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A review on *in vitro* screening assay for inhibitory effect against venom enzymes using medicinal plants

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Abstract

The usage of medicinal or herbal plants has been a particular application throughout human life, whose information, collected through the involvement of countless generations. Medicinal or herbal plants signify a vital source of bioactive constituents which are useful to the treatment of snake bites patient, or indirectly, applicable as boosts to conventional serum therapy. The tribal and local healers use the Indian medicinal plant extract and its active constituents against snake venom has been not established by experimentally. Defensive activity of several of traditional medicinal plants against snake venoms has been scientifically confirmed by enzymatic assays. In general, enzymatic and biochemical analysis of these plants inhibitors, as useful another or supplemental treatments to conventional serum therapy. However, some assays should be considered to evaluate antivenom efficiency of medicinal plants against a variety of snake venoms. The current review deals with the *in-vitro* screening assay work performed in the recent years involving use of different natural plants extracts and products against snakebite.

Keywords: Snake venom, Medicinal Plants, Anivenom, Enzymatic Assay

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1. Introduction

Snakebite is an enormous reason of morbidity and mortality especially in South Asia, WHO recognized snakebite is a common acute medical emergency in most rural areas of the tropical and subtropical countries like India, Bangladesh, Nepal etc. Recent finding showed that globally there are more than 400,000 envenomation cases annually. In India approximately 35,000–50,000 deaths are supposed because of snakebite every year.[1] Snake venoms contain different compounds typically proteins, polypeptides and enzymes, and numerous other substances with poisonous and fatal properties.[2] The established symptom of snakebites is irresistible anxiety, which shows to other signs, including nausea, vomiting, vertigo, fainting, diarrhea, tachycardia, cold, and clammy dermis.[3]

Currently, antivenom immunotherapy is the solitary treatment existing against snake envenomation. Antivenom immunotherapy moreover show lateral effects like anaphylactic shock, allergy serum sickness and pyrogen symptoms are probably consequences through hypersensitivity reaction, non-immunoglobulin proteins and other chemicals existing in higher quantity in antivenom.[4] In rural and tribal areas, the snakebite patient is not immediately reaches a dispensary or hospital for first-aid treatment. Another disadvantage of antivenom immunotherapy is the high cost of production and shelf life. In this perspective, alternative way of snakebite treatment is to develop.[5]

The massive variety of vegetation and wildlife is the result of thousand years of evolution, large proportion of world's population depends on herbal medicine for their main health care needs throughout human history, gathered through the experience of many generations.[6] Since ages men have faith in plants product as a solitary basis of herbal medicine. The art of natural healing has very profound in Indian culture and folklore.[7]

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Even these days in most of the rural and tribal areas, individuals are depending on native herbal medicine systems for their major health care including snakebites in table 1. Medicinal plants signify source of bioactive pharmacological compounds able to benefit directly in the snakebites treatment or indirectly, use as supplements with antivenom immunotherapy. Therefore, medicinal plant extracts develop a capable alternative treatment.[8]

In recent science, there have been numerous screening methods to study these medicinal plants to elucidate their effectiveness against snake bites. Currently, however some of medicinal plants are reported to be very effective against envenomed patients. In this review, some of the enzymatic *in-vitro* assays with inhibitory activity on the binding venoms proteins are discussed.

Phospholipase A_2 (EC 3.1.1.4)

Phospholipase A2 (PLA2) catalyzes the hydrolysis of the middle position (sn-2) of membrane glycerophospholipids to free arachidonic acid and then these can be metabolized to form numerous eicosanoids and related bioactive lipid mediators.[9] Phospholipase A2 harm cell membranes and can lead to long-lasting damage or even lysis of cells. The several physiological effects of Phospholipase A2 are determined by the type of receptor (include neurological) to which it binds. This recommends that there are variances in affinity of the binding in different muscle types. Respiratory failure often accompanies the paralysis because there is likely a high affinity for Phospholipase A2 in phrenic nerve-diaphragm endplate receptors. All snake venoms detected these enzyme activity specially Elapidae, Viperidae and Crotalidae present in a comparatively higher concentration.[10]

Assay: Phospholipase A2 assay was done by acidimetric method, in this method 1% lecithin, 18 mM calcium chloride, and 8.1 mM sodium deoxycholate was mixed in equal proportions and the pH was adjusted to 8.0. About 0.1 mL of venom solution was added to egg yolk suspension. The initial decrease in pH was measured. A decrease of 1 pH unit corresponded to 133 μmoles of fatty acid release. Phospholipase A2 activity was expressed as μmoles of fatty acid released/ minute.[11] For the inhibition studies, venom was preincubated with extracts for 30 minutes at 37°C.

5' nucleotidase (EC 3.1.3.5)

A specific phosphomonoesterase, 5'-nucleotidase is present in all snake venoms. 5'-nucleotidase is an enzyme that catalyzes the phosphorylytic cleavage of 5'nucleotides and is ubiquitously present in snake venoms.[12] The 5'-nucleotidase activity of the viperid and crotalid venoms is relatively high compared to elapid venoms.

Assay: 0.25 mL of 0.1% crude venom was incubated at 37°C for 15 minutes with the solution containing 1 mL of Tris–HCl buffer, 100 μl of 100 mM magnesium chloride and 800 μl of 0.15% 5′ AMP. At the end of incubation the reaction was quenched by addition of TCA and filtered. The filtrate was assayed for inorganic phosphate using potassium dihydrogen phosphate as standard at 625 nm.[13] One unit of 5'-nucleotidase activity was defined as the amount that yielded 0.01 μmole of inorganic phosphate/ minute. Venom was preincubated with extracts for 30 minutes at 37°C for the inhibition studies.

Hyaluronidases (EC 3.2.1.35)

Hyaluronidases are a ubiquitously available group of very different enzymes in snake venom that obviously cleave hyaluronic acid (high molecular weight glycosaminoglycan) which is a foremost component of extracellular matrix of vertebrates that attaches collagen fibers, protein filaments and the connective tissue cells.[14] Hyaluronidases enzyme is generally present in mammalian testes, some invasive bacteria, leech heads, snakes venoms, scorpions, spiders, and some poisonous fishes.[15] All snake venoms contain hyaluronidase, but it is present in a comparatively higher amount in the viperid and crotalid venoms than in the elapid venom.[10]

Assay: Turbidometric method was used for hyaluronidase assay. The assay mixture contained buffer Tris–HCl (pH 8.0), hyaluronic acid (0.5 mg/mL in buffer) and enzymes and incubated for 15 minutes at 37°C, the reaction was quenched by adding cetyltrimethylammonium bromide (2.5%) in sodium hydroxide (2%) and absorbance was read at 400 nm (within ten minutes) against a control solution. Reduction in turbidity was expressed as a percentage of hyaluronic acid remaining. Reduction of approx 50% turbidity is indicative of one unit of enzyme activity. Specific activity was defined as turbidity reducing units per milligram of enzyme.[16] Venom was preincubated with extracts for 30 minutes at 37°C for the inhibition studies.

L-amino acid oxidase (EC 1.4.3.2)

L-amino acid oxidase is a flavor-enzyme that catalyzes the stereo-specific oxidative deamination of an L-amino acid

substrate to a α-keto acid and the production of hydrogen peroxide and ammonia. The L-amino acid oxidase activity is shows in all the venoms examined belonging to Elapidae, Viperidae and Crotalidae, except in white venoms of Denisonia textilis, Bothrops itapetiningae and Vipera ammodytes. L-amino acid oxidase is accountable for the yellow color in snake venoms, for the reason that it contains FAD as the prosthetic group. When L-leucine is used as the substrate, a turnover number of the venom enzyme is about 400 times higher than that of mammalian L-amino acid oxidase.[17]

Assay: L-amino acid oxidase was performed by stereo-specific oxidative deamination method. The assay mixture consisted of L-leucine, Tris–HCl buffer (pH 8.0), dianisidine hydrochloride (0.1%), horseradish peroxidase (0.1%) and crude venom solution (0.5%) and allowed to stand for 10 minutes at 37°C and absorbance was read at 415 nm. One unit (U) was defined as the amount of enzyme that catalyzed the formation of 1 μmol H_2O_2 per minute.[18] Venom was preincubated with extracts for 30 minutes at 37°C for the inhibition studies.

Acetylcholinesterase (EC 3.1.1.7)

Acetylcholinesterase is an enzyme which catalyzes the breakdown of acetylcholine and certain other choline esters that function as neurotransmitters.[19] An acetylcholine-hydrolyzing enzyme, so-called acetylcholinesterase, was first demonstrated in cobra venom by Iyenger. This enzyme is only found in the venoms of Elapidae and Hydrophiidae, not in those of Viperidae and Crotalidae. Since the enzyme is involved in nerve transmission, venom acetylcholinesterase was at one time thought responsible for the neurotoxic action of elapid venoms.[20]

Assay: The assay mixture consist of phosphate buffer (pH 8.0), DTNB (10 mmole/L) and acetylethiocholine iodide (158.5 mmol/L). Crude venom (0.1%) and 3 mL of prepared buffer solution was incubated at 37°C for 5 minutes. Then, DTNB (a strong oxidizing agent) and acetylethiocholine iodide were added in order to reach a final concentration of 1 mmole/L. The increase in absorbance at 412 nm was measured.[21] Venom was preincubated with extracts for 30 minutes at 37°C for the inhibition studies.

Phosphodiesterase (EC 3.1.4.1)

Phosphodiesterase is characterized by phosphodiesters are known to catalyze the hydrolysis of phosphodiester bonds in a liberal manner beginning at the 3′end of polynucleotides liberating 5′mononucleotides at basic pH.[22] Of the four phosphatases available in snake venoms, phosphodiesterase is one of the most well-known enzymes. Phosphodiesterase enzyme, also known as venom exonuclease and its commonly present in numerous venoms from all three families of venomous snakes.

Assay: Phosphodiesterase activity was determined by phosphodiester bonds hydrolysis method. The assay mixture consist of venom solution, Na-p-nitrophenyl phosphate (0.0025 M), $MgSO_4$ (0.01 M) and Tris–HCl (0.17 M, pH 8.0) and absorbance was read at 415 nm. Phosphodiesterase activity was expressed in nanomoles of product released/minute. Molar extinction coefficient at 400 nm was 8100 Cm^{-1} M^{-1}.[23] Venom was preincubated with extracts for 30 minutes at 37°C for the inhibition studies.

Phosphomonoesterase

Phosphomonoesterases shows two nonspecific enzymatic activities at pH 5.0 and at 8.5. They appear to be catalysts with a dissimilar enzyme entity, because some of the venoms contain only one type acid or alkaline phosphatases, whereas others contain both acid and alkaline phosphatases.[24] Phosphatase action is related to shock symptoms due to venomation of snakes.[20] Phosphomonoesterases hydrolyzed number of biologically important compounds such as ATP, glucose-I- and glucose-6-phosphates, and mononucleotides. Many venoms belonging to Elapidae, Hydrophiidae, Viperidae, and Crotalidae show both acid and alkaline phosphatase activities and some venom shows only acid or alkaline phosphatase activity.

Assay: The assay mixture consist of Tris–HCl buffer (pH 8.0), disodium-p-nitrophenol phosphate, crude venom and was incubated at 37°C for 3 hours. The absorbance was read at 425 nm. p-Nitrophenol was used as the standard. One unit of enzyme activity was defined as the amount that yield 0.1 μmole of p-nitrophenol/hour.[25] Venom was preincubated with extracts for 30 minutes at 37°C for the inhibition studies.

Table 1. Medicinal plants with *In vitro* enzymatic activity

Plant Species	Plant Source(s)	Snake Species	In vitro Enzymatic Activity
Abutilon indicum	Leaves	Echis carinatus	Phosphomonoesterase, Phosphodiesterase, L-amino acid oxidase, Acetylcholinesterase, Hyaluronidase, Phospholipase A2, Protease
Andrographis paniculata	Whole plant	Daboia russelli	Phospholipase A2 activity
Aristolochia indica	Whole plant	Daboia russelli	Phospholipase A2 activity
Azadirachta indica	Leaves	N. naja, N. kaouthia and D. russelli	Phospholipase A2
Azima tetracantha	Leaves	Bungarus caeruleus, Vipera russelli	5' nucleotidase, Phosphomonoesterase, Phosphodiesterase, L-amino acid oxidase, Acetylcholinesterase, Hyaluronidase, Phospholipase A2, Protease
Casearia sylvestris	Leaves	Brazilian snakes, *B. asper*	Phospholipase A2
Emblica officinalis	Root	Naja Kaouthia, Vipera russellii	Phospholipase A2
Hemidesmus indicus	Root	Daboia russellii Naja kaouthia	Phospholipase A2
Mandevilla illustris	Roots, leaves	C. durissus terrificus	Phospholipase A2
Mandevilla velutina	Rhizomes	Brazilian snakes	Phospholipase A2
Mangifera indica	Fruits	Naja kaouthia, Calloselasma rhodostoma	Phospholipase A2, Hyaluronidase, L-amino acid oxidase,
Mangifera indica	Stem bark	Russell's viper	5' nucleotidase, Phosphomonoesterase, Phosphodiesterase, Hyaluronidase, Phospholipase A2, Protease
Mimosa pudica	Roots	*N. naja, N. kaouthia, V. russelli and E. carinatus*	Hyaluronidase,
Pouzolzia indica	Leaves	V. russellii	Phospholipase A2
Tamarindus indica	Seed	Vipera russelli	Hyaluronidase, Phospholipase A2, Protease, L-amino acid oxidase, 5' nucleotidase,
Vitex negundo	Leaves	*Daboia russelli, Naja naja*	Phospholipase A2
Borassus Aethiopum	Fruit	*Naja mossandica*	Phospholipase A2
Ficus Iteophyla	Leaves	*Naja mossandica*	Phospholipase A2
Withania somnifera	Roots	*N. naja*	Phospholipase A2
Jatropha curcas	Seeds	Brazilian snakes	Phospholipase A2

2. Conclusion

The application of medicinal or herbal plant extracts as antivenom for snake venom is a public practice in the rural and tribal areas where a quick access to conventional serum therapy is not available, and is also applied as an additional with conventional serum therapy. In overall, numerous deactivating actions of herbal plant extracts may be due to the occurrence of enzyme or chemical inhibitors.

Rural and traditional healers have been applying herbal treatments subsequently years to treat snake bites. Even though there are a lots of research on herbal plants from different tribal and geographical areas and it is the responsibility of the scientific society to do much superior work for the effective herbal medications that are capable to deactivate snake venoms. Further research on the extraction, isolation, structural validation and action mechanism of these herbal inhibitors should be carried out.

3. Acknowledgement

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4. Conflict of Interest

None Declared.

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