CHAPTER- I

INTRODUCTION
1. INTRODUCTION

➢ Prevasiveness of Snake bites:

Snakes have created interest and analogies in various fields of human life by use of it as a symbol, in religion representing evil and sin and in the field of pharmacy and in medicine. Now a day’s snake bite envenoming is a major public health issue. It is very difficult to estimate the mortality due to snakebite envenomation. A public survey conducted by researchers estimated 421,000 cases of envenoming and 20,000 deaths yearly (Kasturiratne et al., 2008). This made researchers to study the characteristics of venoms (Calvete et al., 2009). Poisonous species of snakes are identified in India and among them Ophiophagus hannah (king kobra), Naja naja (spectacled cobra), Bangarus caeruleus (common krait), Daboia russelli (russell’s viper) and Echis charinata (saw scaled viper) are found to be responsible for most of the snake bites (Perumal Samy et al., 2012).

➢ Antivenoms and their disadvantages:

Use of antivenoms is the only treatment for the snake bite. Albert Calmette, a Frech scientist from Pasteur Institute was responsible for developing the first antivenom called as “Anti-ophidic serum” against Indian Cobra (Naja naja) in 1895 (Gomes, et al., 2010). Adequate treatment of snakebite envenoming is critically dependent on the ability of antivenoms to reverse venom-induced coagulopathy, hemorrhage, hypotensive shock and other signs of systemic envenomings (Gomes, et al., 2010). But antivenom binds and neutralize the venom but donot reverse the damage caused. First generation antivenoms, described 100 years ago consist of unpurified serum from animals, that are hyperimmunised with venom (Calmette, 1894). But these antivenoms show hypersensitivity reactions in sensitive individuals (Gomes, et al., 2010). Current antivenoms consist of purified immunoglobulins which have reduced the incidence and severity of treatment-induced serum-sickness, anaphylactic shock, and other adverse reactions (Calvete, et al., 2009). However, the
venom-immunization protocols have no changes and no attempts are made to direct the immune response to the most pathogenic venom proteins. The reason for less effectiveness is that many of the venom proteins are non-toxic and the low molecular weight venom proteins are highly toxic but weakly immunogenic. Consequently, the dose-efficacy of antivenoms suffer from the presence of redundant antibodies to non-toxic molecules and a lack of potent neutralizing antibodies to small molecular weight toxins.

This in turn is the cause for the need for high volumes of antivenom to effect treatment which in turn causes a consequent increase in the risk of serum-sickness and anaphylactic adverse effects (Chippaux et al., 1991). On the other hand, the immunization mixtures used for antivenom production are specific for every country or region, due to the intraspecific venom variability and to the fact that different snake species are responsible for the majority of envenomings in different countries (Shashidharamurthy and Kemparaju, 2010). The inter and intra-species heterogeneity in venom composition may account for differences in the clinical symptoms observed in human victims of envenoming by the same snake species in different geographical regions (Warrell, 1997).

Understanding the variation in antigenic constituents of venoms from snakes of distinct geographic origin represents a key challenge towards the design of novel, toxin-specific approach for the immunotherapy. To have a more specific immunogens a large number of protein toxins have been purified and characterized from snake venoms. Only few centers in India like Central Research Institute, Kasauli, Simla and the Haffkine Corporation, Parel, Mumbai, prepare polyvalent antivenoms. Hence antivenom therapy is costly and of limited supply. Other drawback is that, antivenom should be given only if the range of specificity is known i.e. species and must be given immediately. Problem of storage of these antivenoms is another factor that intensifies the risk.

➢ The need for herbal antivenoms:

Other alternative therapy for the snakebite is the use of Folk and traditional medicine in which various important plants have been included. Finding healing powers in plants is an ancient thought. Plant derived substances have recently gained
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great interest of many researchers owing to their versatile applications (Baris et al., 2006). Medicinal plants have been used for centuries as remedies for human diseases and offer a new source of biologically active chemical compound as antimicrobial agent. Medicinal plants are the richest bio-resources of drugs of traditional medicinal systems, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceuticals, intermediate and chemicals entitled for synthetic drugs (Hammer et al., 1999). It has been estimated that 14 - 28% of higher plant species are used medicinally and that 74% of pharmacologically active plant derived components were discovered after following up on ethno medicinal use of the plants (Ncube et al., 2008). Recently traditional medicine is accepted as an alternative form of health care (Hammer K.A. et al., 1999; Bisigano G. et al., 1996; Lis-Balchin and Deans, 1996). Out of the several hundred thousand medicinal plant species around the globe, only a small portion has been investigated both phytochemically and pharmacologically (Kurt Hostettmann, 1999).

➢ Importance of the work:

Ayurvedic system of medicine describes many plants as potent anti-venomic agents. But these medical plants have no scientific validation. Only few compounds of them have been studied upto SAR. Hence Pergularia daemia which is commonly available in the farming areas and which is also a source of biodynamic agents is selected for the work.

➢ Selection of venom:

The Indian cobra (Naja naja) feeds mainly on rodents which are commonly associated with human habitats. It is therefore a common cause of the snake bite. People from many parts die due to the bite of this species (Ability Dey and Jitendra Nath De, 2012). Pharmacology of the death in most untreated cases is postsynaptic neuromuscular blockade caused by one or more neurotoxins which cause respiratory paralysis. Local necrosis also complicates the treatment. Hence the venom of this snake has been selected for the work.

Many plants have been used to treat various effects induced by snake bite (Martz, 1992, Otero et al., 2000, Coe and Anderson, 2005, Sofowara, 1993, Nunez, et
al., 2005, Raman and Barua 1958). Therefore, much of the research efforts have been published since years (Soares et al., 2005, Ticili et al., 2005, Mukherjee et al., 2008), on the potential use of medicinal plant extracts to reduce or to evaluate compounds that could efficiently minimize the activity of phospholipase A$_2$ (PLA$_2$) enzymes which constituted a rich source in snake venoms. Several PLA$_2$ inhibitors have been isolated from plants. A compound AIPLAI purified from the methanol leaf extract of Azadirachta indica (Neem) inhibits the Cobra and Russell's viper venoms (RVVs) PLA$_2$ enzymes in a dose-dependent manner (Lizano et al., 2003). Chemical constituents like alkaloids, flavonoids, sitosterol or glucoside, lupeol, gymnemagenin, phenolics, triterpenes like oleanoic acid, tannins, α and β amyrin have been reported for anti-snake venom activity. All these classes of chemical compounds are capable of interacting with macromolecular targets with enzymes or receptors and it can effectively inhibit the toxic effect of snake venoms in vitro than in vivo (Borges et al., 2005).

Hence the basic aim of the study was to demonstrate the inhibitory ability of the compounds present in the leaf extract of Pergularia daemia on enzymatic activities of cobra venom i.e. PLA$_2$ activity, L-amino oxidase and hyaluronidase and other important activities associated. Geographically it is found in the plains throughout India and in tropical and sub tropical areas (Pullaiah et al., 2000) worldwide.

➢ Other activities studied:

At the same time antimicrobial and antioxidant activities are also studied as over the last two decades, intensive effort has also been made to discover chemically useful antibacterial or antifungal drugs of plant origin (Sofowara 1993, Valsaraj et al., 1997, Perumalsamy et al., 1999). But, only few of these chemical substances have been isolated and are tested for their activity (Nunez et al., 2005, Soares et al., 2005, Marcussi et al., 2007). Polyphenols are found to be present in considerable number in all these active compounds (Pithayanukul et al., 2005, Leanpolchareanchai et al., 2009, Ticli et al., 2005). Polyphenols are the group of chemical compounds found in all the plants and microorganisms. They are characterized by the presence of more than one phenol unit per molecule. Polyphenols are divided into hydrolysable tannins and phenylpropanoids. Hydrolysable
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tannins include Gallic acid esters of glucose and other sugars and phenylpropanoids include lignins, flavonoids and condensed tannins. These compounds are one of the most versatile group compounds present in the plant kingdom, with diverse effects such as antiophidians, antimicrobial (mostly on highly pathogenic bacteria and virus), antioxidant, anthelminthic and antihepatoxic activities (Haslam et al., 1996). So for humans, plants natural products are the best bio-rational alternatives today (Tiwari et al., 2007). Ethno pharmacologists, botanists, microbiologists and natural product chemists are searching the world for phytochemicals which could be developed for treatment of infectious diseases (Marjorie, 1999). Hence these activities are also described for the extract.

➢ Novelty of the work:

• In this work an attempt had been made to develop an extraction procedure in which all the important constituents required for the activity can be extracted without any toxic components.

• Molecular docking studies for the determination of anti-venom property for Pergularia daemia was performed for the first time.
1.1. AIM:

The study preliminarily aims at identifying the plant at genomic level. The second basic aim of the study is to comparatively analyse the qualitative and quantitative secondary metabolite components and to determine the biological activities of the leaf extract of widely distributed and used weed *Pergularia daemia* of Asclepediaceae member in order to establish a theoretical relationship between its constituents, biological activity and use.

The study aims at identifying anti-venom potential of this plant against *Naja naja* snake venom by using *in vitro* studies after the extract is characterised for its phytoconstituents using chemical tests and GC-MS. It also aims at determining structure activity relationship (SAR) of the identified compounds against venom components by using *in silico* docking studies. The components of the extract will be docked against the venom enzymes which are said to be potent toxins of the venom ie. Phospholipase A₂ (PLA₂), L-Amino acid oxidase (LAAO) and proteases. The basic aim was to identify *Pergularia daemia* as a potent anti-venom plant by determining interaction energies which can be calculated by protein-protein docking. Hence the work aims at providing new insights for the development of novel anti-venom plant.

The present work also aims at determining antimicrobial properties of the extract *in vitro*. Dilution methods are used to determine the minimum inhibitory concentrations of antimicrobial agents. The lowest concentration of an antimicrobial agent that will inhibit the growth of microorganism termed as MIC and MBC are determined. The method described in this work is based on that described in report of an international collaborative study of antimicrobial susceptibility testing and is very similar to those described and recommended in many countries (Kokwar 1981). The most important sources of antioxidant compounds and their medicinal value is due to their antioxidant property (Samuelsson et al., 1991, Nagaraju et al., 1990) hence antioxidant property will also be determined.

This work also aims at developing molecular marker for the identification of plant by using trn-intron chloroplast genome so that adultration of the plant material can be identified easily.
Finally the work aims at determining toxicity of the extract by using animal model. Acute toxicity has to be determined for the total extract.

1.2. OBJECTIVE:

The basic objectives of the work are as follows:

i) To isolate Chloroplast Trnl-F gene for the species identification.

ii) To extract the leaves by using solvent extraction procedure, isolate and to identify the compounds by GC-MS analysis.

iii) To determine in vitro anti-snake venom activity of the extract by performing Anti-PLA₂ activity, Protease inhibition test, anti- L- amino oxidase activity and anti-Hyaluronidase activity.

iv) Further the potentiality of identified compounds as anti-snake venoms will be described by using Molegro Virtual Docker (MVD) software, which is basically aimed at docking the actual binding sites of the isolated compounds required to perform the identified functions.

v) To screen the extract for its in vitro antimicrobial activities against some of the pathogenic agents known to cause many of the gastrointestinal tract related ailments and extra intestinal infections. These organisms are also reported to cause central nervous system, blood stream, skin and opportunistic infections in animals as well as in humans.

vi) To subject the extract for MIC and MBC determination.

vii) To determine antioxidant properties of the extract.
viii) Finally the extract will be checked for its *in-vivo* acute toxicity using animal model i.e. (albino mice).