

Original Research Article

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Study of the Optimal Physical Parameters and Action of the Proteolytic Enzyme on the Antimicrobial and Antioxidant Potential of the Protein Isolated from Different Pulses

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ABSTRACT

Keywords

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In the present study, proteins were extracted and purified from germinated seeds viz. *Vigna radiata* (mung bean), *Cicer arietinum* (gram white and gram black). The protein extracted was evaluated for any antimicrobial effects against the different pathogens and to determine antioxidant potential. The studies were performed to assess the effect of pH, temperature and proteolytic enzymes on the antimicrobial nature of the protein extracted. It was found that the extracted antimicrobial protein had prominent antimicrobial effect on the different microbial pathogens at the range of pH 4-7. The studies also showed the strong antioxidant potential of the different seeds protein isolated and purified. It was also observed that the temperature from 25°C-30°C has no adverse effect on the antimicrobial action of the proteins extracted. The results of the present study thus help to explore and formulate the potent antimicrobial agent against different variety of pathogens and to formulate strong antioxidant agent.

Introduction

Plants have been a precious source of natural products for sustaining human health. According to World Health Organization (Anonymous, 2008) more than 80 % of the world's population relies on traditional medicine for their primary healthcare needs.

Therefore medicinal plants need to be investigated for better understanding in respect to their properties, safety and efficiency. One of such properties like antimicrobial activity can be of great

significance in therapeutic treatments. Especially with the emergence of antibiotic resistant microbes which have become a major health threat over the past decades (Wright, 2000). According to Marx (2005), the continuous use of analogous antibiotics has resulted in multi-resistant bacterial strains all over the world. Presently it is being broadly predictable that in the very near future antibiotic resistance will make healthcare professionals helpless toward effective therapies for bacterial infections.

Consequently there is an urgent need to search for unconventional antibiotics. Therefore as an upshot, interest in peptide antibiotics has increased greatly during the past decade, as these are believed to be very potent, showing higher activity, higher specificity, having few toxicology problems, do not accumulate in organs, few drug-drug interaction challenges and are biological and chemical diverse.

It has been shown in certain studies that plants protect themselves against microbial pathogens by various defense responses including production of antimicrobial peptides (AMPs), secondary metabolites, lytic enzymes and membrane-interacting proteins (Feng *et al.*, 2003).

Materials and Methods

Protein extraction

The leguminous seeds were collected from the local markets of Uttarakhand (Images 1 and 2).

The modified strategy used for protein extraction (Gao *et al.*, 2000; Pasha *et al.*, 2016) is described in the following steps-

Seeds were washed with HgCl₂ and further taken for the study (10g-100 g)

Crushed and blended with 150 ml of PBS at 4°C.

Frozen and thawed 3-4 times.

Centrifuged at 10,000 rpm for 10 minutes at 4°C.

After centrifugation, pellet and supernatant fractions were obtained. Supernatant fractions were subjected to 75% ammonium sulphate precipitation while pellet fractions were

discarded. Further centrifugation (10,000 rpm for 1 hour at 25°C) of the ammonium sulphate precipitated fractions was done to obtain the pellet.

The pellet fractions were solubilized in deionized water and were followed for dialysis and SDS PAGE.

Further Gel-column chromatography of the pellet fractions was performed in order to obtain the pure crude fractions.

Furthermore SDS-PAGE analysis of the pure fractions eluted was performed.

Column gel chromatography of eluted protein fractions

About 2 ml having ~500 µg/ml protein concentration of this sample was applied onto the 1.5 x 70 cm column of Sephadex G-100 pre-equilibrated with 0.02M Sodium acetate buffer (pH 4.5). The column was eluted with the same buffer at the rate of 1ml/1min. The elution pattern was monitored by taking absorbance of collected fractions at 280 nm (Jaladi *et al.*, 2015).

SDS-PAGE analysis of eluted protein fractions and Quantitative estimation by Folin-Ciocalteu's reagent

The fractions along with crude extract and solubilized ammonium sulphate precipitated protein were run on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% - 14% gel by method described (Laemmli, 1970). The gels were stained with Coomassie brilliant blue R-250 and destained with methanol: acetic acid: water (30:60:10 v/v). The approximate molecular weight of fractions activity was determined by plotting R_f versus molecular weight of known standard proteins. Protein fractions isolated and purified from seeds was

determined (Lowry *et al.*, 1951). Absorbance was measured at 750 nm in UV-VIS spectrophotometer.

Determination of antibacterial activity

Culture Media

For antibacterial test, Soyabean Casein Digest agar/broth of Hi Media Pvt. Bombay, India was used.

Inoculum

The bacteria viz. *Bacillus subtilis*, *Bacillus (spreader)* and *Micrococcus luteus* were inoculated separately into Soyabean casein digest broth and incubated at 37 °C for 18 h and suspension was checked to provide approximately, 10⁵ CFU/ml.

Determination of diameter of zone of inhibition by well diffusion method

The agar well diffusion method (Perez *et al.*, 1993) was modified. Soyabean casein digest agar medium (SCDM) was used for bacterial cultures. The culture medium was inoculated with the bacteria separately suspended in nutrient broth.

A total of 8 mm diameter wells were punched into the agar and filled with protein extracts prepared in N-saline at 1.0 mg/ml concentration. Standard antibiotic (Erythromycin, 1 mg/ml) was simultaneously used as the positive control. The plates were incubated at 37 °C for 18 h.

The antibacterial activity was evaluated by measuring the diameter of zone of inhibition observed. The procedure for assaying antibacterial was performed in triplicates to confirm the readings of diameter of zone of inhibition observed for each of the test organism.

Determination of antioxidant activity

Estimation of Total Phenolic Content (TPC) of protein (s) isolated

The total phenolic content of the protein fractions were determined by the method of Singleton and Rossi (1965). The phenolic content was expressed as mg/g gallic acid equivalents. In brief 100 µl aliquots of the sample were added to 2 ml of 0.2 % (w/v) Na₂CO₃ solution.

After 2 minutes of incubation, 100 µl of 500 ml/1 Follin-Ciocalteu reagent were added and the mixture was allowed to stand for 30 minutes at 25 °C. The absorbance was measured at 750 nm using a UV-VIS Systronics spectrophotometer. The blank consist of all reagents and solvents but no sample. The total phenolic content (TPC) was determined using the standard gallic acid calibration curve.

Determination of superoxide anion radical scavenging activity

Superoxide anion radical scavenging activity was measured with some modifications (Duan *et al.*, 2007). The various fractions of proteins were mixed with 3 ml of reaction buffer solution (pH, 7.4) containing 1.3 µM riboflavin, 0.02 M methionine and 5.1 uM NBT separately.

The reaction solution was illuminated by exposure to 30W fluorescent lamps for 20 minutes and the absorbance were measured at 360 nm using a spectrophotometer. Ascorbic acid was used as positive control and the reaction mixture without any sample were used as negative control. The percent inhibition of superoxide anion radical scavenging activity (%) was calculated as:

$$[(A_o - AS) / A_o] \times 100$$

Where, A_0 = absorbance of positive control;
 A_S = absorbance of sample

Determination of total antioxidant activity

Total antioxidant activities of protein fractions isolated and standard ascorbic acid were determined. An aliquot (0.1M) of these fractions were combined with 1ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate).

The tubes were then after capped and incubated at 95°C for 90 minutes. After that the sample were cooled at 25°C, the absorbance were measured at 695 nm against blank. The blank contained 1ml of reagent solution without sample. The total antioxidant activity was expressed as an absorbance value at 695 nm. Higher absorbance value indicates the maximum antioxidant activity.

Effect of enzyme, temperature and pH on the antimicrobial and antioxidant activities of eluted protein fractions

Protein enzymes (Corolase/Neutral protease and papain) were used for treating the protein fractions eluted in order to check their effect on antimicrobial and antioxidant activities (Carol *et al.*, 2005). Purified peptide samples of 3-20µg/10µl were mixed in 0.02 M Tris-HCl, pH 6.8 and then 1-2 U/2µl of enzyme was added into the mixture having final reaction volume 100µl and kept for 5 minutes.

The activity of enzyme was stopped by heating the solution in thermo mixer for 5 minutes. The effect of various temperature and pH was also checked on antimicrobial and antioxidant activities of purified peptide(s). The purified peptides were mixed in 0.02M Tris-HCl, pH 6.8 and were heated to different temperatures viz. 0, 4, 25, 50°C for 10 minutes and then bioassayed for both the

activities. Similarly, the pH stability of purified peptides was determined between the different ranges of pH viz. 4, 5, 6, 7 and 8 using 0.02M sodium acetate and Tris-HCl as buffer solution.

Results and Discussion

In the present study, the protein extracted from the seeds of leguminous plants viz. Mung bean, White and brown gram were screened for antimicrobial activity against the selected contaminants found in molasses and food material. The protein extracted was also check for stability profile by treatment at variable temperature, pH and proteolytic enzymes.

It was found that the proteins extracted from seeds showed prominent antimicrobial and antioxidant potential against the pathogens studied. It was also observed that the antimicrobial and antioxidant potential of these isolated protein samples changes as per the variation in pH, temperature and action of proteolytic enzymes.

The results are shown in tables 1-8 and figures 1-7. Previous studies have reported many antimicrobial peptides isolation from common vegetables and spices (Ngai and Ng, 2004; Hu *et al.*, 2004; Oard *et al.*, 2004; Xia and Ng, 2005; Ngai *et al.*, 2005; Mariângela *et al.*, 2006).

Previous studies by our group have already described the isolation, characterization and antimicrobial nature of the protein isolated from *Vigna radiata* (Jabi and Mathur, 2017). Plants are constantly challenged by pathogens, but the onset of plant disease is the exception rather than the rule. The reason for this being that the constant arms race between pest and plant host has led to the development of an efficient plant defense system.

Table.1 Standard biochemical features of the protein isolated from pulses seeds

Types of seeds protein	Strains	Diameter of zone of inhibition (mm)	
		Enzymes	
		After treatment with Papain	After treatment with Neutral Protease
Mung bean seed protein	<i>Micrococcus luteus</i>	40.0	40.0
	<i>Bacillus subtilis</i>	40.0	30.0
	<i>Bacillus spreader</i>	30.0	25.0
Gram white seed protein	<i>Micrococcus luteus</i>	30.0	11.0
	<i>Bacillus subtilis</i>	20.0	16.0
	<i>Bacillus spreader</i>	11.0	13.0
Gram brown seed protein	<i>Micrococcus luteus</i>	35.0	15.0
	<i>Bacillus subtilis</i>	25.0	11.0
	<i>Bacillus spreader</i>	20.0	15.0

Table.2 Antimicrobial activity of the protein samples extracted

Parameters determined	Inference
Colour	Dirty white as appeared after ammonium sulphate precipitation
Solubility	100% soluble
pH	6.23

Table.3 Effect of proteolytic enzymes on the antimicrobial activity of the extracted protein

Types of seeds protein	Strains	Diameter of zone of inhibition (mm)
Mung bean seed protein	<i>Micrococcus luteus</i>	40.0
	<i>Bacillus subtilis</i>	59.0
	<i>Bacillus (spreader)</i>	49.0
Gram White seed protein	<i>Micrococcus luteus</i>	40.0
	<i>Bacillus subtilis</i>	25.0
	<i>Bacillus (spreader)</i>	25.0
Gram brown seed protein	<i>Micrococcus luteus</i>	40.0
	<i>Bacillus subtilis</i>	28.0
	<i>Bacillus (spreader)</i>	25.0
Erythromycin (1 mg/ml)	<i>Micrococcus luteus</i>	45.0
	<i>Bacillus subtilis</i>	32.0
	<i>Bacillus (spreader)</i>	27.0

Table.4 Effect of temperature on the antimicrobial activity of the extracted protein

Types of seeds protein	Strains	Temperature			
		4°C	25°C	30°C	50°C
Mung bean seed protein	<i>Micrococcus luteus</i>	12.0	22.5	24.0	9.0
	<i>Bacillus subtilis</i>	13.0	26.0	27.5	5.0
	<i>Bacillus spreader</i>	10.0	28.0	28.0	7.0
Gram White seed protein	<i>Micrococcus luteus</i>	12.0	25.0	26.0	10.0
	<i>Bacillus subtilis</i>	13.0	26.0	28.0	8.0
	<i>Bacillus spreader</i>	10.0	25.0	27.0	6.0
Gram Brown seed protein	<i>Micrococcus luteus</i>	15.0	18.0	19.0	10.0
	<i>Bacillus subtilis</i>	11.0	18.0	17.0	8.0
	<i>Bacillus spreader</i>	16.0	19.0	17.0	10.0

Table.5 Effect of pH on the antimicrobial activity of the extracted protein

Types of seeds protein	Strains	pH			
		2	4	5	7
Mung bean seed protein	<i>Micrococcus luteus</i>	20.0	22.0	24.0	23.0
	<i>Bacillus subtilis</i>	20.0	25.0	25.0	22.0
	<i>Bacillus spreader</i>	11.0	24.0	25.0	13.0
Gram White seed protein	<i>Micrococcus luteus</i>	NA	20.0	25.0	30.0
	<i>Bacillus subtilis</i>	NA	20.0	25.0	30.0
	<i>Bacillus spreader</i>	NA	18.0	20.0	22.0
Gram Brown seed protein	<i>Micrococcus luteus</i>	12.0	15.0	17.0	18.0
	<i>Bacillus subtilis</i>	12.0	14.0	16.0	17.0
	<i>Bacillus spreader</i>	20.0	21.0	23.0	25.0

Table.6 TPC ($\mu\text{g/g}$ gallic acid equivalents) of extracted protein samples

Protein samples/Standard	TPC ($\mu\text{g/g}$ gallic acid equivalent)
Standard (Gallic acid)	55.56
Mung bean seed protein	25.0
Gram White seed protein	28.0
Gram Brown seed protein	22.2

Table.7 Percent inhibition of superoxide anion radical scavenging Activity of extracted protein samples

Protein samples/Standard	Percent inhibition of superoxide anion radical scavenging activity
Ascorbic acid	65.67
Mung bean seed protein	56.45
Gram White seed protein	58.23
Gram Brown seed protein	62.34

Table.8 Total antioxidant activity of extracted protein samples

Protein samples/Standard	Total antioxidant activity (A695 nm)
Ascorbic acid	0.06
Mung bean seed protein	0.04
Gram White seed protein	0.07
Gram Brown seed protein	0.05

Image.1 Mung bean seeds



Image.2 Bengal gram (white and brown)



Fig.1 (a) Graphical representation of the protein samples isolated and purified

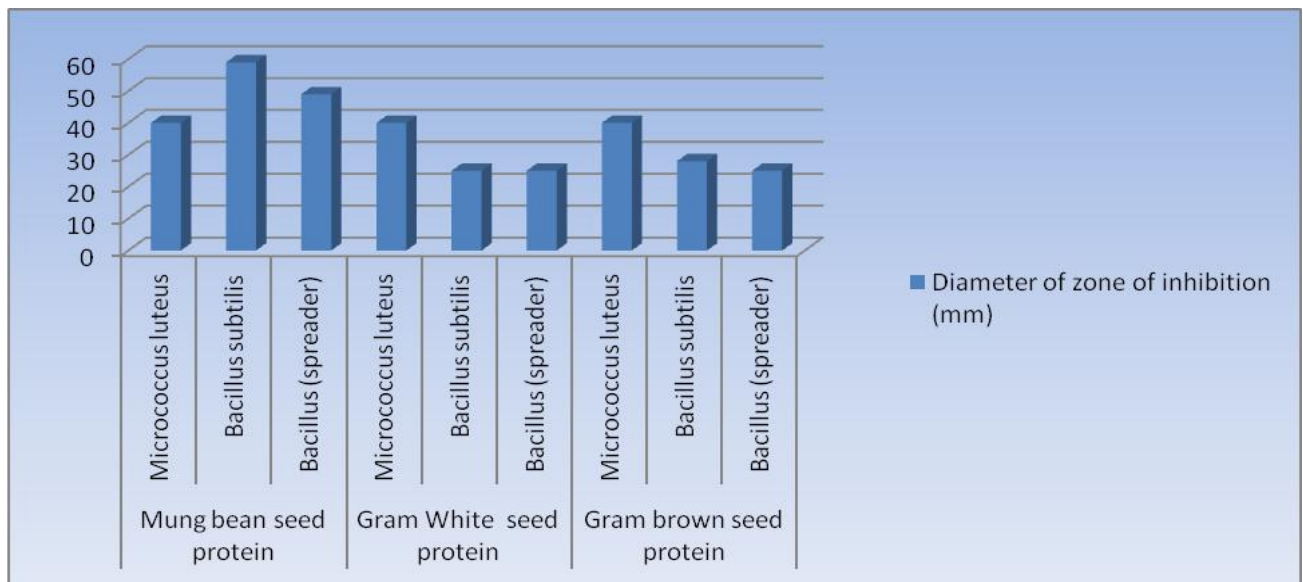


Fig.1 (b) Antimicrobial activity of protein samples isolated and purified

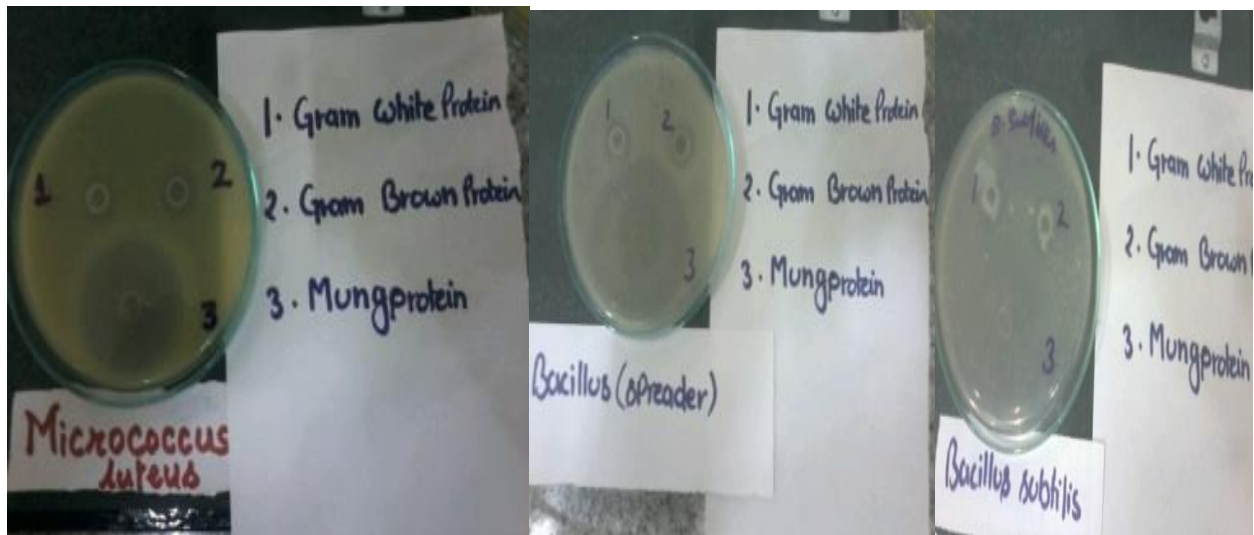


Fig.2 Graphical representation of the effect of proteolytic enzymes on the antimicrobial activity of the extracted protein

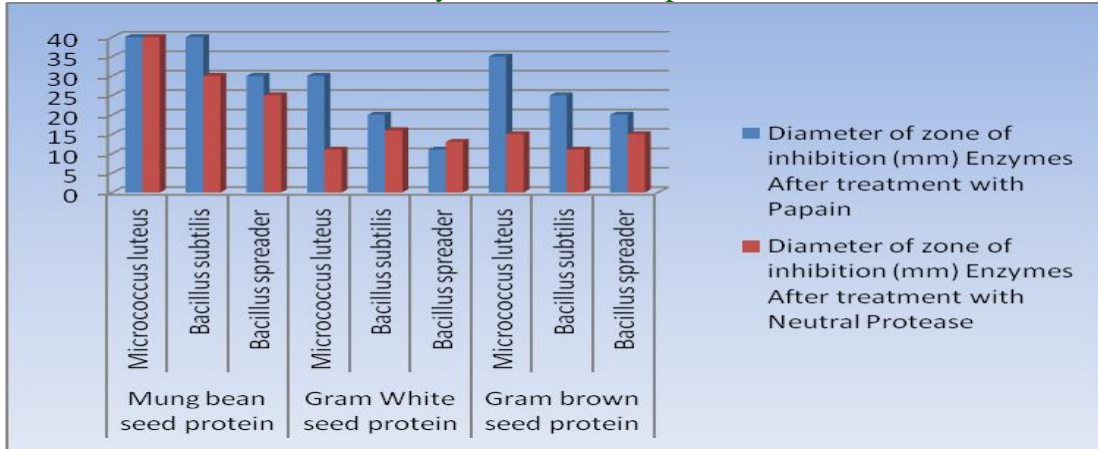


Fig.3 Graphical representation of effect of temperature on the antimicrobial activity of the extracted protein

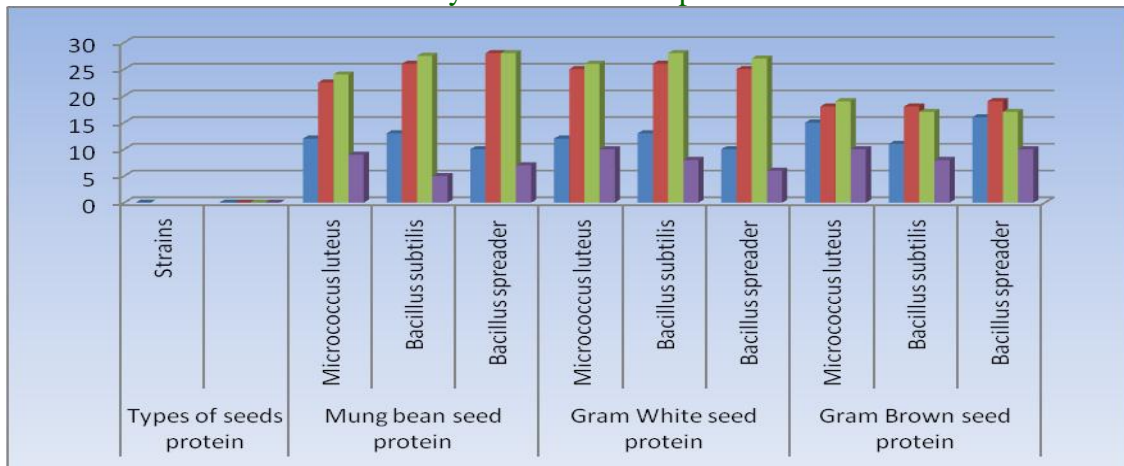


Fig.4 Graphical representation of the effect of pH on the antimicrobial activity of the extracted protein

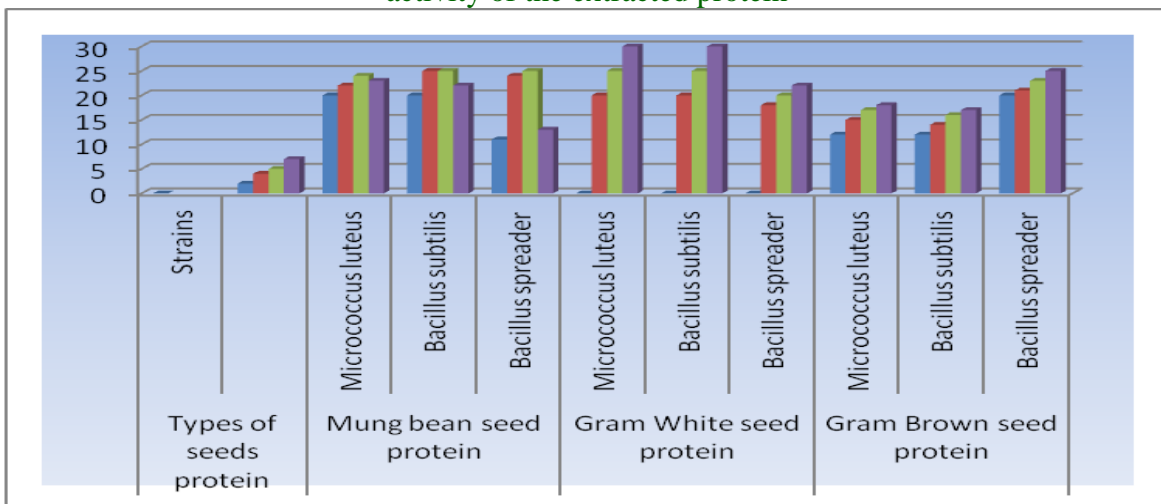


Fig.5 Graphical representation of TPC ($\mu\text{g/g}$ gallic acid equivalents) of extracted protein samples

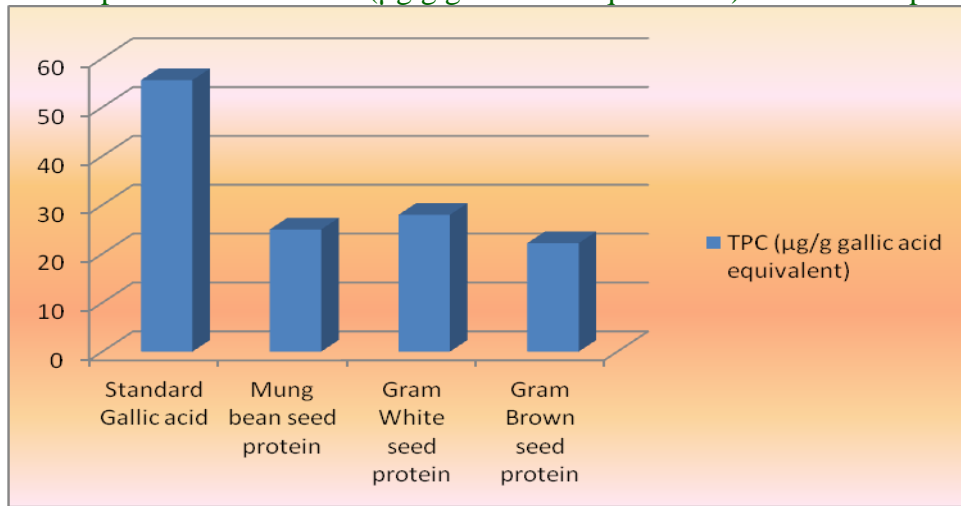


Fig.6 Graphical representation of percent inhibition of superoxide anion radical scavenging activity of extracted protein samples

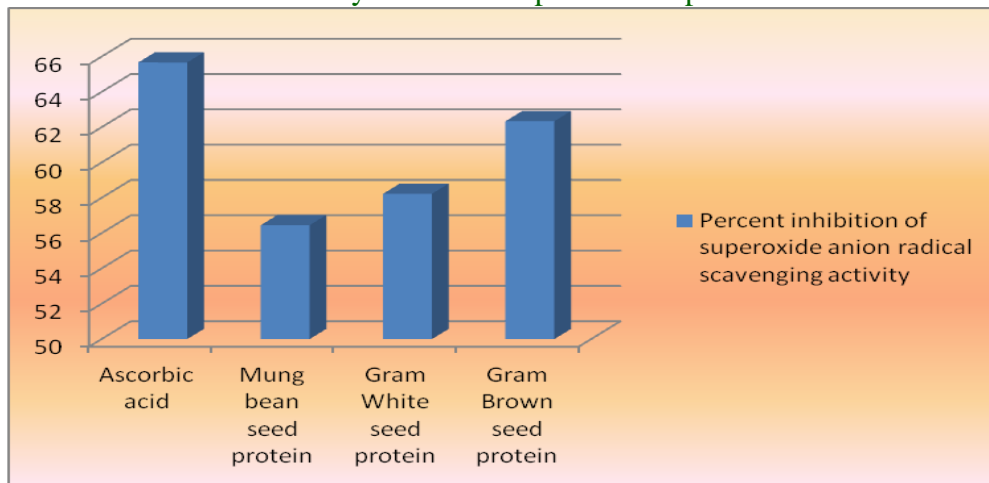
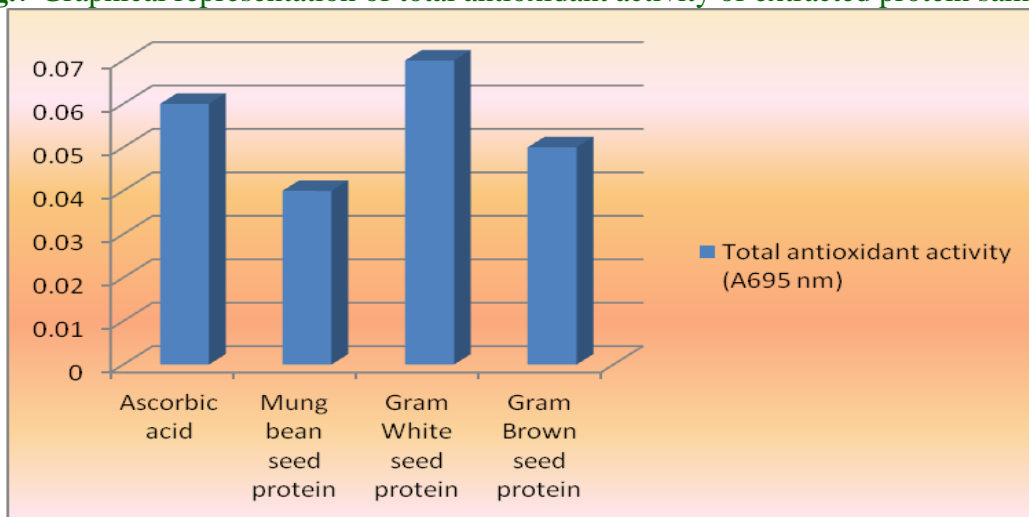


Fig.7 Graphical representation of total antioxidant activity of extracted protein samples



In conclusion, the present study suggests the importance of protein in leguminous pulses seeds. The results describe the potent antimicrobial and antioxidant properties in these proteins. The results thus suggested that, these proteins after purification can act as strong antimicrobial and antioxidant agent. The identification of sequence of amino acids in these proteins and their comparative representation can tell us the types of amino acids responsible for such pharmacological action. However structure elucidation of such proteins will also be a strong parameter to establish the structure-activity relationship of these proteins.

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