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Antimicrobial Activity of a Novel Secondary Metabolite from *Streptomyces* sp. and Molecular Docking Studies against Bacterial Leaf Blight Pathogen of Rice

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A B S T R A C T

The metabolite 2-(1,1-diallyl-but-3-enyl)-5-nonyl-phenol isolated from *Streptomyces* sp. TC1 exhibited antimicrobial activity against bacterial leaf blight pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). The *in vitro* antimicrobial activity was found to be between 1.8-2.5 cm in agar well diffusion assay against the tested *Xoo* isolates and 200 µg/ml in terms of minimum inhibitory concentration. The isolated compound showed well binding with the virulence proteins of *Xoo* by producing a better docking score in molecular docking studies.

Introduction

Bacterial leaf blight (BLB) of rice, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the oldest known diseases and was first noticed by the farmers of Japan in 1884. Subsequently, its incidence has been reported from different parts of Asia, northern Australia, Africa and USA (Gnanamanickam, 2002). The disease is known to occur in epidemic proportions in many parts of the world, incurring severe crop loss of up to 50%. Crop loss assessment studies have revealed that this disease reduces grain yield to varying levels, depending on the stage of the crop, degree of cultivar susceptibility and to a great extent, the conduciveness of the environment in which it occurs (Muralidharan and Venkatarao, 1979). Over 55% of antibiotics have been isolated from genus *Streptomyces* and therefore this genus is one of the several biological control agents which are widely studied and used to control various plant pathogens (Embley and Stackebrandt, 1994). Many of the seed, soil-borne and foliar diseases were efficiently managed by biocontrol means using *Bacillus* sp. *Pseudomonas* sp. *Streptomyces* sp. and

Trichoderma sp. A numbers of antimicrobial metabolites were produced by these strains which have different mode of actions in suppression of plant pathogens.

Recent studies revealed the antimicrobial action of some Streptomycetes against plant pathogens including *Xoo* (Ndonde and Semu, 2000; Rizk *et al.,* 2007). Park *et al.,* (2011) reported the suppression of bacterial blight pathogen by the compounds isolated from *Streptomyces bottropensis* sp. *viz.,* bottromycin A2 and dunaimycin D3S. Xanthostatin is a novel depsipeptide antibiotic produced by *Streptomyces spiroverticillatus* (Cheng *et al.,* 1987) has bacteriostatic activity against *Xanthomonas* sp.

Molecular modeling is more important in cellular biology to study the interaction between the proteins and also with other molecular components. The molecular docking is also used to predict the action of drugs against specific diseases. Knowledge of the preferred orientation in turn may be used to predict the strength of association or binding affinity between two molecules using scoring functions. *Xoo* has so many virulence proteins like Lip A, XoFabV, Fab H, Xoo2316, XometC, XoMCAT, XometB, Xoo0352, GlmU etc. These virulence factors are responsible for establishment of bacterial leaf blight disease in rice. The type II protein secretion system (T2S) of *Xoo* involved in secretion of several protein including xylanase required for its virulence function (Ray *et al.,* 2000). By studying the interaction of molecular components against these virulence proteins the possible antimicrobial spectrum can be obtained.

In this present study the novel compound 2-(1,1-diallyl-but-3-enyl)-5-nonyl-phenol isolated from *Streptomyces* sp. TC1 (Jaivel *et al.,* 2014) was analyzed for their antimicrobial activity against *Xanthomonas oryzae* pv. *oryzae* and their molecular docking results with *Xoo* specific virulence proteins were also reported.

Materials and Methods

Microbial culture

The *Streptomyces* sp. TC1 (GenBank accession number: KC954629) was grown in Ken knight agar slants for five days at 28ºC and maintained under refrigerated conditions. The test culture *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) from various sources were obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India.

Fermentation and isolation

A seed culture of *Streptomyces* sp. TC1 was prepared by inoculating a loop of biomass into a 200 ml Erlenmeyer flask containing 100 ml of Ken knight broth and then incubating at 28 ºC for 3 days. A 10 % level of this inoculum was transferred into 1000 ml of production medium contained in 3 l Erlenmeyer flasks (15 in number). The production medium having the composition of soluble starch 1.0%, casein 0.03%, KNO_3 0.2%, NaCl 0.2%, K_2HPO_4 0.2%, $CaCO_3$ 0.002%, $MgSO_4.7H_2O$ 0.005% and $FeSO_4.7H_2O$ 0.001% with pH 8.0. The inoculated production flasks were incubated for 7 days at 28ºC. After the fermentation period, the metabolite 2-(1,1-diallyl-but-3-enyl)-5-nonyl-phenol isolated from the fermentation broth by standard solvent extraction and column chromatographic separation procedure (Jaivel *et al.,* 2014).

Screening of antimicrobial activity

In vitro antimicrobial activity was studied against various isolates of *Xoo* strains by agar well diffusion method (Perez *et al.,* 1990).

The metabolite was diluted in 100% ethanol at the concentration of 1 mg/ml. The antimicrobial activity was evaluated by loading 50 µl/well. The standard antibiotic streptomycin used as a positive control at the concentration of 1 mg/ml. The antimicrobial spectrum was determined in terms of zone of inhibition.

Ethanol was used as negative control. The control zones were subtracted from the test zones and the results are arrived. The minimum inhibitory concentration (MIC) assay was performed to test the antimicrobial activity using modified version of broth microdilution method recommended by NCCLS (National Committee for Clinical Laboratory Standards, 2000).

Docking studies

Docking is a methodology used in molecular modeling to predict the preferred orientation of one molecule to a second when bound to each other to form a stable complex in three dimensional spaces. The bioinformatics Software used for the docking study was "GLIDE". The target enzymes/ template proteins used for docking studies were cell wall-degrading esterase (Lip A, PDB I.D.: 3H2G), enoyl-ACP reductase (Fab V, PDB I.D.: 3S8M), D-alanine-d-alanine ligase A (DdlA, PDB I.D. 3R5F) and peptide deformylase (PDF, PDB I.D. 3DLD).

All of these were virulence protein secreted by *Xanthomonas oryzae* pv. *oryzae* at different stages of bacterial blight disease development in rice. The details of the target proteins were obtained from Protein Data Bank (PDB) database. The docking work was carried out at Centre for Advanced Studies in Crystallography and Biophysics, University of Madras. The best docking scores indicates the strong binding of the molecule with the protein.

Induced fit docking

Induced Fit Docking studies was carried out using GLIDE (Singh and Randhawa, 2011) software v5.5, developed by Schrodinger, running on Red Hat Enterprise Linux 5 (RHEL5) workstation and Maestro v9.0. Graphical User Interface (GUI) workspace was used for all the steps involved in ligand preparation, protein preparation and Induced Fit Docking (IFD).

Preparation of the ligand

The ligand used in this study was prepared using Ligprep module of v2.3 of Schrodinger Suite 2009. Ligprep follows OPLS-AA (Optimized Potential Liquid Simulations for All Atoms) force fields for energy minimization. The proteins taken for the study was retrieved from PDB (Protein Data Bank) database.

The optimized structure was then energy minimized to remove the steric clashes between the atoms. The energy minimization was done till it reached a Root Mean Square Deviation (RMSD) cutoff of 0.18 °A and the resulting structure was used for docking (Vinuchakkaravarthy *et al.,* 2011).

Induced Fit Docking (IFD) protocol

IFD of the prepared ligand with the prepared protein was performed using IFD protocol of GLIDE v5.5 from Schrodinger Suite 2009 (Vinuchakkaravarthy *et al.,* 2011). Both the ligand and the receptor were flexible which enabled the ligand to dock at the receptor's binding site and generate multiple poses of the receptor-ligand complex. Each docking included unique structural conformations of the receptor needed to fit the ligand pose. The IFD gave the best structure of the docked complex based on the Glide score (G-score) of the dockings.

Visualization and analysis

The hydrophobic interactions and hydrogen bond interactions were obtained as ligplot diagram by submitting the docked complexes to the online PDB sum server http://www.ebi.ac.uk/pdbsum/.

Results and Discussion

Antimicrobial activity of 2-(1,1-diallyl-but-3-enyl)-5-nonyl-phenol against various isolates of *Xoo*

The antimicrobial activity in terms of zone of inhibition against various isolates of *Xoo* was carried out for 2-(1,1-diallyl-but-3-enyl)-5-nonyl-phenol. The compound produced a zone of inhibition of 1.8-2.5 cm against the tested *Xoo* isolates (Table 1). The highest activity of 2.5 cm inhibition zone was found against the *Xoo* isolate obtained from TN1 variety (Fig. 1). Whereas the lowest activity of 1.8 cm zone of inhibition was observed for *Xoo* isolate obtained from CO50 variety. The MIC experiments carried out using NCCLS protocol showed that the isolated compound exhibited an MIC value of 200 µg/mL against the tested *Xoo* isolate.

Docking studies

The antimicrobial compounds inhibit the growth of bacteria by several mechanisms like cell wall inhibition, protein synthesis inhibition, inhibition of DNA synthesis etc. In the present study the possible mode of action of isolated antimicrobial compound was analyzed by molecular docking studies. Docking studies were performed to predict the preferred orientation of isolated molecule to the selected virulence protein of *Xoo* which bound to each other to form a stable complex. The binding strength of the isolated molecule against the tested virulence proteins was also predicted.

Induced fit docking results with the target proteins

Docking was performed for 2-(1,1-diallyl-but-3-enyl)-5-nonyl-phenol with 4 target proteins namely Lip A, Fab V, Ddl A and PDF. The docking score and glide energy of compound with the respective target proteins was given in Table 2. The lower docking score indicates better docking of compound with the desired proteins. The compound with Lip A obtained the Glide energy of -54.46 with a docking score of -11.98 kcal mol^{-1} and there was 1 hydrogen bond interaction with ASN 228 (Fig. 2). Likewise, the compound with Fab V obtained the Glide energy of -42.65 with a docking score of -8.11 kcal mol^{-1}. There was 1 hydrogen bond interaction with THR 276 (Fig. 3). The compound with Ddl A obtained the Glide energy of -36.36 with a docking score of -3.52 kcal mol^{-1}.

There was 1 hydrogen bond interaction with GLU 228 (Fig. 4). The compound with PDF obtained the Glide energy of -41.22 with a docking score of -5.94 kcal mol^{-1}. There were 1 hydrogen bond interactions with TYR 69 (Fig. 5).

Among the four target proteins, Lip A was found to be possess more binding towards the compound (Fig. 6) followed by Fab V. Hence it was determined from this hypothetical way that compound binds well with rice cell wall degrading protein and thus prevents systemic infection caused by the entry of *Xoo*.

Antimicrobial activity of isolated compounds against *Xoo* isolates

The improved rice varieties released in India, though having a certain degree of resistance to bacterial blight became susceptible due to emergence of new races of the pathogen (Khush *et al.*, 1989). In the present study, the isolated compound produced an inhibition

zone of 1.8-2.5 cm in terms of zone of inhibition against the tested *Xoo* isolates. The *Xoo* isolate from TN1 rice variety showed increased susceptibility, whereas the isolated compound produced a moderate inhibition zone against *Xoo* isolate obtained from CO50 rice variety.

The variation may be due to the different levels of susceptibility to the antimicrobial compounds by various isolates of *Xoo*. This was supported by Leach *et al.,* (1995), who reported that the *Xoo* pathotypes posses varied levels of susceptibility to antimicrobial compounds due to their resistance genes.

Larrainzar *et al.,* (2012) reported the antimicrobial and antioxidant activities of many phenolic compounds isolated from natural sources. In the present study, the highest antimicrobial potential of compound was observed against *Xoo* due to the presence of phenol and diallyl moiety in its structure. Similarly the antimicrobial action of natural phenolic compounds against the gram negative bacteria *Enterococcus faecalis* was reported by Fernandez *et al.,* (2013). Further Tsao and Yin (2001) reported the antimicrobial activity of diallylsulphide against methicilin resistant *Staphylococcus aureus*.

Table.1 Antimicrobial activity of 2-(1,1-diallyl-but-3-enyl)-5-nonyl-phenol against *Xoo*

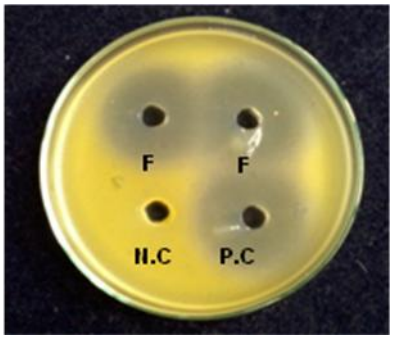
Source of *Xoo* isolate	**Diameter of inhibition zone (in cm)**
TN 1	2.5 ± (0.12)
TNRH 180	2.2 ± (0.10)
CO 47	2.1 ± (0.06)
CO 50	1.8 ± (0.15)
ADT 39	2.2 ± (0.06)
ADT 43	2.0 ± (0.12)
SEd	0.086
CD(.05)	0.188

Values are mean ± SD of three replicates

Table.2 Induced fit docking results of 2-(1,1-diallyl-but-3-enyl)-5-nonyl-phenol with the target proteins

Sl. No.	**Target protein**	**PDB ID**	**Docking Score**	**Glide Energy (kcal/mol)**	**Number of hydrogen bonds**	**Interaction**	**Distance (Å)**
1.	Lip A	3H2G	-11.98	-54.46	1	(ASN 228)O–H· · ·O	3.1
2.	Fab V	3S8M	-8.11	-42.65	1	(THR 276)O–H· · ·O	2.9
3.	Ddl A	3R5F	-3.52	-36.36	1	(GLU 228) O–H· · ·O	2.7
4.	PDB	3DLD	-5.94	-41.22	1	(TYR 69) O–H· · ·O	2.9

Fig.1 Antimicrobial activity of 2-(1,1-diallyl-but-3-enyl)-5-nonyl-phenol against *Xoo*



F- 2-(1,1-diallyl-but-3-enyl)-5-nonyl-phenol
N.C. – Negative control (100% ethanol)
P.C. – Positive control (Streptomycin)

Fig.2 Docking of 2-(1,1-diallyl-but-3-enyl)-5-nonyl-phenol with esterase LipA protein (PDB I.D. 3H2G)

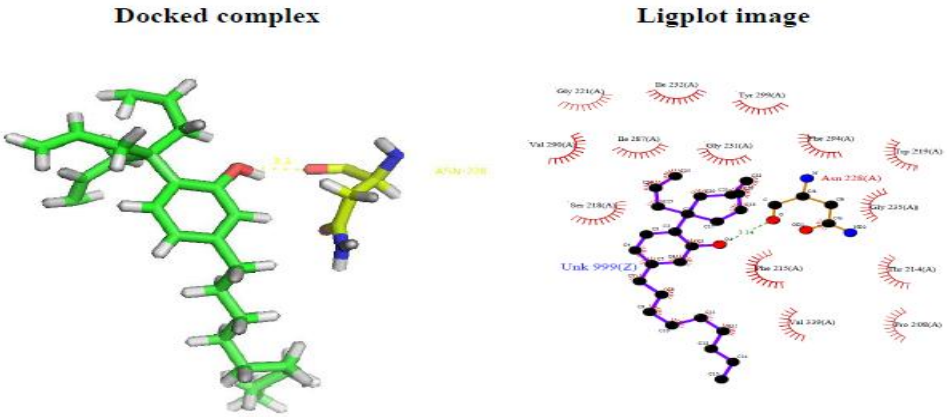


Fig.3 Docking of 2-(1,1-diallyl-but-3-enyl)-5-nonyl-phenol with Enoyl-ACP reductase (FabV) protein (PDB I.D. 3S8M)

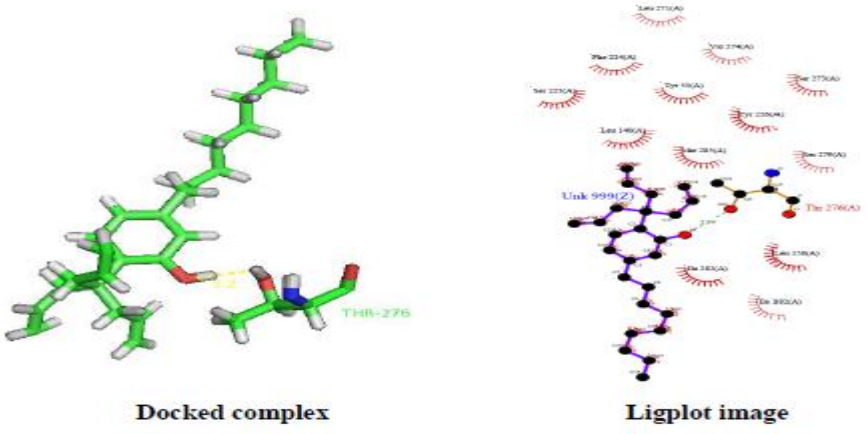


Fig.4 Docking of 2-(1,1-diallyl-but-3-enyl)-5-nonyl-phenol with D-alanine-d-alanine ligase A (DdlA) protein (PDB I.D. 3R5F)

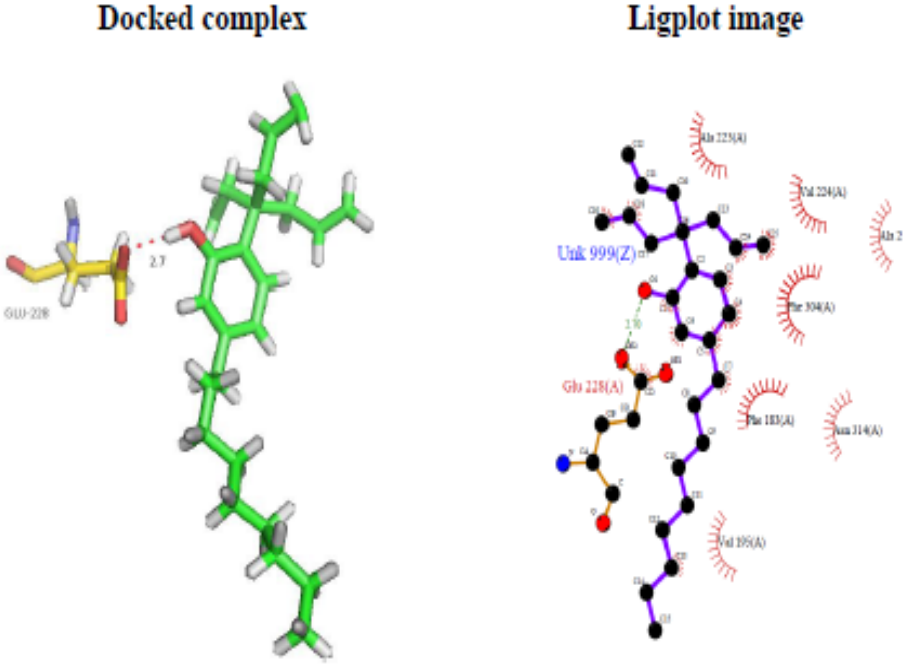


Fig.5 Docking of 2-(1,1-diallyl-but-3-enyl)-5-nonyl-phenol with Peptide deformylase (PDF) protein (PDB I.D. 3DLD)

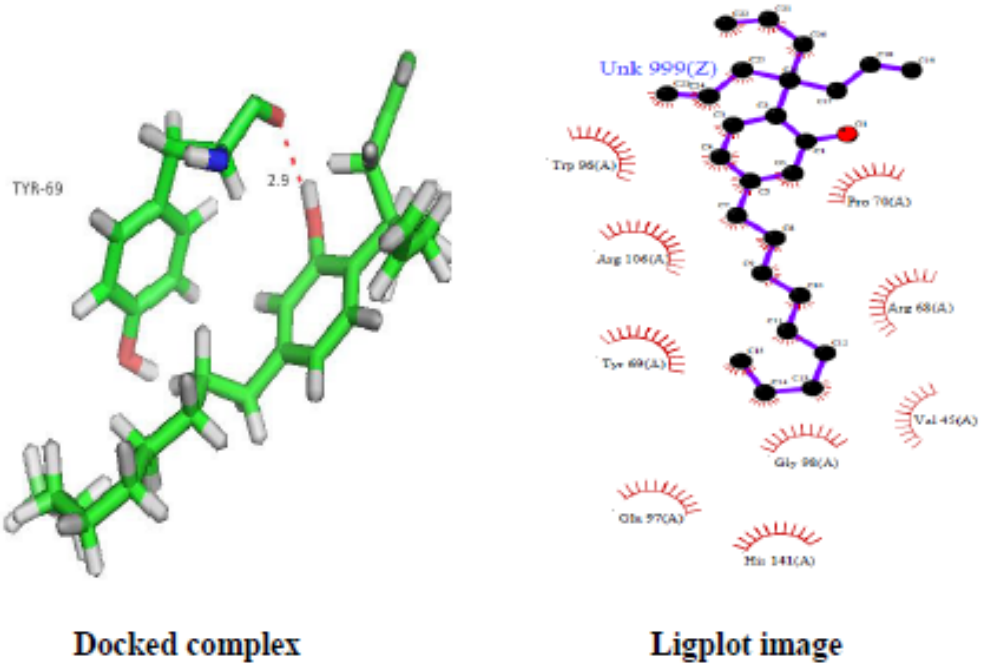
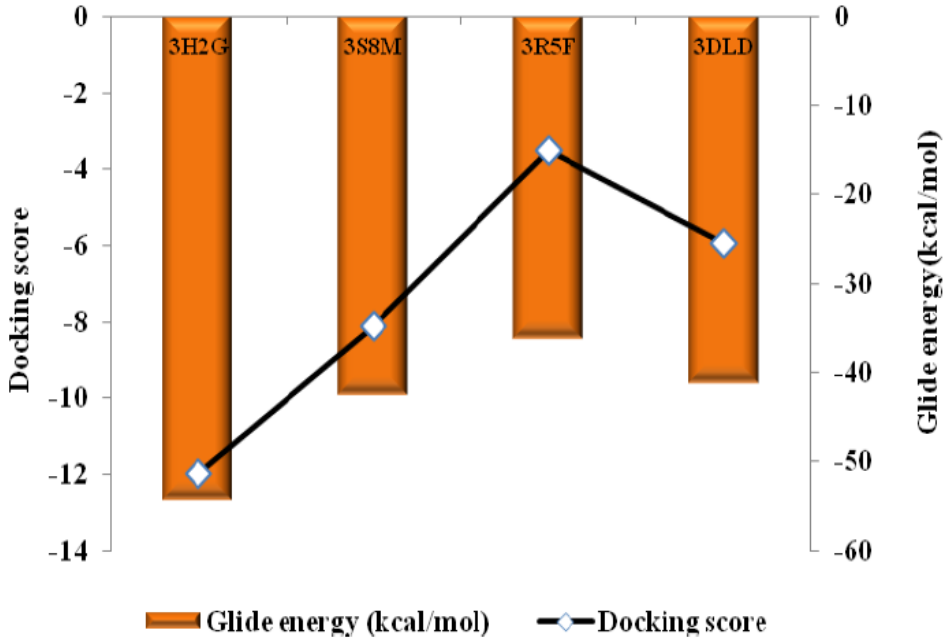


Fig.6 Docking score and glide energy of 2-(1,1-diallyl-but-3-enyl)-5-nonyl-phenol with target proteins



Possible mode of action of isolated antimicrobial metabolites

Understanding the mode of action is a preliminary approach to study the properties of any bioactive molecules. The antimicrobial compounds can affect microbial cells by several ways including cell wall synthesis inhibition, cytoplasmic membrane damage, inhibition of specific enzyme system, inhibition of nucleic acid and protein synthesis etc. In this present study the isolated molecules are studied for their possible mode of action by molecular docking studies. The aim of molecular docking is to achieve an optimized conformation for both the protein and ligand and relative orientation between protein and ligand (Kitchen *et al.*, 2004).

The docking is a method which predicts the preferred orientation of one molecule with other one when bound to each other to form a stable complex. Docking small, mostly organic compounds to proteins is relevant to both understanding biological processes and designing drugs. In the past 25 yrs a large set of different methods have been developed for screening the databases of ligands and accurately analyzing individual molecular interactions (Thomas and Matthias, 1996).

Hughes *et al.*, (1990) reported that molecular docking is a key tool in structural molecular biology and computer-assisted drug design. The goal of ligand-protein docking is to predict the predominant binding model(s) of a ligand with a protein of known three-dimensional structure. Shanthi *et al.*, (2011) carried out docking studies for an antimicrobial compound isolated from cow urine extract of *Pongamia pinnata* against four different virulence proteins of *Xoo*. The antimicrobial compound dihydropyrano-flavanoid produce an binding energy of -7.87, - 6.67, -6.45, -7.98 kcal/mol against Malonyl CoA-ACP transacylase, D-Alanine-d-alanine ligase A, 3-oxoacyl-(acyl carrier protein) synthase III and N-acetylornithine

transcarbamylase respectively. The target enzymes are mainly involved in fatty acid metabolism and peptidoglycan biosynthesis. A number of 1-4 hydrogen bonds are formed between the ligand and protein complex.

In this present study the isolated antimicrobial compound was docked against four different virulence proteins of *Xoo*. The compound exhibited good binding potential with Fab V protein responsible for fatty acid elongation cycle during cell wall synthesis and Lip A protein responsible for rice cell wall degradation. Whereas, binding of isolated compound with peptide deformylase (PDF) and D-alanine-d-alanine-ligase A (Ddl A) was found to be low compared to other two proteins studied. Hence, the compounds might have less influence on inhibiting the protein and DNA synthesis machinery.

This was also supported by poor binding of 2-(1,1-diallyl-but-3-enyl)-5-nonyl-phenol with CT-DNA in previously conducted DNA binding experiments (Jaivel *et al.,* 2014). As the compound binds well with the Lip A protein which is an extracellular esterase aids in rice cell wall degradation, thus the systemic infection of *Xoo* can be prevented at entry level itself. Whereas the isolated compounds bind well with good docking score against Fab V protein, thereby it is inferred that the compounds might have the property of cell wall inhibition of target pathogen.

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