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Research Article

Antifungal Activity of Phenyl Derivative of Pyranocoumarin from *Psoralea corylifolia* L. Seeds by Inhibition of Acetylation Activity of Trichothecene 3-O-Acetyltransferase (Tri101)

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Antifungal activity of petroleum ether extract of *Psoralea corylifolia* L. seed, tested against *Fusarium* sp. namely, *Fusarium oxysporum, Fusarium moniliforme,* and *Fusarium graminearum*, was evaluated by agar well diffusion assay. The chromatographic fractionation of the extract yielded a new phenyl derivative of pyranocoumarin (PDP). The structure of the PDP was confirmed using spectroscopic characterization (GC-MS, IR, and NMR), and a molecular mass of *m/z* 414 $[M\text{-}2H]^+$ with molecular formula $C_{27}H_{28}O_4$ was obtained. The PDP had a potent antifungal activity with a minimum inhibitory concentration of 1 mg/mL against *Fusarium* sp. Molecular docking using Grid-Based Ligand Docking with Energetics (GLIDE, Schrodinger) was carried out with the Tri101, trichothecene 3-O-acetyltransferase, as target protein to propose a mechanism for the antifungal activity. The ligand PDP showed bifurcated hydrogen bond interaction with active site residues at TYR 413 and a single hydrogen bond interaction at ARG 402 with a docking score −7.19 and glide energy of −45.78 kcal/mol. This indicated a strong binding of the ligand with the trichothecene 3-O-acetyltransferase, preventing as a result the acetylation of the trichothecene mycotoxin and destruction of the "self-defense mechanism" of the *Fusarium* sp.

1. Introduction

All over the world, a significant portion of agriculture production is lost due to fungal diseases and different mycotoxins produced by the phytopathogens like *Aspergillus* sp., *Fusarium* sp., and *Penicillium* sp. To overcome these losses in agricultural field, the common procedure is to use synthetic pesticides and fungicides [1, 2]. These synthetic pesticides and fungicides cause several side effects which include carcinogenicity, teratogenicity, and residual toxicity [3, 4]. Plant metabolites and plant-based pesticides and fungicides appear to be one of the better alternatives as they are known to have minimal environmental impact and danger to consumers in contrast to the synthetic pesticides [5, 6]. To promote the proper use of botanicals and to determine their potential as a source for new fungicides, it is essential to study plants of medicinal value. Natural products of higher plants may be a new source of antimicrobial agents with possibly novel mechanism of action contrary to synthetic drugs [7]. The seed of *Psoralea corylifolia* L. (Fabaceae), well known as traditional Chinese medicine "Buguzhi," is widely used for the treatment of various kinds of human disorders and contains good antioxidative, antimicrobial, antiinflammatory, antitumor, antimutagenic, and insect hormonal activities [8]. *P. corylifolia* L. seed has been reported to contain several phytoconstituents mainly coumarins, flavone components [9], and lactone as well as terpenoids like psoralen, isopsoralen, psoralidin, and bavachalcone [10].

With the aim of obtaining a bioactive principle, antifungal screening and activity-based fractionation of seed extract of *P. corylifolia* were carried out, which led to the isolation of the new phenyl derivative of pyranocoumarin (PDP). The compound had *in vitro* antifungal activity against the *Fusarium* sp. and molecular docking studies carried out

Table 1: Antifungal activity of seed extracts of *Psoralea corylifolia* L. against plant pathogens.

Extracts	Zone of inhibition (diameter in cm)		
	Fusarium moniliforme	*Fusarium oxysporum*	*Fusarium graminearum*
Petroleum ether extract (100 mg/mL)	**2.5 (±0.06)**	**2.9 (±0.06)**	**3.03 (±0.03)**
Chloroform extract (100 mg/mL)	1.8 (±0.07)	2.13 (±0.03)	1.5 (±0.03)
Ethanol extract (100 mg/mL)	1.6 (±0.09)	2.03 (±0.03)	1.30 (±0.06)
Ketoconazole (1 mg/mL)	3.2 (±0.03)	3.16 (±0.03)	2.97 (±0.03)
Ethanol (100%)	0.33 (±0.03)	0.26 (±0.03)	0.33 (±0.03)

* Mean of three replications.

Table 2: Antifungal activity of secondary fraction of petroleum ether extract against plant pathogens.

Secondary fractions	Zone of inhibition (diameter in cm)		
	Fusarium moniliforme	*Fusarium oxysporum*	*Fusarium graminearum*
SF1 (10 mg/mL)	0.26 (±0.03)	0.46 (±0.03)	—
SF2 (10 mg/mL)	—	0.4 9 (±0.06)	—
SF3 (10 mg/mL)	0.33 (±0.03)	1.6 (±0.10)	—
SF4 (10 mg/mL) (PDP)	**1.8 (±0.06)**	**2.37 (±0.07)**	**2.43 (±0.03)**
SF5 (10 mg/mL)	1.3 (±0.06)	1.43 (±0.03)	1.13 (±0.09)
Ketoconazole (1 mg/mL)	3.2 (±0.03)	3.16 (±0.03)	2.97 (±0.03)
Ethanol (100%)	0.33 (±0.03)	0.26 (±0.03)	0.33 (±0.03)

* Mean of three replications, (—): no zone.

with the X-ray crystal structures of Tri101, trichothecene 3-O-acetyltransferase (PDB ID: 2RKV) available in the Protein Data Bank (PDB; http://www.pdb.org/pdb/) proposed a hypothetical mechanism for antifungal activity against *Fusarium* sp.

The target protein selected for the molecular docking studies was a Tri101 gene product which catalyzes the transfer of an acetyl group from acetyl coenzyme A to the C3 hydroxyl moiety of several trichothecene mycotoxins. It has been reported that Tri101 acetylation was the primary defense mechanism against 3-hydroxylated trichothecenes in *Fusarium* sp. and that a mutation or a modification, resulting in a loss of Tri101, would be lethal to the organism [11].

When the pathway of the mycotoxin production in *Fusarium* sp. was analyzed, Tri101 gene product, that is, trichothecene 3-O-acetyltransferase catalyzes the acetylation of the C3 hydroxyl group of the toxin. The acetylation at C3 had been proposed to protect the *Fusarium* sp. through the remainder of the biosynthetic pathway. Tri101 will acetylate and reduce the toxicity of the final products of the biosynthetic pathway including trichothecene (T-2 toxin), nivalenol and deoxynivalenol [12–14]. The last step in the biosynthetic pathway is removal of the protecting C3 acetyl group before being secreted by the organism [15]. Control of the toxicity of trichothecene mycotoxins by modification of the C3 hydroxyl moiety has been identified as a self-defense mechanism of the organism.

2. Materials and Methods

2.1. Plant Material. The plant specimen with the seed of *Psoralea corylifolia* was identified and certified by the Botanical Survey of India, Southern Circle, Coimbatore, Tamil Nadu, India. Voucher specimens were maintained in laboratory for future reference.

2.2. Microorganisms. The fungal isolates of *Fusarium* sp. namely, *Fusarium oxysporum*, *Fusarium graminearum*, and *Fusarium moniliforme* from infected maize, paddy, and sorghum seeds were used for the study.

2.3. Extract Preparation. The dried and powdered seed samples of *P. corylifolia* L. were extracted by overnight percolation with ethanol (polar solvent), chloroform (medium polar solvent) and petroleum ether (least polar solvent), at the rate of 1 : 5 at room temperature. The extracts were then filtered with country filter paper and concentrated under vacuum in a rotary evaporator to obtain a gummy residue [16].

2.4. Antifungal Studies by Agar Well Diffusion Assay. The antifungal activity was tested for the prepared extracts against the selected pathogens. The sterilized sabouraud dextrose agar medium was poured into the petri plates and allowed to solidify. The fungal culture 200 μL was transferred to the petri plates. Using a sterile cork borer, wells of 6 mm in diameter were made in the plate containing the media. For each organism, 20 μL of the prepared sample was loaded in each well using sterilized dropping pipette. Three replications were maintained for each treatment. For each microorganism, the positive control (ketoconazole) and the negative control (ethanol) were also loaded in a separate well. The plates were incubated for 2-3 days and the observations were taken. The diameter of inhibition zone (DIZ) was measured and the mean D1Z was calculated [17, 18].

Table 3: Minimum inhibitory concentration of PDP against fungal plant pathogens.

Compound	Fungal plant pathogens		
	Fusarium moniliforme	*Fusarium oxysporum*	*Fusarium graminearum*
Phenyl derivative of pyranocoumarin			
10 mg/mL	−	−	−
1 mg/mL	−	−	−
0.1 mg/mL	+	+	+
0.01 mg/mL	+	+	+
Ketoconazole			
10 mg/mL	−	−	−
1 mg/mL	−	−	−
0.1 mg/mL	+	+	+
0.01 mg/mL	+	+	+
Solvent control	+	+	+
Cells	+	+	+

"+" Growth, "–": no growth.

Table 4: IFD results of ligand PDP and native ligand ZBA with the target protein trichothecene 3-O-acetyltransferase.

Sl. no.	Ligand name	Docking score	Glide energy kcal/mol	Hydrogen bond D–H···A	Distance Å
(1)	Phenyl derivative of pyranocoumarin (PDP)	−7.19	−45.78	(TYR 413)O–H···O	3.03
				(TYR 413)O–H···O	3.33
				(ARG 402)N–H···O	3.06
(2)	Native ligand ZBA	−7.79	−48.81	O–H···N(HIS 156)	2.67
				(TYR 413)O–H···O	2.89

2.5. Fractionation and Antifungal Assay. The fractionation was carried out by column chromatography using a 22 cm long column having 1.6 cm diameter packed with 20 g of silica gel (60–120 mesh) using petroleum ether solvent. The active extract was loaded to the packed column and eluted with a 90 : 10 mixture of petroleum ether and ethyl acetate at a rate of 2.6 mL min^{-1}. The polarity of mobile phase was gradually increased using ethyl acetate to give different fractions. Fractions with similar thin layer chromatogram were pooled together which were labeled serially and then subjected to antifungal assay [17, 18] to obtain the active fraction.

2.6. Minimum Inhibitory Concentration (MIC) by Tube Dilution Method. The assay was performed to obtain the MIC of the active fraction isolated from petroleum ether extract of *P. corylifolia* L. seed against the selected fungal pathogens using tube dilution method. The MIC is defined as the lowest concentration of antibiotics or plant extracts that did not show any growth of tested pathogens. The entire test sample was dissolved in ethanol and diluted to the highest concentration (10 mg min^{-1}) to be tested and then serial dilutions were made in a concentration range from 1 mg min^{-1} to 0.01 mg min^{-1} in sterile test tubes containing standardized inoculums. The growth of the organism for each dilution was observed and thus the MIC was evaluated. Ketoconazole and ethanol were used as positive and negative control, respectively [16].

2.7. Spectroscopic Studies. The active fraction obtained was subjected to GC-MS, NMR, and IR spectroscopic studies to identify the structure of the active compound. GC-MS was done using THERMO GC-TRACE ULTRA VER: 5.0, THERMO MS DSQ II. The column used was TR 5-MS Capillary Standard Non-Polar Column with a dimension of 30 Mts, Id of 0.25 mm, film of 0.25 μm and helium as a carrier gas. The flow maintained was 1.0 mL min^{-1} with oven temperature 80°C raised to 250°C at the rate of 8°C min^{-1}. The volume of sample injected was 1 μL. ^{1}H-NMR and ^{13}C-NMR were recorded at 400 and 100 MHz, respectively, in $CDCl_3$. The NMR spectroscopic studies were done by Bruker 400 spectrometer. IR confirmation studies were carried out in BRUKER OPTIK GMBH with Model no-TENSOR 27, opus version 6.5 software, resolution 4 cm^{-1}, sample scan time 64 scans and data taken from 4000 cm^{-1}–400 cm^{-1}.

2.8. Molecular Modelling Studies. Induced Fit Docking studies have been carried out using GLIDE [19] 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).

2.8.1. Preparation of the Ligand "Phenyl Derivative of Pyranocoumarin" and Protein "Trichothecene 3-O-Acetyltransferase". The ligand used in this study was prepared using Ligprep

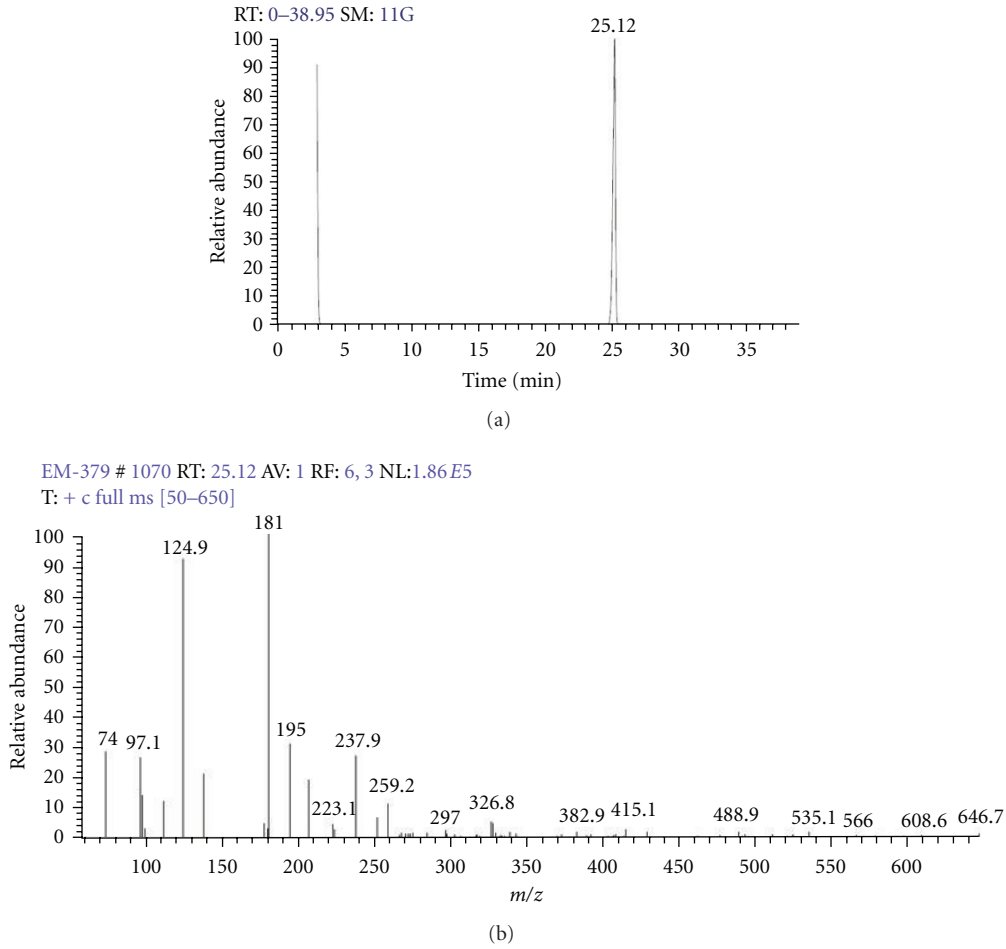

Figure 1: (a) Gas chromatogram of the PDP. (b) Mass fragmentation spectrum of PDP.

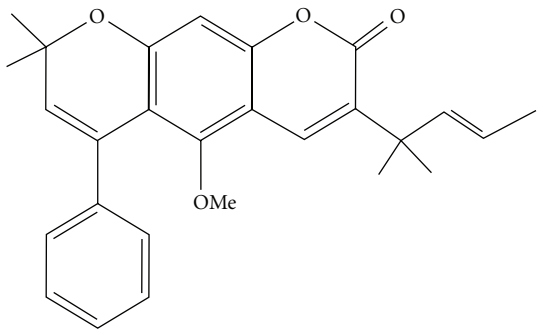

Figure 2: Phenyl derivative of pyranocoumarin (PDP).

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 protein taken for the study was 2RKV (Tri101 Complexed with Coenzyme A and T-2 Mycotoxin) retrieved from PDB 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 Å and the resulting structure was used for docking [20].

The native ligand used was ZBA, a T2 mycotoxin which readily binds with protein trichothecene 3-O-acetyltransferase during the mycotoxin production and activates the self-defense mechanism of the *Fusarium* sp.

2.8.2. Induced Fit Docking (IFD). IFD of the prepared ligand with the prepared protein was performed using IFD protocol of GLIDE v5.5 from Schrodinger Suite 2009 [20]. 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 gives the best structure of the docked complex based on the Glide score (G-score) of the dockings.

2.8.3. 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/).

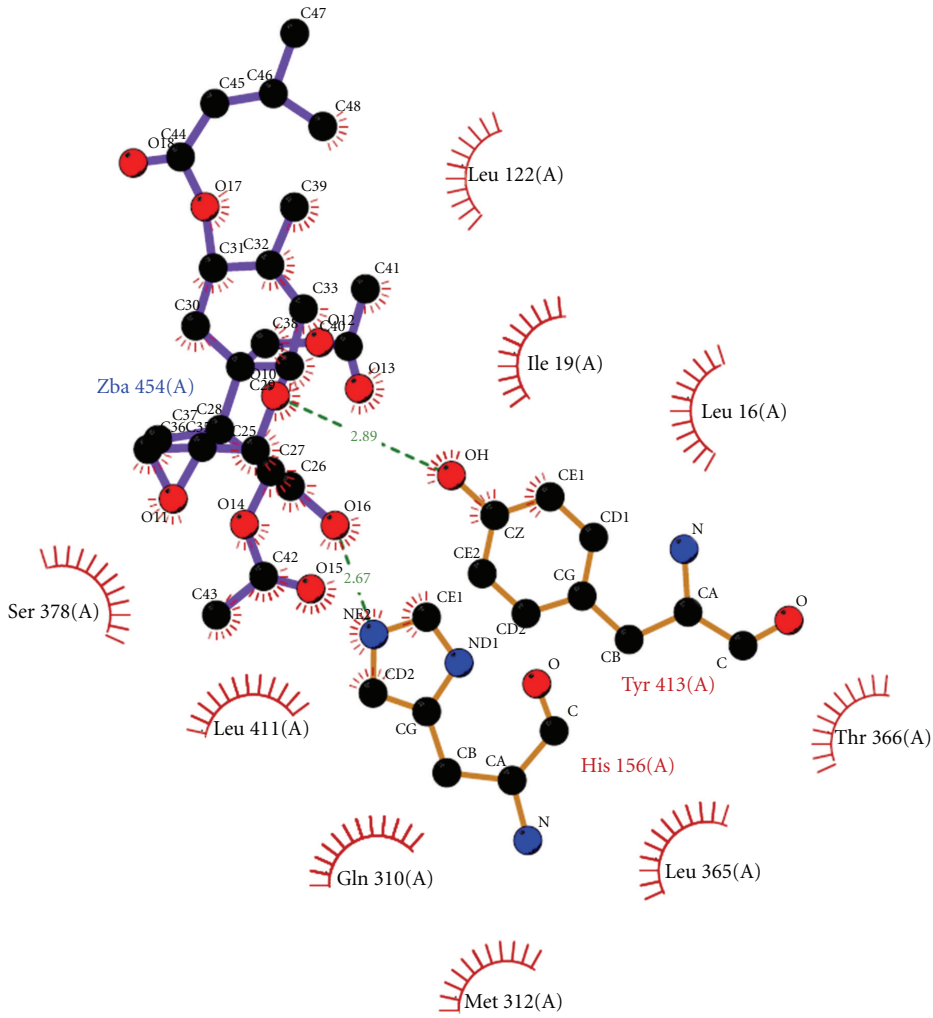

FIGURE 3: Ligplot image of interaction between ZBA (T2 mycotoxin) with trichothecene 3-O-acetyltransferase. Green dotted lines are hydrogen bond interactions and red semicircles are the hydrophobic interactions.

3. Results and Discussion

The seed of *P. corylifolia* L. possesses several secondary metabolites with remarkable biological properties [9]. With this background the present research has been initiated to identify the active secondary metabolite compound produced by *P. corylifolia* L. seed against *Fusarium* sp.

3.1. Antifungal Activity and Fractionation. Among the three extracts tested, petroleum ether extract exhibited highest zone of inhibition when compared to chloroform and ethanol extract at a concentration of 100 mg/mL against the selected pathogens. The DIZ produced by the petroleum ether extract (Table 1) was comparable to that of the positive control ketoconazole. The petroleum ether extract was fractionated and repeated fractionations yielded five different secondary fractions. The secondary fraction four (SF4) showed potent antifungal activity when compared to other fractions (Table 2). There is growing evidence that most of the secondary metabolites from plants are involved in the defense of the plant from plant pests and diseases. Thus, secondary compounds represent a large reservoir of chemical structures with biological activity. This resource is largely untapped for use as pesticides and fungicides [1].

3.2. Minimum Inhibitory Concentration. The bioactive fraction SF4 showed inhibition at a concentration of 10 mg/mL and 1 mg/mL against the selected pathogens, while growth was observed in the other two dilutions 0.1 mg/mL and 0.01 mg/mL (Table 3). For the management and protection from plant diseases, exploitation of naturally available chemicals from plants would be a ecologically sound method and a prominent commercial fungicide can be obtained in future, replacing toxic fungicides [21, 22].

3.3. Spectroscopic Studies. The antifungal compound isolated from the seed of *P. corylifolia* L. was obtained as whitish slight yellow colored compound. The spectroscopic studies confirmed the presence of new phenyl derivative of pyranocoumarin. The molecular formula was determined

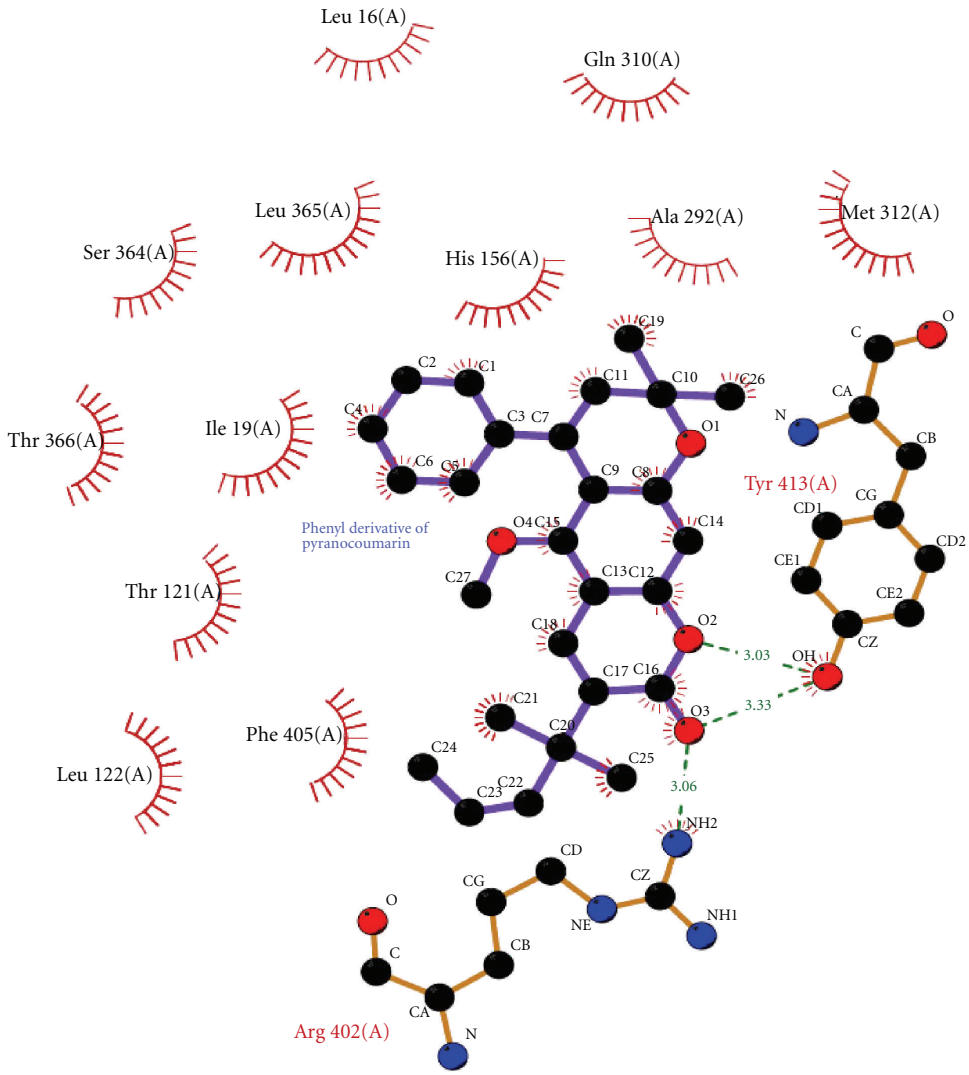

Figure 4: Ligplot image of interaction between PDP with trichothecene 3-O-acetyltransferase. Green dotted lines are hydrogen bond interactions and red semicircles are the hydrophobic interactions.

as $C_{27}H_{28}O_4$ by GC-MS (Figure 1) *m/z* 414[M-2H]$^+$. The fragmentation pattern confirmed the presence of pyranocoumarin [23]. The IR spectrum showed absorption at λ_{max} 1717 and 1174 cm^{-1} indicating the presence of C=O and C–O–C groups, which was confirmed by the presence of δ_C 161.2 in ^{13}C spectrum. Moreover, the ^{13}C NMR spectra displayed 29 carbon signals differentiated as five methyl, one methoxyl, 10 methine (including olefins) and 11 quaternary carbons (including oxygenated at δ_C 68.9, 154.6, 145.9 and characteristic coumarin carbonyl at 161.2) indicating that the structure should be a pyranocoumarin. The ^{1}H NMR spectrum suggested the presence of four methyls (δ 1.65 (3H, s), δ 1.69 (3H, s), 1.32 (3H, s), 1.28 (3H, s), 1.21 (3H, s)), olefinic protons δ_H 5.83 and 9.93 (each 1H, d, J = 8.56 Hz), a methoxyl δ 3.8 (s) and two 1H singlets were observed at about δ_H 6.33 and 6.23 and down field 6 aromatic protons observed between δ_H 6.78 and 7.28. On the basis of these observations and spectral data, the compound was predicted as "(E)-5-methoxy-8, 8-dimethyl-3-(2-methylpent-3-en-2-yl)-6-phenylpyrano [3, 2-g] chromen-2(8H)-one", a PDP (Figure 2) [24–26].

3.4. Docking Studies. The native T2 mycotoxin exhibited docking score of −7.79 and glide energy of −48.81 kcal/mol (Table 4) with hydrogen bond interactions TYR 413 and HIS 156 (Figure 3). The PDP was found to exhibit favorable bifurcated hydrogen bond interactions with the active site residue TYR 413 and ARG 402. It exhibited a docking score of −7.19 and glide energy of −45.78 kcal/mol. In the ligplot image, the hydrophobic interactions with twelve protein residues LEU 16, GLN 310, LEU 365, ALA 292, MET 312, HIS 156, SER 364, ILE 19, THR 121, LEU122, PHE 405, and THR 366 surrounding the PDP were also observed, indicating a stable docked complex of the protein and ligand (Figures 4 and 5).

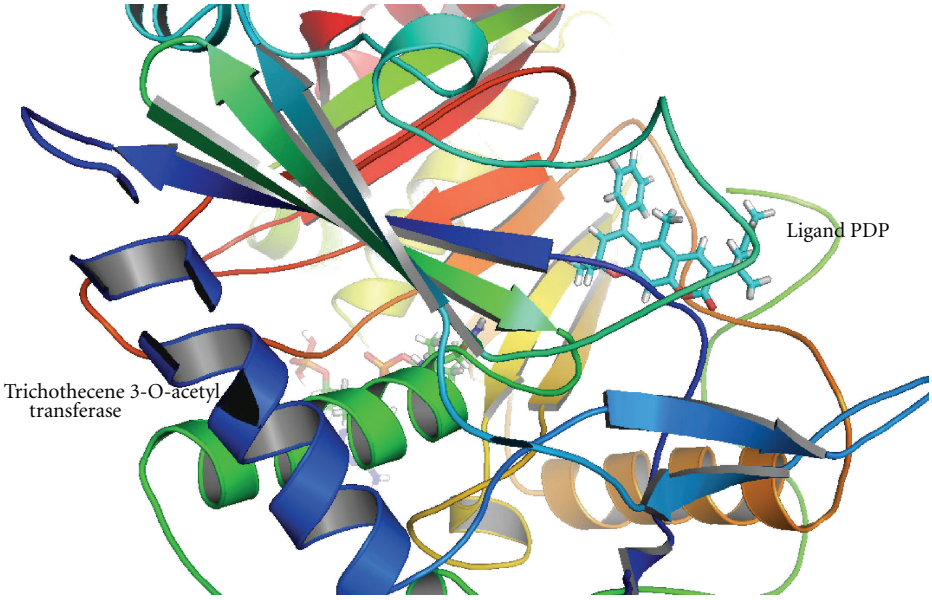

Figure 5: The docked complex of the ligand PDP in the active site residue cleft of the protein trichothecene 3-O-acetyltransferase.

This docking study proposes that the PDP has a similar affinity towards the trichothecene 3-O-acetyltransferase as that of the ZBA (T2 mycotoxin). Hence PDP can easily dock in the active site residue of the protein and form a docked complex and as a result prevent the activation of Tri101. This leads to the inhibition of acetylation of the T2 mycotoxin and disturbs the self-defense mechanism of the *Fusarium* sp.

Coumarins possess several biological activities, but they also play an important role in defense mechanisms against insects and plant pathogens. An increasing body of evidence indicates that the chemical interactions between plant-pathogenic fungi and higher plants are both complex and highly integrated. For instance, as fungi have developed toxins that increase their virulence on plant tissues, plants have developed a variety of ways to limit the effectiveness of these fungal toxins. Biosynthesis of fungal toxins can, however be blocked *in vitro* by the addition of certain naturally occurring plant metabolites at concentrations inhibitory to fungal growth [27, 28].

4. Conclusions

The seed of *P. corylifolia* L. is a commonly used botanical medicine. The present study has explored the use of *P. corylifolia* in the field of agriculture, by using it against plant pathogen *Fusarium* sp. A new antifungal compound, PDP, was identified with a minimum inhibitory concentration of 1 mg/mL against the selected pathogens namely, *Fusarium oxysporum*, *Fusarium moniliforme, and Fusarium graminearum.*

The molecular docking study with trichothecene 3-O-acetyltransferase proposes a hypothesis that the PDP has the affinity towards the target protein and it is able to bind with the protein and inhibit the acetylation mechanism of the protein leading to death of the *Fusarium* sp. The mechanism of how the antifungal compound PDP interacts with the 2RKV protein is revealed here. Nonetheless, this data require confirmation by appropriate experimental data. Our theoretical prediction may lead to the establishment of future molecular biology approaches.

Exploitation of naturally available chemicals from plants, which retards the reproduction of undesirable microorganisms, would be a more realistic and ecologically sound method for plant protection and will have a prominent role in the development of future commercial pesticides and fungicides for crop protection strategies, with special reference to the management of plant diseases [22, 29]. The PDP can be used for developing a commercial formulation based on its field trial and toxicological experiments in future.

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