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In vitro anti snake venom potential of *Abutilon indicum* Linn leaf extracts against *Echis carinatus* (Indian saw scaled viper)

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ABSTRACT
Envenomings by snake bite involves medical emergencies and its clinical management is by the administration of antivenom. The antivenom/antisera induce early or late adverse reactions. In this regard, the plant kingdom is explored to minimize the side effects. The present study evaluates the *in vitro* anti venom potential of *Abutilon indicum* leaves extract against *Echis carinatus* (Saw Scaled Viper) venom**.** The leaf extracts of *A. indicum* were used to evaluate the enzyme inhibiting activity of protease, phosphomonoesterase, phosphodiesterase, acetylcholinesterase, phospholipase A_2, hyaluronidase and L-amino acid oxidase toxic enzymes present in snake venom. The methanolic extract inhibited the activity of all enzymes present in the venom. The extract showed promising results in inhibiting enzymes and would be further confirmed by *in vivo* and pharmacological studies.

Keywords: *Abutilon indicum*, *Echis carinatus*, phosphomonoesterase, acetylcholinesterase, phospholipase A_2,

1. Introduction

Snake bite is an occupational hazard in tropical and sub-tropical countries like India [1]. It is estimated that 2.5 million people envenomed each year on a global basis [2-3] and approximately 35,000- 50,000 deaths were reported in India per annum [4-5]. An accurate measure of snake bite envenoming remains elusive as victims approach traditional healers for aid and treatment. Higher death rates in India are due to the four poisonous snakes namely Indian cobra, Saw scaled viper, Russell's viper and common Krait [6].

Antivenom immunotherapy is the specific treatment available against snake bite and was developed by Albert Calmette in 1895 against the Indian Cobra [7]. It is associated with various side effects like pyrogen reactions [8]; serum sickness along with that supply of antivenom has logistical, marketing, storage and economic difficulties [9]. Hence it is important to derive an alternative treatment that involves the usage of different venom inhibitor, synthetic or natural, that could be a substitute for the action of antivenins [10]. Over the years attempts have been made to develop snake venom antagonists especially from medicinal plants. In this regard, several plants are scientifically studied, *Andrographis paniculata* and *Aristolochia indica* plant extracts inhibits snake venom and could be used for therapeutic purposes in case of snakebite envenomations [11]. The aqueous extract of *Mucuna pruriens* has inhibited the activity of Cobra and Krait venoms [12].

Abutilon indicum (Linn), a medicinal plant of Malvaceae family is used for various properties such as demulcent diuretics, anti-diabetic, anthelmintics, astringent, laxative, expectorant, antibacterial, antifungal activities [13-14] and for snakebite [15]. However, the efficacy of the plant as an inhibitor of snake venom was not scientifically evaluated. In the present study the leaf extracts of *A. indicum* (Linn) were evaluated for *in vitro* inhibitory potential against toxic venom enzymes of *Echis carinatus*.

1.2 Materials and Methods

1.2.1 Chemical and reagent

Venom: The lyophilized venom of *Echis carinatus* (Saw scaled viper) 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 ºC. The protein concentration

was estimated by Lowry *et al*., method [16].
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.

1.2.2 Preparation of extract
A. Indicum Linn leaves were collected in January 2014 from Chikanahalli village, Shira taluk, Tumkur district, Karnataka, India. The plant was authenticated at National Ayurveda Dietetics Research Institute, Bangalore, Karnataka, India (RRCBI/5485). The leaves were washed, shade dried, powdered and stored in airtight container for future use. 30 g of powdered leaves were extracted in a soxhlet extractor using hexane and methanol solvents [13, 17]. Extracts were concentrated by rotary vacuum evaporator and the residue obtained was dried, weighed.

1.2.3 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 coloured 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 colour 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 extract was mixed with 2 mL of chloroform. Then 2 mL of concentrated sulphuric acid (H_2SO_4) was added carefully and shaken gently. A reddish brown colour indicated the presence of steroidal ring,

d. Test for proteins (Ninhydrin test): Crude extract when boiled with 2 mL of 0.2% solution of Ninhydrin, violet colour 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 H_2SO_4 was added and heated for about 2 minutes. A grayish colour 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 colour, which becomes colourless 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 H_2SO_4 was added sidewise. A red colour 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 H_2SO_4 and acetic acid were poured into the mixture. The development of a greenish coloration indicated the presence of steroids [18-20].

1.2.4 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 was chloroform and methanol (9.2:0.8).

1.2.5 Snake Venom Toxic Enzymatic Inhibition Studies
1.2.5.1 Protease
Protease assay of crude venom was carried out by the method of Greenberg (1955). The reaction mixture composed 0.5% Casein, 1.0 mL Tris- HCl buffer (pH 8.0), 0.5 mL 0.25% of crude venom was added and the reaction mixture incubated for 4 hr at 37 ^{0}C. At the end of 4 hr the reaction was terminated by adding trichloroacetic acid and then filtered. 1.0 mL filtrate was used for protein estimation by the method of (Lowry *et.al.,* 1951) using L-tyrosine as a standard [21]. Inhibition study was carried out by pre incubating venom with plant extract for 45 min.

1.2.5.2 Phosphomonoesterase
Method of Bessey *et al.,* (1946) was employed to determine phosphomonoesterase activity with slight modifications. To a mixture 1.0mL Tris –HCl buffer (pH 8.0), 1.0 mL disodium - p - nitrophenol 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 [22]. Inhibition study was carried out by pre incubating venom with plant extract for 45 min.

1.2.5.3 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-nitrophenyl phosphate, 0.3 mL 0.01 M $MgSO_4$, 0.5 mL 0.17 M Tris – HCl (pH 8.0). The reaction was monitored by absorbance measurement at 400 nm [23]. Inhibition study was carried out by pre incubating venom with plant extract for 45min.

1.2.5.4 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 mL0.1% 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 [24]. Inhibition study was carried out by pre incubating venom with plant extract for 45 min.

1.2.5.5 Acetylcholinesterase
The activity was determined by the method of Ellman *et al.,* (1961). 50 µL 0.1% crude venom and 3 mL phosphate buffer (pH 8.0) was incubated at room temperature for 5 min and 10 µL DTNB (5,5'-dithiobis-(2-nitrobenzoic acid) and 20 µL substrate acetylthiocholine iodide was added. The increase in absorbance at 412 nm was measured [25]. Inhibition study was carried out by pre incubating venom with plant extract for 45min.

1.2.5.6 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) cetyltrimethylammonium 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) cetyltrimethylammonium bromide in 2% (w/v) NaOH [26]. Inhibition study was carried out by pre incubating venom with plant extract for 45 min.

1.2.5.7 Phospholipase A_2

Phospholipase A_2 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 [27]. Inhibition study was carried out by pre incubating venom with plant extract for 45min.

1.3 Result and Discussion

The leaf extracts were dried using a vacuum evaporator and the residue obtained was weighed (table1).

Table 1: Weight of the extracts

Solvent	Residue (g)
Hexane	3.55
Methanol	4.37

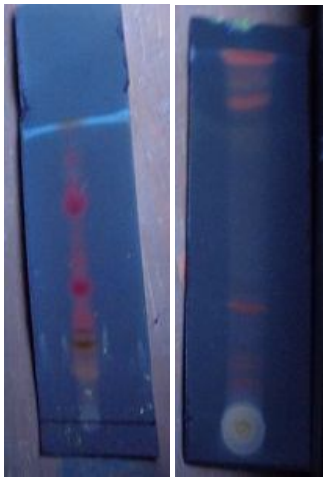
The phytochemical analysis revealed that alkaloids, saponins and proteins are absent in both the extracts, whereas the other metabolite are present in both (table 2).

Table 2: Qualitative phytochemical analysis of the extracts

Test	Hexane	Methanol
Alkaloids	_	_
Phenols	+	+
Steroids	+	+
Glycosides	+	+
Saponins	_	_
Protein	_	_
Flavonoids	+	+
Terpenoids	+	+
Carbohydrates	+	+

+ indicates present, - indicates absent

Earlier studies by Dashputre *et al.*, have reported the presence of metabolites which were not observed in our analysis [28]. The contradiction of results may be due to the habitat, time and season of sample collection.



a **b**

Fig 1: Thin layer chromatography; a-hexane extract, b- methanol extract

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 methanolic extract with chloroform and methanol (9.2:0.8). The plates were visualized under ultra violet at 365nm (Figure 1).The enzymatic inhibition studies were carried in triplicates and mean ± standard deviation was calculated. The extracts were able to inhibit acetylcholinesterase (Fig: 2), phospholipase A_2 (Fig: 3), hyaluronidase (Fig: 5), L- amino acid (Fig: 6), phosphomonoesterase (Fig: 7) and phosphodiesterase (Fig: 8) enzymes. The protease (Fig: 4) enzyme was inhibited only by methanolic extract. Since the methanolic extract inhibited all enzymes it is considered as an active extract. The maximum inhibition was observed in Phosphomonoesterase and phosphodiesterase (100%) and minimum in phosphomonoesterase (14%) at a concentration of 250 µg/mL of the extract. The dose dependent inhibition would determine the minimum inhibitory concentration (IC_{50}) value of the extract.

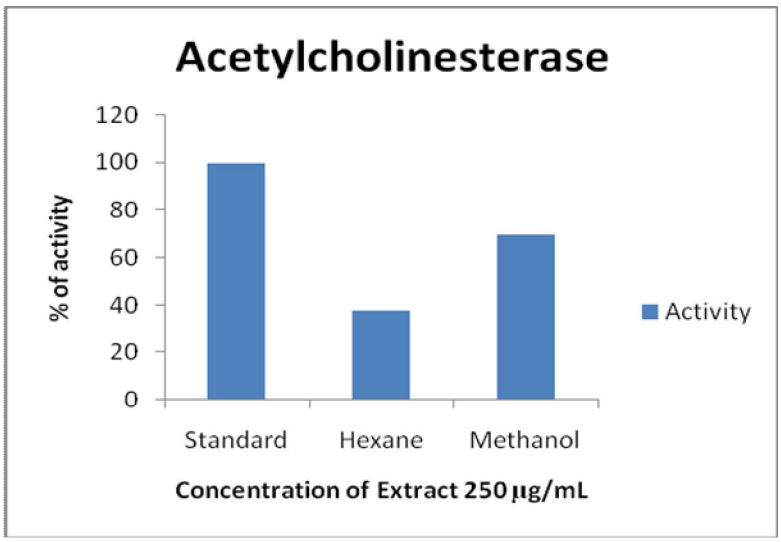


Fig 2: Graphical representation of % activity of acetylcholinesterase enzyme

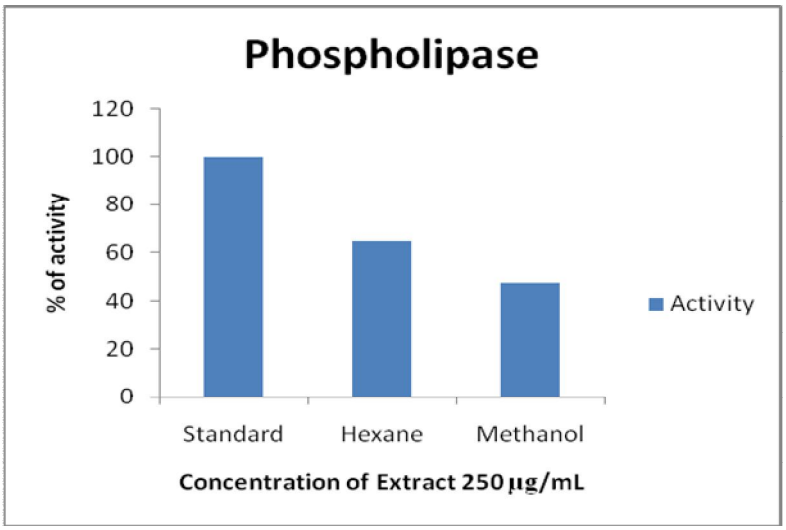


Fig 3: Graphical representation of % activity of phospholipase A_2 enzyme

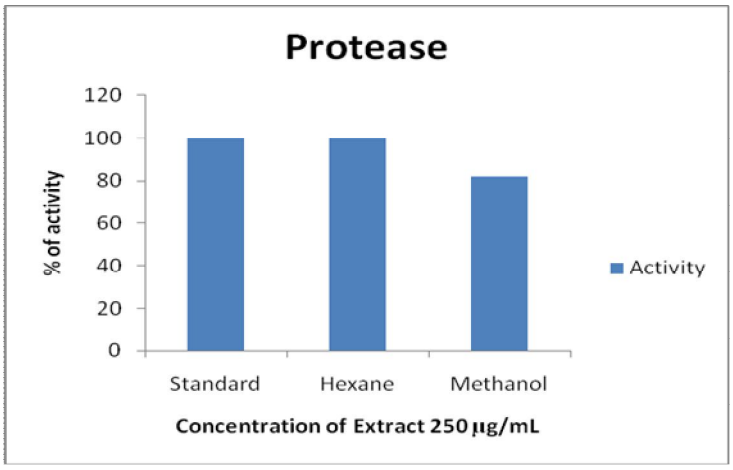


Fig 4: Graphical representation of % activity of Protease enzyme

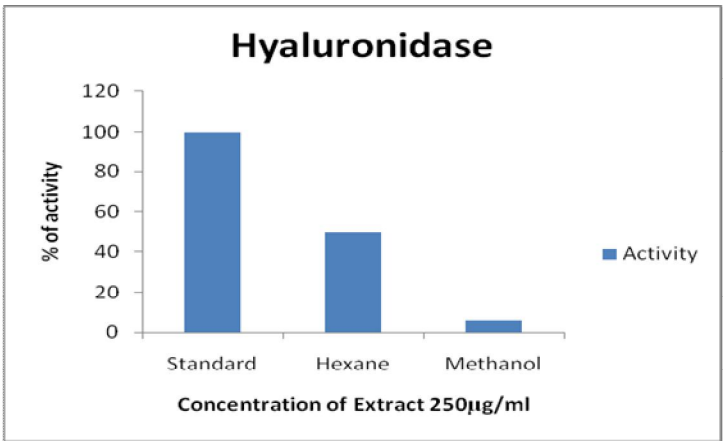


Fig 5: Graphical representation of % activity of hyaluronidase enzyme

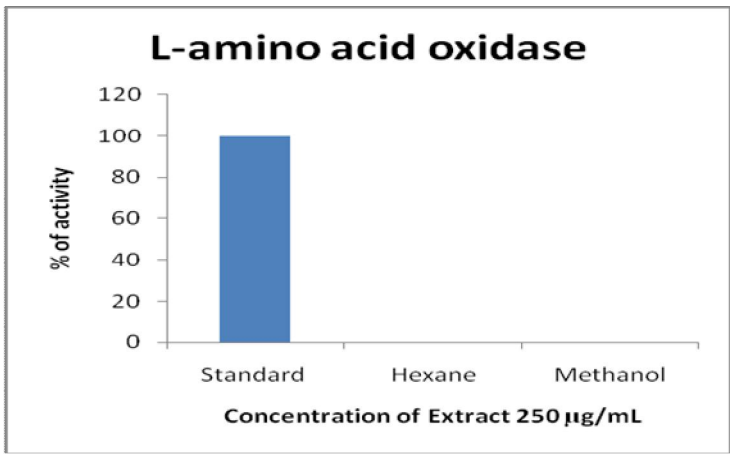


Fig 6: Graphical representation of % activity of L-amino acid oxidase enzyme

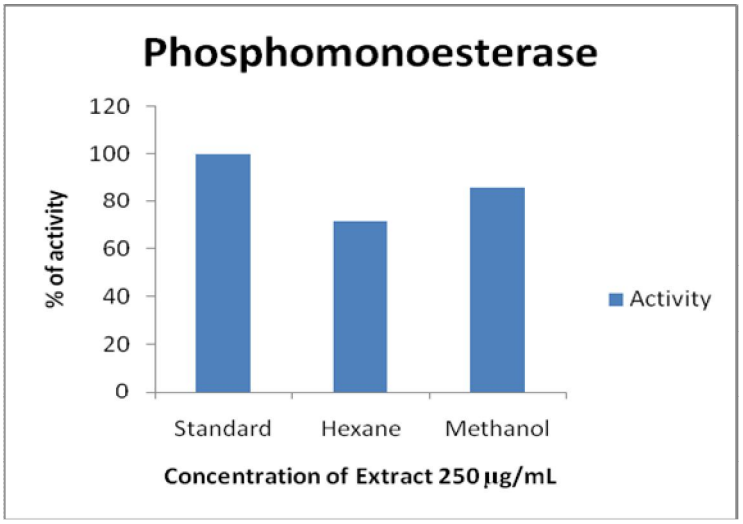


Fig 7: Graphical representation of % activity of phosphomonoesterase enzyme

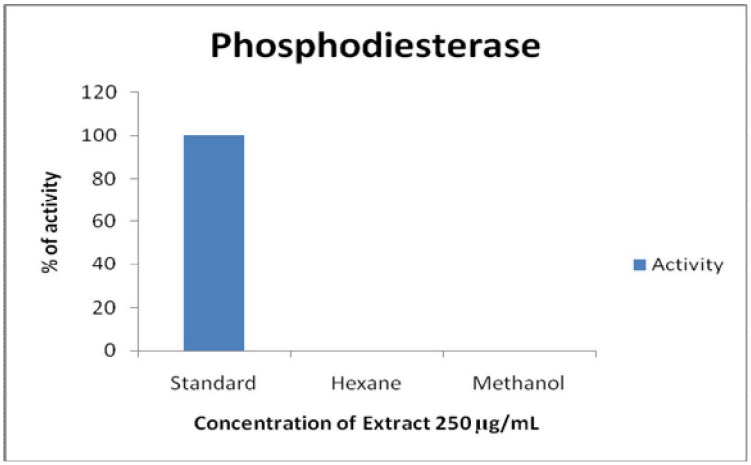


Fig 8: Graphical representation of % activity of phosphodiesterase enzyme

1.4 Conclusion

The *in vitro* enzymatic analysis reveals that the methanolic leaf extract of plant could inhibit most of the toxic enzymes of the *Echis carinatus* which could be further studied for its anti-venom potential by pharmacological and *in vivo* studies.

1.5 Acknowledgments

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