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Homology modeling and molecular docking studies of INF1 protein of *Phytophthora infestans* in Potato

Balakrishnan M*, Vemulapalli Bhavana, Tammana Sharma, SK Soam, Supriya P, Vyshnavi M & Srinivasa Rao CH

ICAR-National Academy of Agricultural Research Management, Hyderabad-500 030, Telangana, India

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INF1, an elicitor protein found in the pathogen *Phytophthora infestans* responsible for causing late blight and tuber blight disease in potato, triggers a hypersensitive response and serves as a virulence factor during the pathogen-plant interaction. As the structural information for the INF1 protein of *P. infestans* was unavailable, homology modelling was conducted using the most closely related template. Various models of the INF1 protein were generated through different homology-based software tools. Subsequently, the optimal model was chosen and validated using the Ramachandran Plot. For further *In silico* investigations, docking was executed, selecting commonly used fungicides as ligands. Owing to its better performance in the Ramachandran plot analysis, the MODELLER software-generated model was chosen. Among the tested ligands, chlorothalonil exhibited the lowest binding energy of -7.7 kcal/mol, it is found to be the best ligand that interacts with INF1 protein. Despite its notable binding capacity, it is notorious for being corrosive and detrimental to the survival of other organisms as proved by evaluation of its ADMET properties. Wise formulations and strict regulations should be practiced ensuring safety of life and environmental sustainability.

Keywords: ADMET properties, Binding affinity, Fungicide, *In silico*, Pathogen

Potato is the crop with economic importance all over the world due to its huge production, consumption, affordable and easy availability in the market[1]. According to 2nd Advance Estimates (2021-2022), production of Potato in India was 53575 metric tonnes in the area of 2200 hectares[2,3]. A variety of pathogenic organisms can cause significant quality and productivity losses in potatoes[4]. Late blight (*Phytophthora infestans*), early blight (*Alternaria solani*), potato wart (*Synchytrium endobioticum*), stem canker (*Rhizoctonia solani*), and powdery scab (*Spongospora subterranea*) are a few of the most significant diseases in the world[5-8]. Because of its aggressiveness and genetic diversity, the oomycete *P. infestans*, which causes late blight and tuber blight, continues to be one of the major biotic limitations on potato output worldwide.

The pathogen generates few sporangia on the stem and leaves of potato, in leaves they are commonly observed at the lower surfaces. The symptoms involve dark blotches displayed at the end of the leaf and on the plant's stem. A grey/dark patch developed on the affected tuber, which covers the skin and rapidly decomposes it resulting in an unpleasant odour as seen in (Figs 1 & 2). Healthy tubers may later become rotten while stored[9].

P. infestans produces 10-kD extracellular protein elicitors, collectively considered as elicitins. One such protein is INF1 elicitin, which is a protein present in the pathogen *P. infestans*. INF1 induces a hypersensitive response in a restricted number of plants. It functions as a virulence factor in the interactions. In an experiment, the highest levels of expression of INF1 were observed in the grown mycelium and late stages of infection when profuse sporulation and leaf necrosis occur[10].

Due to the dominance of cultivars with poor or moderate resistance to late blight resulting from low marketability and acceptability of resistant cultivars, fungicide application remains the most popular method of control management[11,12]. While metalaxyl is infrequently used, mancozeb is one of the most often used fungicides. There are two interesting observations regarding metalaxyl resistance in the Mexican population. First, the pathogen population has a baseline level of resistance even in the absence of metalaxyl exposure. Secondly, once exposed the population experiences selection but quickly returns to threshold levels before exposure. Resistance to metalaxyl evolved rapidly in

*Correspondence:
E-mail: balakrishnan@naarm.org.in

Fig. 1— A leaf afflicted with leaf blight disease

Fig. 2 — Potato affected by leaf blight disease

the Mexican population of *P. infestans* after introduction of metalaxyl but then reverted to resistance levels found before the introduction[13-15]. Apart from these two fungicides, other ones that are utilised are cymoxanil alone or in combination with famoxadone, propamocarb, zoxamide with EBDC, ametoctradin in combination with dimethomorph, cyazofamid, mandipropamid in combination with difenoconazole *etc*[16,17]. In the present study, homology modelling of protein INF1 and docking experiments are carried out to find the fungicide that exhibits the best interaction.

Materials and Methods

Sequence retrieval and template selection

The amino acid sequence of the INF1 protein of *Phytophthora infestans* pathogen, which causes late blight disease in potato, was retrieved in FASTA format from NCBI database (https://www.ncbi.nlm.nih.gov/) with the GenBank accession number: AAV92913.1. To search for the homologous sequences, the retrieved sequence from NCBIwas used as query in BLASTp (https://www.ncbi.nlm.nih.gov/BLAST/) against PDB database (https://www.rcsb.org/) to perform homology modelling. All the parameters were set to default to ensure a maximum number of confident hits. A template for homology modelling was chosen by identifying and selecting the sequence that had the highest sequence similarity and the lowest E value[18].

Sequence alignment and homology modelling (Model building)

Sequence alignment

Sequence Alignment was carried out using CLUSTALW multiple sequence alignment online tool service provided by GenomeNet[18,19].

Method to perform multiple sequence alignment

A better quality of information on molecular evolution can be achieved from an enormous amount of sequence data. One widely used technique in the current scenario is the construction of a phylogenetic evolutionary tree, which is made with the aid of multiple sequence alignment[38]. The sequences obtained on BLASTp were uploaded as input files in FASTA format in the online multiple sequence alignment tool CLUSTALW (https://www.genome.jp/tools-bin/clustalw) hosted by Genome Net (https://www.genome.jp/).Other formatssuch as GCG, EMBL, GenBank PIR, NBRF, Philip or SWISS-PROT can also be used for input files. Multiple sequence alignment was then submitted with the remaining parameters set to their default values such as gap penalty – 10, gap extension penalty-0.05 and BLOSUM (protein) matrix to find evolutionarily divergent protein sequences. The job's results were obtained, and the most similar match was determined by analysis.

Homology modelling

After finding a confident match to the protein of interest, homology modelling was performed using SWISS-MODEL, MODELLER, IntFold, Robetta.

SWISS-MODEL

The protein ID of INF1 protein from *P. infestans* pathogen was submitted to SWISS-MODEL (https://swissmodel.expasy.org) web server to develop a model with sufficient query sequence coverage and sequence identity. The most reliable 3D structure was selected based on the Global Model Quality Estimation (GMQE) and Qualitative Model Energy Analysis (QMEAN) values. While QMEAN values below 4.0 indicate reliability, GMQE values typically range from zero to one, with higher numbers indicating greater reliability of the predicted structure. The similarity identity between the amino acid sequences of the homology model of INF1 protein and the template structure used for the homology model was confirmed using Clustal Omega version[19].

MODELLER

Modeling was performed using MODELLER 9.22v application. INF1protein sequence from *P. infestans*

pathogen was selected as the target sequence and then converted to PIR format (as INF1.ali file) which is the readable format by MODELLER, potentially related sequences of known structure searched using script file build_profile.py. 1LRI was chosen as the template based on sequence identity. The script align2d.py was used to align INF1 Protein to 1LRI (PDB ID) then the target sequence is aligned with the template and is written out in two formats, PIR ("INF1-1LRI.ali") and PAP ("INF1-1LRI.pap"). MODELLER uses the PIR format in the subsequent model building stage, while the PAP alignment format is easier to inspect visually. In the PAP format, all identical positions are marked with the"*" symbol. The script "model-single.py" was used to generate five similar models of INF1 protein based on 1LRI template structure and the alignment in the file "INF1-1LRI.ali". Evaluation of the model is done based on the lowest value of the DOPE score. The script "evaluate_model.py" was used to evaluate an input model with the DOPE potential. The script "plot_profiles.py" was used for the plot creation[21].

IntFold & Robetta

In IntFold (https://www.reading.ac.uk/bioinf/IntFOLD/), the target protein was entered as a sample sequence into the server for homology modelling in single-letter format. The results generated were different 3D models ranked according to decreasing global model quality scores. The range of global model quality scores was between 0 and 1. Based on the global model quality score five structures were selected[22-24]. In Robetta (https://robetta.bakerlab.org/), protein sequence was uploaded in FASTA format and five structures were obtained from homology modeling[25].

Protein model validation

The quality of models generated by MODELLER 9.22v, SWISS-MODEL server, IntFold, Robetta was validated. Assessment of the modelled structures and the stereo chemical analysis of the models was performed using the Ramachandran plot. The model with the highest number of residues in the favoured region and the least number of residues in the outlier region was selected for further analysis[36]. Ramachandran plot was generated using SAVESv6.0 server along with that ERRAT, VERIFY3D, PROCHECK tools were used to analyse the overall quality of the model[26,27]. While the Ramachandran plot is used in accessing the quality of a modelled protein or an experimental structure, the plot statistics provides information on the total number of amino acid residues found in the favourable, allowed and disallowed regions[28]. Finally, one of the models generated by MODELLER was selected for subsequent studies[28].

Selection of ligand molecules

The highly documented FDA-approved and commonly used fungicides utilized for the prevention of late blight and tuber blight disease in potato, were selected as ligands for performing docking with the model protein. Their structures were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and their 2D structures were downloaded in SDF format[28].

Blind docking

To start docking of protein, it is essential to prepare the protein since the crystal structure obtained from Protein Data Bank consists of issues such as improper bond orders, missing side chains, *etc*. Hence, the process of protein preparation is very helpful in assigning proper bonds, bond order, and addition of hydrogen, detection of disulphide bonds and correction of mislabelled elements[29]. The protein preparation procedure was carried out using UCSF ChimeraX. Molecular docking was performed using Auto Dock Tools 1.5.7 software. AutoLigand was used to detect the ligand binding sites within the model protein. To reduce the computational cost and time, a rigid docking protocol was considered through rigid receptor and rotatable ligands[28]. AutoDock Tools assigned polar hydrogens, united atom Kollman charges, solvation parameters. AutoDock saved the prepared file in PDBQT format. AutoGrid was used for the preparation of the grid map using a grid box. The grid size was set to 126 x 126 x 126 xyz points with grid spacing of $0.375A^0$ and grid centre was designated at dimensions (x, y and z): 9.085, 11.165, 2.56[29]. Then after running AutoGrid, the docking was performed and the results were analysed using AutoDock Tools for estimation of binding energies, hydrogen bonds formed, and bond length of formed hydrogen bonds[35]. The Lamarckian genetic algorithm[39,41] was used to explore the best conformational space of the ligand. The algorithm was set to run 50 times for each ligand and the number of energy evaluations is set to 25, 00,000. They are both fixed to control the computational resources a genetic algorithm can utilize while other parameters were set to default[41]. After collecting binding free energies of each ligand, the top results were selected[28]. Twenty-one different conformations were generated for each ligand scored using AutoDock scoring functions and were ranked according to their binding energies[27].

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CLUSTAL O(1.2.4) multiple sequence alignment

2POS_1|Chains      ----------------WEETKEC----AFTEFFKLAPLASNPALSVCQDASGWQMLPPAG 40
AAV92913.1         MNFRALFAATVAALVGSTSATTCTTSQQTVAYVALVSILSDTSFNQCSTDSGYSMLTATS 60
1LJP_1|Chains      --------------------TACTATQQTAAYKTLVSILSESSFSQCSKDSGYSMLTATA 40
1BXM_1|Chain       --------------------RGTCTATQQTAAYHTLVSILSDASFNQCSTDSGYSMLTAKA 41
1BEG_1|Chain       --------------------TACTATQQTAAYKTLVSILSDASFNQCSTDSGYSMLTAKA 40
1LRI_1|Chain       --------------------TACTASQQTAAYKTLVSILSDASFNQCSTDSGYSMLTAKA 40
                                        *      . :  *. : *: ::. *.  **:.**   .

2POS_1|Chains      YPTPEQLKLMCGTAECFTLIDAIKALNPNDCILVFGDV--RLNVKKLVTEFEPSCF--   94
AAV92913.1         LPTTEQYKLMCASTACKTMINKIVSLNAPDCELTVPTSGLVLNVYSYANGFSSTCASL   118
1LJP_1|Chains      LPTNAQYKLMCASTACNTMIKKIVALNPPDCDLTVPTSGLVLDVYTYANGFSSKCASL   98
1BXM_1|Chain       LPTTAQYKLMCASTACNTMIKKIVTLNPPNCDLTVPTSGLVLNVYSYANGFSNKCSSL   99
1BEG_1|Chain       LPTTAQYKLMCASTACNTMIKKIVTLNPPNCDLTVPTSGLVLNVYSYANGFSNKCSSL   98
1LRI_1|Chain       LPTTAQYKLMCASTACNTMIKKIVTLNPPNCDLTVPTSGLVLNVYSYANGFSNKCSSL   98
                    **  * ****.:: * *:*. * :**  :* *..      *:* . .. *. .*
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Fig. 3— Result of multiple sequence alignment

ADMET properties

The SMILES format of the selective fungicides has been downloaded using the public ligand database PubChem. Their ADMET (Absorption, Distribution, Metabolism, Excretion, Toxicity) properties were evaluated using the admetSAR program (http://lmmd.ecust.edu.cn/admetsar2/)[42].

Results and Discussion

Template selection

Generally, a high sequence identity is preferred for high-quality models, although to establish a protein model of adequate quality, more than 30% similarity between the target and template sequences is required. Accordingly, BLASTp result of INF1 Protein (GenBank Accession Number: AAV92913.1) as shown in (Fig. 3) in the PDB database showed that the crystal structure of Chain A, Beta-Elicitin Crytogein of *Phytophthora cryptogea* (PDB ID: 1LRI) has the most query coverage and sequence identity (82.95%) among all other proteinsas depicted in (Fig. 4). Hence, the 3D structure of Beta-Elicitin Crytogein of *Phytophthora cryptogea* was used as a template for model generation. The MODELLER 9.12, SWISS-MODEL, IntFold, and Robetta were used for homology modelling of INF1 Protein as illustrated in (Figs 5-7).

Structure validation using SAVESv6.0 server

Predicted model quality was evaluated by SAVESv6.0 server (https://saves.mbi.ucla.edu/), it is a complete package of five programmes that test the overall consistency of a protein structure. ERRAT, VERIFY3D, PROCHECK tools were used for the analysis and evaluation of the predicted models. Further evaluation of the model by PROCHECK demonstrated the residues in the allowed regions, showing the acceptable quality of the constructed

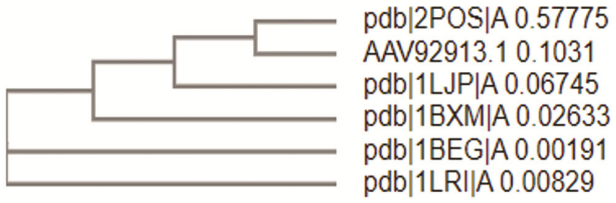

Fig. 4 — Cladogram of Phylogenetic tree the selected protein showing 82.9% sequence identity with protein 1LRI

model[28]. In the ERRAT plot (which is used to evaluate and validate the crystal structure of a protein in which the error values are plotted as a function of the sliding 9-residue window location)[34]. The Ramachandran plot statistics derived through PROCHECK which is the gold standard to determine the stereo chemical quality of a protein structure. The results implied that the modelled 3D structure of INF1 Protein has 95.4% of its residues in the most favoured regions, 4.6% of its residues in additional allowed regions, 0.0% of its residues in the generously allowed regions and 0.0% of its residues in disallowed regions of the Ramachandran plot. As a rule of thumb, the aggregation of ϕ and ψ angles in most favoured regions is >90%, ensures a good quality protein model, this validates that the modelled 3D structure is a good quality model. The final 3D-structure was validated through VERIFY3D. The successful outcome of molecular docking protocol relies on the optimized three-dimensional (3D) structure of protein and ligand for the precise binding affinity prediction [40]. Hence, the best conformation of the model after evaluation was selected is shown in (Figs 8 & 9).

Blind docking Analysis to predict the ligand binding sites

The binding mechanism of proteins with ligands from last few years have shed light on different areas of research and its important applications in numerous

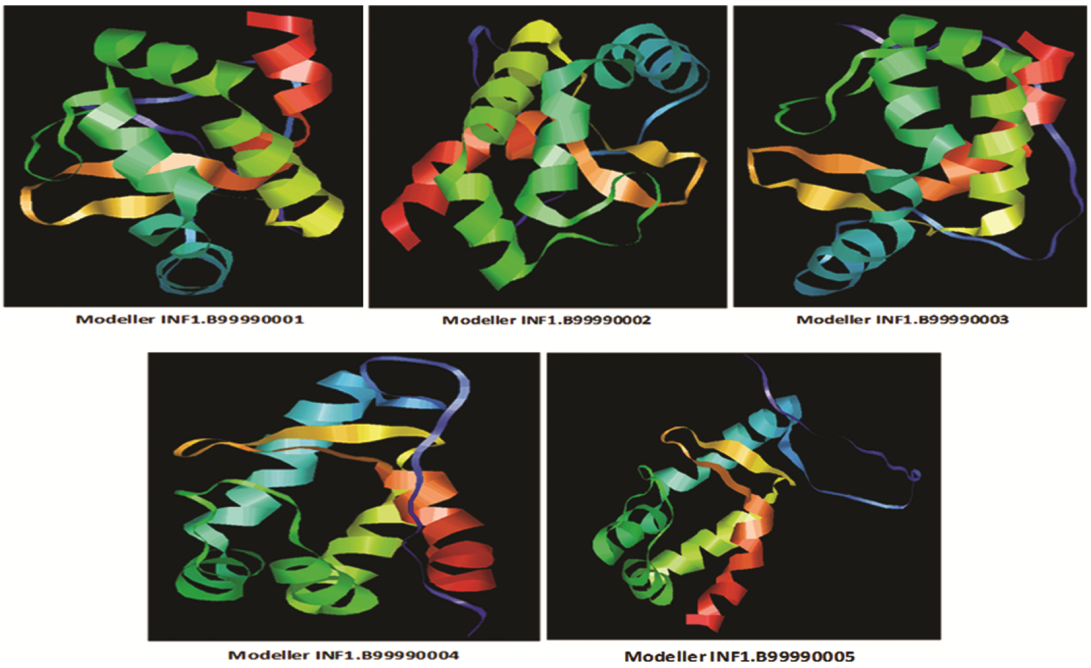

Fig. 5 — Protein models generated from MODELLER depicted in decreasing order of similarity

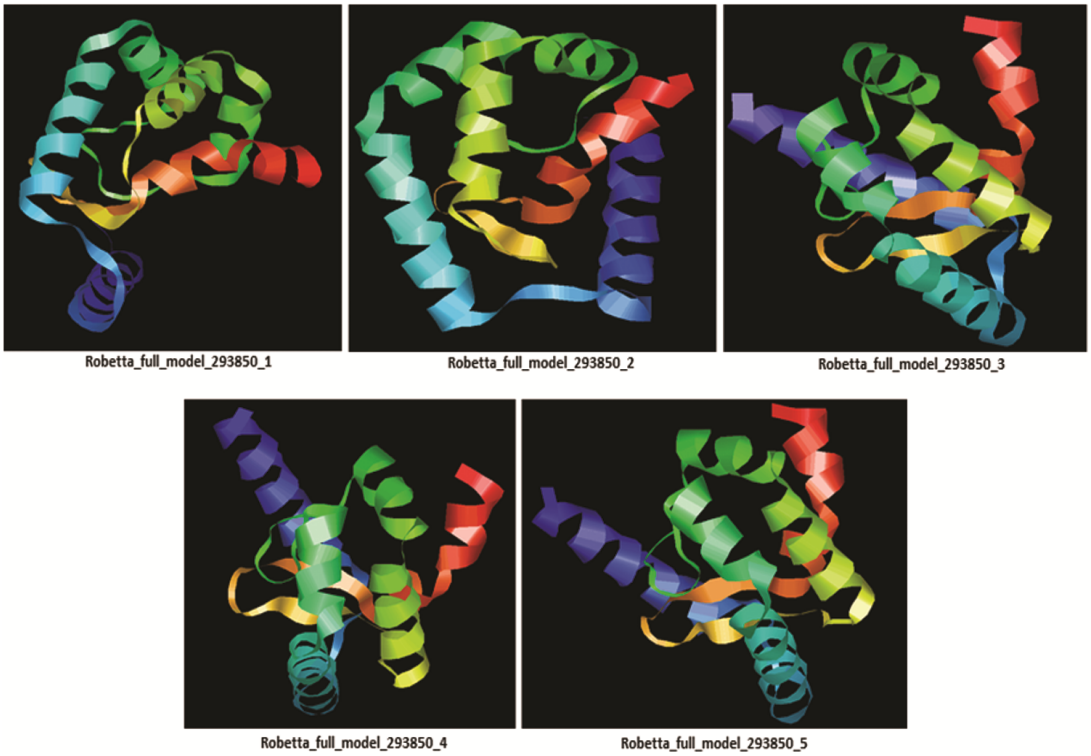

Fig. 6 — Protein models generated from Robetta represented in decreasing order of similarity

fields of science, such as the development of new biomaterials, biochemistry, food chemistry or pharmaceutical science[37]. Blind docking is a reliable and useful approach to explore the possible ligand binding sites of a protein. It has been used in a variety of docking studies to explore unknown binding sites on different proteins. It was employed in the present study to perform docking of different fungicides considering the undefined

ligand binding sites on INF1 Protein. The fungicides registered for the control of blight disease in potato include mancozeb, dimethomorph, chlorothalonil, ametoctradin, cyazofamid, mandipropamid, difenoconazole, famoxadone, cymoxanil, propamocarb, zoxamide, fenamidone were obtained from thorough literature and extensive database search[41]. The above-mentioned ligands were blindly docked within the limits of a grid and the potential binding areas were predicted. The blind docking approach yielded 50 binding conformations for each ligand. The conformations with most frequent binding sites and the highest binding affinities were filtered. The highest binding affinity of each compound belonged to a binding site that had the highest overlap among all ligands[28]. Three ligands *i.e.*, mancozeb, dimethomorph, chlorothalonilas illustrated in (Fig. 10) although structurally unrelated were selected for docking studies as these ligands were found to have interacted well and had highest binding affinity with the model protein.

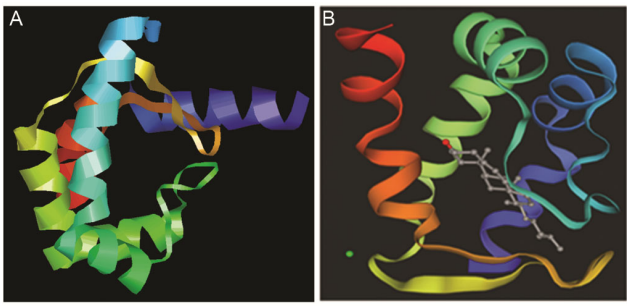

Fig. 7 — Protein model generated by IntFold

Docking results

Using the AutoDock Vina program, the screened ligands were docked with INF1 protein. The ligands were then sorted according to their minimal binding affinity. The optimum posture was determined based on the lowest binding affinity. Highest binding affinity with INF1 protein was found to be of chlorothalonil, followed by dimethomorph and mancozeb. The docking results of the ligands along with their binding energies are illustrated in (Table 1). The best-docked structures of the individual ligands with INF1 protein are shown in (Figs 11-13).

ADMET prediction

Before introduction into a natural environment, evaluation of ADMET (Absorption, Distribution, Metabolism, and Excretion) properties and environmental hazard assessment of a fungicide is performed to assess their efficacy and functionality. The ADMET profile has a direct impact on physiochemical properties such as hydrophobicity, lipophilicity, gastrointestinal environment, and blood-brain barrier integrity. Computer-based prediction of ADMET properties of chlorothalonil is represented in (Table 2) was performed using admetSAR webserver.

Chlorothalonil is an acute, non-systemic fungicide. The results suggest that it is an eye-irritant, corrosive to skin. It enters the body through oral route andgetseasily absorbed in the intestine. It localizes in the mitochondria and affects several biological processes.

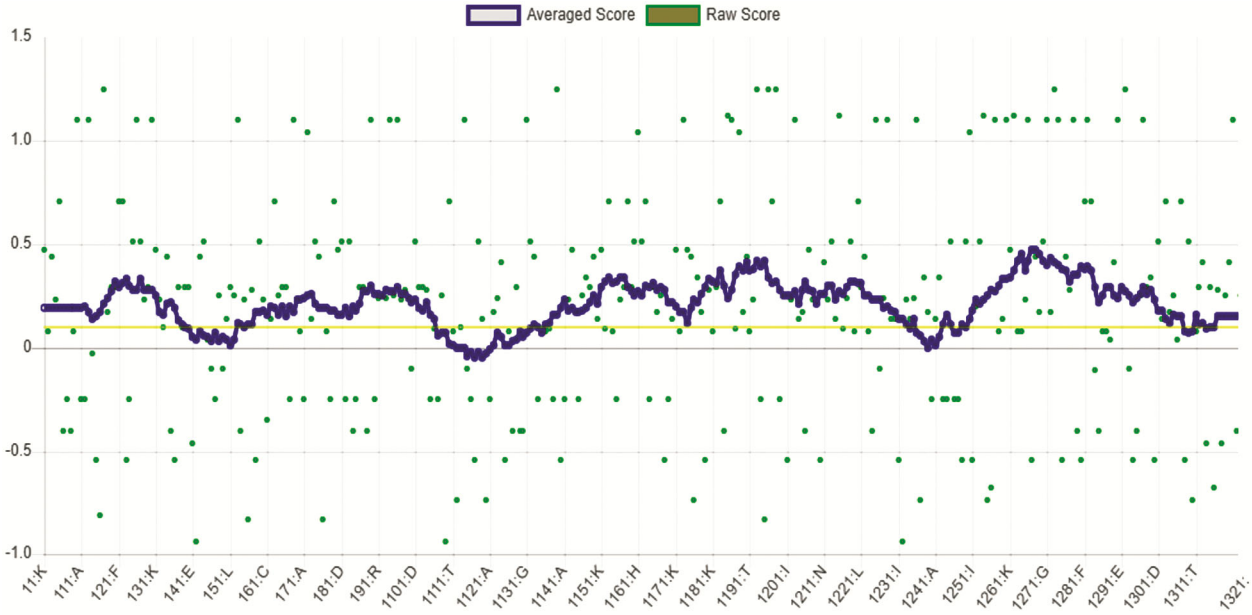

Fig. 8 — Validation results from Verify3D plot showing at least 80% of the amino acids scored ≥ 0.4 in the 3D/1D profile

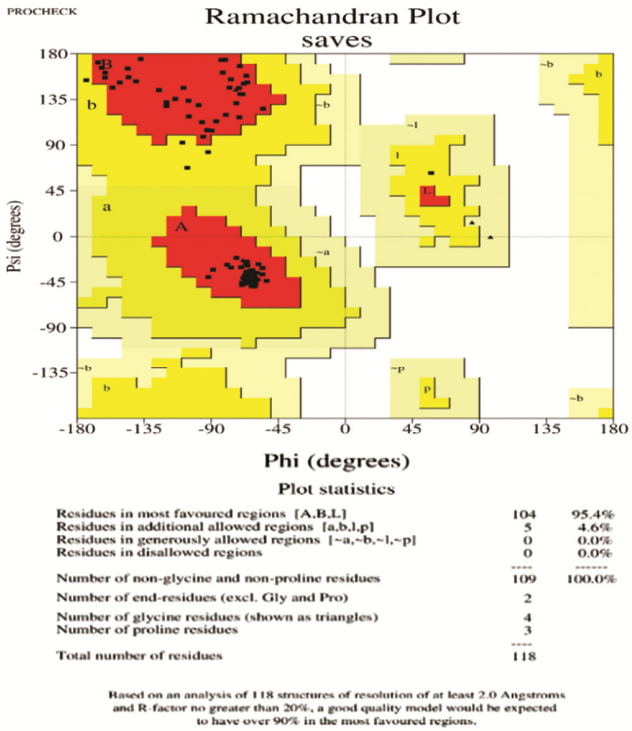

Fig. 9 —Ramachandran Plot of INF1 Protein shows that 95.4% of the amino-acid residues are in the most favoured regions and 4.6% of the amino-acid residues are in allowed regions

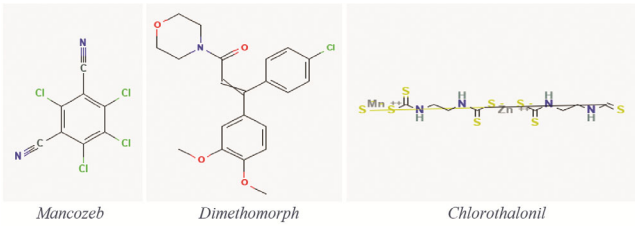

Fig. 10 — Ligands selected for docking

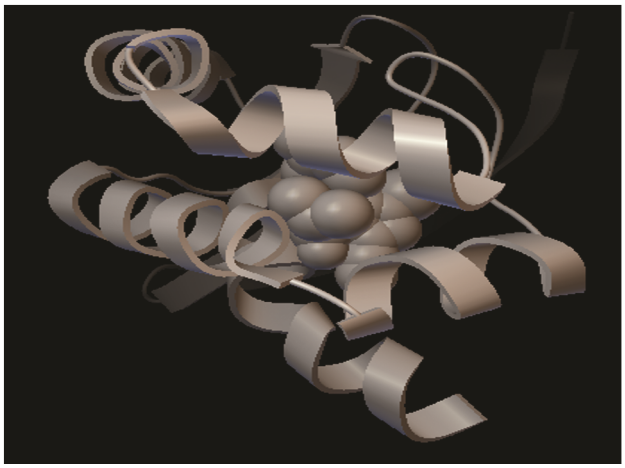

Fig. 11 — Docked structure of INF1 Protein &Dimethomorph

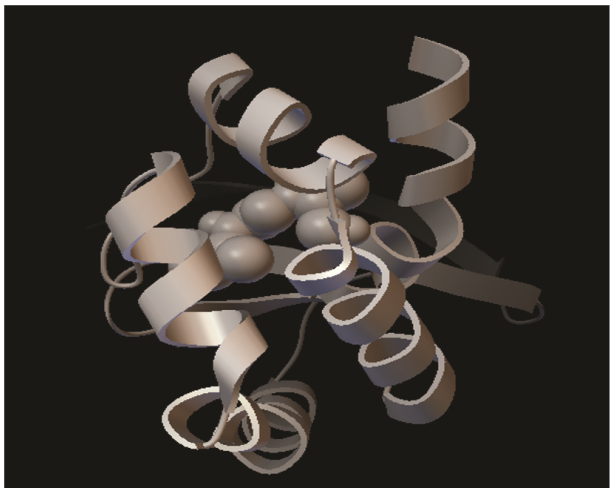

Fig. 12 — Docked structure of INF1 Protein & Mancozeb

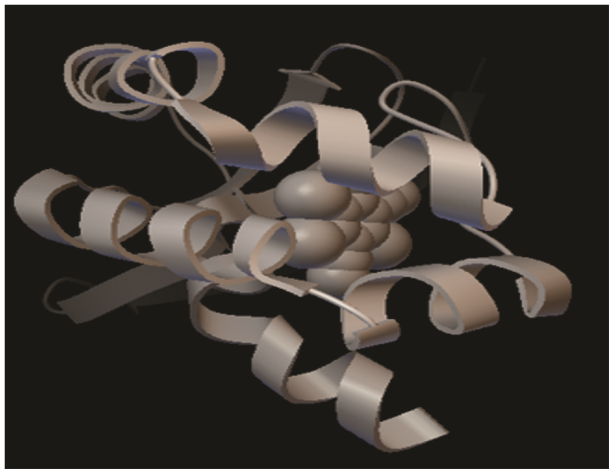

Fig. 13 — Docked structure of INF1 Protein & Chlorothalonil

Table 1 — Docking Analysis of Ligands with respective docking energies

S. No.	Ligand	AutoDock Docking Energy (kcal/mol)
1	Chlorothalonil	-7.77
2	Dimethomorph	-6.24
3	Mancozeb	-5.32

More research is required to establish its carcinogenicity in humans, previously proved in mice. It affects aquatic life; honey bees and can disrupt human health because of bio-magnification. It has exhibited reproductive, respiratory, mitochondrial, acute oral and nephrotoxicity. Immediate effects of chlorothalonil are not noticeable as it accumulates in the human body and causes long-term health defects[42]. Hence, strict regulations should be implemented to allow the permissible use of the fungicide.

Table 2 — ADMET properties of Chlorothalonil

S. No	ADMET Prediction	Value
1.	Ames mutagenesis	0.9000
2.	Acute Oral Toxicity (c)	0.6259
3.	Androgen receptor binding	0.8586
4.	Aromatase binding	0.6175
5.	Blood Brain Barrier	0.8750
6.	Biodegradation	0.9750
7.	BSEP inhibitor	0.6175
8.	Caco-2	0.7156
9.	Carcinogenicity (binary)	0.5219
10.	Carcinogenicity (trinary)	0.6723
11.	Crustacean aquatic toxicity	0.9200
12.	CYP1A2 inhibition	0.8493
13.	CYP2C19 inhibition	0.5435
14.	CYP2C9 inhibition	0.5537
15.	CYP2C9 substrate	0.8153
16.	CYP2D6 inhibition	0.5537
17.	CYP2D6 substrate	0.7370
18.	CYP3A4 inhibition	0.7098
19.	CYP3A4 substrate	0.6796
20.	CYP inhibitory promiscuity	0.6331
21.	Eye corrosion	0.9710
22.	Eye irritation	0.9856
23.	Oestrogen receptor binding	0.8905
24.	Fish aquatic toxicity	0.9690
25.	Glucocorticoid receptor binding	0.7030
26.	Honey bee toxicity	0.9678
27.	Hepatotoxicity	0.8875
28.	Human Ether-a-go-go-Related Gene inhibition	0.7850
29.	Human Intestinal Absorption	0.9870
30.	Human oral bioavailability	0.8571
31.	MATE1 inhibitor	0.8200
32.	Mitochondrial toxicity	0.7000
33.	Micro nuclear	0.6500
34.	Nephrotoxicity	0.7839
35.	Acute Oral Toxicity	0.6259
36.	OATP1B1 inhibitor	0.9490
37.	OATP1B3 inhibitor	0.9569
38.	OATP2B1 inhibitor	1.0000
39.	OCT2 inhibitor	0.8500
40.	P-glycoprotein inhibitor	0.9352
41.	P-glycoprotein substrate	0.9928
42.	PPAR gamma	0.8707
43.	Plasma protein binding	1.013
44.	Reproductive toxicity	0.8667
45.	Respiratory toxicity	0.7111
46.	Skin corrosion	0.8876
47.	Skin irritation	0.5176
48.	skin sensitisation	0.9333
49.	Subcellular localization	0.8398 (in mitochondria)
50.	Tetrahymena pyriformis	2.311
51.	Thyroid receptor binding	0.6299
52.	UGT catalysed	0.0000
53.	Water solubility	-4.704

Conclusion

This study provides first-hand theoretical insight on the homology based structural determination of INF1 protein present in the pathogen *P. infestans,* which is the causal organism of late blight and tuber blight disease in Potato. Different softwares and tools were employed for structure prediction such as MODELLER 9.12, SWISS-MODEL, IntFold, and Robetta to build the model of INF1 Protein. Several software programs and techniques were used to help arrive at the finest possible protein structure. Then, the predicted model quality was evaluated by ERRAT, VERIFY3D, PROCHECK tools using SAVESv6.0 server. Further, the binding affinities as well as the binding modes of available fungicides (ligands) with homology-modelled INF1were predicted. The docking output revealed good binding affinity values ranging from − 5.32 to – 7.7 kcal/mol of three ligands chlorothalonil, dimethomorph and mancozeb, which confirms their resilience potency. This is an indication that the ligands possess better conformation with the active site of the modelled protein when compared with other ligands of the data set. The research findings have confirmed the strong affinity between chlorothalonil and the modelled protein. The output of this study proved that it is the best-suited fungicide to combat the pathogenicity of the disease. Owing to the toxicity of chlorothalonil, its broad use should be limited and regulated in a controlled manner. A toxicological assessment of fungicides and PAINS filter to remove the false leads were not included in the study. Combinatorial application of less-lethal fungicides will foster an overall resistance to the disease in plant without leaching into other organisms. Understanding the global expression and the regulatory pathways of INF1 protein in cellular processes will sensitise the need for the application of apt fungicides and better management of the disease. However, to validate these results, further pharmacological and *in vivo* studies are required. Structure modification strategies involving chlorothalonil needs further exploration. These two aspects represent the future scope of the study.

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Conflict of interest

All authors declare no conflict of interest.

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