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ISOLATION AND CHARACTERIZATION OF ANTIMICROBIAL PEPTIDES FROM *DATURA INOXIA* LEAVES HAVING ANTIMICROBIAL ACTIVITY AGAINST SELECTED BACTERIA

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ABSTRACT

The rapid emergence of multidrug-resistant infections has presented a serious challenge to antibiotic treatment posing major health threat over the past decades. Antimicrobial peptides (AMPs) play an important role in host defense mechanisms and can be used as an alternative source to develop more potent antibiotic against drug-resistant microbes. AMPs are ubiquitous and found in diverse organisms ranging from microorganisms to animals. AMPs have been described as an evolutionary ancient weapon against microbial infection. Plants are the precious source of natural antimicrobial molecules including antimicrobial peptides known as plant antimicrobial peptides (PAMPs). PAMPs exert multiple antimicrobial activities, which includes membrane permeabilization and interference with DNA, RNA and protein synthesis that might provide a suitable approach to prevent bacteria from developing resistance. The present research work was aimed at isolation and characterization of antimicrobial proteins from *Datura inoxia* leaf extract. Leaf extract of *Datura inoxia* showed antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis* whereas no activity was observed against *Escherichia coli* and *Candida albicans.* Maximum zone of inhibition (10 mm) was found against *S. aureus* and minimum (2 mm) against *B. subtilis* using 95% protein pellet. In Tricine SDS-PAGE, six, four and two bands lower than 26.6 kDa were observed in dialyzed samples of 50%, 75% and 90% protein pellet of the extract respectively. Protein bands lower than 26.6 kDa were observed in all the above-mentioned protein pellet of the extract that showed antimicrobial activity. These protein bands are putative AMPs which may be used or modified to develop antibiotics against drug-resistant microbes.

KEYWORDS: Plant antimicrobial peptides, Thionins, Defensins, Lipid transfer protein, Tricine SDS-PAGE gel electrophoresis.

INTRODUCTION

The ever-growing antibiotic-resistant microorganisms have posed a challenge in saving a million lives through decades. The antibiotic resistance has been associated with misuse and overuse of medications, prolonged use of same chemical moieties as drugs, lack of new drug development by pharmaceutical industry, all leading to the development of superbugs.[1-4] Around 170,000 people died globally because of multidrug-resistant tuberculosis infection.[5] Human pathogenic resistant fungal species has created the challenge for planning treatment strategies.[6] Antimicrobial peptides (AMPs) have been described as evolutionary ancient weapons against microbial infection as AMPs can kill microbes using multiple mechanisms, which include membrane permeabilization, interference with DNA, RNA and protein synthesis.

Plants have been a valuable source of natural products for maintaining human health in the indigenous system of medicine and modern pharmaceutical worldwide.[7] A vast number of medicinal plants have been recognized as the major source of antimicrobial compounds. Plant antimicrobial peptides (PAMPs) are one of these compounds. PAMPs can be the source of revolution in the development of new antibiotics, especially against Multi-Drug Resistant (MDR) infections, which are currently difficult to treat, by inhibiting MDR pump. Due to its multifarious action on the microbes, PAMPs are the potential candidate for developing new genre antibiotics with a remote chance of microorganisms getting resistant to these peptides.[4,8] PAMPs are small, positively charged, cysteine-rich proteins, comprising of 10 to 50 amino acids and a mass ranging between 2 to 9 kDa, with antimicrobial activities.[4,9] Based on a number of cysteine residues and disulfide bonds, PAMPs are categorized into six main families: Thionins, Defensins, Haveins, Lipid transfer protein, Snakins and Cyclotides. They are broadly classified into anionic (AAMPs) and cationic peptides (CAMPs) depending on their electrical charges.[10] However, proteins more than 9kDa molecular weight were also shown to have antimicrobial activity.[11-14]

The number of plants from which AMPs have been isolated accounts to a meagre percentage of the huge biodiversity existing in this universe. However few antimicrobial peptides have been isolated from roots, seeds, flowers, stems and leaves of a wide variety of species which have demonstrated activities against pathogenic organisms like viruses, bacteria, fungi,

protozoa and parasites.[9] The uncommonness of pathogens between human and plants could make PAMPs better antimicrobial compounds against human pathogens. Therefore, the chance to induce mechanisms of resistance against them reduces. In the present study, we attempted to isolate and characterize peptides less than 20kDa from leaves of *Datura inoxia* and evaluate their antimicrobial activity against selected.

MATERIALS AND METHODS

Chemicals: All the chemicals and media used in this study were from Hi-Media chemicals of A.R. grade. Microdispo DIALYZERS membrane was procured from Sigma.

Biological material

1. **Medicinal plant:** Leaves of *Datura inoxia* were collected from the fields of Mohali, Punjab.
2. **Microorganisms:** The microorganisms used in this study are *Staphylococcus aureus, Escherichia coli, Bacillus subtilis* and *Candida albicans* procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Bacteria and fungi were subcultured at regular intervals on Nutrient agar (NA) and Yeast extract peptone dextrose media (YEPD) respectively and stored at -20^{O}C and -80^{O}C by making their suspension in 10% glycerol.

Preparation of crude extract: Leaves of *Datura inoxia* were freed from dirt by washing with tap water and then by distilled water. The moisture content was removed by air-drying. After air drying, leaves were ground using mortar pestle maintaining the temperature at 4^{O}C by keeping in ice. Antimicrobial proteins and peptides were extracted using phosphate buffer saline (PBS) buffer of pH 7.2. The leaf powder of *Datura inoxia* was added in PBS buffer and froze at -20^{O}C followed by thawing at 4^{O}C and these steps were repeated thrice. Freeze-thawing treatment was carried out for 3-4 days for better extraction of proteins. The leaf extract was centrifuged at 10,000 RPM at 4^{O} C for 30 min. The supernatant was filtered to remove debris present in the supernatant. The filtrate was stored at 4^{O}C for further use.[15]

Ammonium sulfate precipitation: Crude extract containing soluble proteins was treated with 25%, 50%, 75%, 90% and 95% ammonium sulfate cuts maintained at 4^{O}C until the salt dissolved completely and then centrifuged at 10,000 RPM for 30 min. Precipitated protein pellets were dissolved in PBS buffer and store at -20^{O}C for further use.

Cut off separation: The protein samples were dialyzed using Microdispo DIALYSER ™ (Sigma) (MWCO of 100 to 30,000 Daltons).

Protein estimation: The protein content of different protein pellet and supernatant obtained from ammonium sulfate precipitation after dissolving in PBS buffer (pH 7.2) were determined by Bradford method.[16] Bovine serum albumin (BSA) solution of 1mg/ml concentration was used as the standard.

Agar well diffusion assay: The antimicrobial activity of protein pellet and supernatant before and after dialysis were determined by agar well diffusion assay.[17] For antibacterial and antifungal activity, NA and YEPD media were used respectively. A lawn was prepared with activated culture using sterile cotton swabs. Wells of 8 mm diameter were created using back side of sterile autopipette tip and different volume of protein pellet and supernatant were added to the wells (Table: 1). Petri plates were incubated at 37^{O}C and 27^{O}C for bacterial and fungal culture respectively for 24 hours. The extracts having antimicrobial activity inhibit the microbial growth and clear zones were found. The zone of inhibition was measured in millimetres.

Table 1: Different volume of protein samples used in agar well diffusion method.

S.No	Samples	Volume of sample added in different wells (µl)
1	Protein, Supernatant	100
2	Protein, Supernatant	80
3	Protein, Supernatant	60
4	Protein, Supernatant	40

Calculation for the zone of inhibition

Zone of inhibition (mm) = Radius of clear zone from centre of the well (mm) - Radius of well (mm).

Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE): The fractions having antimicrobial activity were run on Tricine- Sodium dodecyl sulfate Polyacrylamide gel electrophoresis (Tricine SDS-PAGE) using 12% gel.[18] The gel was stained with Coomassie Brilliant Blue G-250 dye and destained with 10% acetic acid (v/v). The approximate molecular weight of fractions having antimicrobial activity was determined by plotting Relative mobility (Rm) versus molecular weight of known standard proteins (Fig. 4).

RESULTS AND DISCUSSION

The crude extract of *Datura inoxia* leaf was screened for antimicrobial activity. The antimicrobial activity was determined in terms of inhibition. The protein pellets showing antimicrobial activity were subjected to Tricine SDS-PAGE to determine their molecular weight.

Protein determination using Bradford method: The concentration of protein in the samples ranged between 150 µg/ml to 520 µg/ml. Protein pellets from *Datura inoxia* leaf extract of 50% and 75% ammonium sulphate cuts showed total protein concentration of 520 µg/ml which was maximum, whereas protein pellet of 95% ammonium sulphate cut was found to have minimum concentration of protein (150 µg/ml) (Table:2, Fig. 1). Protein content in the leaves is comparatively less than the seeds.

Table 2: Estimation of total protein concentration using Bradford method.

S.No	Samples	Protein concentration (µg/ml)
1	25% Pellet	450
2	50% Pellet	520
3	75% Pellet	520
4	90% Pellet	370
5	95% Pellet	150
6	Supernatant	160

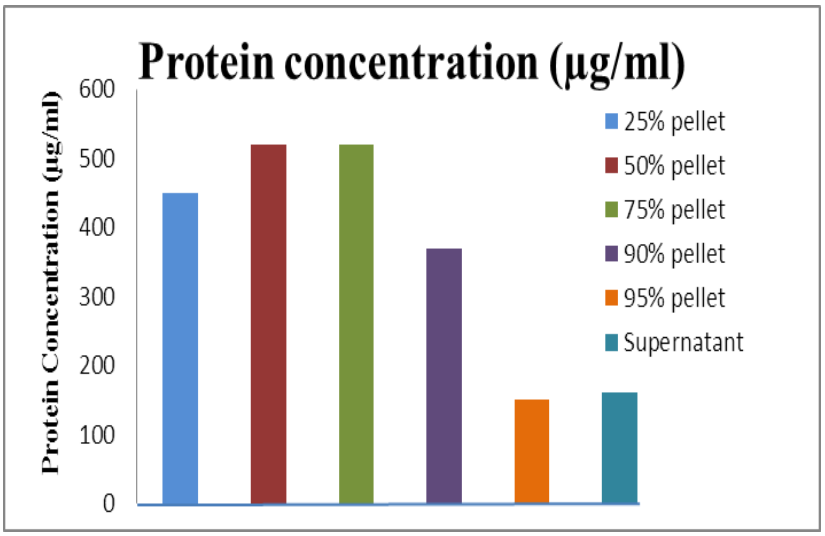

Figure 1: Graphical representation of amount of protein present in different protein samples of *Datura inoxia*.

Antimicrobial activity of ammonium sulfate precipitation: Antimicrobial peptides extracted from *Datura inoxia* leaf extract were screened for antimicrobial activity using agar

well diffusion method against Gram-positive bacteria namely *Bacillus subtilis* and *Staphylococcus aureus*, Gram-negative bacteria *Escherichia coli* and fungi *Candida albicans.* The clear zone of inhibition was observed and the diameter of the zone was measured in millimeter (mm). Antimicrobial activity was performed with the positive and negative control sample. For positive control samples, Ampicillin (60µg/ml) against *Escherichia coli* and *Staphylococcus aureus*, Chloramphenicol (50µg/ml) against *Bacillus subtilis* and Fluconazole (70µg/ml) against *Candida albicans* were used (Table 3 and Fig. 2). PBS buffer was used as negative control. The antimicrobial activity of different protein pellets and supernatant indicated that *Datura inoxia* leaf extract had antimicrobial activity against selected gram-positive bacteria i.e. *Staphylococcus aureus* and *Bacillus subtilis* whereas no antimicrobial activity was observed against selected gram-negative bacteria (*Escherichia coli)* and fungi (*Candida albicans*) (Table 4 and Fig. 3). The negative result obtained against gram-negative bacteria and fungi might be due to the absence of action against these microbes or due to a low concentration of an active compound or due to loss of its activity in the presence of the microorganism.[11] In the previous reports, PAMPs isolated from leaves with antimicrobial activity the pathogens used were different from that of ours except *E. coli*.[11,19] Small peptides having antimicrobial properties of less then 10kDa were reported from germinated and non-germinated 50 different types of seeds.[20] Four novel cyclotides (macrocyclic knotted proteins) was isolated from *Viola hederaceae* (vhl). vhl- 1 was leaf-specific cyclotides with 31-residue. EC50 for vhl-1 was found to be 0.87M against HIV- virus.[21]

Table 3: Antimicrobial activity of antibiotics against various microorganisms.

S.No	Antibiotic	Microorganism	Working Concentration (µg/ml)	Zone of inhibition in millimetres (mm)
1	Ampicillin	*Escherichia coli*	60	12
		Staphylococcus aureus		21
2	Chloramphenicol	*Bacillus subtilis*	50	14
3	Fluconazole	*Candida albicans*	70	10

Table 4: Antimicrobial activity of different samples of *Datura inoxia* leaves extract against various microorganisms.

S. No	Samples	Antimicrobial activity against selected Micro-organisms (Zone of inhibition in millimetres (mm))															
		Staphylococcus aureus (µl)				*Bacillus subtilis* (µl)				*Escherichia coli* (µl)				*Candida albicans* (µl)			
		100	80	60	40	100	80	60	40	100	80	60	40	100	80	60	40
1	25% Pellet	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
2	50% Pellet	4.0	4.0	--	--	3.0	2.0	--	--	--	--	--	--	--	--	--	--
3	75% Pellet	--	--	--	--	2.0	2.0	2.0	--	--	--	--	--	--	--	--	--
4	90% Pellet	10.0	6.0	4.0	2.0	--	--	--	--	--	--	--	--	--	--	--	--
5	95% Pellet	10.0	8.0	6.0	8.0	2.0	1.0	1.0	1.0	--	--	--	--	--	--	--	--
6	Supernat-ant	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--

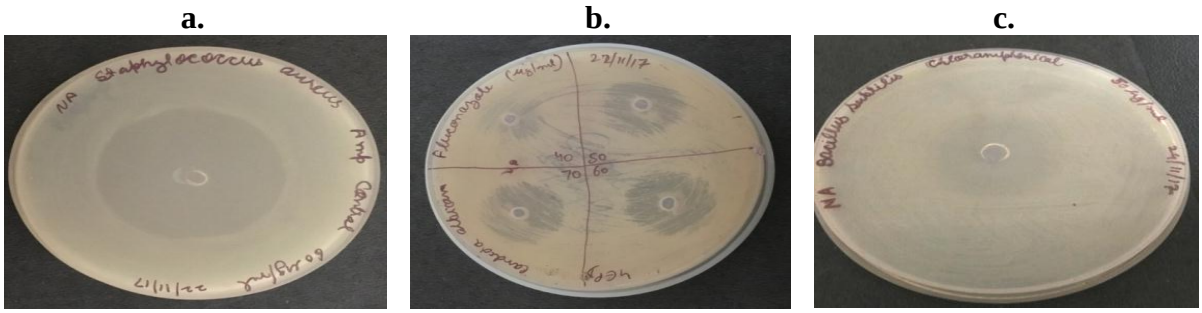

Figure 2: Zone of inhibition (a.) zone of inhibition on *Staphylococcus aureus* against Ampicillin (b.) zone of inhibition on *Candida albicans* against Fluconazole (c.) zone of inhibition on *Bacillus subtilis* against Chloramphenicol.

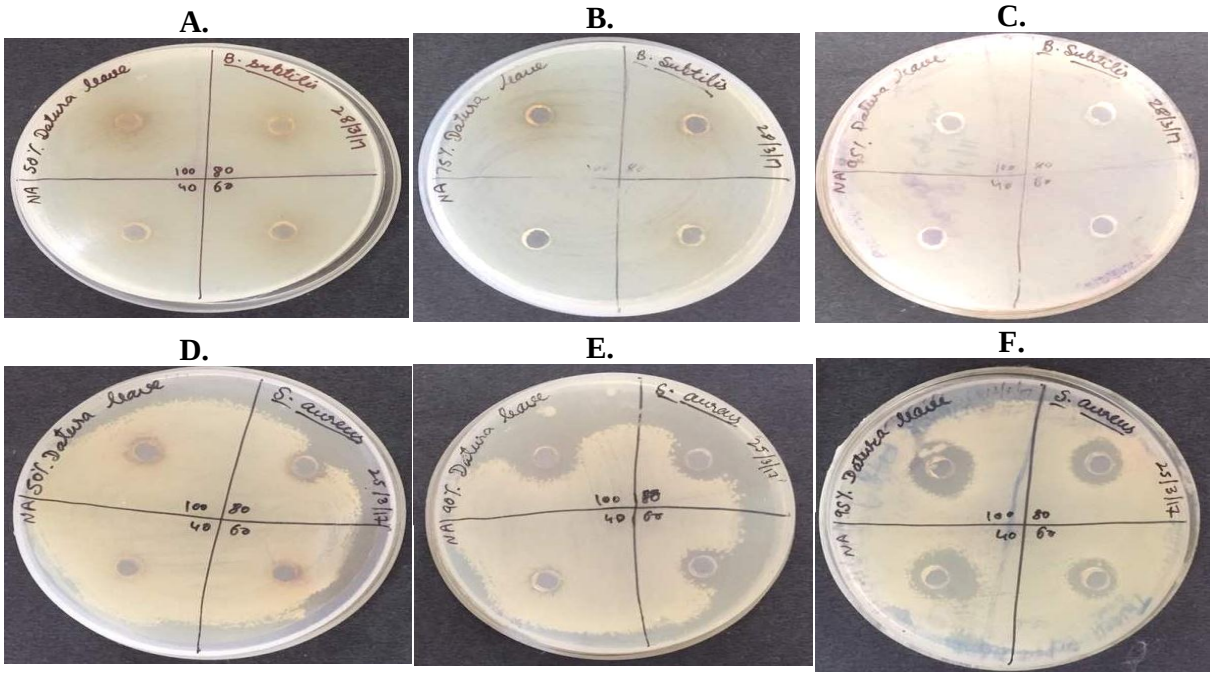

Figure 3: Antimicrobial peptides showing antimicrobial activity against selected microorganisms. (A, B, C) 50%, 75% and 95% protein pellets of *Datura inoxia* leaf extract showing antimicrobial activity against *Bacillus subtilis*. (D, E, F) 50%, 90% and 95% protein pellets of *Datura inoxia* leaf extract showing antimicrobial activity against *Staphylococcus aureus*.

Tricine SDS-PAGE: The dialyzed protein samples having antimicrobial activity were subjected to 12% Tricine SDS-PAGE to estimate the molecular masses of the antimicrobial peptides. Different bands were observed in 50%, 75% and 90% protein pellets of *Datura inoxia* leaf extract (Fig 5). Several bands were observed in Tricine SDS-PAGE gel. Six, four and two bands were observed below 26.6kDa in 50%, 75% and 90% protein pellet respectively. The peptides lower than 26.6kDa molecular weight was the putative AMPs that showed antimicrobial activity. A molecular weight of less than 26.6kDa protein bands was calculated from the standard graph as shown in Fig 4.

To determine the molecular weight of unknown samples

Calculate the Relative mobility (R_m) of protein samples (ladder and protein samples)

R_m value = Distance migrated by protein (cm)

Distance migrated by tracking dye (cm)

Plot log(MW) vs R_m for standard protein ladder and interpolate the unknown protein from its R_m value.

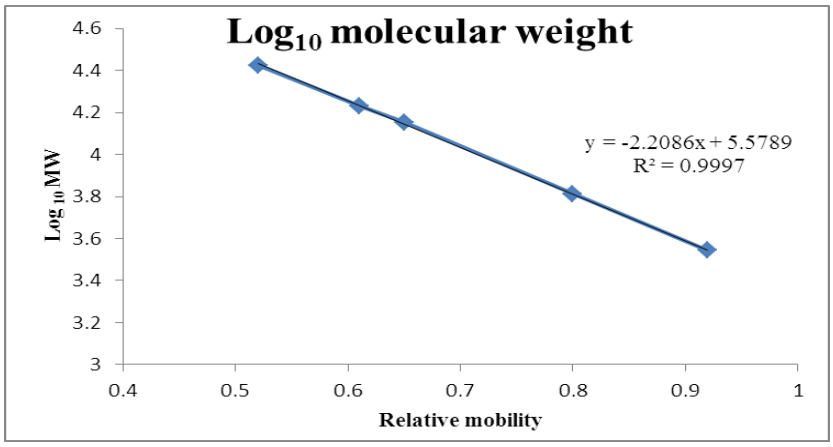

Figure 4: Standard curve for estimation of molecular weight of unknown protein samples using Tricine SDS-PAGE.

The different protein samples of *Datura inoxia* leaf extract showed multiple bands on Tricine SDS-PAGE. The molecular weight of proteins less than 26.6kDa was analyzed using standard graph obtained from protein marker because only these peptides play important role in the antimicrobial activity. Six peptides of 23.98kDa, 18.62kDa, 13.18kDa, 11.74kDa,7.07kDa and 3.89kDa molecular weight protein bands were observed in 50% dialyzed protein pellets whereas 75% dialyzed protein pellet had peptides of 23.98kDa, 12.30kDa, 7.07kDa and 4.07kDa molecular weight. In 90% dialyzed protein sample, two

bands were observed below 26.6kDa molecular weight which had the molecular weight of 14.45kDa and 13.18kDa (Table 6). The protein samples of less than 26.6kDa molecular weight were foremost peptides present in these samples that may be responsible for antimicrobial activity due to the small size of these peptides. Their small sizes enable them to easily penetrate into the cell through membrane permeabilization and lead to the death of microorganisms. There is a clear advantage of isolating AMPs from leaf compared to seeds. Former being available all throughout the year while seeds are available once or twice a year. Leaves can be harvested only when they are required thereby maintaining the sustainable conservation of biodiversity. Similarly, 35kDa protein/peptide having antimicrobial activity against bacterial pathogen was reported from *Ficus glomerata* leaf.[14]

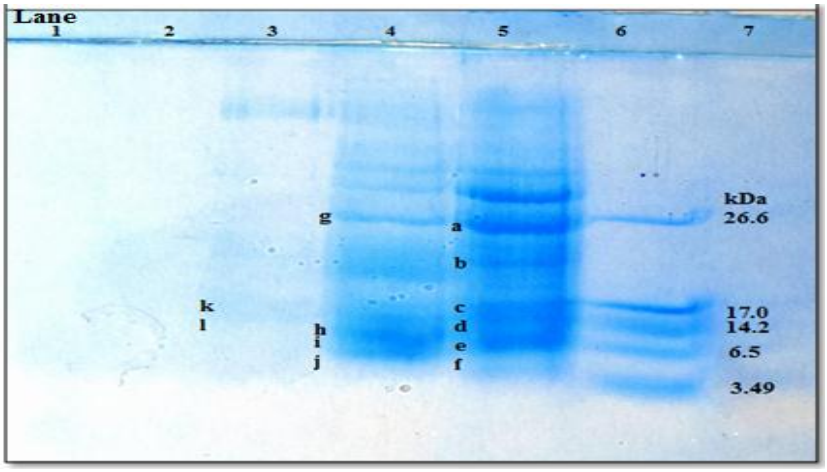

Figure 5: Tricine SDS-PAGE analysis of different dialyzed protein samples of *Datura inoxia* leave extract. Lane 1- supernatant, Lane 2- 95% protein pellet, Lane 3- 90% protein pellet, Lane 4- 75% protein pellet, Lane 5- 50% protein pellet, Lane 6- ladder and Lane 7- blank. a to l - represent different protein bands less than 26.6kDa in 50%, 75% and 90% protein pellets.

Table 6: Approximate molecular weight of protein samples.

S.No	Sample	Number of bands less than 26.6kDa	Relative mobility (R_m)	Molecular weight of protein (kDa)
1	50% Protein pellet	6	0.54	23.98
			0.59	18.62
			0.66	13.18
			0.68	11.74
			0.78	7.07
			0.9	3.89
2	75% Protein pellet	4	0.54	23.98
			0.67	12.30
			0.78	7.07
			0.89	4.07
3	90% Protein pellet	2	0.64	14.45
			0.66	13.18

CONCLUSION

Synthetic peptide drugs obtained from chemical synthesis are very expensive, so isolation of naturally occurring Antimicrobial peptides (AMPs) from medicinal plants are highly desirable and beneficial. Therefore, the present research work was designed to isolate and characterize antimicrobial peptides from *Datura inoxia* that can be used to discover novel antibiotic moieties that can serve as selective agents against infectious diseases. Such screening of various antimicrobial peptides or proteins from medicinal plants leads to promote proper conservation and sustainable use of such plant resources. AMPs from leaves have an advantage over seeds, being available all throughout the year, while seeds can be obtained only once or twice a year, thereby ensuring the continuous supply of raw material. Based on the antimicrobial activity of isolated peptides, these AMPs have been found to be the novel compound used against the treatment of infectious diseases or these AMPs can be used in food preservation as a natural antimicrobial active compound. Further research work is underway to purify the antimicrobial peptides from plants.

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