ANDROGRAPHIS PANICULATA LEAF EXTRACTS AS POTENTIAL NAJA NAJA ANTI-SNAKE VENOM

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ABSTRACT
Traditional medicinal plants from the local region of Javvadu hills, Tamil Nadu, India were used as an antidote for snake bites by irrula tribes. One such a plant Andrographis paniculata leaves were collected from the traditional healers and explored for the for anti-snake venom activity. It was observed that the methanolic leaf extract of Andrographis paniculata significantly neutralized the Naja naja venom in vitro. The leaf extract of Andrographis paniculata was used to evaluate the enzyme inhibiting activity of protease, phosphomonoesterase, phosphodiesterase, acetyl cholinesterase, phospholipase A2, hyaluronidase and L-amino acid oxidase toxic enzymes present in snake venom. In-silico results of individual plant metabolites inhibiting PLA2 correlates with the results of plant extract inhibiting enzymes.

Keywords: Andrographis paniculata, Naja naja, acetyl cholinesterase, phospholipase A2.

INTRODUCTION
In India mortality rate is very high, 40,000 - 50,000 die annually.[1][2] World Health Organization (WHO) estimates place the number of bits to be 83,000 per annum with 11,000 deaths.[3] Snake bite is a neglected public health issue in many tropical and subtropical countries. About 5 million snake bites occur each year, resulting in up to 2.5 million envenomings (poisoning from snake bites), at least 100 000 deaths and around three times as many amputations and other permanent disabilities.[3] Daboia russelli, Echis carinatus, Naja kaouthia and Naja Nagoya are the most common snakes found throughout Tamil Nadu and a large number of deaths occur due to envenomation by them. In allopathic medicine the antiserum is the only therapeutic agent for the treatment of snakebite and its efficacy is limited due to several reasons. Even after anti snake venom antiserum treatment, hemorrhage,
necrosis, nephro-toxicities were reported and often produces hypersensitive reactions.\textsuperscript{[4]} In several cases, death reported after antiserum treatment with enough numbers of antiserum vials. One of the reasons reported by clinicians was inconsistency in polyvalent anti-snake venom antiserum, batch to batch variations, etc. Antiserum development in animal is time consuming, expensive. It is associated with various side effects like pyrogen reactions.\textsuperscript{[5]} Serum sickness along with that supply of anti-venom has logistical, marketing, storage and economic difficulties.\textsuperscript{[6]} Antivenom immunotherapy is the specific treatment available against snake bite and was developed by Albert Calmette in 1895 against the Indian Cobra.\textsuperscript{[7]} This is the high time to derive an alternative treatment that involves the usage of different venom inhibitor, synthetic or natural. Over the years attempts have been made to get the knowledge of medicinal plants that were traditionally used as anti-snake venom therapy by tribes to develop snake venom antagonists. In this regard, several plants are scientifically studied; \textit{Andrographis paniculata} leaf extracts inhibit snake venom and could be used for therapeutic purposes in case of snakebite envenomations.\textsuperscript{[8]}

\textit{Andrographis paniculata} grows erect to a height of 30–110 cm in moist, shady places (Fig. 1). The slender stem is dark green, squared in cross-section with longitudinal furrows and wings along the angles. The lance-shaped leaves have hairless blades measuring up to 8 cm long and 2.5 cm wide. The small flowers are borne in spreading racemes. The fruit is a capsule around 2 cm long and a few millimeters wide. It contains many yellow-brown seeds. \textit{Andrographis paniculata}, a medicinal plant of Acanthaceae family is used for various properties such as treatment of upper respiratory infection, ulcerative colitis and rheumatic symptoms. \textit{Andrographis paniculata} extracts may have the potential to be used as a mosquito repellent. In the present study the leaf extracts of \textit{Andrographis paniculata} were evaluated for \textit{In-vitro and In-silico} inhibitory potential against toxic venom enzymes of \textit{Naja naja}.

\begin{figure}[h]
\centering
\includegraphics[width=0.4\textwidth]{Fig1.jpg}
\caption{Andrographis paniculata.}
\end{figure}
1. MATERIALS AND METHODS

1.1 Chemical and reagent

Venom

The lyophilized venom of *Naja naja* was procured from Irula Snake Catcher’s Co-operative Society, Kancheepuram, Chennai, Tamil Nadu, India. The venom was (5 mg/ml) suspended in physiological saline and centrifuged at 2000 g for 10 min. The supernatant was used for further analysis and they are stored at 4°C. The protein concentration was estimated by Lowry *et al.*, method.[9] Disodium-p-nitrophenol phosphate, L-leucine, diansidine hydrochloride, horseradish peroxidase, 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide, hyaluronic acid, cetyltrimethylammonium bromide, lecithin were purchased from Himedia Laboratories and casein from Sigma Aldrich laboratories, USA. All the other reagents are analytical grade.

1.2 Preparation of extracts

*Andrographis paniculata* leaves were collected in January 2015 from villages in Polur Taluk, Thiruvannamalai district, Tamil Nadu, India. The plant was authenticated by Botanist of VIT University, Vellore. The leaves were washed, shade dried, powdered and stored in airtight container for future use. 30 gm of powdered leaves was extracted in a Soxhlet extractor using methanol as solvent.[10,11] Extracts were concentrated and the residue obtained was dried, weighed. Before use, it was dissolved in saline and centrifuged at 2000 rpm for 10 min at room temperature. The supernatant was used for further investigation and kept at 4°C. The plant extracts were expressed in terms of dry weight.

1.3 Qualitative phytochemical analysis

Detection of alkaloids

Extracts were dissolved individually in dilute Hydrochloric acid and filtered. Mayer’s Test: Filtrates were treated with Mayer’s reagent (Potassium Mercuric Iodide). Formation of a yellow colored precipitate indicates the presence of alkaloids.

a. Test for phenol (Ferric Chloride Test)

Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black color indicates the presence of phenols.

b. Test for saponins

Crude extract was mixed with 5 ml of distilled water in a test tube and it was shaken
vigorously. The formation of stable foam was taken as an indication for the presence of saponins.

c. **Test for glycosides (Salkowski’s test)**
Crude extracts were mixed with 2 ml of chloroform. Then 2 ml of concentrated sulfuric acid (H₂SO₄) was added carefully and shaken gently. A reddish brown color indicated the presence of steroid ring.

d. **Test for proteins (Ninhydrin test)**
Crude extract when boiled with 2 ml of 0.2% solution of Ninhydrin, violet color appeared suggesting the presence of amino acids and proteins.

e. **Test for carbohydrates (Benedict’s test)**
Crude extract when mixed with 2 ml of Benedict’s reagent and boiled, a reddish brown precipitate formed which indicated the presence of the carbohydrates.

f. **Test for terpenoids**
Crude extract was dissolved in 2 ml of chloroform and evaporated to dryness. To this, 2 ml of concentrated H₂SO₄ was added and heated for about 2 minutes. A grayish color indicated the presence of terpenoids.

g. **Detection of flavonoids (Alkaline Reagent Test)**
Extracts were treated with a few drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on the addition of dilute acid, indicates the presence of flavonoids.

h. **Test for steroids**
Crude extract was mixed with 2 ml of chloroform and concentrated H₂SO₄ was added sidewise. A red color produced in the lower chloroform layer indicated the presence of steroids.

Another test was performed by mixing crude extract with 2 ml of chloroform. Then 2 ml of each of concentrated H₂SO₄ and acetic acid was poured into the mixture. The formation of a green color indicates the presence of steroids. [12-14]
1.4 Thin layer chromatography
The extracts were analyzed by thin layer chromatography (TLC) on analytical plates over silica gel (TLC grade – Merck India). The solvent system used for separation of hexane extract was hexane and methanol (9:1) and for methanolic extract it was chloroform and methanol (9.2:0.8).

1.5 Snake Venom Toxic Enzymatic Inhibition Studies
1.5.1 Phosphomonoesterase
Method of Bessey et al., (1946) was employed to determine phosphomonoesterase activity with slight modifications. To a mixture 1.0 ml Tris –HCl buffer (pH 8.0), 1.0 ml disodium - p –nitro phenol phosphate, 0.5 ml 0.25% crude venom was added and incubated at 37°C for 3 hrs. The absorbance was measured at 425 nm.\textsuperscript{[5]} Inhibition study was carried out by the pre-incubating venom with plant extract for 45 min.

1.5.2 Phosphodiesterase
Phosphodiesterase activity was determined by a method modified from Lo et al., (1966). First, 0.1 ml of venom solution/fraction was added to an assay mixture containing 0.5 ml 0.0025 M Sodium-p-nitro phenyl phosphate, 0.3 ml 0.01 M MgSO\textsubscript{4}, 0.5 ml 0.17 M Tris – HCl (pH 8.0). The reaction was monitored by absorbance measurement at 400 nm.\textsuperscript{[15]} Inhibition study was carried out by the pre-incubating venom with plant extract for 45min.

1.5.3 L Amino acid Oxidase
Method of Li et al., was employed in the determination of L-amino acid oxidase activity. The reaction mixture consisting of 1.0 ml L-leucine, 2.0 ml Tris-HCl buffer (pH 8.0), 0.25 ml 0.1% of o-dianisidine hydrochloride, 0.15 ml 0.1% horseradish peroxidase and 0.04 ml 0.5% crude venom solution was allowed to stand for 10 minutes at room temperature and then the absorbance was measured at 415 nm.\textsuperscript{[16]} Inhibition study was carried out by the pre-incubating venom with plant extract for 45 min.

1.5.4 Acetyl cholinesterase
The activity was determined by the method of Ellman et al., (1961). 50 L 0.1% crude venom and 3 ml phosphate buffer (pH 8.0) was incubated at room temperature for 5 min and 10 L DTNB (5,5’-dithiobis-(2-nitrobenzoic acid) and 20 L substrate acetylthiocholine iodide was added. The increase in absorbance at 412 nm was measured.\textsuperscript{[17]} Inhibition study was carried out by the pre- incubating venom with plant extract for 45 min.
1.5.5 Hyaluronidase

Hyaluronidase assay of crude venom was determined turbidometrically by the method of Pukrittayakamee et al., (1988). The assay mixture contained buffer of Tris – HCl (pH 8.0), 50 mg hyaluronic acid (0.5 mg/ml in buffer) and enzyme in the same buffer in a final volume of 1.0 ml. The mixture was incubated for 15 min at 37°C and the reaction was stopped by the addition of 2 ml 2.5% (w/v) cetyltrimethyl ammonium bromide in 2% (w/v) NaOH. The absorbance was read at 400 nm (within 10 min) against a blank containing 1mL of the same buffer and 2 ml 2.5% (w/v) cetyltrimethyl ammonium bromide in 2% (w/v) NaOH [18]. Inhibition study was carried out by the pre-incubating venom with plant extract for 45 min.

1.5.6 Phospholipase A₂

Phospholipase A₂ assay was determined according to the acidimetric method of Tan and Tan (1988) with little modification. Briefly, a Lecithin suspension was prepared by mixing proportionately 1% lecithin, 18 mm calcium chloride and 8.1 mm sodium deoxycholate. The pH of the suspension was adjusted to 8.0 with 1 M sodium hydroxide and stirred for 10 minutes to ensure homogenous mixing. Next, 0.1 ml venom solution/fraction was added to 15 ml of egg yolk suspension to initiate the hydrolysis. The initial decrease in pH was measured by a pH meter. Inhibition study was carried out by the pre-incubating venom with plant extract for 45min.

2. Docking Studies

2.1 Protein – Ligand Interactions With Autodock 4.2

Auto Dock calculations were performed in several steps: 1) preparation of coordinate files using Auto Dock Tools, 2) precalculation of atomic affinities using Auto Grid, 3) docking of ligands using Auto Dock and 4) analysis of results using Auto Dock Tools. The primary method for conformations searching is a Lamarckian Genetic Algorithm and Auto Dock is shown to be an effective tool capable of quickly and accurately predicting binding conformations and binding energies of ligands with macromolecular targets.

Step 1—Coordinate File Preparation

Auto Dock 4.2 is parameterized to use a model of the protein and ligand that includes polar hydrogen atoms, but not hydrogen atoms bonded to carbon atoms. An extended PDB format, termed PDBQT, is used to coordinate files, which includes atomic partial charges and atom types. The current Auto Dock force field uses several atom types for the most common atoms, including separate types for aliphatic and aromatic carbon atoms and separate types
for polar atoms that form hydrogen bonds and those that do not. PDBQT files also include information on the torsional degrees of freedom. In cases where specific side chains in the protein are treated as flexible, a separate PDBQT file is also created for the side chain coordinates. In most cases, AutoDockTools will be used for creating PDBQT files from traditional PDB files.

Step 2—AutoGrid Calculation

Rapid energy evaluation is achieved by pre-calculating atomic affinity potentials for each atom type in the ligand molecule being docked. In the AutoGrid procedure the protein is embedded in a three-dimensional grid and a probe atom is placed at each grid point. The energy of interaction of this single atom with the protein is assigned to the grid point. AutoGrid affinity grids are calculated for each type of atom in the ligand, typically carbon, oxygen, nitrogen and hydrogen, as well as grids of electrostatic and desolvation potentials. Then, during the AutoDock calculation, the energetics of a particular ligand configuration are evaluated using the values from the grids.

Step 3—Docking using AutoDock

Docking is carried out using one of several search methods. The most efficient method is Lamarckian genetic algorithm (LGA), but traditional genetic algorithms and simulated annealing are also available. For typical systems, AutoDock is run several times to give several docked conformations and analysis of the predicted energy and the consistency of results is combined to identify the best solution.

Step 4—Analysis using AutoDock Tools

AutoDock Tools includes a number of methods for analyzing the results of docking simulations, including tools for clustering results by conformational similarity, visualizing conformations, visualizing interactions between ligands and proteins and visualizing the affinity potentials created by AutoGrid.

The crystal structure proteins were retrieved from the Protein Data Bank (PDB entry code 1POB). All polar hydrogen atoms were added and partial charges were placed with the help of GROMACS package. The formatted molecule was energy minimized by keeping heavy atoms fixed at their initial crystal coordinates and the added hydrogen atoms were made free to move. Minimization was effected under vacuum medium (mehler&solmajer). Electrostatic interactions were calculated using the cutoff method. The solvation parameters were added
using the ADDSOL utility of Auto Dock 4.2. Default values of atomic solvation parameters were used throughout the calculations. The grid maps of the protein were calculated using the AutoGride program.

For docking studies in Auto Dock 4.2 ligand molecules were drawn using MDL ISIS draw 2.5 and saved as ‘mol’ file and were imported to ACD/chemsketch for making the file formats compatible for use in Arguslab and 3D optimization done and saved as ‘pdb’ file. All possible flexible torsions of the ligands were defined by AUTOTORS. The prepared ligands were used as input ligands for Auto Dock 4.2. Docking simulations were done with Auto Dock 4.2 using Lamarckian genetic algorithm. The standard docking procedure was used for rigid protein and flexible ligand whose torsion angles were identified. A grid of 100, 100 and 100 points in x, y and z directions was built cantered on the catalytic site of the protein. The default settings were used for all other parameters. All calculations were carried out on PC based machines running Windows XP as operating systems. The resultant structures were analyzed using Auto Dock Tools.

2.2 Analysis using Auto Dock Tools
The ‘dlg’ file from the results of in silico analysis was opened using auto dock analyze (Analyze> dockings > open). The PDBQT file of the receptor molecule was opened using open macromolecule option (Analyze> macromolecule > open). The configurations were arranged according to the order of binding energy (Analyze> conformations > play ranked by energies). The binding energy, H- bond information were derived using (open panel to change play options > show info > build H-bonds). Finally the residues interacting with ligand was observed using (Analyze> dockings >show interactions).

3. Statistical Analysis
All biological in vitro and in silico experiments results were expressed as percentage decrease with respect to control values and compared by one-way ANOVA with Dunnett’s post test was performed. GraphPad Prism version 6.07 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com was used for statistical analysis. A difference was considered statistically significant if p≤0.05.
RESULTS AND DISCUSSION

4.1 Methanolic extract of *Andrographis paniculata*

The leaf extracts were concentrated and dried and the residue obtained was weighing 3.0 gm. The extracts were subjected to thin layer chromatography. The solvents were selected based on the phytochemical separations; each extract was separated in a different solvent system, in different ratios. The spots well separated in hexane with the solvent system hexane and methanol (9:1) and in the methanolic extract with chloroform and methanol (9.2:0.8). The plates were visualized under ultra violet at 365nm (Figure 1). Qualitative phytochemical analysis of the extracts were analysed and tabulated (Table 1). The enzymatic inhibition studies were carried in triplicates and mean ± standard deviation was calculated. The extracts were able to inhibit acetylcholinesterase, phospholipase A₂, hyaluronidase, L-amino acid, phosphomonoesterase and phosphodiesterase enzymes (Fig 2). The maximum inhibition was observed for Phospholipase A₂ (57%) and phosphomonoesterase (54%) in 250µg/ml of the extract (Table 2).

### Table 1: Qualitative phytochemical analysis of the extracts.

<table>
<thead>
<tr>
<th>Test</th>
<th>Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+</td>
</tr>
<tr>
<td>Phenols</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>-</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>-</td>
</tr>
<tr>
<td>Protein</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>-</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>+</td>
</tr>
</tbody>
</table>

+ indicates present, - indicates absent.

Fig 2: Thin layer chromatography of methanol extract.
4.2 *In silico* studies

Methanolic extract of *Andrographis paniculata* leaves was having several phytochemicals, we have selected major six compounds (5-Hydroxy 7,8,2’,3’-tetramethoxyflacone, 14-deoxy-11-dihydroandrographolide, andrographolide, Paniculide a, Paniculide b, Paniculide c) which were expected to have *Naja naja* enzyme inhibition activity. These selected compounds interact with active site residues as mentioned in table 4: Key Amino acid residues required for PLA2 enzymatic activity were found interacting with most of these selected phytochemicals. Selected phytochemicals binding pose in the active site of PLA 2.

![Graphical representation of % enzyme activity for 250μg/ml concentration of methanolic extract.](image)

**Fig 2:** Graphical representation of % enzyme activity for 250μg/ml concentration of methanolic extract.

**Table 2:** % enzyme activity of methanolic extract*.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Standard % of enzyme inhibition</th>
<th>Test % of enzyme inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetylcholinesterase</td>
<td>100</td>
<td>43±3</td>
</tr>
<tr>
<td>phospholipase A2</td>
<td>100</td>
<td>57±2</td>
</tr>
<tr>
<td>hyaluronidase</td>
<td>100</td>
<td>32±6</td>
</tr>
<tr>
<td>L-amino acid oxidase</td>
<td>100</td>
<td>28±7</td>
</tr>
<tr>
<td>phosphomonoesterase</td>
<td>100</td>
<td>54±4</td>
</tr>
<tr>
<td>phosphodiesterase</td>
<td>100</td>
<td>54±3</td>
</tr>
</tbody>
</table>

*Data analyzed by one way ANOVA followed by Dunnett’s ‘t’ test, (n = 3), **P<0.05 significant from control.
Fig 3: 5-Hydroxy 7,8,2',3'-tetramethoxyflacone.

Fig 4: 14-deoxy-11-dehydroandrographolide.

Fig 5: Paniculide-b.

Fig 6: Andrographolide.
In the *Naja naja* PLA2 structure the HIS47 is found interacting with all of the selected phytochemicals mostly it forms a hydrogen bond with the hydroxyl (-OH) groups of ligand molecules. Amino acid PHE100 the main-chain residue also found in the bound conformation with most of ligands. TYR 63 forms hydrogen bonds with oxygen (=O) molecules of ligands. If the charged group of Arg30, as observed in the cubic *Naja naja* structure, moves away from Asp48, which would occur upon binding with ligand molecules, a packet would be created that would have good chemical and shape complementarities to the head group of a PLA2 molecule.

**Table 3: Free energies of binding and In-silico predictions of phytochemicals with PLA 2.**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>BE (Kcal/mol)</th>
<th>LE</th>
<th>Ki (Inh. Const)</th>
<th>Ref. RMSD</th>
<th>H-bond</th>
</tr>
</thead>
<tbody>
<tr>
<td>5h</td>
<td>-8.19</td>
<td>-0.32</td>
<td>999.59 nM</td>
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<td>2</td>
</tr>
<tr>
<td>14de</td>
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<td>-0.34</td>
<td>995.53 nM</td>
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<tr>
<td>Andg</td>
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<td>2.1 uM</td>
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<tr>
<td>P a</td>
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<td>28.32 uM</td>
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<tr>
<td>P b</td>
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<td>1.04 uM</td>
<td>1.362</td>
<td>3</td>
</tr>
<tr>
<td>P c</td>
<td>-7.35</td>
<td>-0.35</td>
<td>4.12 uM</td>
<td>1.63</td>
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5h= 5-Hydroxy 7,8,2’,3’-tetramethoxyflacone, 14de=14-deoxy-11-dihydroandrographolide Andg= andrographolide, Pa=Paniculide a, Pb=Paniculide b, Pc= paniculide c.

**Table 4: Active site Amino acid residues in interaction with the phytochemicals.**

<table>
<thead>
<tr>
<th>AAs</th>
<th>P.I</th>
<th>5h</th>
<th>14d</th>
<th>Andg</th>
<th>P a</th>
<th>P b</th>
<th>P c</th>
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<td>PHE100</td>
<td>-</td>
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</tr>
</tbody>
</table>
5h= 5-Hydroxy 7,8,2’,3’-tetramethoxyflacone, 14de=14-deoxy-11-dydroandrographolide Andg= andrographolide, Pa=Paniculide a, Pb=Paniculide b, Pc= paniculide c.

The pocket could accommodate the charged group of PLA2 by making favorable electrostatic contact with aspartate 48, the carbonyl oxygen atoms of residues 21 and 28 and the peptide-bond nitrogen of residue 30.

5. CONCLUSION
The in vitro enzymatic analysis reveals that the methanolic leaf extract of the plant could inhibit most of the toxic enzymes of the Naja naja. The selected phytoconstituents of Andrographis paniculata leaves were having good enzyme inhibition activities In-silico against PLA2. This could be further studied for its anti-venom potential by pharmacological and in vivo studies.

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REFERENCES


