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Tryptic and chymotryptic inhibitory study of selected vigna protease inhibitors

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Abstract
Plant Protease inhibitors (PIs) are the mostly studied defense proteins of the Plants. They are highly present in seeds and other storage tissues which represents up to 10% of the total protein (Casaretto and Corcuera 1995). Protease inhibitors carry out some physiological changes in the insect pest by inhibiting their gut tryptic and chymotryptic proteinases. This adversely affect their protein digestion. The insect resorts the overproduction of proteases to compensate for the inhibited activity leading to deficiency of essential amino acids. This exerts additional physiological stress resulting in inhibition of growth.
The results obtained in the present study opens new perspectives for utilization of protease inhibitors from the Vigna species in agriculture sector. Total 16 Vigna accessions were studied to find out its tryptic and chymotryptic inhibitory activity. The electrophoretic study of the 16 different Vigna genotypes was performed out of which 3 genotypes found to be the potent tryptic and chymotryptic inhibitors.

Keywords: *Electrophoretic,* protease Inhibitors, vigna

Introduction
Every year, repeated practices of cultivation of the similar population, considerable agricultural losses occur. Such cultivation practices are prone to more incidence of insect pests (Hilder and Boulter, 1999; Oerke *et al*., 1994; Smith, 1999) [6, 7, 8]. To solve such problems, high use of synthetic chemicals as fertilizers, insecticides, herbicides, fungicides are in practice. This creates excessively high pressure on the surrounding environment and destabilizes the ecological balance. Due to the use of traditional pest control method and conventional pesticides, most of which are non-specific and affects the entire community, pollutes the agricultural-ecosystem, and increases the cost of production.
As PIs are abundant proteins in plant tissues and are highly active against insects, bacteria & fungi, the genes encoding PI may be transferred in plants which may yields promising results in the field of agriculture. The approach has several advantages over the standard method to control insect pests and is ecologically safe (Dunaevsky *et al*., 2005) [3].

Material and Methods
The genus Vigna belongs to Fabaceae, formerly called Leguminosae was used as the source material to screen for protease inhibitory activity. Some of the wild Vigna seeds were collected from Melghat Region of Maharashtra and rest of the Vigna accessions were provided by National Bureau of Plant Genetic Resources Regional Station, Vellanikkara, Thrissur-680656, Kerala (Ref. No. IV-8/96-Seeds/1363). The seeds of selected Vigna accessions were studied electrophoretically to find out the protease inhibitors activity.

Defatting & extraction of proteinase inhibitors
The seeds of different genotypes of *Vigna* were washed, dried and grounded to a fine powder in a pestle & mortar separately. The seeds powder of each genotype was dehydrated, depigmented and defatted with the help of acetone and is followed by hexane for six times. The solvents were removed by filtration and tissue powder was air dried. The defatted seed sample was suspended in 0.1 M sodium-phosphate buffer (SPB) (pH 6.8) for 24 hours at 4^0C for extractions of seed proteins with intermittent shaking. The flour to buffer ratio was 2:3 (W/V). 10 ml of 1% PVP (Poly Vinyl Pyrrolidine) was added to the above mixture for removal of phenols of the flour. The resulting suspension was centrifuged at 12,000 rpm speed for 15 minutes at temperature 4^0C and the supernatant was used for protein analysis and also stored at -20^0C. The proteins estimation was done by Bradford method (Bradford, 1976).

Estimation of Protein
The protein was estimated by the Bradford method (1976). This method involves the addition of an acidic dye to protein solution and with the help of ELISA reader subsequent measurement at 595 nm was done.

Standard curve
Bovine Serum Albumin (BSA) was used as a standard protein to get standard curve.

1. 1 mg/ml solution of BSA was prepared & taken in wells of microplate in different volumes ranging from 1 µl to 10 µl, to make the concentrations of BSA from 10 µg to 100 µg; each concentration was replicated three times.
2. Volume of the (BSA) was adjusted to 20 µl by adding 0.15 Normal NaCl.
3. 200 µl ready use Bradford reagent (diluted 1:4 with distilled water) was added in each well.
4. Microplate was incubated for 15 min at room temperature.
5. Absorbance of the proteins was recorded at 595 nm on ELISA Reader
6. Calibration curve was plotted between mean value of concentration on X – axis and mean value of absorbance on Y – axis.

Quantification of protein from unknown sample

The dye reagent was prepared by diluting one part Bradford dye reagent (5X) with 4 parts of distilled water.
1. 10 µl of enzyme extract was added to each well.
2. 10 µl of 0.15 N NaCl was added in to that.
3. After, 200 µl 1X Bradford dye agent was added to each well.
4. The above mixture was incubated at room temperature for 15 minutes.
5. The absorbance was recorded at 595 nm on (Parkin elmer) Microplate reader.

Three experimental replications were maintained in this experiment.

Electrophoretic visualization of PIs

PIs extracted from different Vigna in 0.1 M sodium-phosphate buffer (SPB) (pH 6.8) were diluted each in 100 µl of 1X sample buffer and 25 µl of this sample mixtures were separately electrophoresed on non-reducing 10 % SDS- polyacrylamide. SDS-polyacrylamide gel was washed in 2.5% (v/v) Triton X-100 for 10 min to remove SDS after electrophoresis. The gel was stained with Coomassie brilliant blue R-250 (CBB R-250) for 1 hour and destained till the bands became clear.

Visualization of Tis and Cis

Visualization of Trypsin inhibitor isoforms (TIs) and Chymotrypsin inhibitor isoforms (CIs) was carried out on Gelatin-PAGE same as that of Non-reductive SDS-PAGE having gel co-polymerized with 1% gelatin. PIs extracted from different Vigna in 0.1 M sodium-phosphate buffer (SPB) (pH 6.8) were diluted each in 100 µl of 1X sample buffer and 25 µl of this sample mixtures were separately electrophoresed on 10 % SDS-polyacrylamide (Gujar *et al* 2004) [4].

The gel was be equilibrated in 0.1M Tris-HCl buffer, pH 7.8 for Trypsin and chymotrypsin inhibitory activity after electrophoresis. The respective gels were transferred to the solutions containing 0.1% trypsin and 0.1% chymotrypsin proteases and incubated for 1 to 2 Hour with constant shaking. Later on, the gels were washed with warm water, fixed in 10%TCA, stained with Coomassie Brilliant Blue R-250, and then destained. Dark blue bands of unhydrolyzed gelatin appeared at site of PI activity against light blue background (Harsulkar *et al*., 1999) [5].

Results and Discussions

Protein estimation in different *Vigna* genotypes

Quantification of proteins in different *Vigna* genotypes by Bradford's method extracted in 0.1 M sodium-phosphate buffer, pH 6.8. In the present investigation, it was found that the proteins found in *V. unguiculata cylindrica, V. unguiculata sps., V. sublobata, V. radiata* and *V. unguiculata* (Red Seed) was more as compared to remaining *Vigna* genotypes. Lowest amount of protein was found in *V.vexillata.*

Table 1

Sr. No.	Vigna Genotypes	Protein Concentration ± SE (µg g^{-1}seed flour)
1	*V. hainiana*	69.14
2	*V. unguiculata (Red Seed)*	72.04
3	*V. unguiculata cylindrica*	85.82
4	*V. aconitifolia*	69.13
5	*V. unguiculata sps.*	80.0
6	*V. sublobata*	78.35
7	*V. radiata*	77.05
8	*V. mungo*	70.56
9	*V. pilosa*	66.04
10	*V. vexillata*	30.13
11	*V. mungo var. sylvestris*	66.03
12	*V. trilobata*	55.40
13	*V. dalzelliana*	49.20
14	*V. radiata var. setulosa*	43.1
15	*V. umbellata*	48.21
16	*V. bourneae*	57.29

Electrophoretic visualization of protein profile of *Vigna*

Using gel documentation system molecular weight of each band was characterized. The alpha imager 2000 was the computer software used for the analysis purpose. The comparisons were made between the control set of protein bands with the bands obtained from the sample. On the basis of electrophoretic visualization, it was observed that sample 3 has highest protein concentration followed by sample 1, 2, 4, 5, 6, 7, 8, 9 and 11. Sample 16 has less protein concentration.

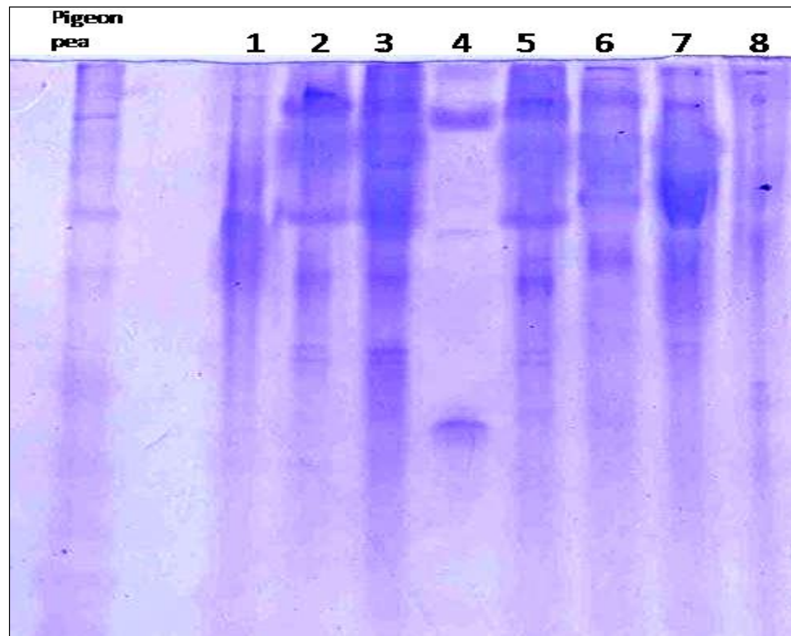
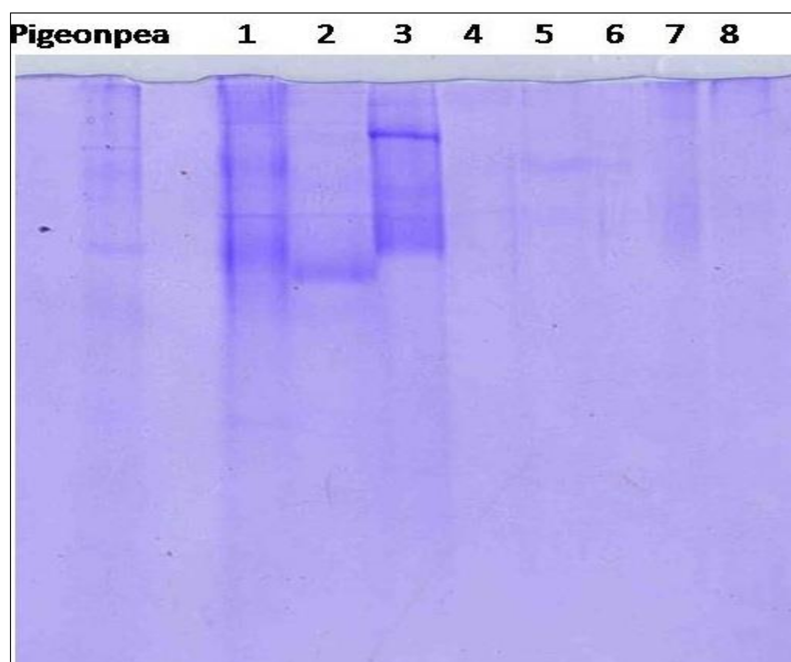


Fig 1: Electrophoretic visualization of PIs of *Vigna*

Standard: Pigeon Pea Markers
Lane 1: Sample 1: *V. hainiana*
Lane 2: Sample 2: *V. unguiculata (Red Seed)*
Lane 3: Sample 3: *V. unguiculata cylindrica*
Lane 4: Sample 4: *V. aconitifolia*
Lane 5: Sample 5: *V. unguiculata sps.*
Lane 6: Sample 6: *V. sublobata*
Lane 7: Sample 7: *V. radiata*
Lane 8: Sample 8: *V. mungo*



Purified PI (Trypsin assay)

Fig 2: Electrophoretic visualization of PIs of *Vigna*

Standard: Pigeon Pea Markers
Lane 1: Sample 9: *V. pilosa*
Lane 2: Sample 10: *V. vexillata*
Lane 3: Sample 11: *V.mungo var. sylvestris*
Lane 4: Sample 12: *V. trilobata*
Lane 5: Sample 13: *V. dalzelliana*
Lane 6: Sample 14: *V. radiata var. setulosa*
Lane 7: Sample 15: *V. umbellata*
Lane 8: Sample 16: *V. bourneae*

Visualization of Trypsin inhibitor isoforms (TIs) from *Vigna*

Visualization of Trypsin inhibitor isoforms (TIs) was carried out on Gelatin-PAGE same as that of Non-reductive SDS-PAGE having gel co-polymerized with 1% gelatin. The results are shown in the banding patterns of the TIs of *Vigna*. Sample 1, 2, 4, 6, 7, 8, 9, 10, 11, 14, 15 and 16 showed presence of dark bands of the trypsin inhibitor isoforms whereas sample 3, 12 and 13 showed very light TIs bands. The slow-moving bands showed smearing effect in the gel.

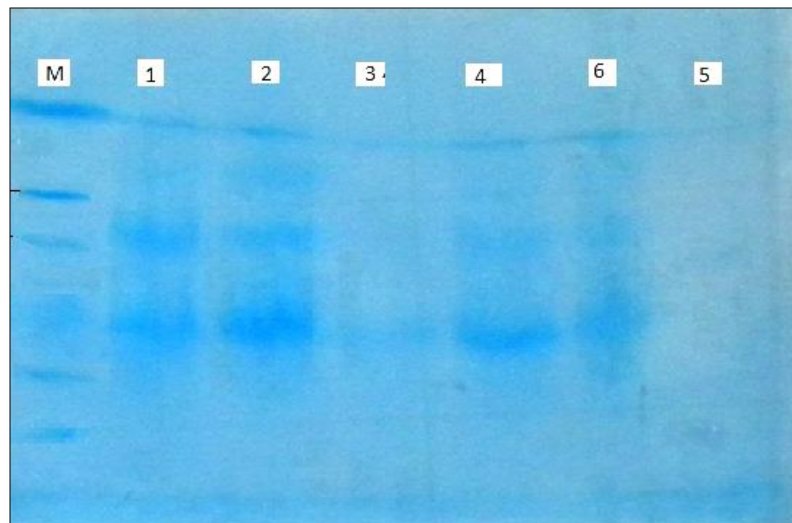


Fig 3: Electrophoretic Visualization of Trypsin inhibitor isoforms (TIs) from *Vigna* genotypes

Standard: Markers
Lane 1: Sample 1: *V. hainiana*
Lane 2: Sample 2: *V. unguiculata (Red Seed)*
Lane 3: Sample 3: *V. unguiculata cylindrica*
Lane 4: Sample 4: *V. aconitifolia*
Lane 5: Sample 5: *V. unguiculata sps.*
Lane 6: Sample 6: *V. sublobata*

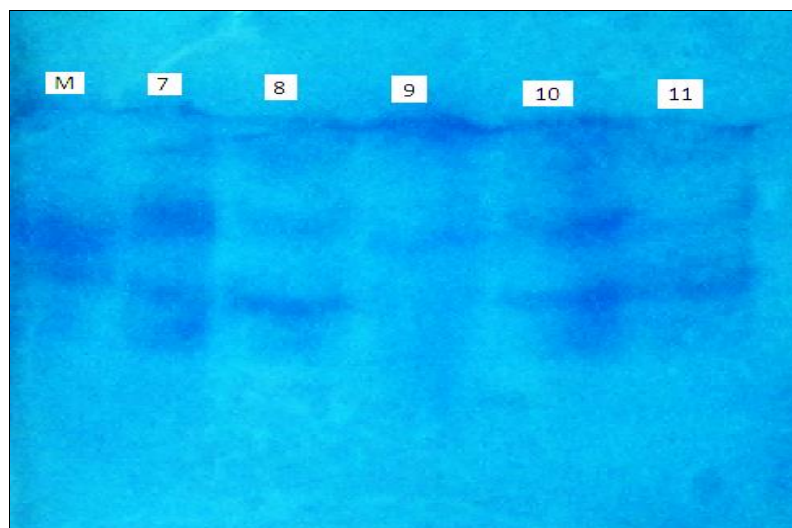


Fig 4: Electrophoretic Visualization of Trypsin inhibitor isoforms (TIs) from *Vigna* genotypes

Standard: Markers
Lane 7: Sample 7: *V. radiata*
Lane 8: Sample 8: *V. mungo*
Lane 9: Sample 9: *V. pilosa*
Lane 10: Sample 10: *V. vexillata*
Lane 11: Sample 11: *V.mungo var. sylvestris*

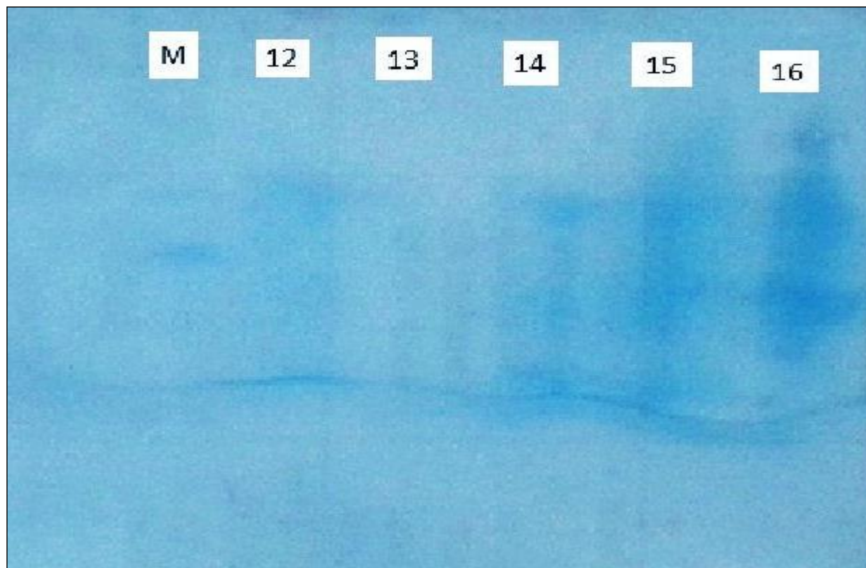


Fig 5: Electrophoretic Visualization of Trypsin inhibitor isoforms (TIs) from *Vigna* genotypes

Standard: Markers
Lane 12: Sample 12: *V. trilobata*
Lane 13: Sample 13: *V. dalzelliana*
Lane 14: Sample 14: *V. radiata var. setulosa*
Lane 15: Sample 15: *V. umbellata*
Lane 16: Sample 16: *V. bourneae*

Visualization of Chymotrypsin inhibitor isoforms (CIs) from *Vigna*

Visualization of Trypsin inhibitor isoforms (CIs) was carried out on Gelatin-PAGE same as that of Non-reductive SDS-PAGE having gel co-polymerized with 1% gelatin described in the chapter, "Materials and Methods". The results are shown in the banding patterns of the CIs of *Vigna*. Sample 1, 2, 4, 5, 7, 8, 10, 11, 12, 13 and 14 showed presence of dark bands of the chymotrypsin inhibitor isoforms whereas sample 3, 6, 9, 15 and 16 showed very light CIs bands. The slow-moving bands showed smearing effect in the gel.

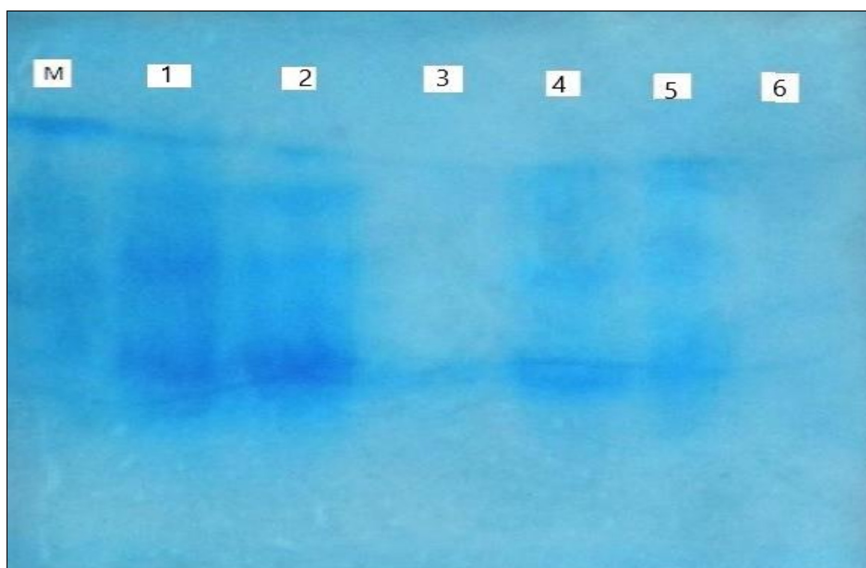


Fig 6: Electrophoretic Visualization of Chymotrypsin inhibitor isoforms (CIs) from *Vigna* genotypes

Standard: Markers
Lane 1: Sample 1: *V. hainiana*
Lane 2: Sample 2: *V. unguiculata (Red Seed)*
Lane 3: Sample 3: *V. unguiculata cylindrica*
Lane 4: Sample 4: *V. aconitifolia*
Lane 5: Sample 5: *V. unguiculata sps.*
Lane 6: Sample 6: *V. sublobata*

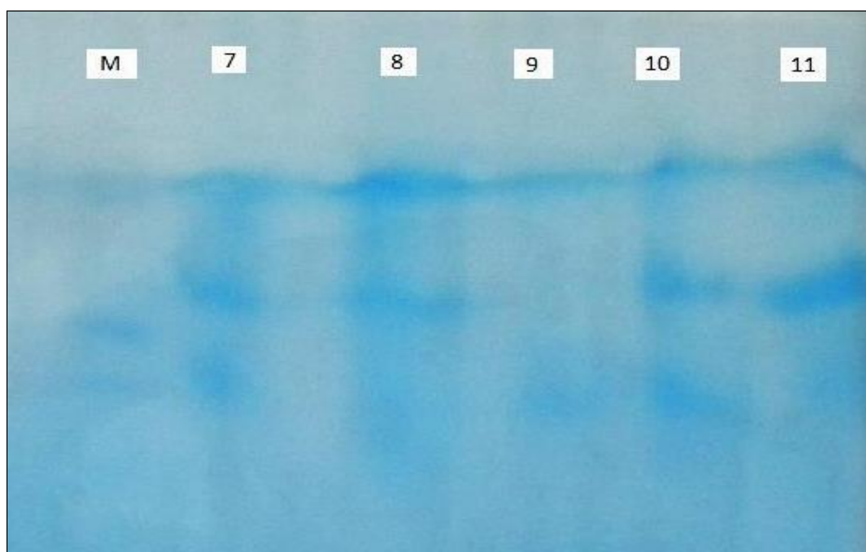


Fig 7: Electrophoretic Visualization of Chymotrypsin inhibitor isoforms (CIs) from *Vigna* genotypes

Standard: Markers
Lane 7: Sample 7: *V. radiata*
Lane 8: Sample 8: *V. mungo*
Lane 9: Sample 9: *V. pilosa*
Lane 10: Sample 10: *V. vexillata*
Lane 11: Sample 11: *V.mungo var. sylvestris*

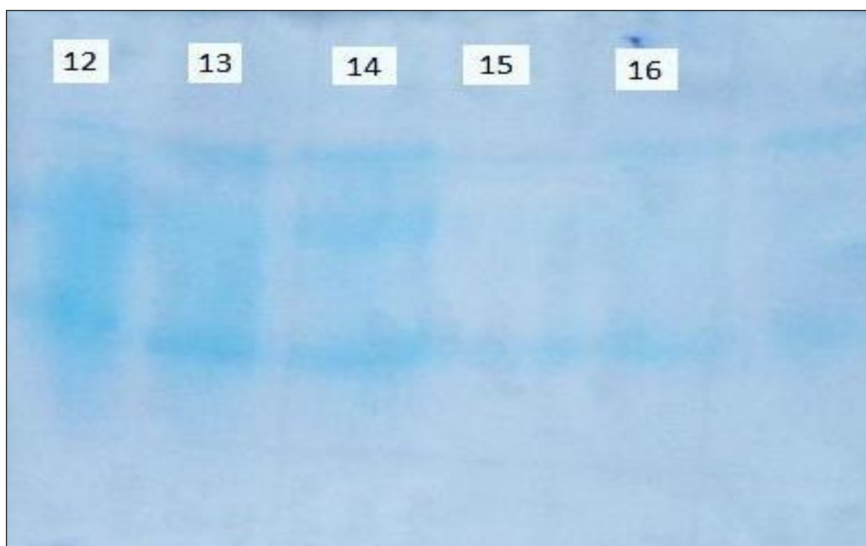


Fig 8: Electrophoretic Visualization of Chymotrypsin inhibitor isoforms (CIs) from *Vigna* genotypes

Standard: Markers
Lane 12: Sample 12: *V. trilobata*
Lane 13: Sample 13: *V. dalzelliana*
Lane 14: Sample 14: *V. radiata var. setulosa*
Lane 15: Sample 15: *V. umbellata*
Lane 16: Sample 16: *V. bourneae*

Conclusion

Protease inhibitors are important tools for regulating the target proteases. They play a crucial role in controlling many physiological functions. The results obtained in the present study opens new perspectives for utilization of protease inhibitors from the Vigna species in agriculture sector. Out of the 16 different Vigna genotypes studied, 3 genotypes found to be the potent tryptic and chymotryptic inhibitors. Therefore, it is important to study Plant Proteases as the potential source of protease inhibitors that may results in overcome of the problem of insect adaptation to the defense mechanism of host plant. There is also a lot of scope for further research which involves protein engineering as well as development of transgenic crops encoding genes for protease inhibitors.

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