Harnessing Nature's Defence: Investigating Plant Extracts as Inhibitors of Russell's Viper Venom for Promising Ancillary Antivenom Development

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CERTIFICATE

This is to certify that the dissertation work entitled **"Harnessing Nature's Defence: Investigating PlantExtracts as Inhibitors of Russell's Viper Venom for Promising Ancillary Antivenom Development** compiled in this thesis was carried out by **Miss Nisha Sharma** herself , and I consider that it is a good segment of original work performed on observation of Inhibitory potentials of different extracts on Russell's viper venom. I have personally gone through all the data/material/photos/results reported in the manuscript and certify their correctness/authenticity. It is further certified that Miss Nisha Sharma has fulfilled all the necessities laid down by the Thapar Institute for the submission of dissertation thesis for the partial fulfilment of the degree of Master of Science in Biotechnology in accordance with UGC rules and regulations. It has not formed the basis for any degree or diploma prior to this. Therefore, he is permitted to submit this dissertation thesis for the partial fulfilment of the degree of Master of Science in Biotechnology of the Thapar Institute of Engineering and Technology, Patiala, Panjab, India.

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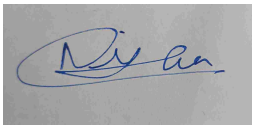
DECLARATION

I hereby declare that the work which is being presented in this dissertation thesis, entitled ""**Harnessing Nature's Defence: Investigating PlantExtracts as Inhibitors of Russell's Viper Venom for Promising Ancillary Antivenom Development** in the fulfilment of the requirements for the award of the degree of Master of Science in Biotechnology and submitted in Thapar Institute of Engineering and Technology, Patiala is an authentic record of my own work carried out during dissertation period (January-July) under the supervision of **Dr Kartik Sunagar.**

The data put forth is based on my observation during the period of January-July. Whenever I have quoted written materials from other sources and due credits are given to the sources in reference or by citing them .

The matter embodied in this thesis has not been submitted by me for the award of any other degree of this or any other University/Institute.



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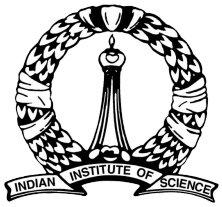

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- I owe my profound gratitude to **Dr Rudresha and Rashmi** for their encouragement, support, guidance and always being there for me.
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I. ABSTRACT

India is renowned for its incredible range of geographic and ecological diversity. The vast expanse of land, diverse terrain, and varying climatic conditions throughout the country give rise to numerous unique ecological regions.

Snakebites, a neglected tropical disease affecting millions worldwide, pose a significant public health concern, with India bearing the unfortunate title of the snakebite capital of the world. The venomous "Big Four" snakes, notably Russell's Viper *(Daboia russelii)*, contribute to the majority of snakebite incidents in Asia(Suraweera et al 2020). Despite the administration of antivenom as the primary treatment, the intra-population variation in venom composition remains inadequately addressed, limiting the efficacy of conventional antivenom therapies. Exploring alternative approaches, traditional plant-based remedies, such as *Cryptolepis buchanani* and *Kalanchoe pinnata,* have long been employed by local healers. While in-vitro studies revealed limited anti-venom potential, including PLA_2 inhibition by *Kalanchoe pinnata*, these plants exhibit notable healing qualities, encompassing antioxidant, anti-inflammatory, antibacterial, and antifungal properties. Their historical utilization and presence in ancient Ayurvedic texts endorse their potential as agents to prevent infections caused by external agents. Further research is warranted to explore the therapeutic potential of these plants and harness their beneficial properties in developing supplementary therapies for snakebite management.

II. INTRODUCTION

1. "Biogeography of India: Exploring the Ecological Diversity and Distribution Patterns"

India is situated in the continent of Asia. It lies completely in the Northern hemisphere and Eastern hemisphere between latitudes 84′ N and 37°6'N and longitudes 68°7′ E and 97°25′ E. India is divided by Tropic of Cancer 23°30′ N in almost two equal parts. Biogeographic distribution of India is the division of India on the basis of different biological factors like distribution of species, flora, fauna and ecosystems in geographic space through geological time. India is a tropical country having different ecological zones and houses a large number of indigenous flora and fauna. India has a rich heritage of natural diversity thanks to its varied topography, climate and geological features. India harbours nearly 11% of the world's floral diversity comprising over 17500 documented flowering plants, 6200 endemic species, 7500 medicinal plants and 246 globally threatened species in only 2.4% of world's land area. India is also home to four biodiversity hotspots—Andaman & Nicobar Islands, Eastern Himalaya, Indo-Burma region, and the Western Ghats. Hence the importance of biogeographical study of India's natural heritage. The first initiative to classify the forests of India was done by Champion in 1936 and revised by Seth in 1968.

2. SNAKES – THE BIG FOUR:

Snakes (suborder Serpentes) also called serpents are a group of elongated, limbless and carnivorous reptiles found in every continent except Antarctica, and on most smaller land masses; exceptions include some large islands, such as Ireland, Iceland, Greenland. Snakes exhibit a wide range of characteristics and adaptations, making them one of the most fascinating creatures on earth. Like all other reptiles, snakes are ectothermic, amniote vertebrates covered in overlapping scales. Snakes have a very good eye -sight and they continually flicker their tongue to inspect their surroundings, they lack voice but are

capable of hissing. Most of the species of snakes are terrestrial /live in burrows but some species are arboreal or aquatic (freshwater and marine), tree dwelling etc.

The class Reptilia consists of 520 genera and more than 3000 species of snakes. Most species of snake are non-venomous and those that have venom use it primarily to kill and subdue prey rather than for self-defence. Some possess venom that is potent enough to cause painful injury or death to humans.

The Indian subcontinent is a home to more than 300 species of snakes out of which 60 species are medically important(Whittaker R and Captain A 2004). India bears the guilt of being the snakebite capital of the World, having annually 50,000 deaths and more than 1.5lakh morbidities/disability (Suraweera et al.,2020). The big four are the main culprit for all these envenomations. The BIG FOUR snake species of India are

1. Indian Spectacled Cobra *(Naja naja*): The king cobra or *Naja naja* also known as the spectacled cobra and one of the Big Four snakes of India possesses highly potent neurotoxic and cardiotoxic venom, belongs to family Elapids.This cobra species can easily be identified by its relatively large and quite impressive hood, which expands when threatened. *Naja naja* shows a wide Indian distribution but they may not occur in some parts of Assam, Kashmir or above the altitude of 2,000 meters.The cobra inhabits a wide range of habitats throughout its geographical range. It can be found in dense or open forests, plains, agricultural lands (rice paddy fields, wheat crops), rocky terrain, wetlands.

Naja naja are oviparous and lays eggs in the months of April and July and at a time can lay as many as10 to 30 eggs. Indian cobra or spectacled cobra make their homes in burrows, termites mounds, tree hollows and small animal dens.Symptoms of envenomation include pain, headache, blurred vision, nausea and vomiting, breathing problems, paralysis, and in some cases respiratory failure and cardiac arrest.

Figure 1a: *Naja naja* or king Cobra or Spectacled cobra (photo courtesy Ajay Kartik)

2. Russell's Viper (*Daboia russelii*): Russell's viper is one of the most venomous snake species prevalent in India and also known as the "Indian chain viper" because of the chains like patterns covering its entire body. Russell's viper possesses a very potent hemotoxic,myotoxic and cytotoxic venom, a major culprit of deaths and disability in India(Suraweera et al 2020).This snake has a near pan-India distribution. Russell's viper is terrestrial and ovoviviparous.

The initial symptoms of envenomation are severe pain, bleeding from gums, sputum and in urine, followed by swelling of the affected extremity. In cases of severe envenomation it can lead to disseminated intravascular coagulation.

Figure 1b*: Daboia russelii* or Russell's viper (picture courtesy Kartik Sunagar)

3. Common krait or *Bungarus caeruleus*: The common krait or *Bungarus caeruleus* is one of the Big Four snakes of India also known as the Bengal krait. It has the distinctive long cylindrical body, black-blueish in colouration with white bands running all over the body. It has a wide spread distribution in India from Sindh to West Bengal. It lives in a wide variety of habitats, from fields and low scrub jungle to settled areas.

It belongs to the family elapids and possesses a very potent neurotoxin which induces muscle paralysis. Clinically, its venom contains presynaptic and postsynaptic neurotoxins, which generally affect the synaptic cleft. Frequently, little or no pain occurs from its bite, which can provide false reassurance to the victim. Typically, victims complain of severe abdominal cramps, accompanied by progressive paralysis.

Figure 1c*: Bungarus caeruleus* or common Krait (Picture courtesy Vivek Sharma)

4. Saw-scaled viper or *Echis carinatus:* *Echis carnitaus* or the Indian saw scaled viper is the smallest member of the Big Four snakes of India.They possess a highly potent haemotoxic venom, of the more dangerous systemic symptoms, hemorrhage and coagulation defects are the most striking. Hematemesis, melena, hemoptysis, hematuria and epistaxis also occur and may lead to hypovolemic shock. In some cases, kidney dialysis is necessary due to acute kidney injury (AKI).

Figure 1d*: Echis carinatus* or Indian saw-scaled viper (picture courtesy Vivek Sharma)

3. Daboia russelii or Russell's Viper (Indian chain viper)

Daboia russelii or Russell's viper is one of the most venomous snakes of India and a part of 'The Big Four' snakes of India. It belongs to the class of Viperidae native to the Indian sub-continent. This species of viper shows a pan -India distribution (found in nearly all states of India , but is uncommon to rare in the Gangetic plains, northern Bengal and Assam).

In the coming part we will discuss the physical features, habitat, distribution, venom composition, symptoms, reproduction, antivenom treatment, prey and behaviour of Russell's viper in more depth and broader scale.

Figure 2*: Daboia russelii* or Indian chain viper (pictures courtesy Vivek Sharma)

Description/Morphology: Russell's viper has a deep brown, yellow tan colouration with circular patterns running all over the body. These rings are black at the centre and the intensity shifts to yellow or

white at the outer end. They resemble or give a chain like appearance, therefore the name Indian chain viper. The head has a pair of distinct dark patches, one on each temple, together with a pinkish, salmon, or brownish V or X marking that forms an apex towards the snout. Behind the eye is a dark streak, outlined in white, pink, or buff. The center is white, whitish, yellowish, or pinkish, often with an irregular scattering of dark spots.

The viper grows to a maximum body length and tail length of 160 cm and average about 120 cm. It is much slender then most vipers.

The head of Russell's viper, its key feature of identification, is triangular in shape and distinct from the neck. The snout is blunt, rounded, and raised. The nostrils are large, each in the middle of a large, single nasal scale. The body is stout, the cross-section of which is rounded to circular. The dorsal scales are strongly keeled; only the lowest row is smooth. Mid-body, the dorsal scales number 27–33. The ventral scales number 153–180. The tail is short about 14% of the total length with the paired subcaudals numbering 41–68.

***Distribution and Habitat:** This species is very abundant in all of the Indian subcontinent. Russell's viper is found in India, Sri Lanka, Bangladesh, Nepal and Pakistan. Populations from South-East Asia previously assigned to this species are now considered to be part of a different species, *Daboia siamensis. Daboia russelii* has a Pan – India distribution. It is present in abundance in the state of Punjab, very common along the west coast and its hill, southern India especially in Karnataka and Tamil Nadu. It is rare to uncommon in Gangetic plains, Northern Bengal and Assam.Russell's viper is not restricted to any particular habitat but it tends to avoid dense forest, prefers plains, grasslands or bushy areas, farmlands, coastal lowlands and hills or suitable habitat. Habitat /areas of high humidity are avoided like marshes, rainforest and swamps.

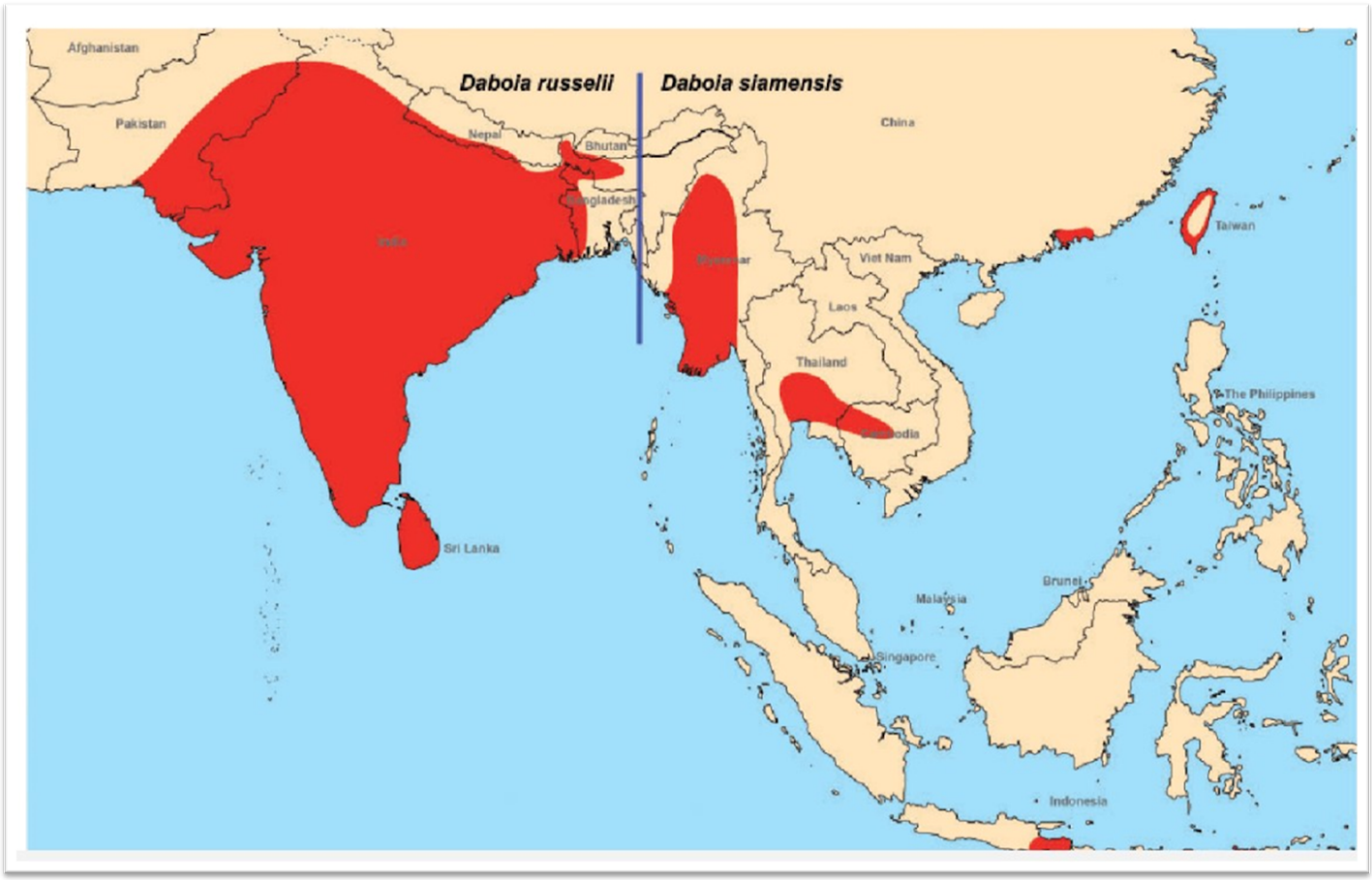

Figure 3: The distribution of two species of *Daboia (Daboia russelii and Daboia siamensis* , C N Antonypillai et al 2010)

***Behaviour/Ecology:** *Daboia russelii* or Russell's viper are terrestrial.

a. **Reproduction:** Russell's viper is ovoviviparous. Mating generally occurs early in the year and the birth takes place around June-July. The gestation period is 6 months. The litter size is 20-40.
b. **Prey:** It majorly feeds on small rodents, although especially it will also eat small reptiles, land crabs, scorpions, and other arthropods. Juveniles are crepuscular, feeding on lizards and foraging actively.

***Venom/Symptoms/Treatment**: Venoms are secretory cocktail /mixture of different components which can be enzymatic or non-enzymatic proteins, used by organisms for different purposes like defence (bees, wasps, toads) or prey capture (snakes).

Daboia russellii possesses a very potent hemotoxic venom. Bleeding in gums, urine and sputum are common symptoms. The blood pressure drops, and the heart rate falls. Blistering occurs at the site of the bite, developing along the affected limb in severe cases. Vomiting and facial swelling occur in about one-third of all cases. Severe disseminated intravascular coagulation (formation of blood clots throughout the body) also occurs in cases of severe envenomations.

The venom of Russell's viper is a composition of different toxin superfamilies like PLA_2, SVMPs, SVSPs, LAAO, NGF, CTL, CRISP, C-3FTx, N-3FTx, VEGF, disintegrin, snaclec, kunitz. Out of which PLA_2 is present in the highest amount and is briefly explained in the coming sections. The local tissue damage is usually restricted to the region of bite but in extreme cases can spread all across the body. Kidney failure (renal failure) also occurs in approximately 25–30 percent of untreated bites.

In later studies it was discovered that the people who survived the bite of *Daboia russelii* suffered severe damage to their pituitary gland causing hypopituitarism. In India a polyvalent antivenom is produced against The Big Four snakes of India, antivenom is the primary treatment of snakebite. The methodologies for the production has not changed since the dawn of a decade, animals like horses are hyper immunised with the venom at sublethal and subtoxic concentration. In the end the blood is pooled/purified for antibodies. The efficacy of conventional antivenom is restricted to the immunogenic potential of venoms used in its manufacture and the concentration of antibodies present in the animal serum (N.Kaur et al 2021). For the production of antivenom the venom is collected from only one population of 'Big Four' found in the districts of Tamil Nadu, which increases the chances of intra population variation. A single polyvalent antivenom is produced from the venom of 'Big Four' snakes of India neglecting the other medically important snakes which are capable of causing serious envenomation or harm to humans. This leads to great loss to livelihood.

Insights to *Daboia Russelii* Venom composition:

Venom is a secretory cocktail composed of different 'exo-physiological' proteins and peptides (collectively 'toxins') that interfere with the functioning of target molecules in key regulatory pathways (Surnase V et al 2022).Venom or toxins are used by organisms for survival (Protection from predators) or prey. Composition of venom varies on the basis of different abiotic or biotic factors. Abiotic factors being geographic variations, temperature, seasonal changes, precipitation and altitude whereas biotic factors include prey availability, competition among species and mutations. Snake venom is composed of 7 toxin super families, both enzymatic and non- enzymatic proteins. The in -depth description of *Daboia russelii* venom composition is discussed in the following.

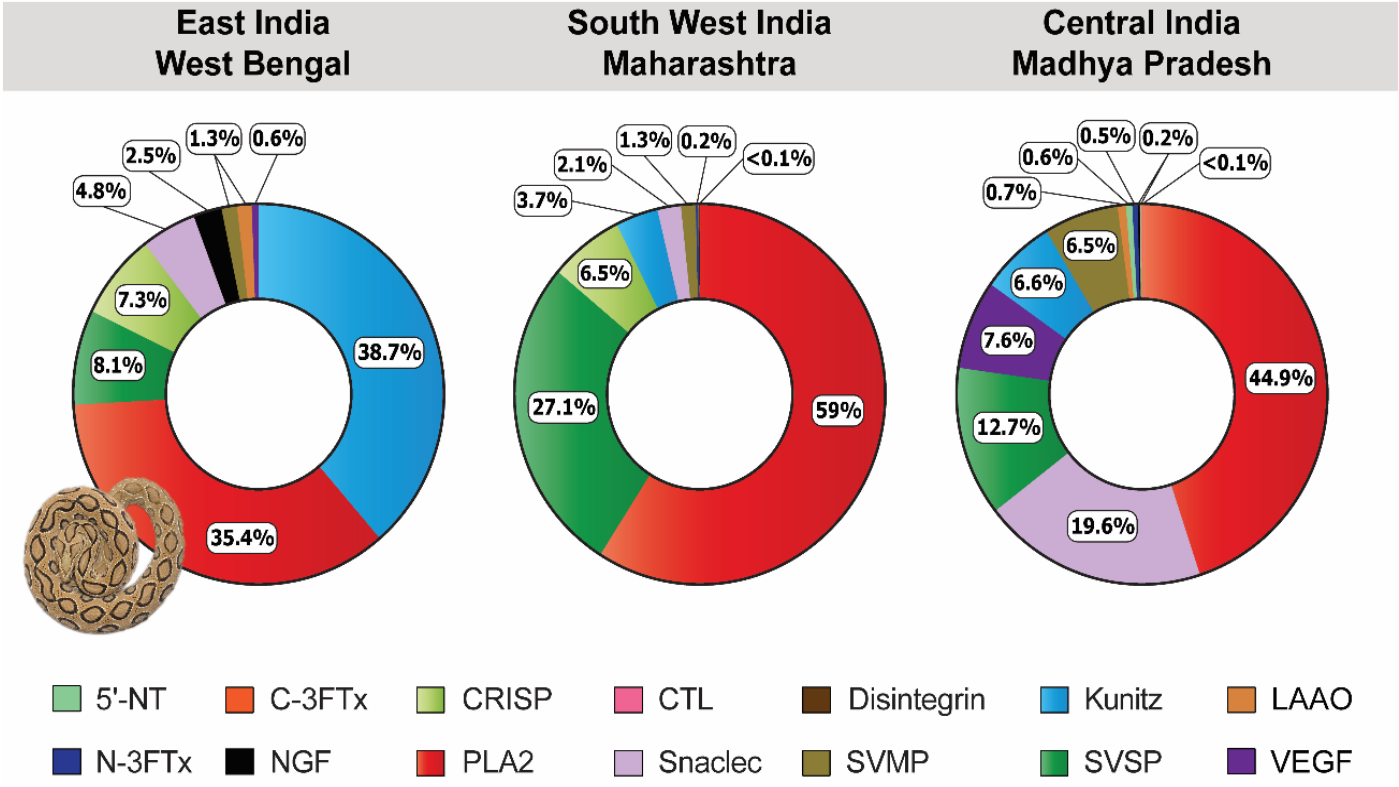

Figure 4: Proteomic composition of *Daboia russelii* venom from different states (Senji et al 2021)

1. PLA_2 or Phospholipase A_2: They are the enzymatic toxin superfamily of proteins that are present in the highest concentration *in Daboia russelii* venom. Apart from being the catalytic enzyme they also elicit many pharmacological effects that play a vital role in snakebite related damage.

PLA_2 belongs to a family of proteins that catalyse the hydrolysis of phospholipids by cleaving the 2-acyl ester linkage of 3-sn-phospholipids in a calcium-dependent manner. As phospholipase acts on the phospholipids present in the cell membrane, arachidonic acid is released from the cell membrane disproportionately. This leads to initial symptoms like internal bleeding, pain and disintegration of cell membrane. $PLA_2$is a major culprit of different pathophysiological symptoms like cytotoxicity, myotoxicity, neurotoxicity, inflammation and anticoagulatory effects (Surnase V et al 2022).

The pain caused by phospholipase is primarily influenced by inflammatory reactions and the activation of sensory neurons. One significant player in the inflammatory pain triggered by phospholipase is bradykinin, which has been highlighted in studies by Moreira et al. (2014), Urs et al. (2014), Mamede et al. (2016), and Zambelli et al. (2017b).

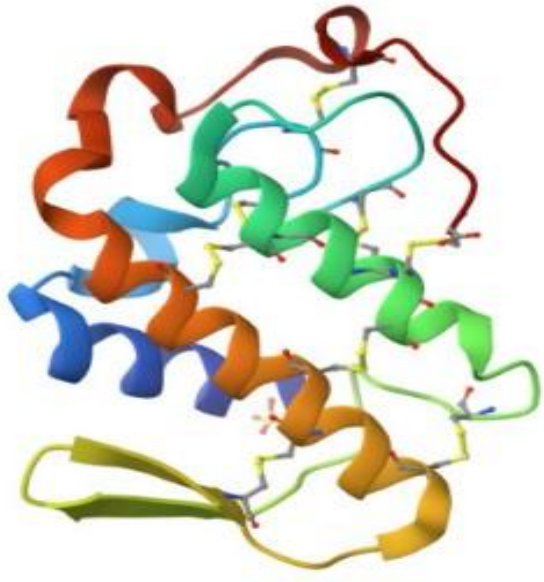

Figure 5a) 3-D structure of *Daboia russelii* Phospholipase (P59071 · PA2B8_DABRR from PDB)

2. **SVMP or Snake Venom metalloproteinases:** SVMP or snake venom metalloprotease are a group of enzymatic zinc rich proteins that can induce a large number of pathophysiological conditions like ability to induce hemorrhage, proteolytic degradation of fibrinogen and fibrin, induction of apoptosis and inhibition of platelet aggregation.

Adam-G-family is the human counterpart or precursor of SVMP. It belongs to a family of transmembrane proteins called type 1, which consists of subunits including metalloproteinase and disintegrin. This family plays a vital role in facilitating cell-cell communication, promoting cell adhesion, and facilitating signal transmission within the body (Seegar TC et al, 2019).

SVMP, or snake venom metalloproteinase, has been categorized into three groups based on its structure and domains. P-1 SVMP consists solely of a catalytic metalloproteinase domain, which is rich in zinc. P-2 SVMP exhibits a slightly more intricate structure, featuring both a catalytic metalloproteinase domain and a disintegrin domain. P-3 SVMP showcases a more diverse structure, incorporating three domains: metalloproteinase, disintegrin, and cysteine-rich domains.

During a snakebite, SVMP interacts with fibrinogen present in the extracellular matrix (ECM) of the human body. This interaction results in the disruption of the ECM, proteins, platelet aggregation, and programmed cell death (apoptosis).

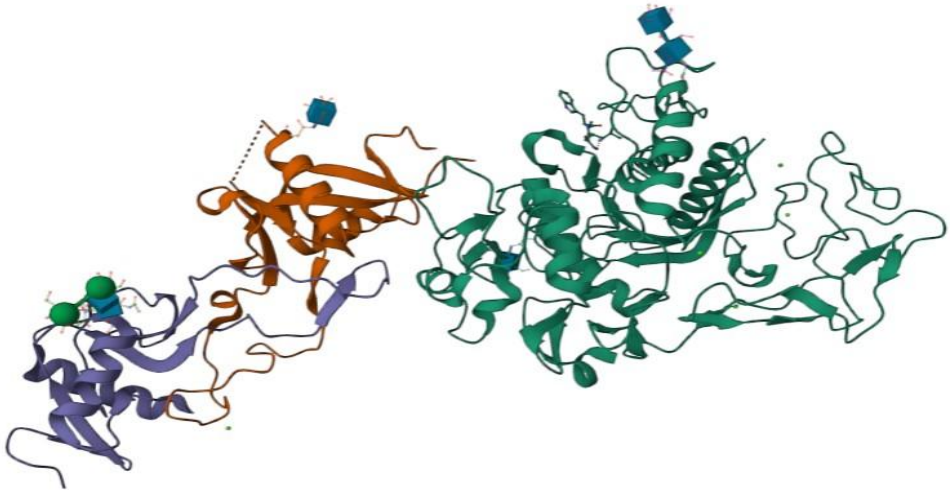

Figure 5b) Daboia russelii SVMP 3-D structure (source PDB)

3. SVSPs or Snake Venom Serine Proteinases: Snake venom serine proteases belong to the S1 family of serine proteinases and have two structural distinct domains.

SVMPs have their origins in kallikrein-like serine proteases, and after being recruited for use in the venom gland, they have undergone gene duplication events, resulting in the emergence of various isoforms (Fry et al., 2008; Vaiyapuri et al., 2012). These venomous proteins can inflict substantial damage to capillary vessels. Their main toxicity lies in disrupting the hemostatic system of their victims, leading to the development of edema and increased sensitivity to pain (hyperalgesia), although the underlying mechanisms are not yet fully understood.SVSPs exert their harmful effects by affecting various aspects of blood function, including blood clotting, edema formation, fibrinolysis (breakdown of blood clots), platelet aggregation, and blood pressure regulation. They achieve this by catalyzing the cleavage of polypeptide chains at specific sites following positively charged or hydrophobic amino acid residues (Page and Di Cera, 2008; Serrano, 2013).

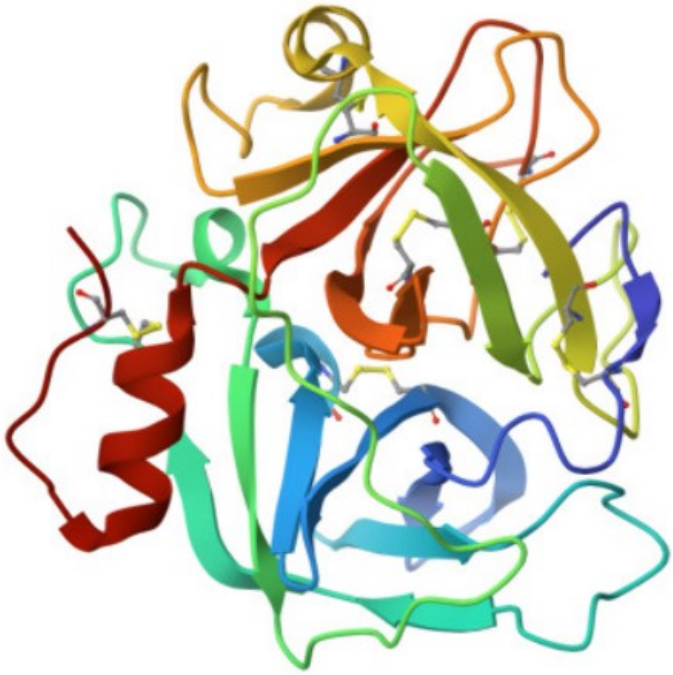

Figure 5c) *Daboia* SVMP or Snake venom serine proteinase (P18965 · VSPG_DABSI from PDB)

4. Kunitz type serine protease inhibitors:Kunitz-type inhibitors are a group of serine protease inhibitors that exist in the venoms of snakes belonging to the Elapidae and Viperidae families (Tasoulis T., Isbister et al .,2017) . These inhibitors are believed to disrupt the balance within the prey's body, particularly by interfering with the blood clotting process (Mukherjee A.K., Mackessy S.P. et al.,2014). Researchers have conducted proteome analyses on various snake venoms and have observed a considerable presence of Kunitz-type inhibitors. For example, in the venom of the *Daboia russelii* russelii snake found in Pakistan, these inhibitors make up around 28% of the venom's protein content(Mukherjee A.K.,et al 2016). It's worth noting that a single snake species can produce multiple variations of Kunitz-type inhibitors(Munawar A.,et al.,2011).

Kunitz-type inhibitors are divided into two major groups: non-neurotoxin (trypsin and chymotrypsin inhibitors) and neurotoxin (potassium and calcium blockers) snake venom Kunitz-type inhibitors (Zupunski V., Kordis D., Gubensek F.et al.,2003).

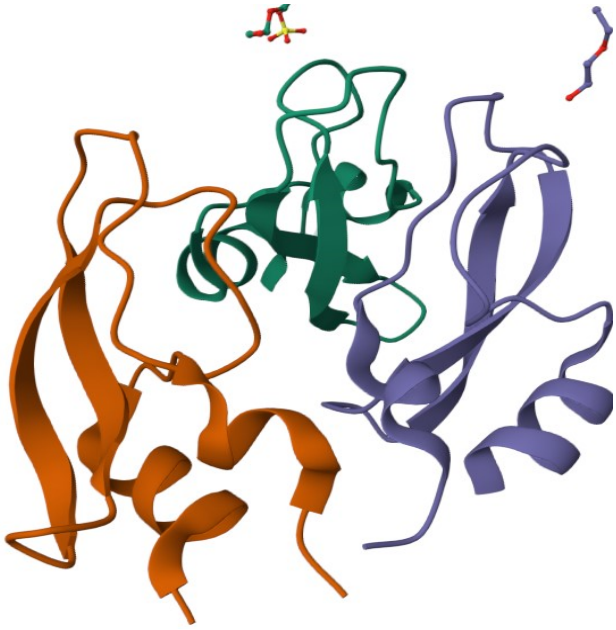

Figure 5d: Kunitz type serine protease inhibitor from the Australian common brown snake(Q90WA1 · VKT1_PSETT source PDB)

III. Literature review

Antivenom -Production, Problems with the First Generation Antivenom

India is home to more than 300 species of snakes (Whitaker and Captain 2004) out of which 60 are medically important. In India annual deaths caused by snake deaths 58000 and disability 1,40,000(Suraweera et al.,2020). This makes India the snakebite capital of the world. Our nationally representative mortality study documents about 1.2 million snakebite deaths from 2000 to 2019 (Suraweera et al 2020). Most of these cases come from the rural areas where there are no proper hospitals or proper treatment. Most of the time these patients have to travel long distances to get proper diagnosis or medication, which significantly increases the risk of disability or even death.

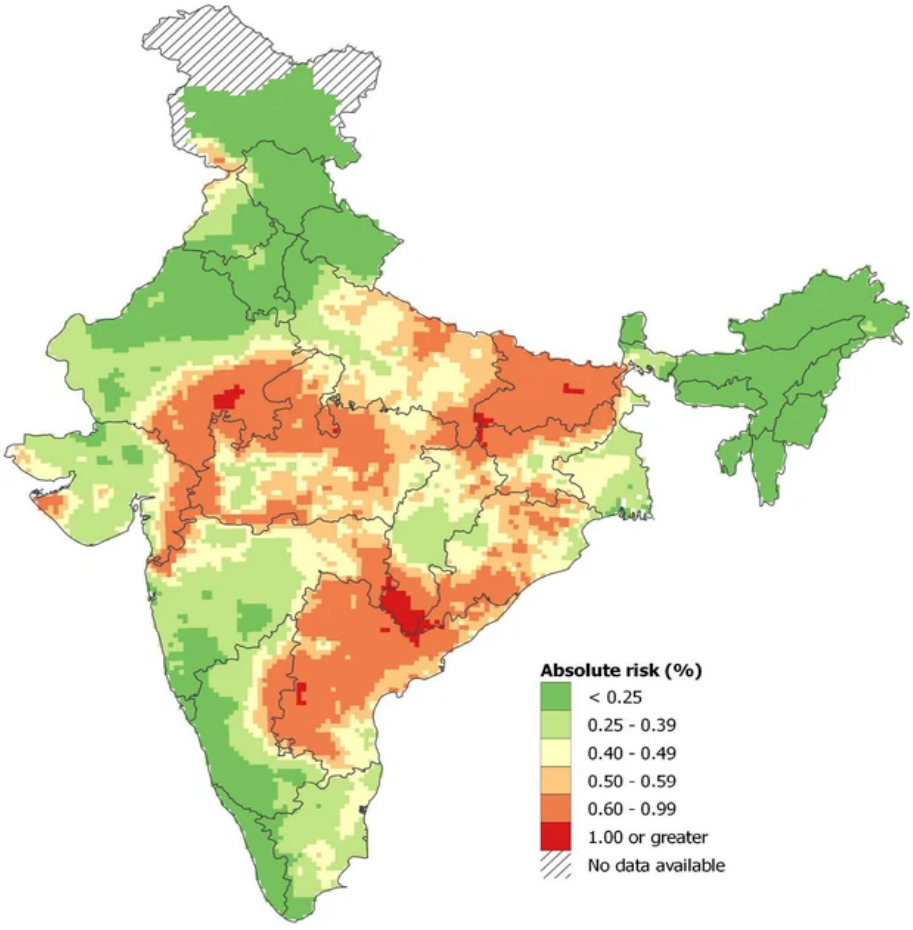

Figure 6: Spatial distribution of snakebite mortality risk in India for 2004-13(Suraweera et al 2020)

Antivenom is the sole solution for snake envenomation, whose methodologies haven't changed since a decade. Antivenom are highly purified antibodies produced by hyperimmunization of equines by sublethal and subtoxic doses of venom. The efficacy of the antivenom depends upon the immunogenic potential of the venom along with the venom composition. Venom composition within the same species has significantly shown major variations along the biogeography (Senji et al 2021). This exhibits a dramatic inter and intra population or specific variability and reduction in the efficacy of antivenom. For the production of antivenom the venom is only collected from one district of Tamil Nadu from the Big-Four snakes of India. In India a polyvalent antivenom is produced against the 'The Big Four' snakes. Apart from the big four there are numerous medically neglected medically important species that have the potential to inflict serious envenomation, *E. c. sochureki, B. sindanus, B. fasciatus* and *N. kaouthia*. There is no antivenom against these neglected species, for their envenomation the polyvalent antivenom is administered. Since this antivenom isn't produced against the neglected species resulting in serious damage and even death of the patient. The serum of animals hyperimmunized with the crude venom often contains other antigens apart from the antibodies, resulting in the contamination in the finished product. Sometimes the antibody percent in the antivenom can be as low as 15% which results in decreased efficiency of the antivenom. In some cases the body shows allergic reaction towards the antivenom or components present in the antivenom; this leads to a serious allergic response by the immune system and can cause systemic anaphylactic shock. All these issues account for modifications, development and more research in the field of antivenom production to increase the effectiveness and efficacy of the conventional antivenom or 'The Second Generation Antivenom'.

Scientists are actively researching recombinant antivenoms as potential alternatives to traditional antivenoms. These recombinant antivenoms offer advantages such as enhanced potency, broader effectiveness against different snake species, and improved cost-effectiveness. However, it is important to acknowledge that these advanced antivenoms are still a considerable time away from being accessible for treating snakebite victims, possibly taking several years to a decade to become available.Therefore, alongside the development of recombinant antivenoms, it is crucial to focus on enhancing the efficacy of existing antivenoms. This ensures that snakebite treatment remains effective and accessible in the

immediate future. The efficacy of conventional antivenom can be enhanced by using chromatographic purification of the antivenom in bulk.This resulted in the enhanced performance of the conventional antivenom in in-vitro as well as in-vivo preclinical assays(: Attarde, S.; Iyer, A.; Khochare, S.; Shaligram, U.; Vikharankar, M.; Sunagar, K.et al.,2022).

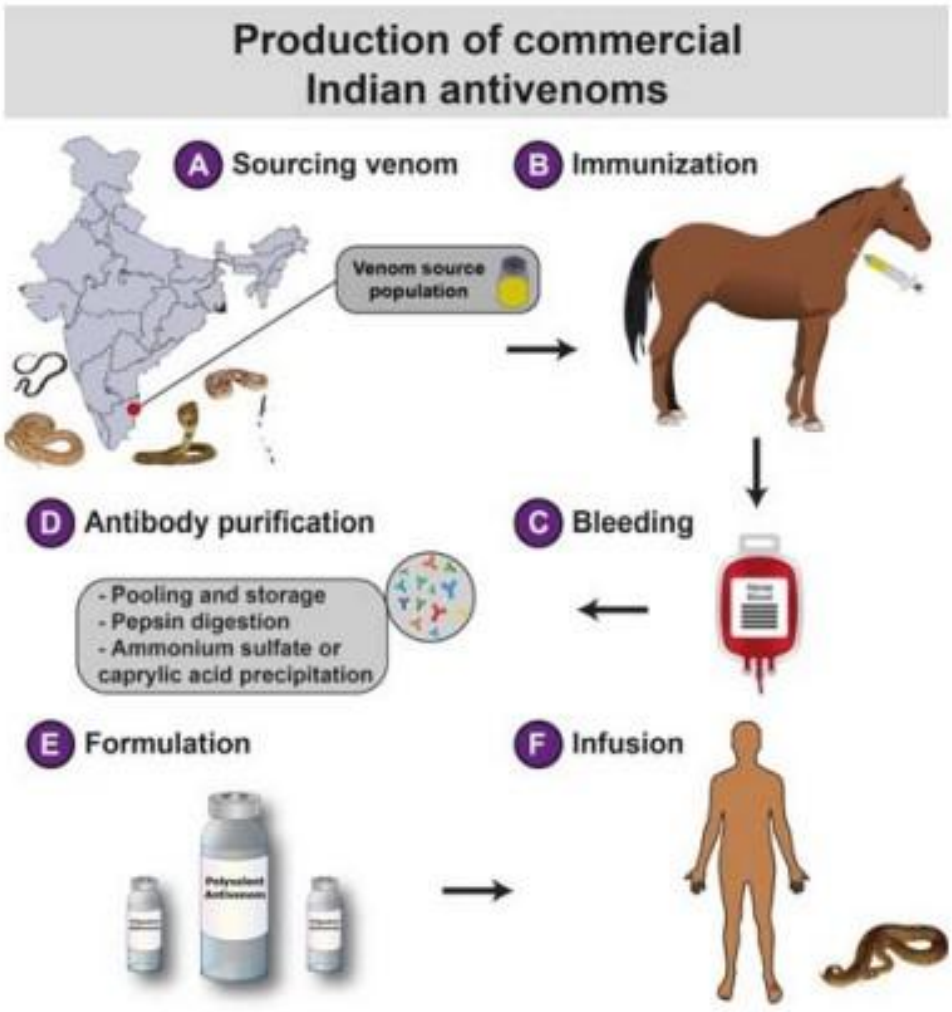

Figure 7: Production of Conventional Indian Antivenom (N.Kaur et al 2021)

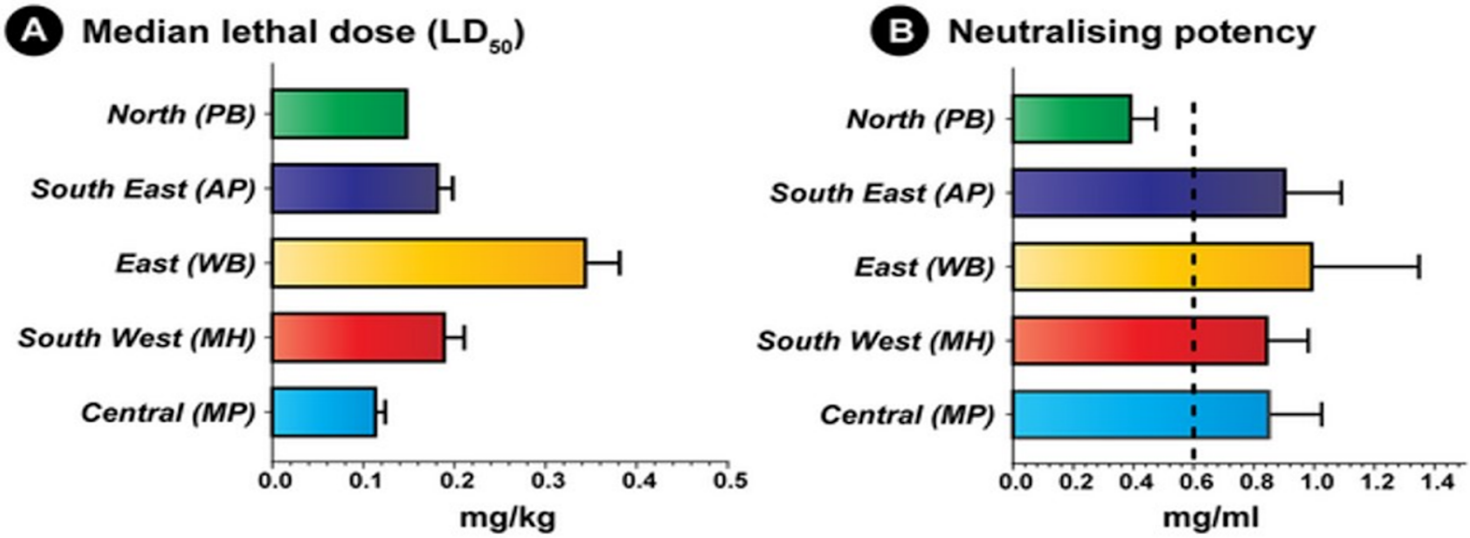

Figure 8: Venom potencies of the pan-Indian populations of *D. russelii* and the neutralisation potencies of the Premium Serums antivenom (Senji Laxme RR et al., 2021) .

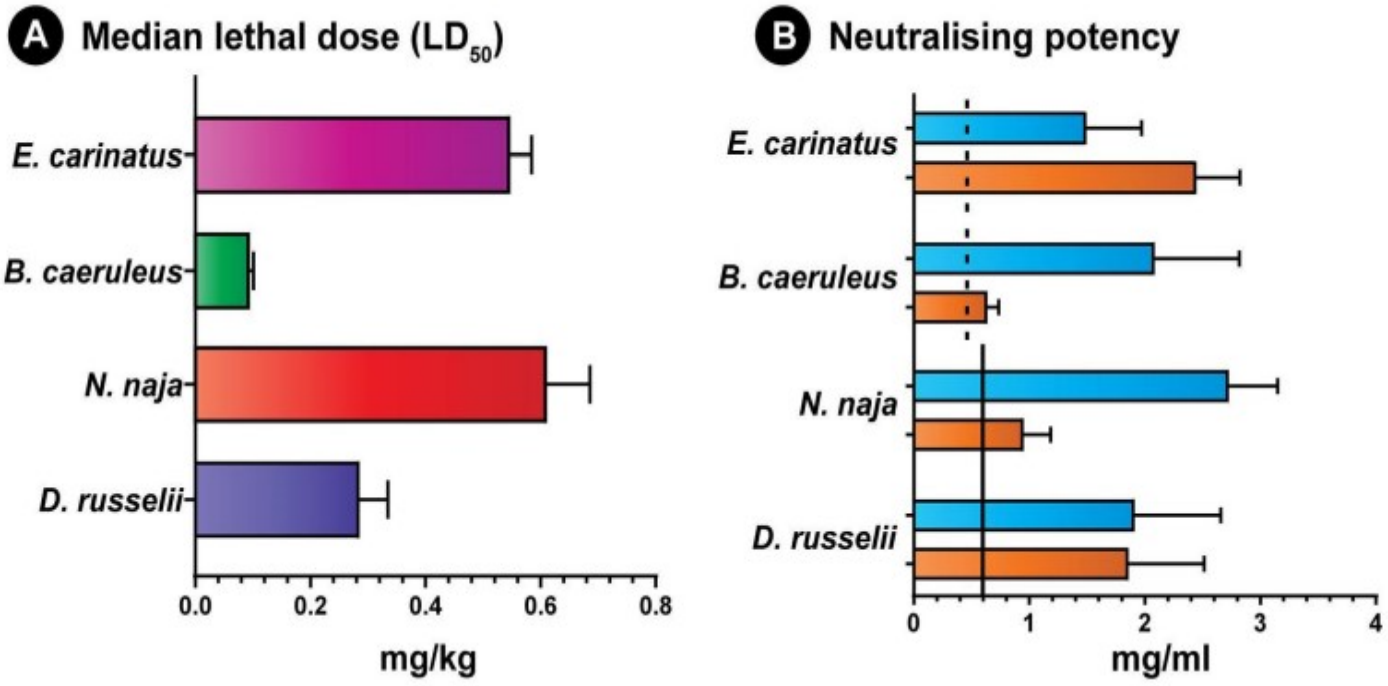

Figure 9 : (A) Venom potencies of the 'big four' snakes from Tamil Nadu (B)The neutralization potencies of second generation antivenom (Blue) and conventional antivenom (Orange) against the "The Big Four" snakes(.Attarde, S.; Iyer, A.; Khochare, S.; Shaligram, U.; Vikharankar, M.; Sunagar, K.et al.,2022)

Ancillary Snakebite therapy. What can be the potential agents?

Ancillary snakebite therapy refers to the development of additional medications used alongside the primary treatment for snakebites, with antivenom being the primary form of treatment. However, it is disheartening that traditional antivenom fails to effectively counteract the local tissue necrosis or cytotoxicity caused by the venom of the Russell's viper or *Daboia russelii*. This unfortunate situation often results in severe disability or even amputations. Compounding the problem is the fact that many affected individuals come from rural backgrounds and belong to the working class.

Considerable research efforts are currently focused on exploring potential ancillary treatment options for snakebites. Scientists have made promising discoveries in the form of compounds, polymers, and peptides that show potential for serving as complementary therapeutic approaches.

For generations, local healers in rural areas have relied on plant-based remedies and concoctions to combat snakebites. These medicinal plants have been found to possess a range of immunological activities. Interestingly, many modern-day medicines are derived from secondary metabolites found in plants. Secondary metabolites are compounds produced by plants that may not play a significant role in biochemical processes but serve secondary functions such as protection against grazers and attraction of pollinators. Phytochemicals, which are chemicals produced during primary or secondary metabolism, are responsible for shielding plants from fungal, bacterial, and viral infections.Plants contain several significant phytochemicals, including Phenolics, Terpenoids, Flavonoids, Isoflavones, Curcumin, Isothiocyanates, Carotenoids, and various aliphatic compounds. Among these, Phenolics exhibit antioxidant properties and effectively inhibit the reactive oxygen species generated as a result of snake venom components. The utilization of plant-based therapies offers numerous advantages. They are associated with minimal side effects, specifically target the local tissue necrosis caused by snakebites, remain stable at room temperature, and can be easily stored without requiring specialized facilities. Furthermore, these therapies can be administered conveniently, allow for mass production with simplified upstream and downstream processing, and are generally considered more ethically acceptable.

***Cryptolepis buchananii* and *Kalanchoe pinnata* :**

Extensive studies were conducted, literature reviewed, meetings with local healers were conducted to learn more about these medically important. After considering a lot of aspects two plants were selected for testing their potentials against the *Daboia russelii* venom. These plants were collected with the help of tribal people. These were selected on the basis of availability in the region, seasonal availability, use by the local healers and ease of downstream processing. The two plants selected were

- *Cryptolepis buchananii(*Indian wax leaved climber)
- *Kalanchoe pinnata (*Bryophyllum)

Cryptolepis buchananii is a large climber shrub from family Asclepiadaceae having large glabrous leaves, its common name is Indian wax leaf climber or Karanta in Hindi.Since ancient times this plant has been used and mentioned in Indian Ayurveda. This plant bears small pale greenish coloured flowers in short axillary panicled cymes and bracteate. This plant shows tropical and subtropical distribution across the Indian subcontinent. It generally grows in moist, damp and shady places, and needs support for climbing and crawling. It also grows well in warm tropical weather with well distributed rainfall. This plant is rich in

- alkaloids, buchananine identified as 6-Isonicotinoyl-alpha-glucopyranose and 1,3, 6-o tri nicotinoyl -L-glucopyranose
- Cryptolepian is a stable glycosylated serine protease with a molecular weight of 50.5 KDa. It has 15 tryptophan residues, 41 tyrosines and 8 cysteine residues forming four disulphide bridges. This component has carbohydrate residue of 6-7%.(Pande M, Dubey VK, Yadav SC, Jagannadham MV et al., 2006)
- Along with all these a cardenolide name Cryptosin is also found in the leaves of *Cryptolepis buchanani.*

This plant has a variety of therapeutic uses, roots of this plant are used as a demulcent, tonic is useful in loss appetite, fever and skin diseases. It is considered an excellent blood purifier and extensively used in skin diseases, leprosy and prescribed to children having rickets. This plant is also used as a remedy for fractured bone by the tribal people of Arunachal Pradesh and as an antivenom by the tribal people of Uttar Kannada for domestic animals. Apart from these medicinal uses it shows antibacterial potential (C.

Sittiwet and D. Puangpronpitag et al 2009), Analgesic, Anti-Inflammatory, and Chondroprotective potentials (Hanprasertpong N et al 2014) and antimicrobial potential of the root extracts (Priyanka, Ajay Singh Bisht and Divya Juyal et al 2018).

Figure 10*)* *Cryptolepis buchanani*i or Indian wax leaved climber (Source Useful Tropical Plants)

Kalanchoe pinnata, commonly known as cathedral bells, air plant, life plant, miracle leaf of Bryophyllum is a common ornamental.It is a popular house plant and has become naturalized to the tropical and subtropical areas.This plant is distinctive and well known for miniature plantlets that grow on the margins of the phylloclades. It is a succulent, perennial plant with fleshy cylindrical stems and young leaves that has a reddish tinge.

Kalanchoe is rich in alkaloids, triterpenes, glycosides, flavonoids, steroids and lipids. The leaves contain a group of chemicals called bufadienolides which are very active and have sparked the interest of scientists. They are very similar in structure and activity as two other cardiac glycosides, digoxin and digitoxin. Bryophyllum is rich in fatty acids, amino acids and minerals.

- Fatty acids: Palmitic acid, stearic acid, arachidic acid, behenic acid, oxalic acid, citric acid, malic acid, succinic acid, oxaloacetate, isocitric acid.

- Amino acid: ascorbic acid, riboflavin, thiamine, niacin, pyridoxine, glycine, cysteine, casein hydrolysate, glutamic acid, protein hydrolysate, methionine, tyrosine, phenylalanine.
- Mineral elements: Na, Ca, K, P, Mg, Mn, Fe, Cu, Zn.
- Sugars: raffinose, lactose, sucrose, glucose, galactose, fructose.

Bryophyllum displays a variety of therapeutic characteristics and is used to create a variety of medications in Ayurveda. Bryophyllum is said to be highly beneficial for kidney stones, urinary disorders, migraines, hemorrhoids, menorrhagia, wound, cuts, boils, ulcers, diarrhea, vomiting, blood vomiting. Along with these it shows antivenom potential (Fernandes JM et al 2016), antibacterial properties (Akinpelu Da et al 2000), antioxidant (Tatsimo, S.J.N., Tamokou, J.d.D., Havyarimana, L. et al 2012), antifungal and immunomodulatory activity (Jacinta Edesiri Okpoho, L. Evbuomwan, Fortune Itojie Ebiala et al 2018).

Figure 11*)* *Kalanchoe pinnata* or Bryophyllum (Source Useful Tropical plants)

IV. OBJECTIVES

Determination of anti-ophidic potentials of *Cryptolepis buchananii* and *Kalanchoe pinnata* plant extracts against hemotoxic venom, *Daboia russelii.

The aim of this study is to find a novel ancillary treatment against *Daboia russelii* envenomation. *Daboia* being the member of big four causes the highest number of deaths and morbidities annually. As discussed previously *Daboia russelii* shows major intra-population variation in the venom composition which leads to reduced efficacy of the conventional antivenom. The production of antivenom is a challenging task for which the venom is collected only from one region of India. It doesn't help that conventional antivenom has no restorative effect against the local tissue necrosis caused by the cytotoxicity of *Daboia russelii* venom. This thesis is dedicated to finding plant based snakebite therapy, isolation and characterisation of these compounds that can be a potential ancillary snakebite therapy.

V. MATERIALS AND METHODS

1. Venom

Venom was collected from rescued snakes from the districts of Tamil Nadu and Karnataka under the supervision and permission of the Forest Department. Venom was lyophilized and kept at -80 degree centigrade. Required amount of venom was weighed (10mg/1mg) and resuspended in HPLC grade water. Aliquots of the venom were stored at -20 degree centigrade to remove debris.

2. Protein estimation in the Venom samples : A standard curve with the help of BSA (Bovine serum albumin)and Bradford assay (Bradford MM et al 1976)was prepared. For the standard curve 1mg/ml of BSA was taken and using dilutions in increasing order (2,4,6,8 and 10 ul). The OD was measured at 595 nm in an EPOCH 2 microplate spectrophotometer (BioTek Instruments, Inc., United States). This curve is used for the estimation of protein concentration in the Venom samples.

3. Sample preparation /Plant extracts :

***Cryptolepis buchananii* or Indian wax leaf climber:**Fresh leaves were collected and dried in an incubator at 37 Degree Celsius for 2 weeks until completely dried. After complete loss of moisture from the leaves, crushed in pestle and mortar till a fine consistency was achieved.

* Exactly 3g of dried plant extract was suspended in 36 ml of water to make a stock solution of 80 ug/ul. Sample was subjected to centrifugation at 9000 rpm for 15 mins. The supernatant was collected and stored at -20 Degree Celsius

.*Exactly 3g of dried plant extract was suspended in 26 ml of absolute ethanol(100%) to make a stock solution of 100 ug/ul. Further the sample was subjected to centrifugation at 9000 rpm for 15 mins. The supernatant was collected and stored at -20 degree celsius.

***Kalanchoe pinnata* or Bryophyllum:**Fresh leaves were collected and dried in an incubator at 40 Degree Celsius for 3 weeks until completely dried. After complete loss of moisture from leaves were crushed in pestle and mortar till a fine consistency was achieved.

*Exactly 1.8g of plant extract was suspended/mixed in 40 ml of HPLC grade water to make a stock solution of 45 mg/ml.

*Exactly 1.8g of plant extract was suspended in 30ml of Ethanol (absolute) to make a stock solution of concentration 60 mg/ml.

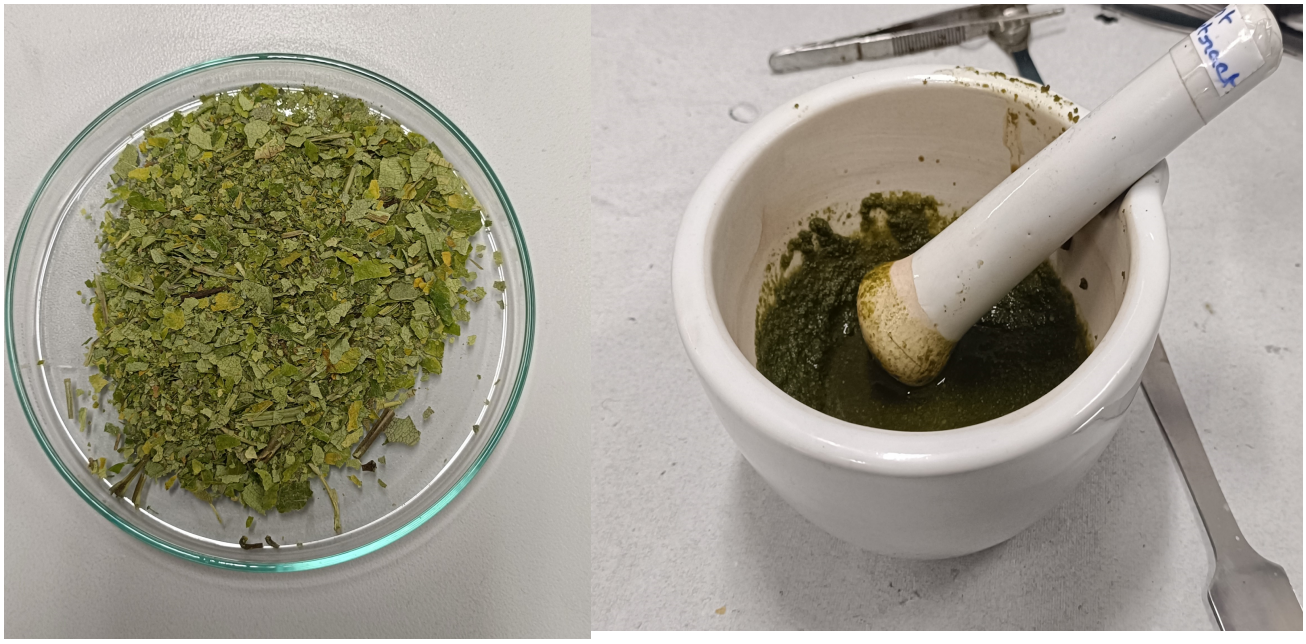

Figure 12: Dried plant samples were subjected to crushing in pestle and mortar till a fine consistency

Before every assay , eppendorf with a particular concentration of plant extract was prepared. The plant extract in the ratio (50,100,200 and 500ug) was aliquoted from the main stock solution. After this the sample was heat fixed at 100 degree celsius for 10-15 mins. For each assay according to protocol different concentrations were used. This was done to

1.Heat, fix the sample in the tube properly and remove the solvent.

2.To destroy the endoproteases present in the plant sample as phytochemicals can endure even high temperatures.

4. *Daboia russelii* Venom Protease Inhibition Assay

Cryptolepis buchananii: Proteolytic activity was assayed with the help of Azocasein as a substrate. For inhibition studies 10ug venom protein from *Daboia russelii* (DaRuKA10 10 mg/ml) was preincubated with plant extracts at increasing concentration (50,100,200,500ug/ul) at 37 Degree Celsius for 30 mins. One unit of enzyme activity is defined as the concentration of plant extract at which the venom activity is least. The absorbance was taken /read at 440 nm in an EPOCH 2 microplate spectrophotometer (BioTek Instruments, Inc., United States)

Bryophyllum: Proteolytic activity was assayed with the help of Azocasein as a substrate. For inhibition studies 10ug venom protein from *Daboia russelii* (DaRuKA10 10 mg/ml) was preincubated with plant extracts at increasing concentration (50,100,200,500ug/ul) at 37 Degree Celsius for 30 mins. One unit of enzyme activity is defined as the concentration of plant extract at which the venom activity is least. The absorbance was taken /read at 440 nm in an EPOCH 2 microplate spectrophotometer (BioTek Instruments, Inc., United States).

5. *Daboia russelii* Phospholipase assay:

Cryptolepis buchananii:In order to assess the activity of phospholipase, we conducted an assay using chicken egg yolk, which is rich in phospholipids. For inhibition studies 10ug venom protein from *Daboia russelii* (DaRuTN01 1mg/ml) was preincubated with plant extracts at increasing concentration (50,100,200,500 ug/ul). Absorbance was measured at 740 nm in an EPOCH 2 microplate spectrophotometer (BioTek Instruments, Inc., United States) and decrease in absorbance was checked at every 1 min interval followed by a gentle shake for 15 seconds. The graph was plotted taking account the activity of venom as 100%.

Kalanchoe pinnata: For inhibition studies 10ug venom protein from *Daboia russelii* (DaRuTN01 1mg/ml) was preincubated with plant extracts at increasing concentration (50,100,200,500 ug/ul). Same ratio was taken for different solvent. Absorbance was measured at 740 nm in an EPOCH 2 microplate

spectrophotometer (BioTek Instruments, Inc., United States) and decrease in OD was checked at every 1 min interval followed by a gentle shake of 15 seconds. The graph was plotted taking account the activity of venom as 100%.

6. Haemolytic assay : The haemolytic assay was only done for the Bryophyllum.To evaluate the haemolytic effects of *Daboia russelii* venom, an Blood assay was conducted (Maisano et al., 2013; Laxme et al., 2019). Varying concentration of venom (30ug) was treated with a fixed concentration of RBCs (2% v/v solution) from healthy volunteers.The venom was pre-incubated with varying concentrations of plant extract (50ug,100ug,200ug and 500ug) at 37 degree celsius for 30 min. Briefly, blood was centrifuged to separate RBCs from the plasma, followed by resuspension of the separated RBCs in phosphate buffer saline (PBS; pH 7.4). This was followed by an overnight incubation, after which the absorbance was measured at 540 nm in an EPOCH 2 microplate spectrophotometer (BioTek Instruments, Inc., United States). The relative haemolytic activities were calculated using 0.5% Triton X as a positive control (treated as 100% activity).

7.Statistical data: The results are represented as the mean ± standard deviation(SD) of three independent experiments performed in triplicates. Experimental data were analyzed using the statistical package GraphPad Prism version 5.03 (GraphPad,La Jolla, CA).The comparison between the groups was considered significant if $P < 0.05$.Independent student's t-test or the two sample t-test was performed to compare the statistical significance between the average inhibitory value of both the test plants considering both the solvents.(The average values were taken in triplicates).

The null hypothesis is “No inhibition by the plant extracts on the specific activity of Venom”.

Alternative hypothesis is “Inhibition by plant extracts on the specific activity of Venom”.

VI. RESULTS

1. Protein Estimation in the Venom sample: The standard Bradford assay was performed on the venom samples collected from different states i.e Karnataka and Tamil Nadu. The protein concentration was discovered to be 2.5050 mg/ml in the sample collected from Tamil Nadu(DaRuTN01) and 3.053mg/ml in the Karnataka sample (DaRuKA10).

2. Protease activity :To evaluate the protease activity of the *Daboia russelii* venom, we conducted assessments on the plant extracts prepared using different solvents.Specifically, we used venom sourced from Tamil Nadu (referred to as DaRu TN01) since it is known to display robust protease activity.

The experiment was firstly done by speed drying the sample(plant extracts)in the tubes.Then the experiment was performed according to the standard protocol.But the activity of venom incubated with the plant extracts was even greater then the activity of venom taken as standard. After this observation it was concluded that *Cryptolepis buchananii* possess endoproteolytic activity which shows an increased proteolytic activity of the venom pre-incubated with plant extracts.

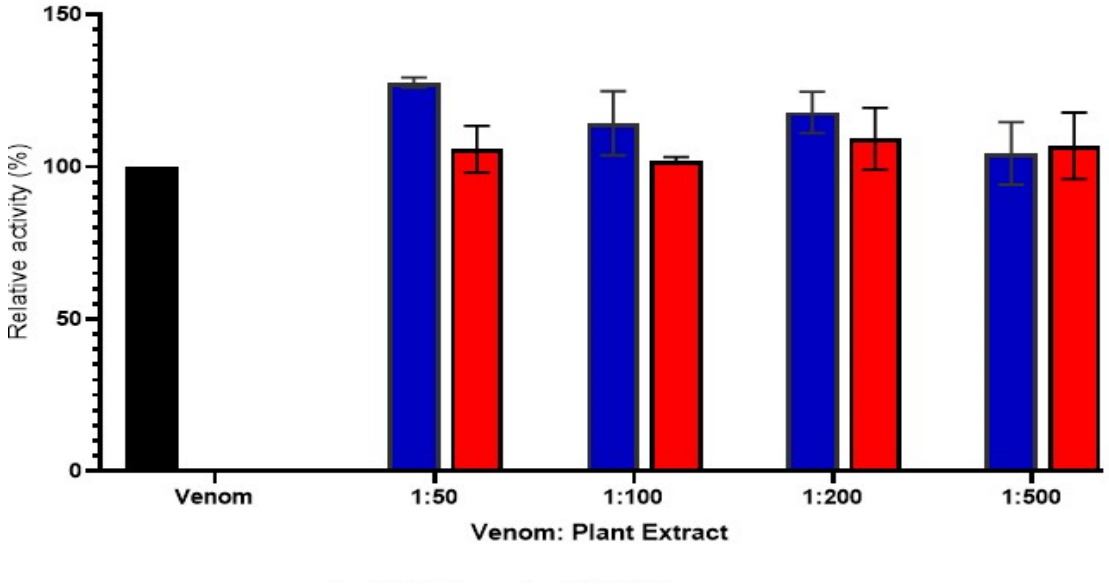

Figure 13 : Protease activity inhibition by *Cryptolepis buchananii.* This is the observation before the heating of the

sample at 100 degree celsius. The venom here is the control(100 %proteolytic activity).The plant extract is showing more activity than the control which tells that maybe *Cryptolepis buchananii* possess some endoproteases.

***Cryptolepis buchananii* :** In this study, we used the activity of the venom alone as our control, which was determined to be 100%. To measure the inhibition caused by the plant extract, we calculated the percentage inhibition by comparing it to the control.A lower value indicates a higher degree of inhibition. When we incubated the venom with the plant extract, we found that its activity was just as high as the control, as shown in the figure.This suggests that the plant extract does not possess any potential to inhibit the proteases present in *Daboia russelii* venom.

The T-test with 2 variable systems was done on GraphPad Prism version 5.03 (GraphPad,La Jolla, CA).Statistical significance determined using the Holm-Sidak method, with alpha = 0.05.The value of p was greater than 0.05(p>0.05).This proves the null hypothesis to be correct i.e there is no inhibition by *Cryptolepis buchanan* on the proteolytic activity of Russell's viper venom.

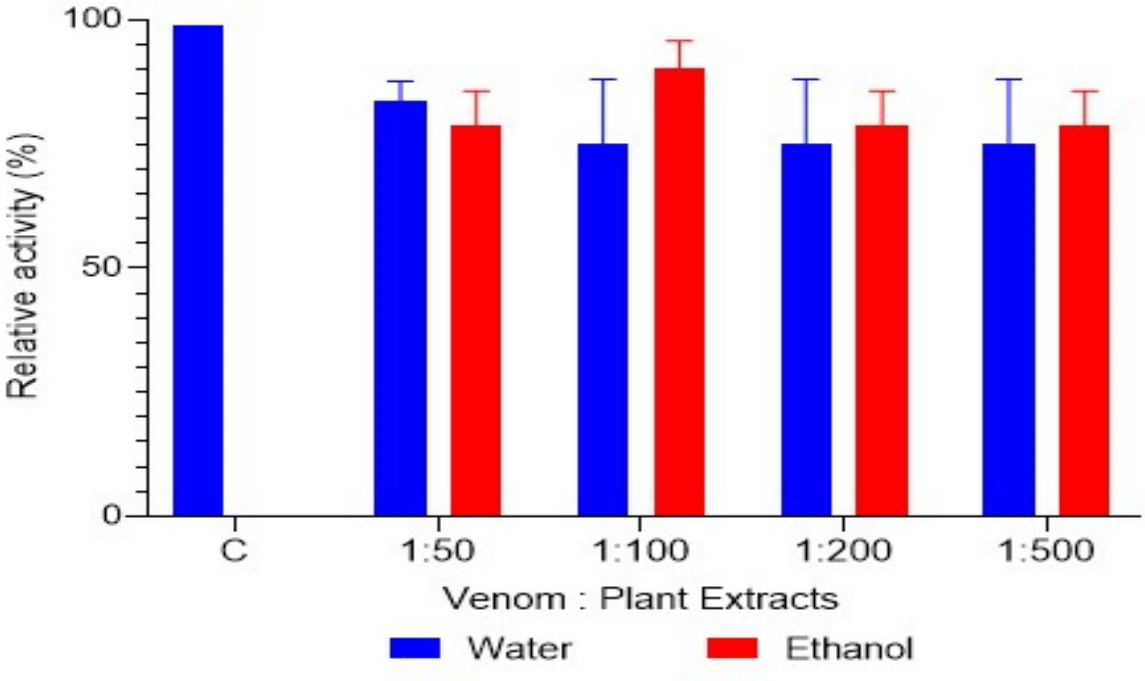

Figure 14: Protease activity inhibition by *Cryptolepis buchanani* prepared in different solvent.**C** here is the control i.e Venom alone showing 100% activity.Red bars represent the Plant extract prepared in EtOH and blue represents plant extract prepared in water

Kalanchoe pinnatum: The Venom from the *Daboia russelii* snake was used as the control in this study, representing 100% activity. By examining the figure 15, we can draw the conclusion that when Venom was incubated with plant extracts dissolved in water, its activity remained as high as the control. This indicates that the plant extracts do not possess any inhibitory potential against the proteases found in Daboia russelii venom.However, when the plant extracts were dissolved in ethanol, they exhibited some level of concentration-dependent inhibition. At a concentration of 500ug, the inhibition was around 50%. It is important to note that this level of inhibition is not significantly effective since 500ug of plant extract is considered a very high concentration and could lead to undesirable consequences.

The T-test with 2 variable systems was done on GraphPad Prism version 5.03 (GraphPad,La Jolla, CA).Statistical significance determined using the Holm-Sidak method, with alpha = 0.05.For the concentration 50 ug the value of **p** observed to be more than 0.05, therefore showing no significance.But for other concentrations (100 ug,200ug and 500ug) the p was less than 0.05.Proving the alternate hypothesis.

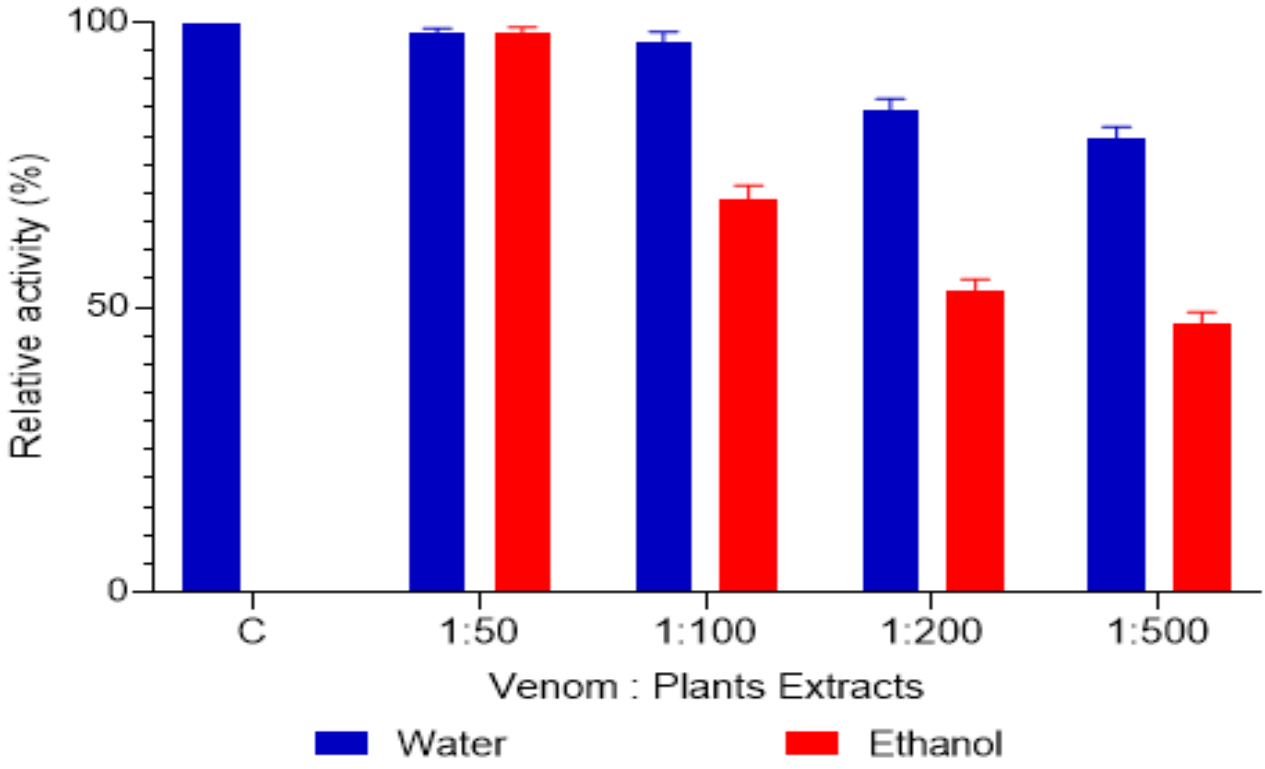

Figure 15: Protease activity inhibition by *Bryophyllum* prepared in different solvent.**C** here is the control i.e Venom alone showing 100% activity.Red bars represent the Plant extract prepared in EtOH and blue represents plant extract prepared in water.

3. Phospholipase activity: Phospholipase A is one of the major components of hemotoxic venom of *Daboia russelii*. This is also known as the Turbidity assay.

Cryptolepis buchananii: In this assay, the activity of the venom alone is taken as control, which was determined to be 100%. The concept of this assay relies on the observation of turbidity, the more turbidity there is, the greater the inhibition caused by the plant extract. On the other hand, a clear solution indicates higher phospholipase activity in the venom.

In the case of *Cryptolepis buchananii,* after the 60-minute recording protocol, all the dilutions were completely transparent for both solvent types. Based on the figure 16 presented, we can conclude that the extract does not possess any inhibitory potential against PLA_2.

The T-test with 2 variable systems was done on GraphPad Prism version 5.03 (GraphPad,La Jolla, CA).Statistical significance determined using the Holm-Sidak method, with alpha = 0.05.The value of p was greater than 0.05($p>0.05$).This proves the null hypothesis to be correct i.e there is no inhibition by *Cryptolepis buchanan* on the phospholipase activity of Russell's viper venom.

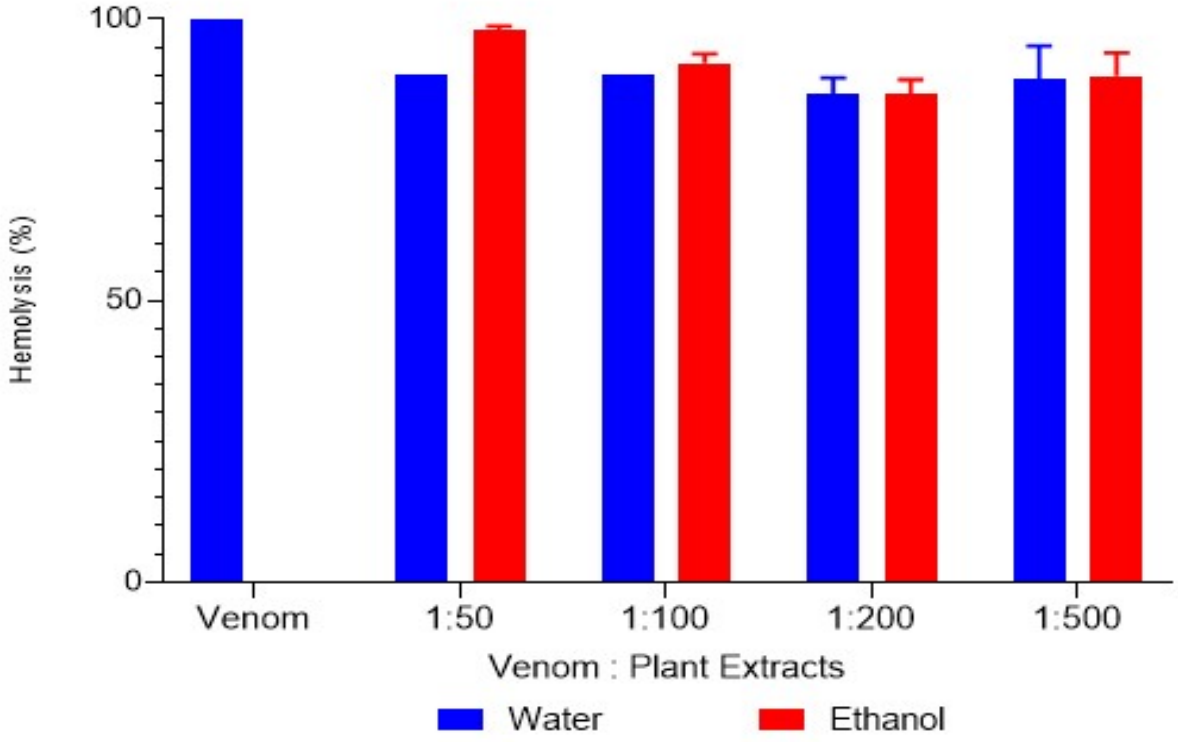

Figure 16: Phospholipase inhibition assay assessed by Cryptolepis buchanani.C here is a control depicting 100% cleavage.The relative activity of Venom incubated with plant extracts is almost identical to the Control,reason behind this can be the presence of a Serine protease in *Cryptolepis buchanani* leaves.

Kalanchoe pinnatum:According to the information provided in the preceding paragraph, during the assay, we noted a notable reduction in the relative activity of the venom when different concentrations of plant extract were introduced. After a recording period of 60 minutes, we were able to visually observe the turbidity.

By analyzing the values of relative activity , we found that the plant extract prepared in water exhibited significant inhibition at a concentration of 500 ug, which was better than the inhibition observed in EtOH extracts. The T-test with 2 variable systems was done on GraphPad Prism version 5.03 (GraphPad,La Jolla, CA).Statistical significance determined using the Holm-Sidak method, with alpha = 0.05.For the concentration 50 ug, 100 ug and 200 ug the value of **p** was observed to be more

than 0.05. Supporting the null hypothesis. But for the concentration 500ug the value of **p** is <0.001. Supporting the alternative hypothesis. Therefore showing positive phospholipase inhibition.

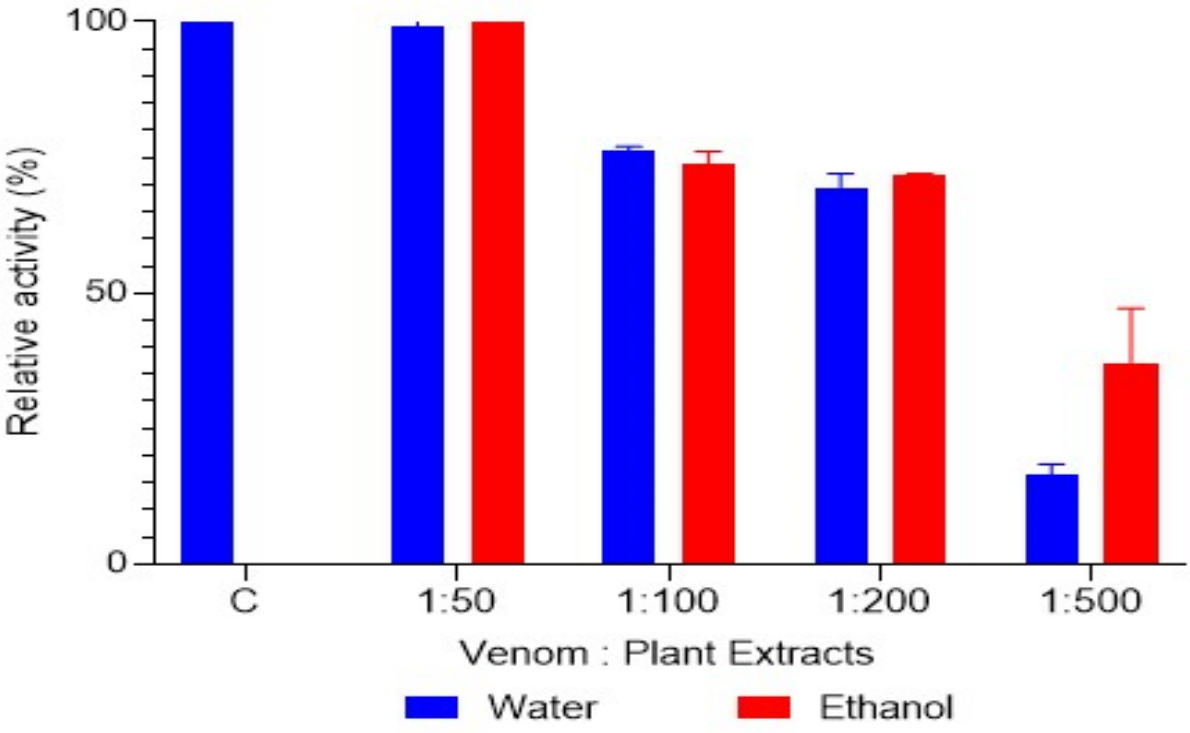

Figure 17: PLA_2 inhibition of *Daboia russelii* by Bryophyllum in two different solvent**. 'C**' is control which is venom alone. The Blue coloured bar depicts the Plant extract in water and Red one d'picture Plant extracts in Ethanol.

3. Haemolytic activity :Haemolysis is a characteristic property of Viperidae venom due to its rich composition of enzymatic toxin superfamilies, including PLA_2, SVMPs, SVSPs, and LAAO. In the study, Cryptolepis buchananii was found to have no positive inhibition for PLA_2.Therefore, the assessment of hemolytic activity focused solely on *Kalanchoe pinnatum* or bryophyllum. To establish a baseline, a control was set using a 0.5% Triton X 100 solution, which exhibited 100% haemolytic activity. After incubating the red blood cells (RBCs) in a 2% solution of plant extract for 24 hours, complete hemolysis of RBCs was observed for all concentrations of the plant extract tested. These

findings led to the conclusion that the plant extracts, including *Kalanchoe pinnatum*, did not possess any anti-haemolytic potential against *Daboia russelii* venom.

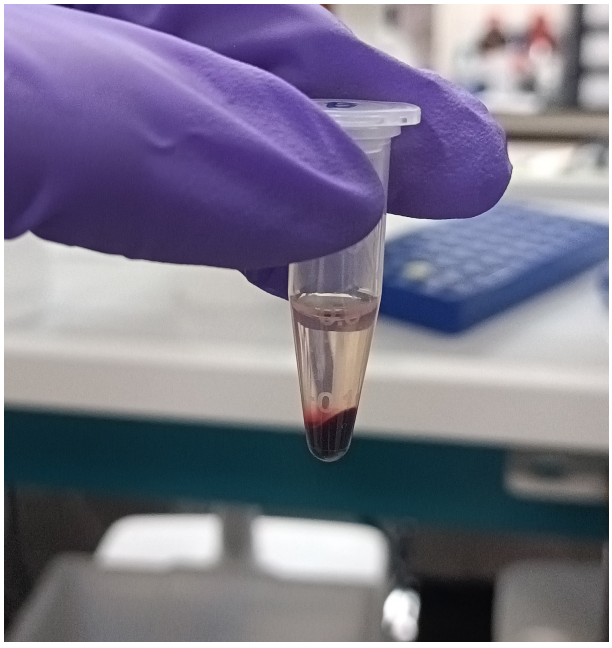

Figure 18:Human RBC in PBS solution.This image was taken after the purification and 24 hour incubation of RBC with venom and plant extracts.Checking at this step is very important because of the risk of RBC rupturing because of external forces.

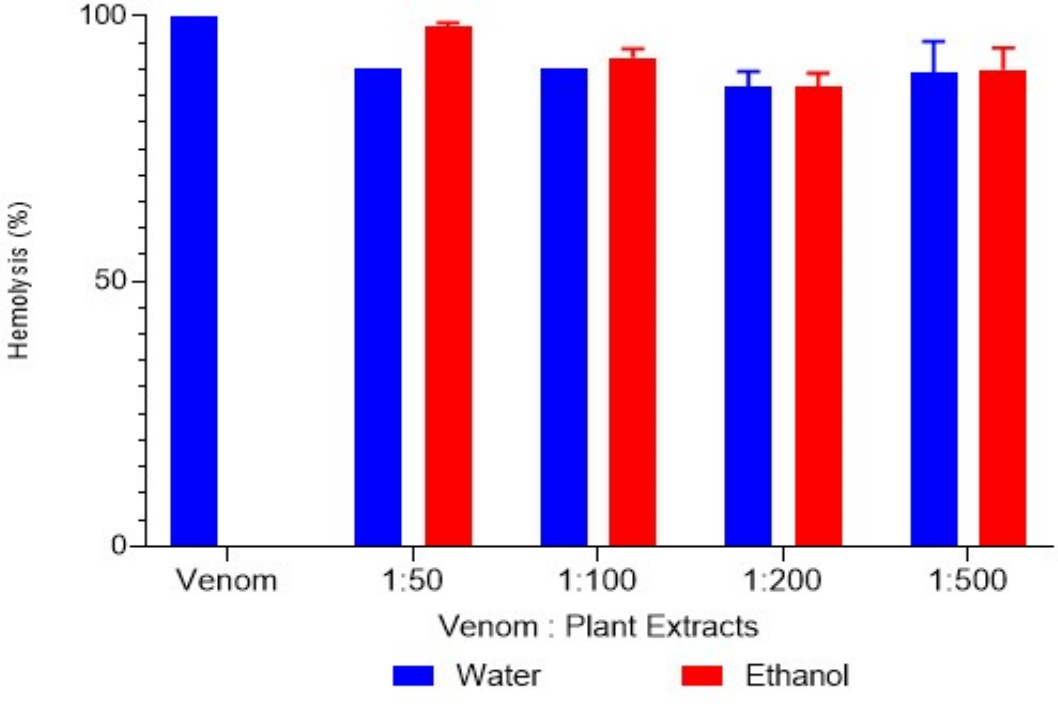

Figure 19: % Haemolysis shown by the venom.The venom was preincubated with plant extracts at different concentrations for 24 hours.The activity of venom alone on the RBCs is taken as 100%.Along with venom, Triton X 100 is also used as an positive control which shows the highest hemolysis.

The T-test with 2 variable systems was done on GraphPad Prism version 5.03 (GraphPad,La Jolla, CA).Statistical significance determined using the Holm-Sidak method, with alpha = 0.05.The value of p was greater than 0.05($p>0.05$).This shows proves the null hypothesis to be correct i.e there is no inhibition by *Bryophyllum* on the haemolytic activity of Russell's viper venom.

VII. DISCUSSION

1.**Protease activity**:Despite the extensive use of *Cryptolepis buchananii* in topical medication against *Daboia russelii,* as reported by local healers and past literature, the in-vitro experiments did not demonstrate any inhibition against the proteolytic activity caused by the two major toxin families: SVMPs and SVSPs. These toxin families are known for their involvement in tissue necrosis, disintegration of the extracellular matrix, and venom-induced coagulopathy.These findings suggest that while *Cryptolepis buchananii* may be traditionally used for topical applications against *Daboia russelii* bites, its effectiveness in inhibiting the proteolytic activity of the venom, was not observed in the in-vitro experiments.

When examining *Kalanchoe pinnata*, the results differed depending on the solvent used for the plant extract. When water was used, the percentage activity or inhibition was similar to the control, indicating no positive results in terms of inhibiting proteolytic activity. However, when an ethanolic extract of *Kalanchoe pinnata* was analyzed, it showed concentration-dependent inhibition. At a concentration of 500 µg, it exhibited approximately 50% inhibition. This suggests that the extract contains components with potential anti-venom properties, albeit at very low concentrations.

Additionally, some plants are known to possess endo-proteases. In the case of *Cryptolepis buchananii*, it is noteworthy that it contains a unique serine protease **Cryptolepain**(Pande M, Dubey VK, Yadav SC, Jagannadham MV et al., 2006) with a molecular weight of 50.5 kDa. This particular compound could potentially be a contributing factor to the plant's ineffectiveness against the proteolytic activity of *Daboia russelii* venom.

2. Phospholipase activity :When analyzing *Cryptolepis buchananii*, the results clearly indicate a high cleavage rate of phospholipids when incubated with both the venom and plant extracts. These rates are comparable to the control group. This finding suggests that *Cryptolepis buchananii* is not effective as an ancillary therapy for snakebite.When investigating *Kalanchoe pinnata*, it was found that the plant extract

prepared in water exhibited a notably low rate of phospholipid cleavage, indicating effective inhibition of the phospholipase activity caused by the venom.The degree of inhibition was found to be dependent on the concentration of the plant extract, with the highest concentration of 500 µg showing the maximum/optimal inhibition of the venom. Similar results were observed with the ethanolic plant extracts, although the rate of inhibition was slightly lower compared to the water extract. Once again, the inhibition demonstrated concentration-dependent behavior.

3. Haemolytic assay: In cases of *Daboia russelii* envenomation, intravascular hemolysis is a significant clinical manifestation. This occurs primarily due to the action of secretory PLA_2 enzymes, which hydrolyze the phospholipid bilayer present in cellular membranes.Following a 24-hour incubation period, it was observed that the percentage of haemolysis caused by venom preincubated with *Kalanchoe pinnata* was nearly identical to the control. This finding is intriguing since the plant is known to be a good inhibitor of PLA_2 activity. There are several possible explanations for this observation. One significant factor could be that *Kalanchoe pinnata* is primarily utilized against Elapid venom, which predominantly consists of neurotoxins and lacks significant haemolytic potential. Additionally, it is plausible that the plant contains certain components with haemolytic properties, considering its rich composition of amino acids, phytochemicals, and trace elements.

VIII. CONCLUSION

The study finds that even though the traditional use of *Cryptolepis buchananii* and *Kalanchoe pinnata* for snakebite since centuries, these plants displayed no inhibition against the Russell's viper venom in the in-vitro assays. The reason can be attributed to the presence of endoproteases in the plants.*Kalanchoe pinnata* did show concentration-dependent inhibition of phospholipase activity of venom but no inhibition against the haemolysis.The reason can be low concentrations of compounds promoting the inhibition of venom.The study also notices difference in the results on the basis of solvent which concludes that the choice of solvent is also important.

Overall the study provides valuable insights into these medicinal plants and their uses.Even though these plants showed no antivenom potential still they are used as a remedy for many diseases.

IX. SUMMARY

Snake bites affect approximately 5.4 million individuals worldwide, with over 70% of cases occurring in Asia. As a result, it is classified as a neglected tropical disease by the World Health Organization (WHO, 2009). Among all countries, India bears the unfortunate title of being the snakebite capital of the world, witnessing the highest number of annual deaths and injuries caused by snake envenomation. These alarming circumstances can be attributed to the "Big Four" snakes (Whitaker et al., 2006), which are primarily responsible for these incidents. India boasts a remarkable diversity of wildlife, including more than 300 species of snakes, of which 60 are medically significant(Whitaker et al., 2006). These snake species cause the highest number of envenomations(Suraweera et al., 2020). The primary treatment for snake envenomation is the administration of antivenom. However, despite these efforts, the considerable variation in venom composition within the snake population is not given sufficient attention. This lack of focus on intra-population variation is a major reason why conventional antivenom treatments are still not displaying desired efficacy .Throughout history, plants have been utilized as a source of remedies and medications, a practice dating back to ancient times (UNESCO, 1996). Many plants possess remarkable properties and have served as valuable resources for discovering drugs to combat various infectious diseases.In fact, some of these plants have even demonstrated potential antivenom properties, offering hope for alternative treatments.For centuries, local healers have relied on *Cryptolepis buchanani* and *Kalanchoe pinnata* as traditional remedies for snake envenomation. However, when these plants were subjected to in-vitro studies, no significant anti-venom potential was observed, except for *Kalanchoe pinnata's* ability to inhibit PLA_2. Nevertheless, this PLA_2 inhibition was not significant enough to be considered a new supplementary therapy for snakebite.Despite the lack of prominent anti-venom properties, these plants do possess various healing qualities, such as antioxidant, anti-inflammatory, antibacterial, and antifungal properties. They may serve as valuable agents in preventing infections caused by external agents. These possibilities are associated with these plants due to their historical usage and references found in ancient Ayurvedic texts.

X. REFERENCES

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CERTIFICATE

This is to certify that **Ms. Nisha Sharma**, final year MSc. Biotechnology student of **Thapar Institute of Engineering and Technology** has successfully completed her project thesis work entitled, "**Harnessing Nature's Defence: Investigating Plant Extracts as Inhibitors of Russells's Viper Venom for Promising Ancillary Antivenom Development** "under my supervision at the Centre for Ecological Sciences, Indian Institute of Science during the period of January 2023 to July 2023.

Yours Sincerely,

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