Original Article

Screening and Identification of Plant Metabolites against Snake Venom Enzymes using *in vitro* to *in silico* Approach

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ABSTRACT

Aim/Background: Snakebites are generally a neglected tropical disease that have harmful impact on thousands of people globally. Although antiserum therapy is the only management available for snakebites, several side effects of this therapy have raised the requirement of an alternative to treat snakebite or boost antiserum efficacy. The present study aimed to provide a scientific explanation for the use of plants extracts in neutralizing snake venom enzymes phospholipase A_2, hyaluronidase, DNase and RNase. **Materials and Methods:** Twelve plant species were selected, which were traditionally used in tribal region of Madhya Pradesh to manage snakebites. Ethanolic and aqueous extracts of these plants were tested for *in vitro* enzyme neutralization. Further chemical investigation of *Dryopteris cochleata* rhizome was carried out followed by Molecular docking studies using Glide software tools. **Results:** *D. cochleata* extract showed the highest enzyme-neutralizing activity when compared to other plants extracts and exhibited significant antioxidant activity. Molecular docking studies of GC-MS identified that constituent 12-phenyl-2,3,7,8-tetramethoxy-5H-(1)-benzopyrano[4,3-c]isoquinoline had a Glide score of −7.455 and formed hydrogen bonds with ASP 49 amino acid residue of phospholipase A_2 molecule. **Conclusion:** The study concludes that extract of *D. cochleata* rhizomes inhibit activity of toxic enzymes of snake venom.

Key words: Phospholipase A_2, Hyaluronidase, Deoxyribonuclease, Ribonuclease, *Dryopteris cochleata*.

Key Messages: Medicinal plants have been traditionally used for the management of snakebites. 12-phenyl-2,3,7,8-tetramethoxy-5H-(1)-benzopyrano[4,3-c]isoquinoline was identified in *Dryopteris cochleata* and exhibited significant phospholipase A_2 inhibitory activity.

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INTRODUCTION

Snakebite results in significant morbidity and mortality especially in South Asia. The World Health Organization recognized snakebite as a common acute medical emergency in most rural areas of tropical and subtropical countries like India, Bangladesh, Nepal, etc. Recent findings showed that more than 420,000-1,800,000 envenomation and 94,000 deaths occur globally per annum.[1] In India, approximately 35,000-50,000 deaths every year through snakebite.[2] Overall, 0.47% of total deaths has been assigned to snakebites. Snakebite deaths were more frequent in rural areas (97%), were more common in men (59%) than women (41%) and peaked at 15-29 years of age (25%) and during June-September.[3] Major snake families in the Indian subcontinent are Viperidae (Russell's viper, pit viper and saw scaled viper), Elapidae (common cobra, king cobra and krait) and Hydrophidae (the sea snakes).[4] The majority of poisonous snake species in India widely responsible for bites and consequent mortality comprise "The big 4" medically signif-

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icant snake species *Naja naja*, *Bungarus caeruleus*, *Daboia russelii* and *Echis carinatus*.

Almost 90% of the dry weight of snake venom includes typically more than 100 proteins, non-enzymatic polypeptide toxins, polypeptides, enzymes and other poisonous/ fatal substances.[5] The snake venom enzymes include digestive hydrolases, hyaluronidase (spreading factor), yellow L-amino acid oxidases, phospholipases A_2 and peptidases. Snake venom metalloproteases damage the basement membranes, causing endothelial cell damage and spontaneous systemic bleeding. Procoagulant enzymes are thrombin-like, splitting fibrinogen, or activators of factors V, X, prothrombin and other clotting factors, thereby causing consumption coagulopathy and incoagulable blood.[6] DNase and RNase are also present in snake venom and have very high deoxyribonucleolytic and ribonucleolytic activity that may reflect a sure role of these enzymes in the toxic effects of snake venom.[7,8] Phospholipases A_2 damage the mitochondria, red blood cells, leucocytes, platelets, peripheral nerve endings, skeletal muscle, vascular endothelium and other membranes, producing presynaptic neurotoxic activity, cardiotoxicity, myotoxicity, necrosis, hypotension, hemolysis, anticoagulation, hemorrhage, plasma leakage (edema formation) and auto pharmacological release of histamine and other autacoids.[9]

A traditional medicine is widely used in the home of tribals and villagers of Madhya Pradesh (Vindhyanchal, Aravali Ranges, Amarkantak, Pachmarhi and Patalkot areas), which includes many plant constituents that act against various effects induced by snakebite.[10] The toxicity caused by proteins and enzymes present in snake venom can be neutralized by the natural inhibitors present in those plants.[11,12] The natural components like phenolic compounds, alkaloids, acids, proteins, etc., can be utilized to create alternative treatments and therapies for snake envenomation.[13] Therefore, the continuous search and identification of new compounds that may be useful as alternative or complementary therapies for snakebite envenomation is a relevant task.

MATERIALS AND METHOD

On the basis of a survey, 12 medicinal plant parts were collected from the tribal region of Madhya Pradesh including *Dryopteris cochleata* (Jatashankari) rhizome, *Radermachera xylocarpa* (Garudphal) fruit, *Aegle marmelos* (Bael) leaf, *Sansevieria trifasciata* (Naagphan) leaf, *Clitoria ternatea* (Aparajita) leaf, *Annona squamosal* (Sitaphal) leaf, *Moringa oleifera* (Sojana) leaf, *Enhydra fluctuans* (Helencha) leaf, *Calotropis gigantea* (Aakundo) tuber, *Ervatamia coronaria* (Safedphool) flower, *Zingiber capitatum* (Jangli Adrak) rhizome and *Curculigo orchioides* (Kaali Moosli) tuber.

Phospholipase A_2, hyaluronidase, DNase, RNase, hyaluronic acid, cetyltrimethyl ammonium bromide and lecithin were purchased from Sigma Aldrich Laboratories and Himedia Laboratories (India). All other reagents were of analytical grade.

Extraction

All 12 plant materials were thoroughly washed in order to remove dust, air dried under shade and ground into coarse powder for further extraction by 2 solvents (ethanol and water).

Aqueous extraction was carried out by the standard method using deionized water as the solvent;[14] 100 g of coarse powdered sample of each plant material was extracted by soaking in 250 mL of deionized water in a beaker with continuous shaking for 48 h. Thereafter, the aqueous solution was filtered using filter paper and the extracts were evaporated by rotary vacuum evaporator.

Ethanol extraction was carried out by Soxhlet method.[15] Coarse powder of all plant material was refluxed with ethanol for 48 hr in a Soxhlet apparatus. The ethanolic extract was concentrated in rotary vacuum evaporator, kept in a dry place and expressed in terms of dry weight.

Preliminary phytochemical analysis of extracts

Preliminary phytochemical analysis of each plant extract was performed to identify the presence and absence of plant constituents such as terpenoids, phenols, alkaloids, flavonoids, saponins, carbohydrates, proteins and other metabolites.[16]

Antioxidant assays

ABTS radical scavenging activity

The radical scavenging activity of all plant extracts was measured by 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).[17] Ascorbic acid was used as reference and the following formula was applied to determine the percentage inhibition of ABTS radical scavenging activity:

% inhibition = [(control absorbance – sample absorbance)/ (control absorbance)] × 100

DPPH radical scavenging activity

The free-radical scavenging activity of all plant extracts was measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH).[18] Ascorbic acid was used as reference and the following formula was applied to determine the percentage inhibition of DPPH radical scavenging activity:

% inhibition = [(control absorbance – sample absorbance)/ (control absorbance)] × 100

Cupric-reducing antioxidant activity

The cupric-reducing antioxidant activity was based on the conversion of cupric ions (Cu^{3+}) to cuprous (Cu^{2+}) in the presence of extracts.[19] Ascorbic acid was used as reference. Absorbance was measured at 450 nm against the blank. The increase in absorbance was proportional to cupric-reducing antioxidant activity.

Estimation of total flavonoid content

The total flavonoid content of all plant extracts was estimated by colorimetric method;[20] the content was calculated against rutin, a calibration standard curve. The total flavonoid content was expressed as microgram of rutin equivalents per milligram of extract.

Estimation of total phenolic content

Total phenolic content of all plant extracts was determined by spectrophotometric method.[19] Concisely, total phenolic content of the extracts was measured against gallic acid calibration standard curve. The total phenolic content was expressed as microgram of gallic acid equivalents per milligram of extracts.

In vitro enzyme neutralization

Phospholipase A_2 neutralization

Phospholipase A_2 activity was observed by an indirect hemolytic assay on erythrocyte egg yolk agar gel plate.[21] Briefly, the minimum indirect hemolytic dose of phospholipase A_2 was determined, which corresponded to a hemolytic halo of 11 mm in diameter; 10 µL of phospholipase A_2 from 0.001 mg/mL to 1.0 mg/mL was added to 3 mm wells in agar gels (1% in PBS; pH = 8.1) containing 1.2% sheep erythrocytes, 1.2% egg yolk as a source of lecithin and 10 mM $CaCl_2$. Plates were incubated at 37°C overnight and a hemolytic halo (diameter in millimeter) was measured. The efficacy of all plant extracts in neutralizing phospholipase A_2 activity was carried out by mixing 20 µL of phospholipase A_2 0.05 mg/mL with 20 µL of 10 mg/mL of the plant extract and incubating for 1 hr at 37°C; 10 µL of treated phospholipase A_2 was added to 3 mm wells in agarose gels. Plates were incubated at 37°C overnight and a hemolytic halo was measured. Control wells contained 10 µL of untreated phospholipase A_2 (0.05 mg/mL). The assay was performed in triplicates.

Hyaluronidase neutralization

Hyaluronidase activity was determined by turbidimetric method.[22] The efficacy of plant extract in neutralizing hyaluronidase activity was assessed by mixing 20 µL of hyaluronidase 0.01 mg/mL with 20 µL of 10 mg/mL of all plant extracts and incubating for 1 hr at 37°C. To measure the hyaluronidase activity, 10 µL of treated hyaluronidase was mixed with 70 µL of 0.2 M acetate buffer and 20 µL of 1 mg/mL hyaluronic acid and incubated for 15 min at 37°C. The reaction was stopped by adding 0.08 M CTAB (100 µL). The OD was measured immediately at 405 nm (EPOCH2 Microplate Reader; BioTek, USA). Turbidity activity was expressed as a percentage of the residual hyaluronic acid, taking the OD of a well to which no hyaluronidase (deionized water as control) was added. The assay was performed in triplicates.

DNase neutralization

Neutralization of DNase activity on DNA was measured by radial diffusion method with slight modifications.[23] A plate was prepared with 40 mL of 1% agarose in assay buffer (10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$; pH = 8.0) containing 0.1 mg/mL calf thymus DNA and 1 µg/mL ethidium bromide. Plates were incubated for 20 h at 37°C and then visualized in ultraviolet transilluminator. For neutralization, equal volume of DNase (0.02 mg/mL) and plant extract (10 mg/mL) were pre-incubated for 1 hr at 37°C. Control wells contained 10 µL of untreated DNase (0.02 mg/mL). The assay was performed in triplicates.

RNase neutralization

Neutralization of RNase was measured by radial diffusion. A plate was prepared with 40 mL of 1% agarose in assay buffer (10 mM Tris-HCl, 0.1 mM NaCl, 1 mM EDTA; pH = 8.0) containing 0.2 mg/mL RNA and 1 µg/mL ethidium bromide. Plates were incubated for 20 h at 37°C and then visualized in ultraviolet transilluminator. For neutralization, equal volume of RNase (0.01 mg/mL) and the plant extract (10 mg/mL) were pre-incubated for 1 hr at 37°C. Control wells contained 10 µL of untreated RNase (0.01 mg/mL). The assay was performed in triplicates.

GC-MS analysis

For the GC-MS analysis, a 30 mL, 0.25 mm, 0.25 µm DB 35–MS Capillary Standard Non–Polar column was used in Trace Ultra Thermo MS DSQ II gas chromatograph. For MS detection, an electron ionization system with ionization energy of 70 eV was used; helium gas was used as the carrier gas at a constant flow rate of 1 mL/min and an injection volume of 1 µL was given in splitless mode. Injector temperature was 250°C; ion-source temperature was 280°C. The oven temperature was programed to 70°C, with an increase of 6°C/min upto 260°C and ending with a 9 min isothermal phase at 280°C. Mass spectra were taken at 70 eV with a scan interval of 0.5 sec. Total GC running time was 37.50 min. The relative percentage amount of each component was calculated by comparing its average peak area

to the total areas. Interpretation of mass spectra of GC-MS was done using the database of National Institute Standard and Technology library search.

Molecular docking studies

The identified constituents of *D. cochleata* were computationally docked with phospholipase A_2 enzyme pockets (PDB Code: 4QER) retrieved from the protein data bank and molecular docking was performed using Glide XP module (Schrodinger, 2016-1 Maestro 10.5.014).[24] The enzyme was prepared with the "protein preparation wizard" workflow. The ligand-binding site was defined using Glide Grid Generation module. LigPrep module application was used for ligands built-up. All other parameters were kept on default settings.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 (GraphPad, San Diego, CA, USA). Results are expressed as mean ± standard deviation (SD) from at least 3 independent experiments. All data were analyzed by 1-way analysis of variance, followed by post hoc Dunnett's test. $P < 0.001$ was considered statistically significant.

RESULTS AND DISCUSSION

Among the plants traditionally used in the tribal region of Madhya Pradesh to treat snakebites, 12 were screened for phospholipase A_2, hyaluronidase, DNase and RNase neutralizing activity. Snake venoms are a complex combination of different enzymes such as phospholipase A_2, metalloproteinase, hyaluronidase, 5'-nucleotidase, DNase and RNase, as well as other nonenzymatic components.[25] Phospholipase A_2 is the most common class of venom enzyme and is frequently present in many snakes. Phospholipase A_2 induces a wide spectrum of toxicity such as hypotension, neurotoxicity, myotoxicity, cytotoxicity and cardiotoxicity.[26]

Preliminary phytochemical analysis

The results of preliminary phytochemical screening of all ethanolic and aqueous plant extracts demonstrated the presence of alkaloids, flavonoids, saponins, phlobatannins, phenolics, tannins, terpenoids, carbohydrates, glycosides, fixed oils and fats. The yield of ethanolic extracts was between 4.23% and 28.91% (Table 1) and that of the aqueous extracts was 2.72% and 13.67% (Table 2).

Antioxidant assays

Snake venoms cause a surge in reactive oxygen species that damages vital organs such as the liver, kidneys and heart and causes oxidative stress that affects the mitochondria and macromolecules like lipids, proteins and DNA.[27,28] Therefore, medicinal plants with antivenom and antioxidant potential would inhibit oxidative stress which is induced by the venoms. The ethanolic extract of *C. orchioides* and aqueous extract of *D. cochleata* had the highest total flavonoid content of 507.17 ± 15.69 µg and 550.11 ± 22.36 µg rutin equivalents per milligram of dry weight of plant extract, respectively (Table 3). Ethanolic and aqueous extracts of *D. cochleata* had the highest total phenolic content of 369.99 ± 28.74 µg and 381.29 ± 15.26 µg gallic acid equivalents per milligram of dry weight of plant extract, respectively (Table 3). The presence of flavonoids and phenolic compounds is directly involved in antioxidant activity.[29]

The ABTS scavenging potential of both ethanolic and aqueous extracts of *D. cochleata* exhibited the highest percentage inhibition of 90.07% ± 0.06% and 89.34% ± 0.19%, respectively. In DPPH radical-scavenging activity, ethanolic and aqueous extracts of *D. cochleata* showed 33.78% ± 2.31% and 31.19% ± 2.29% inhibition, respectively. *D. cochleata* showed higher reducing ability by converting Cu^{3+} to Cu^{2+}.[30] The possible reason of higher antioxidant potential or scavenging ability of *D. cochleata* could be the higher content of phenolics and flavonoids along with the presence of hydroxyl group.[17,31]

Phospholipase A_2–neutralization

Phospholipase A_2 enzyme is commonly present in snake venom and is responsible for the hydrolysis of phospholipids, leading to several toxicological effects such as edema, modulation of platelet aggregation and anticoagulant, neurotoxic and myotoxic effects.[32] In hemolysis, phospholipases A_2 convert lecithin into lysolecithin, which is responsible for the disruption of red cell membranes that leads to the release of hemoglobin into the extracellular medium.[33] Medicinal plant extracts are reported to contain rich source of bioactive constituents that can neutralize phospholipases A_2. The ethanolic and aqueous extracts of the selected plants have shown phospholipase A_2–neutralization effect (Figure 1). Neutralization studies can be performed by incubating the enzyme and plant extract prior to testing (pre-incubation method). The results showed that the ethanolic and aqueous extracts of *D. cochleata* significantly decreased halo diameter from 11 to 1.07 and 5.13 mm, respectively, resulting in significant neutralization of phospholipase A_2 ($P<0.001$). *D. cochleata* extract was capable of inhibiting phospholipase A_2-dependent indirect hemolysis of sheep red blood cells. Inhibition of the indirect hemolysis indicates that the plant extract

Table 1: Preliminary phytochemical analysis and yield of ethanolic plant extracts.

Plant	DC-E	RX-E	AM-E	ST-E	CT-E	AS-E	MO-E	EF-E	CG-E	EC-E	ZC-E	CO-E
Extract yield												
Yield (%)	7.17	4.23	12.26	18.33	11.22	18.65	26.96	7.72	10.1	13.66	28.91	12.78
Alkaloids												
Wagner's test	+	+	+	+	+	+	+	+	+	+	+	+
Hager's test	+	+	-	+	+	+	+	+	+	+	+	+
Mayer's test	+	-	-	+	-	-	-	-	-	-	+	-
Sterols and terpenoids												
Libermann's test	-	+	-	-	+	+	+	-	-	-	+	+
Libermann's Burchard test	+	+	+	-	-	+	+	+	+	+	-	-
Salkowaski's test	+	+	+	+	-	-	-	+	+	+	+	+
Phenolics and tannins												
5% $FeCl_3$ test	+	+	+	+	+	+	+	+	+	-	-	+
10% lead acetate test	+	+	-	+	-	+	+	+	+	+	+	+
Acetic acid test	-	-	-	-	+	-	-	-	-	-	-	-
Flavonoids												
Shinoda test	+	+	+	-	-	+	-	+	-	+	-	-
Dilute NaOH test	-	+	+	-	-	+	+	-	+	+	+	-
Carbohydrates												
Molisch's test	+	-	-	+	-	+	+	+	+	+	+	-
Fehling's test	+	+	+	+	+	-	+	-	-	-	-	+
Glycosides												
Raymond's test	-	+	-	-	-	-	-	-	-	-	+	-
Keller Killiani test	+	+	+	-	+	+	+	+	+	+	+	+
Legal's test	+	-	+	-	-	+	-	+	+	+	+	+
Fixed oils and fats												
Oily spot test	+	+	+	+	+	+	+	+	+	+	+	+
Saponins												
Foam test	+	-	-	+	+	+	+	+	-	-	+	-

Preliminary phytochemical analysis indicated the presence (+) or absence (-) of plant metabolites in the test. DC-E: *D. cochleata* ethanol extract, RX-E: *R. xylocarpa* ethanol extract, AM-E: *A. marmelos* ethanol extract, ST-E: *S. trifasciata* ethanol extract, CT-E: *C. ternatea* ethanol extract, AS-E: *A. squamosal* ethanol extract, MO-E: *M. oleifera* ethanol extract, EF-E: *E. fluctuans* ethanol extract, CG-E: *C. gigantea* ethanol extract, EC-E *E. coronaria* ethanol extract, ZC-E: *Z. capitatum* ethanol extract and CO-E: *C. orchioides* ethanol extract.

Table 2: Preliminary phytochemical analysis and yield of aqueous plant extracts.

Plant	DC-A	RX-A	AM-A	ST-A	CT-A	AS-A	MO-A	EF-A	CG-A	EC-A	ZC-A	CO-A
Extract yield												
Yield (%)	13.29	4.66	13.67	12.85	11.87	2.72	8.62	6.88	10.22	12.91	4.64	8.41
Alkaloids												
Wagner's test	+	+	+	+	+	+	+	+	+	+	+	+
Hager's test	+	+	+	+	+	+	+	+	+	+	-	-
Mayer's test	+	-	+	+	-	-	-	-	-	-	+	+
Sterols and terpenoids												
Libermann's test	-	-	-	-	-	+	+	-	-	-	+	+
Libermann's Burchard test	-	+	-	-	-	+	+	+	-	-	-	+
Salkowaski's test	+	-	+	+	+	-	+	+	+	+	+	+
Phenolics and tannins												
5% $FeCl_3$ test	-	-	+	+	-	-	-	+	-	+	+	+
10% lead acetate test	-	+	+	+	+	+	+	+	+	+	+	-
Acetic acid test	+	-	-	-	-	-	-	-	-	-	+	+
Flavonoids												
Shinoda test	-	-	-	+	-	-	-	-	+	-	+	-
Dilute NaOH test	+	+	-	-	-	-	-	-	+	+	+	-
Carbohydrates												
Molisch's test	+	+	-	-	-	-	+	+	-	-	+	+
Fehling's test	+	-	+	+	-	+	+	-	-	+	+	+
Glycosides												
Raymond's test	-	-	-	-	-	-	-	-	+	-	-	-
Keller Killiani test	+	_	+	-	-	+	+	+	-	-	+	+
Legal's test	+	-	-	-	-	+	+	+	-	-	-	+
Fixed oils and fats												
Oily spot test	+	+	+	+	+	+	-	+	+	+	+	+
Saponins												
Foam test	+	-	+	+	+	+	-	-	+	-	-	+

Preliminary phytochemical analysis indicated the presence (+) or absence (-) of plant metabolites in the test. DC-A: *D. cochleata* aqueous extract, RX-A: *R. xylocarpa* aqueous extract, AM-A: *A. marmelos* aqueous extract, ST-A: *S. trifasciata* aqueous extract, CT-A: *C. ternatea* aqueous extract, AS-A: *A. squamosal* aqueous extract, MO-A: *M. oleifera* aqueous extract, EF-A: *E. fluctuans* aqueous extract, CG-A: *C. gigantea* aqueous extract, EC-A: *E. coronaria* aqueous extract, ZC-A: *Z. capitatum* aqueous extract and CO-A: *C. orchioides* aqueous extract.

contains some compounds that might be inhibiting the enzymatic activity of phospholipase A_2. Block charts show the level of phospholipase A_2-neutralization activity by other ethanolic and aqueous plant extracts (Figure 2A and B).

Hyaluronidase-neutralization

Hyaluronan is a nonsulfated glycosaminoglycan, which holds large amounts of water and metal ions conferring viscoelasticity and integrity of the extracellular matrix. Degradation of hyaluronan by venom hyaluronidase has been considered as a 'spreading factor'. The venom spreading to the systematic circulation results in structural distortion of extracellular matrix due to hydrolysis of hyaluronan.[34,35] Ethanolic and aqueous plant extracts showed detectable hyaluronidase neutralization with hyaluronic acid as substrate. The block chart showed that 8 extracts exhibited more than 90% neutralization. Ethanolic and aqueous extracts of *D. cochleata, A. marmelos* and *C. ternatea* significantly neutralized hyaluronidase activity (Figure 2C and D).

DNase-neutralization

Hydrolytic enzymes like DNase, RNase and phosphodiesterase are present in almost all snake venoms. These enzymes are involved in digestion and are nontoxic. However, recently these enzymes have been shown to endogenously liberate purines, which act as multitoxins.[7]

Table 3: Antioxidant activities, total flavonoid content and total phenolic content of selected plant extracts.

Plant	Extract name	ABTS radical (% inhibition)	DPPH radical (% inhibition)	Cupric-reducing power (OD 450 nm)	Total flavonoid content (µg RE/mg extract)	Total phenolic content (µg GAE/mg extract)
D. cochleata	DC-E	90.07 ± 0.06	33.78 ± 2.31	1.61 ± 0.13	474.02 ± 25.86	369.99 ± 28.74
	DC-A	89.34 ± 0.19	31.19 ± 2.29	1.01 ± 0.02	550.11 ± 22.36	381.29 ± 15.26
R. xylocarpa	RX-E	27.15 ± 1.18	16.97 ± 4.04	0.13 ± 0.03	139.51 ± 17.64	42.82 ± 4.64
	RX-A	35.53 ± 2.48	13.41 ± 2.25	0.09 ± 0.01	141.32 ± 6.82	71.74 ± 7.58
A. marmelos	AM-E	29.25 ± 2.15	15.75 ± 5.77	0.33 ± 0.02	382.71 ± 32.78	49.56 ± 2.88
	AM-A	50.00 ± 2.53	14.83 ± 1.45	0.72 ± 0.03	414.77 ± 18.95	302.76 ± 12.84
S. trifasciata	ST-E	23.67 ± 3.06	17.64 ± 2.08	0.29 ± 0.05	187.03 ± 12.69	42.70 ± 6.58
	ST-A	26.19 ± 1.65	2.62 ± 3.21	0.17 ± 0.01	205.59 ± 14.89	78.97 ± 32.94
C. ternatea	CT-E	25.62 ± 2.82	12.23 ± 1.15	0.19 ± 0.03	143.13 ± 6.67	35.71 ± 6.61
	CT-A	34.58 ± 2.90	16.79 ± 2.98	0.09 ± 0.01	187.03 ± 10.27	94.12 ± 16.36
A. squamosa	AS-E	67.53 ± 2.24	25.31 ± 0.58	0.44 ± 0.02	336.51 ± 19.63	133.24 ± 15.12
	AS-A	15.05 ± 0.88	4.14 ± 4.11	0.10 ± 0.02	135.89 ± 5.26	71.50 ± 5.85
M. oleifera	MO-E	34.49 ± 1.47	26.77 ± 6.43	0.19 ± 0.03	357.71 ± 34.70	61.98 ± 11.35
	MO-A	22.78 ± 1.98	7.74 ± 0.88	0.18 ± 0.01	289.22 ± 34.76	136.71 ± 14.67
E. fluctuans	EF-E	13.67 ± 1.02	12.18 ± 2.89	0.14 ± 0.03	120.95 ± 25.66	29.20 ± 15.45
	EF-A	87.69 ± 0.29	26.06 ± 2.02	0.06 ± 0.00	173.91 ± 17.85	65.95 ± 9.48
C. gigantea	CG-E	7.90 ± 0.62	13.21 ± 5.77	0.23 ± 0.03	133.17 ± 3.63	17.75 ± 9.65
	CG-A	26.15 ± 1.88	12.21 ± 4.58	0.06 ± 0.01	126.83 ± 13. 38	79.81 ± 14.78
E. coronaria	EC-E	24.91 ± 2.14	5.50 ± 2.08	0.11 ± 0.01	111.90 ± 27.63	47.76 ± 5.84
	EC-A	49.08 ± 3.41	21.16 ± 7.09	0.01 ± 0.01	124.57 ± 26.12	65.95 ± 2.45
Z. capitatum	ZC-E	20.30 ± 1.74	11.38 ± 3.46	0.38 ± 0.12	164.85 ± 5.64	38.84 ± 14.39
	ZC-A	21.43 ± 1.65	2.94 ± 1.21	0.15 ± 0.04	149.46 ± 1.29	83.67 ± 9.41
C. orchioides	CO-E	37.79 ± 2.11	23.28 ± 5.20	1.05 ± 0.05	507.17 ± 15.69	67.88 ± 18.73
	CO-A	39.16 ± 1.43	17.12 ± 8.12	0.09 ± 0.01	271.83 ± 14.52	73.55 ± 8.39

Ascorbic acid was used as standard in ABTS radical, DPPH radical and cupric-reducing power analyses. OD = optical density; GAE = gallic acid equivalents; RE = rutin equivalents. Results are mean ± SD of triplicate assays.

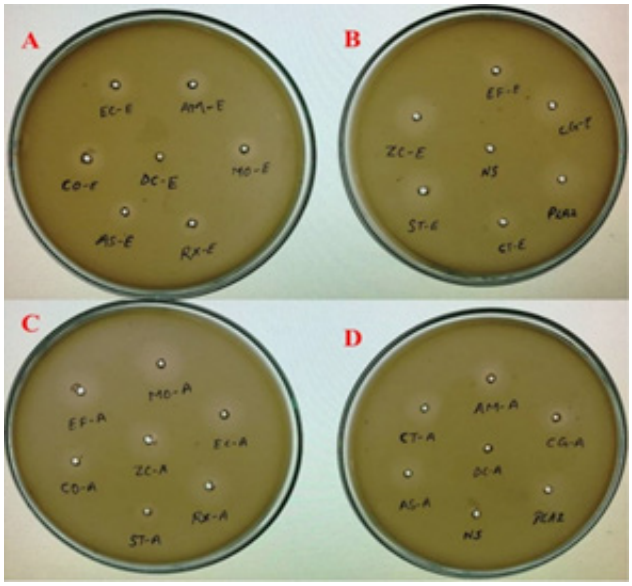

Figure 1: Plant extracts showing phospholipase A_2-neutralization activity by ethanolic (A and B) and aqueous (C and D) extracts.

DNase-neutralization activity is determined by radial diffusion method in agarose gels containing DNA and is expressed as zone of DNA lysis in diameter. Ethanolic extracts of *D. cochleata, E. fluctuans, C. gigantean* and *C. orchioides* showed more than 99% DNase-neutralization activity, with 0.09, 0.12, 0.1 and 0.55 mm diameters, respectively. Among aqueous extracts, *D. cochleata* showed maximum activity (Figure 2E and F).

RNase-neutralization

Similar to DNase, a specific RNase present in *Naja oxiana* and *N. naja* venom hydrolyzes the RNA.[8,36] RNase-neutralization activity was also determined by radial diffusion in agarose gels containing RNA and expressed as zone of RNA lysis in diameter. Ethanolic and aqueous extracts of *D. cochleata* showed significant RNase-neutralization activity among all extracts, with 0.23 and 0.42 mm diameters, respectively (Figure 2G and H).

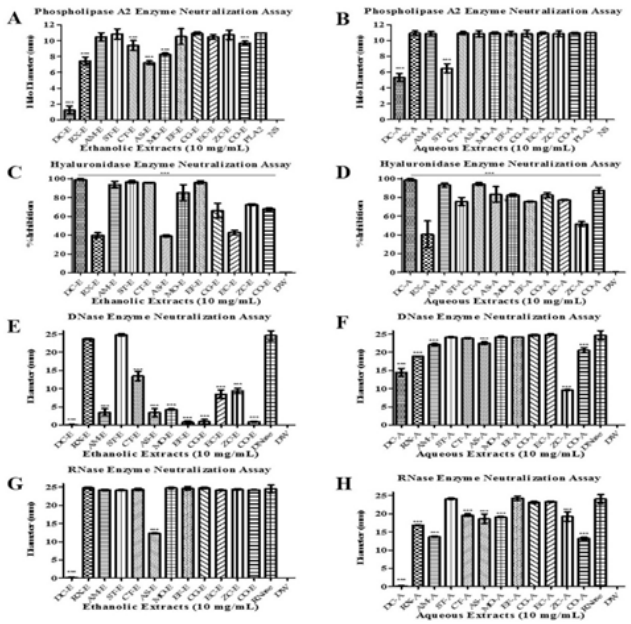

Figure 2: *In vitro* enzyme-neutralization assays were conducted for (A and B) phospholipase A_2 by minimum indirect hemolytic dose of ethanolic and aqueous plant extracts (equal volume of phospholipase A_2 used as a control); (C and D) hyaluronidase by neutralization assay of ethanolic and aqueous plant extracts (equal volume of deionized water used as control); (E and F) DNase by radial diffusion of ethanolic and aqueous plant extracts (equal volume of DNase used as control); and (G and H) RNase by radial diffusion of ethanolic and aqueous plant extracts (equal volume of RNase used as control). Data represent 3 independent experiments and are presented as the mean SD (n=3). ***P<0.001 is significant compared with control. Therefore, the results that are not significant are close to the control values.

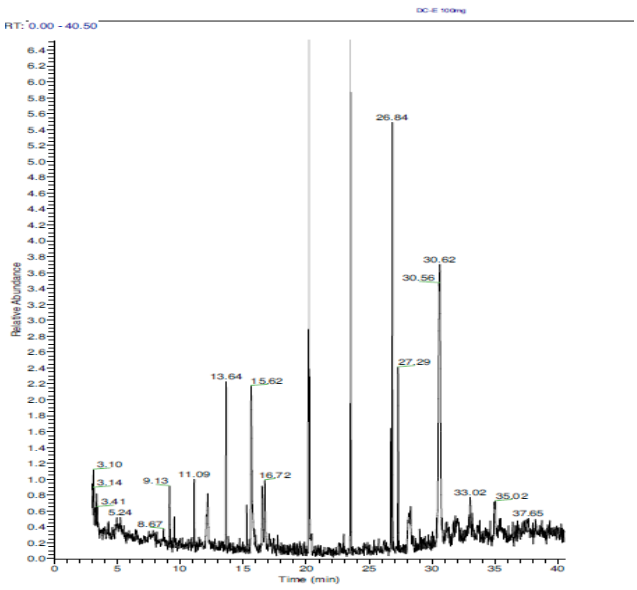

Figure 3: Gas chromatography-mass spectrophotometer chromatogram of *D. cochleata* ethanolic extract.

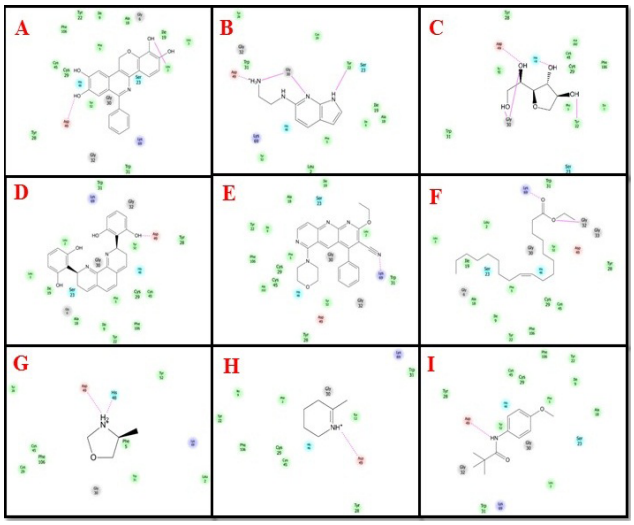

Figure 4: Interaction pose of *D. cochleata* constituents and phospholipase A_2.

Our results showed that plants might have a direct effect on a venom enzyme. Some plants used traditionally for snake bite management shows neutralization effects on minimum one of the venom enzymes. However, *D. cochleata* ethanolic extract showed maximum *in vitro* enzyme-neutralization activity, we selected this plant extract for further analysis to find the phytoconstituents that are responsible for inhibiting the venom enzyme.

GC-MS analysis

D. cochleata ethanolic extract was subjected to GC-MS for the determination of bioactive phytoconstituents. Helium gas was used as carrier and the oven temperature was set initially at 70°C and raised to 260°C at a rate of 6°C/min. One microliter sample was injected to column with splitless mode. Mass spectra of constituents were recorded in the range of 50-650 m/z. The overall run time for extract was 37.50 min. The constituents from the ethanolic extract of *D. cochleata* were identified by relating the retention times of peaks using detector from National Institute Standard and Technology. In total, 30 bioactive phytoconstituents of the ethanolic extract were identified (Table 4) and GC-MS chromatogram with the peak area is shown in Figure 3.

Molecular docking

Phospholipase A_2 is one of the main and highly toxic enzymes found in the snake venom. Several papers have reported that phospholipase A_2 interacts with natural compounds. In the current screening, binding affinities of *D. cochleata* ethanolic extract constituents to phospholipase A_2 were studied using *in silico* molecular docking techniques.

The crystal structure of phospholipase A_2 (PDB ID: 4QER) was prepared for docking with Protein Preparation Wizard workflow of Maestro. All the ligands were

Table 4: Bioactive phytoconstituents of *D. cochleata* ethanolic extract.

RT	Compound name	Probability	Molecular formula	Molecular weight	Area, %
3.12	(E)-4-(1-ethoxyethoxy)-1-(trimethylsilyl)but-1-ene	23.47	$C_{11}H_{24}O_2Si$	216	0.08
3.37	1,4-anhydro-d-galactitol	41.19	$C_6H_{12}O_5$	164	0.24
4.96	4,6-dimethyl-3-octanone	46.95	$C_{10}H_{20}O$	156	0.55
9.16	Ethyl N,N-dimethylcarbamate	63.07	$C_5H_{11}NO_2$	117	0.45
9.53	4-methyloxazolidine	29.95	C_4H_9NO	87	0.21
11.09	1-hexadecanol	8.79	$C_{16}H_{34}O$	242	0.30
12.15	Diisopropylfluoroamine	52.85	$C_8H_{18}FN$	147	0.95
13.62	2-tert-butyl-4-trifluoromethyl-1-methylimidazole	18.54	$C_9H_{13}F_3N_2$	206	1.02
15.27	1-dodecanol	27.46	$C_{12}H_{26}O$	186	0.19
15.64	(2R,5S)-2-tert-butyl-5-methyl-2-phenyl-1,3-dioxolane-4-one	46.10	$C_{14}H_{18}O_3$	234	2.32
16.72	Rebemide	21.12	$C_{11}H_{15}NO$	177	1.79
17.06	N-(1H-pyrrolo[2,3-b]pyridine-6-yl) ethylenediamine	81.14	$C_9H_{12}N_4$	176	0.24
17.71	2-propenoic acid, methyl ester (CAS)	6.85	$C_4H_6O_2$	86	0.12
20.20	5-(hydroxymethyl)-2-(1-methyl-2-imidazolyl)-1H-benzimidazole	77.57	$C_{12}H_{12}N_4O$	228	70.40
23.51	Hexadecanoic acid, ethyl ester (CAS)	63.20	$C_{18}H_{36}O_2$	284	5.09
26.84	9-octadecenoic acid (Z)-, ethyl ester (CAS)	22.15	$C_{20}H_{38}O_2$	310	3.21
27.29	Octadecanoic acid, 2-methyl-, methyl ester (CAS)	59.09	$C_{20}H_{40}O_2$	312	1.43
28.19	12-phenyl-2,3,7,8-tetramethoxy-5H-(1)-benzopyrano[4,3-c]isoquinoline	40.66	$C_{26}H_{23}NO_5$	429	1.08
30.58	γ-sitosterol	36.71	$C_{29}H_{50}O$	414	6.32
31.19	3-cyano-2-ethoxy-4-phenyl-6-morpholino-1,7,10-antyridine	55.87	$C_{24}H_{21}N_5O_2$	411	0.39
31.98	2,9-bis(2',6'-dihydroxyphenyl)-1,10-phenanthroline	36.28	$C_{24}H_{16}N_2O_4$	396	0.79
33.02	N-(4',6'-dimethoxy-2',3'-diphenylindol-7'-ylmethylene)methylamine N-oxide	38.97	$C_{24}H_{22}N_2O_3$	386	0.73
33.74	Retronecine	42.44	$C_8H_{13}NO_2$	156	0.32
34.96	1H-purin-6-amine, [(2-fluorophenyl)methyl]-	26.48	$C_{12}H_{10}FN_5$	243	0.68
35.39	8-methyl-1á-trimethylsilyloxy-trans,trans-cyclodeca-3,7-diene	20.45	$C_{14}H_{26}OSi$	238	0.26
36.39	2-(3,4-dichloroà-hydroxybenzyl)-4-(3,4-dimethoxybenzyl) imidazole	10.84	$C_{19}H_{18}C_{l2}N_2O_3$	392	0.13
36.87	Propanamide, N-(4-methoxyphenyl)-2,2-dimethyl	9.80	$C_{12}H_{17}NO_2$	207	0.17
37.61	1,3-bis(4-chlorobenzyl)-5,6-dihydrobenzo[f] quinazoline	82.38	$C_{26}H_{20}C_{l2}N_2$	430	0.18
38.20	2,3,4,5-tetrahydro-6-methylpyridine	3.70	$C_6H_{11}N$	97	0.12
39.20	2-(2-methoxyphenyl)-1-[1-(2-cyclohexylethyl) indol-3-yl] ethanone	7.83	$C_{25}H_{29}NO_2$	375	0.25

Table 5: Interaction details of *D. cochleata* constituents and phospholipase with higher binding energy.

Compound name	Glide score	Hydrogen bonding	Hydrophobic interaction residue
12-phenyl-2,3,7,8-tetramethoxy-5H-(1)-benzopyrano[4,3-c]isoquinoline	–7.455	ASP 49	LEU 2
N-(1H-pyrrolo[2,3-b]pyridine-6-yl)ethylenediamine	–7.315	ASP 49	GLY 30 TYR 22
1,4-anhydro-d-galactitol	–6.681	ASP 49 HIS 48	GLY 30 TYR 22
2,9-bis(2',6'-dihydroxyphenyl)-1,10-phenanthroline	–6.515	ASP 49	-
3-cyano-2-ethoxy-4-phenyl-6-morpholino-1,7,10-antyridine	–4.330	LYS 69	-
9-octadecenoic acid (Z)-, ethyl ester	–3.332	LYS 69	GLY 32
4-methyloxazolidine	–3.177	ASP 49 HIS 48	-
2,3,4,5-tetrahydro-6-methylpyridine	–3.156	ASP 49	-
Propanamide, N-(4-methoxyphenyl)-2,2-dimethyl-	–3.119	ASP 49	-

prepared with the LigPrep tool. Finally, ligand docking was done using receptor grid file and LigPrep out file in the Glide tool of Application view. The docking simulations were performed using the Glide-XP protocol in the Schrödinger software suite (Schrödinger, LLC, USA, 2016-1 Maestro 10.5.014). The docking results showed that among the 30 bioactive phytoconstituents of *D. cochleata* ethanolic extract, 9 had glide score (Table 5) and dock pose (Figure 4). The highest glide score -7.455 was seen for 12-phenyl-2,3,7,8-tetramethoxy-5H-(1)-benzopyrano[4,3-c]isoquinoline, forming hydrogen bonds with ASP 49 and having hydrophobic interaction with LEU 2 of phospholipase A_2 molecule. HIS 48, ASP 49 and LYS 69 are active site residues of phospholipase A_2 and any hydrogen bonds can possibly neutralize its effect.[37]

CONCLUSION

Among the 12 plant species used to manage snakebites in the tribal region of Madhya Pradesh, the ethanolic extract of *D. cochleata* rhizome showed phospholipase A_2, hyaluronidase, DNase and RNase neutralization activities. Subsequently, GC-MS chromatogram revealed the presence of a majority of bioactive phytoconstituents in *D. cochleata* ethanolic extract. The molecular docking interaction of *D. cochleata* bioactive phytoconstituents and phospholipase A_2 with higher binding energy also disclosed 9 constituents that showed well-defined interaction with phospholipase A_2 amino acid residue, leading to inhibitory effect. Our screening and evaluation established that isoquinoline, an alkaloid present in *D. cochleata,* has inhibitory property against snake venom.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

DC-E: *D. cochleata* ethanol extract; **RX-E:** *R. xylocarpa* ethanol extract; **AM-E:** *A. marmelos* ethanol extract; **ST-E:** *S. trifasciata* ethanol extract; **CT-E:** *C. ternatea* ethanol extract; **AS-E:** *A. squamosal* ethanol extract; **MO-E:** *M. oleifera* ethanol extract; **EF-E:** *E. fluctuans* ethanol extract; **CG-E:** *C. gigantea* ethanol extract; **EC-E:** *E. coronaria* ethanol extract; **ZC-E:** *Z. capitatum* ethanol extract; **CO-E:** *C. orchioides* ethanol extract.

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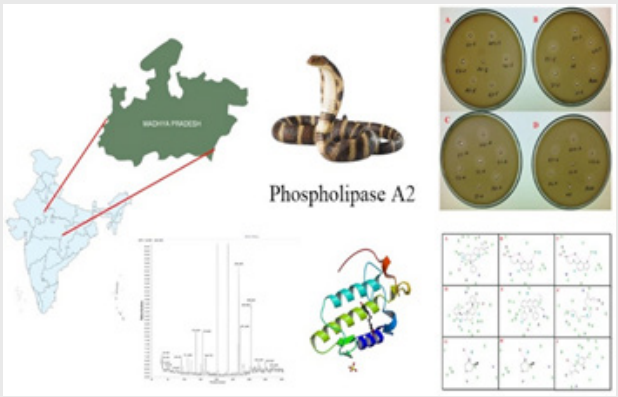
PICTORIAL ABSTRACT



SUMMARY

Nowadays, in rural and tribal areas, individuals are depending on native herbal medicine systems for their major health care including snakebites. Medicinal plants signify source of bioactive pharmacological compounds able to benefit directly in the snakebites treatment or indirectly, use as supplements with antivenom immunotherapy (ASV). In this study screening of medicinal plants for snake venom enzyme neutralization was carried out. Among the selected plant species, the ethanolic extract of *D. cochleata* rhizome showed phospholipase A_2, hyaluronidase, DNase and RNase neutralization activities. An alkaloid, isoquinoline present in *D. cochleata,* has been identified against snake venom.

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