



# Inhibitory potential of important phytochemicals from *Pergularia daemia* (Forsk.) chiov., on snake venom (*Naja naja*)



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## KEYWORDS

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Molecular docking

**Abstract** *Pergularia daemia* (Forsk.) chiov., is a milk weed of Asclepiadaceae family. In the present study  $\beta$ -sitosterol,  $\beta$ -amyirin,  $\alpha$ -amyirin and lupeol were identified in the leaf by GC–MS. Molecular docking studies were performed to evaluate their activities on phospholipase A2 (PLA2) and L-amino acid oxidase enzymes which constituted a rich source in snake venoms (*Naja naja*). Snake venom Phospholipase A2 with PDB code 1A3D devoid of co-crystallized ligand was extracted from Protein Data Bank. Using Molegro Virtual Docker two cavities are formed by cocrystallization. L-Amino acid oxidase (PDB code 4E0V) was a receptor model with a co-crystallized ligand FAD. Among the phytochemicals analysed,  $\beta$ -sitosterol displayed high affinity of binding to the active site regions of phospholipase A2 and L-amino acid oxidase, respectively. The affinity of binding was  $-125.939$  and  $-157.521$  kcal/mole identified by gold scores.  $\alpha$ -Amyrin and  $\beta$ -amyirin had two hydrogen bond interactions with PLA2. Hence this study suggests that  $\beta$ -sitosterol identified in *P. daemia* can antagonize PLA2 and LAAO activities and forms a theoretical basis for the folk use of the plant against snake venom.

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## 1. Introduction

*Pergularia daemia* is also called as Yugmaphala a perennial, small, twining herb with major pharmacological activities being reported on different parts of the plant. Aerial shoots

are known to possess anti-emetic and expectorant properties [1]. Singh et al. reported the probable use of plant parts as anti-asthmatic, rheumatic swellings and anti snake venom [2]. The plant also acts as an Anthelmintic and antiseptic [3–5]. Dried leaves are Anti rheumatic, anti bronchitic, have wound healing properties, reduce body pains, infantile diarrhoea, amenorrhoea and dysmenorrhoea [1,6–8]. Fresh root is used as abortifacient and can treat gonorrhoea [10]. Shoots treat whooping cough [9,11], stem bark acts against malaria, and twig as an antipyretic and appetizer [12,13]. Moreover, Aqueous, ethanolic and petroleum ether extracts of *P. daemia*

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leaves exhibited significant analgesic, antipyretic activities and antibacterial properties [14,15]. Phytochemicals like glucosides and cardenolides (calotoxin, calotropagenin, dihydrocalotropagenin, calotropin, uscharidin) in seed, coroglaucigenin, corotoxigenin, uscharidin and uzarigenin in stem are identified [16]. Roots were reported to contain  $\beta$ -sitosterol, lupeol, lupeol acetate,  $\alpha$ ,  $\beta$ -amyrin and its acetate [17–19]. Apart from this, Jalalpure et al. reported leupol-3- $\beta$ -transcrotonate along with the acetate of  $\alpha$ -amyrin,  $\beta$ -amyrin, oleanolic acid and  $\beta$ -sitosterol from the hexane extract of whole plant [15]. Organic esters, fatty acids and phenolic compounds were identified by GC MS analysis of the ethanolic extract of the plant [20].

Though the literature shows the folk use of the plant to treat snake bite, there exists no theoretical evidence. The present work, reports the isolation and structural elucidation of  $\beta$ -sitosterol,  $\beta$ -amyrin,  $\alpha$ -amyrin and lupeol simultaneously from the leaf powder of *P. daemia* using GC–MS for the first time. Further computer-aided analysis was initiated to evaluate the activity of these compounds against snake bite after their *in vitro* studies thus aiming at developing a theoretical evidence for folk use of the plant.

## 2. Material and methods

### 2.1. General experimental procedure

All the chemicals in the study were purchased from Sigma and Merck Pvt. Ltd and are used without further purification. The venom was purchased from Calcutta Snake Park, Calcutta, India, in its pure form. GC–MS, column, protein data, docking was performed in Ohmlina Centre for Molecular Research, India. The whole plant of *P. daemia* (Forsk) chiov., was collected in the month of August, 2010, from Krishna district, India. The sample was identified using trnL-trnF intergenic spacer sequence based phylogenetic tree (Fig. 2) and a voucher specimen No. 02 was deposited in the herbarium of Chalapathi



**Figure 1** Twig of *Pergularia daemia* bearing flowers and fruit. The leaves are almost glabrous above and velvety below. The flower corolla forms a greenish-yellow or dull white tube. The fruits release ovate seeds covered with velvety hairs.

Institute of Pharmaceutical Sciences, Lam, Guntur, India (Fig. 1).

### 2.2. Extraction, isolation and analysis by GC–MS

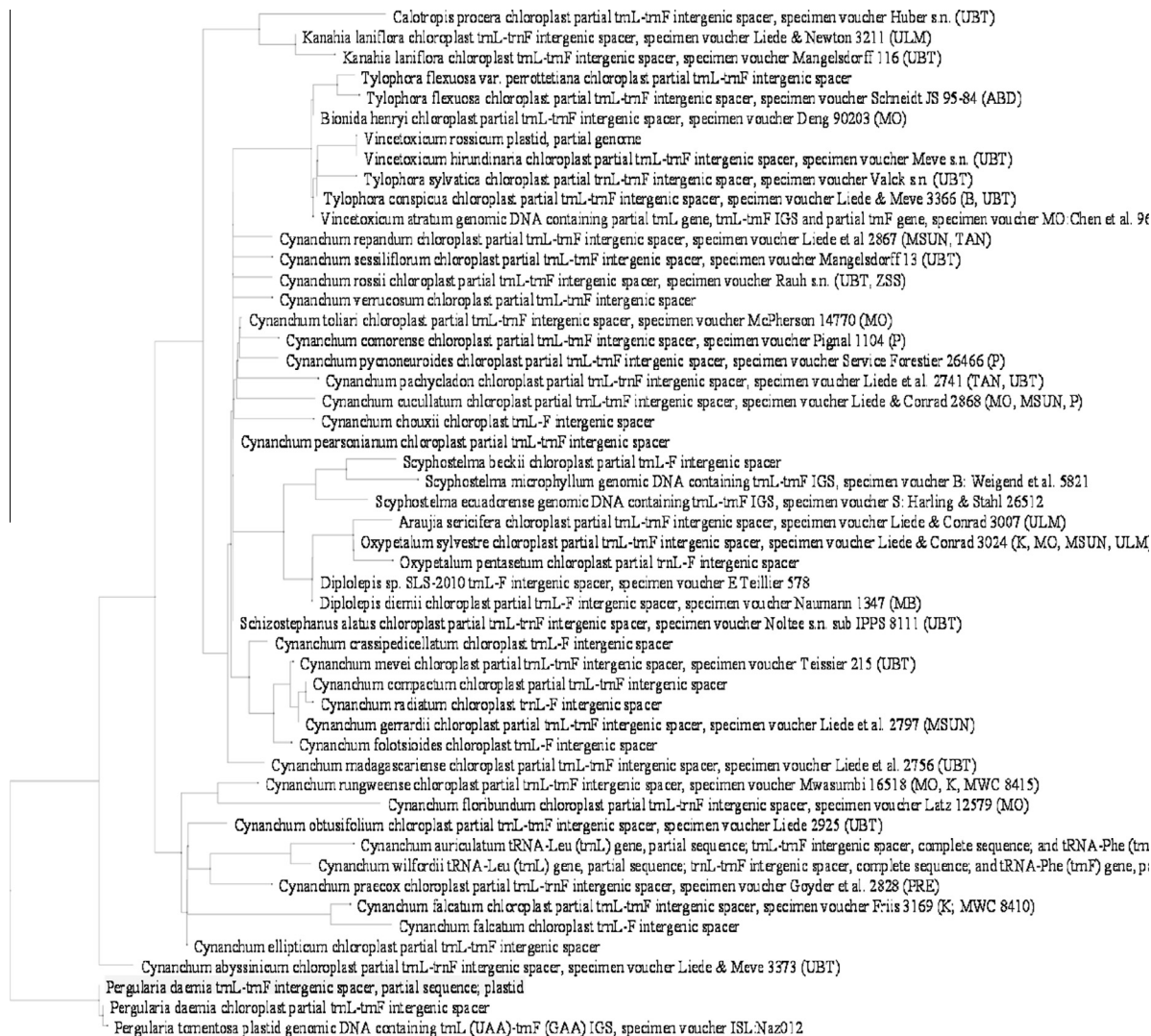
As the plant was selected based on the traditional use, the initial extract was prepared as described by the traditional healer. 10 gms of the leaf powder was extracted using 200 MilliQ-water at 50 °C for 4h and cooled to room temperature, filtered and treated with methylene chloride ( $3 \times 100$  ml) to extract more lipophilic components. The Leaf residue (remained on filter paper) was dissolved in 200 mL of n-Hexane and sonicated for 30 min to dissolve any lipophilic substance that is not removed from the leaf powder [21,22]. Methylene chloride and n-Hexane extracts are combined and passed through a sodium sulphate plug. The combined extract was concentrated in a rotary evaporator up to 10 mL and then further upto 1 mL using nitrogen concentrator and was analysed using GC–MS (5975C Inert MSD) system equipped with a splitless capillary injection port using HP-5MS, 0.25 mm  $\times$  0.25  $\mu$ m  $\times$  30 m column [23]. Helium (99.999%) was used as a carrier gas at a constant flow of 1 mL/min and an injection volume of 0.5 EI was employed with an injector volume of 1  $\mu$ L under splitless mode. Ion source temperature was maintained at 280 °C. The oven temperature was programed from an initial temperature of 70 °C hold for 5.0 min with an increase of 10 °C/min to 320 °C for a total run time of 40 min. Mass spectra were taken at 70 eV, a scan interval of 0.5 s and with MS scan mode from 25 to 550 m/z. The chromatograms thus obtained were recorded (Fig. 4).

### 2.3. In-vitro tests for the inhibition of PLA2 activity

PLA2 activity of *N. naja* venom was performed using an indirect haemolytic assay on agarose–erythrocyte–egg yolk gel plates [24]. Increasing doses of the venom were added to the wells in the agarose gels with 1.2% sheep erythrocytes, 1.2% egg yolk as a source lecithin and 10 mM CaCl<sub>2</sub>, incubated at 37 °C overnight and haemolytic haloes were measured (Fig. 3). Control wells contain 15  $\mu$ L of saline. The minimum indirect haemolytic dose (MIHD) corresponds to the dose that induced a haemolytic halo of 20 mm diameter. 1 MIHD of *N. naja* venom was used. Test solutions and dose of venom (0.005 mL each) were pre-incubated for 1 hour at 37 °C. After centrifugation at 10,000 rpm for 10 min, the supernatant (20  $\mu$ L) was tested for PLA2 activity by incubating the plates at 37 °C for 20 h. Results were expressed as percentage inhibition of enzymatic activity (Table 1 and Fig. 7), where 100% inhibition produced no clear zone. The total phenol and flavonoid content of the extract was estimated using tannic acid and quercetin standard graphs (Figs. 5 and 6).

### 2.4. In vitro tests for the inhibition of protease activity of the venom

Venom Proteolytic activity was assayed as described by Ibrahim et al. [25] with a slight modification. 1 mL of *N. naja* (10–2500  $\mu$ g) with 1 mL of 1% casein in 0.1 M Sorensen's phosphate buffer of pH 7.6 was incubated for 30 min at 37 °C. Undigested casein was precipitated and the reaction was terminated by adding 3 ml of 5% trichloroacetic acid

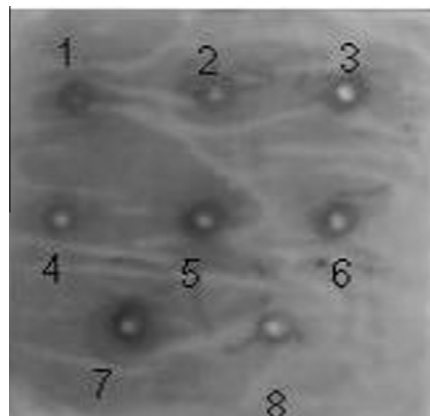


**Figure 2** By using the *Pergularia daemia* trnL-trnF intergenic spacer, partial sequence; plastid a phylogenetic tree was constructed. Maximum likelihood phylogram was discovered in 8 independent ratchet analyses. Note the broken branch to the outgroup, *Cynanchum*, with the exception of a few weakly supported nodes, this topology is identical to the strict consensus of most parsimonious trees discovered.

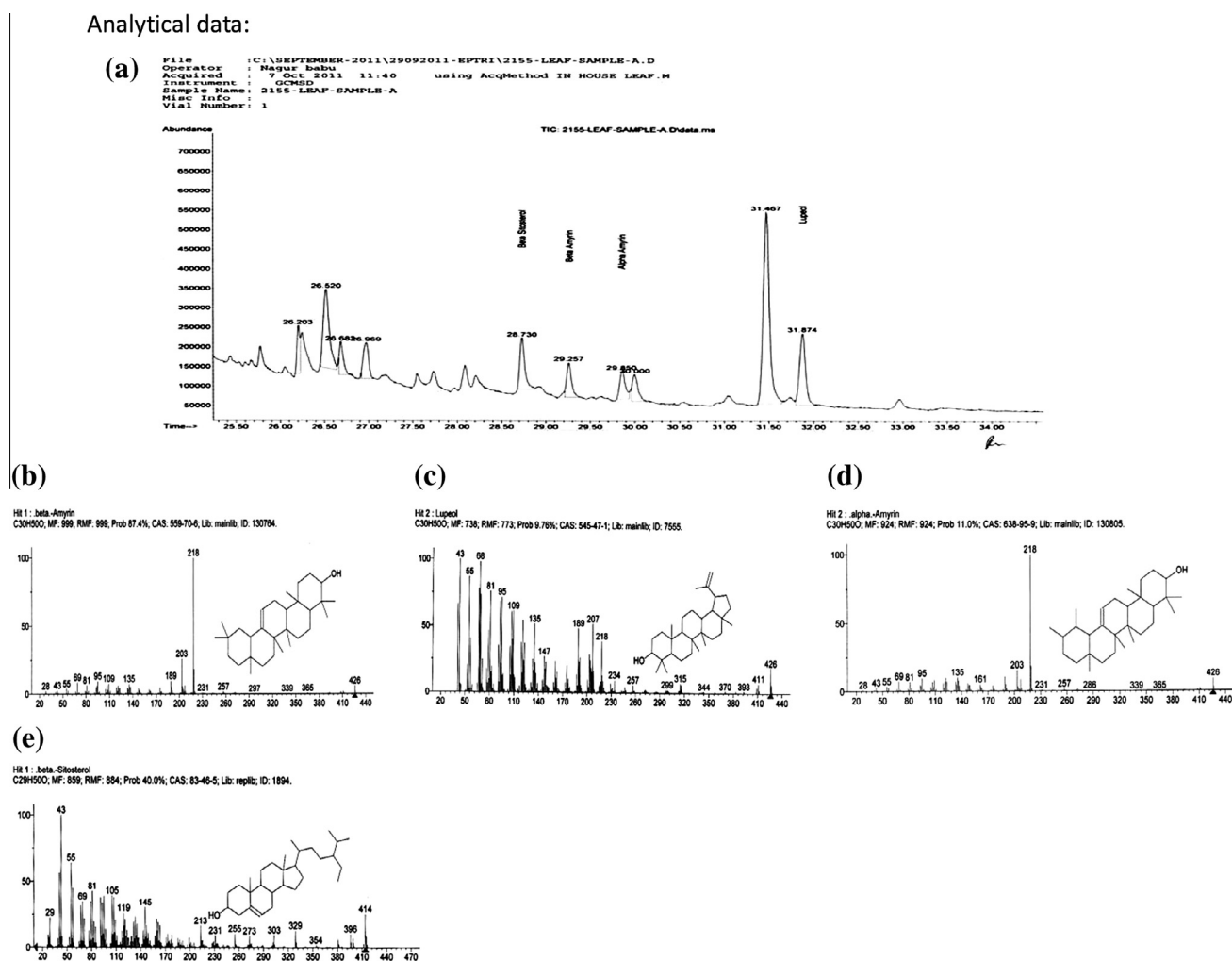
centrifuged at 5000 rpm for 15 min, the absorbance of the supernatant was measured at 366 nm. The activity of protease was defined as the amount of enzyme that hydrolyses 1  $\mu$ mol of amino acids (in terms of tyrosine) from casein per minute under the standard assay conditions. *P. daemia* extract was evaluated for its anti-proteolytic potential against the venom by incubating each 0.5 mL of the test solution (100, 250, 500, 1000  $\mu$ g w/v) with 200  $\mu$ L of venom for 1 h at 37 °C. Blanks containing only the buffer and extract were run in parallel. The optical density was measured at 366 nm (Table 2 and Fig. 8).

### 2.5. Inhibition of hyaluronidase activity

Inhibition of hyaluronidase enzyme activity was determined turbidometrically [26]. NN venom (25–1000  $\mu$ g) in phosphate buffer saline (PBS) of pH 7.4 was incubated with 50  $\mu$ g of hyaluronic acid in 1 mL 0.2 M sodium acetate buffer (pH 5) with 0.15 M sodium chloride for 25 min at 37 °C. The reaction was



**Figure 3** *In-vitro* PLA2 activity of the extract in agarose gel impregnated with 1.2% sheep erythrocytes and 1.2% egg yolk and 10 mM CaCl<sub>2</sub>. 1–8 Cavities show inhibition of haemolytic activity of naja venom by increasing dose of plant extract (5, 10, 15, 20, 25, 30, 35, 40  $\mu$ l of plant extract) in series.



**Figure 4** GC–MS chromatogram of *P. daemia* leaf extract showing  $\beta$ -sitosterol,  $\alpha$ -amyrin,  $\beta$ -amyrin and lupeol Chromatogram b, c, d, e shows compounds identified compared to inbuilt NIST library.

stopped by adding 2 mL of 2.5% cetyl-trimethyl-ammonium bromide in 2% sodium hydroxide. Optical density was read at 400 nm after 30 min against blank. Turbidity-reducing activity was expressed as a percentage of the remaining hyaluronic acid, taking the absorbance of the blank as 100%. The venom concentration that produced a reduction in turbidity of  $\sim 50\%$  was used in inhibition experiments. The percentage inhibition of venom hyaluronidase activity by the extracts was determined after pre-incubating an equal volume (100  $\mu$ L) of each test sample in acetate buffer with NN venom in PBS [0.01–100  $\mu$ g] for 30 min at 37  $^{\circ}$ C, then hyaluronidase activity was measured. Blanks were run in parallel (Table 3 and Fig. 9).

### 2.6. Molecular docking studies

The potential binding site of the target protein and lead candidates are identified by a molecular docking algorithm Mol Dock, based on a novel search algorithm that combines differential evolution techniques with a cavity prediction algorithm. Genetic algorithm, Mol Dock SE with default parameters such as 1500 iterations, population size 50, with maximum 300 steps was used. The scoring scheme was derived from PLP

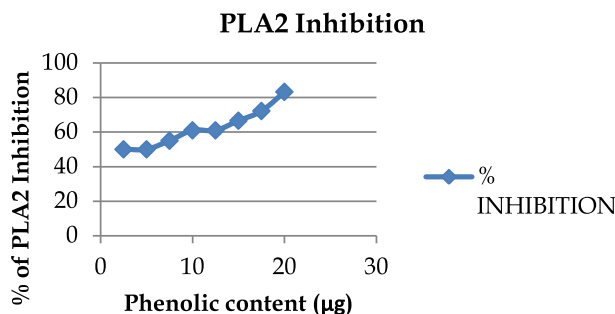
(Piecewise Linear Potential) scoring functions [27]. The compounds used for docking are drawn as 2-D structures using ISIS Draw software and converted to 3-dimensional Mol2 files using ProDRG2 server [28]. We tested three different scoring functions such as GOLD score, M E Dock server and Mol Dock score of Molegro software were tested. The X-ray crystal structures of Phospholipase A2, 1A3D, L-amino acid oxidase and 4E0V were downloaded from PDB database [29–32]. Ligand cavities were detected in all enzymes and by default the major cavity was selected for analysis before docking (Tables 4 and 5 and Figs. 10 and 11).

### 3. Results and discussion

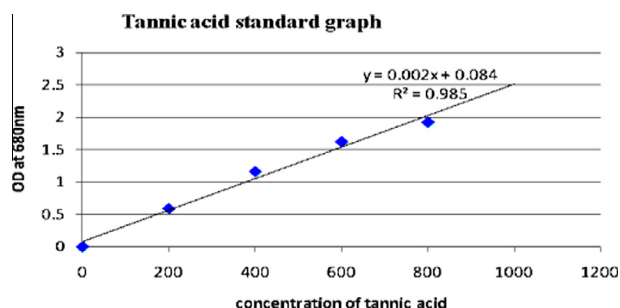
The resulting GC–MS chromatograms are compared with the standard and were found to contain  $\beta$  sitosterol,  $\alpha$ -amyrin,  $\beta$ -amyrin and lupeol. About 5  $\mu$ g of the *N. naja* venom produced 20 mm diameter haemolytic haloes that are considered to be 1 U (U/5  $\mu$ g) in agarose sheep erythrocyte gel. This confirms the PLA2 activity of *naja* venom. *P. daemia* extract was capable of inhibiting PLA2 dependent haemolysis in a dose dependent manner. 40  $\mu$ L of plant extract corresponding

**Table 1** Percentage inhibition of PLA2 activity of venom by plant extract using an indirect haemolytic assay on agarose-erythrocyte-egg yolk gel plates method and corresponding phenol content.

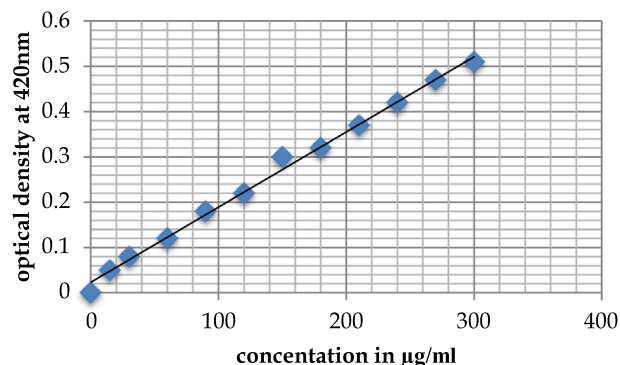
S. no	Extract (µl)	Concentration of phenols (µg)	Inhibition (%)
1	5	2.5	50
2	10	5.0	50
3	15	7.5	55
4	20	10.0	61
5	25	12.5	61
6	30	15.0	66.6
7	35	17.5	72.2
8	40	20.0	83.3



**Figure 7** Graph shows percentage inhibition of PLA2 activity corresponding to phenolic content of the extract.



**Figure 5** Tannic acid standard graph for the estimation of phenols from *Pergularia daemia* extract using the Folins method.



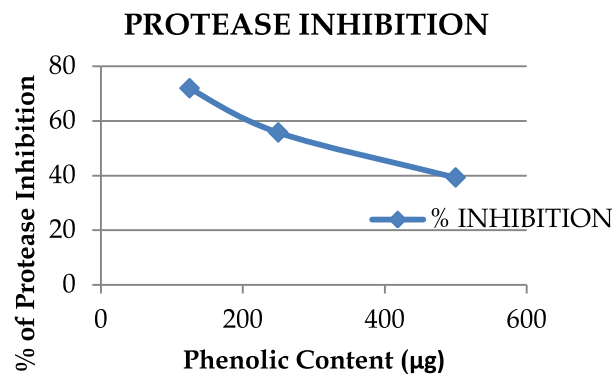
**Figure 6** Standard graph of quercetin for the estimation of flavonoids from *Pergularia daemia* extract.

to 20 µg of phenolic content inhibits 80% of haemolysis of sheep RBC's induced by *naja* venom. The ED<sub>50</sub> of *P. daemia* was found to be 5 µL corresponding to 2.5 µg of the phenolic content. 250 µL of the extract shows 72% of activity. The inhibition of protease activity at low concentration confirms the basic role of triterpenes and sterols identified in the extract.

Snake venom Phospholipase A2 with PDB code 1A3D extracted from Protein Data Bank was devoid of co-crystallized ligand. Two cavities were detected using cavity detection algorithm in Molegro Virtual Docker by comparing the cavity formed by co-crystallized bound ligand of 4DBK. Nearly 50% of residues in both the cases are identical.

**Table 2** Venom (200 µL) protease inhibition activity in of *Pergularia daemia* in terms of percentage.

S. no	Extract µl	Concentration of phenols (µg)	O.D 366 nm	Inhibition (%)
1	100		-0.66	-
2	250	125	0.088	72
3	500	250	0.139	55.8
4	1000	500	0.191	39.3

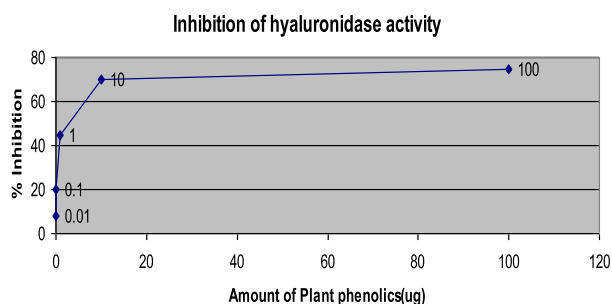


**Figure 8** Venom protease inhibition activity of *Pergularia daemia* plotted against the corresponding concentration of the phenolic component in the extract.

**Table 3** Percentage inhibition of venom hyaluronidase activity of *Pergularia daemia* extract and its corresponding phenol content.

S. no	Concentration of phenols (µg)	Inhibition (%)
1	0.01	8
2	0.1	20
3	1	45
4	10	70
5	100	75

Moreover, the volume of each cavity was 100.352 (1A3D) and 197.12 Å (4DBK) respectively. Binding affinities and probable mode of interactions of four ligands, viz.

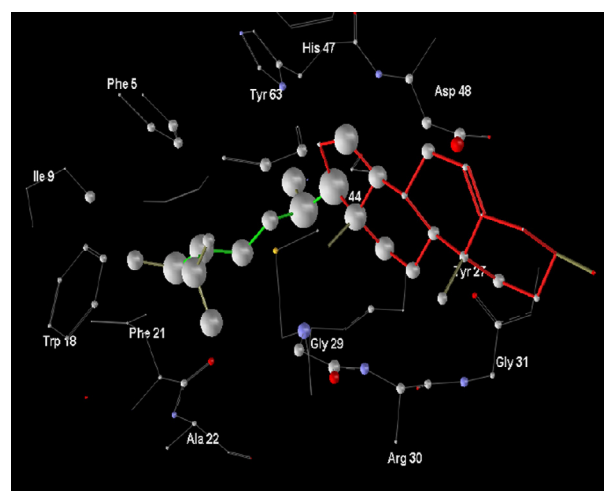


**Figure 9** Percentage inhibition of hyaluronidase activity of *Pergularia daemia* plotted against corresponding concentration of phenolic component in the extract.

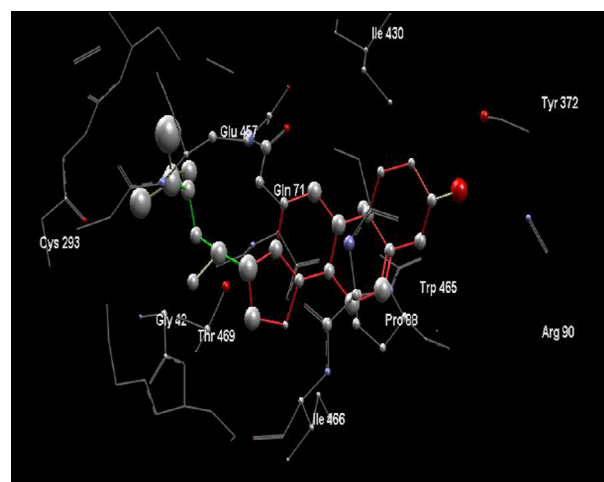
$\beta$ -sitosterol,  $\beta$ -amyrin,  $\alpha$ -amyrin and lupeol with active site residues of 1A3D resulted in dock scores between  $-92.18$  kcal/mol and  $-125.94$  kcal/mol respectively (Table 4).  $\beta$ -sitosterol displayed high affinity ( $-125.94$  kcal/mol). However, this ligand and lupeol are without H-bond interactions. Hydrophobic and steric interactions might exist. On the other hand, ligand efficiency (moldock score divided by heavy atoms) resulted in  $-4.198$  kcal/mol of ligand efficiency for  $\beta$ -sitosterol that was much better than the remaining ligands. The energy contributions of active site residues with ligand molecule,  $\beta$ -sitosterol, are predominantly from Cys, Gly, Phe, and Tyr amino acids.

L-Amino acid oxidase, 4E0V selected as receptor model has a co-crystallized ligand, FAD with one cavity. Hence ligand docking was initiated by choosing the cavity as active site. The resultant dock scores (Table 5) suggest the fact that almost all ligands present similar competitive inhibition (around  $-133$  kcal/mol) at the active site except  $\beta$ -sitosterol ( $-157.521$  kcal/mol). This high affinity of  $\beta$ -sitosterol might be attributed to the ligand efficiency ( $-5.2507$  kcal/mol) as well as hydrogen bond interactions with Arg90 and Tyr372 respectively. The energy contributions of active site residues with ligand molecule,  $\beta$ -sitosterol, were predominantly from Gly, Thr and Tyr amino acids.

An attempt has been made to isolate a few compounds from *P. daemia* that are expected to have inhibitory potential against snake venom phospholipase A2 and L-amino acid oxidase. The work complies with the literature, which states that most of the phenolic acids, terpenoids, and flavonoids from the plant extract are responsible for snake venom neutralizing ability [33]. There exists a strong correlation between total phenols, flavonoids and their biological activities. Of the four compounds,  $\beta$ -sitosterol,  $\beta$ -amyrin,  $\alpha$ -amyrin and lupeol that were identified by GC-MS, it was observed from molecular docking studies that  $\beta$ -sitosterol has high affinity of binding to the active site regions of alpha cobra toxin, phospholipase A2 and L-amino acid oxidase. Computational analysis revealed



**Figure 10** Energy contributions of active site amino acids of 1A3D with  $\beta$ -sitosterol. Atoms contributing energy are represented as spheres (size corresponds to the energy associated).



**Figure 11** Energy contributions of active site amino acids of 4E0V with  $\beta$ -sitosterol. Atoms contributing energy are represented as spheres (size corresponds to the energy associated).

the strength of binding and important amino acids that confer the stability of  $\beta$ -sitosterol within the active site regions of these enzymes. It is the abundant phyto-steroid isolated from various plant sources which are identified for their anti-snake venom activity like *Aristolochia serpentaria* (the Virginia snake root) [34], *Cissampelos glaberrima* (snake liana in Brazil) [35] *Marsypianthes chamaedrys* (snake plant) [36] and *Ophiorrhiza mungos* (*Ophiorrhiza* is snake root in Latin) [37].

**Table 4** Dock scores of four ligands against 1A3D with H-bond interacting residues and ligand efficiency (LE).

Ligands	Mol Wt	Mol Dock score (kcal/mol)	H-bonding amino acids	Heavy atoms	Ligand efficiency (kcal/mol)
$\alpha$ -Amyrin	426.717	$-92.1781$	O-Tyr 27, Gly 29	31	$-2.9735$
$\beta$ -Amyrin	425.709	$-96.8369$	O-Tyr 27, Gly 29	31	$-3.1238$
$\beta$ -Sitosterol	414.707	$-125.939$	–	30	$-4.1979$
Lupeol	426.717	$-93.8994$	–	31	$-3.029$

**Table 5** Dock scores of four ligands against 4E0V with H-bond interacting residues and ligand efficiency (LE).

Ligands	Mol Wt	Mol Dock score (kcal/mol)	H-bonding amino acids	Heavy atoms	Ligand efficiency (kcal/mol)
$\alpha$ -Amyrin	426.717	-133.106	O Met 43	31	-4.2937
$\beta$ -Amyrin	425.709	-143.833	O Met 43, N Ser 44	31	-4.6398
$\beta$ -Sitosterol	414.707	-157.521	N Arg 90, O Tyr 372	30	-5.2507
Lupeol	426.717	-131.528	O Met 43	31	-4.2428

#### 4. Conclusion

Hence, it can be concluded that  $\beta$ -sitosterol isolated from *P. daemia* is responsible for anti-snake venom potential of the extract, provided, further experimental investigations on this active principle would highlight the importance of this compound.

The main groups of triterpenoids are represented by tetra cyclic and pentacyclic derivatives. Pentacyclic triterpenoids are all based on a 30-carbon skeleton comprising five, six-member rings (ursanes and lanostanes) or four six-member rings and one five-member ring (lupanes and hopenes) [38]. Pentacyclic triterpenoids  $\alpha$ -amyirin, betulinic acid and betulinaldehyde, and other related triterpenes such as oleanolic acid (oleanic acid), ursolic acid, zeylasteral were reported to possess anti-snake venom activity [39]. But Lupeol,  $\alpha$ -amyirin and  $\beta$ -amyirin belonging to this group isolated from *P. daemia* showed less effectiveness on *Naja* venom. Hence the synergistic activity of all the compounds in the extract might potentiate the anti-venom activity of the plant in its folk use.

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