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EXTRACTION AND QUANTIFICATION OF ANTIMICROBIAL PEPTIDES FROM *RICINUS COMMUNIS, CASSIA FISTULA* AND *ACACIA NILOTICA* THROUGH DIFFERENT PHARMACEUTIAL BUFFERS

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ABSTRACT

Background: As humans are gifted by a defense system, Plants also possess a specialized system of defense that helps them to combat attacking microorganisms and harsh environmental conditions. This defense system is in the form of secondary metabolites that are present in the form of antimicrobial peptides. Antimicrobial peptides are specialized cysteine rich residues that play their marked protective role in plant defense systems. **Methods**: In this study, we have extracted antimicrobial peptides from seeds and roots of three medicinal plants *Ricinus communis, Cassia fistula,* and *Acacia nilotica.* Extraction was carried out in two pharmaceutical buffers Tris NaCl and PBS buffer. **Results**: Results indicated that the extracts with PBS buffer without freeze thaw yield more concentrations of 2353 µg/ml, 1508 µg/ml, and 1553 µg/ml of proteins in *Cassia fistula* roots, *Ricinus communis* (seeds), and *Cassia fistula* (seeds) respectively. These concentrations were higher than the proteins extracted by Tris NaCl and PBS buffer with freeze thaw cycles. **Conclusion**: Extraction of these peptides from important medicinal plants and incorporating them in other plants with lesser immunity, can boost the immune system of receptive plants.

Keywords: Antimicrobial Peptides, Tris NaCl, PBS Buffer, *Cassia fistula*, Medicinal plants.

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INTRODUCTION

Plants are exposed to several conditions, these can be climate change, drought, or a microbial attack **[1]**. Plants have to defend themselves against these harmful conditions to survive in nature. Plants have their own defense system that consists of antimicrobial peptides **[2]**. These peptides are proteins in nature that are made up of cysteine residues. There are several classes of antimicrobial proteins like defensins, chitinases etc. all of these have their specific role in the defense of plants **[3]**. Antimicrobial peptides have been extracted from flower, stems, leaves roots and seeds of plants and these peptides show their activities against parasites, bacteria, viruses, fungi and neoplastic cells. Extraction of these antimicrobial peptides has been carried in many researches to check their role in defense and to appreciate their antimicrobial properties **[4]**. Extraction can be carried out through different buffers. In one of the studies, extraction was carried out in Tris NaCl and PBS buffer **[5]**. Extraction of these antimicrobial peptides is opening new ways for pharmaceutical industries. It can help in treating many diseases with the natural way of plants. Plants have the potential to treat many incurable diseases. Several new techniques and novel drug delivery systems have been introduced in the market which are being utilized for the betterment of human health **[6]**. Synthesis of nanoparticles is also a new approach to therapeutics **[7]**. Since pathogenic

resistance mounting gradually against antibiotics, hence consequentially decreases antibiotics efficacy. There we need to locate a natural antibacterial method to crack this problem. In our study, we aimed to extract these antimicrobial peptides from the seeds and roots of three medicinal important plants *Ricinus communis*, *Cassia fistula*, and *Acacia nilotica* from different pharmaceutical buffers to compare the concentration of given peptides in them. So that these peptides can be used for therapeutic purposes and transgenic plants in the future.

MATERIAL METHODS

Collection and Preparation of Plant's Part

Seeds of *Ricinus communis* and *Cassia fistula* were purchased from the market. In contrast, roots of *Cassia fistula* and leaves of *Acacia nilotica* were collected from the nursery of The Women University Multan, and *Ricinus communis* roots were collected from Jahanian **(Fig 1)**. Fresh leaves of *Acacia nilotica* and collected roots and seeds of other respective plants were washed with distilled water and dried under shade for 24 hours. A sample of each dried plant part was ground in a pestle and mortar to convert them into fine powder and proteins were extracted by grinding the powdered leaf sample of each plant in a given buffer. Temperature was maintained up to four degrees to prevent proteins from degradation.

Selection of Pharmaceutical Buffers for Protein Extraction

Two different pharmaceuticals buffers Tris NaCl and PBS were selected for the extraction of antimicrobial peptides from medicinal plants.

Extraction of Proteins by Tris NaCl Buffer

The ground fine powder of collected samples of seeds, roots, and leaves of *Ricinus communis*, *Cassia fistula*, and *Acacia nilotica* respectively was further processed for protein extraction. 1g of each powdered sample was soaked and further ground in Tris-NaCl (PH 8) buffer **[8].** 1.75ml of 0.5M NaCl and 1.75ml of 1M Tris Base and minced by pestle and mortar for isolation of crude protein. Samples were then refrigerated overnight and centrifuged at 12000rpm for 20 minutes in cooling. The supernatant was collected and stored at a temperature of up to four degrees to prevent proteins from degradation. The presence of proteins in samples was confirmed by Bradford assay **(Fig. 2).**

Extraction of Proteins by Phosphate Buffer Saline

Extraction of antimicrobial peptides was also done through PBS buffer. While carrying out this extraction all the samples were allowed to undergo alternate freeze and thaw cycles. Results were then recorded both with and without passing through freeze and thaw cycles **[9].**

Extraction through PBS Buffer under Freeze and Thaw cycles

0.3g of each sample was ground and blended with 4.5ml PBS buffer. Then the mixture was frozen and thawed 3 times. Centrifuged at 10,000rpm for 10 min at 40 ˚C. A supernatant was collected that contained protein confirmed by Bradford assay **(Fig. 3).**

Figure 1: Plant samples 1and 4 shows seeds of C*assia fistula* and *Ricinus communis* respectively while 2 and 3 contain roots of C*assia fistula* and *Ricinus communis* in ground form respectively.

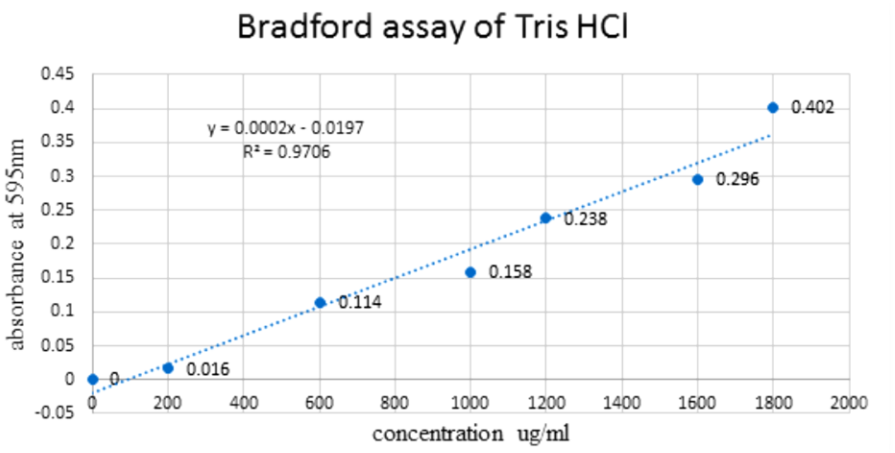

Figure 2: Protein extractions through Tris buffer taking bovine serum albumin as standard.

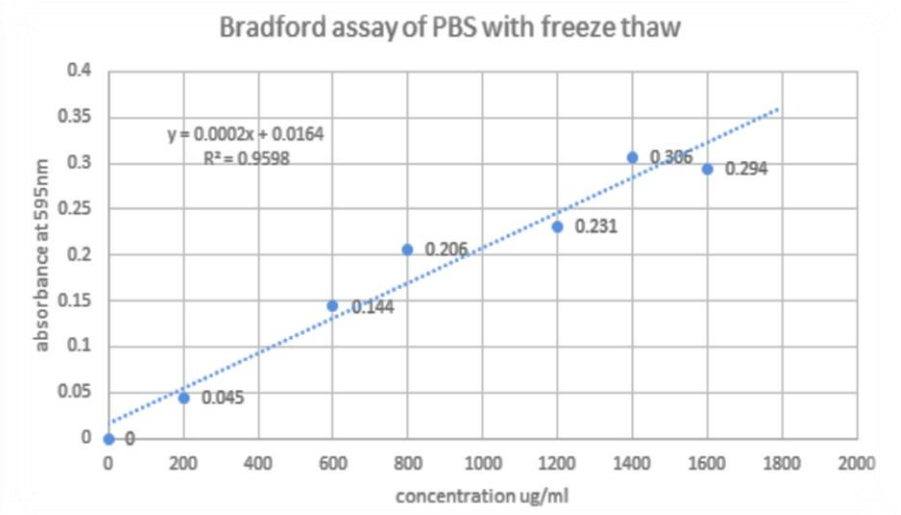

Figure 3: Protein extractions through PBS taking bovine serum albumin as standard.

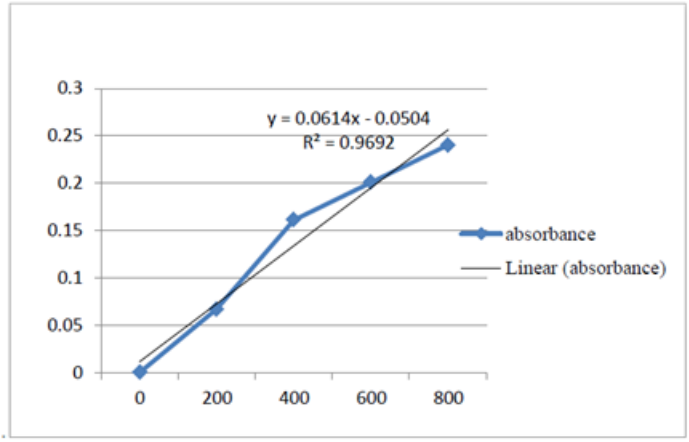

Figure 4: Protein extractions through PBS without freeze thaw taking bovine serum albumin as standard.

Extraction under PBS Buffer without Freeze and Thaw Cycles

0.3g of each sample was taken and passed through the same process but without going through freeze and thaw cycles. The presence of proteins was confirmed by measuring the absorbance of standard bovine serum albumin against plant samples **(Fig. 4).**

Bradford Assay

Bradford assay is performed to quantify the concentration of proteins in an unknown sample. In this study our plant extracts through different buffers had unknown protein concentrations. So Bradford assay was performed to calculate the protein concentrations in a given samples**.** 100 ml of bradford reagent was mixed with 5mg/ml of BSA solution. All the extracted protein samples were passed through the Bradford assay. Bovine Serum Albumin was taken as stock solution and was prepared in the ratio of 2mg/ml for PBS buffer and 1mg/ml for Tris NaCl buffer. Absorbance of the samples was checked at the wavelength of 595nm **[10].**

Quantification of Protein in Samples

To quantify the protein concentration in samples, Bovine Serum Albumin (BSA) was used as standard. BSA stock solution was prepared 5 dilution of BSA was prepared in buffer **(Table 1).** 50µl of each BSA dilution and unknown protein sample were mix with 950µl of Bradford reagent. Then protein concentration was estimated by taking O.D at 595nm in spectrophotometer and draws a graph to find concentration of unknown protein **[11]**.

RESULTS AND DISCUSSION

A number of pathogens spread different diseases in humans as well as in plants. In an attempt to shield from such pathogenic invasions, plants begin to form a network of defense proteins that form part of the general plant defense mechanisms **[12]**. These are proteins that are involved in both antibacterial and anti-fungal roles such as the defensin proteins which are named as antimicrobial peptides. These are effective for both the gram positive and gram negative bacteria **[13]**.

Protein Concentration of Extracts

Crude extracts of seeds and roots of the *Ricinus communis, Cassia fistula* and *Acacia nilotica* leaves were prepared in Tris HCl buffer and in PBS buffer. Protein concentration was estimated by performing Bradford assay using bovine serum albumin as standard. Concentration of proteins was found by using formulae:-

$y = mx + b$

Here x is the concentration of the unknown protein, 'Y' is the absorbance of the sample obtained by spectrophotometer. In the equation 'm' and 'b' represent the coefficients there values are obtained by Microsoft excel software **[14]**.

The absorbance of the samples was checked at the wavelength of 595nm. The maximum concentration of protein was found in the extract of *Cassia fistula* roots by PBS buffer without freeze thaw with the absorption of 0.451 and concentration of 2353 µg/ml, the maximum concentration of *Ricinus communis* (seeds) was also found in extracts with PBS buffer with freeze thaw with the absorption of 0.274 and concentration of 1468 µg/ml. *Cassia fistula* (seeds) also showed a maximum concentration of 1553 µg/ml with the PBS buffer with freeze thaw.

Table 1: BSA dilution for analysis.

Sample	Conc. (µg/ml)	BSA volume (µl)	Buffer volume (µl)
1	0	0	1000
2	200	200	800
3	400	400	600
4	600	600	400
5	800	800	200
6	1000	1000	0

Table 2: Protein concentration of plant samples.

Sample	Tris buffer		PBS buffer with freeze thaw		PBS buffer Without freeze thaw	
	O.D	µg/ml	O.D	µg/ml	O.D	µg/ml
Cassia fistula (roots)	0.187	562.66	0.451	2353	0.426	2228
Cassia fistula (seeds)	0.249	739.33	0.291	1553	0.200	1098
Ricinus communis (roots)	0.078	199.33	0.124	718	0.037	0.056
Ricinus communis (seeds)	0.290	90.6	0.274	1468	0.301	1603
Acacia nilotica (Leaves)	0.282	1508	0.282	1508	0.124	718.5

These results indicated that the extracts with PBS buffer with freeze thaw yield more concentration of proteins than the other two methods of Tris NaCl and PBS without freeze thaw cycles **(Table 2).** Antimicrobial peptides are extracted by different pharmaceutical buffers named Tris NaCl and PBS buffer. These buffers have a significant role in extracting proteins from any sample **[15]**. The quantity of protein extracted by these buffers varies. In the given research the concentration of proteins from PBS buffer was found greater than the Tris NaCl buffer. Similar results were found in the study carried out by Habiba et al., 2021 where they found a higher concentration of proteins in samples extracted by Tris NaCl buffer **[4].** Two processes were utilized for the extraction of proteins from PBS buffer. Extracts were passed through alternate cycles of freeze and thaw for at least three times. While on the other hand, extracts were not passed through the freeze and thaw cycles **[9]**. In order to check the effect of these cycles extraction through PBS was carried out both with and without freeze thaw. Results showed the increased concentration of proteins in extracts with freeze thaw. E.g. the concentration of *Cassia fistula* (seeds) was 1553 µg/ml for PBS with freeze thaw and 1098 µg/ml without freeze thaw cycles. In one of the research extraction of the antimicrobial peptides was done through the leaves of medicinal plants by Tris NaCl and PBS buffer. There extracts showed higher concentrations with Tris NaCl buffer **[4]**. The part of plant used can also affect the amount of concentration in them. Seeds are difficult to crush into fine powder therefore can result in lesser yield of proteins **[16].**

CONCLUSION

Antimicrobial plants though the part of defense system help in defending the plants against several stressful conditions. These antimicrobial peptides can be used for the therapeutic purposes for the cure of many infectious diseases. Isolation of genes of these peptides and their incorporation to other less resistant plants can open the ways towards better crop yield. On the other hand these medicinal plants can also be used against their therapeutic potential for many infectious diseases in humans as well as in plants. Most of these peptides are antibacterial as well as antifungal so they can serve best alternate to hazardous antibiotics.

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