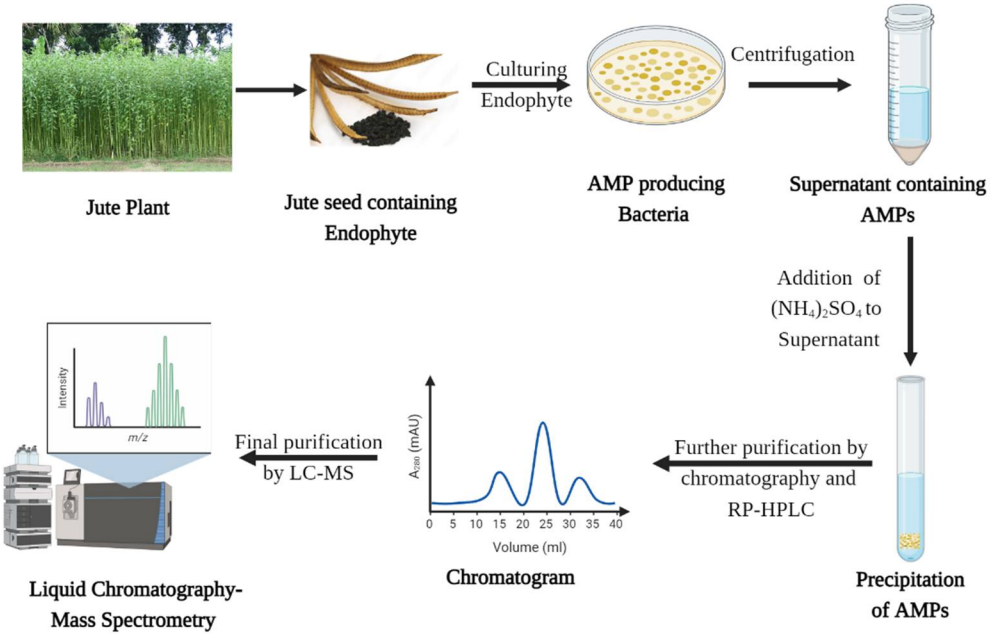

Figure 3. The scheme for isolating bacterial AMPs from a jute endophyte.

Other AMPs: Besides the previous sources, AMPs have been found in other animals too, e.g., fish, cattle, birds, earthworms, and so on. Skin mucous of fish is a vital source of AMPs. A number of AMPs have been isolated from gene expression of the AMPs (Gong *et al*., 2010). These AMPs are extracted by centrifugation, and chemical extraction methods described earlier. For example, Lumbricin PG from *Pheretima guillelmi* (Li *et al*., 2011), Oncorhyncin II from *Oncorhynchus mykiss* (Fernandes *et al*., 2004), Myxinidin from *Myxine glutinosa* (Subramanian *et al*., 2009), Pelteobagrin from *Pelteobagrus fulvidraco* (Su, 2011) are extracted using these techniques.

Potential applications of AMPs

AMPs have shown broad-spectrum antimicrobial activity against several pathogenic microbes such as

bacteria, viruses, fungi, and other parasites (Quiñones-Mateu *et al*., 2003; Albiol Matanic and Castilla, 2004; Chen *et al*., 2007). LL-37 has a significant wound-healing activity (Heilborn *et al*., 2003; Dorschner *et al*., 2001), and has been found in human skin after healing. Not only that, some of them have exhibited cytotoxic activity against different cancer cell lines; notably, they are highly selective against cancerous cells rather than healthy normal cell lines, which may provide fewer side effects and toxicity (Guzmán-Rodríguez *et al*., 2015).

Transgenic expression of the AMPs in animals and plants has shown a resistant activity against several pathogens, even their transmission from vector to host. Transgenic expression of cecropin-A in *Anopheles gambiae* shows the reduction of oocytes of *Plasmodium* in their body, while the expression of both cecropin-A and defensin-A in *A. aegypti* can combinedly prevent the transmission of malaria parasites (Nadal *et al.*, 2012). Transgenic expression of insect defensin in tobacco also enhances the resistance against pathogenic fungi (Mitsuhara *et al*., 2007).

Several AMPs are now investigating the clinical trial to examine their efficacy and acceptability. Omiganan, a synthetic peptide analog of indolicidin discovered from bovine neutrophils (Mookherjee *et al*., 2020) has undergone the phase-III clinical trial against atopic dermatitis and catheter-associated vaginal infection (Fritsche *et al*., 2008). LL-37 has undergone the phase-II trial to treat hard-to-heal venous leg ulcers (Grönberg *et al*., 2014). NP213 (Novexatin) has been investigated for the phase-II trial to treat onychomycosis (fungal nail infection) (Mercer *et al*., 2020). In the future, extensive research and investigation on this sector might show us their activities against various infectious diseases and allergic reactions or even enhance immunity against unforeseeable interactions.

Conclusion

Almost every species from this vast plant and animal kingdom could be a vital source of any novel antimicrobial peptide. These novel host defense peptides could be effective in treating several humans as well as other organism diseases. AMPs have been discovered in a wide range of species, from prokaryotes to humans. They are distributed in various parts of their body, and the extraction, purification, and characterization processes are also widely diverse. A concise knowledge is constructive for isolating new AMPs from various sources that could be an excellent substitute for traditional antimicrobials. This review will shade light on the isolation process of AMPs from different sources.

Authors' contribution

Authors MH and MMI designed the study, and SUZ and MKHA performed the literature review. MMI designed the figures with the help of FA. MMI and SUZ drafted the manuscript along with MMR. All the checked the manuscript and approved for publication.

Conflict of interest

The authors declare that there is no conflict of interest.

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Ethics statement

Not applicable.

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