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Mini Review

Anti Inflammatory Activity of Ayurvedic Oil, Pinda Tailam is Contributed by Trypsin Inhibition

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Keywords

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- Purpurin
- Anti-inflammatory activity
- Multiple binding sites
- Designing of better enzyme inhibitors

Abstract

Degenerative disorders, like rheumatoid arthritis (RA) are generally associated with proteolytic enzymes responsible for cartilage destruction and bone damage. Thus, development of therapeutic approaches against arthritis may include protease inhibition. The present report expresses assessing of trypsin inhibition by purpurin identified by *in-silico* method and assessed by wet-lab experiments. Purpurin is a pharmacologically active anthraquinone, present in Ayurvedic massaging oils, prescribed for inflammatory conditions like rheumatoid arthritis, joint aches etc. Isothermal titration calorimetry experiments identified multiple-sites protein-binding free energies of purpurin. From the present study, it could be concluded that purpurin is capable of inhibiting a serine protease, trypsin that could be of therapeutic value for rheumatoid arthritis treatment.

ABBREVIATIONS

mM: mili Molar; CAL: Calorie; COX2: Cyclooxygenase-2; PLA2: Phospholipases A2; LOX: Lipoxygenases; MMP: Matrix metalloproteinase; IL-1: Interleukin-1

INTRODUCTION

Proteases play a significant role in onset of arthritis [1,2]. Deregulated proteolysis may have causative functions in pathological conditions such as inflammatory disorders [3]. It has been found that precise lysis of proteins by proteases leads to a very subtle method of regulation. Inappropriate proteolysis has been found to have a major role in inflammatory disorders. Excessive proteolysis leading to signals into promotion of inflammatory symptoms can be prevented by blocking the appropriate proteases such as trypsin [4,5]. The action of proteases has been indicated in tumor invasion and metastasis [6]. They are mainly secreted by neutrophils and stored in lysosomes. During inflammation, tissue damage is caused by leukocyte proteases [7]. This would make them a target for inflammation management, by protease inhibitors [8-10].

Therefore, the identification of pharmacologically active small molecule protease inhibitors from natural sources would facilitate developing of drugs with lesser side effects [11].

In silico analysis and identification from both *Rubia cordifiolia* and pinda oil with liquid chromatography followed by mass spectrometry yielded purpurin, a trypsin inhibitor. Purpurin (1,2,4-trihydroxy-9,10-anthraquinone) comes under the class of anthroquinones with a distinct red colour, as in madder root (Rubiacordifiolia) [12-14]. It has potent antifungal activity against Candida species [15]. Purpurin is known to act as a potent Monoamine oxidase (MAO) inhibitor, a key enzyme in the onset of inflammation [16,17]. The present study expresses *in vitro* and *in silico* anti-trypsin activity of this compound.

MATERIALS AND METHODS

The present study deals with some of the plants used in medicinal oils which are used against inflammatory disease as per traditional ayurvedic preparation. Pinda tailam is widely used ayurvedic medicinal oil for the inflammatory ailments. The chemical structures of the compounds present in the ingredients of pinda tailam were considered for therapeutically significant target enzyme inhibition study. One of the ingredients of pinda tailam is *Rubia cordifolia* [18]. Structures of the major compounds present *Rubia cordifolia* were collected from PUBCHEM database and systematically studied by *in silico* methods for their inflammation markers inhibition. Since most of the inflammatory symptoms were up-regulated by protease activity, interactions of these compounds against protease enzyme were studied. In order to further understand the mode of action underlying these Ayurvedic preparations, trypsin was found suitable as one of the enzyme and its ligand interactions were analyzed by docking studies.[9,10] From the results purpurin emerged as

a high scoring compound. LC-MS study of pinda oil confirmed the presence of purpurin in it. Further, mechanism of enzyme inhibition by purpurin was characterized by enzyme kinetics studies and its thermodynamic aspects were revealed by ITC experiments.

Molecular docking studies

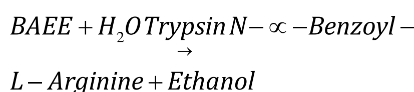
To identify the inflammation marker enzyme inhibitory compounds, molecular docking was carried out with phyto compounds reported in *Rubia cordifolia* by molecular docking method. The crystal structure of trypsin in complexation with an inhibitor (PDB ID 3LJJ) was taken as the target protein for the docking studies. Water molecules in the crystal structure were removed, and a minimization protocol was performed by setting a RMSD cut off of 0.30 Å. The force field assigned was OPLS 3 [19]. The residues corresponding to the active site were identified and grids was set over these selected residues with a dimension of 10 Å [20,21]. Induced fit docking, coupled with extra precision (XP) method implemented in Schrodinger 10.4 was used for docking studies [22-24].

LC-MS analysis

The samples were filtered through appropriate filters for application on to the Acquity UPLC system fitted with C18 column and connected to Xevo G2-S Q-Tof MS/MS system (Waters). The UPLC run conditions were standardized for a 15-min run with mobile phase A (0.1 % Formic Acid in Water) and B (100 % Acetonitrile), the total run time includes column equilibration and post run washing. The gradient elution starting from 95(A):5(B) to 5(A):95(B) in the end. The masses and corresponding Retention time were compared with the standards. The MS analysis was performed with a capillary voltage of 3 kV. The desired masses were then undergone collision induced dissociation in the same instrument. The CID was also standardized by applying varying collision energies (25V to 50V). The fragmentation patterns were then compared with the standard compounds using Massfrag software (Waters).

Trypsin inhibitory activity

The trypsin inhibitory activity of the purified compound was calculated by measuring the change in hydrolysis of the substrate BAEE (N-benzoyl-L-arginine ethyl ester) at the ester linkage causing an increase of absorbance.



The final volume of reaction mixture (3.4 ml) includes 67 mM phosphate buffer (pH 7.6), 0.25 mM BAEE in Phosphate buffer and 0.05 mM trypsin in ice cold .001 M Hcl. Purpurin (Sigma-Aldrich, St. Louis, MO, USA) used for the assay was prepared in DMSO. The mixture of 200 µl of trypsin and 200 µl of test solution was kept for 10 minutes incubation. The reaction was initiated by the addition of 3 ml of substrate and the absorbance was measured at 253 nm for 10 minutes, using a UV visible Spectrophotometer. PMSF (Phenyl methylulphonyl Fluoride) a known trypsin inhibitor was taken as positive control. Trypsin

Table 1: Glide Scores of purpurin with different inflammatory marker enzymes.

Si.No	Target Enzyme	Glide Score
1	COX-2	-9.483
2	Trypsin	-6.722
3	PLA-2	-6.637
4	LOX	-6.472
5	MMP	-5.773
6	IL-1	-1.395

Abbreviations: COX-2: Cyclooxygenase-2; PLA-2: Phospholipases A2; LOX: Lipoxygenases; MMP: Matrix Metalloproteinase; IL-1: Interleukin-1

inhibitory activity was expressed as its percentage-inhibition, calculated by following equation:

$$\%inhibition = \left(1 - \frac{B}{A}\right) x100$$

Where A is the change in absorbance without test sample and B is the change in absorbance with the test solution.

Enzyme kinetics

In order to gather the kinetics of inhibition, assay experiments were performed using different substrate (BAEE) concentrations (0.0625 mM to 1 mM). The substrate was prepared in phosphate buffer at pH 7.6. 200 µl of trypsin (0.05 mM in 1 mM HCl) was added to each aliquot. Absorbance was measured at 253 nm. The experiment was repeated in the presence of 0.75 mM of purpurin. The reduction in the activity was monitored and plotted against respective substrate concentration. Michaelis-Menten constant (Km) and maximal velocity (Vmax) were determined from the Line-Weaver-Burk plot.

From the Km and Vmax obtained, the inhibitor constant, Ki, was calculated using the following equation derived from Michaelis-Menten Equation [25]:

$$K_m^{'} = K_m\left(1 + \frac{I_0}{K_i}\right)$$

calculated using Cheng–Prusoff equation

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}}$$

Isothermal titration calorimetric assay

0.005 mM of trypsin and 0.1 mM of ligand was prepared for isothermal titration calorimetric assay. Purpurin was dissolved in 2 % DMSO solution in distilled water. Both protein and ligand solutions were degassed before loading on to the ITC. Calorimetric titrations were performed at 298.15 K using VP-ITC isothermal titration calorimeter (Microcal Northampton, MA, USA), as described in the manufacturer's instructions manual. 10 µl of purpurin was added from the rotating syringe to the cell which contained the protein solution.

Time was set at 10 seconds for each injection and a time

Table 2: Thermodynamic parameters for the binding of purpurin to trypsin calculated from ITC analysis.				
	***K*(*mol*$^{-1}$)**	***ΔH*(*cal mol*$^{-1}$)**	***ΔS*(*cal mol*$^{-1}$ *deg*)**	***ΔG*(*kcal mol*$^{-1}$)**
1	1.00 x 105	1.403 x 107	-4.71 x 104	-12.865
2	9.97 x 104	-3.052 x 106	-1.02 x 104	-10.87
3	1.01 x 105	-1.198 x 106	-4.00 x 103	-5.4
Abbreviations: K: Different Binding Constants; ΔH: Changes in enthalpy; ΔS: Changes in Entropy; ΔG: Binding Energy				

Table 3: Glide score of purpurin at each binding clefts.				
Sites	**Glide score (kcal/mol)**	**Volume of the sites Å^3**	**Site Score**	**Dscore**
A	-6.722	1863.42	0.853971	0.839280
B	-5.505	567.42	0.765089	0.637932
C	-4.362	401.20	0.529870	0.468772
Abbreviations: kcal: Kilocalorie; Å: Angstrom; Dscore: Druggability Score				

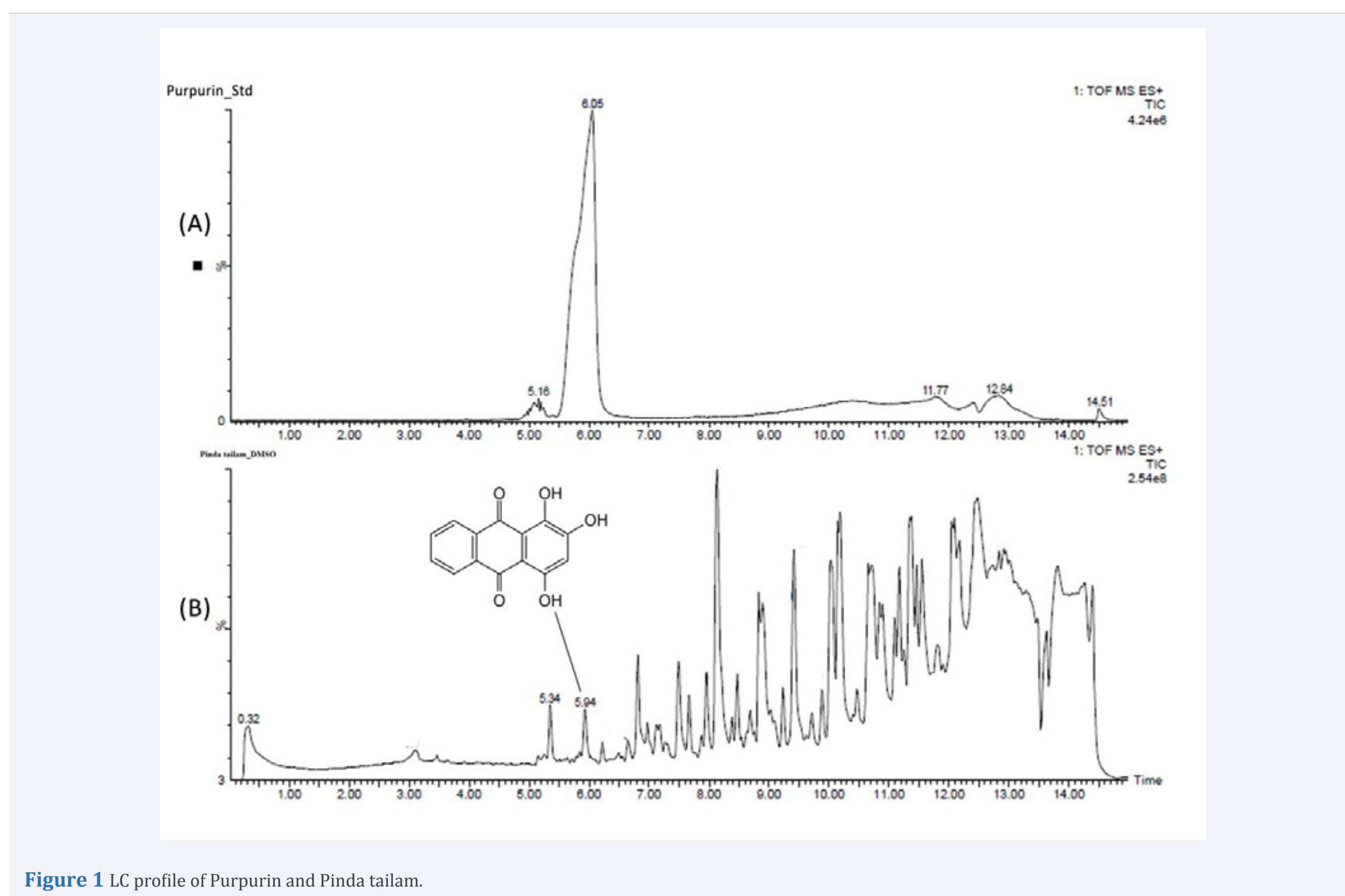


Figure 1 LC profile of Purpurin and Pinda tailam.

interval of 180 seconds was also set between injections to allow the peak resulting from each injection to return to the baseline.

A programme of 29 injections was made. The reference power was set at 10 µcal, and the stirring speed was kept at 307 rpm. The volume of the 1st injection was set as 3 µL to avoid inaccuracy. The heat changes between the trypsin and the solvents used to dissolve the compounds were subtracted from the original value. The final data was fitted by a nonlinear least-squares method with the software ORIGIN from the Microcal. Using ORIGIN binding constant (K), entropy change (ΔS), binding free energy (ΔG), and enthalpy change (ΔH) were calculated.

RESULTS AND DISCUSSION

The docking simulation studies suggested that purpurin could interact with different enzymes involved in inflammatory pathway. Along with COX, PLA2 and LOX, Trypsin also plays a prominent role in the manifestation of arthritic symptoms. The results are tabulated in Table. 1. The presence of purpurin was confirmed in both *Rubia cordifolia* and pinda tailam by LC MS method. The presence of purpurin in pindathailam is confirmed by comparing the total ion chromatogram with that of the standard purpurin, the isolated peak at 5.94 minutes (Rt) in

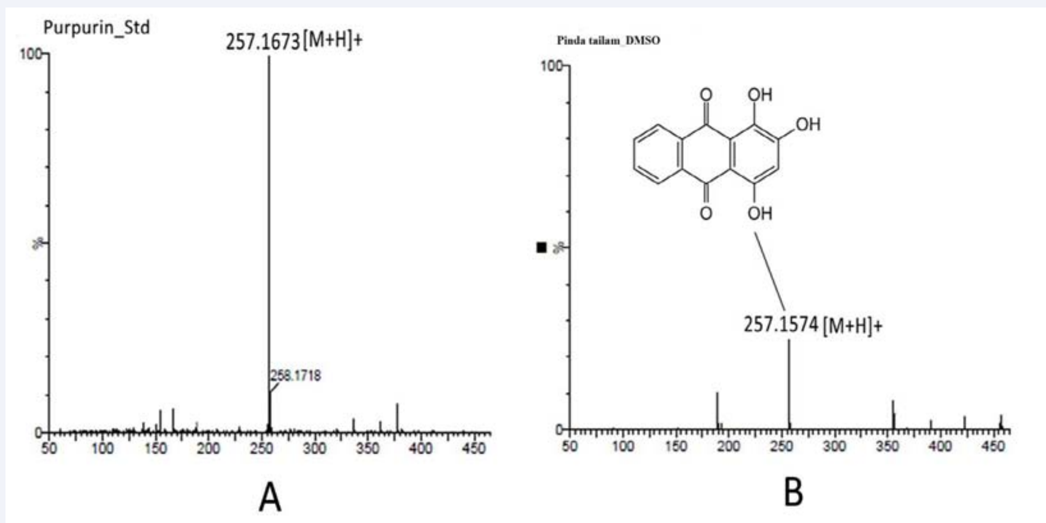


Figure 2 MS-MS Spectrum of Purpurin (A) and Pinda tailam (B) shows the presence of Purpurin.

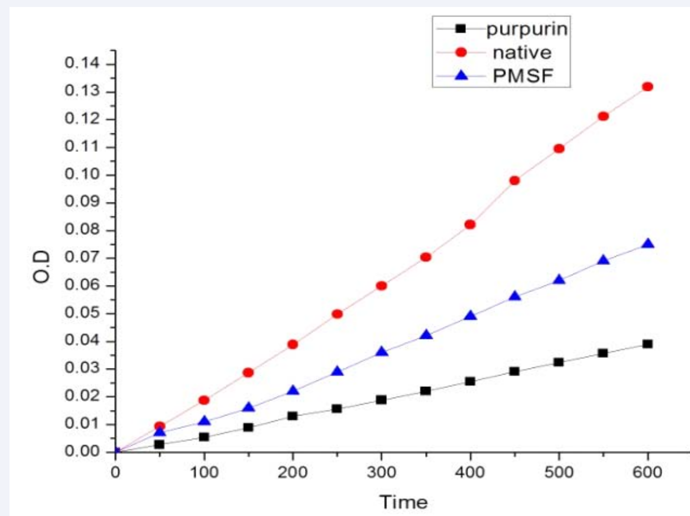


Figure 3 Trypsin inhibitory activity of purpurin and PMSF (Phenyl methylulphonyl fluoride).

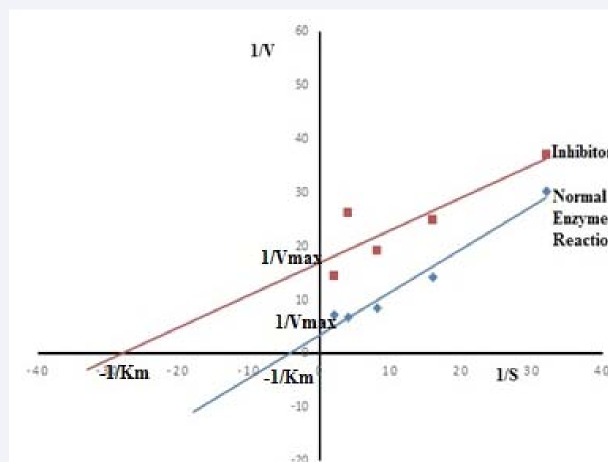


Figure 4 The Lineweaver-Burk plot of Trypsin inhibition by purpurin.

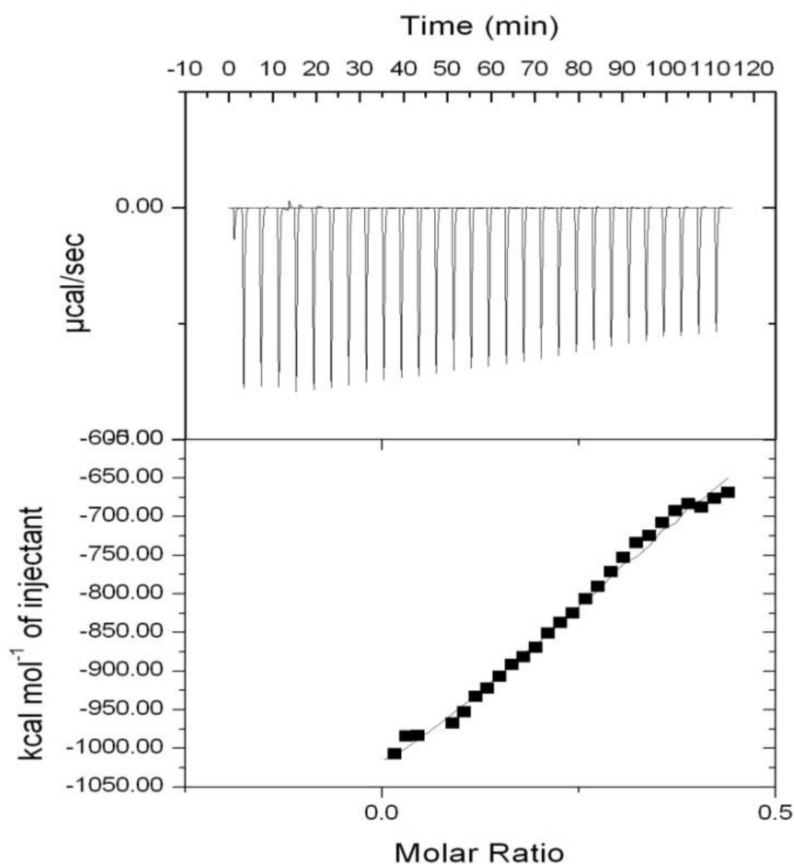


Figure 5 Isothermal titration calorimetric analysis of trypsin with purpurin. The curve represents the nonlinear least squares fit of the energy released as a function of the compounds added during the titration. Raw thermal power signal (top) and plot of integrated heat versus ligand/ protein molar ratio (bottom).

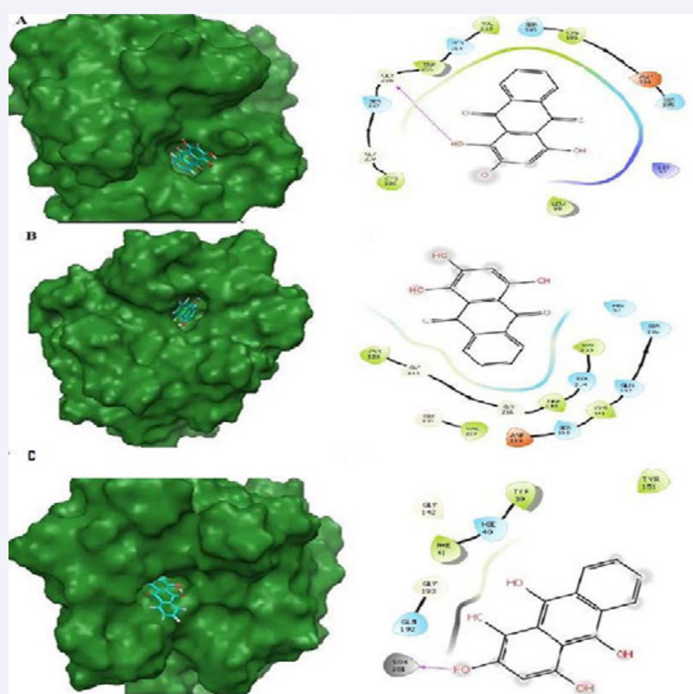


Figure 6 Purpurin at different binding pockets of trypsin and their corresponding protein ligand interaction diagrams.

pinda tailam corresponding to the Rt of standard purpurin (Rt =6.05) (Figure 1). Purpurin content in panda tailam has been quantitatively assed by HPTLC method and has found varying () in samples of different manufacturers. The Mass of the respective peaks was determined by Mass spectrometry analysis (Figure 2). In the present study we selected trypsin for further investigation of active entity in Pinda tailam. Influence of pinda tailam on LOX and COX was under investigation their *in-silico* studies are only highlighted in this paper. Purpurin, as suggested by molecular docking studies, could inhibit trypsin and was confirmed by the inhibition assay (Figure 3). From the assay inhibition was found to be 70 %. The Lineweaver-Burk plot (Figure 4) which does not have intercepting point displayed the uncompetitive mode of inhibition. As a confirmatory study for the impact of DMSO on the trypsin activity, the kinetics experiments were repeated with trypsin in 100 % DMSO, and the difference was found to be insignificant.

It was perceived that the raw thermal power signals from ITC analysis were exothermic in nature (Figure 5). Twenty-nine injections were made and the data fitted with a non-linear least squares fitting using sequential method with a stoichiometry, n=3(Figure 5). Normally, the stoichiometric value correlates with the available binding sites for the ligand to interact with receptor. Hence, it was assumed from the ITC experiments that purpurin can interact at three different sites on trypsin, with three different binding constants (K), changes in enthalpy (ΔH), and changes in entropy (ΔS). Binding free energies at different sites were deduced from the equation $\Delta G = \Delta H - T.\Delta S$ and tabulated (Table 2). From the evaluation of binding free energies and binding constants, the binding at the active site contributes high binding energy ($\Delta G = -12.865$) than other two sites.

The enzyme inhibition studies proposed an uncompetitive mode of inhibition. The possibilities of interaction of purpurin at different sites, other than the active site of the trypsin, were deduced by molecular docking methods as well. Also, it was inferred from the stoichiometry values obtained from the ITC experiments that Purpurin could bind at three different sites on Trypsin. The binding cavities on trypsin were predicted using PDBSUM (http://www.ebi.ac.uk/pdbsum/), and their volumes were comparable with that of Purpurin. The Site Map (Schrodinger 10.4) suite also used to validate the cleft for the drug ability of ligand. The ligand was docked at different clefts including active site. (Figure 6), and the scores were tabulated in Table 3. Docking study revealed that purpurin binds to three different clefts of trypsin with high affinity and this result justified the ITC data obtained.

CONCLUSION

The topical anti-inflammatory activity of pindatailam has been already established by clinical studies [26]. Purpurin is widely found in food and in many poly-herbal drug preparations of Ayurvedic medicine. The present work demonstrates the molecular mechanism of the anti-inflammatory activity elicited by purpurin. It may be noted that purpurin binds to trypsin at two locations other than the active site. Until purpurin is structurally modified to bind only at the active site of trypsin, assessment of trypsin inhibition by the two non-active site bindings, would render elusive. It is suggested that the structural modification of this compound may further enhance its binding affinity to trypsin. It is suggested that simply rendering it more water soluble may also enhance its trypsin inhibition property and such a change could be assumed to take place by subjecting purpurin to fermentation [27].There are many fermented ayurvedic herbal medicines, medicinal wines, containing *Rubiacordifolia*. Those medicinal wines may contain biotransformed derivatives of purpurin with higher trypsin inhibitory activity.

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