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IN SILICO MODELING AND DOCKING OF SALICYLIC ACID REVEALING THE SOD LIKE ACTIVITY OF AN ANTIMICROBIAL SEED VICILIN OF *CAPSICUM ANNUUM* L. VAR. *GROSSUM*

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Abstract

This work is presenting a thorough *in-silico* modeling and salicylic acid docking in the predicted structure of *Capsicum annuum* var. *grossum* (bell pepper) seed vicilin along with antimicrobial activities. The *Capsicum annuum* var. *grossum* vicilin (*Cag*V) was purified by ammonium sulfate precipitation (60%) followed by anion exchange chromatography and gel filtration. SDS-PAGE showed an intact 50 kDa band of *Cag*V under both non-reduced and reduced conditions. LC-MS/MS analysis produced one fragment (14 residues) which showed 100% sequence homology with already reported vicilin of *Capsicum annuum* var. *grossum* in the NCBI database. The primary sequence of this highly homologous *C. annuum* var. *grossum* vicilin (NCBI Accession ID: KAF3621967.1) along with crystallographic coordinates of PDB ID: 5YJS were used for prediction of the *in-silico* model. The predicted cartoon model (P*Cag*V) showed an intact copper-binding extended loop having polar residues as well as the presence of non-mutated sites (Gly-Phe-Gly). Low Root Mean Square Deviation (RMSD) values indicated high structural homology of P*Cag*V with 5YJS, 6L4C, and 6L4M vicilins. Additionally, the docking experiment confirmed the binding of salicylic acid to the C-terminal localized binding pocket of P*Cag*V. Numerous hydrogen bonds and hydrophobic interactions took part in stabilizing the ligand binding. Purified *Cag*V (15 µg) showed strong antibacterial activity against pathogenic bacterial stains. Similarly, *Cag*V (60 µg) exhibited strong mycelial inhibition of phytopathogenic fungi. Thus, it is concluded that *Cag*V is an effective antibacterial and antifungal agent.

Key words: *Capsicum annuum*; Vicilin; Docking study; Salicylic acid; Superoxide dismutase, Antimicrobial activity.

Introduction

Seed proteins are known to maintain their structural features and metabolism e.g., immunoglobulin-binding protein, heat shock protein, and β-amylase (Shewry *et al*., 1995) while other seed proteins are involved in defense against pests and pathogens (Tapia *et al*., 2013). Some proteins are only known as a nutritional source for the seed but have no significant association with any physiological activity. Nevertheless, such proteins are named as storage proteins are worthy candidates for further investigations as they may have essential physiological potentials which are yet to be discovered (Shewry *et al*., 1995). Seed storage proteins are classified into albumins, globulins, and prolamins. Globulins contribute as a prime part of seed proteome and these are subdivided into 7S and 11S globulins based on their sedimentation rates (Danielsson, 1949). The 7S globulins (vicilins) are members of the cupin superfamily and a main source of nutrition during seed germination (Kriz, 1999). Vicilins are considered enzymatically inactive protein but recently, a comparative study of two vicilins has shown the functional variability in different plant species. The structural similarity does not impose similar functions and a large number of proteins are known to have a different function in different species (d Moore, 2004). However, the fate of this functional diversity of proteins is not yet clear and it could be feasibly due to small differences in their structures, sequences, and environments in which they functioned (Dunwell *et al*., 2000).

Capsicum annuum L. var. *grossum* (bell pepper) is a member of family Solanaceae and native to South and Central America. It is used as a vegetable and widely cultivated around the World (Dias *et al*., 2013; Wahyuni *et al*., 2013). Chilli (bell pepper) is an important economic cash crop globally with 7 million tons from an area of 1.5 million hectares. Pakistan is also a major producer where Sindh province is a major one followed by Punjab and Balouchistan (Arain, 2019) along with 7.2% of total global production (2 lac tons). It is an important source of biologically active compounds that are associated with pharmaceutical properties like analgesic, counterirritant, anticarcinogenic, antiarthritics, anti-inflammatory, antimicrobial, and antioxidant activities (Giuffrida *et al*., 2013; Srinivasan, 2016; Olatunji & Afolayan, 2018). Seed storage proteins are synthesized during seed development and localized inside the cells as membrane bounded proteins bodies (Derbyshire *et al*., 1976). Vicilins are seed reserved protein during seed germination and support seedling growth. Although vicilins are defined as reserve proteins, studies suggest that they have an active role in defense processes (Wang *et al*., 2001; Wang *et al*., 2002). Germin is a dimer of vicilin (7S globulins) and single manganese ion is buried inside each monomer of germin that is analogous to manganese superoxide dismutase (MnSOD). Germin has SOD activity which is involved in the defensive mechanism of extracellular superoxide radicals (Woo *et al*., 2000). Similarly, the germin, *Lupinus*

angustifolius seed vicilin (NLL β-conglutin) exhibit functional interplay between storage protein mobilization and regulation of oxidative metabolism during seed germination and seedling growth (Lima-Cabello *et al*., 2017; Lima-Cabello *et al*., 2019). The vicilin (NLL β-conglutin) is involved to mediate the ROS production in response to oxidative brust which stimulate the signaling responses against pathogen attack (Jimenez-Lopez, 2020).

In this study, we are reporting the predicted model *Cag*V and docking with Salicylic Acid (SA) to predict the SOD activity followed by the evaluation of the antimicrobial activity of seed vicilin of *C. annuum* var. *grossum*. We propose that the defense against extracellular superoxide radicals is an important additional role for vicilins and related proteins.

Materials and Methods

Isolation and purification of vicilin: *Capsicum annuum* seeds (10 g) were ground into fine powder in an electric blender and powder was dissolved in 100 mM sodium phosphate buffer (pH 7.5). The mixture was stirred for 2-3 hours and centrifuged (13000 rpm, 20 minutes at 4°C). The resultant supernatant was separated and filtered through a 0.8 µm pore size filter paper (Whatman Cat. No. 1441125). The clear filtrate was subjected to an optimized 60% ammonium sulfate saturation constant and centrifuged (2500 rpm) for 5 minutes at 4°C. The supernatant was collected and dialyzed against the same phosphate buffer by using a dialysis membrane of 3.5 kDa MWCO (Spectra/Por 3; Catalog No. 132724). The dialyzed sample was loaded on to pre-equilibrated anion exchanger-Hi Trap Q FF column at a flow rate of 0.5 ml/min and eluted with NaCl gradient of 0–1M in the same phosphate buffer. The fractions underlying the peaks of the anion exchanger chromatogram were subjected to SDS-PAGE. Based on molecular weight analysis, highly purified fractions were pooled together and were further loaded on Hi-load 16/60 Superdex 200 pg column at a flow rate of 0.5 ml/min while a UV detector (280 nm) was used for the recording of eluent absorbance. The fractions with high purity and quantity were pooled together and loaded on SDS-PAGE (Laemmli, 1970). The molecular mass was determined by comparing the electrophoretic mobility of the *Cag*V and protein marker (Thermo Scientific™ Catalog No. 26616). The gel was stained with 0.25% Coomassie Brilliant Blue R-250 (CBB R-250) in methanol/acetic acid/water and destained with the same solution without dye (Wilson, 1979). Purified *Cag*V was quantified through the standard procedure of Bradford assay by using Bovine Serum Albumin (BSA) as a standard (Bradford, 1976).

LC-MS/MS analysis: Multiple bands of 50 kDa *Cag*V were cut and reduced with Dithiothreitol (10 mM, 55°C for 30 min) and alkylated with iodoacetamide (5 mM, 20 min, room temperature, in absence of light). The protein in-gel was digested overnight with trypsin as per the described protocol (Shevchenko *et al*., 2006). The digested gel pieces were extracted by using a 50% acetonitrile/5% formic acid solution and were dried in a vacuum concentrator. Nano LC-MS/MS measurements were performed by injecting the reconstituted samples to a Nano liquid chromatography system (Dionex UltiMate 3000) coupled via electrospray-ionization source to an orbitrap mass spectrometer (Orbitrap Fusion, Thermo, Germany). For chromatography, buffer A was 0.1% formic acid in MS-grade water (Thermo) and buffer B was 0.1% formic acid in acetonitrile. The digested sample was loaded on a trapping column at 5 µL/min (Acclaim PepMap C18, µ-precolumn) with 2% buffer B, and the peptides were eluted with gradient program of 2-30% B in 35 minutes at (300 nl/min) on the separation column (Acclaim PepMap 100, C18, 75 µm × 250 mm). LC-MS/MS analysis was carried out in data-dependent acquisition mode (DDA). Raw data files were processed with Proteome Discoverer 2.0 (Thermo Scientific, Germany). For identification, MS/MS spectra were searched using Sequest HT against the *Arabidopsis* and the UniProtKB databases (10/Nov/2015), while identifications were confirmed manually. The spectral searches were performed using precursor mass tolerance of 10 ppm, product mass tolerance 0.5 Da, 02 missed cleavages allowed, carbamidomethylation of cysteine (fixed modification), oxidation of methionine (variable modification), and acetylation of protein N-terminal (variable modification).

Model prediction: The LC-MS/MS residual sequence was BLAST on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) which showed high homology (100%) with the primary sequence of putative vicilin of *Capsicum annuum* var. *grossum* (Accession ID: KAF3621967.1). The primary amino acid sequence of putative vicilin of *Capsicum annuum* var. *grossum* was taken and processed for model building into the Swiss-Model online server (Biasini, 2014). The predicted model (P*Cag*V) was generated by using the crystal coordinates of vicilin from *Capsicum annuum* (PDB ID: 5YJS) as a template (Benkert *et al*., 2011). The quality of the predicted structure model was determined by the Ramachandran plot by using PROCHECK online server (Laskowski *et al*., 2006). The portray of the structure details was made as a cartoon model by using PyMOL (Bramucci *et al*., 2012).

Molecular docking of P*Cag*V: Molecular docking was performed between the Predicted *Capsicum annuum* var. *grossum* Vicilin (P*Cag*V) model and the salicylic acid (CID 338) by using the AutoDock 4.2 software. The program performed the ligand docking using a set of pre-defined 3D grids of the target protein. Molecular docking was initiated by adding polar hydrogens and assigning of kollman charges along generation of PDBQT file through Auto Dock Vina (Seeliger & de Groot, 2010; Trott & Olson, 2010). Two dimensional structure of salicylic acid (CID 338) was obtained from PubChem database (https://pubchem.ncbi.nlm.nih.gov) and transformed to PDBQT files. PDBQT files of both P*Cag*V and ligand were subjected to Auto Dock Vina for docking calculations. A docking grid of 40 Å × 40 Å × 40 Å in size, centered on a pre-positioned ligand at the active site, was used for docking. AutoDock software was used to obtain the minimum energy conformation of salicylic acid in complex with the P*Cag*V model protein. For docking result analysis, web server Protein-Ligand Interaction Profiler (https://projects.biotec.tu-dresden.de/plip-web/plip) was

used to visualize the non-covalent interaction between the P*Cag*V and salicylic acid complex. Hydrogen bond interaction along the salicylic acid and P*Cag*V was visualized in 2D form by Protein Plus (https://proteins.plus/) web server (Stierand *et al*., 2006).

Antibacterial activity: The antibacterial activity of *Cag*V was checked by microtiter assay (Almeida *et al*., 2006) against bacterial pathogens i.e., *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. The 96-well microtiter plates were used to inoculate all bacteria in Luria Bertani (LB) medium and incubated at 37°C with agitation (200 rpm). The bacterial growth was optimized and OD_{600} was checked until it reached up to 0.1 absorbance unit. The two-fold serial dilutions of the bacterial culture medium were incubated (37°C) with different concentrations of *Cag*V (5, 10, 15, 20, and 25 µg). The OD values were recorded at 600 nm after each hour and continuous up to 8 hours. Antibiotic [Amoxicillin (1 mg ml^{-1})] and extraction buffer (100 mM sodium phosphate buffer, pH 7.5) were used as positive and negative controls, respectively. The experiment was carried out in triplicate and repeated twice. The optimum effective concentrations of *Cag*V obtained from microtiter assay were further confirmed by the Kirby-Bauer disc diffusion method using the same bacterial species (Boyle *et al*., 1973). In the disc diffusion test, the bacterial growth bed was formed on an agar plate and four discs were arranged on it at equal distance. Disc A was used as a positive control (Amoxicillin 5 µg/disc) and disc B was soaked in extraction buffer (100 mM sodium phosphate buffer; pH 7.5) as a negative control. Two concentrations (20 and 15 µg/disc) were loaded on disc C and D respectively and plates were incubated at 37°C for 8 hours.

Antifungal activity: The phytopathogenic fungal strains *Aspergillus niger* (FCBP-PTF-720), *Aspergillus flavus* (FCBP-PTF-862), and *Fusarium oxysporum* (FCBP-PTF-866) were purchased from First Culture Bank of Pakistan (FCBP), University of Punjab, Lahore, Pakistan. The spores were taken from mature fungal cultures; which were grown on PDA plates (at 28°C), by adding 15 ml pre-chilled distilled water. The sterile loop was used to stir the fungal culture before filtering through cheesecloth and filtrate was collected into a sterile falcon tube. The spores were counted at 400x magnification of binocular microscope (Ernst Leitz Wetzlar GMBH, Germany) by using the hemocytometer (NeubauerHausser Bright-Line; Catalog No. 3100) and were counted and fixed at a standard cell concentration of 2×10^4 cells/ml. The fungal growth inhibition was tested by incubating (at 28°C) the fungal spores with 30, 40, 50, 60, and 70 µg concentrations of *Cag*V in a 200 µl 96 well microtiter plate. The extraction buffer (100 mM sodium phosphate buffer; pH 7.5) was used as a mock (control). The optical density (OD) was calculated by using the wavelength of 600 nm after 0, 24, and 48 h. The experiment was done in triplicate and the mean values, standard errors, and coefficients of variance were calculated by using MS excel. Similarly, three sterilized discs (15 mm) were place in a culture plate and one disc in each culture plate was loaded with *Cag*V (60 µg). The other two discs in each culture plate were loaded with extraction buffer (negative control) and fungicide TOPSIN® 4.5 FL (positive control) respectively. The fungal spores were inoculated at the center of the culture plate and kept at 30°C. The fungal growth was monitored after 48 hours of incubation.

Results

Isolation and Purification of *Cag*V: *Cag*V was extracted from seeds of bell pepper in 100 mM sodium phosphate buffer (pH 7.5) and partially purified by subjecting to 60% ammonium sulfate precipitation. The *Cag*V remained in the supernatant and other storage proteins were precipitated into a pellet. Further purification was done by using an anion exchanger column and ultimate purity was obtained after size exclusion chromatography. Moreover, the purification strategy produced 10 mg *Cag*V of 10 fold with a 6.66% yield from one gram of seeds. Further, purification folds are summarized in (Table 1). The highly purified *Cag*V on SDS-PAGE showed a single band of 50 kDa under both reduced and non-reduced conditions (Fig. 1).

Identification of *Cag*V: The purified 50 kDa band of *Cag*V was subjected to mass spectrometric analysis resulting in the identification of a single major 14 residual peptide fragment (QDQSYFVTGPGQQR). The residual sequence was BLAST in NCBI online server (https://blast.ncbi.nlm.nih.gov/Blast.cgi) which showed 100% sequence identity with already reported vicilin of *Capsicum annuum* and other different plants (Table S1).

Model prediction of *Cag*V: The primary sequence of vicilin was used for the calculation of the predicted model. The 3D monomeric ribbon structure of P*Cag*V was thoroughly analyzed. Structural features are represented as cartoon models (Fig. 2a and b). The predicted model was validated through ProCheck online server and generated a Ramachandran plot which showed 94% residues in the most favored region, 5.7% in the additional allowed region, and only 0.3% in the disallowed region (Fig. S1). Structure alignment of P*Cag*V with 5YJS 6L4C and 6L4M showed 0.063, 0.914 and 0.617 Å RMSD values respectively (Fig. 2b).

Table 1. Purification steps of CagV from one gram of seed powder.

Purification steps	Total protein (mg/g)	Purification (times)	Recovery (%)
Crude extract	150	1	100
Ammonium sulfate precipitation (60%)	90	1.66	60
Hi Trap Q FF column	13	11.53	8.66
Hi-Load 16/60 Superdex column	10	15	6.66

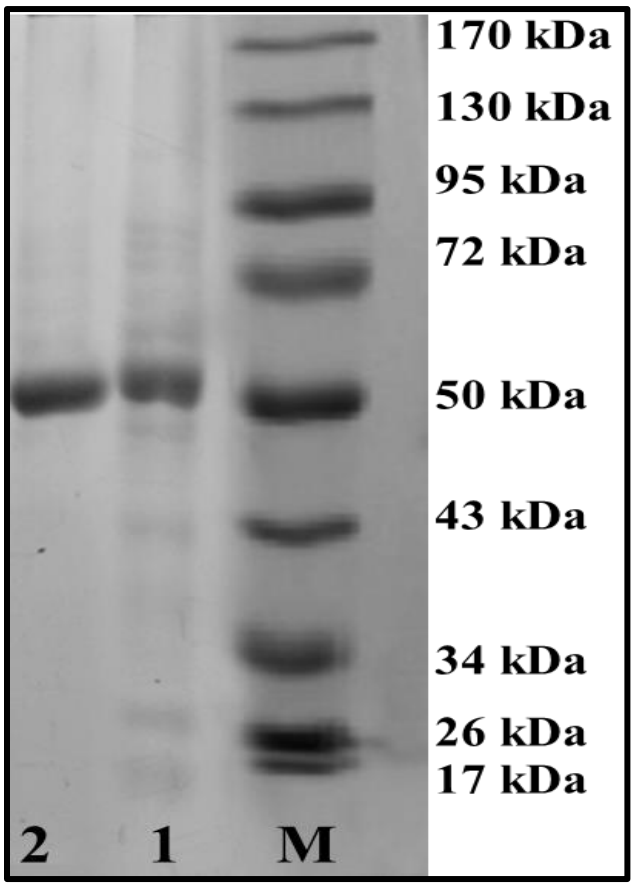

Fig. 1. SDS-PAGE is showing purification of 50 kDa vicilin from seeds of *Capsicum annuum* L. var. *grossum*. Lane 1 is showing *Cag*V band after anion chromatography under non-reduced conditions while lane 2 is loaded with purified vicilin after gel filtration in the presence of *β*-mercaptoethanol. Lane M is showing a protein marker (Thermo Scientific™ Catalog No. 26616).

Molecular docking studies of P*Cag*V: Docking studies showed that salicylic acid-binding pockets were detected outer and inner surface of the P*Cag*V protein. It clearly showed that the amino acids involved in salicylic acid-binding are present within the β-sheets and at the C-terminal which is the basic characteristic of all known plant vicilin proteins. The docking analysis of P*Cag*V complexed with salicylic acid (Fig. 3) showed the hydrogen bonds and hydrophobic interactions surrounded by β-sheets and C-terminal of P*Cag*V. Salicylic acid formed hydrogen bonds with Tyr-250, Asn-252, Arg-257, and Lys-347 residues of P*Cag*V protein (Fig. 4). Salicylic acid showed hydrophobic interactions with Phe-219, Met-239, and Phe-325 residues, and distance was calculated in angstrom (Å) and is described in (Fig. S2). Protein-Ligand Interaction Profiler also found salt bridges with Arg-257 and Lys-347 at a distance of 2.78 Å and 2.69 Å respectively of P*Cag*V protein and salicylic acid complex. From the docking analysis, -7.14 kcal/mol and 4.17 µM free binding energy and inhibition constant (ki) respectively were calculated to dock the salicylic acid with P*Cag*V protein and are presented in (Table S2). Therefore, it is confirmed that these interacting residues of P*Cag*V play important role in superoxide dismutase (SOD) activity.

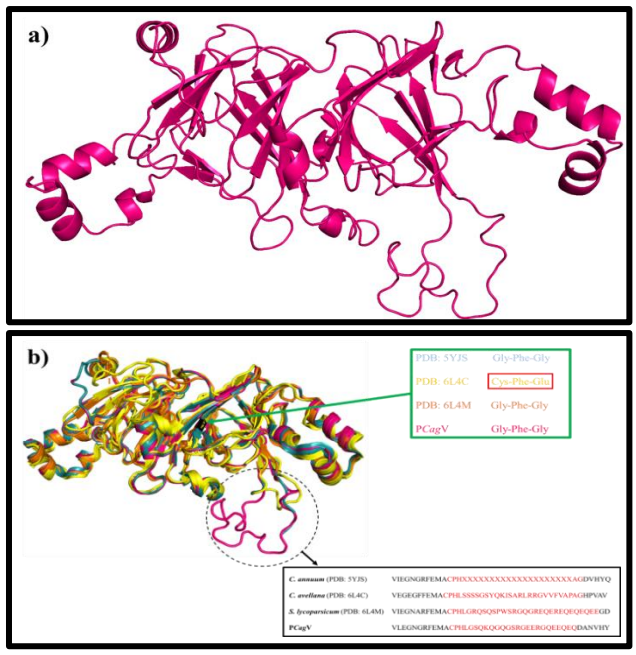

Fig. 2. Cartoon diagrams are showing the structural details and alignment of P*Cag*V. Figure (a) is the predicted 3D ribbon model of P*Cag*V; which is rich in beta sheets, loops and alpha helices (b) is the structural alignment of P*Cag*V (Hotpink) with other plant vicilins [PDB: 5YJS (Deepteel), PDB: 6L4C (Yellow), PDB: 6L4M (Orange)]. The green square inset is showing the two-point mutations cysteine and glutamate in the SA binding pocket of Hazelnut vicilin (PDB: 6L4C) which makes it enzymatically inactive as compared with the other three enzymatically active vicilins having conserved glycines. The splitted circle is representing the comparison of the copper-binding loop of all four vicilins while the black box is showing their corresponding residual sequences inside of loops.

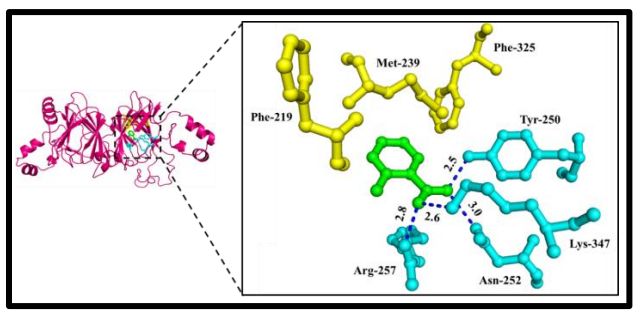

Fig. 3. Molecular Docking of SA inside the binding pocket of P*Cag*V. P*Cag*V residues involved in hydrogen bondings and hydrophobic interactions with salicylic acid (green) have been highlighted in cyan and yellow colors, respectively. Corresponding lengths of hydrogen bonding are shown through blue dotted lines and distances are mentioned in Angstrom (Å) unit.

Antibacterial activity of *Cag*V: *Cag*V strongly inhibited the growth of all tested bacterial strains. Two concentrations (15 and 20 µg) significantly inhibited the growth of both Gram-positive and Gram-negative bacteria. The maximum concentration of 25 µg did not significantly decrease bacterial growth in comparison to the 20 µg treatment. Two effective concentrations of *Cag*V (15 µg and 20 µg) were further confirmed by a disc diffusion test against the same bacteria. *Cag*V soaked discs developed zones of growth inhibition (Fig. 5).

Antifungal activity of *Cag*V: Similarly, *Cag*V showed strong antifungal activity against *Aspergillus flavus*, *Aspergillus niger* and *Fusarium oxysporum* (Fig. 6). *Cag*V concentration of 60 µg produced 50% reduction in mycelial growth after 24 hours of incubation but with no significant improvement of inhibition at 70 µg concentration. The disc diffusion assay also validates the data showing the same growth inhibition pattern at 60 µg after 72 hours of incubation. These results are confirming that *Cag*V has a strong potency against phytopathogenic fungi.

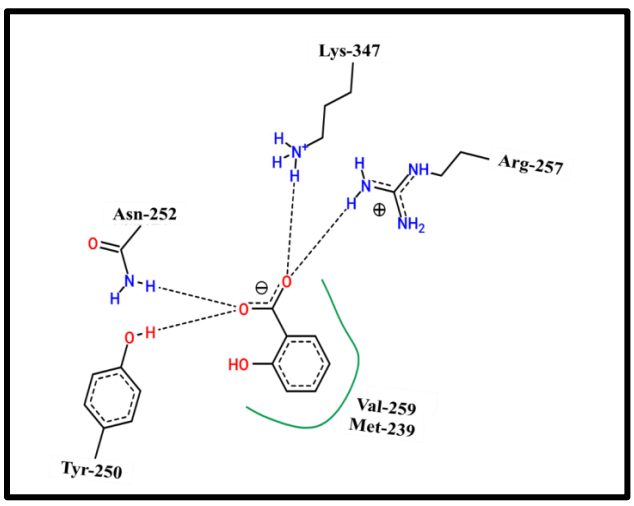

Fig. 4. Ligplot is showing the 2D image of Hydrogen bonds and hydrophobic interactions between SA and P*Cag*V residues. Black dotted lines are indicating the hydrogen bondings between P*Cag*V residues and oxygen atoms of salicylic acid while the green line is marking the site for hydrophobic interactions between P*Cag*V residues and SA.

Discussion

Vicilins are seed storage proteins of plants and member of the 7S globulin class and are comprised of two cupin domains each of which are further composed of 6 stranded short beta-barrel conformations (Derbyshire *et al*., 1976; Fritsch *et al*., 1997; Ballmer-Weber *et al*., 2001; Hiemori *et al*., 2004). An antimicrobial vicilin (50 kDa) had been characterized from the seeds of *Capsicum annuum* var. *grossum* (*Cag*V) and molecular structure were also predicted (P*Cag*V). Both P*Cag*V and 5YJS have shown high structural homology. The comparative analysis indicated the presence of a copper-binding extended loop with polar residues in P*Cag*V (Hotpink colored in fig. 2b). However, a smaller loop is present in *Corylus avellana* vicilin (PDB: 6L4C) which makes its SOD property inactive (Shikhi *et al*., 2020). It is important to note that an extended polar residue filled copper-binding loop is important for guided entry of superoxide radicals into the active site of vicilins similar to the loop of CuZnSOD (EC 1.15.1.1). Copper is naturally present in the native protein for its SOD activity. SOD activity of vicilins depends on the charge/length of the copper-binding loop and binding of SA in the C-terminal pocket (Shikhi *et al*., 2018). SA is an important plant hormone that takes part in various signaling pathways and gives defensive strength to plant against the abiotic and biotic stresses (Alonso-Ramírez *et al*., 2009). SA is present in the plant cell in both free and conjugated forms and is synthesized through a phenylalanine or isochorismate pathway (Mustafa *et al*., 2009; Boatwright & Pajerowska-Mukhtar, 2013). Salicylic acid is bounded with antioxidant enzymes to enhance their activities like carbonic anhydrase (Slaymaker *et al*., 2002), catalase (Chen *et al*., 1993), lipase (Kumar & Klessig, 2003), ascorbate peroxidase (Durner & Klessig, 1995), and Superoxide dismutase (Song *et al*., 2014; Shikhi *et al*., 2020). Shikhi *et al.* (2020) indicated that SA binding pocket was also necessary for the SOD activity of vicilins as well. *In-silico* study of P*Cag*V confirmed the docking of SA into the C-terminal binding pocket and further the SOD activity. The structural comparison further revealed two point mutations (Gly-X-Gly→Cys-X-Glu) in SA binding pocket of *C. avellana* vicilin (6L4C) which makes it inactive towards its SOD activity (Fig. 2b) while Gly is conserved in other active vicilins of *Solanum esculentum* (6L4M; Gly-Phe-Gly), *C. annuum* (5YJS; Gly-Phe-Gly) and as well as in *C. annuum* var *grossum* (P*Cag*V: Gly-Phe-Gly). These mutations reduced the binding cavity of 6L4C making it more difficult for access for SA (Shikhi *et al*., 2020). The binding of SA imposed allosteric effect on SOD active vicilins which could stimulate the evolution of H_2O_2 directly or indirectly by inhibiting the catalases and peroxidases those scavenge H_2O_2 (Durner & Klessig, 1995) along with triggering the immune system against abiotic stresses and microbial attacks (Shikhi *et al*., 2020). The high level of H_2O_2 in cells is responsible for the induction and expression of PR-1 genes which are defensive against the microbial infections (Niderman *et al*., 1995). The expression of PR-1 genes led towards the abundance of PR-1 antimicrobial proteins in the apoplastic regions that known to be a potential site for plant-microbe interaction. The PR-1 inhibits the microbial growth by sterol sequestration from microbial membranes and cause cellular leakage as well as ceased sterol biosynthesis (Gamir *et al*., 2017). Other PR-proteins that are upregulated through the salicylic acid are PR-2 (Beta-1,3-glucanase) and PR-5 (Thaumatin-like protein), which have enzymatic activities known to inhibit microbial growth (Leah *et al*., 1991; Selitrennikoff, 2001).

Likewise other vicilins of Solanaceae, *Cag*V has antimicrobial activities against different pathogens. It showed the significant growth inhibition of bacterial pathogens and fungal strains at a concentration of 15 and 60 µg respectively. Soybean vicilin exhibited good antibacterial activity against *Bacillus subtilis*, *Listeria monocytogenes*, and *Salmonella* at a concentration of 100 µg (Sitohy *et al*., 2014). Similarly, another cowpea vicilin has been reported as a strong antibacterial agent against pathogenic bacteria such as *Klebsiella pneumonia*, *Listeria ivanivii*, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Streptococcus pyogenes* (Abdel-Shafi *et al*., 2019). The vicilins isolated from legume interact with the negative charge region of bacterial cell wall or membrane by the positively charged region of vicilin or hydrophobic

interactions which is the major cause of bacterial death (Sitohy & Osman, 2010). Additionally, the Brownian motion of vicilin macromolecules is also responsible for the development of large-sized pores, channels, the disintegration of the bacterial cell wall and cell membrane which is finally responsible for cell lysis, emptiness, and death (Zhang & Lewis, 1997; Murzyn *et al*., 2005). *Cag*V significantly inhibited the mycelial growth of phytopathogenic fungi *Aspergillus flavus*, *Aspergillus niger*, and *Fusarium oxysporum*. Vicilin has been reported as an antifungal agent from many leguminous and non-leguminous plants e.g., *Vigna unguiculata*, *Vigna radiata*, *Glycine max*, and *Capsicum baccatum* (Casey *et al*., 1986; Doyle *et al*., 1986; Gomes *et al*., 1998; Vieira Bard *et al*., 2014). The vicilin isolated from *Capsicum baccatum* exhibited strong antifungal activity at 40 µg/ml dose against *Candida albicans*, *Candida tropicalis*, *Kluyveromyces marxiannus*, and *Saccharomyces cerevisiae* (Vieira Bard *et al*., 2014). The *Cag*V showed low inhibition of fungal growth below 40 µg/200 µl dose but maximum conidial inhibition at 60 µg/200 µl. It has already been reported that vicilin binds to the fungal cell wall and inhibits conidia germination (Gomes *et al*., 1997). In another report, vicilin from cowpea also binds to the fungal cell wall and such binding interfere with the plasma membrane which leads to inhibition of the H^+ pump and causes cell death due to the acidity inside the cell (Gomes *et al*., 1997). Vicilin also binds with the glycol-conjugates of membrane surface containing GlcNAc residues leading to the death of fungal cells (Ribeiro *et al*., 2007).

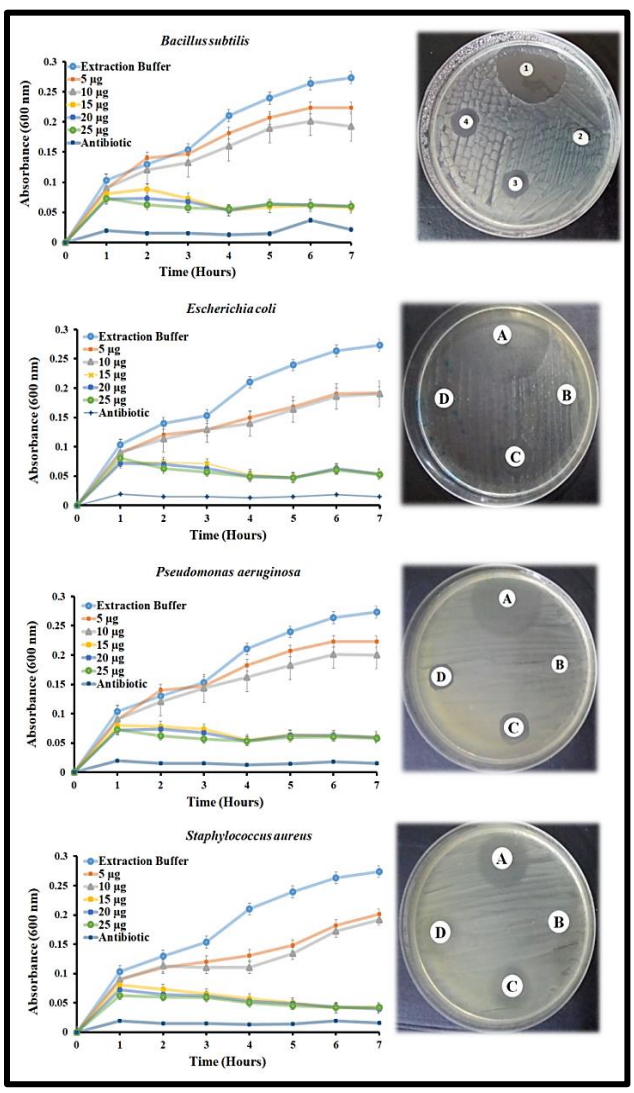

Fig. 5. Time-course study of the effects of different concentrations of *Cag*V on pathogenic bacteria. The microtiter assay showed little reduction in bacterial growth at a 10 µg concentration of *Cag*V. Significant growth inhibition was observed at 15 µg concentration. Additionally, two effective concentrations of *Cag*V (15 and 20 µg) were used against the same bacteria by using the disc diffusion method. Amoxicillin (5 µg/disc A) was used as a positive control; Phosphate buffer (100mM, pH 7.5; disc B) as negative control while discs C and D were soaked with 20 and 15 µg of *Cag*V respectively.

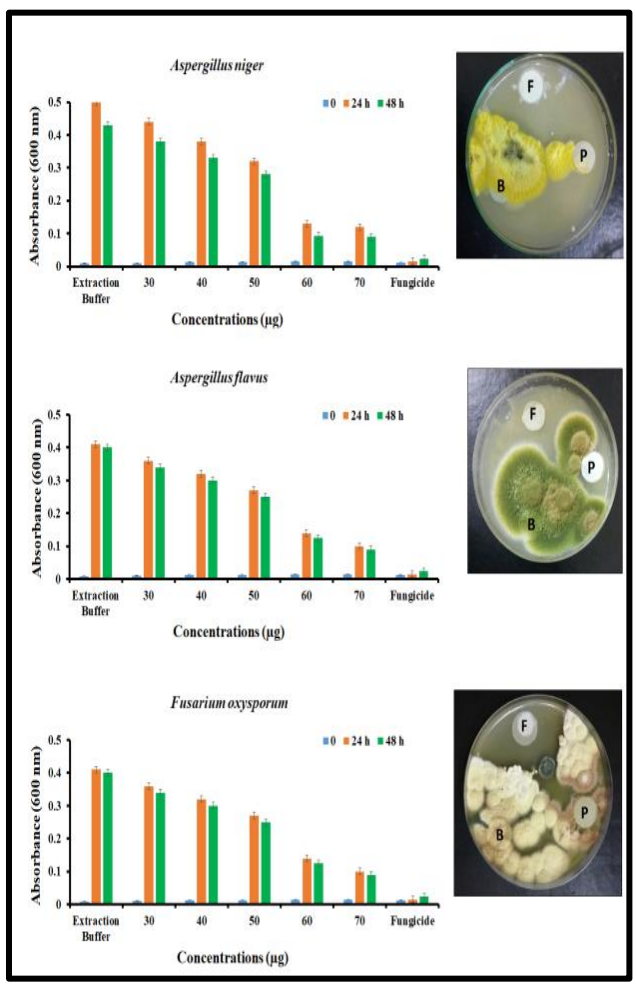

Fig. 6**.** Antifungal activity of *Cag*V against three phytopathogenic fungal strains (*Aspergillus flavus*, *Aspergillus niger*, and *Fusarium oxysporum*). A time-course study (0, 24, and 48 hrs) of fungal growth against different concentrations of *Cag*V was performed and it showed significant mycelial growth inhibition at 60 µg after 24 hours of fungal incubation where 50% conidial germinal inhibition was observed. The negative control (0.1 M, Phosphate buffer) had maximum conidial germination of all tested fungi. The effective concentration (60 µg) was used to test the mycelial inhibition on agar plates by the disc diffusion method. Disc F was used as a positive control (fungicide). Disc B was used as a negative control whereas disc P is indicating the *Cag*V concentration of 60 µg/disc. Fungal growth inhibition was observed after 48 hours of inoculation on petri dishes.

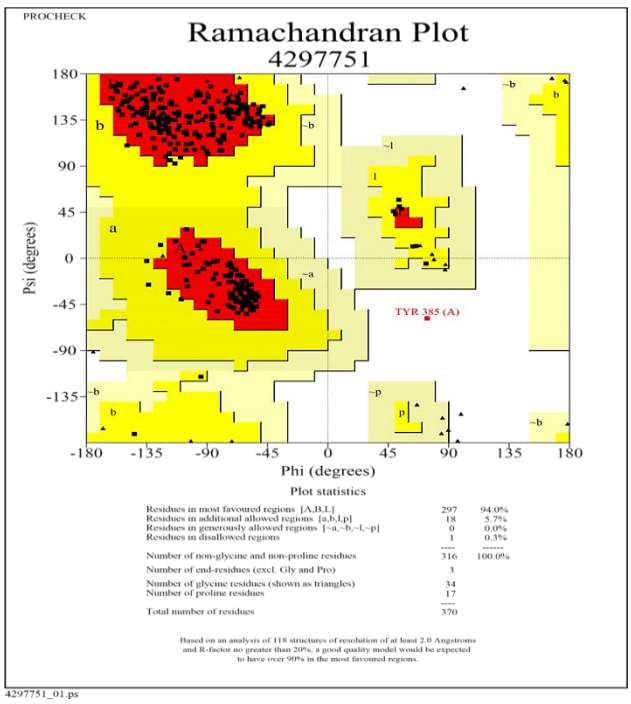

Fig. S1. The Ramachandran plot is confirming the structure validity of P*Cag*V.

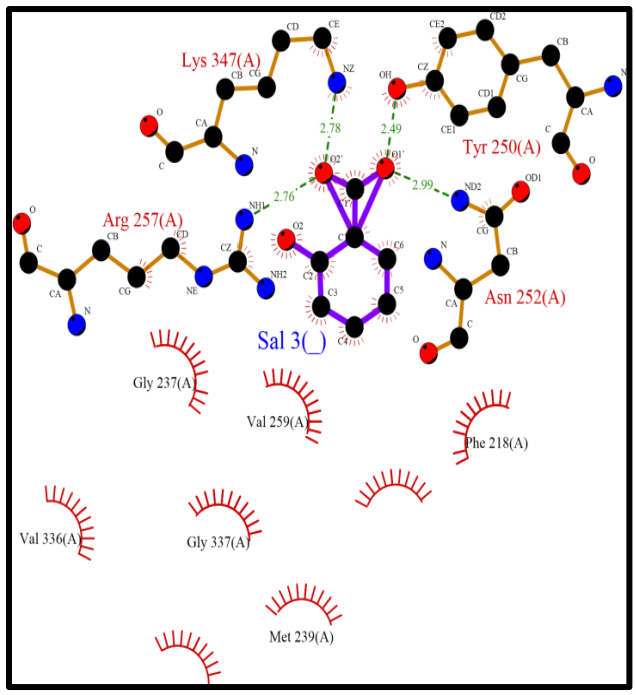

Fig. S2. The Ligplot is showing the hydrogen bonding and hydrophobic interaction between P*Cag*V with SA.

Table S1. Vicilin was identified by using LC–MS/MS generated residual sequence through BLAST in NCBI online server and showed 100% sequence identity with already reported vicilin of *C. annuum* var. *grossum*.

Sr. No.	Organism name	Protein	Sequence homology (%)	Accession ID
1.	*Capsicum annuum* var. *grossum*	Vicilin	100	KAF3621967.1
2.	*Capsicum annuum*	Vicilin-like peptide 2-2	100	XP_016556976.1
3.	*Capsicum baccatum*	Hypothetical protein	100	PHT38931.1

Table S2. Hydrogen bonds, hydrophobic interactions and free binding energy between SA and P*Cag*V residues.

Index	Salicylic acid			
	Amino acid residues	**Interacting atoms**	**Ligand atoms**	**Distance (Å)**
		Hydrogen bond		
1.	Tyr-250A	OH	O1′	2.5
2.	Asn-252A	NH	O1′	3.0
3.	Arg-257A	NH	O2′	2.8
4.	Lys-347A	NH	O2′	2.8
		Hydrophobic interaction		
5.	Phe-219A	CE1	C4	3.49
6.	Met-239A	CB	C5	3.42
7.	Phe-325A	CZ	C7	3.34
Free Binding energy		-7.34 kcal/mol		
Inhibition constant (ki)		4.17 µM		

Conclusions

It is concluded that the *in-silico* study confirms the presence of copper-binding and SA-binding loops reveals that *Cag*V is an enzymatically active and also exhibiting strong antibacterial and antifungal activities. SOD activity through copper and salicylic acid-binding sites indicated that vicilins were far more important proteins in terms of defensive agents than being of nutritional importance to plants. Hence, it can be considered to be an effective bioactive compound. These findings suggest that the binding of different ligands to vicilins can open exciting avenues of functional diversity of these important proteins.

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