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Antibacterial activity of antimicrobial peptide extracted from *Trianthema portulacastrum* Leaves

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Abstract
Multidrug-resistant (MDR) infections have posed a major health threat over the past decades. The presently available antibiotics are unable to work against these MDRs. New strategies are being developed and developing potent antibiotic from antimicrobial protein peptides (AMPPs) can be one of the important strategies to tackle MDRs as an alternative to synthetic antibiotic. AMPPs are the crucial factors that play an important role in host defense mechanisms. Apart from secondary metabolites, plants are the major source of antimicrobial peptides mainly known as plant antimicrobial peptides (PAMPs). PAMPs mainly interact with phospholipids present in the cell wall of microorganisms that lead to membrane permeabilization. The present research work was an attempt to isolate novel antimicrobial peptides (AMPs) from the leaves of *Trianthema portulacastrum*. These AMPs showed antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis* whereas no activity was observed against *Escherichia coli* and *Candida albicans*. Maximum zone of inhibition of 8mm and 4mm was found with 75% protein pellet against *Staphylococcus aureus* and *Bacillus subtilis* respectively. In Tricine SDS-PAGE, three bands lower than 26.6kDa were found in both 25% and 50% protein pellet and four bands were observed in 75% and 90% protein pellets. The antimicrobial peptides lower than 26.6kDa are the putative antimicrobial peptides, which may be used or altered to evolve new antibiotics against drug-resistant microbes.

Keywords: Plant antimicrobial peptides, thionins, defensins, tricine SDS-PAGE, *Trianthema portulacastrum*

1. Introduction
According to the World health organization (WHO) report 2002 and 2003, on average about 55% of the world population relies on the traditional system of medicines, mainly on plant source for their health care. Therefore, such medicinal plants should be explored to access a variety of drug and active compounds [1, 2]. Treatment of resistant microbes presents a serious challenge in the development of antibiotics. In the last decade, only few new moieties have been developed for the infectious microbes, however they have several side effects [3]. Plants have been a valuable source of natural products for maintaining human health in the indigenous system of medicine and modern pharmaceutics worldwide [4]. Antimicrobial peptides have received great attention in the recent past due to their strong killing effect on microbes. An enormous number of medicinal plants have been known as leading source of antimicrobial compounds. Plant antimicrobial peptides (PAMPs) are one of these compounds. PAMPs can be used or altered to evolve new antibiotics, especially against multidrug-resistant (MDR) infections that are currently difficult to treat, by hindering MDR pump. PAMPs kill microorganisms using multifarious actions that include interference with the synthesis of DNA, RNA, protein and membrane permeabilization [5]. PAMPs are small, cysteine-rich proteins with antimicrobial activity comprising of 10-50 amino acids, positively charged and a molecular weight ranging between 2 to 9kDa [6, 7]. PAMPs are broadly classified into six main families based on the number of cysteines and disulfide bonds: Thionins, Defensins, Haveins, Lipid transfer protein, Cyclotides and Snakins [8]. However, protein molecules more than 9kDa were also reported to have antimicrobial property [9-1]. During the course of evolution, it had been observed that human pathogenic bacteria had been able to develop resistance against human antimicrobial peptides [13, 14]. Plant can act as better alternative source of isolating antimicrobial peptides other than human because plant antimicrobial peptides have rare chance of contact with human pathogen. Therefore, chances of acquiring resistance by the human pathogens against these PAMPs will be rare [15]. The plant diversity being vast, only 273 PAMPs were characterized and reported in the Phyt AMP database [16]. There lies a huge opportunity to explore many plants in the search of new AMPs.

In this endeavor, we attempted to isolate and characterize novel peptides of molecular weight less than 26.6kDa from leaves of *Trianthema portulacastrum* and assess their antimicrobial activity against selected microorganisms. *T. portulacastrum* belonging to the family Aizoaceae is considered to be a weed, with immense medicinal properties.

2. Material and Methods

2.1 Chemicals

All the chemicals of A.R. grade used in this study were obtained from Hi-Media chemicals, Mumbai, India. Microdispo DIALYZERS membrane was procured from Sigma, United States of America.

2.2 Biological material

1. Medicinal plant: Leaves of *T. portulacastrum* were collected from the fields of Mohali, Punjab.

2. Microorganisms: The microorganisms used in this study are *Bacillus subtilis*, *Candida albicans*, *Escherichia coli* and *Staphylococcus aureus* procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Fungi and bacteria were subcultured at regular intervals on Yeast extract peptone dextrose media (YEPD) and Nutrient agar (NA) respectively and ampoules were preserved as 10% glycerol stock stored at -80°C.

2.3 Preparation of crude extract

Leaves of *T. portulacastrum* were freed from dirt by washing with tap water, followed by distilled water. The moisture content was removed by drying at 37°C. Dried leaves were ground using mortar pestle maintaining the temperature at 4°C by keeping in ice. Antimicrobial proteins and peptides were extracted using phosphate buffer saline (PBS, pH 7.2). The leaf powder of *T. portulacastrum* was added in PBS buffer and frozen at -20°C followed by thawing at 4°C. 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°C for 30 min. The supernatant was filtered using Whatman filter paper to remove debris and then stored the filtrate at 4°C for further use [17].

2.4 Ammonium sulfate precipitation

Crude extract obtained from the above step containing soluble proteins was treated with 25%, 50%, 75%, 90% and 95% ammonium sulfate cuts while maintaining at 4°C until the salt dissolved completely, followed by centrifugation at 10,000 RPM for 30 min. Precipitated protein pellets were dissolved in PBS buffer and store at -20°C for further use [18].

2.5 Cut off separation

The protein samples were dialyzed using sigma Microdispo DIALYSER ™ of MWCO 0.1 to 30kDa.

2.6 Protein quantification

Bradford method was used to determine the protein content present in different protein pellet and supernatant obtained from ammonium sulfate precipitation after dissolving in PBS buffer (pH 7.2) [19]. Bovine serum albumin (BSA) solution of 1mg/ml concentration was used as the standard.

2.7 Agar well diffusion assay

The antimicrobial activity of protein pellet and supernatant were determined by agar well diffusion assay [20]. For antifungal and antibacterial activity, YEPD and NA 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 auto pipette tip, in which different volume of protein pellet and supernatant were added (Table:1). Petri plates were incubated at 27°C and 37°C for fungal and bacterial culture respectively for 24 hours. Antimicrobial activity was also carried out with the positive and negative control sample. For positive control samples, Fluconazole (70µg/ml) against *C. albicans*, Chloramphenicol (50µg/ml) against *B. subtilis* and Ampicillin (60µg/ml) against *E. coli* and *S. aureus* were used. PBS buffer was used as negative control. The clear zone of inhibition was observed around the wells having extracts with antimicrobial protein or peptides. The zone of inhibition was measured in millimetres.

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

Samples	Volume of each sample added in different wells (µl)
Dissolved Protein Pellets and Supernatant	100
	80
	60
	40

Calculation for the zone of inhibition

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

2.8 Tricine-Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (Tricine SDS-PAGE)

The protein pellets and supernatant having antimicrobial activity were run on 16% Tricine SDS-PAGE gel [21]. During electrophoresis, the initial voltage was 50V and as the sample reached separating gel the voltage was increased to 120V. The gel was dipped in the fixative solution to fix the bands for 30 min. The gel was stained with Coomassie Brilliant Blue G-250 dye for 3-4 hrs or overnight and then destained with 10% Glacial acetic acid (V/V).

Determination of the molecular weight of unknown samples

$$\text{Rm value} = \frac{\text{Distance migrated by protein (mm)}}{\text{Distance migrated by tracking dye (mm)}}$$

The approximate molecular weight of bands present in different protein pellet and supernatant having antimicrobial activity was determined by interpolating and extrapolating the relative mobility (Rm) on the standard graph plotted between Rm and log molecular weight (Log MW) of standard proteins (Fig. 4). Standard protein ladder comprising of four (26.6kDa, 17.0kDa, 14.2kDa and 6.5kDa) molecular weight proteins was procured from the Sigma, USA.

3. Results and Discussion

The crude extract of *T. portulacastrum* leaf was screened for antimicrobial activity. The antimicrobial activity was

determined in terms of the zone of inhibition. The protein pellets showing antimicrobial activity were subjected to Tricine SDS-PAGE to determine their molecular weight.

3.1 Determination of protein content using brad ford method

The concentration of protein in the samples ranged between 31.1 µg/ml to 812 µg/ml. Maximum total protein (812 µg/ml) was found in the protein pellet of *T. portulacastrum* leaf extract in 50% ammonium sulphate cuts, whereas minimum concentration (31 µg/ml) was found in the supernatant (Table:2, Fig. 1).

Table 2: Estimated total protein concentration in different protein samples of *T. portulacastrum* using Bradford method.

S. No	Samples	Protein concentration (µg/ml)
1	25% Pellet	609.4
2	50% Pellet	812.0
3	75% Pellet	585.1
4	90% Pellet	214.8
5	95% Pellet	24.5
6	Supernatant	13.1

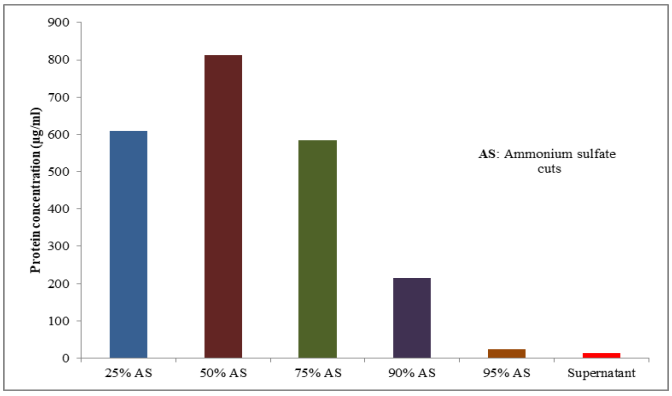

Fig 1: Protein concentration of pellets and supernatant after ammonium sulfate cuts from the leaves of *T. portulacastrum.*

3.2 Antimicrobial activity of ammonium sulfate precipitation

Antimicrobial peptides extracted from *T. portulacastrum* leaf extract were screened for antimicrobial activity using agar well diffusion method against selected fungus and bacteria. The clear zone of inhibition was observed and the diameter of the zone was measured. The zone of inhibition were also recorded with the positive and negative control samples (Table 3 and Fig. 2). The antimicrobial activity of different protein pellets and supernatant showed that *T. portulacastrum* leaf extract had antimicrobial property against selected gram-positive bacteria i.e. *B. subtilis* and *S. aureus* whereas no antimicrobial activity was recorded against selected fungi (*C. albicans*) and gram-negative bacteria (*E. coli)* (Table 4 and Fig. 3). The negative result recorded against *C. albicans* and *E. coli* suggests these proteins/ peptides might be unable to interact with the membrane proteins or lipids of the said microorganisms. Due to this non-interaction, probably further permeabilization of the membrane of microorganisms doesn't occur [9]. It has been reported that glucosylceramides (GlcCer) present in the membrane of yeast cells and fungi, binds with the peptides causing oxidative damage to cell, related to induction of reactive oxygen species (ROS) and nitric oxide (NO) production [22-24]. The peptides isolated from *T. portulacastrum* leaves might be unable to bind with such molecules in case of *C. albicans*. Omptins are the proteases present in the outer membrane of gram-negative bacteria like *E. coli*. These proteases interact with lipopolysaccharide of the membrane. The bacterial proteases had ability to convert active AMPs into inactive fragments that made host bacteria resistant to these active AMPs [25, 26]. Isolated peptides from *T. portulacastrum* leaves showed no antimicrobial activity with *E. coli* and this might be due to these proteases like molecules present on the membrane, which convert active AMPs to inactive molecules. Another reason may be insufficient availability of protein due to low concentration of protein/ peptide.

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: Different protein samples of *T. portulacastrum* leaves extract showing the zone of inhibition (millimeters) against selected 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	5.0	3.0	3.0	2.0	--	--	--	--	--	--	--	--	--	--	--	--
2	50% Pellet	4.0	3.0	3.0	2.0	1.0	1.0	1.0	--	--	--	--	--	--	--	--	--
3	75% Pellet	8.0	6.0	4.5	4.0	4.0	2.0	2.0	1.0	--	--	--	--	--	--	--	--
4	90% Pellet	3.0	2.0	2.0	1.0	3.0	1.0	--	--	--	--	--	--	--	--	--	--
5	95% Pellet	3.0	3.0	2.0	1.0	3.0	1.0	--	--	--	--	--	--	--	--	--	--
6	Supernatant	4.0	4.0	2.0	1.0	1.0	--	--	--	--	--	--	--	--	--	--	--
7	PBS buffer	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--

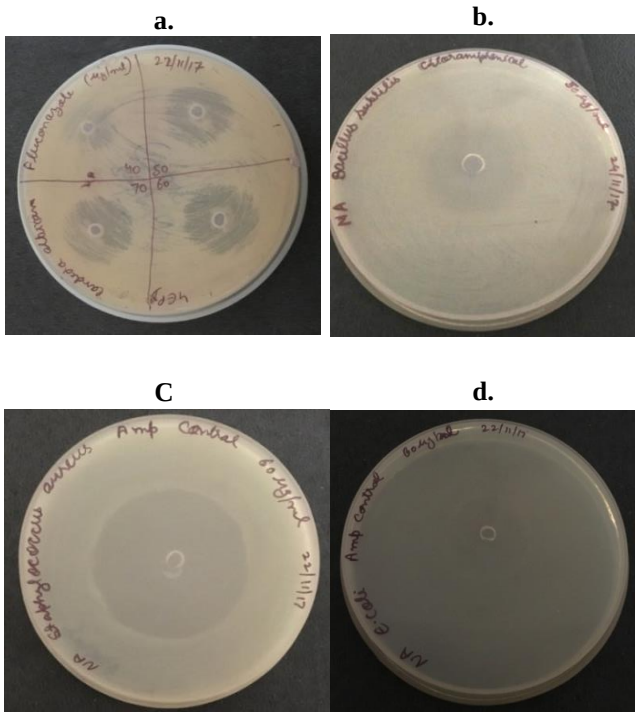

Fig 2: Antimicrobial activity with positive control showing the zone of inhibition (a) *C. albicans* against Fluconazole (70µg/ml) (b.) *B. subtilis* against Chloramphenicol (50µg/ml) (c.) *S. aureus* against Ampicillin (60µg/ml) (d.) *E. coli* against Ampicillin (60µg/ml)

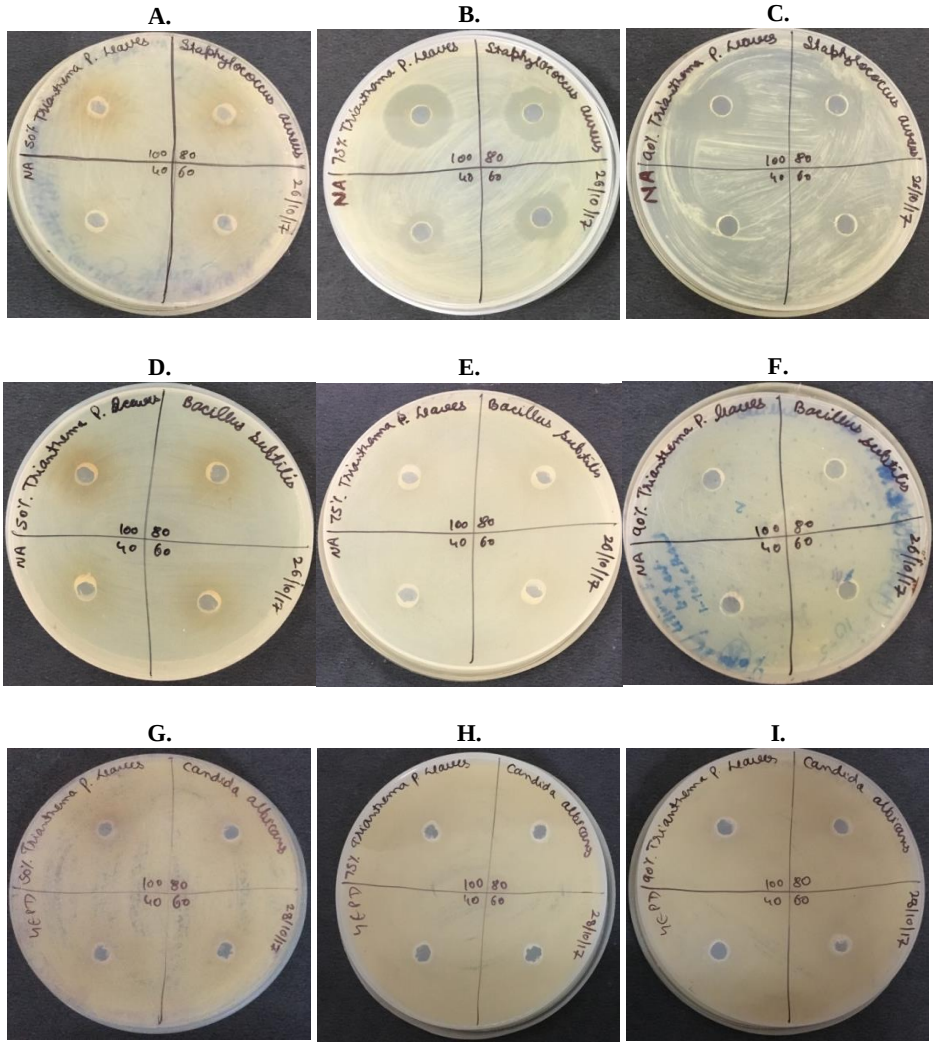

Fig 3: Antimicrobial peptides from *T. portulacastrum* leaf extract showing the zone of inhibition against selected microorganisms. (A, B, C) 50%, 75% and 90% protein pellets showing clear zone against *S. aureus*. (D, E, F) 50%, 75% and 90% protein pellets showing clear zone against *B. subtilis* (G, H, I) 50%, 75% and 90% protein pellets showing no antimicrobial activity against *C. albicans.*

3.3 Tricine SDS-PAGE

Dialyzed samples having antimicrobial activity were subjected to 16% Tricine SDS-PAGE to estimate the approximate molecular weight of the antimicrobial peptides. Several different bands were observed in 25%, 50%, 75% and 90% protein pellets of *T. portulacastrum* leaf extract (Fig 5) which were less than 30kDa. Since the proteins lower than 30kDa play important role and are responsible for antimicrobial activity, molecular weight of proteins less than 26.6kDa were calculated from the standard graph (Fig. 4). Three protein bands of molecular weights 23.44kDa, 19.14kDa and 18.28kDa were observed in 25% protein pellets whereas 50% protein pellet showed proteins of 23.44kDa, 15.06kDa and 9.37kDa. In 75% protein sample, four bands were observed below 26.6kDa which had the molecular weight of 22.75kDa, 17.78kDa 14.06kDa and 6.05kDa. Four bands were recorded in 90% protein pellet with molecular weight of 20.94kDa, 17.94kDa, 14.06kDa and 5.57kDa (Table 6). Therefore, these peptides may be the putative AMPs that actively exhibited antimicrobial activity.

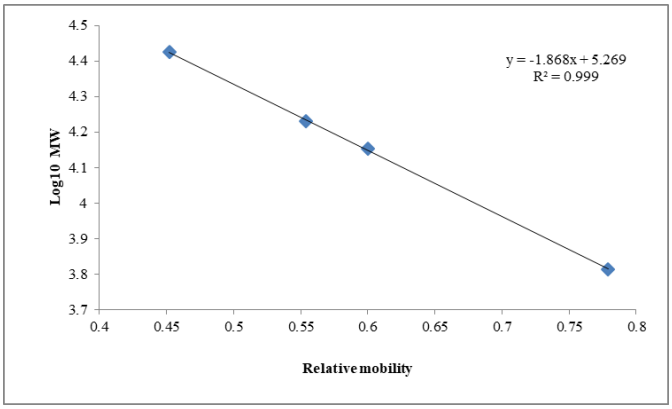

Fig 4: Standard curve for estimation of the molecular weight of unknown protein samples using Tricine SDS-PAGE.

The antimicrobial activity of protein/peptide depends upon many factors, which include charge, size and folding pattern of peptides [27]. Their small size renders them to easily penetrate into the cell through membrane permeabilization leading to the death of microorganisms. Due to the multifarious activity of AMPs on microbes, the chances of the microbes becoming MDRs are less. The source of these proteins being eukaryotic, their adverse effect on humans is also minimal.

In the previous reports, PAMPs isolated from *Datura inoxia* leaf extract showed antimicrobial activity against *S aureus* and *B. subtilis* whereas no activity was recorded against *E. coli* and *C. albicans*. Protein bands lower than 26.6kDa were observed in different protein pellets of *D. inoxia* leaf extract [3]. Similarly, 35kDa protein/peptide having antibacterial activity against selected bacterial pathogen was reported from *Ficus glomerata* leaf [18].

Using plants indiscriminately may hamper the biodiversity. However, in the present study only leaves were used which can be harvested when they are required thereby maintaining the sustainable conservation of biodiversity. Also peptide drugs obtained from chemical synthesis are very expensive and time consuming. Therefore, isolation of naturally occurring Antimicrobial peptides (AMPs) from plants are highly desirable and beneficial as their isolation and characterization are less time consuming and cost effective. The characterized peptides are also amenable to modifications by genetic engineering to increase their efficiency. Since most of these proteins/ peptides are produced by the plants as their self defense mechanism and are generally expressed under stress condition, therefore there is a good possibility of enhancing these proteins/ peptides within the plant by providing specific biotic and abiotic stress.

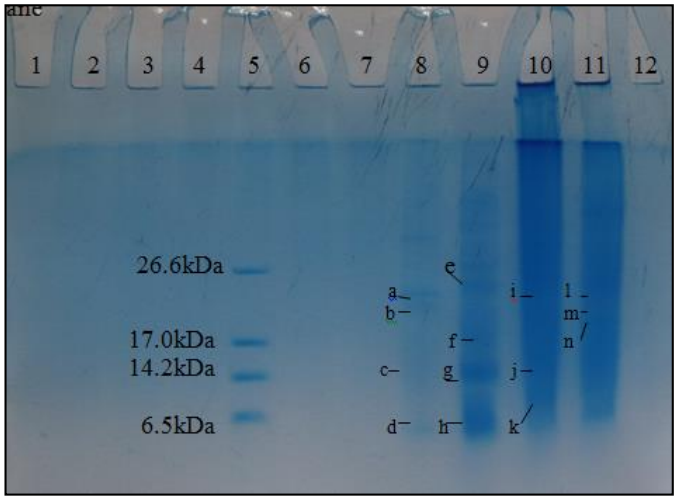

Fig 5: Tricine SDS-PAGE analysis of different dialyzed protein samples of *Trianthema portulacastrum* leaf extract. Lane 1- supernatant, Lane 2- 95% protein pellet, Lane 3- 90% protein pellet, Lane 4- 75% protein pellet, Lane 5- 50% protein pellet, Lane 6- ladder and Lane 7- blank. a to l - represent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

Table 6: Approximate molecular weight of protein bands present in different protein samples.

S. No	Sample	Number of bands less than 26.6kDa	Relative mobility (Rm)	Molecular weight of protein (kDa)
1	25% Protein pellet	3	0.481	23.44
			0.528	19.14
			0.539	18.28
2	50% Protein pellet	3	0.481	23.44
			0.584	15.06
			0.694	9.37
3	75% Protein pellet	4	0.488	22.75
			0.545	17.78
			0.600	14.06
			0.796	6.05
4	90% Protein pellet	4	0.507	20.94
			0.543	17.94
			0.600	14.06
			0.815	5.57

4. Conclusion

In the present research work, twelve different bands less than 24kDa were isolated with antimicrobial activity from *Trianthema portulacastrum* that can be used to discover novel antibiotic moieties that can serve as selective agents against infectious diseases. Further, the characterization of these putative proteins/ peptides is being carried out to analyze the sequence and folding patterns of proteins/ peptides.

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