CYTOTOXICITY AND IN-VITRO ANTIVIRAL ACTIVITY OF LECTIN FROM *CROCUS VERNUS* L. AGAINST POTATO VIRUS Y

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Abstract. Plant lectins are the potential proteins that specifically bound and cross linked with carbohydrates and target particular glycans present on the cell surface of viruses through a pathway termed as a lectin activation pathway. They have a role in plant defense system. In this study, we evaluated anti-*PVY* (potato virus Y) activity of plant derived lectin in in-vitro assay. A tetramer lectin was isolated from the corms of *Crocus vernus* and purified to sub fractions through ammonium sulfate precipitation, dialysis, filtration on the PD 10 column, cation exchange chromatography on SP-Sepharose gel. In-silico studies predicted stable interaction between *Crocus vernus* lectin (CVL) and targeted protein of *PVY*. Cytotoxicity tests performed on HepG2 cells indicated IC50 value for CVL was 770 µg/ml. The IC50 values indicated the safe limit and non-cytotoxic effect of the CVL extract and worth for further testing. The hemagglutination activity of CVL against the rabbit erythrocytes was revealed to be 100 µg/ml. The anti-*PVY* activity displayed in the CVL lectin was evaluated through quantitative real-time PCR assay. It was revealed that CVL was effective against targeted gene of *PVY* at 30, 60 and 90 µg/ml concentration and up to 100% inhibition of the *PVY* mRNA was achieved in all three tested concentrations in comparison with control. Our results clearly indicate the CVL gene can be a used for transformation studies in potato to control the potato virus Y as an effective tool, in the future.
Keywords: *Crocus vernus lectin (CVL), potato virus Y, real time PCR, coat protein, antiviral activity, in silico analysis*

Introduction

Mannose-binding lectins prove to be of immense importance and potency in preventing and treating virus-mediated infections as they specifically target particular glycans present on the cell surface which are required by virus for entry (Balzarini et al., 2007). A variety of lectins have already been reported from different organisms belonging to a wide range of taxonomical groups, including plants, animals, bacteria, fungus and algae (Golotin et al., 2019; Singh et al., 2018; Sreeramulu et al., 2018; Muslim et al., 2018; Zhang et al., 2017a). The structure, efficacy and specificity of these lectins may vary from source to source. Till to date, lectins have been published from different plant families, including *Alliaceae, Amaryllidaceae, Araceae, Bromeliaceae,*

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Iridaceae, Liliaceae, and *Orchidaceae* and their activity has been reported against Human Immunodeficiency Virus, Simian Immunodeficiency Virus and Feline Immunodeficiency Virus (Lam and Ng, 2001; Davidson et al., 2000).

Potato virus Y (*PVY*) belongs to the genus *Potyvirus* of the *Potyviridae* family (Kitajima et al., 1997). Worldwide a number of crops are affected in a severe damaging way by this virus, especially solanaceous plants like potato (*Solanumtuberosum* L.), tobacco (*Nicotianatabacum* L.) and pepper (*Capsicum annuum* L.). *PVY* symptoms vary from barely visible mosaic patterns to an extreme necrosis and premature death of plants (Tabassum et al., 2016). *PVY* infection is difficult to control because it is vegetatively propagated, making primary viral infection more destructive and consistent generations after generations (Gargouri-Bouzid et al., 2005). In Pakistan, due to early infection of *PVY* loss is about 70% (Abbas et al., 2017). Among the crucial genes of *PVY*, capsid protein (CP) gene is involved in the encapsulation, in replication of the viral genome, movement across host cells, and transfer from infected to uninfected plants using mobile biological vectors (Callaway et al., 2001).

Several approaches have been tested to date to produce high yielding commercial varieties with advance levels of resistance, including genetic amendments of the *PVY* coat protein (CP) gene or P1 gene (Gargouri-Bouzid et al., 2005; Mäki-Valkama et al., 2000). Resistance exhibited by gene silencing is more effective as compared to protein mediated resistance (Mäki-Valkama et al., 2007).

In Pakistan, commercially offered high yielding potato lines fail to show resistance against viruses, especially the potato virus Y. In the current study, we aimed to find out the effect of mannose-binding lectin from the bulbs of *C. vernus* activity against the *PVY* coat protein gene.

Materials and methods

Sequence retrieval and modeling of CP protein

The full-length amino acid sequence of potato virus Y, coat protein (Accession # ABK13679.1) was retrieved from the NCBI database and functional domains of Coat Protein (CP) were determined using an online InterPro server (www.ebi.ac.uk/interpro/).

Crocus vernus lectin (CVL) molecular structure, coordinate information (PDB ID: 3MEZ) was downloaded from the protein database (http://www.rcsb.org/structure/3MEZ) and the CP structure was generated using the online tool (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). The validity of the predicted model was checked by calculating the Ramachandran plot which was produced by online tool RAMPAGE (http://mordred.bioc.cam.ac.uk/~ rapper/rampage.php).

Refinement, evaluation, and validation of CP protein model

Using the ModRefiner online tool (https://zhanglab.ccmb.med.umich.edu/ModRefiner/), the CP model was further refined. Using Ramachandran plot and by determining the physiochemical properties of the CP protein through ProtScale, the model was evaluated and authenticated.

Protein-protein docking analysis of CP and CVL

Online server Z-DOCK (http:// zdock.umassmed.edu/) was used for docking of receptor model to fusion protein and ten coordinate files were obtained. PDBePISA

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online tool (http://www.ebi.ac.uk/pdbe/pisa/) was used to find the interactions between the CP and CVL. For the visualization of structures showing the interactions between the two proteins the PDB viewer was used.

Protein source, extraction and fractionation of extract

Crocus vernus (L.) Hill corms were kindly provided by Dr. Ahmed Akrem (Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan-Pakistan). Briefly, 10 g of corms were reduced to powder and homogenized in 0.1 M phosphate buffer (pH 6.5). The slurry was stirred continuously at 4 °C for 2 h. Further, the suspension was filtered through the muslin cloth and centrifuged at 15000 × g for 15 min at 4 °C. Supernatant was collected and subjected to 60% ammonium sulfate precipitation. The pellet was re-suspended in 20 mM phosphate buffer (pH 6.5) and dialyzed overnight by dialyzing tube (Spectra/Por RC Biotech membrane, 6-8 kDa MWCO) against 10 mM phosphate buffer (pH 6.5).

Crude protein extracts were fractionated through cation exchange chromatography where desalted protein was subjected to Hi Trap SP FF column (GE Healthcare). The column was equilibrated with 5 cv of 50 mM of phosphate buffer (pH 6.5) and then partially purified protein was loaded onto the column. Protein was eluted with 0.5 M NaCl gradient and different fractions were obtained which were analyzed by SDS-PAGE. The protein was quantified through the Bradford method (Bradford, 1976).

Construction of pCP-PVY clone

Full length coat protein (CP) gene of potato virus Y (*PVY*) was amplified from *PVY* infected potato samples. A fragment of full-length CP gene 807 bp was amplified with specific forward 5′-ATGGCAAATGACACAATCGAT-3′ and reverse primer 5′-ATCACCCTGCCACCTCTATC-3′ and the deduced sequence was submitted in NCBI database under accession # MK130988. For directional cloning in pCDNA3.1 (+) (mammalian expression vector), the amplified fragments were generated with *HindIII* and *EcoRI* restriction sites at 5 and 3 ends. The recombinant vector was named as pCP-PVY.

Cell line

The HepG2 cell line was grown and maintained in Dulbecco's modified Eagle medium (DMEM) containing 100 U/ml penicillin, 100 µg/ml streptomycin and 10% Fetal Bovine Serum (FBS) at 37 °C in 5% CO2 atmosphere at 37 °C.

Cytotoxicity assay for CVL

Cell survival and proliferation was quantified by using the MTT assay kit (Millipore, USA). Using the HepG2 cell model, Cell viability and proliferation was evaluated. HepG2 cells were cultured in 96 well plate containing DMEM supplemented with 10% FBS, 100 µg/ml streptomycin and 100 IU/ml penicillin. CVL protein was diluted in different concentration ranges (110 to 770 µg/ml, 3 wells per concentration) in 96-well plates and incubated at 37 °C in the humidified CO_2 incubator.

After incubation, the concentration range of CVL drawn from 110 to 770 µg/ml and were added to the wells of the plate in triplicates and the plate was again incubated at 37 °C for 24 h in CO_2 incubator. After incubation, the exhausted media was discarded

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and fresh media along with MTT reagent (5 mg/ml in PBS) were added to each well according to the manufacturer's instructions. The plate was then incubated at 37 °C in a CO_2 incubator for 4 h. To dissolve the formazen crystals produced after incubation, DMSO was added in the wells. To quantify the MTT formazen product, the optical density of contents of plate was analyzed through ELISA plate reader as per described protocol.

Cell viability was attained by means of following formula (Rehman et al., 2011):

Percent cell viability = (Test 570 nm – 620 nm / Control 570 nm – 620 nm) × 100.

Hemagglutinating activity

The hemagglutinating activity of the CVL was evaluated against the rabbit erythrocytes using the U-shaped bottom 96 wells microtiter plate as described by Ynalvez et al. (2015). Briefly, twofold serial dilutions of the CVL were made in phosphate buffer saline (PBS) and was incubated with 2% erythrocytes suspension in equal volume and kept at room temperature for 1 h, while control contains the PBS in spite of the CVL. Positive results indicated the red-carpet layer while negative results show the red button formation in the well.

Co-transfection

The HepG2 cells were cultured at a cell density of 1×10^6 cells per well in a 6-well plate. CVL protein extract and pCP-PVY plasmid DNA were co-transfected at 50-70% cell confluence. Lipofectamine 2000 (Invitrogen, CA) was used as transfection reagent. For the down regulation of CP-PVY mRNA, 50 ng – 1 μg construct DNA was used along with three different CVL concentrations; 30, 60 and 90 μg/ml. Experiments were carried out in triplicate.

Real-time PCR analysis

To measure the anti-PVY potential of CVL, the mRNA expression of CP gene was revealed through real time PCR assay. Post co-transfection, total RNA was isolated from HepG2 cells by using TRIzol reagent (Invitrogen) and 1 μg was used to synthesize complementary DNA (cDNA) by using the cDNA synthesis kit (Thermo Scientific, Lithuania). Specific forward 5′-TGTGGGTTTAGCGCGTTATG-3′ and reverse 5′-GTGCCTCTCTGTGTTCTCCT -3′ were used to amplify 172 bp amplicon. The assay was run in triplicate in the PikoReal™ Real-Time PCR system (Thermo Scientific). Reactions were prepared in a total volume of 10 μl containing: 1 μl cDNA, 0. 5 μl of each 10 pmoles primer, 5 μl of Maxima SYBR Green qPCR Master Mix (2X) (Thermo scientific) and 2.5 μl RNase/DNase-free sterile water. The cycle profile comprised of 30 cycles with denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 30 s after an initial denaturation at 94 °C for 10 min. For normalization, β-actin was used as a control. The relative gene expression analysis was measured by using Cq values in different samples. Each real-time PCR assay was performed in triplicate.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 7. Triplicate studies were performed. Descriptive statistics were used to evaluate the mean and standard deviation

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of the results. One-way ANOVA was used was to investigate a significant reduction in the cell viability and MRNA expression of the CP gene at $P \leq 0.05$.

Results

In silico modeling, refinement, evaluation, and validation of CP gene was performed with online available tools as indicated in *Figure 1A* and *B*. The verified in silico model of CP was used for docking analysis.

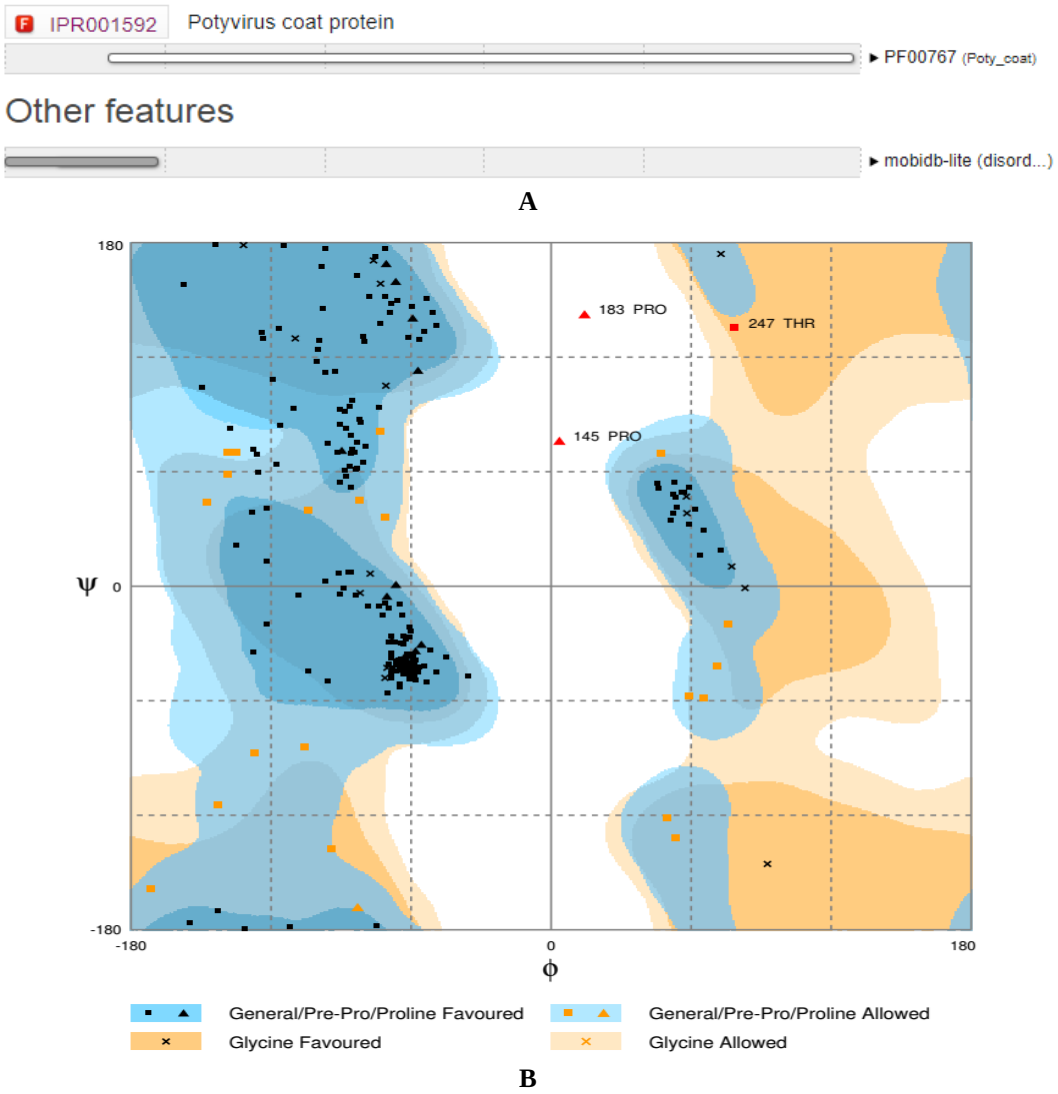

Figure 1. A *Functional domains analysis of CP sequence through InterPro is showing Potyvirus CP domain.* ***B*** *Ramachandran plot analysis of Capsid protein model to visualize dihedral angles; ϕ against ψ. Only two residues indicated with a red triangle in the figure are in the outlier region except these two, all other residues are in favored and allowed regions*

Primary structure examination of the CP model was executed utilizing Protparam (https://web.expasy.org/protparam/). It was found that the CP has an aggregate length of 267 amino acids and hypothetical *pI* of 6.61. The instability index was 43.03, ordering it

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as not steady protein. The evaluated half-life in mammalian reticulocytes was 30 h, whereas in yeast and *Escherichia coli* is over twenty and 10 h, on an individual basis (*Fig. 2A*). Similarly, the CVL protein model was taken as a PDB format from the online protein data bank. CVL protein contains four chains in which chain 1 and 3 were the same and chain 2 and 4 were same as given with the amino acid details (*Fig. 2B*).

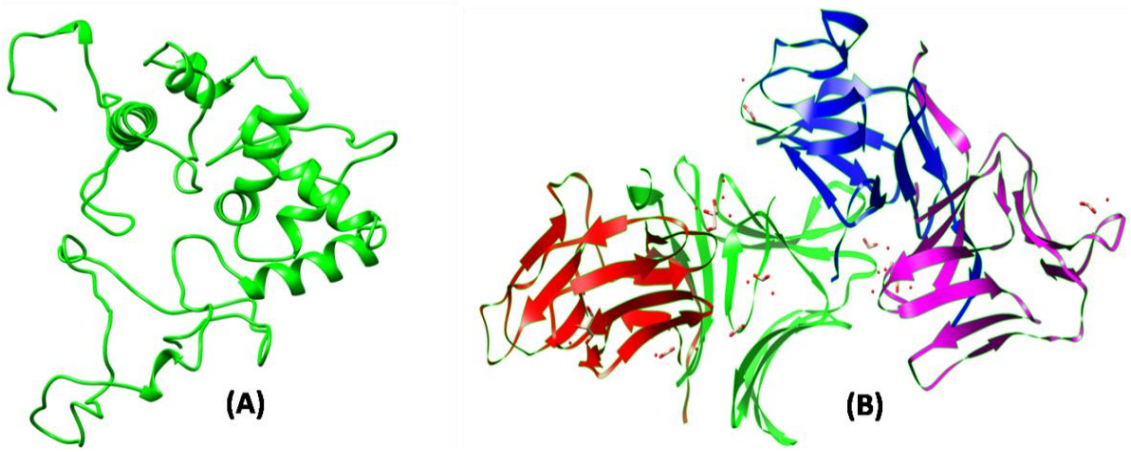

Figure 2. *Three-dimensional protein models of (**A**) PVY coat protein and (**B**) CVL protein, predicted by I-TASSER. In (B), the red color chains present A chain, green shows B, magenta C and blue presenting D chain*

Docking analysis of coat protein with CVL

The amino acids and molecules concerned in interchain H bonds, while not choosing any residue within the CP, were assessed utilizing the ZDOCK server. The interactions created through the ZDOCK server between CP and CVL protein are shown by the hydrogen bonds between the two proteins. Details of hydrogen bonds are given as in *Tables 1* and *2*.

Table 1. *Hydrogen bonds between CP and CVL (A and C domain) obtained from PDBePisa. Interactions with a value less than 3.0 were considered to have highlighted bond distances*

Sr. No.	CP	Dist. [Å]	CVL
1	:GLY 131 [N]	3.16	A: Tyr 76 [OH]
2	**:Asn 130 [N]**	**2.94**	**A: Tyr 76 [OH]**
3	:Tyr 185 [OH]	3.09	A: Ser 79 [O]
4	**:Tyr 181 [OH]**	**2.73**	**A: Val 97 [O]**
5	:Arg 184 [NH1]	3.83	A: Val 97 [O]
6	:Tyr 181 [N]	3.79	A: Tyr 100 [OH]
7	:Arg 189 [N]	3.03	A: Gly 101 [O]
8	:Tyr 181 [OH]	3.47	A: Val 97[N]
9	:Leu 187 [O]	3.11	A: Gly 101 [N]
10	:Ile 188 [O]	3.00	A: Tyr 87 [OH]
11	:Ala 224 [O]	3.20	A: Asn 81 [ND2]
12	**:Ala 225 [O]**	**2.94**	**A: Asn 81 [ND2]**
13	**:Asp 254 [OD2]**	**2.56**	**A: Arg 84 [NH2]**

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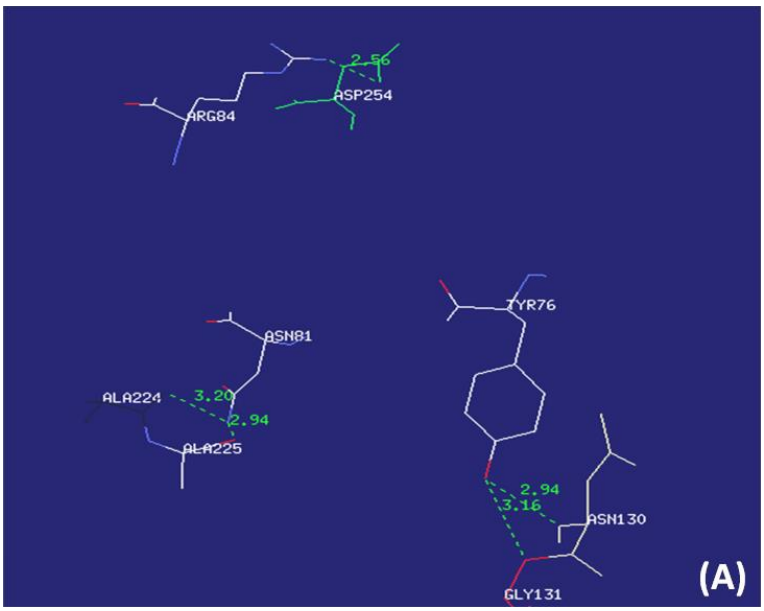
Table 2. *Hydrogen bonds between CP and CVL (B and D domain) obtained from PDBePisa. Highlighted bond distances were considered as an interaction whose value was less than 3.0*

Sr. No.	CP	Dist. [Å]	CVL
1	:Lys 177 [NZ]	3.76	B: Asn 1 [O]
2	:Lys 147 [NZ]	3.16	B: Asp 17 [OD2]
3	**:Tyr 144 [OH]**	**2.58**	**B: Gln 57 [O]**
4	:Val 134 [O]	3.84	B: Asn 1 [ND2]
5	:Tyr 181 [OH]	3.17	B: Arg 4 [N]
6	:Gly 138 [O]	3.71	B: Ser 12 [OG]
7	:Gly 138 [O]	3.61	B: Arg 62 [NH2]
8	:Glu 140 [[OE1]	3.72	B: Thr 72 [OG1]
9	:Asp 103 [OD1]	3.86	B: Leu 76 [N]

The two proteins interacted only through hydrogen bonds and no disulphide or covalent bonds were observed. The PDBePISA tool was utilized to locate the most effective interaction between coat protein and the two chains, i.e. chain A and B of CVL as mentioned before as CVL has four chains and chain 1 and 3 are same as chain 2 and 4. The outcomes demonstrated that various residues of the CP are predictable to act with the chain of CVL. In the case of A and C domain of CVL interaction with CP, totally 13 hydrogen bonds were observed from which three bonds, which are highlighted in *Table 1* has been shown in *Figure 3A*. In the case of B and D domain of CVL interacting with CP, totally 9 hydrogen bonds were observed from which two bonds, which are highlighted in *Table 2* has been shown in *Figure 3B*.

CVL protein purification and cytotoxic activity

CVL protein was purified through cation exchange chromatography using the column Hi Trap SP FF (GE Healthcare). On SDS-PAGE, two final fractions were obtained with their exchange profiles; one was of 10.5 kDa and the other fraction was about 11.5 kDa (*Fig. 4A* and *B*).



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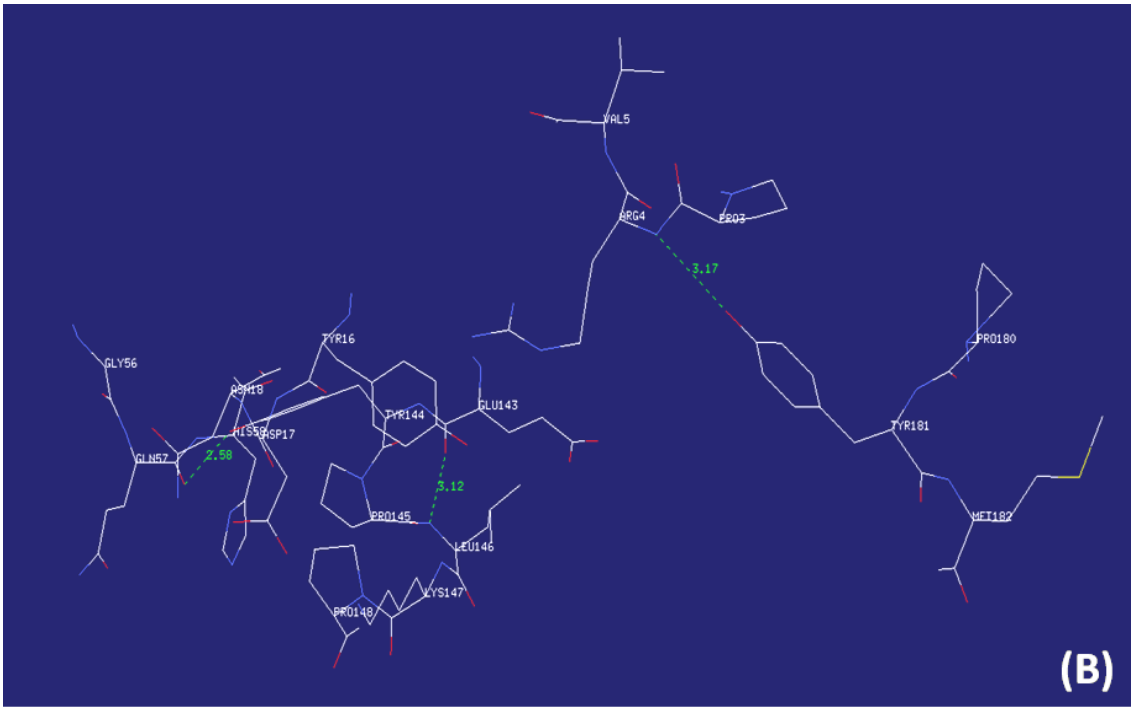

Figure 3. *In-silico protein-protein docking analysis.* ***A*** *Interactions of CVL A and C chains with CP visualized by PDBViewer. Asn 130, Ala 225 and Asp 254 residues of CP were interacting with Tyr 76, Asn 81 and Arg84 residues of CVL.* ***B*** *Interactions of B and D chains of CVL with CP visualized by PDBViewer. Lys 147, Tyr 144 and Tyr 181 residues of CP were interacting with Asp 17, Gln 57 and Arg 4 residues of CVL*

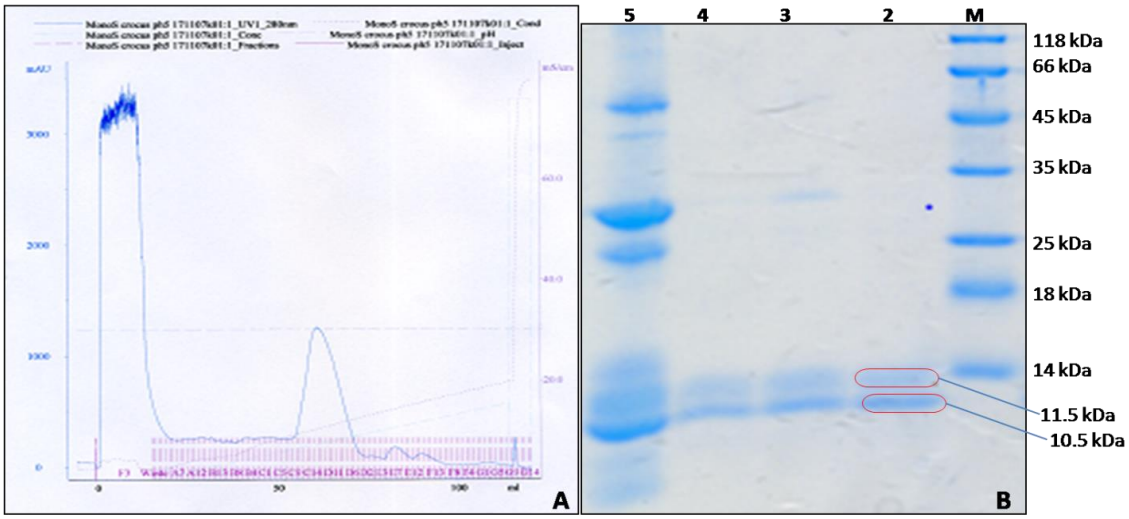

Figure 4. *SDS PAGE shows purified fractions of the CVL. Lane 1: PageRuler™ unstained Protein Ladder (Catalog number: 26610); lane 2, 3 and 4: purified CVL fractions obtained after cation exchange chromatography; lane 5: crude CVL protein after desalting*

The cytotoxic concentration that inhibited 50% of cell growth (CC50) showed that the CVL protein extract had the highest cytotoxicity with CC50 value of 770 µg/ml (*Fig. 4*). It was found that at 110 µg/ml CVL concentration, the cytotoxicity was

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111.47%. Similarly, at 220, 330, 440, 550, 660 µg/ml CVL concentrations, the cytotoxicity was recorded as 71.87, 62.39, 65.13 and 54.00% as compared to the control (*Fig. 5*).

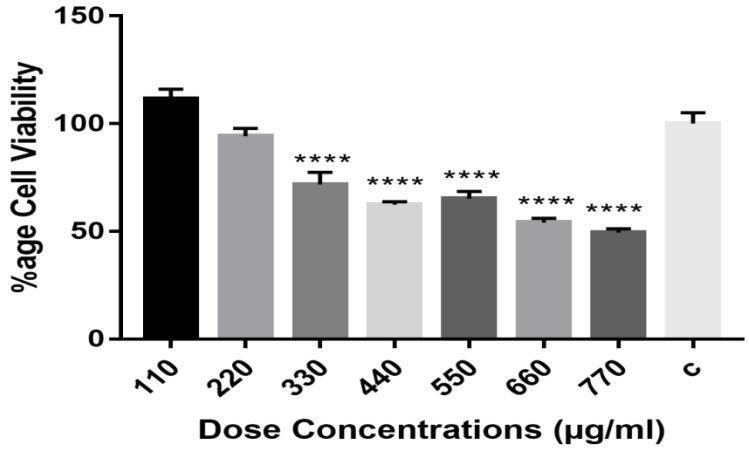

Figure 5. *Cytotoxicity of CVL to reveal a safe dose limit. The dose concentration of CVL purified protein extracts was ranging between 110 -770 µg/ml. Percentage cell survivals were calculated for each dose. Bars indicate the standard deviation. One-way ANOVA (see tables in the Appendix) shows the significant reduction of cell viability at the concentrations 330, 440, 550, 660 and 770 µg/ml at P < 0.0001. Three biological replicated were processed to obtain data*

Hemagglutination activity

The minimum concentration of purified CVL was observed at the end of the 1-h incubation period, which shows hemagglutination activity against the rabbit erythrocytes was high approximately 100 µg/ml as described by Van Dam et al. (2000).

Antiviral activity of CVL purified fractions against CP-PVY

In the antiviral screening assay, the CVL purified fractions were found to be active against the CP-PVY in in-vitro assays. Briefly, 30 µg/ml CVL displayed 98% inhibition of CP-PVY, 99% inhibition at 60 µg/ml, while up to 100% inhibition at 90 µg/ml CVL concentration (*Fig. 6*). However, the control sample did not exhibit any inhibition of CP-PVY expression.

Discussion

Potato is central crop and it is rigorously affected by Potato Virus Y (*PVY*). The essential point of the present work was to utilize CVL to effectively build firm PVY-resistance in in-vitro assays. Lectins derived from different plants have shown the antiviral activity against different plant viruses (Lusvarghi and Bewley, 2016; Liu et al., 2014). CVL is a tetrameric protein comprising of four subunits with two chains of 10.5 kDa, while other two of 11.5 kDa and is capable of agglutinating the rabbit erythrocytes. Previous studies have shown that CVL and lectin from *C. sativus* has identical N-terminal sequences and moreover, CVL showed reasonable similarity with respect to the two domain lectins of tulip and *Arum maculatum,* which reinforce that the

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CVL belongs to monocot mannose-binding lectins (Van Dam et al., 2000). There are numbers of pathogens, including bacteria, fungi, viruses and parasites through, which mannose binding lectins interact and provide protection against these to the host (Kilpatrick, 2002).

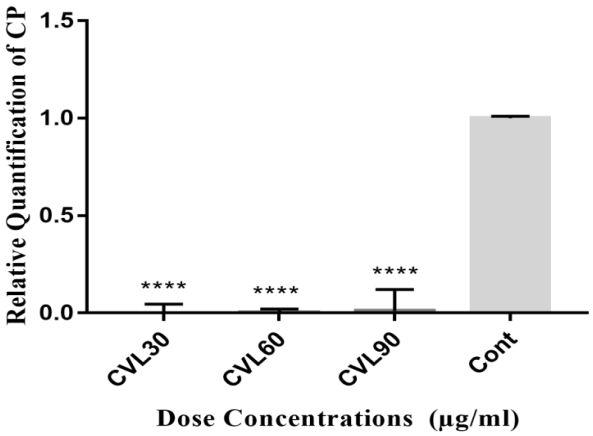

Figure 6. *Real time PCR study of down regulation of CP-PVY against CVL. Antiviral activity of CVL dose against CP-PVY. Three dose concentrations of CVL were tested; 30, 60 and 90 µg/ml. Percent inhibition of CP-PVY mRNA was measured, bars in the graph depict the standard deviation, while a significant decrease in CP gene expression was observed in 30, 60 and 90 µg/ml at P < 0.0001*

Protein docking refers to the scheming of three-dimensional (3D) structure of a protein initiating with the separate structures of protein subunits. The aim of protein docking is to know how proteins interact (Vitkup et al., 2001). Aloy and Russell stated that an estimate of 9 interaction partners are present for each protein and these interactions are one of around 10000 basic types, out of which only 2000 are known till date (Aloy and Russell, 2004) protein docking has emerged as a distinct computational field which make use of techniques and knowledge from diverse spectrum of science with the intention of formulating in silico how macromolecules like proteins act (Camacho et al., 2000). The in-silico protein-protein interaction showed that CP interact with the chain C and chain B of CVL. Our findings correlate with of Zhang et al. (2017b) who reported that Arg90 of *TMV* (tobacco mosaic virus) has a major role of interaction with the antiviral enantiomericα-aminophosphonate derivatives for the loss of virus ability to cause infection. In a similar study, mannose binding lectin from *Lycorisradiate* indicated that Gln80, Asp82, Asn84 and Tyr88 are the main residues which are involved in the interaction with mannose (Zhu et al., 2013).

We adopted in-vitro assay to reveal the antiviral effect of CVL, which is based on transient expression of the CP gene in the mammalian cell line. Several reports support the adoption of in-vitro assay to screen potential plant extracts against plant viruses like Li et al. (2012) showed that tobacco mosaic virus (*TMV*) virions were transfected into HeLa cells to generate the expression of the CP gene of *TMV*. The possibility of plant capsid uptake and release in mammalian cells has been proposed in case of clover necrotic mosaic virus (*RCNMV*) by Lockney et al. (2011). In another study, 480 bp of CP from *PVY* was transfected into CHO cells and its mRNA knockdown assay was conducted with siRNA (Tabassum et al., 2011). Furthermore, plant viral capsids have

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been used as vectors in mammalian cells for gene delivery and expression studies (Azizgolshani et al., 2013). More recently, *Arabidopsis* cryptochrome was successfully expressed in the HEK293T cells to produce a photochemically active product (Yang et al., 2016).

To reveal the activity of lectin (CVL), instead of full *PVY* virus particles, we cloned the CP gene as it is involved in the number of virus infection events like, uncoating and translation of the viral RNA, targeting the site of replication and in the transmission of potyviruses by vectors/aphids (Bol, 2008). There are some studies which clearly indicate involvement of CP transgene in creating resistance in transgenic potato crops (Bukovinszki et al., 2007; Gargouri-Bouzid et al., 2005).

The high antiviral effect of CVL protein fractions (up to 100% in our study) attributed the effectiveness of plant derived lectins in preventing notorious plant viruses like PVY. Plant lectins might show involvement in recognition of pathogenic microorganisms. Lectin from a soybean show high affinity for binding to β-glucan, which is a powerful PAMP of Phytophthorasojae (Mithöfer et al., 2000). Similarly, spread of tobacco etch virus (TEV), a single-stranded RNA plant virus belonging to the genus Potyvirus is restricted by, the *Arabidopsis thaliana* restricted TEV movement 1 (RTM1) lectin gene (Whitham et al., 2000). Moreover, a jacalin-type lectin gene exhibits resistance to potexviruses which belong to family Potexviridae. This resistance is conferred in the early stage of infection caused by plant viruses as compared to another lectinRTM1 which shows resistance at an advanced stage of viral infection, implementing the vital role of lectin-mediated resistance in a multiplicity of plant-virus relation (Yamaji et al., 2012). Conclusively, we have reported the antiviral activity of lectin derived from *C. vernus* against potato virus Y. The purified fractions of CVL exhibited the strong antiviral effect against *PVY*. To the best of our knowledge, this is the first report on the antiviral activity of CVL against *PVY*. Furthermore, as results showing the maximum down regulation of the CP gene due to CVL, which clearly indicate that efficiency of the CVL against PVY. The genetic integration of lectin in the potato genome could provide complete protection from PVY in transgenic potato plants.

Conclusion

Lectins obtained from different plants have been shown to have antiviral activity against various plant viruses. The purified *Crocus vernus* lectin exhibited the strong antiviral effect against *PVY*. This is the first study of CVL's antiviral activity against PVY, as far as we know. Genetic incorporation of lectin into the genome of potatoes could provide comprehensive defense against PVY in transgenic potato plants.

Conflict of interests. The authors have no conflict of interests.

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APPENDIX

Table Analyzed	**Data 1**								
Data sets analyzed	A : CVL30	B : CVL60	C : CVL90	D : Cont					
ANOVA summary									
F	207.9								
P value	<0.0001								
P value summary	****								
Significant diff. among means (P < 0.05)?	Yes								
R square	0.9873								
Brown-Forsythe test									
F (DFn, DFd)									
P value									
P value summary									
Are SDs significantly different (P < 0.05)?									
Bartlett's test									
Bartlett's statistic (corrected)									
P value									
P value summary									
Are SDs significantly different (P < 0.05)?									

ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Treatment (between columns)	2.23	3	0.7432	F (3, 8) = 207.9	P<0.0001
Residual (within columns)	0.0286	8	0.003575		
Total	2.258	11			

Data summary	
Number of treatments (columns)	4
Number of values (total)	12
Number of families	1
Number of comparisons per family	3
Alpha	0.05

Dunnett's multiple comparisons test	**Mean Diff.**	**95.00% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**		**D-?**
Cont vs. CVL30	0.9988	0.8582 to 1.139	Yes	****	0.0001	A	CVL30
Cont vs. CVL60	0.9986	0.858 to 1.139	Yes	****	0.0001	B	CVL60
Cont vs. CVL90	0.9889	0.8483 to 1.129	Yes	****	0.0001	C	CVL90

Test details	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**	**n2**	**q**	**DF**
Cont vs. CVL30	1	0.001217	0.9988	0.04882	3	3	20.46	8
Cont vs. CVL60	1	0.001426	0.9986	0.04882	3	3	20.45	8
Cont vs. CVL90	1	0.01111	0.9889	0.04882	3	3	20.26	8

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Table Analyzed	**Data 1**									
Data sets analyzed	A : 110	B : 220	C : 330	D : 440	E : 550					
ANOVA summary										
F				113.8						
P value				<0.0001						
P value summary				****						
Significant diff. among means (P < 0.05)?				Yes						
R square				0.9803						
Brown-Forsythe test										
F (DFn, DFd)										
P value										
P value summary										
Are SDs significantly different (P < 0.05)?										
Bartlett's test										
Bartlett's statistic (corrected)										
P value										
P value summary										
Are SDs significantly different (P < 0.05)?										
ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**		**P value**				
Treatment (between columns)	11026	7	1575	F (7, 16) = 113.8		P<0.0001				
Residual (within columns)	221.5	16	13.84							
Total	11247	23								
Data summary										
Number of treatments (columns)		8								
Number of values (total)		24								
Number of families		1								
Number of comparisons per family		7								
Alpha		0.05								
Dunnett's multiple comparisons test		**Mean Diff.**	**95.00% CI of diff.**	**Significant?**		**Summary**		**Adjusted P Value**		**H-?**
c vs. 110		-11.48	-20.36 to -2.593	Yes		**		0.0091	A	110
c vs. 220		5.825	-3.058 to 14.71	No		ns		0.2967	B	220
c vs. 330		28.13	19.24 to 37.01	Yes		****		0.0001	C	330
c vs. 440		37.61	28.72 to 46.49	Yes		****		0.0001	D	440
c vs. 550		34.86	25.98 to 43.75	Yes		****		0.0001	E	550
c vs. 660		45.99	37.11 to 54.88	Yes		****		0.0001	F	660
c vs. 770		50.58	41.7 to 59.46	Yes		****		0.0001	G	770
Test details	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**	**n2**	**q**	**DF**		
c vs. 110	100	111.5	-11.48	3.038	3	3	3.778	16		
c vs. 220	100	94.17	5.825	3.038	3	3	1.917	16		
c vs. 330	100	71.87	28.13	3.038	3	3	9.258	16		
c vs. 440	100	62.39	37.61	3.038	3	3	12.38	16		
c vs. 550	100	65.14	34.86	3.038	3	3	11.47	16		
c vs. 660	100	54.01	45.99	3.038	3	3	15.14	16		
c vs. 770	100	49.42	50.58	3.038	3	3	16.65	16		

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