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Original Research

Extraction and quantification of antimicrobial peptides from Medicinal plants through TrisNaCl and PBS buffer

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Abstract

Nature has provided plants with their own specific defence system that protect the plant from several traumatic conditions. These include environmental conditions like drought, harsh climate changes, wounding, pathogen attack and other biological as well as a biological stress. In order to deal with all these harmful occurrences, plants synthesize a wide range of defence factors that include both primary as well as secondary metabolites. Out of these the most popular are the defence proteins which are known as antimicrobial peptides (AMP). These AMPs are actually the pathogenesis-related (PR) defence proteins. These proteins are activated under the control of defence system of plant whenever triggered by the alarming situation. In the current study Crude protein extraction of four medicinally important plants named as Cassia fistula, Albizia lebbeck, Saccharum officinarum & Cymbopogon citratus was performen. Extraction was done in TrisNaCl and PBS buffer. Quantification of the protein content in the extract was done by Bradford assay. Concentration of protein from TrisNaCl buffer extracts was high as compared to the extracts from PBS buffer. As these proteins play their protective role in defence of the plants against pathogen attack so these extracts can better be used to check the antimicrobial activity of these plants in future to treat several infectious diseases in humans.

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Introduction

Every living organism on the earth has a property to defend itself against harmful condition. Plants also possess a specific system of defence in the form of antimicrobial proteins that make plants able to survive under harsh environmental conditions and to combat the pathogen attack. [1]. Although their system is not as much established as humans but they develop a wide range of different molecules, chemicals and proteins that are activated and transcribed in response to any damaging situation[2]. Whenever a threat is detected by the plant they start a defence response and initiate several mechanisms to prevent themselves against harm[3]. Out of these responses production of antimicrobial peptides (AMP) is the most significant one. These AMPs are the part of first line of defence and play their role in a prominent way through different channels. They reside in the innate immunity of plants[4]. AMPs are widely present in plants, they are present inherently as well as produced in response to a stimulus [5]. These AMPs possess several protecting roles like antibacterial as well as antifungal. Structurally these are very small peptides having many amino acid cysteine residues which are basic in nature [6]. These are the most potent and significant part of plant's immune system that provide an army backbone to the plant defence [7]. They are not only involved in protecting the plant by playing their independent role instead they also activate and stimulate several other protecting mechanisms and chemicals involved in defending the plant against harmful stimuli [8]. Once these proteins are activated and have initiated their defence mechanism a cascade of events occur that result in destruction of harmful pathogen like fungi or bacteria[9].

In this study we aimed at extracting the proteins by two different buffers from medicinally important plants. Furthermore, the concentration of proteins was also calculated so that it can be used for their antimicrobial potential and can be helpful in synthesizing antimicrobial drugs.

Material method

Collection of Plant material

Plants were purchased and collected from the market. Identification and confirmation were done by the department of botany **The Women University Multan, Pakistan**. Sampling was carried out during October 2017. Fresh plant leaves of appropriate size were taken and dried. Sample was ground into fine powder and proteins were extracted by grinding the powdered leaf sample of each plant in given buffer. Temperature was maintained upto four degrees to prevent proteins from degradation.

Extraction of Proteins from plant leaf sample

It was done by using following buffers

- TrisNaCl buffer [10]
- Phosphate Buffered Saline(PBS) [11].

Extraction through PBS buffer was carried out by passing the sample solution from freeze thaw cycles and from without freeze thaw cycles to check the effect of these cycles over quantification of proteins extracted from the plants.

Extraction of antibacterial peptide through Tris NaCl buffer [10]

Powdered sample of 1gm leaves of each plant was taken in 3.3 ml of 1 Molar Tris-HCl solution and 0.5 Molar NaCl solution. The pH of the buffer solution was set to pH 7.5. Plant leaves were absolutely homogenized in buffer and was incubated at 4 ^{o}C to avoid protein denaturation for 24 hours. Sample solutions were then subjected to centrifugation at 12,000rpm for 30 minutes.

Extraction of antibacterial peptides through PBS

Extraction of antibacterial peptides through PBS by passing under freeze thaw [11]

Fresh 0.3gm powdered leaves samples of each plant were in 4.5 ml of Phosphate Buffer Saline. After that samples were subjected to alternate 3 freeze thaw cycles at the interval of 24 hours. After these cycles the samples were centrifuged at 12000 rpm for 15 minutes. After the whole procedure the supernatant was collected & stored at 4 ^{o}C for quantification.

Extraction of antibacterial peptides through PBS without passing from freeze thaw

In some research the alternate freeze thaw cycles sometimes leads to the degradation of proteins in sample. In order to check the effect of these cycles the samples were then passed under the same procedure as mentioned above but without repeated freeze thaw cycles.

Bradford assay[12]

After the proteins extracted from the samples, the determination of concentration of proteins in given plant sample with different buffers was done. It was checked through spectrophotometer using Bradford assay. The stock solution of Bovine Serum Albumin was prepared in the ratio 2 mg/ml for PBS buffer & 1 mg/ml for Tris NaCl buffer. The wavelength of spectrophotometer was set at 595nm.

Preparation of Bradford Reagent

The reagent was prepared by following the recipe given in Table 1. Reagent was diluted 1:4 times and filtered immediately to make it suitable for the passage of light rays in spectrophotometer. The reagent was used within 15 minutes after its preparation.

Calculation of absorbance of samples

Glass cuvette was properly sterilized and samples were taken one by one for the measurement of absorbance in spectrophotometer at 595nm.

Results

Absorption protein extarcts

Absorbance of protein extracts of each plant leaf sample was calculated on spectrophotometer at 595nm

Calculation of protein concentration in extracts

Concentrations of protein in each plant sample with different buffer was calculated by the given formula:

$Y = Ax + B$

In this equation the 'Y' indicates the absorbance of sample in spectrophotometer while 'x' shows the concentration of protein in respective sample. 'A' and 'B' are the coefficients there values are obtained by Microsoft excel software.

Bradford assay

It was performed by preparing BSA stock solution at the concentration of 2 mg/ml for Phosphate Buffer Saline buffer and 1 mg/ml for Tris NaCl buffer protein extracted samples. Readings were taken at 595nm.

The highest concentration of protein was present in leaves of *Cassia fistula* extract by Tris NaCl, which showed the absorption of 1.195 nm and concentration of 6073ug/ml. Highest concentration of proteins in *Albizia lebbeck* were also found in the extract through Tris NaCl which showed the absorption of 0.697 nm and concentration of 3583ug/ml. *Cymbopogan citratus* and *Saccharum officinarum* also showed their maximum protein concentrations of 1823 ug/ml and 2043 ug/ml respectively in Tris NaCl buffer samples (Table 3). Protein concentration of the same plants extracts through other buffer was lesser than Tris NaCl buffer.

Protein extraction of all four plant's leaves sample was also done through PBS buffer following two procedures; passing through repeated 3 cycles of freeze thaw and without freeze thaw. The maximum protein concentration through PBS buffer was again found in *Cassia fistula,* it was 2438 ug/ml and 2019 ug/ml with and without freeze thaw cycles respectively.

Hence from the results the greater concentration of proteins was found in *Cassia fistula* among all 4 medicinal plants and the Tris Nacl Buffer yielded better concentrations of protein than PBS buffer.

Discussion

Not only humans but plants are also affected by number of pathogens that cause different diseases in plants. In order to stay protected from these pathogenic attacks plants develop a network of defence proteins that are the integral part of plant defecne system. Sme proteins are specific against fungal attack like chitinases that alter the cell wall structure of fungal organisms. On the other hand, some proteins serve as both antibacterial as well as anti-fungal role like defensin proteins. These are effective both gram positive and gram negative bacteria[13]. Although these plants do not have a well-established system of defence as compared to animals, so plants possess different metabolic compounds like secondary metabolites and proteins that are defending in their mechanism of action[2].

The underlying mechanism involved in the plant defence is actually the recognition of particular structures located on the surfaces of attacking microorganisms, these structures are named as pathogen-associated molecular patterns (PAMPs). These patterns are recognized by the specialized receptors in plants that are termed as plant pattern recognition receptors (PRRs)[14].After this recognition a diverse response is initiated in the host, which halts the growth and infectivity of attacking organisms. As soon as PAMPs are recognized several antimicrobial peptides are also generated that perform their respective function in defence of plants against pathogens[15].

There are several types of proteins, like glucanases, chitinases (specific for antifungal activity), defensins (antimicrobial peptides), and glycoproteins (reduce the pathogenicity of pathogen) etc. All these proteins one in other way are involved in boosting the immune response of the plant against pathogenic assaults [8]. These proteins are synthesized in different plant's parts like leaves, fruits and seeds etc. Once these are transcribed and generated, they potentially kill fungi, inhibit bacterial growth and insect herbivores[16]. These proteins belong to a wide class of antimicrobial peptides that are classified into 17 families. These are named as classified according to their structural composition and functional properties [17]. These proteins follow different mechanisms of action for attacking the invading organism, some halt the cell wall stricture of fungi while others are bacteriostatic [18]. Their immune related properti3es in defending the plants have been reported in many researches. In some researches they have also confirmed their role as insecticides [19].The aim of this study was to extract these potentiating proteins from plants through different buffers so they can further be used in synthesizing natural antimicrobial agents.

Isolation of defence related proteins specially defensins have been done in many researches and their mechanism of action is studies. Their defence related role is due to their ion channel blocking activity, cell wall synthesis inhibition and bacterial pathogenic action [20]. Extraction and identification of two novel antimicrobial peptides has been carried out from the *Arabidopsis thialana* is done. Transgenic transfer of genes for antimicrobial peptides is a one of the most moderinozed technique for the synthesis of antimicrobial peptides in selected plants so they can also develop immunity against their microbe specific disease [21]. After the extraction and transgenic transfer of these proteins in plants the next step is to check the antimicrobial activity of these peptides against different microorganisms. These activities have also been checked and have shown several remarkable anti-fungal as well as antibacterial properties. As it is confirmed that these proteins are extensively present in different plants parts like seeds and leaves, extraction of proteins from seeds of six plants was done and their activity was checked [22].

Conclusion

Antimicrobial proteins are the defence proteins of plants that protect the respective plants from attacking microorganisms. Extraction of these proteins and their quantification through different solvents helps to find that in which buffer the better yield of protein can be carried out, through this research work extraction of proteins was carried out in Tris NaCl and PBS buffer, according to results Tris NaCl give the better results on the quantification of proteins. This research opens furthers doors for checking the antimicrobial activities of theses extracts, so helping us out in synthesizing natural medicines containing these proteins as acting therapeutic agents against infectious diseases. Furthermore, genes of these antimicrobial peptides can also be transferred transgenically in the plants that are found to be poor resistant against the attacking microorganism, hence developing a type of passive immunity in most desiring plants and preventing them from loss done by the microorganisms.

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Table 1: recipe for the preparation of Bradford reagent

Solution	Component	Quantity
Solution A	Coomassie Brilliant Blue	100 mg
	95 % ethanol	50.0 ml
Solution B	absolute phosphoric acid	85 ml
	Distilled water	15 ml

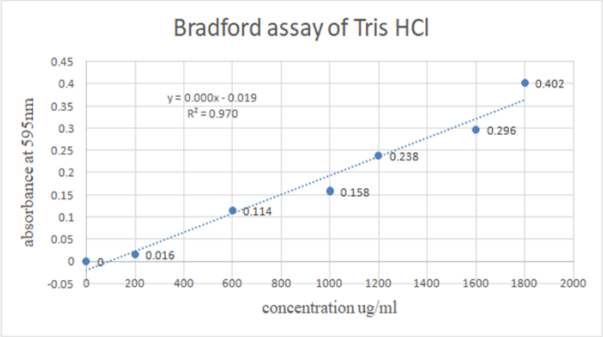
Mixed solution A with solution B and made total volume up to 1000 ml.

Table 2: Absorption of serial dilutions of Bovine Serum Albumin

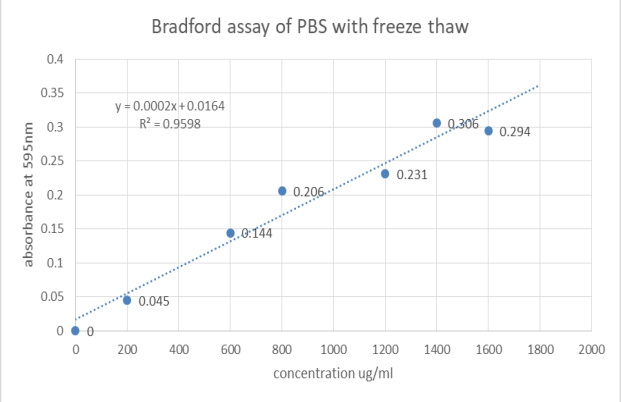
Sample	BSA µg/ml	BSA-Standard µl	amount of buffer	Abs of Tris Hcl	different (PBS)with freeze thaw	buffers (nm) (PBS)without freeze thaw
1 blank	0	0	1000	blank	blank	blank
2	200	100	900	0.016	0.045	0.067
3	400	200	800	0.019	0.183	0.161
4	600	300	700	0.114	0.144	0.201
5	800	400	600	0.127	0.206	0.24
6	1000	500	500	0.158	0.287	0.28
7	1200	600	400	0.238	0.231	0.31
8	1400	700	300	0.167	0.306	0.30
9	1600	800	200	0.296	0.294	0.357
10	1800	900	100	0.402	0.285	0.449

Table 3: readings of the Absorbance and Concentration of proteins

Plants	**Tris buffer HCl**		**PBS buffer with thaw freeze**		**PBS buffer without thaw freeze**	
	abs (nm)	**conc. ug/ml**	**abs (nm)**	**conc. ug/ml**	**abs (nm)**	**conc. ug/ml**
Cassia fistula	**1.195**	**6073**	**0.504**	**2438**	**0.624**	**2019.33**
Albizia lebbeck	**0.697**	**3583**	**0.246**	**1148**	**0.430**	**1372**
Cymbopogon citratus	**0.345**	**1823**	**0.012**	**-22**	**0.107**	**296**
Saccharum officinarum	**0.389**	**2043**	**0.117**	**503**	**0.285**	**889.333**



Graph 1: BSA solution Standard curve in Tris HCl buffer



Graph 2: BSA solution Standard Curve in PBS with alternate freeze thaw cycles