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**Research Article**

# PROTEOMIC ANALYSIS TO IDENTIFY POSSIBLE CALCIUM BINDING PROTEINS IN MAJOR PULSES COLLECTED FROM NAINITAL DISTRICT OF UTTARAKHAND

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**ABSTRACT**

The proteomic analysis for identification of calcium binding proteins (CBPs) could be useful for development of nutraceuticals and food to food fortification. The present investigation was aimed to explore the proteomic study to identify CBPs in major pulses available in Nainital District. Pulses were collected from local market and farmers of Nainital region of Kumaun. All physiological parameters were examined as per standard protocols. Total buffer soluble proteins (TBSPs) were quantified with the help of Bradford assay. TBSPs were checked their quality in discontinuous system of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel with staining Coomassie Brilliant Blue dye. CBPs were examined by the staining with the stains-all staining. In this study the major finding is that the pulses are the rich source of soluble protein (6.57 to 11.18mg/g) and CBPs. One to three distinct bands were observed after stains-all staining of SDS-PAGE gel. This is the first hand report on CBPs report of major pulses collected from Nainital of Kumaun region. The study would be useful to the researchers of this field to generate information on nutraceuticals for curing malnutrition including farmers. Present study generated significant information on CBPs including the hope to convert the staple crop in to cash crop to marginal farmers.

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## INTRODUCTION

Nainital located on 29º00' and 29º05' north latitude, 80º14' and 78º80' east longitude and 1,938 meter asl in Kumaun region of Uttarkhand. Nainital is known as a cold area (Nainital, statmagzine, 2011). The major food of study area for vegetarians is vegetables, pulsed, cereals and millets. However, their survival may be compromised by cooking practices, food handling out and environmental circumstances (Andre *et al.,* 2017). Over 60% of total use of pulses is for human consumption, but the significance of pulses in human diets varies from region to region and country to country, with a general trend of higher consumption in lower income nations. The share of food use in total use of pulses in the developing countries is over 75 percent, compared to 25 percent in the developed countries (Odendo *et al.,* 2011). Major legumes and cereals are better adapted than the crops to extreme soil and climatic circumstances, with high tolerance to abiotic environmental stresses such as drought. As a stress response they can also create compounds with pharmaceutical significance. However, greater investment of resources and manpower are necessary if the latent of orphan legumes is to be unlocked and applied in the prospect (Cullis and Kunert, 2017). Cells are prepared with highly proficient mechanisms to perceive, transduce and counter to a wide variety of inner and outer signals during their development. Perception of signals via receptors results in generation or synthesis of non-protein or less protein molecules often termed messengers such as calcium which control diverse cellular processes through calcium sensors also known as calcium binding proteins (Nath *et al.,* 2010). Calcium binding proteins (CBPs) have a vital role in calcium homeostasis by buffering and probably also have a neuroprotective function. Fluctuations in intracellular calcium ($Ca^{2+}$) are central to orderly neurotransmission and the operation of a wide range of cellular functions (Kris van Kuyck *et al.,* 2009). Attempts were made to identify the CBPs in pulses using proteomics approaches.

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## MATERIALS AND METHODS

***Sample collection:*** Cultivars of pulses (Fig.1) were collected from local farmers from different locations of Kumaun districts of Uttarakhand (Tab.1) during the harvesting seasons.

**Table 1** Name of collected samples and physiological parameters of collected samples (pulses) and colour coding (Munsell color system (1994).

| S. No. | Common Name of Cultivars | Scientific Name | Seed colour with code |
|---|---|---|---|
| 1. | Moong 1 | (Green Gram), *Vigna radiata* | 10YR,8/6 |
| 2. | Hari Moong 2 | (Green Gram), *Vigna radiata* | 2.5Y,6/3 |
| 3. | Soyabean | (Soya Bean), *Glucine max* | 10YR,8/5 |
| 4. | Arhar | (Red Gram), *Cajanus cajan* | 10YR, 8/6 |
| 5. | Lobhiya | (Cow Pea), *Vigna unguiculata* | 2.5Y, 8/4 |
| 6. | Chana | (Chick Pea), *Cicer arietinum* | 2.5Y, 7/6 |
| 7. | Urad | (Black Gram), Vignamungo | 2.5Y, 4/1 |
| 8. | Masoor/Malka | (Red Split Lentil), Lens culinaris | 7.5YR, 7/6 |
| 9. | Chana Daal | (Split Bengal Gram), *Cicer arietinus* | 10YR, 8/6 |
| 10. | Rajma1 | (Kidney Bean), *Cicer arictinum* | 5YR, 6/6 |
| 11. | Rajma2 | (Kidney Bean), *Cicer arictinum* | 10YR, 8/4 |
| 12. | Rajma3 | (Kidney Bean), *Cicer arictinum* | 10YR, 8/6 |
| 13. | Rajma4 | (Kidney Bean), *Cicer arictinum* | 10YR, 8/6 |
| 14. | Rajma5 | (Kidney Bean), *Cicer arictinum* | 10YR, 8/4 |
| 15. | Rajma6 | (Kidney Bean), *Cicer arictinum* | 2.5Y, 8/6 |
| 16. | Rajma7 | (Kidney Bean), *Cicer arictinum* | 10YR, 6/5 |
| 17. | Bhatt1 | (Black Soyabean), *Glycine max* | 10YR, 3/1 |
| 18. | Bhatt2 | (Black Soyabean), *Glycine max* | 10YR, 3/1 |
| 19. | Bhatt3 | (Black Soyabean), *Glycine max* | 10YR, 3/1 |
| 20. | Gahat1 | (Horse gram), *Macrotyloma uniflorum* | 2.5Y, 8/5 |
| 21. | Gahat2 | (Horse gram), *Macrotyloma uniflorum* | 2.5Y, 4/1 |

**Figure 1** Sample collection sites (dotted black) Nainital district of Uttarakhand.

**Figure 2** Total 21 samples of pulses collected from different locations of Nainital, Kumaun region, Uttarakhand.

***Seed diameter analysis:*** Thickness of seeds was measured in contrast of length and width with the help of screw gauge in millimeter (mm). Following formulas were used for calculation:

$$\textbf{Least Count} = \frac{\text{No. of reading on main scale}}{\text{No. of reading on circular scale}} \text{ (mm)}$$

**Thickness** = Reading on main scale + Reading on circular scale X least count (mm)

***Isolation of Total Buffer Soluble Proteins (TBSP):*** 1 g of defatted powder of selected seeds was transferred in Oakridge tubes. 0.1M PMSF (100 µl) with 2ml of chilled extraction buffer (5mM-EDTA, 50mM-NaCl, 25mM-Sodium phosphate, pH-7.2). Mixture was Vortexes by hand tabbing followed by centrifuging all samples 10,000g for 15min at 4°C. The supernatant was collected in fresh tubes and stored at -20°C. The Total Protein was run in 12% SDS-PAGE gel to find good resolution, the banding pattern observed with the gel documentation system (Alpha Innotech).

***Protein quantification and SDS PAGE:*** Total protein was quantified according to Bradford assay (Bradford, 1976) and 20µg/well protein in 12 % SDS-PAGE gel run according to Laemmli, 1970.

***Stains-all based identification of CBPs:*** Stains-all is a metachromatic cationic carbocyanine dye, and used for the identification of CBPs in various organisms (Campbell et al., 1983). The protocol for Stains-all was adapted from Goldberg and Warner (1997). After electrophoresis, the gels were rinsed three times with 25% (v/v) isopropanol followed by washing in 30–50 ml of the same solution on a shaker for 10min. The cycle of rinsing (three times) and washing was repeated three times (30 min. incubation time). Beside this a slight modification was done in time of 25% isopropanol washing in 30–50 ml on a shaker for 30min instead of 10 min (90 min. incubation time). This procedure ensures the removal of all SDS, which, if present, would cause the precipitation of the Stains-all dye. Isopropanol was then replaced by 30 ml of Stains-all solution (30mM Tris, 7.5% (v/v) formamide, 25% (v/v) isopropanol, adjusted to pH 8.8 with HCl, followed by addition of 0.025% (w/v) Stains- all). Due to the photosensitivity of Stains-all, gels were incubated in light-tight containers on an orbital shaker at room temperature normally for at least 2 h.

***Sequential extraction of seed storage proteins:*** All 21 genotypes of pulses were tested for their Seed storage protein (SSP) profiles. Seed storage proteins (Albumin, Globulin, Prolamin and Glutelin) were also extracted and subjected for their protein profiles. Prolamins were extracted from ball-milled seed (10 g), which was defatted with chloroform (100 ml) and air-dried. Albumins and globulins were extracted by stirring with 1 M NaCl (100 ml) for 1 h and centrifuged (10,000rpm for 15 min), the supernatant solutions were dialysed and freeze-dried. The pellet was washed with water and prolamins extracted with 70% (v/v) aqueous ethanol (100 ml for 1 h each), followed by 50% (v/v) aqueous propan-1-ol, 2% (v/v) acetic acid and 2% (v/v) 2-mercaptoethanol (100 ml for 1 h). The respective supernatants were dialysed in a low MW cutoff membrane (LA387-5MT, HIMEDIA) and freeze-dried. Glutelin-alkali soluble fraction, the insoluble residue

obtained after the above extraction was extracted with 20 ml of 0.2% NaOH. Proteins were analysed on 15% (w/v) acrylamide SDS–PAGE gels and quantified with Bradford method, based on the system of Laemmli (Laemmli, 1970; Ayyangar, 1934; Kumar *et al.,* 2011).

***Data analysis:*** Data analyzed to obtain the statistically significant or non-significant with the help of Microsoft excel and Jaccard's coefficients among the genotypes by using NTSYS-pc (version 2.11W; Exeter Biological Software) (Tatham *et al.,* 1996). All values were taken in triplicate for statistical analysis.

## RESULTS

The results of present investigation are summarized in Tab.2 and Bhatt1 and Bhatt3 gave the promising results as increased amount of total buffer soluble protein (TBSP) and significant reduction in respect of seed weight and seed size. Some results showing that the negative correlation between thickness and length of seed (Fig.3) and it was found that positive correlation between number of seeds and ten seed weight (Fig. 4 and 5).

**Table 2** Physiological parameters and protein analysis of collected samples (Pulses). Sample -17 showing significant amount of TBSP. All values taken in triplicate and ±SE were calculated with the help of Microsoft excel. Star (**) indicate highest amount of protein, *n=10.

| S. No. | Cultivars | Seeds*/g | Seeds weight (g) | Seed size (mm) | TBSP (mg/g) |
|---|---|---|---|---|---|
| | | Mean ±SE | | | |
| 1. | Moong (1) | 38.00±0.57 | 0.253±0.010 | 10.65±0.75 | 06.57 |
| 2. | Hari Moong (2) | 59.00±0.57 | 0.177±0.008 | 07.15±1.75 | 07.20 |
| 3. | Soyabean | 07.67±0.33 | 1.297±0.120 | 10.50±2.87 | 09.91 |
| 4. | Arhar | 14.00±0.57 | 0.766±0.040 | 08.16±2.80 | 07.63 |
| 5. | Lobhiya | 04.33±0.33 | 1.716±0.050 | 12.83±1.21 | 07.62 |
| 6. | Chana | 03.67±0.33 | 2.117±0.010 | 12.41±0.61 | 08.13 |
| 7. | Urad | 15.67±0.88 | 0.684±0.020 | 10.50±0.75 | 06.21 |
| 8. | Masoor/Malka | 27.00±0.57 | 0.371±0.005 | 04.25±1.14 | 07.22 |
| 9. | Chana Daal | 14.00±0.00 | 0.823±0.010 | 10.25±1.50 | 08.52 |
| 10. | Rajma1 | 02.67±0.33 | 3.149±0.210 | 12.25±0.90 | 07.33 |
| 11. | Rajma2 | 02.00±0.00 | 6.016±0.110 | 12.58±1.12 | 06.89 |
| 12. | Rajma3 | 03.00±0.00 | 2.713±0.050 | 10.16±3.04 | 07.62 |
| 13. | Rajma4 | 02.33±0.33 | 4.083±0.260 | 08.41±1.67 | 07.92 |
| 14. | Rajma5 | 03.67±0.33 | 2.350±0.030 | 11.83±1.88 | 07.13 |
| 15. | Rajma6 | 03.67±0.66 | 2.901±0.040 | 12.16±2.61 | 06.64 |
| 16. | Rajma7 | 02.33±0.33 | 4.158±0.080 | 11.08±3.70 | 07.25 |
| 17. | Bhatt1 | 08.67±0.88 | 0.999±0.140 | 08.33±2.46 | 11.18** |
| 18. | Bhatt2 | 07.67±0.33 | 1.046±0.200 | 10.00±2.70 | 09.97 |
| 19. | Bhatt3 | 08.67±0.33 | 1.140±0.020 | 11.83±3.19 | 10.85 |
| 20. | Gahat1 | 28.00±1.00 | 0.403±0.007 | 07.91±2.09 | 07.44 |
| 21. | Gahat2 | 28.00±0.57 | 0.372±0.010 | 10.26±3.76 | 07.56 |

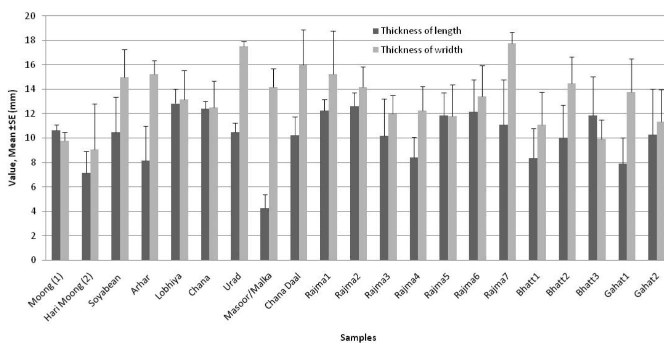


**Figure 3** Bar diagram showing the thickness of seeds. (It was measured in contrast of length and width with the help of screw gauge in millimeter (mm).

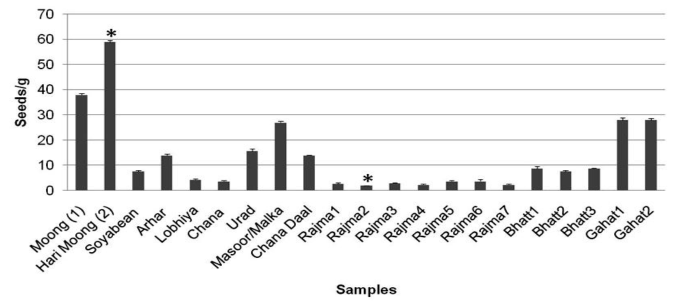


**Figure 4** Number of seeds in one gram of collected pulse samples. Graph plotted of Mean±SE value.

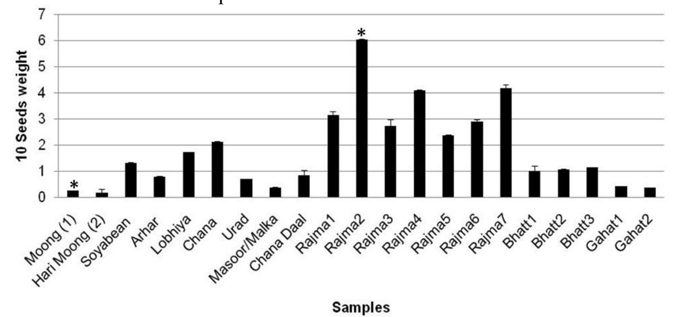


**Figure 5** 10 seeds weight of collected pulse samples. Graph plotted of Mean±SE value. Seed weight of Rajma2 is significantly high in compare to Moong (1), p<0.005.

***Isolation of TBSP:*** The isolated total protein was divided in three groups that are first (Low), Second (medium) and third (high). First group have 14 samples (protein content 6-8 mg/g seeds), second group have three samples (protein content 8-10 mg/g seeds), third group have (protein content 10-12 mg/g seeds) and finally it may say that Bhatt1 (*Glycine max*) has significant amount of TBSP.

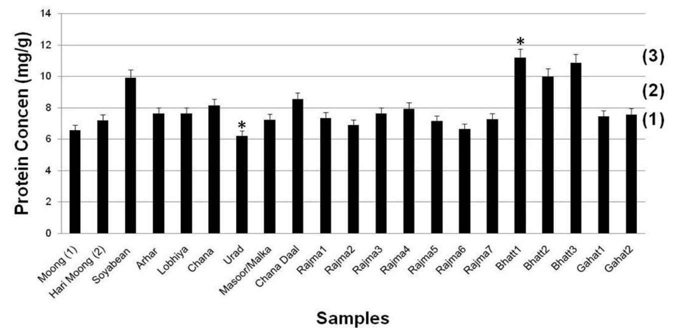


**Figure 6** Total buffer soluble protein (TBSP) isolated from pulses of Nainital district of Uttarakhand. According to above figure TBSP were divided in to three groups that are 6-8 (1), 8-10 (2) and 10-12 (3). 14 samples lie in group one, 03 samples in group two and 04 samples in group three. TBSP of Bhatt1 is significantly high in compare to Urad if degree of freedom is less than 0.005 (p<0.005).

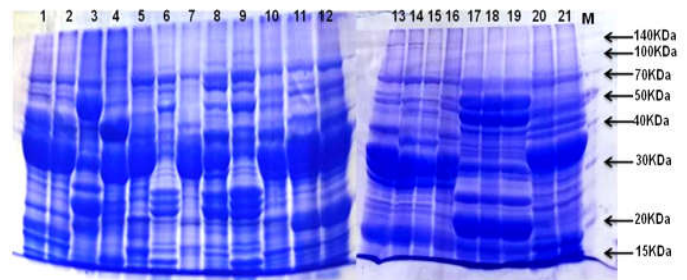


**Figure 7** SDS-PAGE of total buffer soluble protein of isolated from pulses collected from Nainital district of Uttarakhand. Gel loading according to table1 and lane M is denoting the medium range protein molecular weight marker. 20µg/well protein was loaded and run in 12% separating gel.

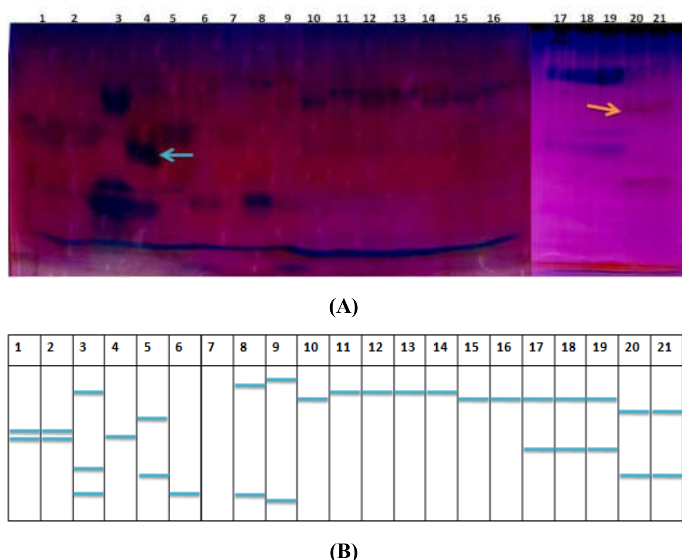


**Figure 8** Stains-all staining the SDS-PAGE gel of total buffer soluble protein of isolated from pulses. (A). Gel loading according to table1 and 20µg/well protein was loaded and run in 12% separating gel. (B). Electrophorogram for CBPs which are present in gel. Arrows indicate the CBPs (Blue) and Non-CBPs (Pink).

***Sequential extraction of seed storage proteins (SSP):*** After extraction of SSP the variation was found in quantity of protein (Fig. 9). According to figure—it is indicated that in between collected samples-17 (Bhatt-1, *Glycine max*) is most prominent in term of SSP and in other term it may say that sample-17 having maximum amount of Albumin and globulin but lower in prolamin and glutelin. In other hand sample-1 (Green Gram, *Vigna radiate*) have highest in albumin but others were lowest. Overall it may say that sample-17 is significantly rich in SSP in compare others (Fig. 9).

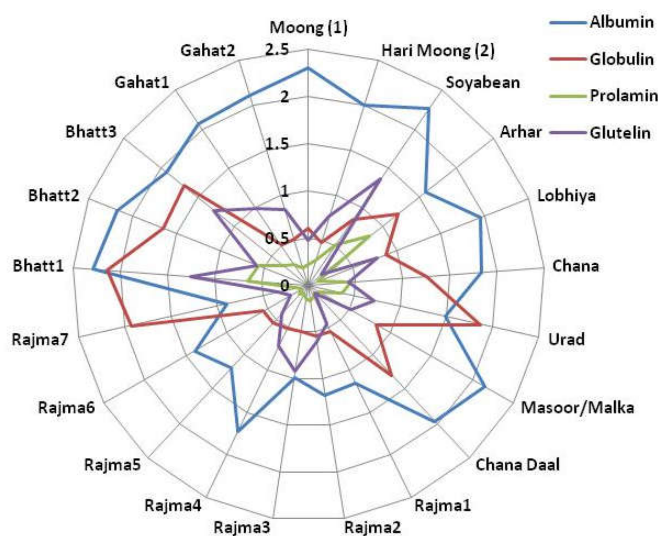


**Figure 9** Spider Web chart showing the concentration of seed storage proteins in collected study materials from Nainital district of Uttarakhand.

## DISCUSSION

The arithmetical evidences pointed out that share of pulses in the per capita food expenditure in India has reduced from 40 to 28 per cent between 2000 and 2010 while that of high value products including fruits and vegetables rose from 36 per cent to 42 per cent during the same period. Therefore, future of agriculture and food sector will rest on crop diversification towards high value crops and higher value addition (Usha Tuteja, 2013). During the present study investigators analyzed that all CBPs were shown molecule weight between ~15KDa to ~94KDa. In a proteomic study a protein (~44KDa) of horse gram showed its distinct appearance when we screened the seeds from high elevation to low elevation. Three blue bands (CBPs) appear in all samples after stains-all staining. One high molecular weight protein (~43KDa) and two low molecular weight proteins (~25.1 and ~17KDa) were observed. SDS-PAGE technique of seed proteins can be economically used to assess genetic variation and relation in Cultivars and also to make distinction mutants from their parent genotype, seed protein based marker can be used for identification of genotype in future (Sharma *et al*., 2018). CBPs contain a highly conserved helix–loop–helix structure or EF hand motif. Typically, EF hand motifs occur in pairs (EF hand domain) and make easy the cooperative binding of two $Ca^{2+}$ ions per domain (Luan *et al*., 2002). Compartmentalization of CBPs such as calretinin and calbindin 28KDa has been noted within cells, suggesting that these proteins perform distinct functions in localized calcium signaling (Mojumder *et al*., 2008). Nutraceuticals have shown a great promise in the recent past with some plant made pharmaceuticals in clinical trials and many other under investigation (Thomas *et al*., 2002). Secondary metabolites which are stress biochemical shaped by the plant systems to defend the plants from the biotic or abiotic stresses have been a great source of pharmaceuticals and typically provide a lower cost of production relative to the cell culture system currently used to produce biological therapeutics. According to the Ahmed *et al*., 2016; most kidney stones are calcium stones, combined with oxalate, phosphate, or occasionally uric acid. Oxalate is a naturally occurring substance found in food. CBPs may bind on calcium and free the calcium from binding with oxalate. Simultaneously CBPs facilitate free calcium for other body metabolic uses for the body like obesity, diarrhea, asthma etc.

## CONCLUSION

Pulses have very important role in nutraceuticals and help in curing the protein malnutrition. Stains-all is a strong dye to preliminary identification of CBPs. The present investigation is able to give the lead in the form of CBPs to evaluate their sequences. This is first hand report on CBPs of pulses of Nainital district. Moreover seed protein based marker can be used for identification of local cultivars in future. This study will be helpful for researchers and farmers too for future research and possible cash cropping and nutraceutical farming in hills of Uttarakhand.


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