Characterization of Plant Based Antidote Against Snake Venom.

By

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Antidotes

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Anti venom

Drugs – Toxicology

Characterization of Plant Based Antidote Against Snake Venom.

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"In the name of ALLAH The Most Gracious and The Most Beneficial"

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FINAL APPROVAL

It is certified that we have read the final thesis entitled "**Characterization of Plant Based Antidote Against Snake Venom.**" submitted by **Mr. Sayyad Waqas Umar** and it is our Judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the MS Degree in Biotechnology.

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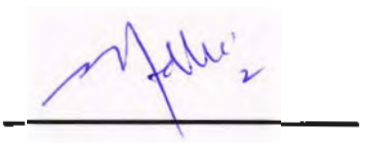

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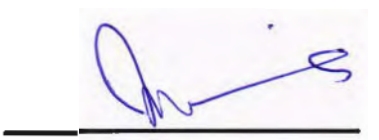

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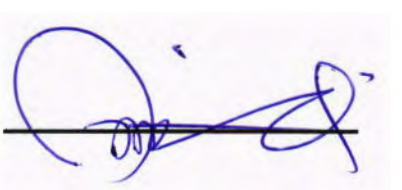

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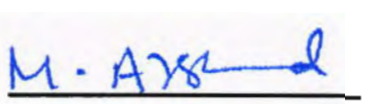

A thesis submitted to Department of Bioinformatics and Biotechnology,
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Master in Sciences of Biotechnology
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This humble effort is

Dedicated

To

My beloved

Parents,

Brothers, Sisters

And

Teachers

Who inspired me for higher ideals of Life

DECLARATION

I hereby solemnly declare that the work **"Characterization of Plant Based Antidote Against Snake Venom."** presented in the following thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Dated: _____

Sayyad Waqas Umar

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Sayyad Waqas Umar

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ABBREVIATIONS

AA	Amino acid
AVS	Standard polyvalent anti venom serum
C. gigantea	*Calotropis gigantea*
GM	Gram
IP	Intraperitoneal
LD	Lethal Dose
M	Meter
ML	Milliliter
N	Number
NIH	National Institute of Health
PLA2	Phospholipase A2
PO	Per oral
WHO	World Health Organization

ABSTRACT

Calotropis gigantea is used traditionally for many ailments such as leaves and outer parts have a good effect as antioxidant activity, anti-Candida activity, antidiarrheal activity and antibacterial activity. Flowers work as antimicrobial, painkiller action and cytotoxic activity. Roots are informed to comprise antipyretic activity and cytotoxic activity. Whole three parts of this plants are well-thought-out to contain therapeutic possessions and used in the cure of syphilis, boils, inflammation, epilepsy, hysteria, fever, muscular spasm, warts, leprosy, gout, snakebites, and cancer.

Venom of all four snakes was collected and stored separately. The snake's venom was centrifuged and lyophilized. After these processes the processed venom was stored at 4°C by collecting in dark glass vessels with rubber seals on the top. Vessels were labeled with Snake specie name and total weight of the venom. Standard polyvalent anti venom serum (AVS) collected from horse plasma was used in all experiments. The mixture of antivenom and venom was prepared and incubated for 30 minutes. After incubation the mixture was injected to test mice, treated with 0.2ml dose which was injected intraperitoneal route of injecting. 4 Swiss albino mice 18-20gms used for each dose. After 24 hrs results were calculated.

Calotropis gigantea's four extracts of chroloform, distilled water, petroleum ether and methanol with three different doses (100mg/kg, 200mg/kg, and 300mg/kg) were incubated with 4 different snakes venom at 37 °C for 30 minutes and applied by in vivo and in vitro methods on test animals. After 24 hrs results were noted for each snake venom and mice were found survived without any indications of toxicity or deaths. The study confirms the antidote characteristics of different extracts of *Calotropis gigantea* with distilled water, chloroform, petroleum ether and methanol against venoms of *Cobra, Vipera russelli, Krait* and *Echis.*

INTRODUCTION

1. INTRODUCTION

From primitive era's snakes are considered as holy and blessed in nations of the world especially by Hindus and Buddhists. They are revered in many regions of the world even these days and much folklore are related to them in different cultures. There is no single country in the world in which snakes are not found. In Himalayas and America snakes are present from sea level up to altitudes higher than 4000 m by little ecological elision. Snakes are not only found in landscapes but there are also aquatic types of snakes. Aquatic snakes plunge more than 100 m to depths in water (Warrell 2010).

Since primeval era snakes are also considered as sign of fear. Because of great proportion of deaths and injuries snake biting is known as one of threatening topics. It is now a health problem worldwide but more serious problem in hot rural regions of the world because of nearly 40,000 deaths annually. Merely two hundred types of snakes have found as poisonous among all snake types found on the earth. These poisonous types are categorized into following groups namely *Viperidae* (old world vipers), *Elapidae* (fixed & front fanged species), *Crotalidae* (pit vipers), *Colubridae* (fixed & rear fanged species) and *Hydrophidae* (sea snakes), families (Matsui *et al.*, 2000; Warrell, 2010).

Among these poisonous families *Elapidae, Viperidae and Hydrophidae* are placed in deadly poisonous zone which are frequently inhabit in southern Asian regions such as Pakistan. Because of these deadly venomous species presence in Asian countries there are much problems faced by developing parts by snake bite envenomation. In the rural areas of the Pakistan poisonous snakes are threatening reality. Medically significant types are available in huge variety with a great health threat to people in towns and undeveloped regions. Pakistan is the habitat of 80 different types of snakes. Among them 15 species are considered as noxious poisonous. Pakistani cobra is a member of family *Elapidaes* and genus Naja is frequently distributed (Wuster, 1996; Feroze *et al.*, 2010) and known as symbol of danger in southern Punjab area of Pakistan (Gutierrez *et al.*, 2006).

In Pakistan it has been reported that per year about 20,000 bits happen and 8,200 people died in the result (Razi *et al.*, 2011). Besides, those preys that persist have many serious health problems such as bleeding gums, tissue necrosis, swelling and blistering, bleeding from wounds (Davidson *et al.*, 1995; Gutiérrez *et al.*, 2009).

Worldwide, snakebite is a recognized health exigency predominant, but those areas of world which are not properly developed with large population and less economic growth are facing more problems, misunderstandings and ignoring to this serious issue. The load of deaths by snake biting is maximum in states that are not able fiscally to treat those patients. In 1869, Joseph Fyrer of Indian Medical services, introduced snakebite first time in Indian subcontinent in which he stated deaths occurred by snakebite nearly half of British India in which Bangladesh, Pakistan and Burma also included (Mohapatra *et al.,* 2011).

Total number of deaths caused contact with poisonous animals reduced from 54,900 (95% uncertainty intervals 30,100-89,300) in 1990 to 47,000 (25,600-84,700) in 2010 is estimated from great ways of altered bases in recently published Global Burden of Disease 2010. While, morbidity from "other neglected tropical diseases" in which snake biting also included higher from 22,900 (14,300-29,500) in 1990 to 23,700 (16,600 - 30,900) in 2010. From last 50 years, globally some efforts have been made to know about occurrence and deaths and disease caused from snake biting (Warrell *et al.,* 2013).

Pakistan is suffering from serious threats of venomous snake biting. According to a report deaths occur in Pakistan per year has a part of 20,000 deaths happen due to biting of poisonous snakes. Many species of venomous and non-venomous snakes are found in Pakisatan's different areas, but *Naja naja* and *Naja oxiana* snakes are scattered all over the Pakistan belonging to genus *Naja* are highly poisonous (Gutiérrez *et al.,* 2006; Asad *et al.,* 2014).

Snakes venom contains special chemicals, biological actions and intricate combination of enzymes, proteins and peptides of low molecular mass. Many lectines, cardiotoxins, neurotoxins, cytotoxins, haemorrhagins, nerve growth factors and disintrigrins. Hypotension, haematurea, bleeding from the gastrointestinal tract, tympanic membrane, genito-urinary tract, gingival bleeding, and ecchymosis. Some studies shows that intracranial and extensive necrosis are the manifestations of snake's envenomation (Chacko *et al.,* 2012).

Besides death, survived victims from snake bites bear several problems such as hematuria, local pain, bleeding gums, swelling, bleeding from wounds (local hemorrhage), and blistering and tissue necrosis. One of hemostatic complication, Local hemorrhage cause after Viperid and Elapid (including cobra) snakes bite. As hemolysis is a causal element of snake envenomation that cannot be ignored therefore, has been noticed, though it is not very common in cobra venom. Anti-sera named Equine benefit in combating cobra bite

envenomation yet do not defend local tissue damage i.e. edema and hemorrhage necrosis (Asad *et al.*, 2014).

Instant administration of specific polyvalent anti-venoms subsequent envenomation is considered the most accepted and effective cure for snakebite patients. Usually, venominduced local tissue damage related with danger of serum reactions and of anaphylaxis cannot be cured with anti anti venoms. May be commercially available hyper immune antivenom consists of excess amounts of non-immunoglobulin proteins which cause these indications. Generally, antivenoms are made of hyper immune sera composed from animals that fix and deactivate venom constituents. Formation of antiserum takes more time, cost and needs perfect packing circumstance. There are different reactions of antivenom such as serum sickness, pyrogen reaction and anaphylactic shock (Meenatchisundaram *et al.*, 2009).

Even though the accessibility of various methods for the detection of medicinally, natural goods still stay as one of the greatest reservoirs of new basic sorts. The standardized extracts of plants, offer limitless chances for new medication findings due to the matchless obtainability of chemical variety (Cos *et al.*, 2006).

Besides, snake biting is a serious issue globally and most countries are not in the condition of affording its treatments per injury Mother Nature contains a full stock of herbs, shrubs and trees for the treatment of living beings. Animals, plants and other natural things of the world from ancient era to modern times have deep effect on daily life, culture and society of human.

As the starting of evolution many sections of human beings has prey to plants. Plants are also used to store as a genomic source and used as fiber, fuel, fodder, fertilizer (Sureshkumar *et al.*, 2012). As pre historic time's plants have been a mutable way of medicins; man inclines to avoid the significance of herbal drugs (Sofowora *et al.*, 1982). Though a great variety of plants is spread throughout the world, a small section has been examined by pharmacologically and phutochemically. When one suppose that a solitary plant may comprise up to thousands of components, the opportunities of making novel detections come to be obvious (Janovik *et al.*, 2012).

Pakistan has extensive past of therapeutic plants where diversity of them have been used locally particularly for snakebite (Asad *et al.*, 2011). For that reason it is essential to assess them scientifically their myth statements as anti-snake venom (anti-hemolytic) (Asad *et al.*, 2014).

From primitive era medically important plants have been used as therapies for venomous nibbles due to the presence of antitoxic material in them naturally and they are cheap source also. Nearly 25000 effectual plant based preparations are projected in the aboriginal medical scripts. In Asian countries traditional treatment method is more successful in countryside inhabitants by healing and upholding the physical and emotional comfort. Traditional medication contains all types of traditional remedies, alternative drugs, herbs and plants and any type of method which is traditionally famous and useful in specific tradition of people. In underdeveloped regions nearly 80% of population hinge on traditional remedies for primary healthcare treatments and plants play an important role in traditional remedies in these cases. A large section of people in various countries favor herbal remedies over artificial antibiotics. Science has a new branch in which collaboration between people and plants comprise the use of medicinal plants traditionally by local societies and organization of plant diversity by the natives, is called Ethnobotany (Ishtiaq *et al.,* 2007).

For the cures of snakebite traditional herbal remedies are frequently accessible in rural regions. These known plants are used to apply on bitten part to less snake venom action either by rubbing whole plant or some of its parts, or rubbing its extract or chewing or dinking plant juice. The practice is carried out in many areas of the world of using medicinal plants either as single for the treatment or as mixture with other plants as antidote for better results (Samy *et al.,* 2008).

Allopathic methods co-occur with traditional methods in many civilizations. Mostly traditional methods used for curing diseases are basically natural and native and usually those in which people have viewpoint on the world and life (Toledo *et al.,* 2009). It has revealed through research work that in Pakistan most of the common medicinal plants for their anti-venom properties have not been scientifically valued. Assortment of medicinal plants for anti-venom properties is very vital and essential work. By using various methods i.e randomized selection or amalgamation, chemical content, traditional use, toxicity of different criteria selection can be made (Rates, 2001).

Numerous ethno botanical papers describe *Calotropis gigantea* as a strong anti-snake venomous plant (Rahmatullah *et al.,* 2010). *Calotropis* are innate to tropical and subtropical regions of Africa and Asia and are commonly known plant in hot regions of the world. It is a common wasteland weed related to Asclepiadaceae family in kingdom Plantae (Sharma, 1934).

Crown flower weed and milk weed are also the names of *Calotropis gigantea*. It is sap producing weed and excrete sap out of it after an internal wound. Various components are found in its latex including proteins, alkaloids, sugars, tannins, starch, gum and resins (Abraham and Joshi, 1979).

Table No.1.1 Synonyms of *Calotropis gigantea* in different languages (Sharma *et al.*, 2011).

S. no.	Language	Common name
1	Sanskrit	Arka, Alaka, Ravi
2	Hindi	Aaka, Aanka, Ak
3	English	Calotropis, Roostertree, Mudar plant
4	Arabic	Oshar
5	French	Calotrope, Pomme de Sodome
6	German	Wahre Mudarpflanzer, Gomeiner
7	Italian	Calotropo
8	Spanish	Algodon extranjero, Cazuela
9	Turkish	Ipekag

Two common species of this plant are *Calotropis procera* (Ait.) R.Br. and *Calotropis gigantea* (Linn.) R.Br. Defined by the Sanskrit writers (Yelne et al., 2000). These species carried same properties and used as alternate for one another. Three types of milk weed are stated in Dhanvantari Nigantu i.e Suklarkah, Sveta and Rajarkah. This plant has been extensively used in therapeutic methods (Kartikar and Basu, 1994).

Its uses are numerous traditionally i.e. it cures common illnesses like cough, fever, cold, asthma, nausea, diarrhea, vomiting and indigestion and also useful medically (Kirthikar and Basu, 1991).

"*Sweta arka*" is also a name of this weed in old time Ayurveda in Indian subcontinent. This plant is experimented for many medicinal purposes lately and described as scientifically important plant having different useful medicinal characteristics such as it is used as anodyne, disinfectant and cytotoxic in action. Several forms of components have been revealed by phytochemical studies on this plant i.e triterpinoids, cardenolide, resins, alkaloids, proteolytic enzymes in latex, anthocyanins, sterol, tannins, cardiac glycosides, saponins and flavonoids. Flower consists cyclisadol, multiflorenol and terpenes (Al-Yahya *et al.*, 1990).

1.1 AIM AND OBJECTIVES OF STUDY

Studies on characterization of plant based antidote against snake's venom.

- Sampling and extraction of metabolites (extracts) from *Calotropis gigantea.*
- Screening and Inhibition studies of Anti-venom activities.
- Venom neutralization studies.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Types of Venomous snakes present in Pakistan

The medical range of snake bite envenomation is also described by the word ophitoxaemia. Only 500 species of the snakes out of 2500-3000, scattered all over the world are venomous. Like animals and other living things snakes are also classified into families on the basis of their physical properties and mode of action including mycology. Dentition, sensory organs, osteology arrangement of scales etc (Makhija and Khamar, 2010).

King Cobra, common Cobra and Krait, members of *Elapidae* family, pit viper, saw-scaled viper and *Russell's* viper belonging to family *Viperidae* and sea snakes (Hydrophidae) are commonly found in the India subcontinent (Makhija and Khamar, 2010).

2.1.1 Family Elapidae

Deadly poisonous snakes are the members of this family such as, Corals, Cobras, Mambas and Kraits. This family is further classified into two chief genera, Naja and Bangarus. Examples are Sindh Krait (*Bungarus s. sindanus),* common Krait (*Bungarus caeruleus),* and Indian Cobra also called Sheesh nag (*Naja naja naja),* Northern Punjab krait (*Bungarus sindanus razai)* and Oxus Cobra (*Naja naja oxiana).* Physically they are less harmful excluding a couple of hollow, fixed short teeth that are generally long from other teeth in the jaw. These two teeth are related with the release of venom by an opining on their tips. Furthermore, loreal scales lacking from head scales is one of their characteristic (www.willifeofpakistan.com; Khan, 1993).

2.1.1.1 Genus *Naja (nags)*

In subcontinent snakes of this genus known as greatest dreaded and lethal snakes. They are lengthy, weighted and compacted bodied. They are able to form a "hood" like structure and can raise it above in vertical position by the expansion of the skin by the enlargement of their ribs. In Pakistan, this genus is signified by two species; Oxus or Brown Cobra (*Naja oxiana*) and Spectacled or Indian cobra (*Naja naja*).

Figure No. 2.1 Cobra (*Naja naja*)

2.1.1.2 Genus *Bangarus*

They are cautious but if they are triggered they moves their body in round shape and producing hissing sounds. Head bent under loops whereas keep tail in moving state back and forth and high to scatter the concentration of prey. They are average sized, small eyed, dark brown or black steel like shinny dorsal cavity having white colored tiny bands, horizontal scales Kraits. Kraits have ability to circumvolve the body in ball shape.

Figure No. 2.2 Common Krait (Bungarus caeruleus)

2.1.2 Family Viperidae (Pitless vipers)

This family is classified into five genera, seven species and subspecies. This family contains most useful snakes in medicine and research i.e. Saw-scaled Viper (*Echis carinatus*) and Russell's or Chain Viper (*Daboia russelii*). It has been reported that Russell's viper is commonly found in the regions of Northern Pakistan, western Himalayas and Sikkim

to Chitral. It is also present in Baltistan, Deosai Plain, eastern Khyber Pakhtoon khwa, Margalla Hills and Nathia Gali according to fresh surveys.

Figure No. 2.3 Russell viper (*Daboia russelii*)

2.1.3 Family Crotalidae (Pit vipers)

In Pakistan Himalayan Pit Viper (*Gloydius himalayanus)* is single symbolic specie of this family. It is found in mountains. It belongs to the genus *Gloydius*. Up to 1500 m altitude its presence has been reported restricted. From Dharmshala Glacier its presence has reported at 5000 m altitude (Khan, 2002). Physically they are sharped muzzle, "a deep loreal quarry between eye and nostril"; generally minor single scales split off from the central head scales; head-top comparatively smooth with large protections; postocular and subocular are joined to form a large scale; nostril fixed between two nasal scales; which does not route the mouth (www.wildlifeofpakistan.com).

Figure No. 2.4 Saw-scaled viper (*Echis carinatus*)

2.1.4 Family Hydrophidae (Sea Snakes)

Though they have been reported as poisonous snakes but they are not commonly encountered. In Pakistan, there 14 species are present. Their examples are short Sea Snake (*Lapemis curtus)*, Stoke's Sea Snake (*Astrotia stokesii),* Annulated Sea Snake (*Hydrophis cyanocinctus),* Common Small-Headed Sea Snake (*Microcephalophis gracilis gracilis)* etc.

Figure No. 2.5 Sea Snake (*Astrotia stokesii*)

2.2 Chemical composition of Snake Venom

Combination of enzymes with different lethal proteins is the basic components of venoms of snakes. Cytotoxins, myotoxins, cardio toxins, neurotoxins hemorrhagic metalloproteinases coagulant components, phospholipases A2(PLA2s) and other proteolytic enzymes are major constituents of snakes venoms enzymes and proteins (Kini, 1997: Soares *et al.,* 2004). Three proteins namely neurotoxins, phospholipase A2 and cardiotoxins are present in Cobra venom therefore they are neurotoxic by nature. These proteins have ability to encounter the prey central nervous system and make insufficient respiration, tissues injury, paralyzing and heart failure (Meenatchisundaram *et al.,* 2009).

2.3 Snakes classification according to their toxins

Snakes venoms have unique mode of action. Some venom victimizes the circulatory system of the victim, other affect the central nervous system. Those venoms which target the central nervous system are called, neurotoxins while those, degrade the circulatory system are haemotoxins. Haemotoxins is the part of rattlesnakes, cottonmouths and copper head snakes

while mambas, kraits, cobras, sea snakes and coral are the snakes which contain neurotoxic venom (Blanchard, 2001).

2.4 Complications/ symptoms

Main symptoms or complications of snake bite include:

- Myotoxicity.
- Hemorrhage.
- Cytotoxicity.
- Fang marks in the skin.
- Dizziness.
- Pain.
- Convulsions.
- Blurred vision.
- Weakness.
- Fever.
- Tingling.
- Edema.
- Burning.
- Necrosis.
- High pulse rate.
- Diarrhea and Excessive sweating. (Razi *et al.*, 2010; Das, 2009).

2.5 Anti-venom medicinal plants

From ancient times people use plants for the treatment of different diseases because of their characteristics against those diseases. Plants show unique behavior against many illnesses, wounds, cuts etc. their unique behavior is due to those components that present in these plants and work on effected area of patient. Plants have different properties from one another against different ailments. Plants are widely used in our cultures because of their better working mechanism in the body and nullifying reactions (Rates, 2001).

Many medicines are produced from plants or plants play a key role as precursor in their formation to make them more powerful against diseases and make them able to inhibit, allay and remedy an infection or modify their infectious and biological cycles. Those types of plants are known as Medicinal plants.

Medicinal plants are used worldwide as therapy against many infectious diseases. There are many uses of medicinal plants. One of their major uses in treating of infections is that they are widely used in case of biting of venomous animals (Martz, 1992; Mors *et al.*, 2000; Soares *et al.*, 2004; Soares *et al.*, 2005; Soares *et al,*. 2009).

Medicinal plants are enriched with those compounds that are pharmalogic dynamic complexes and act against venoms as inhibitor. Some constituents of medicinal plants have best mode of action because physically they look like biological compounds and in this way they perform their work. Therefore, medicinal plants play part in the health of a major proportion of world (Havsteen, 1983). It is proved by medicinal studies that the mode of action of various purified toxins and crude venoms are provoked in customary medicine by using some of the fractions and extracts of medicinal plants Borges *et al.*, 2000; Otero *et al,*. 2000 Borges *et al.*, 2001; Biondo *et al.*, 2003; Biondo *et al.*, 2004; Januário *et al.*, 2004; da Silva *et al.*, 2005; Maiorano *et al.*, 2005; Oliveira *et al.*, 2005; Ticli *et al.*, 2005).

In a survey, it has been shown that 80% of world developing population is depend on medicinal plants in various cases including as a source of earning as well as for the betterment of health (Shah *et al.*, 2009). Medicinal plants have much importance in current pharmacopoeia. Different parts of medicinal plants are used in the production of nearly 25% of medicines; natural pioneers are the sources of 121 of such dynamic combinations are being in current practice or artificial analogue (Shinwari, 2010).

2.6 Use of medicinal plants in Pakistan

80,943 km^2 is the expanse of Pakistan. It has 23° 45" to 36° 50" N lati-tude and 60° 55" to 75° 30" E longitude. Furthermore, 0 to 8611 m is an altitude ranges of Pakistan, by combination of climatic regions and distinguishing biodiversity of medicinal plants. In Pakistan 600 to 700 plants species are used in curative resolutions out of 60,000 higher plants species present in our country. For curing slight and serious diseases Bulk of Pakistan's residents rely on myth therapies. Pakistani medicinal plants are very useful in theraping many ailments. These medicinal plants have abilities to relief many types of pains including headache to stomach pain, and also cure and fill wouds and cuts made by insects or animals (Shinwari, 2010). Yet, conferring to the works examination and to the finest of our information, in Pakistan widely held of medicinal plants prevalent, have not been systematically assessed for their antivenom potentials. One of very important and fundamental steps is the selection of medicinal plants for antivenom action. In a number of

ways collection can be prepared comprising chemical content, traditional use, randomized selection, noxiousness, or union of different measures (Rates, 2001).

2.7 Types of medicinal plants as antivenin snakebites

- *Acacia catechu* Willd,
- *Belamcanda chinensis* DC,
- *Caesalpinia bonduc Roxb,*
- *Daphne mezereum* L,
- *Echinacea angustifolia* DC,
- *Fagopyrum cymosum* Meissn,
- *Gentiana lutea* L,
- *Harpalyce brasiliana Benth,*
- *Impatiens balsamina* L,
- *Liatris squarrosa* Willd,
- *Macfadyena unguis-cati Gent,*
- *Nerium oleander* L,
- *Ocimum basilicum* L,
- *Pentaclethra macroloba* (Willd.) Ktze,
- *Ruta graveolens* L,
- *Serenoa repens* Small,
- *Taraxacum officinale* Weber
- Verbascum thapsus L (Mors et al., 2000) and
- *Calotropis gigantea* (Aarti, 2014) are the medicinal plants that have very affective properties against snake bite.

2.8 *Calotropis gigantea*

2.8.1 Organized Classification of the Plant

Table No. 2.1 Classification of *Calotropis gigantea*

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Gentianales
Family	Apocynaceae
Genus	*Calotropis*
Species	*gigantea*

Figure No. 2.6 Crown flower (*Calotropis gigantean*)

In plant kingdom, fitting in the family of Asclepiadaceae, *Calotropis* species, are inherent to the stifling and subtropical areas of Asia and Africa and famous all over the stifling areas sphere. Generally, people call it crown flower weed or milk weed. It is sap producing weed and after its tissue damage it seeps milk like sap. Tannins, starch, gum, alkaloids, sugars, protein and resins are the main constituents of its sap (Arti, 2014). It was also known as 'sweta Arka' in old times homoeopathic. In modern scientific researches it has been proved that this plant has many characteristics, useful in medical point of view.

2.8.2 Morphology

Calotropis gigantea is a large shrub or small tree, about 4–10 m tall. Its stem is erect, up to 20 cm in diameter. The leaves are broadly oval in shape, with the size of 9–20 cm × 6–12.5 cm but subsessile. The cymes are 5–12.5 cm in diameter. The inflorescence stalk is 5–12 cm long; the stalk of an individual flower is 2.5–4 cm long. Sepal lobes are broadly egg-shaped with a size of 4–6 mm × 2-3 mm. Petal is 2.5–4 cm in diameter. It has clusters of waxy flowers that are either white or lavender in colour. Each flower consists of five pointed petals and a small, elegant "crown" rising from the centre, which holds the stamens. The plant has oval, light green leaves and milky stem. The petal lobes are broadly triangular measuring 10–15 mm × 5–8 mm; they are pale lilac and cream coloured towards the tips (Arti, 2014). *Calotropis* is drought resistant, salt tolerant to a relatively high degree, grows wild up to 900 meters (msl) throughout the country (Sharma and Tripathi, 2009). It is one of the few plants not consumed by grazing animals (Oudhia, 1997). It thrives on poor soils particularly where overgrazing has removed competition from native grasses (Smith, 2002).

2.8.3 Distribution

Malaysia, India and China are the homeland of *Calotropis gigantea* but it is also dispersed in the following parts of the world:Afghanistan, Algeria, Burkina Faso, Cameroon, Chad, Cote d'Ivoire, Democratic Republic of Congo, Egypt, Eritrea, Ethiopia, Gambia, Ghana, India, Iran, Iraq, Israel, Kenya, Kuwait, Lebanon, Libyan, Arab Jamahiriya, Mali, Mauritania, Morocco, Mozambique, Myanmar, Nepal, Niger, Nigeria, Oman, Pakistan, Saudi Arabia, Senegal, Somalia, Sudan, Syrian Arab Republic, Tanzania, Thailand, Uganda, United Arab emirates, Vietnam, Yemen, Exotic: Antigua and Barbuda, Argentina, Australia, Bahmas, Barbados, Bolivia, Brazil, Colombia, Cuba, Dominica, Dominican Republic, Ecuador, French Guina, Grenada, Guadeloupe, Guatemala, Guyana, Haiti, Honduras, Jamaica, Martinique, Mexico, Montserrat, Netherlands Antilles, Nicaragua, Panama, Paraguay, Peru, Puerto Rico, St Lucia, St Vincent, Surinam, Trinidad, Uruguay, Venezuela (Arti, 2014).

2.8.4 Therapeutic Characteristics

Many ailments and conditions can be mediated with several parts of this plant. Each part has huge therapeutic activity. *Calotropis gigantea* has great need in making mixtures of many herbals together to cure the disease (Tenpe, 2007). Each and every part of the plant has

different properties against different health complains. Leaves and outer parts have a good effect as antioxidant activity, anti-Candida activity, antidiarrheal activity and antibacterial activity. Flowers work as antimicrobial, painkiller action and cytotoxic activity. Roots are informed to comprise antipyretic activity and cytotoxic activity. Whole three parts of this plants are well-thought-out to contain therapeutic possessions and used in the cure of syphilis, boils, inflammation, epilepsy, hysteria, fever, muscular spasm, warts, leprosy, gout, snakebites, and cancer. Duke (1992) defined hundred above medicinal characteristics of this plant.

2.8.5 Chemical Conformation

Calotropis gigantea contains a- and b- amyrin, teraxasterol, gigantin (chemical) and giganteol. They are poisonous plants; calotropin, a compound in the latex, is more toxic than strychnine. Cardenolides contents in leaf (2.04 mg/gm) and in latex (162.0 mg/g), mostly calotropagenin – derived cardenolides present from *Calotropis gigantea*, two triterpene esters–3 methylbutanoates of amyrin and taraxasterol are isolated from latex of *C. gigantea*. Calotropins D1 and D2 had been isolated from *C. gigantea* (Pal and Sinha, 1980). The new oxiopregnane- oligoglycosides named *Calotropis* A and B have been isolated from the root of *C. gigantea* and their chemical structure have been elucidated by chemical and spectroscopy methods (Isao, 1992). The cytotoxic principles of *'Akondmul'* (Root of *C. gigantea*) cardenoloids glycosides, calotropin frugoside and 4-O-Beta-Dglucopyranosyl frugoside were obtained as the cytotoxic principles (Kiuchi, 1998). Leaves contain active constituent's like-mudarine resin, calotropin, uscharin and calotoxin. The latex contains a powerful bacteriolytic enzyme, a very toxic glycoside calactin (the concentration of which is increased following insect or grasshopper attack as a defense mechanism), calotropin D I, calotropin D II, calotropin F I, calotropin F II and a non-toxic protealytic enzyme calotropin (2-3%).

2.8.6 Uses of *Calotropis gigantea*

Calotropis gigantea is used can be used in many ways for cure of different ailments. Each part of the plant can be used differently for desired purpose. Finely crumpled roots of the plant can be used by rubbing well on nibbled skin. Leaves can be used on snake bite affected site, periodic temperature, gut insects and abscesses. In case of respiratory asthma flowers of the plant showed useful results. Mouth related complications, inflammations, rat

bite, gonococcal ache and other aching problems are cured by latex of *Calotropis gigantea* (Kumar, 2011).

In the process of low-cost book-mending, sheep and goat lather is used. *Calotropis* is also used in the process of taken away the hair of the skins of goat and sheep by making an agitated fusion of it with salt (Joseph, 2013).

Life threatening diseases can be cured by *Calotropis Gigantea*. To validate its traditional uses scientists have made various in vivo and in vitro tests which exposed its ability to cure ailments by natural means (Joseph *et al.*, 2013). It has been experimented in chemical and thermal models in mice as painkiller agent mixing with fermented extract of flowers (Pathak and Argal, 2007).

Calotropis Gigantea root bark is reported in wound healing in incision, excision and dead space of mice. It has also been reported that the sap of this plant convey procoagulant action. In a quantity reliant way the sap hydrolyzes human fibrinogen, casein and crude fibrin clot.

Distilled water extracts of *Calotropis Gigantea* leaves with ethanol, chloroform and n-butanol showed antipyretic and anti-inflammatory activities. The aerial parts with hydroalcoholic extract showed best results as antidiarrheal product in castor oil based diarrhea modles of mice (Joseph *et al.*, 2013).

2.8.7 Antagonistic Consequence

Further studies needed on *Calotropis* Allelopathic effects. It has been surveyed that juice of its many parts have affect the propagation and sprout potency of many cultivated harvest (Joseph, 2013). Other *Calotropis* spp. have been studied with respect to their side effects. Eruptions, wounds and injuries are their common side effects. Mucosa can be annoying by applying the latex (Arti, 2014).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.0 Materials

3.1 Venom

The venom of four different snakes i.e. Cobra *(Naja naja),* saw scaled viper *(Echis carinatus),* Russell viper *(Doboia russelli)* and Krait *(Bungarus caeruleus)* was extracted that were provided at serpentarium of NIH Islamabad, collected from different areas of Pakistan.

3.1.1 Venom extraction and preparation

Venom was extracted using finger stimulation technique. The snake was held from behind the head and teeth (fangs) were placed over the rim of a wine glass and then the upper part of the head was rubbed for better venom extraction.

The color of venom was slightly different of each snake specie. Venom of *Russell's viper* was yellow in color while Cobra and *echis* venom was yellowish white. While the color of krait venom was reddish yellow due to blood in the venom.

Venom of all four snakes was collected and stored separately. The snake's venom was centrifuged and lyophilized. After these processes the processed venom was stored at 4°C by collecting in dark glass vessels with rubber seals on the top. Vessels were labeled with Snake specie name and total weight of the venom.

3.2 Anti venom

Standard polyvalent anti-venom serum (AVS) collected from horse plasma was used in all experiments. The anti-venom serum was collected from the Sera processing laboratory, BPD, NIH Islamabad.

3.3 Experimental Animals

Swiss albino mice of both sexes weighing between 15-20gms were engaged for all experiments.

3.4 Collection of Plant material

The plant was collected from Swat, Khyber Pakhtunkhwa, and some parts of Islamabad Pakistan and was authenticated by botanist at drugs division of National Institute of Health Pakistan.

3.4.1 Drying and grinding of plant

The fresh plant material of *Calotropis gigantea* was washed and dried in proper shade at room temperature for a period of a month with much care and cautions to avoid any injury at any part of the plant.

The air-dried plant material was finely ground to powder carefully and subjected for maceration.

3.4.2 Plant Extraction

The 200gm powdered plant material was soaked in each of the 4 different solvents i.e. petroleum ether, chloroform, methanol and distilled water and kept aside for 14 days with occasional stirring. After 14 days, the extract was filtered first through muslin cloth (coarse filtration) and then with Whitman No.1 filter paper. The solvent from the total extract was distilled off by the rotary evaporator to a syrupy consistency and then evaporated to dryness by water bath at 50 ^{0}C.

3.5 Qualitative phytochemical screening

3.5.1 Tannins

A portion of the extract was dissolved in water and clarified by filtration. 10% ferric chloride solution was then added to the resultant filtrate. Result was noted then after few minutes.

3.5.2 Alkaloids

0.5g of the extract was stirred in 0.5ml of 1% HCL on steam bath and filtered while hot. Few drops of the distilled water were added. Took 1ml of this filtrate and treated it with few drops of winger's reagent. Results were noted down.

3.5.3 Cardiac Glycosides

0.5% of the extract was dissolved in 2.0ml glacial acid containing drop of ferric chloride solution followed by 2ml concentrated H2SO4.

3.5.4 Flavonoids

In 2ml of extract 2.0ml of diluted NaOH. The color of the solution was observed for the result.

3.5.5 Saponins

0.5g of the powdered plant was boiled in 12ml of distilled water in a water bath and filtered. 10 ml of the filtrate was mixed with 5ml of distilled water. Mixture was shaken vigorously a stable persistent froth.

3.5.6 Phenols

Equal volume of extract and FeCl3 were mixed stirred for few seconds and the results were noted.

3.5.7 Anthraquinones

0.5g of the extract was dissolved by shaking in 10ml of benzene and filtered. 10% of ammonia solution added and filtered and then the mixture was shaken then kept on room temperature for results.

3.5.8 Reducing sugar

3.0ml of extract was dissolved in 5ml of distilled water followed by Fehling solution A and B. it was then boiled. Left over on room temperature for few mints and results were observed.

3.5.9 Carbohydrates

0.5g of the extract was shaken in distilled water and filtered. To the aqueous filtrate few drops of molish reagent were added, then 1.0 ml conc. H2SO4 was added to form aqueous layer.

3.5.10 Volatile oils

The extract was dissolved in 90% ethanol and few FeCl3 drops were added and shaken well for a min.

3.5.11 Steroids

1mg of the extract was dissolved in 5ml of chloroform and filtered. H2SO4 was added carefully and filtered after some time. Then left over the table for ring formation.

3.5.12Amino acids

In 1.0ml of the extract few drops of ninhydrine reagent were added kept for some time for changing the color of the solution.

3.6 Evaluation of LD_{50} of venom

The median lethal dose (LD_{50}) of *Bungarus caeruleus, Doboia russellii, Echis carinatus and Naja naja* venoms were evaluated conferring to the process discribed by Theakston and Reid 1983. The poisonousness of these snakes venoms were measured by IP administration of various amounts of the venoms mixed in 0.2 ml of physical saline to sets (n = 4) of Swiss albino mice (18-20g). The LD_{50} was noted with the assurance boundary at 50% possibility by the observation of deaths happening count 24 hrs. of the venom direction.

3.7 Neutralization of Lethality

The *in-vivo* neutralization influence of *C.gigantea* plant extracts were measured by IP. Administration of $2LD_{50}$ dose of venom into various groups of mice straightaway after the administration of several doses of the plant extract per oral (PO). To evaluate *in-vitro* neutralization, numerous amounts of the plant extracts were mixed with $2LD_{50}$ of the venom

samples and incubated at 37°C for 30 minutes and then injected IP. in to the mice. For each group 4 mice were used. Without plant extracts control mice received same amount of venom. The standard reference group i.e. snake venom anti serum was directed next the administration of $2LD_{50}$ of venom and the results were calculated.

3.8 Dosage

The mixture of antivenom and venom was prepared and incubated for 30 minutes. After incubation, the mixture was injected to test mice, treated with 0.2ml dose which was injected intraperitonealy. Four Swiss albino mice about (18-20gm) were used for each dose. After 24 hrs results were recorded.

RESULTS

4. RESULTS

4.1 Qualitative phytochemical screening

Phytochemical screening revealed that tannins, alkaloids, flavonoids, saponins, phenols, anthraquinones, steroids and volatile oil were the components found in the *C. gigantea* plant. While there was no trace of the cardiac glycosides, reducing sugar, carbohydrates or amino acids as showed in the **(Table # 4.1)**

Table No. 4.1 Phytochemical Screening of *C. gigantea.*

S. No	Constitutes	Results
1.	Tannins	+
2.	Alkaloids	+
3.	Cardiac Glycosides	-
4.	Flavonoids	+
5.	Saponins	+
6.	Phenols	+
7.	Anthraquinones	+
8.	Reducing Sugar	-
9.	Carbohydrates	-
10.	Volatile Oil	+
11.	Steroids	+
12.	Amino Acids	-

4.2 Evaluation of $2LD_{50}$ of the Venom

According to the process practiced by Theakston and Reid 1983, the median lethal doses ($2LD_{50}$) was evaluated of four snakes i.e. *Doboia russeli, echis carinatus, naja naja* and *Bungarus caeruleus*. With the confidence level at 100% possibility of the test animals' death after injection of venom within 24 hrs. the 2LD50 of the above snake's venom are as showed in **(table# 4.2).**

Table No. 4.2 $2LD_{50}$ of four deadly snakes of Pakistan.

S.no	Snake Name	$2LD_{50}$
1	Russel Viper (*Doboia Russeli)*	17.6 µg/ml
2	Saw-Scaled Vipers *(Echis Carinatus)*	12.8 µg/ml
3	Cobra (*Naja Naja*)	18.5 µg/ml
4	Krait *(Bungarus Caeruleus)*	10.8 µg/ml

4.3 In-vitro neutralization of *Doboia Russeli* venom

C. gigantea's four extracts of chloroform, distilled water, petroleum ether and methanol with three different doses of 100mg/kg, 200mg/kg, and 300mg/kg body weight were prepared and then incubated with *Doboia russeli* snake venom at 37 °C for 30 minutes and injected by IP route. After giving the doses by the described route the mice were kept on normal temperature without food and water. Each group of test animals were kept in separate cage along with the dose vial. After 24 hours. Results were noted for each dose and mice were to be found either dead or live. With or without any indications and symptoms of toxicity. All survived mice were feeded and kept for another day for the conformation of the results.

4.3.1 Result of *C. gigantea* Chloroformic extract on *Doboia russeli* venom

Chloroformic extract of *C. gigantea* showed 100% results against $2LD_{50}$ venom of *Doboia russeli.* These results were noted after 24 hours and then conformed after 48 hours of

the injection of venom along with the plant extract. Control was used to check the venom and all the control mice were found dead. While plant extract along with the venom showed no mortality after 24 hours, as like venom along with the AVS. The results of the chloroformic plant extract shows the same results in (table # 4.3).

Table No. 4.3 Effect of *C. gigantea* Chloroform extract against *Doboia russeli* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	4/4	-
2	Venom + AVS (polyvalent antivenom)	0/4	100
3	Venom + *C. gigantea* extract 100mg/kg	0/4	100
4	Venom + *C. gigantea* extract 200mg/kg	0/4	100
5	Venom + *C. gigantea* extract 300mg/kg	0/4	100

0.2ml of chloroform extract was administered from stock solution to neutralize 17.6µg/ml of *Doboia russeli* venom in each dose. The results in this table (table#3) reflects that chloroform extract of plant with three doses on *Doboia russelii* venom was 100%.

4.3.2 Effect of *C. gigantea* distilled water extract on *Doboia russeli* venom

Distilled water extract of *C. gigantea* showed 100% results against $2LD_{50}$ venom of *Doboia russeli.* These results were noted after 24 hours and then conformed after 48 hours of the injection of venom along with the plant extract. Control was used to check the venom and all the control mice were found dead. While plant extract along with the venom showed no mortality after 24 hours, as like venom along with the AVS. The results of the chloroformic plant extract shows the same results in (table # 4.4).

Table 4.4 Effect of *C. gigantea* distilled water extract against *Doboia russelii* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	4/4	-
2	Venom + AVS (polyvalent antivenom)	0/4	100
3	Venom + *C. gigantea* extract 100mg/kg	0/4	100
4	Venom + *C. gigantea* extract 200mg/kg	0/4	100
5	Venom + *C. gigantea* extract 300mg/kg	0/4	100

0.2ml extract with distilled water was administered from stock solution to neutralize 17.6µg/ml of *Doboia russelii* venom in each dose. The results of distilled water extract of plant with three doses on *Doboia russelii* venom was 100%.

4.3.3 Effect of *C. gigantea* Metanolic extract on *Doboia russeli* venom

Metanolic extract of *C. gigantea* showed 100% results against $2LD_{50}$ venom of *Doboia russeli.* These results were noted after 24 hours and then conformed after 48 hours of the injection of venom along with the plant extract. Control was used to check the venom and

all the control mice were found dead. While plant extract along with the venom showed no mortality after 24 hours, as like venom along with the AVS. The results of the chloroformic plant extract shows the same results in (table # 4.5).

Table 4.5 Effect of *C. gigantea* methanolic extract against *Doboia russelii* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	4/4	-
2	Venom + AVS (polyvalent antivenom)	0/4	100
3	Venom + *C. gigantea* extract 100mg/kg	0/4	100
4	Venom + *C. gigantea* extract 200mg/kg	0/4	100
5	Venom + *C. gigantea* extract 300mg/kg	0/4	100

0.2ml of methanolic extract of plant was administered from stock solution to neutralize 17.6µg/ml of *Doboia russelii* venom in each dose. Result was 100%.

4.3.4 Effect of *C. gigantea* petroleum ether extract on *Doboia russeli* venom

Petroleum ether extract of *C. Gigantea* showed 100% results against $2LD_{50}$ venom of *Doboia russeli.* These results were noted after 24 hours and then conformed after 48 hours of the injection of venom along with the plant extract. Control was used to check the venom and all the control mice were found dead. While plant extract along with the venom showed no mortality after 24 hours, as like venom along with the AVS. The results of the chloroformic plant extract shows the same results in (table # 4.6).

Table 4.6 Effect of *C. gigantea* petroleum ether extract against *Doboia russelii* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	4/4	-
2	Venom + AVS (polyvalent antivenom)	0/4	100
3	Venom + *C. gigantea* extract 100mg/kg	0/4	100
4	Venom + *C. gigantea* extract 200mg/kg	0/4	100
5	Venom + *C. gigantea* extract 300mg/kg	0/4	100

0.2ml of petroleum ether extract of plant was administered from stock solution to neutralize 17.6µg/ml of *Doboia russelii* venom in each dose. Result was 100%. *C. gigantea* all extracts showed 100% survival results of mice against *Doboia russelii* venom in all cases by IP route.

4.4 In-vitro neutralization of cobra venom

C. gigantea's four extracts of chloroform, distilled water, petroleum ether and methanol with three different doses of 100mg/kg, 200mg/kg, and 300mg/kg body weight were prepared and then incubated along with cobra (*Naja naja*) venom at 37 °C for 30

minutes and injected by IP route. After giving the doses by the described route the mice were kept on normal temperature without food and water. Each group of test animals were kept in separate cage along with the dose vial. After 24 hours. Results were noted for each dose and mice were to be found either dead or live. With or without any indications and symptoms of toxicity. All survived mice were feeded and kept for another day for the conformation of the results

4.4.1 Effect of *C. gigantea* chloroformic extract on *Naja naja* venom

Cobra venom along with three different chloroformic extract doses in the same ratio were injected after incubation for 30 mints at 37 °C. Control mice were injected only with 2LD50 cobra venom to check the potency of the venom to be right. Control mice showed no mortality. Venom along with the AVS and group #5 showed 0% mortality. Different doses responded differently, at dose 100mg/kg body weight dose (group #3) showed 50% mortality. Group #4 showed 25% mortality as shown in the following table (4.7).

Table 4.7 Effect of *C. gigantea* Chloroformic extract against *Naja naja* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	4/4	-
2	Venom + AVS (polyvalent antivenom)	0/4	100
3	Venom + *C. gigantea* extract 100mg/kg	2/4	50
4	Venom + *C. gigantea* extract 200mg/kg	¼	75
5	Venom + *C. gigantea* extract 300mg/kg	0/4	100

0.2ml of chloroformic extract was administered from stock solution to neutralize 18.5µg/ml of *Naja naja* venom in each dose. In first, second and third dose result was 50%, 75% and 100% respectively.

4.4.2 Effect of *C. gigantea* distilled water extract on *Naja naja* venom

Cobra venom along with three different doses of distilled water extract were injected into 3 mice groups through IP route. Only venom was injected in control group. Control mice showed no mortality. Only venom along with the AVS showed 0% mortality. At dose 100mg/kg body weight dose (group #3) showed 75% mortality. Group #4 and 5 showed 50% mortality as shown in the following table (4.8).

Table 4.8 Effect of *C. gigantea* distilled water extract against *naja naja* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	4/4	-
2	Venom + AVS (polyvalent antivenom)	0/4	100
3	Venom + *C. gigantea* extract 100mg/kg	¾	25
4	Venom + *C. gigantea* extract 200mg/kg	2/4	50
5	Venom + *C. gigantea* extract 300mg/kg	2/4	50

0.2ml of distilled water extract of plant was administered from stock solution to neutralize 18.5µg/ml of *Naja naja* venom in each dose. Result was 25% in first dose and 50% in second and third dose.

4.4.3 Effect of *C. gigantea* methanolic on *Naja naja* venom

Plant methanolic extract showed good results at dose 200mg/kg and 300mg/kg body weight (group #4 and 5) as like venom along with the AVS showed 0% mortality. Control mice showed no mortality. At dose 100mg/kg body weight dose (group #3) showed 25% mortality as shown in the following table (4.9).

Table 4.9 Effect of *C. gigantea* methanolic extract against *Naja naja* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	4/4	-
2	Venom + AVS (polyvalent antivenom)	0/4	100
3	Venom + *C. gigantea* extract 100mg/kg	¼	75
4	Venom + *C. gigantea* extract 200mg/kg	0/4	100
5	Venom + *C. gigantea* extract 300mg/kg	0/4	100

0.2ml of methanolic extract of plant was administered from stock solution to neutralize 18.5µg/ml of *Naja naja* venom in each dose. In first dose result was 75% in second dose 100% and in third dose was also 100%.

4.4.4 Effect of *C. gigantea* petroleum ether on *Naja naja* venom

Plant methanolic extract showed good results at dose 200mg/kg and 300mg/kg body weight (group #4 and 5) as like venom along with the AVS showed 0% mortality. Control mice showed no mortality. At dose 100mg/kg body weight dose (group #3) showed 75% mortality as shown in the following table (4.10).

Table 4.10 Effect of *C. gigantea* petroleum ether extract against *naja naja* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	4/4	-
2	Venom + AVS (polyvalent antivenom)	0/4	100
3	Venom + *C. gigantea* extract 100mg/kg	¾	25
4	Venom + *C. gigantea* extract 200mg/kg	0/4	100
5	Venom + *C. gigantea* extract 300mg/kg	0/4	100

0.2ml of petroleum ether extract of plant was administered from stock solution to neutralize 18.5µg/ml of *Naja naja* venom in each dose. *C. gigantea* four different extracts with three doses in each case showed good results against *Naja naja* venom by IP route.

4.5 In-vitro neutralization of *Echis carinatus* venom

C. gigantea's four extracts of chloroform, distilled water, petroleum ether and methanol with three different doses of 100mg/kg, 200mg/kg, and 300mg/kg body weight were prepared and then incubated with *Echis carinatus* snake venom at 37 °C for 30 minutes and injected by IP route. After giving the doses by the described route the mice were kept on normal temperature without food and water. Each group of test animals were kept in separate cage along with the dose vial. After 24 hours. Results were noted for each dose and mice

were to be found either dead or live. With or without any indications and symptoms of toxicity. All survived mice were feeded and kept for another day for the conformation of the results.

4.5.1 Effect of *C. gigantea* Chloroformic extract on *Echis carinatus* venom

Chloroformic extract of *C. gigantea* showed 100% results against $2LD_{50}$ venom of *Echis carinatus*. All the groups were injected with the same ratio of venom and extract. The results of the chloroformic plant extract shows the same results in table (4.11).

Table 4.11 Effect of *C. gigantea* Chloroformic extract against *Echis carinatus* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	4/4	-
2	Venom + AVS (polyvalent antivenom)	0/4	100
3	Venom + *C. gigantea* extract 100mg/kg	0/4	100
4	Venom + *C. gigantea* extract 200mg/kg	0/4	100
5	Venom + *C. gigantea* extract 300mg/kg	0/4	100

0.2ml of chloroformic extract of plant was administered from stock solution to neutralize 12.8µg/ml of *Echis carinatus* venom in each dose. Results were 100% in all three doses.

4.5.2 Effect of *C. gigantea* distilled water extract on *Echis carinatus* venom

Distilled water extract of *C. gigantea* showed 100% results against $2LD_{50}$ venom of *Echis carinatus*. All the groups were injected with the same ratio of venom and extract. The results of the chloroformic plant extract shows the same results in table (4.12).

Table 4.12 Effect of *C. gigantea* distilled water extract against *Echis carinatus* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	4/4	-
2	Venom + AVS (polyvalent antivenom)	0/4	100
3	Venom + *C. gigantea* extract 100mg/kg	0/4	100
4	Venom + *C. gigantea* extract 200mg/kg	0/4	100
5	Venom + *C. gigantea* extract 300mg/kg	0/4	100

0.2ml of distilled water extract of plant was administered from stock solution to neutralize 12.8µg/ml of *Echis carinatus* venom in each dose. Results were 100% in all three doses.

4.5.3 Effect of *C. gigantea* methanolic extract on *Echis carinatus* venom

Three doses of *C. gigantea* extract with methanol showed 100% results as like AVS showed 100% results against *Echis carinatus* venom. The results are as shown in the following table (4.13).

Table 4.13 Effect of *C. gigantea* methanolic extract against *Echis carinatus* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	4/4	-
2	Venom + AVS (polyvalent antivenom)	0/4	100
3	Venom + *C. gigantea* extract 100mg/kg	0/4	100
4	Venom + *C. gigantea* extract 200mg/kg	0/4	100
5	Venom + *C. gigantea* extract 300mg/kg	0/4	100

0.2ml of methanolic extract of plant was administered from stock solution to neutralize 12.8µg/ml of *Echis carinatus* venom in each dose. Results were 100% in all three doses.

4.5.4 Effect of *C. gigantea* petroleum ether extract on *Echis carinatus* venom

Three doses of *C. gigantea* extract with petroleum ether showed 100% results as like AVS showed 100% results against *Echis carinatus* venom. The results are as shown in the following table (4.14).

Table 4.14 Effect of *C. gigantea* petroleum ether extract against *echis carinatus* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	4/4	-
2	Venom + AVS (polyvalent antivenom)	0/4	100
3	Venom + *C. gigantea* extract 100mg/kg	0/4	100
4	Venom + *C. gigantea* extract 200mg/kg	0/4	100
5	Venom + *C. gigantea* extract 300mg/kg	0/4	100

0.2ml of petroleum ether extract of plant was administered from stock solution to neutralize 12.8µg/ml of *Echis carinatus* venom in each dose. Results were 100% in all three doses. All four different extracts of *C. gigantea* with three doses in each case has shown 100% survival of test animal against *Echis carinatus* venom.

4.6 In-vitro neutralization of *Bungarus caeruleus* venom

C. gigantea's four extracts of chloroform, distilled water, petroleum ether and methanol with three different doses of 100mg/kg, 200mg/kg, and 300mg/kg body weight were prepared and then incubated with *Bungarus caeruleus* snake venom at 37 °C for 30

minutes and injected by IP route. After giving the doses by the described route the mice were kept on normal temperature without food and water. Each group of test animals were kept in separate cage along with the dose vial. After 24 hours. Results were noted for each dose and mice were to be found either dead or live. With or without any indications and symptoms of toxicity. All survived mice were feeded and kept for another day for the conformation of the results.

4.6.1 Effect of *C. gigantea* Chloroformic extract on *Bungarus caeruleus* venom

Only AVS showed 0% mortality in this experiment. Group 2 and three showed 75% mortality while that of group #5 showed 25 mortality. All the results are as shown in table (4.15).

Table 4.15 Effect of *C. gigantea* Chloroformic extract against *Bungarus caeruleus* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	4/4	-
2	Venom + AVS (polyvalent antivenom)	0/4	100
3	Venom + *C. gigantea* extract 100mg/kg	3/4	25
4	Venom + *C. gigantea* extract 200mg/kg	3/4	25
5	Venom + *C. gigantea* extract 300mg/kg	1/4	75

0.2ml of chloroformic extract of plant was administered from stock solution to neutralize 10.8µg/ml of *Bungarus caeruleus* venom in each dose.

4.6.2 Effect of *C. gigantea* distilled water extract on *Bungarus caeruleus* venom

Control mice were all found dead after 24 hours while all the mice in the 2nd group in which venom was injected along with AVS were alive. Group #3 and 4 showed 75% mortality while group #5 showed 50% mortality after 24 hour. The results are as described as shown in the following table (4.16)

Table 4.16 Effect of *C. gigantea* distilled water extract against *Bungarus caeruleus* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	4/4	-
2	Venom + AVS (polyvalent antivenom)	0/4	100
3	Venom + *C. gigantea* extract 100mg/kg	3/4	25
4	Venom + *C. gigantea* extract 200mg/kg	3/4	25
5	Venom + *C. gigantea* extract 300mg/kg	2/4	50

0.2ml of distilled water extract of plant was administered from stock solution to neutralize 10.8µg/ml of *Bungarus caeruleus* venom in each dose. Percentage of survival was:

1st dose= 25%, 2nd dose = 25% and 3rd dose= 50%.

4.6.3 Effect of *C. gigantea* methanolic extract on *Bungarus caeruleus* venom

Group #2 and 5 showed 100% good results while group #1 and 3 showed 100% mortality. And Group #4 showed 50% mortality and 50% survival after 24 hours. As shown in the following table (4.17)

Table 4.17 Effect of *C. gigantea* methanolic extract against *Bungarus caeruleus* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	4/4	-
2	Venom + AVS (polyvalent antivenom)	0/4	100
3	Venom + *C. gigantea* extract 100mg/kg	4/4	0
4	Venom + *C. gigantea* extract 200mg/kg	2/4	50
5	Venom + *C. gigantea* extract 300mg/kg	0/4	100

0.2ml of methanolic extract of plant was administered from stock solution to neutralize 10.8µg/ml of *Bungarus caeruleus* venom in each dose. Percentage of survival was:

1st dose= 0%, 2nd dose= 50%, 3rd dose= 100%.

4.6.4 Effect of *C. gigantea* petroleum ether extract on *Bungarus caeruleus* venom

Two of the petroleum ether plant extracts showed some positive results. Group #1 and 4 showed 100% mortality while AVS along with venom showed 0%mortality. Group #3 showed 25% mortality and group #4 showed 50% mortality as shown in the following table (4.18).

Table 4.18 Effect of *C. gigantea* petroleum ether extract against *Bungarus Caeruleus* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	4/4	-
2	Venom + AVS (polyvalent antivenom)	0/4	100
3	Venom + *C. gigantea* extract 100mg/kg	1/4	75
4	Venom + *C. gigantea* extract 200mg/kg	2/4	50
5	Venom + *C. gigantea* extract 300mg/kg	4/4	0

0.2ml of petroleum ether extract of plant was administered from stock solution to neutralize 10.8µg/ml of *Bungarus caeruleus* venom in each dose. Percentage of survival was:

1st dose= 75%, 2nd dose= 50%, 3rd dose= 0%.

4.7 In vivo neutralization of *Doboia russeli* venom

Four extracts of plant *C. gigantea* that were prepared in chloroform, methanol, distilled water and petroleum ether were orally given instantly after administration of $2LD_{50}$ *Doboia russeli* snake venom. Three doses of each extract were applied separately on each

group of mice's. After 24 hours, results were collected with the confidence limit at 100% possibility of test animal's morality occurring in this time period.

4.7.1 Effect of *C. gigantea* Chloroformic extract orally administered against *Doboia russeli* venom

All the *C. gigantea* Chloroformic extract doses administered orally against *Doboia russeli* neutralized the $2LD_{50}$ venom. 2 mice per dose was used in the oral administration of plant extract. Only control mice were found dead after 24 hours. The results are shown as in the following table (4.19).

Table 4.19 Effect of *C. gigantea* Chloroform extract against *Doboia russeli* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	2/2	-
2	Venom + AVS (polyvalent antivenom)	0/2	100
3	Venom + *C. gigantea* extract 100mg/kg	0/2	100
4	Venom + *C. gigantea* extract 200mg/kg	0/2	100
5	Venom + *C. gigantea* extract 300mg/kg	0/2	100

Neutralization of 17.6µg/ml of *Doboia russeli* venom was done by 0.2ml of chloroformic extract of *C. gigantea* in three doses. Result was 100% in all three doses.

4.7.2 Effect of *C. gigantea* distilled water extract orally administered against *Doboia russeli* venom

Results are the same as the previous extract. All mice survived except control group (group #1).

Table 4.20 Effect of *C. gigantea* distilled water extract against *Doboia russeli* venom ($2LD_{50}$)

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	2/2	-
2	Venom + AVS (polyvalent antivenom)	0/2	100
3	Venom + *C. gigantea* extract 100mg/kg	0/2	100
4	Venom + *C. gigantea* extract 200mg/kg	0/2	100
5	Venom + *C. gigantea* extract 300mg/kg	0/2	100

Neutralization of 17.6µg/ml of *Doboia russelii* venom was done by 0.2ml of distilled water extract of *C. gigantea* in three doses. Result was 100% in all three doses.

4.7.3 Effect of *C. gigantea* methanolic extract orally administered against *Doboia russeli* venom

Methanolic extract showed 100% positive results against 2LD50 venom of *Doboia russeli* venom. All group of mice's survived except control mice's which were supposed to be dead after 24 hours of time. All the results were noted after 24 hours. The results are as shown in table (4.21).

Table 4.21 Effect of *C. gigantea* methanolic extract against *Doboia russelii* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	2/2	-
2	Venom + AVS (polyvalent antivenom)	0/2	100
3	Venom + *C. gigantea* extract 100mg/kg	0/2	100
4	Venom + *C. gigantea* extract 200mg/kg	0/2	100
5	Venom + *C. gigantea* extract 300mg/kg	0/2	100

Neutralization of 17.6µg/ml of *Doboia russeli* venom was done by 0.2ml of methanolic extract of *C. gigantea* in three doses. Percentage of survivors was 100% in all doses.

4.7.4 Effect of *C. gigantea* petroleum ether extract orally administered against *Doboia russeli* venom

Control mice's were all dead. While rest of the groups showed 100% survival rate. After 24 hours the results were calculated. The results are as shown in table (4.22).

Table 4.22 Effect of *C. gigantea* petroleum ether extract against *Doboia russeli* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	2/2	-
2	Venom + AVS (polyvalent antivenom)	0/2	100
3	Venom + *C. gigantea* extract 100mg/kg	0/2	100
4	Venom + *C. gigantea* extract 200mg/kg	0/2	100
5	Venom + *C. gigantea* extract 300mg/kg	0/2	100

Neutralization of 17.6µg/ml of *Doboia russeli* venom was done by 0.2ml of petroleum ether extract of *C. gigantea* in three doses. Result percentage was 100%.

From these results it has proved that *C. gigantea* plant has strong potency against *Doboia russeli* snake venom by in vivo method. Because all extracts from all doses showed 100% survival rate.

4.8 In vivo neutralization of cobra venom

Four extracts of plant *C. gigantea* that were prepared in chloroform, methanol, distilled water and petroleum ether were orally given instantly after administration of $2LD_{50}$ cobra (*Naja naja*) snake venom. Three doses of each extract were applied separately on each

group of mice's. After 24 hours results were collected with the confidence limit at 100% possibility of test animal's morality occurring in this time period.

4.8.1 Effect of *C. gigantea* Chloroformic extract orally administered against *Naja naja* venom

Oral administration of *C. gigantea* Chloroformic extract does not showed any neutralizing activity against cobra (*Naja naja)* venom. It means that the cobra venom act so fast so that oral dose cannot neutralize it. The results are as shown in table (4.23).

Table 4.23 Effect of *C. gigantea* Chloroformic Extract against *Naja naja* Venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	2/2	-
2	Venom + AVS (polyvalent antivenom)	0/2	100
3	Venom + *C. gigantea* extract 100mg/kg	2/2	0
4	Venom + *C. gigantea* extract 200mg/kg	2/2	0
5	Venom + *C. gigantea* extract 300mg/kg	2/2	0

Neutralization of 18.5µg/ml of *Naja naja* venom was done by 0.2ml of chloroformic extract of *C. gigantea* in three doses. It showed 0% result in all three doses.

4.8.2 Effect of *C. gigantea* distilled water extract orally administered against *Naja Naja* venom

Oral administration of *C. gigantea* distilled water extract showed 50% neutralization activity against cobra *(Naja naja)* venom. While control mice were all dead. And venom along with AVS showed 100% survival after 24 hours. The results are as shown in table (4.24).

Table 4.24 Effect of *C. gigantea* distilled water extract against *Naja naja* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	2/2	-
2	Venom + AVS (polyvalent antivenom)	0/2	100
3	Venom + *C. gigantea* extract 100mg/kg	½	50
4	Venom + *C. gigantea* extract 200mg/kg	½	50
5	Venom + *C. gigantea* extract 300mg/kg	½	50

Neutralization of 18.5µg/ml of *Naja naja* venom was done by 0.2ml distilled water extract of *C. gigantea* in three doses. Result was 50% in all cases.

4.8.3 Effect of *C. gigantea* Methanolic extract orally administered against *Naja naja* venom

Group #1, 3 and 4 showed 100% mortality. Group #2 showed 100% positive results while group #5 showed 50% mortality. The results are as shown in table (4.25) below.

Table 4.25 Effect of *C. gigantea* Methanolic Extract against *Naja naja* Venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	2/2	-
2	Venom + AVS (polyvalent antivenom)	0/2	100
3	Venom + *C. gigantea* extract 100mg/kg	2/2	0
4	Venom + *C. gigantea* extract 200mg/kg	2/2	0
5	Venom + *C. gigantea* extract 300mg/kg	½	50

Neutralization of 18.5µg/ml of *Naja naja* venom was done by 0.2ml of methanolic extract of *C. gigantea* in three doses. In 100mg/kg and 200mg/kg result was 0% of survival. In 300mg/kg dose survival of mice was 50%.

4.8.4 Effect of *C. gigantea* petroleum ether extract orally administered against *Naja naja* venom

None of the three *C. gigantea* Petroleum Ether Extract neutralized cobra snake (*Naja naja)* venom. Control mice's were also dead after 24 hours. All the results of petroleum ether extract against *naja naja* venom are as shown in table (4.26).

Table 4.26 Effect of *C. gigantea* Petroleum Ether Extract against *Naja naja* Venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	2/2	-
2	Venom + AVS (polyvalent antivenom)	0/2	100
3	Venom + *C. gigantea* extract 100mg/kg	2/2	0
4	Venom + *C. gigantea* extract 200mg/kg	2/2	0
5	Venom + *C. gigantea* extract 300mg/kg	2/2	0

Neutralization of 18.5µg/ml of *Naja naja* venom was injected through IP route while 0.2ml petroleum ether extract of *C. gigantea* in three doses were given orally. Survival percentage was 0% in all doses.

It has shown that distilled water and methanolic extracts of plant put good potency against *Naja naja*'s venom. While chloroformic and petroleum ether showed poor percentage of survival of mice.

4.9 In vivo neutralization of *Echis carinatus* venom

Four extracts of plant *C. gigantea* that were prepared in chloroform, methanol, distilled water and petroleum ether were orally given instantly after administration of $2LD_{50}$

Echis carinatus snake venom. Three doses of each extract were applied separately on each group of mice's. After 24 hours results were collected with the confidence limit at 100% possibility of test animal's morality occurring in this time period.

4.9.1 Effect of *C. gigantea* Chloroformic extract orally administered against *Echis carinatus* venom

Oral administration of *C. gigantea* Chloroformic extract showed 100% neutralizing activity against *Echis carinatus* venom. All the three doses showed 0% mortality after 24 hours. The results are as shown in table (4.27).

Table 4.27 Effect of *C. gigantea* Chloroformic extract against *Echis carinatus* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	2/2	-
2	Venom + AVS (polyvalent antivenom)	0/2	100
3	Venom + *C. gigantea* extract 100mg/kg	0/2	100
4	Venom + *C. gigantea* extract 200mg/kg	0/2	100
5	Venom + *C. gigantea* extract 300mg/kg	0/2	100

Neutralization of 12.8µg/ml of *echis carinatus* venom was done by 0.2ml of chlorofrmic extract of *C. gigantea* in three doses. Result was 100% in all cases.

4.9.2 Effect of *C. gigantea* distilled water extract orally administered against *Echis carinatus* venom

Distilled water extract of *C. gigantea* showed 100% neutralization against *Echis carinatus* venom at three different doses of 100, 200 and 300mg/kg body weight. The results were calculated after 24 hours of the oral administration and envenomation of mice's by IP injection. Same results are shown below in table (4.28).

Table 4.28 Effect of *C. gigantea* distilled water extract against *echis carinatus* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	2/2	-
2	Venom + AVS (polyvalent antivenom)	0/2	100
3	Venom + *C. gigantea* extract 100mg/kg	0/2	100
4	Venom + *C. gigantea* extract 200mg/kg	0/2	100
5	Venom + *C. gigantea* extract 300mg/kg	0/2	100

Neutralization of 12.8µg/ml of *Echis carinatus* venom was done by 0.2ml of distilled water extract of *C. gigantea* in three doses. 100% result was collected.

4.9.3 Effect of *C. gigantea* methanolic extract orally administered against *Echis carinatus* venom

Methanolic extract of *C. gigantea* showed 100% neutralization against *Echis carinatus* venom at three different doses of 100, 200 and 300mg/kg body weight. The results were calculated after 24 hours of the oral administration and envenomation of mice's by IP injection. Same results are shown below in table (4.29).

Table 4.29 Effect of *C. gigantea* methanolic extract against *Echis carinatus* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	2/2	-
2	Venom + AVS (polyvalent antivenom)	0/2	100
3	Venom + *C. gigantea* extract 100mg/kg	0/2	100
4	Venom + *C. gigantea* extract 200mg/kg	0/2	100
5	Venom + *C. gigantea* extract 300mg/kg	0/2	100

Neutralization of 12.8µg/ml of *Echis carinatus* venom was done by 0.2ml of methanolic extract of *C. gigantea* in three doses. 100% result was collected.

4.9.4 Effect of *C. gigantea* petroleum ether extract orally administered against *Echis carinatus* venom

Methanolic extract of *C. gigantea* showed 100% neutralization against *Echis carinatus* venom at three different doses of 100, 200 and 300mg/kg body weight. The results were calculated after 24 hours of the oral administration and envenomation of mice's by IP injection. Same results are shown below in table (4.30).

Table 4.30 Effect of *C. gigantea* petroleum ether extract against *Echis carinatus* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	2/2	-
2	Venom + AVS (polyvalent antivenom)	0/2	100
3	Venom + *C. gigantea* extract 100mg/kg	0/2	100
4	Venom + *C. gigantea* extract 200mg/kg	0/2	100
5	Venom + *C. gigantea* extract 300mg/kg	0/2	100

Neutralization of 12.8µg/ml of *Echis carinatus* venom was done by 0.2ml of petroleum ether extract of *C. gigantea* in three doses. 100% result was calculated.

C. gigantea potency against *Echis carinatus* venom in four different extracts with three doses in each case gave 100% survival results of test animals by in-vivo method.

4.10 In vivo neutralization of *Bungarus caeruleus* venom

Four extracts of plant *C. gigantea* that were prepared in chloroform, methanol, distilled water and petroleum ether were orally given instantly after administration of $2LD_{50}$ *Bungarus caeruleus* snake venom. Three doses of each extract were applied separately on

each group of mice's. After 24 hours results were collected with the confidence limit at 100% possibility of test animal's morality occurring in this time period.

4.10.1 Effect of *C. gigantea* Chloroformic extract orally administered against *Bungarus caeruleus* venom

AVS showed 100% neutralization potency against $2LD_{50}$ *Bungarus caeruleus* venom. Group #3 and 4 showed 100% mortality while group #5 showed 50% survival. The results are as shown below in table (4. 31).

Table 4.31 Effect of *C. gigantea* Chloroformic extract against *Bungarus caeruleus* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	2/2	-
2	Venom + AVS (polyvalent antivenom)	0/2	100
3	Venom + *C. gigantea* extract 100mg/kg	2/2	0
4	Venom + *C. gigantea* extract 200mg/kg	2/2	0
5	Venom + *C. gigantea* extract 300mg/kg	1/2	50

Neutralization of 10.8µg/ml of *Bungarus caeruleus* venom was done by 0.2ml of chloroformic extract of *C. gigantea* in three doses. Only 300mg/kg of plant extract showed 50% result.

4.10.2 Effect of *C. gigantea* distilled water extract orally administered against *Bungarus caeruleus* venom

Only AVS showed 100% neutralization potency against $2LD_{50}$ *Bungarus caeruleus* venom. Group #3 and 4 showed 100% mortality while group #5 showed 50% survival. The results are as shown below in table (4.32).

Table 4.32 Effect of *C. gigantea* distilled water extract against *Bungarus caeruleus* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	2/2	-
2	Venom + AVS (polyvalent antivenom)	0/2	100
3	Venom + *C. gigantea* extract 100mg/kg	0/2	0
4	Venom + *C. gigantea* extract 200mg/kg	0/2	0
5	Venom + *C. gigantea* extract 300mg/kg	1/2	50

Neutralization of 10.8µg/ml of *Bungarus caeruleus* venom was done by 0.2ml of distilled water extract of *C. gigantea* in three doses. Only 300mg/kg of plant extract showed 50% neutralization.

4.10.3 Effect of *C. gigantea* methanolic extract orally administered against *Bungarus caeruleus* venom

Only AVS showed 100% neutralization potency against $2LD_{50}$ *Bungarus caeruleus* venom. Group #3 and 4 showed 100% mortality while group #5 showed 50% survival. The results are as shown below in table (4.33).

Table 4.33 Effect of *C. gigantea* methanolic extract against *Bungarus caeruleus* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	2/2	0
2	Venom + AVS (polyvalent antivenom)	0/2	100
3	Venom + *C. gigantea* extract 100mg/kg	2/2	0
4	Venom + *C. gigantea* extract 200mg/kg	2/2	0
5	Venom + *C. gigantea* extract 300mg/kg	½	50

Neutralization of 10.8µg/ml of *Bungarus caeruleus* venom was done by 0.2ml of methanolic extract of *C. gigantea* in three doses. Only 300mg/kg of plant extract showed 50% result.

4.10.4 Effect of *C. gigantea* petroleum ether extract orally administered against *Bungarus caeruleus* venom

Group #2, 4 and 5 showed 100% survival against $2LD_{50}$ *Bungarus caeruleus* venom. Group #3 showed 100% mortality. The results are as shown below in table (4.34).

Table 4.34 Effect of *C. gigantea* petroleum ether extract against *Bungarus caeruleus* venom ($2LD_{50}$).

Group	Dose of the drug	Mortality (after 24hrs) [No. of death/no. of mice used]	% survival after 24hrs
1	Control (only venom)	2/2	0
2	Venom + AVS (polyvalent antivenom)	0/2	100
3	Venom + *C. gigantea* extract 100mg/kg	2/2	0
4	Venom + *C. gigantea* extract 200mg/kg	0/2	100
5	Venom + *C. gigantea* extract 300mg/kg	0/2	100

Neutralization of 10.8µg/ml of *Bungarus caeruleus* venom was done by 0.2ml of petroleum ether extract of *C. gigantea* in three doses. Result percentage of survival was:

1st dose= 0%, 2nd dose – 100%, 3rd dose = 100%.

Overall, effects of *C. gigantea* extracts on *Bungarus caeruleus* venom were good. Plant extract with petroleum ether has good effects than other three extracts.

DISCUSSION

5. DISCUSSION

Snake envenomation in Pakistan is responsible for 20,000 deaths per year. All kinds of venomous snakes are scattered all over Pakistan specially Cobra (*Naja naja*), Russell viper *(Doboia russeli*), saw scaled viper (*Echis carinatus*) and krait (*Bungarus caeruleus*). Snake venom contains many toxic proteins i.e. cardio toxins, phospholipase A2, neurotoxins and so many other toxic proteins which causes many complications like dizziness, pain, blurred vision, necrosis, edema and many other complications besides death. And AVS is the only therapeutic available in the market for snake envenomation. National Institute of Pakistan is the only manufacturer of AVS. It's because its production is very expensive and hard.

In the present studies venom of four deadly snakes of Pakistan cobra (*Naja naja*), saw scaled viper (*Echis carinatus),* Russell viper *(Doboia russeli)* and krait (*Bungarus caeruleus*) was neutralized by a wild plant *C. gigantea*. A plant which can be found all around Pakistan. *C. gigantea* is known for many medicinal actions, one of which is anti-venomous property. Four different extracts of *C. gigantea* i.e. chloroformic extract, methanolic extract, distilled water extract and petroleum ether extract were used in the present study to neutralize the previously defined snake's venom.

Both in-vitro and in-vivo techniques were used to neutralize venom. Three doses of the plant extract 100, 200, 300mg/kg body weight were selected on the basis of acute toxicity studies which was conducted previously by other researchers. For the in-vitro and in-vivo neutralizations of venoms $2LD_{50}$ of four different snake's venom was calculated. Which was 17.6µg/ml, 18.5µg/ml, 12.8µg/ml and 10.8µg/ml of *Doboia russeli*, *Naja naja*, *Echis carinatus* and *Bungarus caeruleus* respectively. $2LD_{50}$ of all four snakes' venom was determined by the method described by Theakston and Reid, (1983).

Standard polyvalent anti venom was used as reference serum. The AVS was used with every dose for comparison reasons and also as a positive control. Phytochemical screening of the *C. gigantea* showed presence of flavonoids, alkaloid, tannins, steroids, Phenols, Anthraquinones, Volatile Oil, Steroids and saponins. The aim of the study was studies on characterization of plant based antidote against snakes' venom. Hence, it is proved by the following work that studies on plant based antidote showed positive results by neutralizing four different snakes venom.

In-vitro studies were carried out by premixing of venom with each dose of the plant extract in equal proportion which was then incubated at 37 °C for 30 minutes and then injected through IP route into 4 mice per dose. Four types of plant extracts were used which are described above. Three doses of each extract were prepared for neutralization of the venom. The results were calculated after 24 hours of the injection. All of the extracts showed 100% neutralization potency against $2LD_{50}$ Venom of *Doboia russelli.* And most doses of all extracts showed 100% neutralization against $2LD_{50}$ Venom of *Echis carinatus.* 300mg/kg chloroformic extract, 200, 300mg/kg methanolic and petroleum ether extract showed 100% neutralization against $2LD_{50}$ Venom of cobra (*Naja naja).* Krait (*Bungarus Caeruleus*) $2LD_{50}$ venom was completely neutralized by 300mg/kg dose of distilled water extract, 200, 300mg/kg methanolic and petroleum ether extracts.

In-vivo neutralization method was carried out by injecting 0.2ml of $2LD_{50}$ venom followed by immediate oral dose of plant extract. 2 mice per dose were used for oral administration. $2LD_{50}$ Venom of *Doboia russelli* and *Echis carinatus* was completely neutralized by all of the plant extracts and by all of the doses i.e. 100, 200 and 300mg/kg body weight. While none of the extract showed complete neutralization potency against cobra (*Naja naja*) venom. Some of the extracts were effective to 50% potent against *Naja naja* venom. While $2LD_{50}$ of krait venom was completely neutralized by 300mg/kg petroleum ether extract.

Venom of *Doboia russelli* contains special chemicals, biological actions and intricate combination of enzymes, proteins and peptides of low molecular mass. Many lectines, cardiotoxins, neurotoxins, cytotoxins, haemorrhagins, nerve growth factors and disintrigrins. Hypotension, haematurea, bleeding from the gastrointestinal tract, tympanic membrane, genito-urinary tract, gingival bleeding, and ecchymosis. Some studies shows that intracranial and extensive necrosis are the manifestations of *Doboia russelli* envenomation. *Echis carinatus* bites are responsible more deaths rate globally than other snakes. Systemic and local haemorrhage is the common indications occur in *Echis carinatus* bitten prey (Casewell *et al.*, 2009).

β-bungarotoxins which are presynaptic neurotoxins with phospholipase A_2 action which is supposed to be the main reason of paralysis is found in *Bungarus caeruleus* venom (Harris and Scott-Davey, 2013).

In these studies, it has been revealed that *C. gigantea* is very effective in neutralization of all life-threatening snakes venom. Enough work has not been done on this area to produce plant based antidote against snake venom. Ingredients and components of this plant should be purified and studied individually against every toxic protein found in snake venom. Many studies are conducted on this topic but the present study has a unique aspect which is neutralization of four snake's venom, with four different kind of extracts, and three doses of each extract was tested.

5.1 CONCLUSION

We conducted study by applying a controlled amount of four types of deadliest snake's venom and It has been concluded in this work that *C. gigantean* contains active ingredients against all four snakes venom. As results show that each extract of plant neutralized all snakes venom by both in vivo and in vitro methods. Some extract neutralized venom with 100 percent of results while others showed weak results but if further researches will conduct on them with novel methods by purifying the ingredients their performance will be better within low dose in all types of envenomation's. These all snakes considered life threating and found all over the Pakistan regions with variety of numbers.

Antidote prepared from this plant will be easy manufacture within less time, powerful, low cost and accessible to all people as compared to anti-venom which is prepared only in NIH, Islamabad and is costly, time consuming in preparation not accessible to poor sections of people and has many side effects.

We have found *C. gigantea* active against snake venom and other researches must be making on this and other medically important plants that have been tested against snake venom in the relevant field. The plant has very important and unique components in it against snake bite and other complications. If the ingredients of these medicinal plants used separately for the neutralization of venom proteins then a plant based antidote can be prepared which will more powerful and will be used for several purposes in medicine.

Research programme must be supported on *C. gigantea* and awareness about its properties should be promoted among researchers, doctors and general public. The plant should keep in a valuable portion of research. Researchers should try to introduce novel properties of the plant. Although the plant is famous in homeopathic remedies but attention should give to it's medically use in drugs of snake bite ailments.

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