Journal of Critical Reviews

ISSN- 2394-5125 Vol 3, Issue 2, 2016

Review Article

IN-VITRO ASSAYS FOR NEUTRALIZATION OF SNAKE VENOM USING HERBAL DRUGS: A REVIEW

SIPRA SARKAR*, RAJA CHAKRAVERTY, SUPRIYA DATTA, AMITAVA GHOSH

Bengal College of Pharmaceutical Sciences and Research, BRB Sarani, Bidhannagar, Durgapur 713212
Email: sipra.2000@gmail.com

Received: 14 Jan 2016 Revised and Accepted: 01 Mar 2016

ABSTRACT

Snakebite is a major health hazard that leads to high mortality rate, especially in India. The present review article focuses on the point of view of different *in–vitro* neutralization assays that serve as an index for assessing the status of therapy. For this purpose bibliographic and scientific literature articles in indexed journal, databases were comprehensively researched, and contemporary articles were studied from different abstracting and indexing systems like PubMed using relevant keywords. It was found that some of the in-vitro assays like Phospholipase A2, Hemorrhagic activity assay, agglutination assay for potency assessment, general proteolytic activity assay, snake venom metalloprotease activity (SVMP), hemolytic activity, neutralization of serum inhibitory activity assay, neutralization of fibrinolytic activity, inhibition of venom cardiotoxic activity assay and brine shrimp lethality test are prevalent in use. Performing these *in–vitro* tests is essential for the development of therapy against envenomation. Anti snake venom serum (ASVS) for snake bite therapeutics is available but suffers from many drawbacks; herbal plants provide a solid platform for the natural treatment of this serious issue. Herbal medications have excellent potential to treat snake bite. Herbal medicinal plants are an important element of indigenous medical systems globally. Many of the active plant constituents are promising contenders for the development of antivenom drug molecules. So our objective is to find different alternative *in-vitro* processes for antivenom activity. The rationale behind choosing the *in-vitro* assays is to enable to generate basic data and understand the probable basic mechanism of snake venom and herbal anti snake venom without sacrificing or using invasive procedures on the experimental animal.

Keywords: Envenomation, Neutralization assays, *In–vitro* processes, Hemorrhagic activity, Metalloprotease (SVMP) activity, Brine shrimp lethality test

INTRODUCTION

Many people who survive snakebites have permanent tissue damage caused by venom, leading to disability. Most snake envenoming and fatalities occur geographically in South Asia, Southeast Asia, and sub-Saharan Africa, with India reporting the most snakebite deaths of any country [1]. Most snakebite is caused by non-venomous snakes. Of the roughly 3,000 known species of snake found worldwide, only 15 % are considered dangerous to humans. Snakes are found on every continent except Antarctica [1]. Worldwide, snakebites occur most frequently in the summer season when snakes are active, and humans are outdoors. Agricultural and tropical regions report more snakebites than anywhere else. In the USA, those bitten are typically male and between 17 and 27 y of age. Children and the elderly are the most likely to die [2].

Snake venoms consist of a mixture of enzymatic and non-enzymatic proteins and peptides and small organic compounds such as citrates, nucleosides & acetylcholine [3]. The envenomation includes cytotoxicity, cardiotoxicity, pro-coagulating activity, hemorrhagic, hemolytic, homeostatic, neurotoxicity and edema-forming activity [4]. The venom has been studied for many active principles like procoagulant factors [4], ATPase [5], Phospholipase A_2 [7], trypsin inhibitors [8], daboia toxin like factor [9], platelet aggregation factor [10], hemorrhaging [11], neurotoxic peptide [12]and a heat stable protein drct-I [13].

The most effective and accepted therapy for snakebite patients is the immediate administration of specific or polyvalent antivenom following envenomation. Unfortunately, this therapy carries an associated risk of anaphylaxis and serum reactions. The ubiquity of venom variation in snakes poses special problems for the manufacture of antivenom and has undermined the commercial attractiveness of this class of therapeutic agent. In particular, it has been amply documented that both inter-specific and intra-specific variation in venom composition can affect the neutralization capacity of antivenom [14]. The scarcity of sufficient amount of quality venom from authorized venom dealers also poses a challenge for a reasonable amount of antivenom production to meet the national requirement.

The ASVS development is a costly, time-consuming process requiring ideal storage conditions. Absolute specificity is an issue in management with ASV. The geographic and taxonomic diversity in species leads to a significant variation in composition and antigenic reactivity of venom [14]. In short, due to the complex interplay of economic, epidemiological, therapeutic efficacy and safety issues of antivenom, the mortality of snakebite remains incongruously high in the developing countries. In this context, the only available option for snakebite treatment is herbal treatment as these herbs are common, easily available and cheaper.

In accordance with the recommendations of the report of a WHO Coordination Meeting on Venoms and Antivenoms, methods have been developed for the assessment of lethal, defibrinogenating, procoagulant, hemorrhagic, and necrotizing properties of venoms. The tests will be used to assay the neutralizing potency of both international standard antivenoms (raised using the IRVs) and new and currently available commercial antivenoms. Such studies should result in the production of more potent antivenoms for use in both developing and developed countries, and improve the understanding and management of snake bite throughout the world [15].

Different *in-vitro* assays

Neutralization assay

For phospholipase A_2 (PLA_2) and hemorrhage activities, myo toxicity and lethality assays, venom solutions were mixed with a solution of extract at room temperature immediately before the test, except when otherwise stated. The extract was weighted and dissolved in deionized water before use (1.0 mg/ml). The extract concentration was expressed in terms of dry weight, and the amount of extract used in each experiment was established according to the concentration of the venom indicated for each test [16].

1. **Phospholipase A_2 activity**

PLA_2 enzymatic activity is determined according to De Haas *et al.* (1968), using egg yolk suspension as substrate. The major toxin

from snake venom was used to test PLA_2 enzymatic activity. The neutralizing effect of the extract on PLA_2 activity in snake venom was measured at a ratio1:1 (w/w, venom/toxin: extract). Released free fatty acids were titrated with NaOH (0.12N). PLA_2 activity was expressed in Eq. NaOH/min/mg protein (mean±S. D, *n* = 3) [18-20].

2. Hemorrhagic activity

The in-vitro test for assessing the ability of extract to inhibit venom hemorrhagic action follows a method described by Dunn and Boone in 1976; Sells and others in 1997, 1998, 2001. Day-old fertile eggs were incubated till day 4 in a humid incubator at 37±1 °C. In a laminar flow cabinet, the eggs were disinfected with 70 % ethanol and cracked on day 4 into cling film hammocks and incubated further till day 6. Discs of 2 mm diameter cut from filter paper (Whatman no.2) were impregnated with 1.5 µl volume of various concentrations (1,2, 3 and4 µg/1.5 µl, per egg) of venom and standard haemorrhagic dose (SHD)of the venom was determined. The concentration of venom required to cause a haemorrhagic corona of 2 mm diameter was accepted as SHD of the venom. Filter paper discs were impregnatedwithsetvolumeof1.5 µl of a mixture of venom and two different concentrations of extract incubated at 37±1 °C for30 min such that the first mixture incorporates 1SHD of venom and 100 mg/ml extract, and the other mixture incorporates 1 SHD of venom and200 mg/ml of extract. Concentrated extract solutions (at least 100 mg/ml) are required because of the 1.5 µl/disc volume limit. Each of the discs was carefully placed on the yolk sac membrane over a major bilateral vein and left for 2–4 hour to form a hemorrhagic corona and the diameter of coronas was measured. A similar study was performed using antivenom as a standard reference. Control experiments were performed with saline used in the preparation of extract and venom dilutions [21-23].

3. **Agglutination assay for potency estimation**

For the venom potency estimation, four different sets of flocculation tubes were added with graded (0.02 mg increment, 0.02–0.1 mg) concentrations of venom adsorbed on 0.15 % (w/v) bentonite suspension. The final volume was made up to 1.0 ml with normal saline. Each tube was added with 1.0 ml of unprocessed plasma, un-purified serum or purified serum, incubated in a water bath and observed for agglutination [24].

4. General proteolytic activity

The general proteolytic activity of venom was determined by the method of Palmer of 1993 as modified by Biardi and his associates in 2006. A solution of 1.0 % agarose and 0.75 % gelatin (Type I bovine tendon, Sigma Chemical Co.) was used to create the assay matrix. Plates were incubated at 37 °C for 24 h in a humidified chamber. The area of hydrolysis was calculated from the product of two perpendicular diameters across the clear lysis zone. Each assay was repeated five times per venom per sera sample and the unit of analysis for statistical comparisons was the mean of these five replications [25].

5. Snake venom metalloprotease (SVMP) activity

Collagen hydrolysis was quantified using Azocoll (Sigma Chemical Co.) as a substrate using the method of Biardiet and others in 2006. Assays were incubated at 37 °C for 2 h and then centrifuged for 2 min at 16,000 g to pellet unreached substrate. The absorbance of the supernatant was determined at 520 nm using a Shimadzu UV160 spectrophotometer. Each assay was duplicated three times per venom per sera sample and the unit of analysis for statistical comparisons was the mean value of these three duplications [26].

6. Hemolytic activity

BBL Stacker plates (Carolina Biological Supply) containing Columbia agar and 5 % defibrinated sheep's blood should be used to assay for venom hemolytic activity. After loading, the plates are sealed with Parafilm and incubated at 37 °C for 24 h in a humidified chamber. The area of the hemolytic zone, defined as the transparent zone around the well cleared of red blood cells, was calculated from two perpendicular diameters. Each assay was duplicated three times per venom per sera sample and the unit of analysis for statistical comparisons was the mean value of these three duplications [27].

7. **Neutralization of serum inhibitory activity**

This is measured in order to see if the decreased clotting of *V. russellii* venom on goat plasma was due to venom inhibition in serum. Goat serum should be collected without anticoagulant, incubated in glass tubes for 2 h at 37 °C and then centrifuged at 2000 rpm for 15 min. Pooled sera is keptfrozen at −20 °C. Equal volumes of Tyrode–Hepes buffer (THB) (137 mM NaCl; 2.7 mM KCl; 12 mM $NaHCO_3$; 0.42 mM NaH_2PO_4, 10 mM Hepes; pH 7.4) or goat serum were incubated with venom solution of different concentration for 5 min at 37 °C. Aliquots of 50 ml should be added to 200 ml of goat plasma (maintained for 2 min at 37 °C) and the clotting time recorded. MCDS should be calculated. Neutralization of serum inhibitory activity was estimated by mixing the different amount of venom with fixed amount of plant extract, incubating for 1 h at 37 °X and centrifuged. The supernatant was then added to the experimental group and the clotting time was observed [28].

8**. Neutralization of fibrinolytic activity**

A modified plaque assay was used by Rojas and associates in1987. The minimum fibrinolytic concentration was defined as the concentration of venom that induced a fibrinolytic halo of 10 mm diameter. Neutralization experiments were performed by incubating a constant amount of plant extract with varying amount of venom at 37 °X for 1 h. After incubation, the mixture was applied to the wells in the plaque. After 18 h of incubation at 37 °C, fibrinolytic halos were measured [29].

9. **Inhibition of venom cardiotoxic activity**

Minimum cardiotoxic dose (MCTD) is defined as the least amount of venom which stops auricular contraction within 15 min. Isolated guinea pig auricle was prepared from the freshly sacrificed male guineapig (200±10 g) which was subsequently suspended in oxygenated (95 % O_2 and 5 % CO_2) Ringer's solution (NaCl, 154 mM, KCl 5.6 mM, $CaCl_2$ 2.2 mM, $NaHCO_3$ 6 mM, glucose, 5.5 mM) containing double dextrose (2 g/l). The temperature was maintained at 29±1 °C. The spontaneous contraction was recorded on a smoked drum through lightly sprung heart lever. One cardiotoxic dose (25 µg) stopped 98±0.5 % of the contractions of the isolated guinea pig auricle within 15 min. Inhibition of the cardiotoxic activity was estimated as venom/venom active compound incubated at 37 °C for 60 min centrifuged at 2000 rpm for 10 min and supernatant added to glass bath (4 ml) containing the auricle and nature of the contraction was recorded [30].

10. Brine shrimp lethality test

The brine shrimp lethality test is used to test the activity of the extract and to estimate its toxicity against zoologic systems according to Meyer and others in 1982. The test is performed according to the method described by Mackean and others in 2000 but slightly modified to suit our local laboratory settings. Briefly, 50 mg of brine shrimp (*Artemia saline* Leach) eggs (HOBBY®, Germany) were sprinkled into a 50 ml beaker containing natural seawater (collected at Bar Beach, Victoria Island, Lagos, Nigeria) and placed in a secure place for 48 h to hatch. The phototropic nauplii of the hatched shrimps are harvested with plastic pipette by covering three-fourths of the beaker with black carbon paper, as they move towards a torchlight directed at the uncovered portion. The stock of the extract (10 mg/ml) is prepared using doubly distilled water. From this stock, 1000, 500, 250 and 125 µg/ml of the extract are prepared, and 1 ml of each preparation transferred into a 1 ml, 8×12 vial (Falcon) in triplicate. Ten–fifteen shrimp nauplii are added to each vial. The number of survivors against total over 24 h was recorded, and LC_{50} calculated using Finner's 1971 method-based computer program [31].

Discussions

These studies were carried out to establish the scientific basis for the traditional application of herbal drugs in the treatment of victims of snakebite among the indigenous people. The test is used in

screening agents that can be toxic (cytotoxic) to zoologic systems and to predict potential biological activity in unknown pharmaceutical samples. For instance, agents that elicit LC_{50}≤30µg/ml are considered to have significant bioactive component Meyer and others in 1982 [32] whileLC_{50} of about 650µg/ml towards brine shrimp nauplii was considered to be only mildly toxic [33]. Since the traditional healers sometimes administered these agents by making it into a paste and tying them over incisions made at the point of bites, it is possible that the toxic enzymes might be neutralised through the process. Some plant constituents have the ability to bind venom proteins [17]. Toxicological properties of snakebite are thought to be associated with enzymes as described by Stocker in 1990[18] especially phospholipase A_2(PLA2), which is believed to be its most toxic component [19] implicated in hemorrhage as described by Melo and Own by in 1999 [34]. The effects of the extract on the venom might thus be linked to its activity against the toxic enzymes [34].

Mahanta and Mukherjee in the year 2001postulated that neutralization of these enzymes might lead to inhibition of lethality of venom at the site of application. The results of the *in–vitro* detoxification test suggest that the extract might act by neutralizing the activity of the venomat the site of the bite, thereby reducing the severity of the toxic effects. The sudden change associated with venom poisoning may be linked to the breakdown of biochemical functions of the liver. Current Opinion here is that the liver may be involved directly but perhaps on a cumulative basis. Another possibility may be asymptomatic or physiological antagonism by the extract in which the toxic features, such as convulsion which lead to eventual suffocation and death could be attenuated, thereby reducing the fatality of the venom a symptomatic relief. The various classes of compounds identified in the phytochemicals study of the extract [35] could be studied closely. Some research had find earlier related antivenom activity to alkaloidal glycosides they traced in the plant they studied. Tannins are also known to inactivate unspecifically proteins. The activity of the extract may, therefore, be linked to its tannin contents. Furthermore, phytochemicals constituents of plants used for snakebites tend to have similar functions [35].

CONCLUSION

Many research-based studies employing in-vitro assays on neutralization parameters of snake venom have shown positive results with respect to their anti-venom action of the plant extracts, especially when the venoms are mixed with the extracts before the biological assays. These procedures will show that the efficacy of a plant extract against the intoxication by venom cannot be assured if only based on results obtained *in–vitro*. The utilization of tannins as anti-ophidics is an ancient practice. Moreover, the treatment of local hemorrhage using tannins was described before. After performing the *in–vitro* studies if the results are positive and if the structural elucidation of these molecules will allow the understanding of the interaction between tannins and proteins and, consequently, the inhibition mechanism of proteins by these compounds.

In conclusion, we hope these findings may clarify the supposed efficacy of the use of herbal drugs as an anti-snake venom agent. We also point out to the necessity of a serious evaluation with experiments *in vivo* to validate the possible anti-venom effects of an extract or plant substance. The relevance of these bioactivities in relation to their possible adaptive roles for the snakes is discussed. Finally, experiments designed to assess the validity of such hypothetical roles are suggested, to stimulate future studies in this field.

CONFLICT OF INTERESTS

Declared none

REFERENCES

1. Mc Diarmid RW, Campbell JA, Touré T. Snake species of the world: a taxonomic and geographic reference. Vol. 1. Washington, District of Columbia: Herpetologists' League; 1999. p. 511.
2. Mallow D, Ludwig D, Nilson G. True vipers: natural history and toxicology of old world vipers. Malabar, Florida: Krieger Publishing Company; 2003. p. 359.
3. SD Aris. Ophidian envenomation strategies and the role of purines. Toxicon 2002;40:335-93.
4. R Murthy Sashidhara, Jagadisha DK, Girisha V, K Kamparaju. Variation in biochemical and pharmacological properties of Indian cobra (Naja naja) venom due to geographical distribution. Mol Cell Biochem 2002;229:93-101.
5. S Schffman, I Thareodor, S I Rapaport. Separation from Russell's viper venom of one fraction reacting with factor X and another reacting with factor V. Biochemistry 1969;8:1397-405.
6. Kini RM, Gowda TV. Inhibition of local effects of Indian Daboia/Vipera russellii venom by the methanolic extracts of grape(Vitis vinifera L.) seeds. Indian J Biochem Biophys 1982;19:342-6.
7. Bishwanath BS, Kini V, Gowda TV. Electrophysiological studies of myotoxin a, isolated from prairie rattlesnake (Crotalus viridis) venom, on murine skeletal muscles. Toxicon 1985;6:929.
8. Jayanthi, TV Gowda. An interdisciplinary journal on the toxins derived from animals, plants and microorganisms. Toxicon 1928;6:65-74.
9. Maung PTM, Gopalkrishnakone R, Tan, Yewn V. A major lethal factor of the venom of burmese russell's viper (*Daboia russelli siamensis*): Isolation, n-terminal sequencing and biological activities of Daboia toxin. Toxicon 1995;33:73-6.
10. Prasad BN, Kemparaju K, KGS Bhatt, TV Gowda. A platelet aggregation inhibitor phospholipase A_2 from russell's viper (vipera russelli) venom: Isolation and characterization. Toxicon 1996;4:1173-85.
11. Chakraborty AK, Datta K, Gomes A, Bhattacharya D. Haemorrhagic protein of Vipera russelli with fibrinolytic and esterolytic activities. Toxicon 2000;38:1475-90.
12. Guiterterrez JM, Leon G, Rojas G, Lomanate B, Rucavado A, Chaves F. Neutralization of local tissue damage induced by Bothrops asper(terciopelo) snake venom. Toxicon 1998; 49:1529-38.
13. Stidworthy J. Snakes of the World. Revised Edition. New York: Grosset and Dunlap Inc; 1974. p. 160.
14. Ditmars RL. Reptiles of the World: The Crocodilians, Lizards, Snakes, Turtles and Tortoises of the Eastern and Western Hemispheres. New York: The McMillan Company; 1937. p. 321.
15. MH Borges, DLF Alves, DS Raslan, D Pil´o-Veloso, VM Rodrigues, MI Homsi-Brandeburgo, ME de Limaa. Neutralizing properties of musa paradisiacal L. (Musaceae) juice on phospholipase A2, myotoxic, hemorrhagic and lethal activities of crotalidae venoms. J Economic Perspectives 2000;11;608-17.
16. De Haas GH, Postema NM, Nieuwenhuizen, Van Deenen, L I M. Purification and properties of phospholipase A2 from porcine pancreas. Biochem Biophys Acta 1968;159:103–17.
17. Wen-Chan Cxana, Mux-Lnv Leei, Tunc-Bnv Lol, To~Ccn. Phospholipase a2 activity of long-chain cardiotoxins in the venom of the Banded krait(Bungarus fasciatus). Toxicon 1983;21:163-5.
18. PA Shenoy, SS Nipate, JM Sonpetkar, NC Salvi, AB Waghmare, PD Chaudhari. Anti-snake venom activities of ethanolic extract of fruits of Piper longum L. (Piperaceae) against Russell's viper venom: characterization of piper in as active principle. J Ethnopharmacol 2013;147:373–82.
19. James E Biardia, Richard G Coss. Rock squirrel (Spermophilus variegatus) blood sera affects proteolytic and hemolytic activities of rattlesnake venoms. Toxicon 2011;57;323–31.
20. M I Alam, A Gomes D. Snake venom neutralization by Indian medicinal plants vitexnegundo and emblica officinalis root extracts. J Ethnopharmacol 2003;86;75–80.
21. B Adzua, MS Abubakarb, KS Izebec, DD Akumkaa, KS Gamaniela. Effect of Annonasenegalensis root bark extracts on venom in rats. J Ethnopharmacol 2005;96;507–13.
22. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen L, Nichols DE, McLaughlin JL. Brine shrimp: a convenient in a general bioassay for active plant constituent. Planta Med 1982;45:31-4.
23. Mackean MM, Ali AM, Lajis NH, Kawazu K, Hassan ZH, Habsah M, *et al*. Antimicrobial, antioxidant, antitumour-promoting and cytotoxic activities of different plant part of Garcia altroviridis Griffext T. Anderi. J Ethnopharmacol 2000;72:395–402.

24. Otero R, Nunez V, Barona J, Fonnegra R, Jimenez SL, Osorio RG, *et al*. Snakebites and ethnobotany in the northwest region of Colombia. Part III: neutralization of the haemorrhagic effect of Bothropsatroxvenom. J Ethnopharmacol 2000;73:233–41.
25. Stocker KF. Composition of snake venoms. In: Medical use of snake venom proteins. CRC Press: Boston; 1990. p. 33.
26. Mahanta M, Mukherjee AK. Neutralization of lethality, myotoxicity and toxic enzymes of Najakaouthiavenom by Mimosa pudicaroot extracts. J Ethnopharmacol 2001;75:55–60.
27. Melo PA, Ownby CL. The ability of wedelo lactone, heparin, and para-bromophenacyl bromide to antagonize the myotoxic effects of two crotadine venoms and their PLA2 myotoxins. Toxicon 1999;37:199–215.
28. Haslam E. Plants polyphenols–vegetable tannins revisited. Cambridge University Press: Cambridge; 1989.
29. Adzu B, Abbah J, Vongtau H, Gamaniel K. Studies on the use of Cassia singueanain malaria ethnopharmacy. J Ethnopharmacol 2003b;88:261–7.
30. Akunyili DN, Akubue PI. Schumanniofoside, the antisnake venom principle from the stem bark of Schumanninphytonmagnificum Harms. J Ethnopharmacol 1986;18:167–72.
31. Okonogi T, Hattori Z, Ogiso A, Mitsui S. Detoxification of persimon tannin of snake venom and bacterial toxins. Toxicon 1979;17:524–7.
32. Reyes-Chilpa R, Gomez-Garibay F, Quijano L, Magos-Guerrero G, Rios T. Preliminary results of the protective effect of (−)-edunol, a pterocarpan from Brongniartia podalyrioides (Leguminosae), against Bothropsatrox venom in mice. J Ethnopharmacol 1994;42:199–203.
33. Swinyard EA. Demulcents, emollients, protectives and adsorbents, absorbable hemostatics, astringents, irritants, sclerosing agents, caustics, keratolytic, antiseborrheic, melanizing and demelaninzing agents and certain enzymes. In: Goodman LS, Gilman A. Eds. The Pharmacological Basis of Therapeutics, third ed. Ed. Macmillan, New York, USA; 1965. p. 981–2.
34. Haslam E. Natural polyphenols (vegetable tannins) as drugs and medicine: possible modes of action. J Nat Prod 1996;59:205–15.
35. Bruno Lomonte, Yamileth Angulo, Mahmood Sasa, José María Gutiérrez. The phospholipase A2 homologues of snake venoms: biological activities and their possible adaptive roles. Protein Pept Lett 2009;16:860-76.