UNDERSTANDING THE TOXICITY OF ALPHA NEUROTOXINS AND THE ROLE OF *IN-SILICO* TECHNIQUES IN DECIPHERING THE ANTIDOTE POTENTIAL OF PHYTOCHEMICALS AGAINST ALPHA NEUROTOXINS

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Odisha University of Agriculture and Technology
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By

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CERTIFICATE I

This is to certify that the thesis entitled "**Understanding the toxicity of alpha neurotoxins and the role of in-silico techniques in deciphering the antidote potential of phytochemicals on alpha neurotoxins**"submitted in partial fulfilment of the requirements for the award of the degree of Master of Science to the Odisha University of Agriculture and Technology is a faithful record of bonafide and original research work carried out by Miss Mahashweta Swain under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

It is further certified that the assistance and help received by him/her from various sources during the course of investigation has been duly acknowledged.

Bhubaneswar
Date: 17.07.2020

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DEPARTMENT OF ZOOLOGY
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CERTIFICATE II

This is to certify that the thesis entitled "**Understanding the toxicity of alpha neurotoxins and the role of in-silico techniques in deciphering the antidote potential of phytochemicals on alpha neurotoxins"** submitted by Miss Mahashweta Swain to the Odisha University of Agriculture and Technology, Bhubaneswar in partial fulfillment of the requirements for the degree of Master of Science in Zoology has been approved by the student's advisory committee and the external examiner.

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
AChBP	Acetylcholine Binding Protein
AdmCTX	Adenovirus vector carrying mutated cobratoxin cDNA
ASV	Anti Snake Venom
ADMET	Absorption, distribution, metabolism, excretion and toxicity
Arg	Arginine
Bgt	Bungarotoxin
CASTp	Computed Atlas of Surface Topography of proteins
CHARMM	Chemistry at Harvard Macromolecular Mechanics
cDNA	Complementary DNA
CNS	Central Nervous System
CRiSP	Cysteine-Rich Secretory Protein
CTL	C-Type Lectin
Cbtx	Cobratoxin
DIS	Disintegrin
3FTx	Three-Finger Toxin
KUN	Kunitz-Type Protease Inhibitor
LAAO	L-Amino Acid Oxidase
LD_{50}	Lethal Dose 50%
Lys	Lysine
MD	Molecular Dynamics

nAChR	Nicotinic Acetylcholine Receptor
NP	Natriuretic Peptide
NTD	Neglected Tropical Disease
PDB	Protein Data Bank
PDBTM	Protein Data bank Trans-membrane
PLA_2	Phospholipase A2
PLP	Piecewise Linear Programming
Phe	Phenylalanine
PNS	Peripheral Nervous System
RMSD	Root Mean Square Deviation
SCOP	Structural Classification Of Proteins database
SVMP	Snake Venom Metalloproteinase
SVSP	Snake Venom Serine Protease
Tyr	Tyrosine
WHO	World Health Organization

ABSTRACT

Snake bite envenomation is a significant socio-medical problem of global concern. It is classified as a Neglected Tropical Disease by the WHO. In India, snake bite takes a heavy toll of human lives and therefore warrant urgent attention. To date, Plasma derived Anti Snake Venom have been the only effective treatment option for otherwise intractable envenoming. Indian medicals use a polyvalent antivenom against the “big four” most venomous snakes of India. However, it suffers from major drawbacks such as immunogenicity, affordability and availability. India has a large repository of medicinal herbs that helps mitigate the effects of snake venom but only few are known to cure the α-neurotoxin induced neuromuscular paralysis. This review attempts to provide an insight about the structure and mechanism of action of α-neurotoxin, its clinical manifestations, currently available treatment options and their relevance. It finally concludes with an emphasis on the use of *in-silico* techniques for identifying the phyto-pharmacochemical compounds with potent antivenin activity against α-neurotoxin.

Keywords: Alpha neurotoxin, nicotinic acetylcholine receptor, in-silico methods

CHAPTER - 1

Introduction

1. INTRODUCTION

Snake bite envenomation is a notable health hazard prevalent in the warmer tropical and subtropical countries. It disproportionately, affects the impecunious and impoverished segments of the society, those with limited access to education and well equipped health care. The WHO has re-included snake bite in the category 'A' of neglected tropical disease in June 2017. As reported by the WHO about 1.8-2.7 million people worldwide are affected by snake venom infliction per year. About 46000 deaths have been estimated from India alone (Gutiérrez et al., 2017). However, the true national burden of snake bite remains unknown due to lack of standardized reporting and underreporting (Murray et al., 2015). Having about 66% of rural population, India is the worst hit country by snake bite in Southeast Asia.

The four most venomous snakes of India – The Indian or Spectacled Cobra (*Naja naja*), Common Krait (*Bungarus Caeruleus*), Saw scaled viper (*Echis Cariatus*) and Russell's viper (*Daboia russelii*), commonly known as the "Big Four" are responsible for majority of the inflictions in India (Choudhury, McCleary, Kesherwani, Kini, & Velmurugan, 2017). Arguably, Neurotoxins are the most important component of snake venom found mainly from the venoms of snakes of Elapidae family (*Naja naja* and *Bungarus caeruleus*).

Post Synaptic neurotoxins make about 98% of the venom of Elapid snakes. They bind to the nicotinic acetylcholine receptor (nAChR) present on the post synaptic neuronal or muscular membrane. They act antagonistically at the receptor, inhibiting the Acetylcholine from binding at the receptor (Lewis & Gutmann, 2004). This leads to blockade of neuromuscular transmission and a descending flaccid neuromuscular paralysis (Ranawaka, Lalloo, & de Silva, 2013).

Although, specific Anti Snake Venom (ASV) administration can reverse the post synaptic neurotoxicity (Gutiérrez et al., 2017), inadequate health facilities, difficult transportation, consequent delay in the ASV administration make it quite unachievable in the rural areas of India. Despite of being the only available specific treatment for snake bite, antivenoms come with several other limitations. Species specificity, Immunogenicity, difficulty in availability, affordability and ideal storage conditions are some of the issues with ASV therapy (Gupta & Peshin, 2014).

India uses a polyvalent ASV against the so called "big four" snakes of India (Gutiérrez et al., 2017). The concept of "Big Four" is a big roadblock in the development of an effective Anti-

inSnake Venom. Venom composition variation, low potency, bite by species other than the "big four" are some of the reasons responsible for reported failure of polyvalent ASV in India (Gupta & Peshin, 2014).

Utilization of plants for medicinal purposes by the ethnic groups can be documented long back in the history of India. Moreover, inadequate health care facilities in rural area make people largely dependent on the herbal antidotes. A plethora of herbal medicines have been widely used by the traditional healers in the form of decoctions or paste (Gupta & Peshin, 2014). But lack of definite protocol for their usage, unstable composition and their instability renders them less productive (Gómez-Betancur, Gogineni, Salazar-Ospina, & León, 2019). Even though various conventional and current techniques for extracting active principles have been developed (Gómez-Betancur et al., 2019), these indigenous practices need scientific corroborations (Panghal, Arya, Yadav, Kumar, & Yadav, 2010). Thus, there is a need of an antidote that works in a species specific manner in neutralizing the neurotoxin and at the same time does not stimulate any anaphylactic reactions. The present review is an attempt to sketch a resume of the prevailing issues with the current medications used against the alpha neurotoxic venom. The main purpose of this review is to endeavor insights into the identification of specific antidote for alpha neurotoxin using the *in-silico* analysis approach.*in*

CHAPTER - 2

Review of Literature

2. REVIEW OF LITERATURE

2.1 Epidemiology

Snake bite envenomation is a neglected tropical disease (NTD) (Gutiérrez et al., 2017) requiring prompt treatment (Ainsworth et al., 2018). It is an important cause of mortality and morbidity, (Gutiérrez et al., 2017) disproportionately affecting lower socio-economic segments of the society (Habib et al., 2015; Harrison, Hargreaves, Wagstaff, Faragher, & Lalloo, 2009). It has become a major health hazard in the impoverished areas of the warmer Tropical and Subtropical regions such as Sub-Saharan Africa, South to Southeast Asia, Papua New Guinea and Latin America (Harrison et al., 2009; Mohapatra et al., 2011).

According to WHO reports at least 1.8-2.7 million people worldwide suffer from the snake bite envenomation per year, with mortality ranging from 81,410 to 137,880 deaths (Chippaux, 1998; Kasturiratne et al., 2008). In India, about 45,900 deaths due to snake bite were reported in the year 2005 (Mohapatra et al., 2011). Highest number of deaths occurred in Uttar Pradesh (8,700), Andhra Pradesh (5,200) and Bihar (5,200) (Mohapatra et al., 2011). In India, the bites and fatalities are mostly seen among children in the age group of 5-14 years (Mohapatra et al., 2011). Snakebites peak during the monsoon season in India (Ahuja & Gurkirpal, 1954; Sawai & Honma, 1976) causing nearly 5000-7000 fatalities during monsoons in the year 2005 (Mohapatra et al., 2011).

2.2. Snake venom neurotoxins

Snake venom is a complex cocktail of enzymes, polypeptides, non- enzymatic proteins, nucleotides and other substances, many of which are neurotoxic in nature. These neurotoxins induce a flaccid neuromuscular paralysis when inflicted. Neurotoxins are broadly classified into 2 types based on their site of action in Synaptic transmission. These are as follows:

2.2.1. Pre-Synaptic Neurotoxins (β-neurotoxins)

They produce neuromuscular blockade by inhibiting the release of acetylcholine from presynaptic membrane (Connolly et al., 1995). The blockade occurs in 3 phases: an immediate depression of Acetylcholine (ACh) release followed by a transient increase in ACh release and then complete inhibition of neuromuscular function transmission (Connolly et al., 1995). The PLA2 enzymes cause hydrolysis of phospholipids of the pre-synaptic membrane and the hydrolysis products cause membrane destabilization. These processes are likely the key drivers

in process of pre synaptic blockade (FAURE, 1999). Pre-Synaptic neurotoxins have been isolated from venoms of snake families Elapidae, Viperidae, Crotalidae and Hydrophiidae (FAURE, 1999).

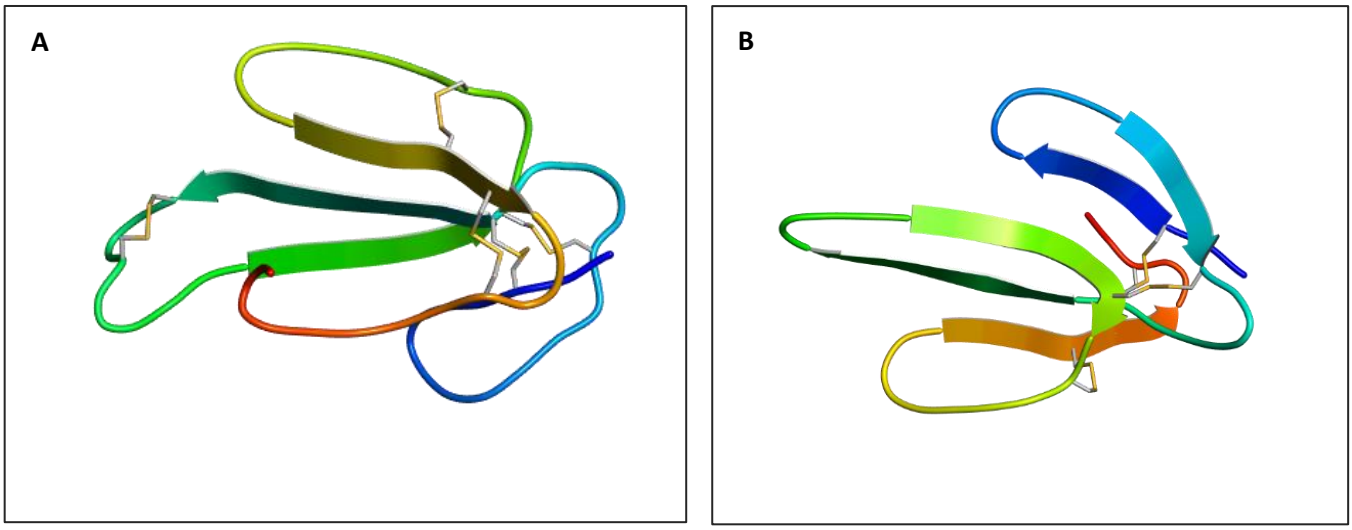
2.2.2. Post-Synaptic Neurotoxins (α-neurotoxins)

They produce neuromuscular blockade by blocking the ACh binding to its receptor nicotinic Acetylcholine receptor (nAChR) (Lewis & Gutmann, 2004). Post-Synaptic neurotoxins have been isolated from the venoms of snake families Elapidae and Hydrophiidae. Some snakes of Colubridae family (Eg: *Coilognathus radiatus*) also possess post-synaptic neurotoxins (Larréché et al., 2008). Alpha Neurotoxins are classified into 3 main groups depending on their amino acid sequence: **Long Chain, Short Chain, Non-Conventional Alpha Neurotoxin** (Larréché et al., 2008). Short chain α- neurotoxins have 60-62 amino acid residues and four disulfide bonds (T Endo, 1991). Their molecular weight ranges between 6-7 kDa (Barber, Isbister, & Hodgson, 2013). Long Chain α-neurotoxins consist of 66-75 amino acids residues and five disulfide bond (Tsetlin, 1999). Four of the disulfide bonds are in similar positions as in the short chain neurotoxins while the fifth disulfide bridge is located between Cys 30 and Cys 34 at the tip of loop 2 (T Endo, 1991). Long chain α-neurotoxins have a smaller loop 1 and a larger C terminal tail region as compared to Short chain α-neurotoxins (Selvanayagam Nirthanan & Gwee, 2004). Short α-neurotoxins tend to associate with nAChR 6-7 fold faster and dissociate 5-9 fold faster than the Long chain α-neurotoxins (Chicheportiche, Vincent, Kopeyan, Schweitz, & Lazdunski, 1975). Despite the differences both long and short chain α-neurotoxins bind to the same site on muscle nAChR with equal affinity and compete with each other for binding (Endo and Tamiya 1991). It has been reported that long neurotoxins can bind to neuronal α7 nAChR with higher affinity than the short chain neurotoxin (Antil-Delbeke et al., 2000; Servent et al., 2000; Servent et al., 1997). These differences in their targeting have been attributed to the presence of the unconserved fifth disulfide bond in the 2nd loop of long chain α-neurotoxins (Servent et al., 1997). Non-Conventional Toxins consist of 62-68 amino acid residues and five disulfide bridges. However, unlike the long chain α-neurotoxins the fifth disulfide bridge is located in loop 1 (N terminus loop) (Servent & Ménez, 2002). They are characterized by a lower order of toxicity (LD_{50} from ~5-80mg/kg) (S Nirthanan, Gopalakrishnakone, Gwee, Khoo, & Kini, 2003; Yu N Utkin et al., 2001; Yuri N Utkin et al., 2001) and hence are referred to as weak toxins (Yu N Utkin et al., 2001; Yuri N Utkin et al., 2001). Some examples of Non-Conventional Toxins are – γ-bungarotoxin (from *Bungarus multicinctus)(Aird, Womble, Iii, & Griffin, 1999)*, Bucandin (TORRES, KINI,

SELVANAYAGAM, & KUCHEL, 2001) and Candoxin (Yu N Utkin et al., 2001; Yuri N Utkin et al., 2001). These post synaptic neurotoxins also known as α-neurotoxins will be the subject of this review.

2.2.2.1. Structure of alpha neurotoxins

The alpha neurotoxins belong to the Three-Finger Toxin (3FTxs) family. The characteristic feature of all 3FTx proteins is their distinct protein fold formed by three β-stranded loops extending from a small, globular, hydrophobic core that is cross linked by four conserved disulfide bridges (Ménez, 1998; Tsetlin, 1999). The three loops that project from the core region resemble three outstretched fingers of a hand and hence the name (Kini & Doley, 2010). All the 3FTxs proteins are basic in nature with isoelectric values lying between pH 9-10. These non-enzymatic polypeptides (Kini & Doley, 2010) have chain length ranging between 61 to 74 amino acids (Walkinshaw, Saenger, & Maelicke, 1980). The 3FTxs proteins have structurally conserved regions like the eight conserved Cysteine residues in the core region and the aromatic residues (Tyr 25 or Phe 27) which contribute to the proper folding and structural integrity of the polypeptide chain (Antil, Servent, & Ménez, 1999; Dufton & Hider, 1983). These conserved regions are also required for the stability of antiparallel β-sheet structure (TORRES et al., 2001). The 3FTxs proteins also consist of some charged amino acid residues (like Arg 39 in Erabutoxin and Asp 60 in α-Cobratoxin) that are also conserved and stabilize the native conformation of the protein by forming a salt link with the C or N-terminus of the toxin(T Endo, 1991).

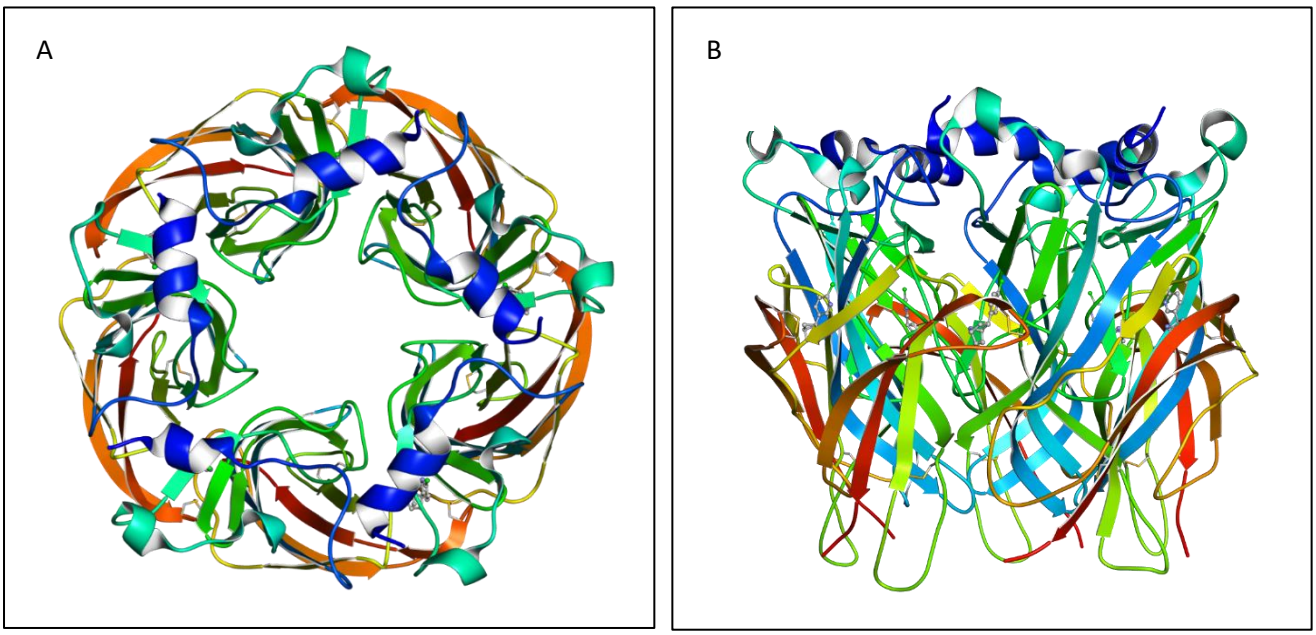


Source:PDBj

Fig.1 Structure of long chain alpha cobratoxin from *Naja naja siamensis* (A- long chain α-neurotoxin B- short chain α-neurotoxin)

2.3. Nicotinic acetylcholine receptor

Nicotinic acetylcholine receptors (nAChR) are ligand gated ion channel present in the post synaptic membrane of skeletal muscles (Hodgson & Wickramaratna, 2002). It is a heteropentameric receptor with either a stoichiometry of $(\alpha 1)_2 \beta 1 \delta \varepsilon$ (adult form) or $(\alpha 1)_2 \beta 1 \delta \gamma$ (foetal form) (Harris, 2009). The neuronal acetylcholine receptors and their subunits alpha 7, 8 and 9 are important receptor proteins of central nervous system. They play an important role in several neuronal functions like signal transduction, modulatory effects on the neurons in the nervous system (Berg & Conroy, 2002). The muscular nicotinic acetylcholine receptors are also involved in nervous system functions by stimulating the Sodium and Potassium ions conductance. The α7 nAChR are considered to be responsible for pre and post synaptic excitation of neurons (Tribollet, Bertrand, & Raggenbass, 2001).



Source: PDBj

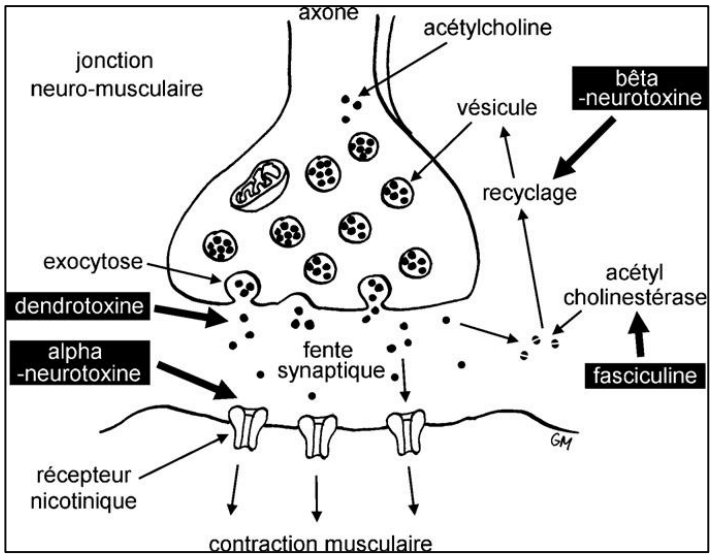
Fig.2 Three dimensional structure of nAChR (A- top view B- side view)

2.4. Mechanism of action of alpha neurotoxins

2.4.1 Molecular targets of α-neurotoxin

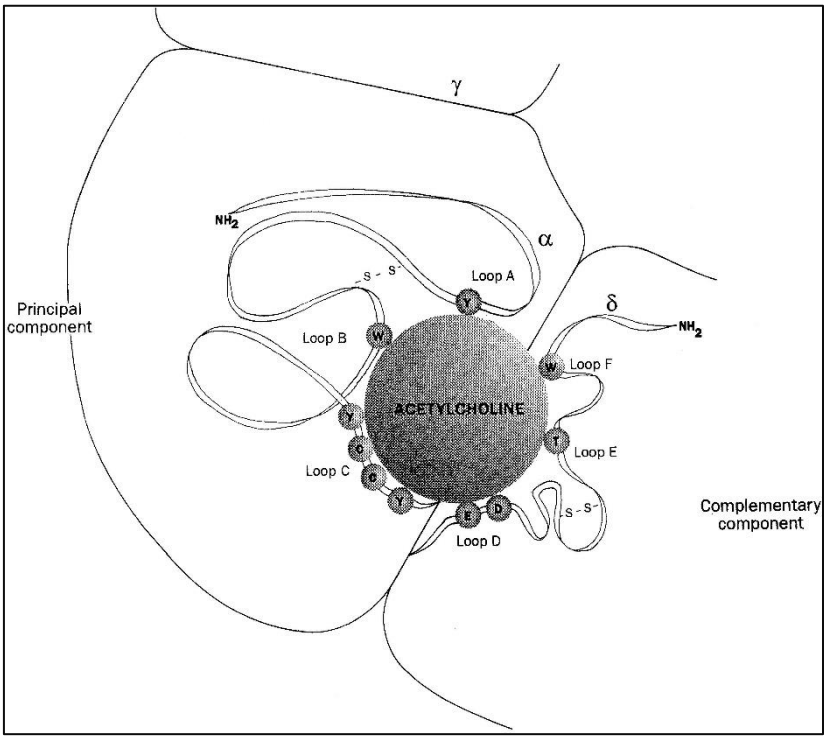
The nAChR receptor consists of 2 acetylcholine (neurotransmitter) binding sites, one of the binding sites being located at the interface between 'δ – α' subunits and the other being located at the interface of 'δ – ε' subunits (Harris, 2009). The two acetylcholine (ACh) binding sites

constitute a single larger toxin binding sites. The two binding sites are spatially so close that toxin binding to even one of the binding site sterically hinders the binding at the second site (Brejc et al., 2001).



Source: S. Larreche et al., 2013

Fig.3 Site of action of post synaptic α-neurotoxin



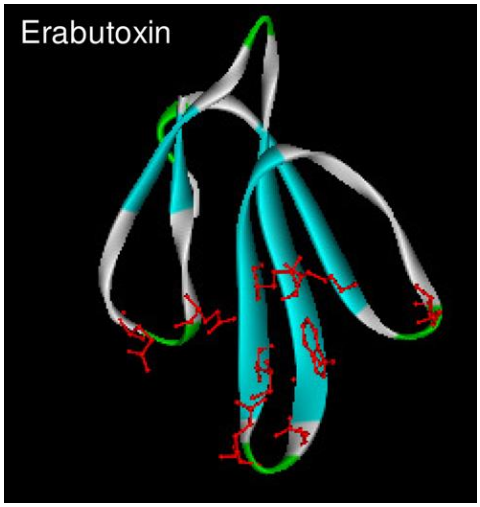
Source: (Arias, 1997)

Fig.4 Schematic model showing the folding of extracellular domain of the nAChR involved in the high affinity binding site for acetylcholine.

2.4.1.2. Residues important for toxin binding

The amino acid residues important for neurotoxin binding with the nAChR are located on the concave face of both long and short α-neurotoxins. The residues important for the binding to nAChR are as follow –

1. **Arginine** Residues –
   It is situated at the tip of loop II at position 33 of short and position 36 of long chain α-neurotoxin. The positively charged Arginine residue mimics the Guanidium group contained in the ACh structure (Samson, Scherf, Eisenstein, Chill, & Anglister, 2002).
2. **Lysine** Residue –
   It is located at the position 26 of both long and short α-neurotoxin. These positively charged residues play vital role in binding of α-neurotoxins to the muscle nAChR (Rosenthal et al., 1999).
3. **Aspartic** Acid Residue –
   These are present at position 21 of short α-neurotoxin and at position 27 of long α-neurotoxin (Barber et al., 2013).



Source: Kini et al., 2010

Fig.5 The functional residues of Erabutoxin(a 3FTx protein) which are involved in binding to muscle nicotinic acetylcholine receptors (nAChR) are located in all the three loops

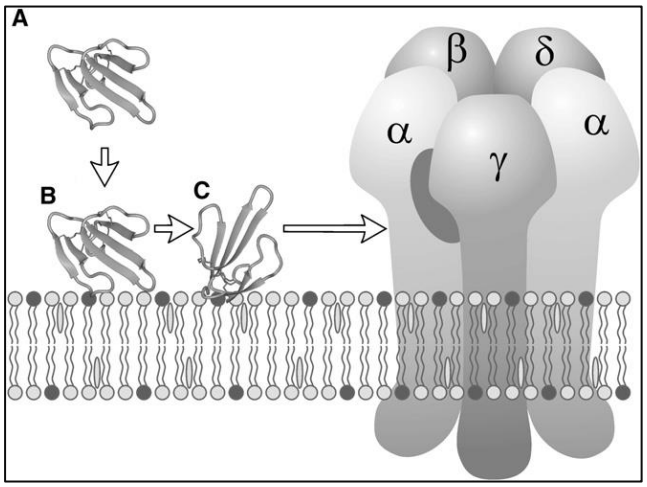
2.4.2 Binding of α-neurotoxin with nAChR

Before the binding of α-neurotoxins to the nAChRs, their positive head region binds with the lipid bilayer close to the nAChR. This results in the formation of hydrogen bond between the polar head groups of the positively charged amino acid residues in the head region of α-

neurotoxins (Lesovoy et al., 2009). This interaction helps in the favorable positioning of loop II of α-neurotoxins in the nAChR binding site(Lesovoy et al., 2009). The nAChR exists in equilibrium between 3 states (Konstantakaki, Changeux, & Taly, 2007):

a. A "basal" resting state / '**B**' state.
b. An active (open channel) state / '**A'** state.
c. One (or several) high affinity "desensitized" state(s) with closed channel / '**D'** state

The alpha neurotoxins (post synaptic neurotoxins) bind exclusively to the '**B**' state of the nAChR when the receptor is in a more 'open' state. Hence it can be correlated with the fact that binding of α-neurotoxins require widening of the binding site (Konstantakaki et al., 2007).



Source: (Lesovoy et al., 2009)

Fig.6 Diagrammatic representation showing the processes involved in the binding of α-neurotoxin to the nAChR

2.4.3 Action of α-neurotoxin

Once bound to the nAChR, it blocks the ACh binding site and prevents the ACh from binding with the receptor and thus, disrupts the neuronal conductivity (Servent et al., 1997)**.** The α-neurotoxins are potent nAChR antagonists, binding with high affinity to the nAChR with Kd values ranging between 10^{9} and 10^{11} M (Servent et al., 1997). While both long and short α-neurotoxins bind with greater affinity with the muscle nAChR (α1 type), but long neurotoxins have higher affinity for the neuronal nAChR (α7 type) (Antil-Delbeke et al., 2000). This difference in binding can be attributed to the presence of the fifth disulfide bridge in the long neurotoxin (Servent et al., 1997). The X-ray structure of AChBP-Cbtx complex has revealed that the Cbtx tightly interacts with the nAChR with the help of its 3 sequence stretches: loop I,

loop II and the C terminus. Loop II of the α-neurotoxin penetrates the nAChR binding site (Konstantakaki et al., 2007). The basic amino acid residues present in the C terminal tail of long chain α-neurotoxin contribute in receptor binding (Toshiya Endo, Oya, Tamiya, & Hayashi, 1987).

2.4.4. Reversibility of action

Lee et al. have shown that the blockade caused by long chain α-neurotoxin is less reversible than the blockade caused by the short chain neurotoxin. They suggested the reason behind this that the primary amino acid sequence of long chain α-neurotoxin contains more hydrophobic amino acid residues like Alanine and Valine. It has also been shown that the short chain neurotoxins associate 6-7 fold faster and dissociate 5-9 fold faster than the long chain neurotoxins.

2.5. Clinical manifestations of α-neurotoxicity

Snake venom neurotoxins leave a person's body in a complete paralytic state as they specifically target and block the neurotransmission at the neuromuscular junction of the skeletal muscle (Johnston et al., 2012; Silva et al., 2016). Rapid onset of neuromuscular paralysis leads to respiratory failure and death if left untreated. Ocular muscles are the first to be hit by the neuromuscular paralytic attack leading to a condition called **Bilateral Ptosis**, followed by **Ophthalmoplegia** and **Diplopia**. From here the neuromuscular paralysis descends to the facial muscles resulting in stiffness around the mouth region which make it difficult to open the mouth and often ends up at **speech impairment** (Malina et al., 2011; Silva et al., 2016**)**. The paralysis then reaches the neck and bulbar muscles where it interferes with the swallowing process and resists the airflows by obstructing the airways. This is followed by the hypoventilation due to reduction in tidal volumes (Manock, Suarez, Graham, Avila-Aguero, & Warrell, 2008; Silva et al., 2016). Severe neuromuscular paralysis often affects all limb muscles (Laothong & Sitprija, 2001; Silva et al., 2016).

2.6. Current Treatment Strategy

2.6.1 Anti-Snake Venom and its limitations

Anti-Snake Venoms are the pharmacological agents used for treating snake bite intoxication (Crosland, 1989). Recent studies have shown that antivenom is the only currently available specific treatment for snake bite envenomation (Gómez-Betancur et al., 2019). The ASV

therapy aims to reverse the damage caused by the venom by binding to the venom proteins and neutralizing them (Heard, O'Malley, & Dart, 1999).

Kini et al have proposed a next generation therapy for improved treatment of snake bites. They have shown that a recombinant antivenom when administered with oligoclonal mixes of human monoclonal antibodies can give efficacious results. The oligoclonal mixes of human antibodies includes about 20-40 neutralizing antibodies against all or most of the significant snake venom toxins (Kini, Sidhu, & Laustsen, 2018).

Another advanced approach for development of effective snake antivenom comprises of **Independent venom immunization techniques**. This approach includes 4 different strategies (Bermúdez-Méndez et al., 2018; Williams et al., 2019)–

- 1st Strategy – Injection of chemically synthesized epitopes of toxins. (The epitopes can be determined using bioinformatics software or epitope mapping studies.)
- 2nd Strategy – Injection of full length recombinant or synthetic toxin with the chemically synthesized epitopes.
- 3rs Strategy – Injection of molecules that mimic the structure of the toxin epitopes, also called mimotopes but have non-identical amino acid sequences.
- 4th Strategy – It involves the use of DNA.

However, the Anti Venom therapy may pose problems related to several antivenom characteristics as follows:

- Immediate hypersensitivity reactions against the non-self-antibodies, which may include anaphylactic and pyrogenic reactions (H. A. de Silva, Ryan, & de Silva, 2016).
- Limited potency of antivenom therapy to protect the affected organ(s) against local tissue damage.
- Geographic variation in the composition of the venom may render the antivenom ineffective.
- Antigenic reactions due to taxonomic diversity of the snakes.
- Inefficient usage due to improper medical management may lead to adverse reactions or a decreased productivity of antivenoms (Gómez-Betancur et al., 2019).

Moreover, Lausten et al. have shown that around 70% of the immunoglobulins are not specific to venom toxins (Gawarammana, Mendis, & Jeganathan, 2009). The instability in the

antivenom market, low profit margins, little financial incentives for pharmacists and health centers and lack of a definite protocol for antivenom usage have contributed to a reduction in viability and interest of pharmaceutical industries in manufacturing them (A. de Silva, Mendis, & Warrell, 1993). Thus there is a deficiency in the availability of antivenom in the areas where snake bite is endemic and there ineffective distribution worsening the consequences of snake bites and leading to increased mortality among the people living in rural and remote areas.

2.6.2 Folkloric medications having potential antivenin activity

Several ethnic groups of Asian, African and Central and South American countries have been using herbal preparations (as decoctions) for treating the snake bite (Gómez-Betancur et al., 2019). Some of the ethnic groups of India like the people of snake charmers community from Haryana, inherit a highly specialized indigenous knowledge of medicinal plants that can treat snake bite (Panghal et al., 2010).The usage of herbal decoctions are not productive enough in mitigating the effects of snake venom due to lack of a definite protocol for their use, unstable composition and stability problems (Gómez-Betancur et al., 2019). The disadvantages of decoctions could be overcome using plant extracts. Many conventional and current techniques are in use for extracting the active ingredients from their natural sources. Plant alkaloids are a major class of compounds out of other 15 chemical groups (alkaloids, flavonoids, tannins, proteins, amino acids, organic acids, enzymes, trace elements, polysaccharides, glucosides, glycosides, volatile oils, resins, Phytochromes and mineral salts among others) (Pan et al., 2013). Several of the previous researches have demonstrated the potential use of plant extracts to negate the effect of neurotoxins.

It has been studied that grape seed extract successfully neutralizes the neurotoxic effect of Post synaptic neurotoxin from Egyptian sand viper (*Cerastus cerastus cerastus*) (Mahmoud, 2013). The purified lupeol acetate from the methanolic root extract of *Hemidesmus indicus* efficiently neutralizes the *Naja Kaouthia* venom induced neurotoxicity (Chatterjee, Chakravarty, & Gomes, 2006). Likewise, β-sitosterol and stigmasterol in the methanolic root extract of *Pluchea indica* nullifies the neurotoxic effects of cobra venom (Gomes, Saha, Chatterjee, & Chakravarty, 2007). Vadiyali *et al* highlighted the antivenin activity of the methanolic root extract of *Leucas aspera (Gopi, Renu, & Jayaraman, 2014)*. Plant metabolites from *Mucrura pruriens* inhibit the non-muscular blockage (Shabbir, Shahzad, Masci, & Gobe, 2014).

2.7. Advent of Bio-informatics in antidote discovery against α- neurotoxins

With the advancement in the field of bioinformatics and availability of protein structural databases, several metabolites isolated from different sources like plants, bacteria, fungi etc. have been evaluated for their ability to neutralize snake venoms (Laustsen et al., 2018).

2.7.1. Molecular modelling

The 3-Dimensional structure is a pre-requisite for identifying the structural and functional information of a receptor (toxin). The 3D structure can be found from the online structural databases like protein data bank (PDB), ModBase, PDBsum, PDBTM, SCOP. When the structure cannot be found in the databases it can be predicted using an automated homology modelling method like Modeller 9v11 (Šali & Blundell, 1993). The obtained 3D model can be validated with Structural Analysis and Verification Server (SAVS) and molecular probity servers by analysing amino acids distribution in ψ and ϕ of Ramachandran Plot (Davis et al., 2007; Laskowski, MacArthur, Moss, & Thornton, 1993). The predicted model can then be subjected to energy minimisation process using steepest decent and conjugate gradient algorithms (Kumar, Suresh, & Priya, 2015).

2.7.2. Molecular dynamics simulation

Then it is allowed for molecular dynamics simulation, (MD simulation). MD simulation enables the description of structure, energy, and function relationships of molecular processes at the sub-atomic, atomic, supra-atomic or supramolecular level. The MD simulation can be done using Standard Dynamics Cascade programme of Accelrys Discovery Studio (ADS). This gives a stabilised model which can be tested for potential energy and root mean square deviation (RMSD). Then a final simulated model is obtained which is used for further computational studies (Kumar et al., 2015).

2.7.3. Identification and selection of antivenomic plants and their compounds

The information about plants with potent antivenomic property can be collected from various literature resources. The pharmacologically active plant compounds and their structural analogues are retrieved from various chemical databases using drug-likeliness filters (Kumar et al., 2015). IMPRAT is a manually curated database which has a collection of 1742 Indian medicinal plants, 9596 phytochemicals and 1124 therapeutic uses comprising 27074 plant phytochemical associations and 11514 plant therapeutic associations (Mohanraj et al., 2018). Pharmacokinetic properties of the retrieved compounds can be analysed using “absorption, distribution, metabolism, elimination and toxicity (ADMET) descriptors analysis” module.

Among these, the compounds sharing common chemical features called pharmacophore and can be listed out (Kumar et al., 2015).

2.7.4. Pharmacophore model generation

The pharmacoactive compounds are used as base structures for pharmacophore generation using "common feature pharmacophore generation" program of ADS 2.0 or any other similar program. The hypotheses generated are subjected for mapping the best pharmacophores among them. The mapping can be done using "Ligand pharmacophore mapping" protocol of ADS. The selection is done based on the fit values. The ligand compounds having comparatively higher structural likeliness are supposed to have higher fit values (Kumar et al., 2015).

2.7.5. Computational Virtual Screening

Computational virtual screening protocols are followed based on the generated pharmacophore models against the drug-like compound database, Mini-maybridge or ZINC drug like database or Druglike Diverse (Jiang, Zhou, Jiang, Sun, & Deng, 2018). The retrieved compounds are validated by checking their drug-likeliness and pharmacokinetics using ADMET descriptors module of ADS (Kumar et al., 2015).

2.7.6. Binding site analysis

The binding site of receptor (toxin) is predicted using three different binding site prediction tools so that definite binding pocket and the constituent amino acid residues can be confirmed. The prediction can be done using Q- Site Finder, CASTp server, and "binding site prediction" tool of ADS module in consequent manner (Kumar et al., 2015; Laurie & Jackson, 2005; Peters, Fauck, & Frömmel, 1996).

2.7.7. Molecular Docking analysis

The predicted binding site is used for Molecular docking analysis. The compounds (ligands) having comparatively good drug-likeliness and ADMET properties are chosen and docked to the predicted binding site of the receptor (toxin) using AutoDoc 4.0 and ADS ligand fit programmes (Morris et al., 1998). The resulting docked complexes are selected based on the binding energy inhibition constant, Van der Waal's and electrostatic energies. The "ADS consensus score protocol" is used to categorise the docked complex on the basis of the measured scores through multiple scoring functions, such as ligand score 1 and 2, Piecewise linear potential (PLP1) and (PLP2), Jain, potentials of mean score, Ludi, Dock score etc. The

top scoring ligands and their conformations are taken for further MD simulations (Kumar & Suresh, 2013b; Venkatachalam, Jiang, Oldfield, & Waldman, 2003).

2.7.8. Molecular Dynamics Simulation on Toxin- Inhibitor Complexes

MD simulation is done for calculating physical properties of biological molecules and predict the essential properties of biological interest using "ADS simulation module" (Karplus & McCammon, 2002; Kumar & Suresh, 2013a). Some initial steps to be done before MD simulation include: stabilisation of each docked complex by CHARMM force field, addition of hydrogen to receptor (toxin) and ligand (pharmacoactive compounds) structures, correction of protein structure conformation using a ADS clean protein tool. After these steps the docked complexes are analysed using MD simulation under periodic boundary conditions in all directions to simulate the entire molecular system. The final production steps are carried out for one nanosecond for each docked complex under constant temperature & volume and atomic coordinates of each complex is updated at one Pico second level. The resultant simulated complex conformational changes and corresponding RMSD are analysed using superimposition tool available in "ADS- Analysed Trajectory and Superimposed Protein tool (Kumar et al., 2015).

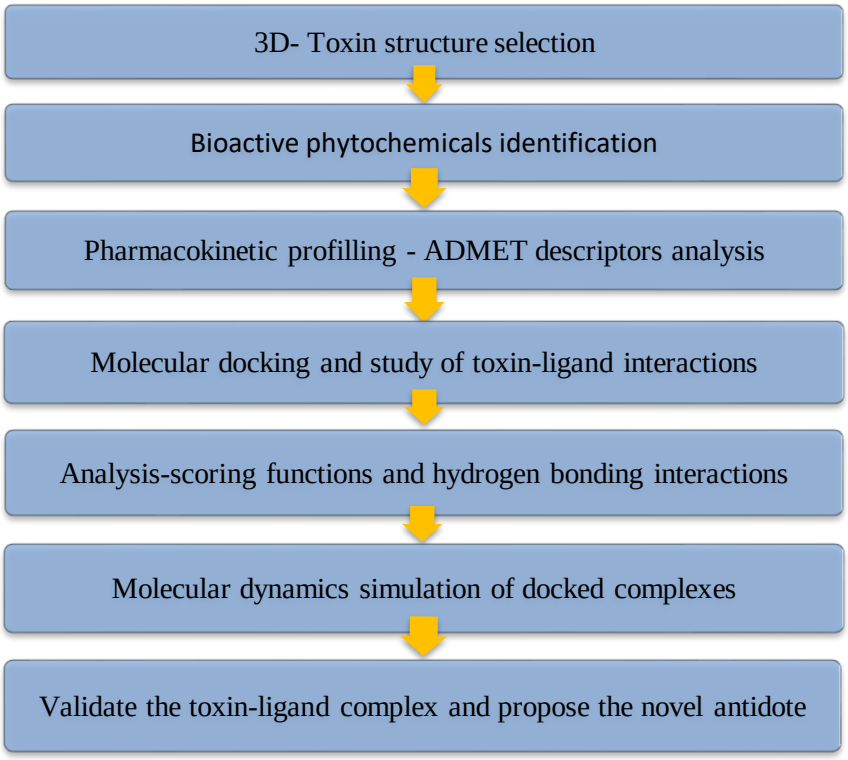
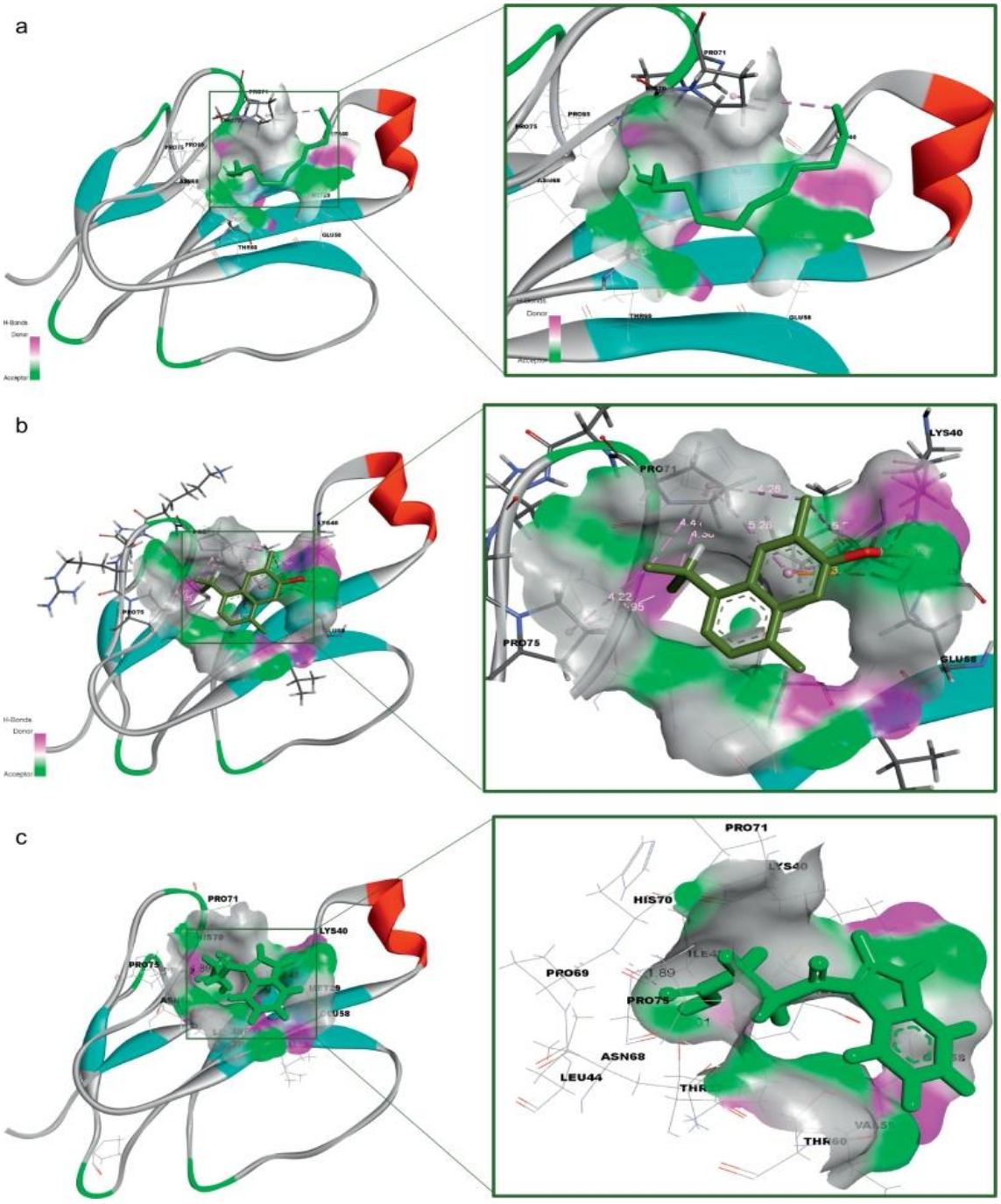
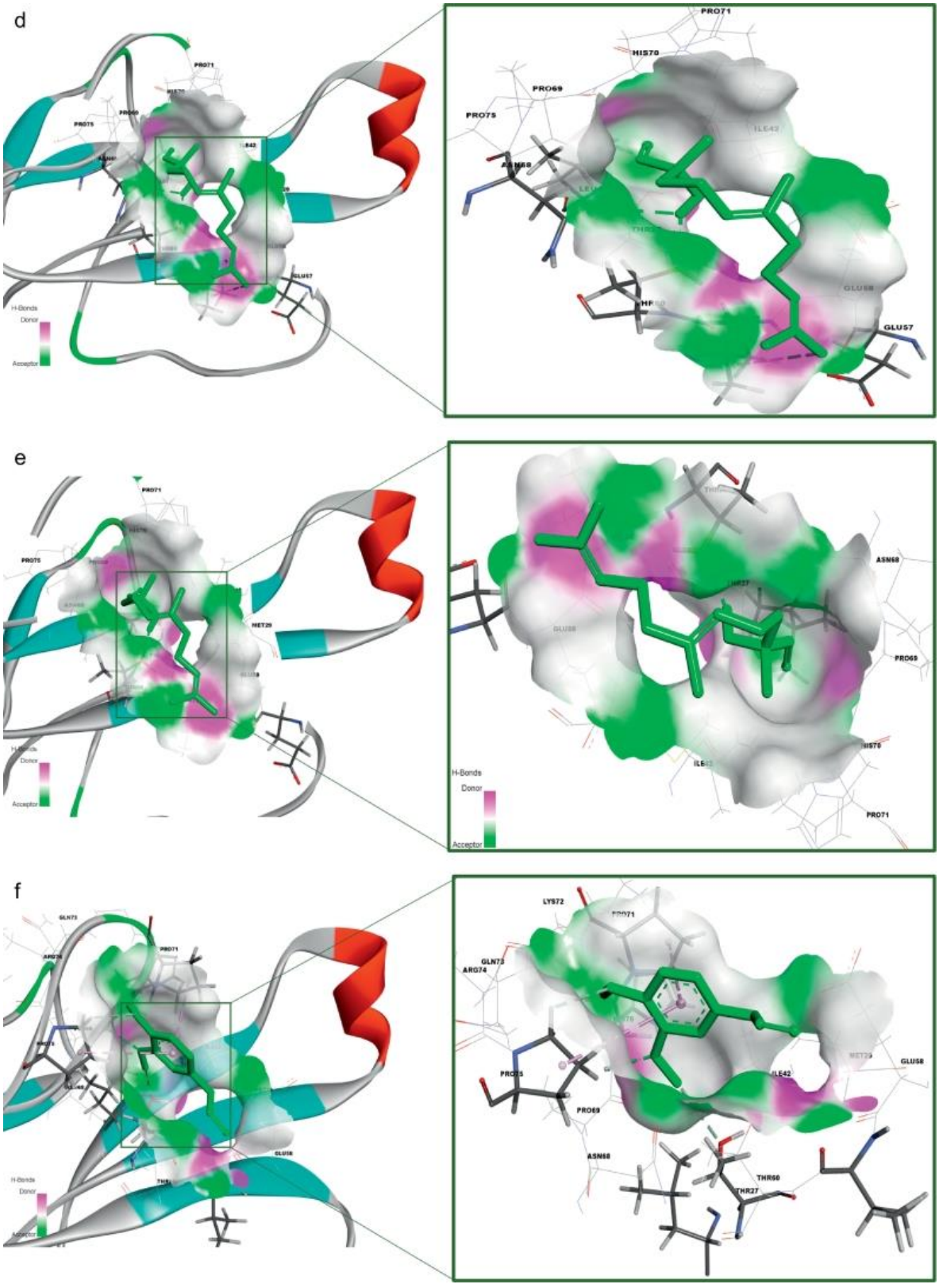


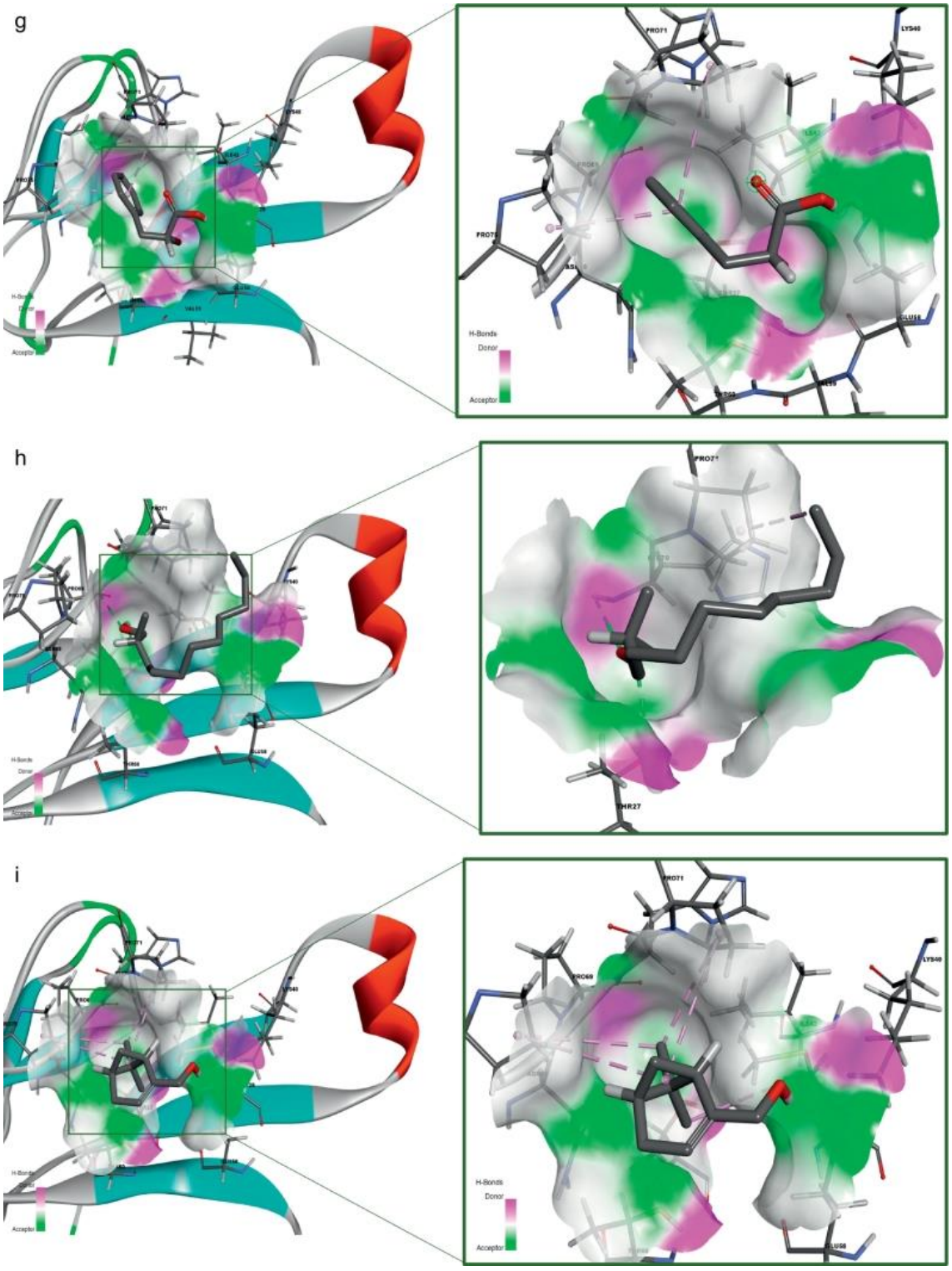
Fig.7 Flowchart showing step by step protocol involved in in-silico antidote identification

A molecular docking work against α-δ Bungarotoxin-4 (α-δ-Bgt-4) has revealed 9 inhibitory bioactive phytochemicals that could be used in designing a specific antidote against α-δ bgtx-4. This study screened about 849 bio active phytochemicals from 82 medicinal plants that have already shown antidote properties against snake venom toxins. Out of 849, 9 phytochemicals that showed best affinity to the α-δ Bungarotoxin-4 were selected. The 9 bio active phytochemicals are as follows : 2- Dodecanol, 7- Hydroxycadalene, Indole-3-(4'- oxo) butyric acid, Nerolidol-2, Transnerolidol, Eugenol, Benzene propanoic acid, 2- methyl- 1 – undecanol, Germacren-4-ol (Rajendran et al., 2018).





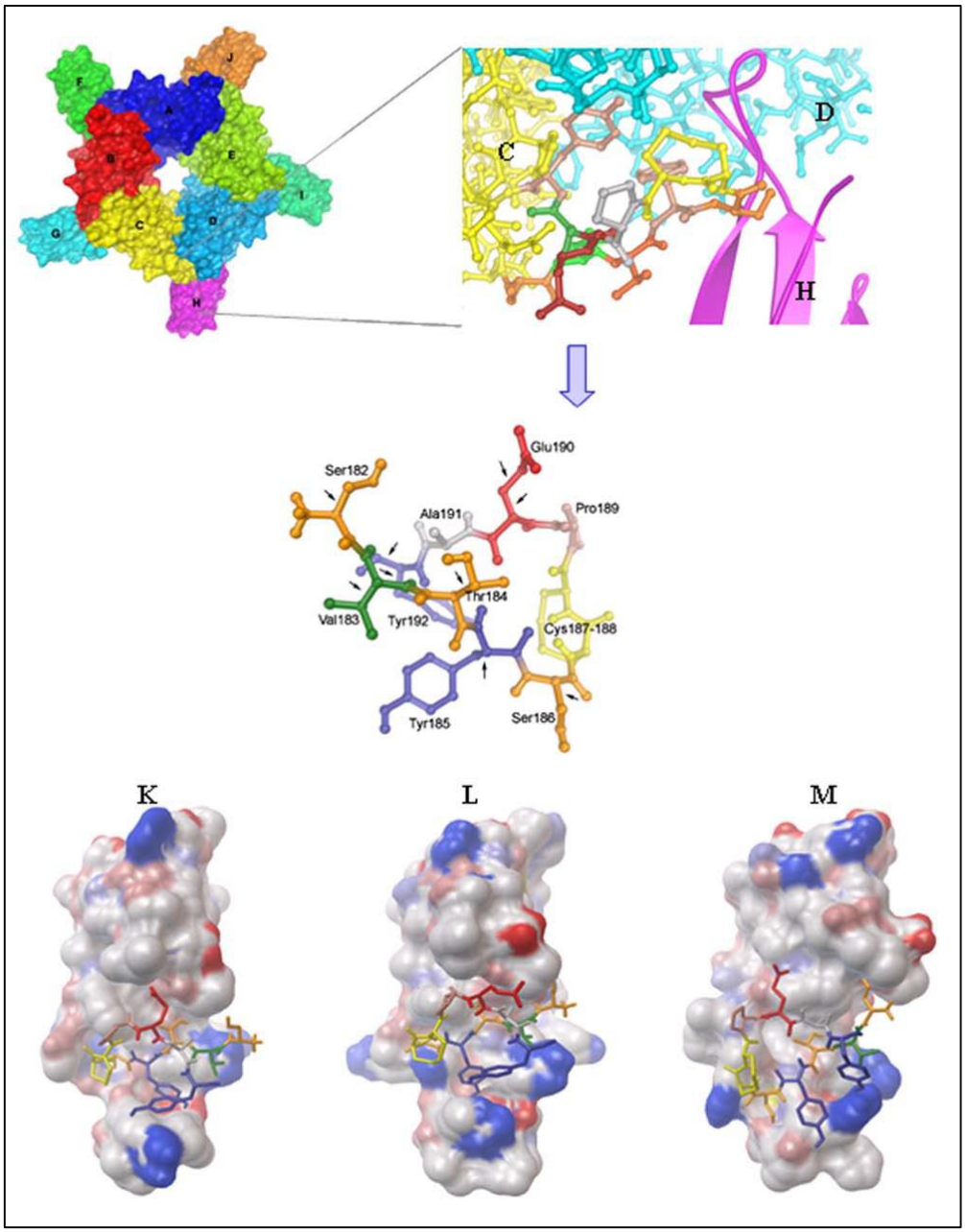
Source: Barani et al.



Source: Barani et al.

Fig.8 Molecular docking results by Barani et al. showing the receptor- ligand interaction between α-δ Bgt-4 and the 9 phytochemicals a)2- Dodecanol, b) 7-Hydroxycadalene, c) Indole-3-(4'- oxo) butyric acid, d) Nerolidol-2, e) Transnerolidol, f) Eugenol, g) Benzene propanoic acid, h)2- methyl- 1 – undecanol, i) Germacren-4-ol

Maleeruk et al, conducted a virtual screening and docking study against the CTX active site, the region directly involved in binding to nAChRs. They revealed that compound NSC121865 is the most potent inhibiters based on the screening docking experiment that can serve as a novel template for development of more specific anti Cobra toxin (Utsintong, Talley, Taylor, Olson, & Vajragupta, 2009).



Source: Maleeruk et al., 2009

Fig.9 Docking results of Maleeruk et al. showing docking between eleven residues from chain C (Ser182-Tyr192) of nAChR and α-cobratoxin

CHAPTER - 3

Discussion

3. DISCUSSION

Contemporary approaches to the treatment of snake bite count upon prompt administration of antivenom and management of the adverse consequences resulting from the venom per se, as well as from the antivenom. For this approach to be propitious, prompt medical treatment is required which is often inaccessible in the areas where snake bite is endemic. This usually brings with it significant discomfort and morbidity even when promptly applied.

In order to address these problems several studies have been carried out recently. Joy et al, proposed a dermato-pharmacologic approach for treating both local and systemic toxicity of snakebite. In their study, they repurposed a well-known dermatologic surgery technique tumescent anesthesia as a 'Contravenom'. They hypothesized that subcutaneous tumescent epinephrine when applied into and around the site of a subcutaneous lethal dose of a snake venom neurotoxin, will delay the systemic absorption of the toxin and prolong survival. This can allow time for the victim to reach a hospital for antivenom therapy (Makdisi, Kim, Klein, & Klein, 2018). However, they only focused on subcutaneous neurotoxin envenomation whereas intramuscular tumescent delivery ought to be studied.

Another study presented a new paradigm for the development of genetic vaccines against α-Cobratoxin which is the major toxic component of venom of *Naja Kaouthia*. The researchers introduced two substitutions in the Cobratoxin (CTX) cDNA at 2 residues critical for binding to the nAChR (Asp 27 to Arg, Arg 33 to Gly). This mutated cDNA was delivered in the context of a replication deficient adenovirus vector (AdmCTX). Their study showed that animals receiving AdmCTX 63 days prior to α-Cobratoxin infliction survived. One drawback of this Ad-based vaccine is that 30-50% of human population have pre-existing immunity to group C adenovirus, including the one studied by them (Pergolizzi, Dragos, Ropper, Menez, & Crystal, 2005).

Last two decades have seen a revolutionary advancement in drug discovery using in-silico techniques. The computational studies like ADMET7, molecular docking and molecular dynamics simulation, the molecular interaction of the pharmacologically active phytochemical compounds with neurotoxin can be investigated. It is evident from the aforementioned studies that the in-silico methods can be used as an effective tool to investigate the toxin-ligand interaction which in turn can be helpful in uncovering the antidote potential of herbal compounds.

CHAPTER - 4

Conclusion

4. CONCLUSION

Despite of having a huge armamentarium of traditional medicines, the venom induced lethality could not have been reduced to a considerable amount. This is indeed not very surprising because snake venoms comprise a diverse and vast multitude of toxins. Just using a polyvalent antivenin can't address or counteract all of the toxins present in the venom appropriately. So, the need of the hour is to develop an effective, species specific antidote against α-neurotoxins and with minimum side effects. Since the plasma derived antivenins put the life of patient at several other risks, plant derived metabolites can prove to be more effective on this front and can replace them promisingly. But even this would not suffice our requirements as we ought to know the specificity of the phytomedicines against the α-neurotoxins and their dosage. This can be achieved with the help of pharmacoinformatic tools.

In this post genomic era, where the scientists have been shifting their work interest towards the phytomedicines, bioinformatics can play a revolutionary role in identifying the phytocompounds with potent antivenin activity. With the help of bioinformatic tools, a lot many phyto-pharmacochemical compounds can be screened at the same time to check their specific interaction with neurotoxins, thus bringing a new hope for the discovery of effective novel anti-venom therapeutics.

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