Antimicrobial activity of methanolic extracts of Vernonia cinerea against Xanthomonas oryzae and identification of their compounds using in silico techniques

Tushar Joshi¹, Satish Chandra Pandey¹,², Priyanka Maiti³, Manish Tripathi⁴, Ashutosh Paliwal¹, Mahesa Nand⁵, Priyanka Sharma⁶, Mukesh Samant², Veena Pande¹, Subhash Chandra ID*¹

¹ Department of Biotechnology, Bhimtal Campus, Bhimtal, Kumaun University, Uttarakhand, India, ² Cell in Molecular Biology Laboratory, Department of Zoology, Soban Singh Jeena University, Almora, Uttarakhand, India, ³ Centre for Environmental Assessment & Climate Change, G. B. Pant National Institute of Himalayan Environment, Kosi-Katarmal, Almora, Uttarakhand, India, ⁴ Computational Biology & Biotechnology Laboratory, Department of Botany, Soban Singh Jeena University, Almora, Uttarakhand, India, ⁵ Environmental Information System on Himalayan Ecology, G.B. Pant National Institute of Himalayan Environment, Kosi-Katarmal, Almora, Uttarakhand, India, ⁶ Department of Botany, Kumaun University, D.S.B Campus, Nainital, Uttarakhand, India

* scjnu@yahoo.co.in

Abstract

Bacterial Leaf Blight (BLB) disease is an extremely ruinous disease in rice, caused by Xanthomonas oryzae pv. oryzae (Xoo). Although various chemicals are available to manage BLB, they are toxic to the environment as well as humans. Hence there is a need to develop new pesticides as alternatives to hazardous chemicals. Therefore, a study was carried out to discover new potent natural pesticides against Xoo from different solvent extracts of Vernonia cinerea. Among all the fractions, the methanolic extract showed the highest inhibition zone. Further, to gain mechanistic insight of inhibitory action, 40 molecules of methanolic extracts were subjected for in silico study against two enzymes D-alanine-D-alanine ligase (Ddl) and Peptide deformylase (PDF). In silico study showed Rutin and Methanone, [1,4-dimethyl-7-(1-methylethyl)-2-azulenyl]phenyl have a good binding affinity with Ddl while Phenol, 2,4-bis(1-phenylethyl)- and 1,2-Benzenedicarboxylic acid, diisooctyl ester showed an excellent binding affinity to PDF. Finally, the system biology approach was applied to understand the agrochemical’s effect in the cell system of bacteria against both the enzymes. Conclusively, these four-hit compounds may have strong potential against Xoo and can be used as biopesticides in the future.

Introduction

The global rice demand was estimated to rise from 439 million tons (milled rice) in 2010 to 496 million tons in 2020 and further, it will increase to 555 million tons in 2035 as per need of
increasing the population, according to the Food and Agricultural Policy Research Institute (FAPRI) (http://ricepedia.org/rice-as-a-crop/rice-productivity) (accessed date- 6 April 2021). The crop is widespread all over the world due to its broad adaptability under different environmental conditions and also an important food crop as a huge chunk of the global population depends on rice for its nutrition. But, the current day the crop is facing a huge amount of yield losses throughout the world due to a disease named Bacterial Leaf Blight (BLB) caused by a bacteria known as \textit{Xanthomonas oryzae pv. oryzae} (\textit{Xoo}) [1]. The disease commonly causes significant yield losses of 20–30% or even up to 80% at the maximum tillering stage. At the booting stage of the plant, the \textit{Xoo} lowers the quality of grains and causes broken kernels. Rice plants in both tropical and temperate environments, particularly in irrigated and rainfed lowland areas, areas that have weeds and stubbles of infected plants with optimum temperature $25–34^\circ C$ and relative humidity above 70% are prone for the disease.

In India, BLB disease occurs from north to west and east to south with approximate yield losses up to 60–80% [2]. This significant loss affects the farmer’s fields along with the industries, and the global population depends on the various product of rice. Therefore, the management of BLB disease is a necessary step towards the protection of rice crops worldwide. Studies showed three different approaches, \textit{viz}, biological control, chemical control, and genetic resistance, are applied for the management of BLB disease [3, 4]. Among these methods, the biological control method is one of the most appropriate for the above disease management, as it is environment friendly and non-toxic not only for humans but also small microorganisms to large mammals [5]. In some of the studies, researchers reported the use of plant growth-promoting rhizobacteria to manage the BLB disease [6]. Several phytochemical extracts from plant species including \textit{Ocimum gratissimum}, \textit{Curcuma longa}, \textit{Acorus calamus}, \textit{Tamarindus indica}, and \textit{Azadirachta indica}, have been reported for the management of BLB disease [7–9].

Previously many technologies have been used to protect rice crops from BLB however, directly targeting the bacterial growth enzymes is an efficient strategy to stop BLB. In this regard, Ddl and PDF are key bacterial growth enzymes. Ddl is an ATP-dependent bacterial enzyme that plays a vital role in the intracellular stages of peptidoglycan biosynthesis [10]. The main product of the Ddl enzyme is D-alanyl-D-alanine, which is the terminal dipeptide of UDP-N-acetylMuramoylpentapeptide [11] and is eventually involved in trans-peptidation. Whereas PDF is important for bacterial cells and catalyzes the removal of the N-formyl group from N-terminal methionine. Hence, Ddl and PDF enzymes have elected the targets for the present study. Thus, due to the importance of BLB disease of rice, the present study aims to find out the effective natural pesticide compounds from \textit{Vernonia cinerea} against \textit{Xanthomonas oryzae pv. oryzae} disease.

The plant \textit{Vernonia cinerea} is used in the component of dasapushpam (in Sanskrit, dasa = ten and pushpam = flowers), an herbal combination of 10 plants that are traditionally used in the Kerala state of India. It is known as purple fleabane and Sahadevi and has been reported to use in traditional medicine for treating various ailments including inflammation, diarrhea, cough, smoking cessation, asthma, Parkinson’s disease, leprosy, and conjunctivitis [12]. The plant has also been reported to show several pharmacological properties including analgesic, anti-inflammatory, antipyretic, antibacterial, anti-oxidant, anti-glycemic, antidiarrheal, anti-tumor, antiplasmodial, and anti-Helicobacter pylori activity. In the current work, four different solvent extracts of the whole plant were tested against \textit{Xoo} bacterium under in-vitro conditions for their anti-BLB activity. Further, Ddl and PDF target-specific phytochemicals were identified against the bacteria by molecular docking, molecular dynamics simulation, and system biology. Hence, in this study, we have evaluated the anti-BLB activity of \textit{V. cinerea} extracts in vitro conditions and identified potential natural agrochemicals targeting two key enzymes, i.e., Ddl, and PDF by \textit{in silico} approaches. Further system biology approach was
applied to understand the possible mechanism of action of these target-specific drug molecules. The network analysis was also conducted to identify key reactions in the Xoo system that could affect cellular reactions after drug interaction.

**Material and methods**

**Plant collection and extraction**

The whole plant of *V. cinerea* was collected from District Sant Kabir Nagar, Uttar Pradesh (specific permission was not required because this plant is wild and can found anywhere). Identification of the plant was done by Forest Research Institute, Dehradun. Further, the collected plant was dried and pulverized by using a mechanical grinder to a coarse powder, and the powder was sifted and stored in airtight containers and kept at room temperature until processing. The preparation of crude extract was done with the help of the Soxhlet apparatus. Four solvents; Methanol, Ethanol, Chloroform, and Chloroform+Ethanol were used in 90% (v/v) concentration for extraction of phytochemicals of *V. Cinerea*. 100 g dry powder of *V. cinerea* was dipped in four different solvents combinations for 24 hrs. The extracts were filtered and concentrated under reduced pressure and the remaining solvents were evaporated. Before using, the dried extracts of plant material were dissolved in 0.7% DMSO (Dimethyl sulfoxide) and stored at 4°C.

**Antibacterial activity of *V. cinerea***

*Xoo* was purchased from the Indian Agriculture Research Institute–ICAR Delhi. The antimicrobial activity of plant extracts was analyzed on agar plates, liquid media, and MIC was calculated according to the modified laboratory protocol of the Clinical and Laboratory Standards Institute [13]. Briefly, 0.1 mL bacterial culture was inoculated on Peptone-sucrose (PS) agar plates and incubated at 28°C with filter discs (5 mm diameter) saturated with different dilutions of plant extracts (25, 50, and 100 μg/mL) for one day. The inhibition zones (mm) were measured by determining the diameter of the clear area. Similarly, the antibacterial activity was also measured by incubating the above-mentioned concentrations of plant extracts into PS broth media at 28°C for 24 hours. For the MICs, different concentrations (2.5, 5, 10, 25, 20, 50, and 100 μg/mL) of plant extracts were added to *Xoo* culture in PS media and incubated for 24 h at 28°C. The lowest concentration of plant extracts that prevented microbial growth (showed no turbidity) was measured by spectrophotometer at OD 600. Each test was performed in triplicate. Tetracycline was employed as a positive control. Cultures without plant extracts or antimicrobials were used as a negative control.

**Statistical analysis**

For statistical analysis, all experimental data were analyzed in triplicates, and results were calculated as mean±SD. The results (pooled data of three experiments) of experiments were calculated by one-way ANOVA. All analysis was done by using Graph Pad Prism (version 3.03) software.

**In silico screening**

Phytochemicals library and ligand preparation. To know the insight activity of methanol extracted compounds of *V. cinerea* against *Xoo*, a library of 40 phytochemicals was constructed by using text mining analysis. Several studies have been reported on GC-MS analysis of phytochemicals of different extracts of *V. cinerea* and from these studies, we collected the phytochemicals found in methanolic extract and constructed a library [14, 15] by downloading
3D structures of these phytochemicals from PubChem in SDF format, and converted into PDB files using Open Babel. The Reference molecule ANP (Phosphoaminophosphonic acid-adenylate ester) and 56V [(3R)-2,3-dihydro[1,3]thiazolo[3,2a]benzimidazol-3-ol] which was co-crystallize with Ddl and PDF proteins, were downloaded from Protein Data Bank.

**Protein preparation.** The 3D structures of Ddl (PDB ID 4L1K) and PDF (PDB ID 5CY8) enzymes of Xoo (Xoo1075) were retrieved from the Protein Data Bank (https://www.rcsb.org). In the protein preparation, all water molecules, ions, and ligands were removed using PyMOL software. After that, the addition of hydrogen atoms to the receptor molecule was carried out by MGL Tools. The preprocessed structures of both proteins were then saved in PDB format for further analysis.

**Molecular docking and visualization.** The docking calculation process was performed to obtain a population of possible orientations and conformations for the ligand at the binding site by using PyRx open-source software (GUI version 0.8 of AutodockVina). This software performs the prediction of the bound conformation based on the binding affinity. The grid center for docking was set for Ddl was X = 12.19, Y = 16.17, and Z = 17.76 and for PDF was X = 7.589, Y = -15.291, and Z = -2.049, and the dimensions of the grid box were set as 25.00 × 25.00 × 25.00 Å having a spacing of 0.375 Å between the grids points. Molecular docking of compounds was performed in the active site of Ddl and PDF proteins. Further, Molecular interactions between protein-ligand complexes, including hydrogen bonds and the bond lengths, were visualized using Ligplot+ v.1.4.5 software.

**Molecular dynamics (MD) simulations.** MD simulations were carried out with docked complexes using a GROMACS 5.0 package and the topologies files were generated using the CHARMM 36 force field. Afterward, complexes were solvated with a water model followed by neutralization by adding the ions. After adding ions, to relax the structure, Energy minimization was performed at 10 KJ/mol with the steepest descent Algorithm by using Verlet cut off-scheme and the total nsteps of protein and protein-ligand complex energy minimization cycle was 50,000. The equilibration step was performed in NVT (constant volume) as well as NPT (constant pressure) ensemble conditions, each with a 100 ps time scale. Further, the production of MD simulation was performed at a constant temperature of 300 K and a constant pressure of 1 atm with a time step of 2 fs, using the Parrinello-Rahman for constant pressure. The final MD simulations were produced using the LINCS algorithm for a 100 ns time scale. The generated trajectories were used to analyze the behavior of each complex in the explicit water environment. The deviations of the protein-ligand complex system were calculated by using Root mean square deviation (RMSD), Root mean square fluctuation (RMSF), Radius of gyration (RG), interaction energy. The short-range Lennard-Jones energy model was used for the calculation of interaction energy between proteins and compounds.

**System biology analysis model construction.** To analyze the effect of different amounts of the agrochemical against two Ddl and PDF in the bacterial cell system, a detailed analysis of the system biology of the bacterial cell was performed. For that, a model of the integrated biochemical levels was developed to understand the reaction mechanism in which these enzymes work as catalyzing agents. For the construction of biological networks, a System Biology Graphical Notation (SBGN) was used to represent cellular components (S1 Fig in S1 File) [16]. The pathway of enzyme reactions was constructed using Cell Designer 4.1 and stored in Systems Biology Markup Language (SBML). SBML is a machine-readable expression for reflecting the biological networks. System biologists use existing signs of DNA, RNA, protein, simple molecule, catalysis, stimulation, inhibition, phosphorylation, activation, and degradation to develop the pathway by using Cell Designer software to study live cells on a computer. In a cell designer, simulation can also be conducted by utilizing SBMLODE Solver and Copasi.
**Kinetic rate equations assignment.** To generate the kinetic rate equations for every reaction in the model, SBML squeezer version 2.1 of Cell Designer was used. SBML squeezer makes the whole process error-free and easy through Cell Designer. Rate laws comprising different forms of general mass action and enzyme kinetics were also generated by the SBML squeezer (S3 Table in S1 File). Kinetic rate equations for various enzymatic reactions include Michaelis-Menten kinetics and hill equation for single substrate reactions, irreversible non-modulated non-interacting reactant and enzymes, bi-uni enzyme reactions, bi–bi enzyme reactions, thermodynamics, and kinetic modular rate laws [17, 18].

**Model simulation.** SBMLbODE Solver Library (SOSlib) of cell designer was employed to simulate the dynamic nature of the developed model. This library allows the management of ordinary differential equations (ODE) based simulations that are common procedures for quantitative investigation of biological networks. SOSlib is a programming library commonly used in the symbolic and numerical interpretation of biochemical reaction network models encoded in the SBML. While working with biological networks, relevant parameters are needed to be evaluated for their dependency on other elements of the model. The native library was run by a simulation engine and obtained results are shown in a Graphical User Interface (GUI) window written in the JAVA programming language [17].

**Network analysis.** Reaction models of Ddl and PDF enzymes were built by using Cell Designer and exported in SBML format to Cytoscape 2.8.3 software by using Biological Network Manager (BiNoM). BiNoM is a Cytoscape plugin to assist numerous biological network activities that are demonstrated in common systems biology formats (SBML, SBGN, BioPAX). It is also used to undertake investigations of the complicated network structure. Various types of plugins are present in Cytoscape software, which enable us to decode the complications of biological networks. Network Analyzer plugin was used to investigate and visualize the crucial elements in enzyme reaction in our model [18].

**Results**

**Antimicrobial activity of V. cinerea**

The antibacterial potential of *V. cinerea* was evaluated according to their zone of inhibition against *Xoo*. The zones of inhibition were compared with the activity of the standards antibiotic Tetracycline as positive control while DMSO was used as a negative control (S1A, S1B, and S1C Fig in S1 File).

It was observed that the methanolic extract of the plant was the most effective among the three solvent extracts (Ethanol, Chloroform, and Chloroform+Ethanol) and showed a better zone of inhibition at different concentrations (25, 50, and 100 μg/ml). The Chloroform, Ethanol, and Chloroform+Ethanol extracts showed no zones of inhibition at low concentrations therefore these extracts were omitted for further study.

The methanolic extract inhibited the growth of *Xoo* bacteria after 24 h of incubation and showed a remarkable zone of inhibition. At the concentration, 25 μg/ml the size of the zone was 16.0±1.0 mm, while at 50 μg/ml concentration, the size of the zone was 18.1±1.0 mm and at 100 μg/ml concentration, the size of the zone was 22.6±2.08 mm as compared to positive control tetracycline (33.17±3.7mm at 5 μg/ml) (S1 Table in S1 File). The methanolic extract showed significant (P < 0.001) growth inhibition activity up to 24 h.

In liquid culture medium, the treatment of *Xoo* at the concentration of 25 μg/ml showed 31.14±0.54% inhibition while at the concentration 50 μg/ml, the percent inhibition was 50.50±0.89% and at the concentration 100 μg/ml the percent inhibition was 74.94±0.78% as compared to positive control tetracycline (92.61±0.80) (S1 Table in S1 File). Thus, the methanolic extracts showed significant (P < 0.001) growth inhibition activity up to 24 h (Fig 1B). The
Methanolic extracts showed significant inhibitory effects within the range of MIC. The MIC value of *V. cinerea* extract for *Xoo* was measured to be 10 μg/ml as compared to 1 μg/mL (tetracycline) as a positive control.

In addition, *in silico* studies were carried out to evaluate the mechanism of the phytochemicals of methanolic extracts and a compound’s library was prepared. Further, these molecules were subjected to molecular docking and dynamic simulation study against two important proteins of *Xoo* viz., Ddl and PDF.

**Molecular docking analysis**

The library of 40 compounds was subjected for molecular docking against the Ddl and PDF receptor. Out of 40 compounds, the top 2 compounds namely; rutin (-8.3 kcal mol \(^{-1}\)) and Methanone, [1,4- dimethyl-7-(1- methylethyl)-2-azul enyl]phenyl- (-7.8 kcal mol \(^{-1}\)) showed a binding affinity with Ddl and Phenol, 2,4-bis(1-phenylethyl)- (-7.5 kcal mol \(^{-1}\)) and 1,2-Benzenedicarboxylic acid, diisooctyl ester (-7.4 kcal mol \(^{-1}\)) showed their binding affinity with PDF. These compounds showed better and significant binding energy with Ddl and PDF as compared to reference molecule ANP (-8.3 kcal mol \(^{-1}\)) and 56V (-7.7 kcal mol \(^{-1}\)). The hydrogen

![Figure 1](https://doi.org/10.1371/journal.pone.0252759.g001)

**Table 1. Binding affinity of methanolic extract compounds against Ddl and PDF enzymes.**

| S. No. | Phytochemicals and Protein name                  | Binding Energy (Kcal/mol) | Hydrogen Bonds                        | Hydrophobic Bonds                          |
|-------|-------------------------------------------------|--------------------------|---------------------------------------|--------------------------------------------|
| 1     | ANP-Ddl (Ref)                                   | -8.3                     | Lys141, Asp302, Glu315, Asn314, Glu221, Ala22a, Val224, Lys185 | Val195, Ser191, Ser192, Glu228, Phe304, Ala223, Phe183 |
| 2     | Rutin-Ddl                                       | -8.3                     | Val327, Asn317, Glu16, His107, Glu112, Gln189, Glu315, Asp302, Glu230, Arg300, Gly321 | Pro320, Ser326, Val19, Leu319 |
| 3     | Methanone, [1,4- dimethyl-7-(1- methylethyl)-2- azul enyl]phenyl- -Ddl | -7.8                     | Asn314                                 | Glu221, Phe304, Phe183, Val224, Gly226, Glu228, Val195, Ser192, Lys185 |
| 4     | 56V-PDF (Ref)                                   | -7.7                     | Glu142, Tyr69, Gly98                   | Gly46, Val45, Leu100, Arg137, Gly97, Phe134, Val138, His141 |
| 5     | Phenol, 2,4-bis(1- phenylethyl)- PDF             | -7.5                     | Leu105                                 | Arg106, Asp164, Arg68, Cys99, Leu100, Gly44, His43, Val145, Gly142, His141, Gly98, Tyr69 |
| 6     | 2,4-bis(1- phenylethyl)- and 1,2- Benzenedicarboxylic acid, diisooctyl ester- PDF | -7.4                     | Cys99, Gly104                          | Pro103, Leu100, Ile5, Leu105, Val45, His141, Phe134, Val138, Arg137, Gly97, Tyr69, Trp96, Arg106, Gly98, Arg68, Asp164 |

https://doi.org/10.1371/journal.pone.0252759.t001
and hydrophobic bonds between protein and ligands have been shown in Table 1 and the binding of ligands to the protein has been shown in Fig 2A and 2B in the 3D model.

**Molecular dynamic (MD) simulation analysis**

To analyze the stability of four compounds with two proteins Ddl and PDF, MD simulation was conducted for protein-ligand complexes. Four compounds namely; rutin and Methanone, [1,4-dimethyl-7-(1-methylethyl)-2-azulenyl]phenyl- with Ddl and Phenol, 2,4-bis(1-phenylethyl)- and 1,2-Benzenedicarboxylic acid, diisooctyl ester with Peptide deformylase, which possessed the best binding energies were subjected for MD simulation. The structural changes in protein-ligand complex and dynamic behavior patterns were analyzed by the RMSD, RG, interaction energy, and RMSF calculations.

**Root Mean Square Deviation & radius of gyration**

Root Mean Square Deviation (RMSD) was analyzed to calculate the deviation among protein-ligand complexes during the 100 ns MD simulation to understand the effect of ligands on protein dynamics. Fig 3A depicts the RMSD plot of protein-ligand complexes (Rutin-Ddl, Methanone, [1,4-dimethyl-7-(1-methylethyl)-2-azulenyl]phenyl- -Ddl, Phenol, 2,4-bis(1-phenylethyl)- PDF, and Benzenedicarboxylic acid, diisooctylester-PDF). In the case of the complex system, all complexes are showing stability with protein in 100 ns simulation. The average value of RMSD is 0.18 nm (red), 0.20 nm (green), 0.25 nm (blue) and 0.26 nm (indigo) (Table 2). Further, The Radius of Gyration (Rg) was calculated to analyze the stably folded or unfolded protein and complexes system. The average Rg value of complexes is 1.79±0.077 nm (red), 1.79±0.080 nm (green), 1.33±0.054 nm (blue), and 1.33±0.053 nm (indigo) (Table 2) (Fig 3B).

**Calculation of interaction energy**

The average interaction energy of compounds with PDF was better than the Ddl enzyme. The interaction energy of Phenol, 2,4-bis(1-phenylethyl) (blue), and Benzene dicarboxylic acid, diisooctyl ester (indigo) showed -148.115 kJ mol⁻¹ and -182.885 kJ mol⁻¹ respectively with PDF enzyme while compounds Rutin (red) Methanone, [1,4-dimethyl-7-(1-methylethyl)-2-azulenyl]phenyl- (green) showed -60.75 kJ mol⁻¹ and -78.95 kJ mol⁻¹ respectively with Ddl enzyme (Table 2) (Fig 3C).

---

**Fig 2.** 3D view of the docked ligands position in the crystal structure of proteins (A) Ddl (B) PDF and 2D structure of screened compounds.

https://doi.org/10.1371/journal.pone.0252759.g002
Calculation of residual components fluctuation

The Root Mean Square Fluctuation (RMSF) calculation was performed to know the fluctuation of residues in protein-ligand complexes during the 100 ns trajectory period and a graph was plotted to compare the flexibility of each residue in the complex. The maximum fluctuations in rutin during the simulation were 0.28 nm in SER192 residue followed by 0.24 nm in Val193 and Gly194 (shown in red color) and Methanone, [1,4-dimethyl-7-(1-methylethyl)-2-azulenyl]phenyl- -Ddl (green), Phenol, 2,4-bis(1-phenylethyl)- PDF (blue), 2,4-bis(1-phenylethyl)- and 1,2-Benzedicarboxylic acid, diisooctyl ester-PDF (indigo). Compounds with PDF enzyme showed very little fluctuation in residues. Phenol, 2,4-bis(1-phenylethyl)- showed fluctuation in only one residue Arg12 i.e., 0.30 nm (blue). Benzene-dicarboxylic acid, diisooctylester is depicted in indigo color and it showed fluctuation between 67 to72 number residues (Fig 3D).

Table 2. RMSD, Rg, and interaction energy value of screened compounds.

| S. No. | Phytochemicals and Protein name | Average RMSD (nm) | Average Rg (nm) | Interaction Energy (kJ mol⁻¹) |
|--------|--------------------------------|-------------------|----------------|-----------------------------|
| 1      | Rutin-Ddl                      | 0.18±0.013        | 1.79±0.077     | -60.75                      |
| 2      | Methanone, [1,4- dimethyl-7-(1- methylethyl)-2- azulenyl]phenyl- -Ddl | 0.20±0.039        | 1.79±0.080     | -78.95                      |
| 3      | Phenol, 2,4-bis(1-phenylethyl)- PDF | 0.25±0.022        | 1.33±0.054     | -148.115                    |
| 4      | Benzenedicarboxylic acid, diisooctyl ester-PDF | 0.26±0.024        | 1.33±0.053     | -182.885                    |

https://doi.org/10.1371/journal.pone.0252759.t002

System biology analysis

**Construct model description.** A model was constructed to understand the mechanism of the agrochemical in a bacterial cell against Ddl and PDF enzymes. This model aimed to get
the information of agrochemical reaction inside the bacterial cell. The pathway of these two enzymes was constructed in this model in which Ddl and PDF enzymes play their essential role. The first pathway of the model shows the PDF enzyme removes N-formyl group in the methionine cycle to form a mature protein. In this pathway, we showed the agrochemical inhibition on the PDF enzyme. The second pathway was for the Ddl enzyme, which is used as a catalyst for the formation of D-alanyl-D-alanine for the biosynthesis of the bacterial cell wall. In this pathway, we applied the agrochemical inhibition on the Ddl enzyme. Both models were constructed using previously available literature describing the mechanism of these two pathways [19, 20]. The model comprises one compartment, 25 species, two genes, 4 RNA, ten proteins, and 14 reactions (Fig 4).

**Dynamic behavior of an agrochemical on PDF and Ddl**

Simulation analysis of agrochemical was carried out to analyze the inhibition effect of agrochemical on enzymes through an integrated systems biology approach. For the prediction of the dynamic behavior of the reaction, SBML squeezer was employed to produce the rate laws. The predicted dynamic behavior can be used to understand the effect of the agrochemical on Ddl and PDF enzymes. The value for each molecular species ranges from 1 to 6.0 (S2 Table in S1 File). The gene, protein, and mRNA values were set at 1.0 due to the basal amount in bacterial cells [21]. The agrochemical amounts were set at 0.5, 1.00, 1.5, 2.00, 3.00, and 4.00 and calculate the enzyme’s productivity on its products. The graph shows that as the amount of the agrochemical was increased, the amount of the Ddl enzyme was decreased (Fig 5A and S3A Fig in S1 File). However, the amount of D-Ala-D-Ala fluctuated very little as the amount of the agrochemical increased only in one amount of agrochemical, the production of D-Ala-D-Ala was found to be decreased (Fig 5B and S3B Fig in S1 File), but the amount of PDF remained the same in every amount of agrochemical (Fig 5C and S3C Fig in S1 File).

**Network analysis of enzyme pathways**

The interactive network of enzyme inhibition and their effect on their product was found to have 40 nodes and 40 edges. All the data were analyzed as scale-free property as predicted from the system biology network [22, 23]. Enzyme pathway and agrochemical inhibition network were utilized to estimate the average path length. For network analysis,
sample parameters were used, which are shown in S4 Table in S1 File. The node size “Degree” and node color “Betweenness Centrality” can be mapped using visual style and may be employed to determine hub nodes in the cell designer. The Betweenness Centrality of each node represents the amount of control that any node imposing over the interactions of other nodes in the network [24] and it is a number between 0 and 1 [18]. The nodes presented in red color are hub nodes and maybe a significant regulatory character in enzyme inhibition (Fig 6). The nodes represented in yellow color show the moderate regulatory effect on enzyme pathways.

**Discussion**

In modern agriculture, natural products are being exploited as biocontrol agents because they are eco-friendly and present a sustainable approach to disease control of crops. In India, in terms of production and use, rice is the second-largest cereal crop, and a lot of chemical fertilizers, pesticides, fungicides, and bactericides are applied to keep the plant healthy disease-free. All these chemicals are very harmful to the environment [25] and keeping this problem in mind, this study was carried out to evaluate the antibacterial activity of *V. cinerea* extracts against *Xoo*. 

Fig 5. Dynamic behavior analysis of drug inhibition activity on different amounts with (A) Ddl (B) D-alanyl-D-alanine and (C) PDF (amount 1.0 and 4.0).

https://doi.org/10.1371/journal.pone.0252759.g005
The plant *V. cinerea* is well established for its anti-bacterial properties against many types of bacteria [26–28] (Table 3). In the present work, we also evaluated this plant against Xoo. The bacterial inhibitory percentage in 25, 50, and 100 μg/ml concentration revealed that the *V. cinerea* is very effective against Xoo. The MIC value (10 μg/ml) was also found to be significant in terms of the inhibition zone. The results of our study show similarity with the previously reported activity of *V. cinerea* against several bacteria viz., the methanolic extract showed 3.13 mg/ml MIC value against gram-negative bacteria *P. aeruginosa* [29], and silver nanoparticles made by leaf extract of *V. cinerea* showed 80 μg/mL MIC value against *Xanthomonas campestris pv. Malvacearum* [30]. The MIC of plant extract in a study was high (3.13mg/mL and 80 μg /mL) against both gram-negative bacteria as compared to our study (10 μg/ml) and it shows that *V. cinerea* methanolic extract has the potential to inhibit a wide variety of bacteria in low concentration.

Through *in silico* approach, four-hit compounds were screened from the methanolic extract having an excellent binding affinity with PDF and Ddl enzymes of the bacteria. Out of four, two compounds Rutin and 1,2-Benzenedicarboxylic acid, dieisoctyl ester have been reported to show antibacterial activity in previous reports against various bacteria [1, 31–33]. Two compounds Methanone, [1,4-dimethyl-7-(1-methylethyl)-2-azulenyl]phenyl- and Phenol, 2,4-bis (1-phenylethyl)- first time reported as an antibacterial against Xoo by *in silico* study. Moreover, these compounds’ binding affinity and RMSD were better against PDF and Ddl enzymes. The interaction energy analysis of the compounds indicates, higher interaction energy with the PDF enzyme and lower interaction energy with the Ddl enzyme compared to the average short-range Lennard-Jones energy, -99.1±7.2 kJ mol⁻¹ [34]. Root mean square fluctuation analysis for residual mobility showed constituent residues’ fluctuation for each complex during 100 ns MD simulations. The fluctuation of residue describes that the ligand binding to protein is not disrupting the original conformation of residues. This result suggests that the complexes have active interaction with their residues in the context of RMSF calculation.
In addition, the system model was created to simulate the mechanism of inactivation of Ddl and PDF enzymes by agrochemical inside the bacterial cell. In the cell system, the PDF enzyme helps to remove N-formyl group from the enzymes [35]. In the bacterial cell, protein synthesis starts with N-formyl methionine, and the newly synthesized polypeptide is converted to mature protein through the sequential removal of the N-formyl group and methionine [36]. The graph (Fig 5A) of our study shows that the inhibition of PDF affects the production of Ddl enzymes. Next inhibition was of Ddl enzyme by the agrochemical which leads to the formation of the dipeptide D-alanyl-D-alanine and involves in bacterial cell wall peptidoglycan biosynthetic pathway [37]. The results of the graph reflect a little fluctuation in the production of D-Ala-D-Ala on an increasing amount of agrochemical. This graph also shows that the PDF amount remains the same in every condition. Further, the reaction was subjected to network analysis, which is one of the most important techniques for decoding the key regulatory element in reaction, essential for controlling the complex biological machinery during diseases [38]. The finding of network analysis demonstrated that Ddl and PDF enzymes are key regulatory elements, and inhibition of these enzymes through agrochemicals can be useful to protect rice crops from Xoo. Finally, the system biology result shows that the agrochemical affects the productivity of enzymes and will do the same effect in the bacterial cell.

The current study results suggest that using the V. cinerea plant as an agrochemical against Xoo might be valuable for plants and disease management and it will not affect the environment and humans. Such type of study can be used to develop new agrochemicals targeting particular enzymes to stop bacteria growth in agriculture.

Table 3. The activity of V. cinerea plant extract against different bacteria.

| Plant Name | Bacterial Pathogen | Extracts showing Antibacterial activity | References |
|------------|--------------------|----------------------------------------|------------|
| V. cinerea (L.) | Escherichia coli | VNHH + VCHHH + VEAH + VCMEH + PECHHH + EECCHH + BENZHE AgNO₃HH | Sonibare et al., 2016, Somasundaram et al., 2010, Gupta et al., 2003 [26–28] |
| | Pseudomonas aeruginosa | VNHH + VCHHH + VHHEHHHH + + | Sonibare et al., 2016, Somasundaram et al., 2010 Gupta et al., 2003, Latha et al., 2010 Gupta, 2003 [26–29] |
| | Proteus vulgaris | VNHH + VCHHH + VHHEHHHH + + | Sonibare et al., 2016 [28] |
| | Klebsiella pneumonia | VNHH + VCHHH + VHHEHHHH + | Sonibare et al., 2016, Somasundaram et al., 2010, Gupta et al., 2003 [26–28] |
| | Xanthomonas campestris pv. malvacearum | VNHH + VCHHH + VHHEHHHH + | Sahayara et al., 2015 [30] |
| | Bacillus subtilis | VNHH + VCHHH + VHHEHHHH + | Sonibare et al., 2016, Somasundaram et al., 2010 Gupta et al., 2003 [26–28] |
| | Staphylococcus aureus | VNHH + VCHHH + VHHEHHHH + | Sonibare et al., 2016, Somasundaram et al., 2010, Gupta et al., 2003 [26–28] |
| | Aspergillus flavus | VNHH + VCHHH + VHHEHHHH + | Sonibare et al., 2016 [28] |
| | Bacillus cereus | VNHH + VCHHH + VHHEHHHH + | Somasundaram et al., 2010 [27] |
| | Shigella dysenteriae | VNHH + VCHHH + VHHEHHHH + | Gupta et al., 2003 [26] |
| | Salmonella typhi | VNHH + VCHHH + VHHEHHHH + | Gupta et al., 2003 [26] |
| | Micrococcus luteus | VNHH + VCHHH + VHHEHHHH + | Gupta et al., 2003 [26] |
| | Staphylococcus epidermidis | VNHH + VCHHH + VHHEHHHH + | Gupta et al., 2003 [26] |

VCME- Vernonia cinerea crude methanolic extract, VNH- V. cinerea n-Hexane fraction, VCH- V. cinerea chloroform fraction, VEA- V. cinerea Ethyl acetate fraction, PEVC (petroleum ether V. cinerea), EEVC (alcoholic extracts V. cinerea), AgNO₃ (Silver nitrate)

Sign “+” (Result positive) “–” (Result negative) “-” (Study not performed)

https://doi.org/10.1371/journal.pone.0252759.t003
Conclusion
The overall analysis of our study indicates that methanolic extract of *V. cinerea* has good activity against *Xoo*. Hence, the extracts of *V. cinerea* can be used as pesticides against *Xoo* bacteria. Further *in silico* study showed that the compounds namely, Rutin, Methanone, [1,4-dimethyl-7-(1-methylethyl)-2-azulenyl]phenyl- have better stability with Ddl while, Phenol, 2,4-bis(1-phenylethyl)- and 1,2-Benzenedicarboxylic acid, diisooctyl ester binds favorably with PDF enzyme. This reveals the phytochemicals of *V. cinerea* could inhibit PDF enzymes as well as Ddl enzyme resulting ineffective growth inhibition of *Xoo*. Therefore, the present study suggests specific molecules of *V. cinerea* that can be utilized to develop potential natural pesticide candidates and can provide better opportunities for further innovation and development of eco-friendly antimicrobial compounds against *Xoo* infection of rice.

Supporting information

S1 File.
(DOCX)

Acknowledgments

The authors are thankful to the Department of Botany and Zoology, Kumaun University, SSJ Campus, Almora (Uttarakhand), India for providing basic facilities to conduct this research work. The authors also acknowledge Kumaun University, Nainital for providing high-speed internet facility. We also extend our acknowledge to Rashtriya Uchchattar Shiksha Abhiyan (RUSA), Ministry of Human Resource Development, Government of India to provide Computational infrastructure for the establishment of Bioinformatics Centre in Kumaun University, S.S.J. Campus, Almora, (Uttarakhand), India. The help provided by Director, Environmental Information System on Himalayan Ecology, G.B. Pant National Institute of Himalayan Environment, Kosi-Katarmal, Almora, Uttarakhand 263643, India is highly acknowledged.

Author Contributions

Formal analysis: Satish Chandra Pandey, Manish Tripathi, Mahesha Nand, Veena Pande.
Methodology: Subhash Chandra.
Resources: Satish Chandra Pandey, Priyanka Maiti, Manish Tripathi, Ashutosh Paliwal.
Software: Tushar Joshi, Priyanka Sharma, Subhash Chandra.
Supervision: Mukesh Samant, Subhash Chandra.
Visualization: Mukesh Samant, Subhash Chandra.
Writing – original draft: Tushar Joshi.
Writing – review & editing: Mukesh Samant, Subhash Chandra.

References

1. Chien CC, Chou MY, Chen CY, Shih MC. Analysis of genetic diversity of Xanthomonas oryzae pv. oryzae populations in Taiwan. Sci Rep. 2019; 9: 316. https://doi.org/10.1038/s41598-018-36575-x PMID: 30670790
2. Mishra D, Vishnupriya MR, Anil MG, Konda K, Raj Y. Pathotype and Genetic Diversity amongst Indian Isolates of Xanthomonas oryzae pv. oryzae. PLoS One. 2013; 8: e61996. https://doi.org/10.1371/journal.pone.0061996 PMID: 24312391
3. Syed-Ab-Rahman SF, Carvalhais LC, Omar D. Development of plant-based emulsion formulations to control bacterial leaf blight and sheath brown rot of rice. Heliyon. 2020; 6: e03151. https://doi.org/10.1016/j.heliyon.2019.e03151 PMID: 32042948

4. Kanugala S, Kumar CG, Rachamalla HKR, Palakeeti B, Kallaganti VSR, Nimmu N V., et al. Chumacin-1 and Chumacin-2 from Pseudomonas aeruginosa strain CGK-KS-1 as novel quorum sensing signaling inhibitors for biocontrol of bacterial blight of rice. Microbiol Res. 2019; 228: 126301. https://doi.org/10.1016/j.micres.2019.126301 PMID: 31422232

5. Kim SI, Song JT, Jeong JY, Seo HS. Niclosamide inhibits leaf blight caused by Xanthomonas oryzae in rice. Sci Rep. 2016; 6: 21209. https://doi.org/10.1038/srep21209 PMID: 26879887

6. Chithrasruee AC, Udayashankar S, Chandra Nayaka MS, Srinivas RC. Plant growth-promoting rhizobacteria mediate induced systemic resistance in rice against bacterial leaf blight caused by Xanthomonas oryzae pv. Oryzae. Biol Control. 2011; 59: 114–122.

7. Govindappa M, Umesh S, Lokesh S. Adhatoda vasica leaf extract induces resistance in rice against bacterial leaf blight disease (Xanthomonas oryzae pv. oryzae). Int J Plant Physiol Biochem. 2011; 3: 6–14.

8. Kavitha HU, Satish S. Eco friendly management of plant pathogens by some medicinal plant extracts. J AgricTechnol. 2011; 7: 449–461.

9. Jabeen R, Ashraf M, Ahmad I, Itikhar T. Purification and bioassays of bioactive fraction from curcuma longa against Xanthomonas oryzae pv. Oryzae causing bidental disease in rice. Pakistan J Bot. 2011; 43: 1335–1342.

10. Vollmer W, Blanot D, De Pedro MA. Peptidoglycan structure and architecture. FEMS Microbiol Rev. 2008; 32: 149–167. https://doi.org/10.1111/j.1574-6976.2007.00094.x PMID: 18194336

11. Barreteau H, Kovác A, Boniface A, Sova M, Gobec S, Blanot D. Cytoplasmic steps of peptidoglycan biosynthesis. FEMS Microbiol Rev. 2008; 32: 168–207. https://doi.org/10.1111/j.1574-6976.2008.00104.x PMID: 18266853

12. Maiti P, Nand M, Joshi T, Ramakrishnan MA, Chandra S. Identification of luteolin-7-glucoside and epi- catechin gallate from Vernonia cinerea, as novel EGFR L858R kinase inhibitors against lung cancer: Docking and simulation-based study. J Biomol Struct Dyn. 2020. https://doi.org/10.1080/07391102.2020.1784791 PMID: 32579072

13. Kirkpatrick WR, Atee RKMC, Revankar SG, Fothergill AW, Carthy DIMC, Rinaldi MG. Comparative Evaluation of National Committee for Clinical Laboratory Standards Broth Macrodilution and Agar Dilution Screening Methods for Testing Fluconazole Susceptibility of Cryptococcus neoformans. 1998; 36: 1330–1332.

14. Abirami P, Rajendran A. GC-MS analysis of methanol extracts of Vernonia cinerea. Eur J Exp Biol. 2012; 2: 9–12. Available: http://www.imedpub.com/articles/gcms-analysis-of-methanol-extracts-of-vernonia-cinereai.pdf

15. Rajamurugan R, Selvaganabathy N, Kumaravel S, Ramamurthy C, Sujatha V, Suress Kumar M, et al. Identification, quantification of bioactive constituents, evaluation of antioxidant and in vivo acute toxicity property from the methanol extract of Vernonia cinerea leaf extract. Pharm Biol. 2011; 49: 1311–1320. https://doi.org/10.3109/13880209.2011.604334 PMID: 22077167

16. Kitano H. A Graphical Notation for Biochemical Networks. BIOSILICO. 2003; 1: 169–176.

17. Autiero I, Costantini S, Colonna G. Modeling of the bacteria mechanism of methicillin-resistance by a systems biology approach. PLoS One. 2009; 4: e6226. https://doi.org/10.1371/journal.pone.0006226 PMID: 19593454

18. Pathak RK, Baunthiyal M, Pandey N, Pandey D, Kumar A. Modeling of the jasmonate signaling pathway in Arabidopsis thaliana with respect to pathophysiology of Alternaria blight in Brassica. Sci Rep. 2017; 7: 1–12. https://doi.org/10.1038/s41598-016-0028-x PMID: 28127051

19. Aubart K, Zalachain M. 3 Peptide Deformylase Inhibitors. Prog Med Chem. 2006; 44: 109–143. https://doi.org/10.1016/S0079-6468(05)44403-3 PMID: 16697896

20. Kitamura Y, Agari Y, Shinkai A, Hirotsu K, Kuramitsu S. Structure of D-alanine- D-alanine ligase from Thermus thermophilus HB8: cumulative conformational change and enzyme–ligand interactions. Acta Crystallogr. 2009; 65: 1098–1106. https://doi.org/10.1107/S0907444909029710 PMID: 19770507

21. Bhatt P, Pal K, Bhandari G, Barh A. Modelling of the methyl halide biodegradation in bacteria and its effect on environmental systems. Pestic Biochem Physiol. 2019; 158: 88–100. https://doi.org/10.1016/j.pestbp.2019.04.015 PMID: 31378365

22. Barabási AL. Scale-free networks: A decade and beyond. Science (80-). 2009; 325: 412–413. https://doi.org/10.1126.science.1173299 PMID: 19628854

23. Neumann A, Siebert A, Trescher T, Reinhardt S, Wohlfarth G, Diekert G. Tetrachloroethene reductive dehalogenase of Dehalospirillum multivorans: Substrate specificity of the native enzyme and its
corrinoid cofactor. Arch Microbiol. 2002; 177: 420–426. https://doi.org/10.1007/s00203-002-0409-3 PMID: 11976751

24. Yoon J, Blumer A, Lee K. An algorithm for modularity analysis of directed and weighted biological networks based on edge-betweenness centrality. Bioinformatics. 2006; 22: 3106–8. https://doi.org/10.1093/bioinformatics/btl533 PMID: 17060356

25. Meena RK, Mishra P. Bio-pesticides for Agriculture and Environment Sustainability. Resources Use Efficiency in Agriculture. 2020. pp. 85–107. https://doi.org/10.1007/978-981-5-6953-1_3

26. Gupta M, Mazumder UK, Manikandan L, Haldar PK, Bhattacharya S, Kandar CC. Antibacterial activity of Vernonia cinerea. Fitoterapia. 2003; 74: 148–50. https://doi.org/10.1016/s0367-326x(02)00291-5 PMID: 12628412

27. Somasundaram A, Velmurugan V, Senthilkumar GP. In vitro Antimicrobial Activity of Vernonia Cinerea (L) Less. Pharmacology online. 2010; 960: 957–960.

28. Sonibare MA, Aremu OT, Okorie PN. Antioxidant and antimicrobial activities of solvent fractions of Vernonia cinerea (L) Less leaf extract. Afr Health Sci. 2016; 16: 629–639. https://doi.org/10.4314/ahs.v16i2.34 PMID: 27605981

29. Latha LY, Darah I, Kassim MJNM, Sasidharan S. Antibacterial activity and morphological changes of Pseudomonas aeruginosa cells after exposure to Vernonia cinerea extract. Ultrastruct Pathol. 2010; 34: 219–25. https://doi.org/10.3109/019131211003651513 PMID: 20594042

30. Sahayaraj K, Roobadevi M, Rajesh S, Azizi S. Vernonia cinerea (L) Less. silver nanocomposite and its antibacterial activity against a cotton pathogen. Res Chem Intermed. 2015; 41: 5495–5507. https://doi.org/10.1007/s11164-014-1676-8

31. Arima H, Ashida H, Danno G. Rutin-enhanced antibacterial activities of flavonoids against Bacillus cereus and Salmonella enteritidis. Biosci Biotechnol Biochem. 2002; 66: 1009–14. https://doi.org/10.1271/bbb.66.1009 PMID: 12092809

32. Adamczak A, Ozarrowski M, Karpinski TM. Antibacterial Activity of Some Flavanoids and Organic Acids Widely Distributed in Plants. J Clin Med. 2019;9. https://doi.org/10.3390/jcm9010109 PMID: 31906141

33. Yang MT, Kuo TF, Chung KF, Liang YC, Yang CW, Lin CY, et al. Authentication, phytochemical characterization and anti-bacterial activity of two Artemisia species. Food chem. 2020; 333: 127458. https://doi.org/10.1016/j.foodchem.2020.127458 PMID: 32673952

34. Lemkul J. From Proteins to Perturbed Hamiltonians: A Suite of Tutorials for the GROMACS-2018 Molecular Simulation Package [Article v1.0]. Living J Comput Mol Sci. 2019; 1: 1–53. https://doi.org/10.33011/livecoms.1.1.5068

35. Verma SK, Jat RK, Nagar NL, Saharan R, Sharma V, et al. A novel antibacterial target: Peptidyl deformylase. Pharmacophore, 2011; 2: 114–123.

36. Groche D, Becker A, Schlichting I, Kabsch W, Schultz S, Wagner AFV. Isolation and crystallization of functionally competent escherichia coli peptide deformylase forms containing either iron or nickel in the active site. Biochem Biophys Res Commun. 1998; 246: 342–346. https://doi.org/10.1006/bbrc.1998.8616 PMID: 9610360

37. Neuhaus FC, Lynch JL. The Enzymatic Synthesis of d-Alanyl-d-alanine. III. On the Inhibition of d-Alanyl-d-alanine Synthetase by the Antibiotic d-Cycloserine. Biochemistry. 1964; 3: 471–480. https://doi.org/10.1021/bi00892a001 PMID: 14188160

38. Lu X, Jain V, Finn PW, Perkins DL. Hubs in biological interaction networks exhibit low changes in expression in experimental asthma. Mol Syst Biol. 2007; 3: 98. https://doi.org/10.1038/msb4100138 PMID: 17437023