"Evaluation of *Daboia russelii* Venom Induced Biochemical Changes in *Calotropis gigantea* (L). R.Br Treated Mice"

Thesis submitted to
BLDE (Deemed to be University)
Vijayapur, Karnataka, India.

Faculty of Medicine

For the award of the degree of

Doctor of Philosophy in
Medical Biochemistry

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DECLARATION BY THE CANDIDATE

I hereby declare that this thesis entitled ***"Evaluation of Daboia russelii Venom Induced Biochemical Changes in Calotropis gigantea (L). R.Br Treated Mice"*** is bonafide and genuine research work carried out by me under the supervision of **Dr. Basavaraj Devaranavadagi**, (Guide, Professor and Head, Department of Biochemistry, Shri B. M. Patil Medical College, Hospital and Research Centre, BLDE (Deemed to be University), Vijayapur, Karnataka, India). and **Dr. Achuthan Raghava Menon** (Co guide, Associate Professor, Department of Biochemistry, Amala Cancer Research Center, Amalanagar, Thrissur, Kerala, India). No part of this thesis has been formed the basis for the award of any degree or fellowship previously.

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Dedication

This thesis is dedicated to my late Father

'In loving memory of

P.G. Parthasarathy*'*

Whose love and care

has been a pillar of support to me

Acknowledgement

First and foremost, I take this opportunity to express my deep and profound gratitude to my research guide **Dr. Basavaraj Devaranavadagi**, Professor & Head, Dept of Biochemistry, Shri B. M. Patil Medical College, Hospital and Research Centre, Vijayapur. His dedication, patience, constant optimism, and wise counsel have been a source of strength for me. I express my gratitude to my Co-guide **Dr. Achuthan Raghava Menon**, Associate Professor, Department of Biochemistry, Amala Cancer Research Center, Thrissur, Kerala. This initiative has been guided by his technical knowledge, attitude to tackling scientific challenges, and originality. This association with my mentors will remain an encouraging and enabling force in all my future professional endeavors.

I thank **Dr. Kusal K Das**, Distinguished Chair Professor, Laboratory of Vascular Physiology and Medicine, Shri. B. M. Patil Medical College Hospital & Research Centre, Vijayapur**.** His valuable inputs pertaining to my field of study has helped me refine the idea and provided a new perspective to my scientific thought. I am grateful to **Dr. Ramadasan Kuttan** and **Dr. Girija Kuttan**, a prominent scientific couple, for introducing me to research and encouraging me to undertake research at the Amala Cancer Research Center in Thrissur, Kerala. I sincerely thank **Dr. Deepu Mathew**, Assistant Professor-CPBMB, Kerala Agricultural University- KAS, Thrissur, Kerala and **Ms. Sangeetha** for helping me with *in silico* part of this project. I thank **Dr. Rajkumar Chemudugunta** Associate Professor, Department of Pathology, KMCT Medical College, Calicut, Kerala for diligently observing all histopathological sections pertaining to this study. **Dr. Vijaya Sorangavi**, Associate Professor, Department of Community Medicine, Shri. B. M. Patil Medical College Hospital & Research Centre, Vijayapur, provided important statistical inputs for my research.

I extend my sincere gratitude to the Hon. Chancellor **Dr. M. B. Patil**, BLDE Deemed to be University, President BLDE Association, Vijayapur for his persistent commitment towards research and innovation.

Special thanks to late and former Honourable Vice chancellor **Dr. M.S Biradar**, and current Vice chancellor **Dr. R.S. Mudhol** BLDE (Deemed to be University), Vijayapur. **Dr. J.G Ambekar**, Registrar, BLDE (Deemed to be University), Vijayapur. **Dr. Aravind V Patil**, Principal and Dean Faculty of Medicine, Shri. B. M. Patil Medical College Hospital & Research Centre, Vijayapur. **Dr. S. V. Patil**, Vice Principal (Academic), Shri. B. M. Patil Medical College Hospital & Research Centre, Vijayapur, **Dr. Satish B. Patil,** Deputy Registrar BLDE (Deemed to be university), Vijayapur for their unwavering support and prompt administrative help.

I acknowledge, faculty members of Department of Biochemistry, B.M Patil Medical College, Vijayapur. **Dr. B.S. Aski, Dr. Indira. Hundekari, Dr. Deepa Sajjanar, Dr. Shivaleela Biradar, Dr. S.S. Walvekar, Dr. R. Chandramouli Reddy and Mr. G.V. Naregal** for their constant support and suggestions. I appreciate all the non-teaching staff members of Department of Biochemistry for their help and assistance.

I acknowledge with gratitude, valuable inputs received from **Dr. Babu.T** and **Dr. Suraj**. **K** of Amala Cancer Research Center, Thrissur, Kerala. I thank **Mr. Vishak Nair**, Research fellow at Amala Cancer Research Center for his suggestions and timely help during the course of this project. **Dr. Arunaksharan, Gislin Davis, Shruthi,** and **Surya P**, have enriched my knowledge by sharing their research experiences. I thank **Ms. Preetha** and **Ms. Usha** for helping me with animal experiments in particular.

I thank **Dr. Nilima Dongre** , Department of Biochemistry BLDE (Deemed to be University) Vijayapur and all esteemed members of PhD committee for providing regular inputs of my work.

This work would not be complete without the support of my classmate and friend **Dr. M. Govind Pai**. Lastly, I am eternally indebted to my late father **P.G Parthasarathy** and Mother **Hemalatha**, for my upbringing, which has imparted a positive outlook in life and enriched it. Besides fostering a happy home, I am grateful to my wife **Kavya** for help with typesetting this thesis and my younger sister **Veena** for providing software packages essential for this piece of work. My little daughter **Akaanksha** deserves all the love and praise for creating a cheerful atmosphere and cooperating during my thesis writing.

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Abbreviations

CG- *Calotropis gigantea*

CGMR= *Calotropis gigantea* methanolic root extract

DRSV- *Daboia russelii* snake venom

Hb- Hemoglobin

RBC- Red blood cell

MCV- Mean corpuscular volume

MCH- Mean Corpuscular Hemoglobin

MCHC-Mean Corpuscular Hemoglobin Concentration

PCV- Packed cell volume

SGOT-Serum Glutamate Oxaloacetate Transaminase

SGPT-Serum Glutamate Pyruvate Transaminase

ALP-Alkaline Phosphatase

Bilirubin (T) -Bilirubin total

Bilirubin (D) - Bilirubin direct

Bilirubin (I)-Bilirubin indirect

PLA2-Phospholipase A2

GC- Gas chromatography

LC-Liquid chromatography

MS- Mass spectrometry

IC_{50}- Half maximal inhibitory concentration

LD_{50}- Lethal dose 50

Kgb.wt- Kg body weight

Ki-Binding energy

Abstract

Objective

To study the venom neutralization ability and prophylactic effect of *Calotropis gigantea* methanolic root extract (*CGMR*) against *Daboia russelii* envenomation using *in vitro, in silico,* and *in vivo* methods.

Background

Snake venom is composite, that contains neurotoxins, disintegrins, hemotoxins, and proteases in complex with phospholipase A2 [PLA2] enzymes. Hence toxicity of venom is mainly attributed to PLA2 enzymes or their protein complexes per se. Roots of the plant, *Calotropis gigantea* are used extensively as a phyto-antidote by tribal communities to treat snake bite victims. However the antivenom property of root has not been comprehensively studied so far. This study scientifically reasserted the use of *Calotropis Gigantea* R.Br (root extract) in neutralization of *D.russelii* venom, alongside investigated the protection rendered by the extract.

Materials and Methods

Phytochemicals of *Calotropis gigantea* methanolic root (*CGMR*) extract were fractionated into hexane (non-polar fraction) and methanol (polar fraction) based on polarity. The *in vitro* PLA2 inhibitory action of crude extract and both fractions was determined using biochemical assay. Since significant PLA2 inhibition was observed in non-polar fraction, it was subjected to GC/MS analysis. The compounds obtained through GC/MS analysis were virtually docked to PLA2 macromolecule by protein-ligand binding simulation programs. The acute and sub-acute toxicity of the extract was determined using guidelines 423 and 407 respectively in mice. The LD_{50} dose of *Daboia russelii* snake venom (*DRSV*) was determined using standard protocol. The *in vivo* neutralization ability and the *in vitro* neutralization (pre-incubated)

ability of the *CGMR* extract was determined using 24hr survivability of the animal as endpoint. The group receiving antivenom was used as positive control. Animals pretreated with *CGMR* were used to study the prophylactic effect of the extract against venom insult.

Results

The total phenols and flavonoids were estimated to be higher in crude extract, when compared to non-polar and polar fraction. The non-polar fraction recorded the least in amount of total phenols and flavonoids. The methanolic crude extract (IC_{50}=33.531±5.630 μg/ml), non-polar fraction (IC_{50}=59.586±1.491 μg/ml) and polar fraction (IC_{50}= 77.505±3.772 μg/ml) of the root extract effectively inhibited PLA2 activity in-vitro. Non-polar fraction inhibited PLA2 with more efficacy than polar fraction (*P=.0037*).

The GC/MS profile of non-polar fraction of methanolic root extract indicated the presence of phytochemicals like n-Hexadecanoic acid, Oleic acid, 9, 12 Octadecadienoic acid and allyl octadecyl ester oxalic acid. n-Hexadecanoic acid ($Ki=1.58 \times 10^{-5}$) was found to be competitive inhibitor of PLA2. Docking studies revealed that n-Hexadecanoic acid (ligand) interacted with catalytically important residues (His48, Asp49, and Gly30) of PLA2 (PDBID-3CBI, macromolecule). Interestingly, three other phyto-chemicals found in the extract, oleic acid, 9, 12 Octadecadienoic acid and allyl octadecyl ester oxalic acid showed similar type of chemical interactions with active site residues of PLA2. The *in vitro* and *in silico* experiments conducted in this study indicate the presence of potential PLA2 inhibitors in the extract.

In toxicity assessment a significant increase in food intake, bodyweight, water consumption and anti-platelet activity was observed at higher dose (400 mg/kg b.wt). Histopathological analysis of organs indicates hepatotoxic and cardiotoxic property of the extract at higher dose.

LD_{50} of venom was determined to be 11µg/dose and the % survival of group receiving only venom was 50%. High dose of *CGMR* effectively neutralized LD_{50} dose of venom in both *in vivo* (%survival = 67.67%) and *in vitro* neutralization experiment (% survival =100%). Neutralization of venom was better in *in vitro* neutralization (pre-incubation). The histopathological analysis of organs necropsied from *CGMR* pre-treated animals demonstrated significant tolerance against venom.

Conclusion

Together these results indicate that *CGMR* has significant venom neutralizing potential as traditionally claimed. Further the extract showed significant venom neutralization ability and protection effects against venom insult. The high venom neutralization ability may be due to PLA2 inhibitors present in the extract.

Keywords: *Daboia russelii*, *Calotropis gigantea*, docking, neutralization, Phospholipase A2

CHAPTER 1
INTRODUCTION

Snakebite is a neglected global public health concern that impacts around 1.8 million people each year. Approximately 6.94% percent victims succumb to death, while surviving victims suffer from long term morbidities (Slagboom *et al*. 2017). The number of deaths recorded from India accounts for little more than half of all snakebite deaths worldwide. The Indian Russell's viper has been regarded a notorious snake, causing roughly 43% of bites.

The provision of first aid, according to a WHO report on the control of neglected tropical diseases, is a critical factor in reducing the number of fatalities. Though many first aid strategies like use of constriction bands, tourniquets, electric shock therapy, ice therapy, incision and vacuum suction therapy exist for snakebite treatment, they are reported to be contraindicative (Blackman and Dillon 1992). The use of suction pumps for removing venom is avoided since other bloody fluids are removed instead (Gellert GA, 1992). In several snakebite instances plasmapheresis is recommended as an alternative (Yildirim et al. 2006). However the risk of developing cross reactivity is inevitable.

Several studies have indicated that polyvalent antivenom administration results in adversities like serum sickness, severe pyrogenic reaction and anaphylaxis. On the other hand, monovalent antivenom have restricted neutralization ability due to lesser volume. Furthermore, no evidence exists that its use is superior to polyvalent antivenom. (Ariaratnam *et al*. 1999) (H. A. de Silva, Ryan, and de Silva 2016). Snakebites are more common in isolated places and remote tribal areas with limited access to immediate first aid. As a result, traditional healers are still sought by the majority of victims today. Traditional healers use plant extracts for treating aliments and their folkloric knowledge and practices are appreciated even today. Some of the plant extracts used by traditional healers as antivenom has drawn the attention of researchers to study their ethanopharmacological properties

(Kumarapppan, Jaswanth, and Kumarasunderi 2011). Few of the plants like *Aristolochia shimadai, Rouwalfia serpentina* and *Schumanniophyton magnificum* have been thoroughly studied for their antivenom property (Martz 1992) (Sivaraman *et al*. 2020). Pharmaceutical researchers are focused on isolating the exact secondary metabolite responsible for antivenom property present in these plant extracts to develop active compounds. These plant based active compounds are known to possess lesser side effects, cheaper and socially accepted in many cultures, including India. Previously a number of plant based metabolites having antivenom properties have been isolated like sitosterol, rosmaric acid and beta-amyrin (P. Singh *et al*. 2017). However their clinical utility is limited due to lack of scientific evidence.

Calotropis gigantea is a dry land shrub that is commonly found in wastelands. Traditionally the root of this plant are crushed and applied on to the snakebite site as antidote. Even today, *Bagata* tribes inhabiting parts of Orissa and Andhra Pradesh use it as a medication for snakebite(Jain *et al*. 2011) (Sri and Reddi 2011). The antivenom property of leaf and latex extract of the plant is documented previously (Chacko *et al*. 2012). However the root extract which the tribes specifically use to neutralize venom has been neglected and scientific studies are required to corroborate the traditional claim. Also many venom neutralization studies involving plant extract have failed to ascertain the possible role of phytochemicals responsible for producing desired biological activity. So in this study the neutralization ability of the extract and the prophylactic effect of the extract have been studied using *in silico*, *in vitro* and *in vivo* experiments.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Global statistics

Snakebite is a neglected tropical disease world over and affects around 1.9 million people yearly. A current study estimates that approximately 6.94% percent snakebite victims succumb to death, while surviving victims suffer from long term morbidities (Slagboom *et al.* 2017). The number of annual deaths in India due to snakebite is alarming, as it accounts for more than 50% of annual deaths worldwide (C. Arnold 2020). Snake bite fatalities in India are mostly linked to the following four species (Chauhan and Thakur 2016). King Cobra (*Naja naja*) envenomation results in severe local pain, edema, ecchymosis, decrease Spo_2, confusion arrhythmia and CNS impairment. Edema, hypokalemia and glossopharyngeal discomfort are few common observations following Common krait (*Bungarus caeruleus*) bites. Russell's viper (*Daboia russelli*) bites causes blisters and a bigger punctures compared to saw scaled vipers. The victim suffers from severe pain edema and ecchymosis. Saw scaled viper (*Echis carinatus*) bites are discolored and easy to identify. The pathological features like anemia and cyanosis are evidently seen (Kumar, Maheshwari, and Verma 2006). Among these species, Russell's viper has been reported as the single most deadly species, causing death in roughly 43% of instances, followed by unknown species (21%), krait (18%) and cobra (12%). As a result, snakebite has become one of the most dangerous tropical diseases in India and the rest of the world.

2.2 Biting problem

In 2017, World health organization (WHO) has launched a global initiative to halve the number of snakebite deaths by 2030. India's role in this regard is crucial, as it accounts for more than 50% of snakebite deaths. Adequate focus on managing, preventing and treating Russell's viper envenomation (43%) alone may significantly lower the snakebite statistics for India and improve the global average. In a study significant association is made between the

snakebite deaths and Human development index (HDI), GDP per capita, quantum of labor force engaged in occupations like agriculture, and state expenditure on health. Another study found a link between snake bites and gender (males are more likely to be bitten), rural inhabitants, and people who work in jobs where they are not compelled to wear shoes. (Harrison *et al*. 2009). Also the quantity of venom injected and the site of bite affect the fatality rate (Alirol *et al*. 2010).

2.3 Antivenom therapy

Researchers, pharmaceutical enterprises, nongovernmental organizations and state sponsored bodies have been working towards better awareness of snakebite among public, adequate first aid care and better treatment strategies. But the number of snake bite episodes is less uncommon and the number of new complications and post treatment consequences are ever increasing (Gutiérrez *et al*. 2017). The only treatment available for snakebite is administration of antivenom that is polyvalent antibodies raised by hyper immunizing the animal. The drawbacks of using polyvalent antibodies are manifold. Firstly, the variation of venom is not addressed with polyvalent antivenom treatment. The snake responsible for the bite may have a different composition while the polyvalent antibodies may be specific to snake venom belonging to a different species. This results in cross reactivity, restricts the cross efficacy and para-specific (Chippaux and Goyffon 1991). The administered antivenom due to above reasons may not neutralize the venom completely leading to increased fatality (Isbister 2010). To circumvent this problem of non specific reaction and incompatibility the antibodies are raised against a cocktail of venoms, however in this case the antibodies specific to the venom are diluted and results in incomplete neutralization (I. S. Abubakar *et al*. 2010). For effective neutralization of venom sufficient quantities of venom specific antibodies are required and therefore it mandates more vials of antibodies which may

increase the treatment cost. In addition, entry of large quantities of high molecular weight foreign antibodies may increase the risk of hyper immune reactions (S. B. Abubakar *et al*. 2010). In roughly 55% of instances, adverse reactions are reported, demonstrating the danger of not only snakebite but also snakebite therapy. (Deshpande *et al*. 2013). The preceding talks have brought to light the international health emergency that is snakebite, which requires immediate attention. A strategy to develop low cost, highly specific and effective antivenom mandated.

2.4 Venom and its components

Venoms are complex mixtures of toxic proteins. However the composition of venom varies between snakes of different families, genera and species. In fact, the venom composition of the same snake varies dramatically over the course of its life. This variation in venom composition and toxicity is mainly due to evolutionary changes that have occurred by different molecular processes like gene duplication, positive selection, recombination and alternative splicing (Slagboom *et al*. 2017). These many toxin components are ultimately responsible for the functional toxicity of venom as well as the underlying diseases. Snake venom, because of its diverse variation can The WHO has categorized venoms as haemotoxic, neurotoxic and cytotoxic. Snake venom can show cytotoxic haemotoxic neurotoxic or mytoxic properties, some venoms show a combination of these properties. Historically, viper venoms are considered to be predominately hemorrhagic while elapid venoms are neurotoxic. However several studies have reported vipers causing neuronal dysfunction and elapids causing haemostatic dysfunction (Tednes and Slesinger 2021)**.**

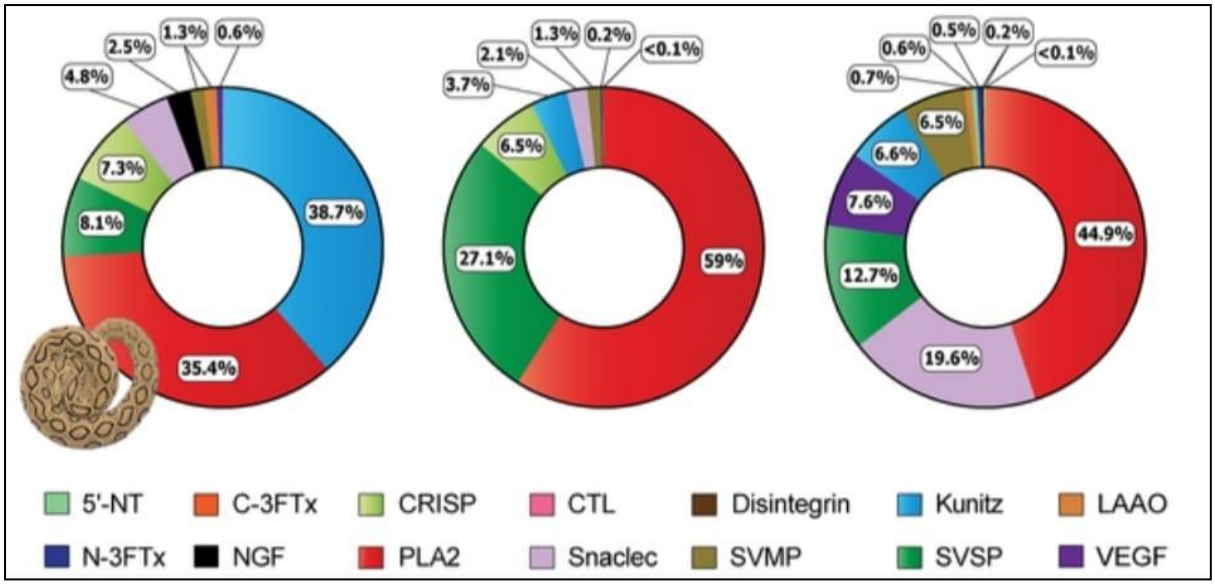


Figure 2.1: Depicts the variations and toxin composition present in *Daboia russelii* snake venom sourced from three distinct geographical locations from India (Laxme *et al*. 2021).

In **Figure 2.1** the first inset shows composition of *DRSV* from eastern parts of India. A high concentration of Kunitz proteins is noted in this venom compared to south western India (inset 2) and central India (inset 3). On the other hand in south western India concentration of SVSP is predominant. PLA2 is the most abundant protein in all three snakes. Hemotoxic venom in general decreases the blood pressure by damaging the capillary basement membrane. Snake venom Metalloproteinase (SVMP) is mainly responsible for this damage which results in vascular permeability. Another toxin Snake venom serine protease (SVSP) functions like kallikrien and results in production of bradykinin peptides that potentiates vasodilatation. Hemorrhage is a typical clinical manifestation of snake bites, particularly viper bites. Bleeding from gums, gastrointestinal tract and the urinary tract are commonly presented within few hours of envenomation. In addition if hemorrhages occur in the brain the lethal effects are exaggerated. In the instance of viper envenomation, however, shock causes systemic hypertension, which might lead to mortality.

Envenomation by viper causes modulation in the activity of blood clotting factors that result in venom induced consumption coagulopathy it is similar to disseminated intravascular coagulation (DIC) like syndrome. Due to the deficiency of coagulation factors the blood is unable to clot and becomes incoagulable. In case of VLCC induced by viper bite deficiency of clotting factors such as factor II, and factor X are observed along with fibrinogenolytic activity. In addition Factor V and factor VIII deficiency is seen. Consequently, assessments of the bleeding time and the clotting time have become diagnostically important. A 20 minute whole blood clotting test is performed to detect the extent of envenomation. In case of VICC, prolonged prothrombin time increased partial thromboplastin time are detected. Few venom toxins act on platelets they either induce aggregation of platelets via von willebrand factor or they may inhibit aggregation of platelets by arresting the integrin receptors found on platelets. In venom induced consumptive coagulopathy thrombocytopenia is observed along with hemorrhages and bleeding clotting disorders.

SVMPs (snake venom metalloproteases) are a key component of viper venom(Markland and Swenson 2013). Venom containing multiple SVMPs is present in a single snake species. The composition and variety exhibited in SVMPs is primarily due to gene duplication. SVMPs are classified as P1, P2 and P3 types. SVMPS are implicated in pathological conditions such as haemorrhages, platelet aggregation inhibition, and prothrombin activation. P3 is the most hemorrhagic of the three, and P2 has higher hemorrhagic activity than P1. They mainly affect the endothelial cell lining of the basement membrane of capillaries and cause vascular leaks leading to extravasation. Further studies have shown that the SVMPs act on collagen type IV to destabilize the capillary basement membrane(Swenson and Markland 2005). P1 is shown to posses fibrinogenolytic activity resulting in defibrination induces coagulopathy and other bleeding disorders.

By inducing a conformational change in factor X, RVV activates it to Xa, which inturn activates factor V to Va. Factor Xa and Va form the prothrombinase complex, that is required for activating prothrombin to thrombin. Phospholipases and Ca^{2+} are required as cofactors for the functioning of prothrombinase complex. Thrombin acts on fibrinogen resulting in formation of fibrin clots. Some SVMPs directly act on prothrombin, some SVMP require Ca^{2+} for their activity and some function devoid of calcium (Slagboom *et al*. 2017). Ecarin, a SVMP for instance doesn't require Ca^{2+} for its activity while Carinactivase-1 requires Ca^{2+} for its activity. Through this mechanism the clotting factors are used up resulting a deficiency, this form the pathological bases for venom induced consumptive coagulopathy.

Disintegrins are key component of viper venom that mainly functions as integrin receptor blockers. They are cysteine rich peptides that are either obtained from SVMP cleavage or by direct synthesis from disintegrins gene (Juárez *et al*. 2008). Few of these integrin receptors blocked by disintegrins are having immense pharmacological value and are well researched in recent times. Researchers are interested in a2b3 integrin, which is associated in coronary disease, and a9b1, which is involved in inflammatory diseases. In viper envenomation in particular disintegrins bind a2Bb3 integrin, which inhibits platelet aggregation and restricts fibrinogen association with platelets (Calvete *et al*. 2005). Snake venom serine proteases (SVSP) are important component of viper venom showing fibrinogenolytic activity similar to thrombin. They act on fibrinogen, which results in aggregation of fibrin monomer. But unlike thrombin action SVSP do not stimulate Factor XIII which results in formation clots that are not intact (Latinović *et al*. 2020).

2.5 Phospholipase A2

PLA2 (E.C- 3.1.1.4) catalytically hydrolyzes the Sn-2 acyl bond of phospholipids to release arachidonic acid and lysophospholipids. Oxidation of arachidonic acid by cyclooxygenases

generates active inflammatory mediators called eicosanoids- prostaglandins (PG), thromboxanes (TX) and leukotrienes (LT) (Sales *et al*. 2017). Phospholipase A2 [PLA2] is present in mammalian tissues, snake venom, bee venom and frog venom (Shukla *et al*. 2015). The toxicity of venom is mainly attributed to PLA2 enzymes and their integral protein complexes (Manjunatha Kini 2003). Pre-synaptic neurotoxins, for instance exist as complexes of PLA2 (Xiao *et al*. 2017). Multiple sequence alignment of PLA2 sequences from different species has reported a conserved active domain spanning the catalytic residue HIS 48. Though PLA2 enzymes posses diverse biological activities they share high degree of identity in amino acid sequence which suggestive of similar 3D structures and foldings (Hiu and Yap 2020). The focus of this study is to screen for potential PLA2 antagonists from *C.gigantea* roots, which are traditionally used to neutralize snake venom. Since the toxicity of venom is mainly attributed to PLA2, we hypothesize that strong inhibitors of PLA2 may be present in *C.gigantea* root extract.

2.6 Calotropis gigantea

Calotropis gigantea (L.) R.BR (Apocynaceae) is a dry land weed found in Asia and Africa (Singh 2012). The plant belongs to *Calotropis* genus and is commonly called as milky weed or crown flower. Traditionally the plant leaves, latex and roots are attributed to possess immense medicinal value. The root extract, in particular possesses wound healing activity, rheumatoid arthritis, insomnia, pregnancy interception, skin cancers, anxiolytic, sedative, analgesic, and anticonvulsant properties. The antioxidant property of *C. gigantea* leaf, flower, and latex extracts are reported in previous studies (Rathod *et al*. 2009). The traditional practice has been well substantiated by several *in vivo* studies. *Calotropis gigantea* root extract, in particular is traditionally used for inducing abortions and healing wounds (Srivastava *et al*. 2007) (Deshmukh *et al*. 2009). It has been shown that, single oral dose of

C.gigantea root extract (100mg/kg), administered on first day of post coitum completely intercepted pregnancy in rats. While dose administered post implantation resulted in resorption of implants. Further a decrease in body weight in treated rats was noted, compared to increased body weight in vehicle control group. In another study, *C.gigantea* root bark extract was known to increase wound healing ability in excision and incision types of wounds. *In vivo* studies have investigated analgesic, anticonvulsant, anxiolytic, and sedative properties of the extract (Argal and Pathak 2006) (I. N. Khan, Sarker, and Ajrin 2014). An *in vivo* experiment, using writhing techniques and hot plate method has ascertained analgesic and anxiolytic effect of extract. In the same study anticonvulsant and sedative effects of the extract have been established. The extract has been shown to inhibit chronic myelogenous leukemia K562 cell. The methanolic bark extract of *C.gigantea* showed insecticidal activity against several inster of larvae and adult of *Tribolium castaneum.* In Ayurveda, the powdered roots are used in asthma and bronchitis treatment (Warrier, Nambiar, and Ramankutty 1993). The list here may not be exhaustive owing to the diverse pharmaceutical properties present in the extract. These studies have scientifically substantiated the traditional practice of using *C.gigantea* root extract for curing several ailments, apart from investigating new pharmacological properties endowed in the extract. However, there are not enough studies that provide comprehensive knowledge for elucidation of specific phytochemical constituents in *C.gigantea* root. Analyzing the phyto-chemical profile of *C.gigantea* root extract may provide insights for identification of specific bioactive compounds, which may be responsible and may contribute to diverse pharmacological properties exhibited by this extract.

2.7 Plant based medicines

Over the decades, the public interest and social acceptance of plant based medicines are rapidly increasing in both developed and developing countries(Ekor 2014). The WHO global

report on Traditional and Complementary medicine (TCM), 2019, indicates that at least 80% of surveyed member states (170) recognize and use TCM, including indigenous medicine (World Health Organization 2019). The increasing patronage and inclination towards herbal medicines is mainly due to easy availability, low cost, and effectiveness of treatment (Vaidya and Devasagayam 2007). Also herbal medicines are generally considered safe and less toxic compared to allopathic drugs. Contrary to this view, recent studies have acknowledged the adverse effects of few commonly used medicinal plants, and have commented critically on their spurious and unsafe use (Mensah *et al*. 2019). For example, Ginger (*Zingiber officinale* Roscoe, Zingiberacae) is generally considered as safe herbal medicine and is traditionally used to treat wide array of ailments such as arthritis, ulcers and cancers. However, at a higher dose (600mg/kg b.wt), ginger is known to increase serum testosterone levels and cause androgenic activity in Wistar male rats(Rong *et al*. 2009). In addition, there exists inadequate knowledge about mechanism of their action, dosage, specificity and cross reactivity. On the other hand medicinal plants and herbal medicines cannot be neglected in toto, as they contribute significantly to human health worldwide, particularly in developing countries(Ekor 2014). So, regulatory authorities across the world are striving to evolve a structured control model to ensure licensing, manufacturing, trading and safety of TCM medications. According to WHO global report on Traditional and Complementary medicine (TCM), 2019, about 99 out of 133 member states cited limited research data on safe usage of TCM as a top challenge for regulation and registration of herbal medicines (World Health Organization 2019). Plant based products such as phytonutrients and nutraceuticals are widely used across the world. The past decade has witnessed a tremendous surge in herbal medicinal products. This change in trend may be dangerous, because it can lead to self-medication without understanding the associated risks that follow its use. In addition, toxicological studies of herbal products are limited, as opposed to synthetic drugs (Karimi,

Majlesi, and Rafieian-Kopaei 2015). Therefore, there exists an imperative need to evaluate the toxicity associated with herbal drugs.

2.8 Toxicity of plant extracts

Few studies have demonstrated that traditionally used plant extracts have potential to cause toxicity when used at higher dose. For instance, medicinal plants such as *Callilepsis laureola* DC and *Larrea tridentata* DC that are traditionally used to ameliorate GI tract anomalies have been reported to cause hepatotoxicity at high dose (Steenkamp, Stewart, and Zuckerman 1999) (Arteaga, Andrade-Cetto, and Cárdenas 2005) (Pelkonen *et al*. 2017). In general, milkweed plants (Asclepias spp.) such as *Calotropis gigantea, Asclepias syriaca,* and *Calotropis procera* produce up to 200 structurally unique cardenolides that are conventionally used as cardiotonic steroids (Züst *et al*. 2019). Hence using milkweed plants in herbal medications poses a high risk. On account of these reasons toxicity assessment of traditionally used therapeutic plants has become a necessity. And determining toxicity of a widely used milkweed plant such as *Calotropis gigantea* has become a priority.

On the other hand, folkloric knowledge and few medico-legal investigations suggest that the root extract, in particular was extremely harmful. Serious clinical implications like vomiting, irritation, arrhythmias, and lethal consequences including death, following its ingestion have been recorded as experiential evidence from the past(Chandra *et al*. 2015). Despite its adverse effects, the toxicity of the extract has not been comprehensively studied so far and is still recommended for asthma, bronchitis, dyspepsia and wound healing treatment, making its use controversial.

2.9 Plant based snake venom antidotes

The usefulness of plant-based herbal remedies as efficient antidotes against snake venom has been highlighted in several ethanopharmacological research (Okot *et al*. 2020). Snakebite, which is frequent in tribal and hilly locations, is not an exception. Since ancient times, tribes have used specialized plant extracts to treat snakebite victims. Few of these plant extracts have been researched for their antivenom property and proven to be beneficial with a significantly high rate of venom neutralization. *Aristolochia indica* plant for instance is traditionally used to treat snake bites has been shown to posses significant antivenom potential (Bhattacharjee and Bhattacharyya 2013). *Tamarindus indica* a traditionally used plant to treat snakebite has been demonstrated for its venom neutralization property(Ushanandini *et al*. 2006). *Vitis vinifera* seed extract has been shown to inhibit necrotic and procoagulant activity of venom(Mahadeswaraswamy *et al*. 2008). Though polyvalent anti-snake venom is widely used for treatment, indigenous tribes apply *C.gigantea* root paste locally on bite sites (Sri and Reddi 2011). Traditionally, leaves, roots and latex of *C. gigantea* plant are used as phyto-antidote to treat snake bite (Jain *et al*. 2011). However the roots, in particular are extensively used by Indian tribes. The traditional practice has been well substantiated by *in vivo* neutralization study wherein 400 mg/Kg b.wt of *C.gigantea* leaf extract administered to Balb/c mice, substantially neutralized $2LD_{50}$ and $3LD_{50}$ dose of *Viper russelli* venom (Chacko *et al*. 2012). But, the anti-venom property of *C.gigantea* root has not been comprehensively studied, so far. Hence in this study an attempt is been made to investigate the antivenom potential of *C.gigantea* root extract.

CHAPTER 3
AIMS AND OBJECTIVES

3.1 Aim of the study

Aims of the present study include

1. Phytochemical screening of *C.gigantea* methanolic root extract.
2. Screening of phytochemical responsible for PLA2 inhibition using *in vitro* and *In silico* tools.
3. Safety evaluation of *C.gigantea* root extract in mice.
4. Investigate anti-venom property of *C.gigantea* root extract against *D.russelii* envenomation in mice.
5. Assess the protection rendered by *C.gigantea* extract in circumventing these biochemical changes.

3.2 Objectives of the study

Objectives of the present study includes

1. Identification, isolation, authentication, extraction and fractionation of *CGMR*
2. Phytochemical screening of *C.gigantea* root extract.
3. GC/MS and LC/MS analysis of *CGMR.*
4. Molecular docking study to identify phyto-chemicals responsible for PLA2 inhibition.
5. Determination of *in vitro* PLA2 enzyme inhibition
6. Evaluation of oral acute toxicity and sub acute toxicity of extract in mice
7. Determination of LD_{50} of venom
8. Evaluation of neutralization of *D.russelii* venom by *C.gigantea* methanolic root extract.

9. Evaluation of protection rendered by *C.gigantea* aqueous root extract in *D.russelii* envenomation.

CHAPTER 4

MATERIALS AND METHODS

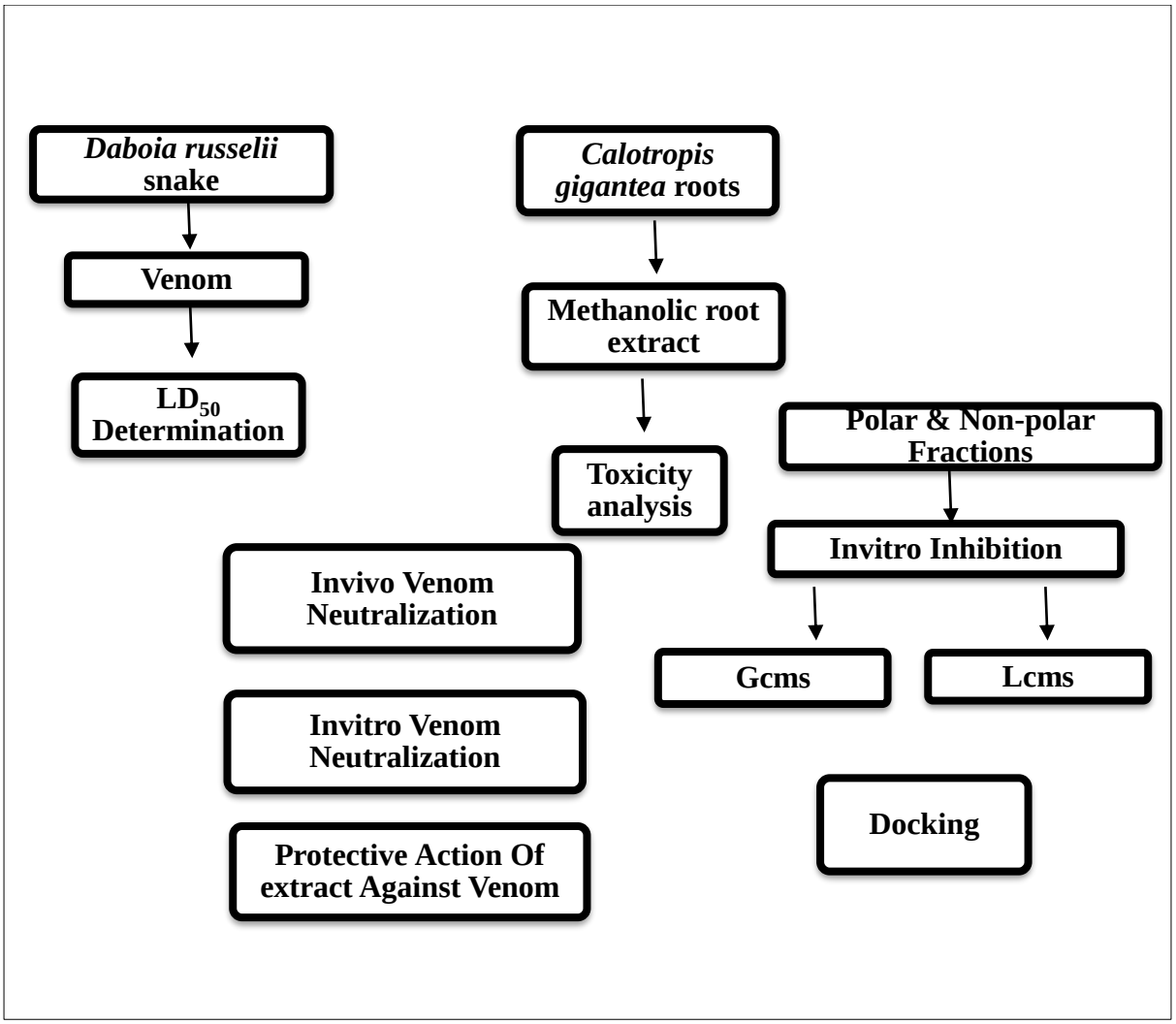


Figure 4.1- Overview of methods and techniques used for the study

4.1 Plant collection

C.gigantea plant was identified and its roots were collected from medicinal garden located within Amala Medical College campus, Trichur. The co-ordinates of sampling site were $10^0$33′44.8″N and $76^0$09′56.4″E Southern India. The average elevation is 6 m and 19.7 feet above sea level. A herbarium specimen of the plant (voucher no- KFRI/17703) was deposited at Kerala Forest Research Institute [KFRI], Trichur and the authenticated proof is kept in Annexure II.

4.2 Extract preparation

Fresh roots of *C.gigantea* were obtained after thorough washing with tap water followed by distilled water wash. The roots were shade dried and powdered using mechanical grinder. The powdered root was extracted in methanol. Root powder (10 g) was soaked in 100ml methanol and allowed to stir overnight at a speed of 120 rpm for 24 hrs at room temperature. The mixture was centrifuged at 2000 rpm for 15 min to get a clear supernatant. This procedure was repeated till maximum yield was obtained; supernatant was collected, pooled and evaporated to dryness. The dried extract was collected, weighed and reconstituted in adequate volume of methanol (A. R. Abubakar and Haque 2020).

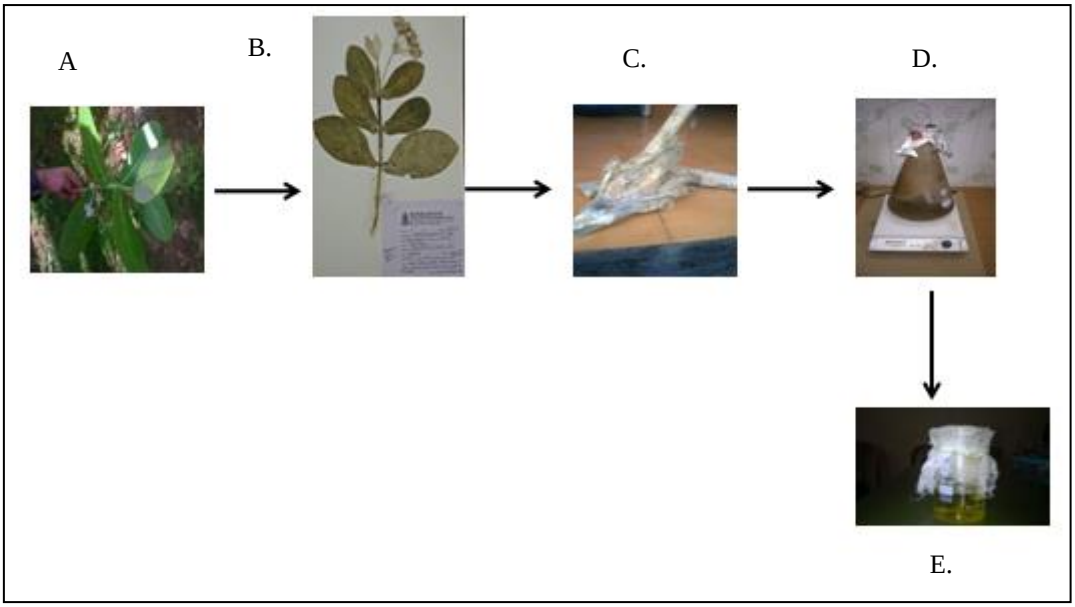


Figure 4.2: (A) Identification, (B) authentication, (C) isolation, (D) extraction and (D) fractionation of *C.gigantea* root extract.

4.3 Extraction of polar and non-polar compounds

C. gigantea root extract (20 mg) was dissolved in a solvent mixture containing hexane: water: methanol in (2:1:2) ratio. The mixture was vigorously mixed to ensure uniform distribution of constituents. Later the mixture was allowed to stand for 10 min to allow separation of polar and non polar constituents into polar and non –polar phases. Two immiscible solvent phases were separated and labeled as polar and non-polar extract respectively (Zhang, Lin, and Ye 2018).

4.4 Phytochemical Screening

The methanolic root extract of *C.gigantea* roots were screened for the presence of specific constituents according to standard procedure.

4.4.1 Tests for alkaloids

About 0.5 g of the extract was mixed and stirred continuously with 5 ml of 1% aqueous HCl on a water bath. The contents were filtered and the obtained filtrate was divided into 3 portions. To the first portion, a few drops of Dragendorffs reagent (Part A: 0.85 g of bismuth subnitrate, 40 ml water, and 10 ml glacial acetic acid, Part B: 8 g KI in 20 ml water) was added and observed for the formation of brownish precipitate. Mayer's reagent (prepared by mixing mercuric chloride (1.36 g) and KI (5 g) in 100 ml water) was added to the second potion and observed for the formation of white precipitate. To the third portion a drop of Wagners reagent (2.5 g iodine dissolved in 12.5 g of potassium iodide in 250 ml water) was added and observed for the formation of brown precipitate (Harborne and Williams 2000).

4.4.2 Tests for flavonoids

4.4.2.1 Sodium hydroxide test

Plant extract (2 ml) was dissolved in 10% aqueous NaOH solution and filtered to give yellow color solution. Diluted HCl was added in drops until the solution turns colorless, indicating the presence of flavonoids.

4.2.2.2 Ferric chloride test

Extract (1 mg/ml) was boiled in water and filtered. 2 drops of freshly prepared $Fecl_3$ solution was added to the filtrate and observed for greenish blue color formation.

4.2.2.3 Sulphuric acid test

The extract was dissolved in concentrated sulphuric acid and was observed for the formation of yellow color.

4.4.3 Tests for phenols

4.4.3.1 Ferric chloride test

1 ml of the extract was diluted in 1 ml of water and 1 ml of $Fecl_3$ and was observed for the formation of blue color.

4.4.3.2 Ammonium hydroxide test

1 ml of the extract was mixed with 1% gelatin solution containing 10% NaOH and observed for the formation of white precipitate.

4.4.3.3 Lead acetate test

10% lead acetate solution was added to the extract and was observed for the formation of white precipitate.

4.4.4 Tests for tannins

4.4.4.1 Potassium hydroxide method

About 4 ml of freshly prepared 10% KOH solution was added to 4 ml of 5% concentrated extract and was observed for the formation of white precipitate.

4.4.4.2 Lead acetate method

To 2 ml of the extract, few drops of 1% lead acetate was added and observed for yellow precipitate.

4.4.4.3 Ferric chloride method

1ml of the extract was diluted with 1ml of water and heated on water bath. The solution was filtered and 4 ml of 5%ferric chloride was added to the filtrate. The solution was mixed gently and was observed for the formation of green precipitate.

4.4.5 Test for phlobatannins

2 ml of the extract was diluted with 2 ml of distilled water and filtered. The filtrate was boiled with 2% HCl and was observed for the formation of red precipitate.

4.4.6 Test for anthraquinones

About 10 ml benzene was added to 2 ml of extract and mixed vigorously. The solution was filtered and 5 ml of 10% ammonia solution was added to the filtrate and was observed for the formation of pink color in the lower ammoniacal layer.

4.4.7 Test for resins

The extract (0.5g) was diluted with 10ml distilled water and shaken vigorously for 3 min. The solution was kept aside for 1 min and was observed for the formation of turbid solution.

4.4.8 Test for coumarins

About 3 ml of 10% NaOH was added to 2 ml of extract and was observed for the formation of yellow color.

4.4.9 Test for emodins

The extract (2 ml) was mixed with a mixture of 2 ml of NH_4OH, 3 ml of benzene and observed for the appearance of red color.

4.4.10 Test for saponins

0.2 g of extract was diluted in 5 ml of distilled water and heated to boil. The tubes were observed for the appearance of frothing.

4.4.11 Test for leucoanthocyanins

About 5 ml of extract was mixed with 5 ml of isoamyl alcohol. The upper layer of the solution was observed for the appearance of red color.

4.4.12 Test for glycosides

Extract was hydrolyzed with dilute HCl for few hours on water bath and the hydrolysate was subjected to the following tests.

4.4.12.1 Legals test

To the hydrolysate 1ml of pyridine and few drops of sodium nitroprusside was added and then made alkaline with sodium hydroxide solution. The solution was observed for color change from pink to red color.

4.4.12.2 Borntragers test

To the hydrolysate, chloroform was added and chloroform layer was separated. To this equal volume of dilute ammonia was added and the ammoniacal layer was observed keenly for the development of pink color.

4.4.13 Test for carbohydrates

4.4.13.1 Molischs test

About 2-3 drops of 1% alcoholic α-naphthol was added to the extract and mixed well. To this, 2ml of conc. H_2SO_4 was added along the sides of the tube. The tube was carefully observed for the appearance of brown ring at the junction of two liquids.

4.4.13.2 Benedicts test

To the extract, Benedict's reagent is added and the tube was observed for color change from blue to brick red color.

14.4.13.3 Fehlings test

To the extract, Fehlings A and Fehling B was added and heated on water bath. The tube was observed for the appearance of red precipitate.

4.4.14 Test for proteins and free amino acids (Sofowora, 1993)

4.4.14.1 Millons test

To the extract millons reagent was added and the tube was observed for the appearance of red color.

4.4.14.2 Biuret test

To the extract equal volume of 5% NaOH and 1% $CuSO_4$ is added. The tube was observed for the appearance of blue color.

14.4.14.3 Ninhydrin test

To the extract ninhydrin reagent is added and the tube was observed for appearance of purple color.

4.4.15 Test for terpenoids

4.4.15.1 Salkowskis test

Acetic anhydride and concentrated H_2SO_4 was added to 2 ml of extract. The tubes were observed for the formation of blue green ring.

14.4.15.2 Sulphuric acid test

Added 5 drops of conc. H_2SO_4 to 1 ml of extract and lower layer was observed for the appearance of yellow color.

4.4.16 Tests for steroids

4.4.16.1 Liebermann Buchard test

About 1 ml acetic anhydride was added to 1 ml chloroform and cooled to 0^0C. Then one drop of conc. H_2SO_4 was added to the cooled mixture followed by the extract. The solution was observed for blue, green, red color that changes with time.

4.4.16.2 Salkowskis test

Acetic anhydride and conc. H_2SO_4 was added to 2ml of extract formation of red color indicates the presence of steroids.

4.5 Total Phenolic Content

Total phenolic content was analyzed using Folin-Ciocalteu colorimetric method with some modifications (Singleton and Rossi 1965) . An aliquot of 0.3 ml of CGMR was mixed with Folin-Ciocalteu phenol reagent (2.25 ml). After 5min, 6% sodium carbonate (2.25 ml) was added and the mixture was allowed to stand at room temperature for 90 min. The absorbance of the mixture was measured at 725 nm in a spectrophotometer (Systronics 119, Madras, India). A Calibration curve for gallic acid in the range 20-80 μg/ml was prepared in the same manner. Results were expressed as mg Gallic Acid Equivalent (GAE) per gram extract.

4.6 Total Flavonoid Content

Total flavonoid content was determined using the aluminium chloride colorimetric method with some modifications (Chang *et al*. 2002). A calibration curve for quercitin in the range 20-80 µg/ml was prepared. CGMR (0.5 ml) and standard (0.5 ml) were placed in separate tubes and 10% aluminium chloride (0.1 ml), 1M potassium acetate (0.1 ml), 80% methanol (1.5 ml) and distilled water (2.8 ml) added and mixed. A blank was prepared in the same manner but 0.5 ml of distilled water was used instead of sample or standard. All the tubes were incubated at room temperature for 30 min and the absorbance was read at 415 nm. The concentration of flavonoid was expressed as mg Quercetin Equivalent (QE) per gram extract.

4.7 *In vitro* PLA2 inhibition assay

Standard lecithin curve – The protocol for standardization of lecithin was obtained from Sigma Aldrich (Bengaluru). Briefly, 2.5 ml mixture consisting of Lecithin or Phosphatidyl choline (PC), 5mM Tris Hcl (pH=8.5), 10mM $CaCl_2$ (0.05 ml), 1.5 percent triton 100x (0.7ml) was prepared to obtain final lecithin concentration of (0.24, 0.37, 0.617, 0.864, 1.11 and 1.48 µmol) respectively. The mixtures were incubated for 5 min at 37ºC. Exactly 20 µl of lecithin was pipetted from each aliquot and mixed with 25% ether in isopropyl alcohol, 14% NaOH and 2M hydroxylamine and incubated at 25^{o}C for 20 min. To this incubated mixture 3N Hcl and 10% $Fecl_3$ added and mixed. The OD was recorded at 570 nm against a blank.

Enzyme assay- Phosphatidyl choline (Lecithin) and Phospholipase A2 (1 mg/ml in deionized water) were procured from Sigma Aldrich, Bengaluru. Lecithin was equilibrated with 0.5 mM Tris Hcl; pH 8.5, 1.5% TritonX 100, and 10 mM $CaCl_2$, at 37^{o}C for 5 min. Equal volume of mixture was taken into two tubes labeled Test (T) and Blank (B). Exactly, 50 µl of PLA2 enzyme was added to T while 50 µl of $CaCl_2$ was added to B. The mixtures were incubated at 37^{o}C for 5 min. Sample was collected at 1 min intervals. Reaction was stopped by addition of

1.5 ml ethanol-ether mixture. Then, 2 M hydroxyl amine and 14% w/v NaOH were added simultaneously for the formation of hydroximic acid derivatives. Mixture was incubated at room temperature for 20 min. FeCl3 (10%w /v) and HCl (3N) were mixed with the incubated reaction mixtures, and the optical density (OD) was measured at 570 nm. The OD was proportional to the hydroxamic acid derivative formed. The enzyme activity was determined by estimating the amount of product formed in 1 min (Aparna *et al*. 2012).

4.8 GC/MS analysis

GC-MS analysis of the methanol extract of *C. gigantea* root was performed at SAIF, IITB facility, using Thermo Scientific Triple Quadrupole GC-MS (Trace 1300 GC, Tsq 8000 triple quadrupole MS) equipped with TG 5 MS (30 m X 0.25 mm, 0.25 µm) column. Helium was used as the carrier gas at a flow rate of 1ml/min. using an injection volume of 1.0 µL. Injector temperature was kept at 250^{o}C and ion source temperature was 230^{o}C. The oven temperature was maintained at 50^{o}C isothermal at 280^{o}C (Safaei-Ghomi *et al*. 2009) .

4.9 LC/MS analysis

LC-MS analysis of the methanol extract of *C. gigantea* root was performed at SAIF, IITB facility, using Hewlett-Packard 1100 chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a quaternary pump and a diode array detector (DAD). The column was coupled with an MSD Ion Trap XCT mass spectrometer (Agilent Technologies, Santa Clara, CA) equipped with an electrospray ionization interface (ESI). Fractions were injected onto a C-18 column (Agilent, 4.6 × 25 cm, 5 µm) at a constant flow rate of 200 µl/min (I. Khan et al. 2017).

4.10 Active site prediction

The active site of the macromolecule was predicted using metapocket (v2.0) online server (Huang 2009). It utilized a multi computational consensus approach by employing 8 different algorithms for active site prediction viz. LIGSITE, PASS, QsiteFinder, SURFNET, Fpocket, GHECOM, ConCavity and POCASA. Based on z-score, the top three pocket sites in each predictor are obtained and clustered based on spatial similarity.

4.11 Preparation of macromolecule and ligands

The crystal structure of PLA2 enzyme of snake venom (*Daboia russelii*- PDBID-3CBI) structure was downloaded from the Protein Data Bank. Heteroatoms were removed in Argus lab tool and energy minimization was done using Swiss PDB Viewer. The phytochemical structures of ligands, naturally occurring in *Calotropis gigantea* root, were downloaded from the PubChem database in SDF format. The structures were checked for torsion count, amide bonds if present were considered non rotatable, non-polar hydrogen were merged, and energy minimization was done using the mmff94 force field (Sastry *et al*. 2013).

4.12 Molecular docking

The 3D structures of ligands (compounds obtained from GC/MS and LC/MS) were docked with *Daboia russelii* snake venom Phospholipase A2 (PDB ID-3CBI) using molecular docking software-Autodock Vina version 4.1, to obtain 9 different binding modes of the ligand with CAII (Ramírez and Caballero 2018). The predicted binding affinity was obtained for each ligand (Lagarde *et al*. 2019). The docked pose having RMSD<2 and least binding affinity (more negative) was included for analyzing intermolecular interaction using PyMOL, Molecular Graphics System, (Version 1.8) Schrödinger, LLC and Protein Ligand Interaction Profiler (PLIP) server (Fährrolfes *et al*. 2017).

4.13 *In vivo* study

4.13.1 Study design: Animal study

4.13.2 Inclusion criteria: Female Swiss albino mice (7-8 weeks old) weighing around 25-30 g were included in all animal experiments pertaining to this study.

4.13.3 Exclusion criteria: Diseased, old age, pregnant and new born mice were excluded from the study.

4.13.4 Ethical approval : All the animal experiments were carried out with prior permission from Institutional Animals Ethics Committee (Ref: IAEC/ ACRC/ 18(2)-P7) and strictly conducted according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by animal welfare division, Government of India.

4.13.5 Source of Data: Laboratory bred Swiss albino mice (25-30 g) were purchased from Small Animal Breeding Station, College of Kerala Veterinary and Animal Science, Thrissur, Kerala. The animals were housed in well ventilated polypropylene cages under standardized conditions (25-30oC temperature, 60-80% relative humidity and 12 hrs of light/dark cycle) and fed with standard feed (Sai Durga Feeds and Foods, Bangalore, India) and water ad libitum. The animals were allowed to acclimatize to laboratory condition for a period of 7 days before the onset of experimental study.

4.14 Procurement of Venom

Daboia russelii venom (200 mg) was procured from Irula Co-operative Society, (Madras Crocodile Bank), Guindy, Chennai. The venom sample was diluted appropriately (25 mg/ml) with 0.9 % NaCl and stored in vacutainer tubes at -20^{o} C for future use.

4.15 Acute toxicity study

The acute toxicity studies on Swiss albino mice were carried out according to guidelines 423 of the Organization for Economic Co-operation and Development (OECD) with a few minor modifications (Yang *et al*. 2019). Twelve female Swiss albino mice, were randomly assigned to control and treatment groups (n=6/ group). Feed was removed 4 hrs prior to drug administration and replaced after 2 hr of administration, but water supply was kept unlimited. Following the period of 4 hr fasting, animals were weighed and test material was administered orally in a single dose using an oral gavage. The treatment group mice received a single oral dose of 2000 mg/kg bodyweight of *CGMR* dissolved in 200 µl of 1% propylene glycol v/v (vehicle). The control group mice received 200 µl of 1% propylene glycol (v/v) as vehicle. Following the administration of *CGMR*, animals were individually observed for any indication of mortality, morbidity or other abnormal changes during the first 30 min, repeatedly for 24 hours, with special attention given during first 4hrs. Animals were then observed twice daily for a 14 day period to assess the toxic impact of the extract like changes in body weight, food intake, water consumption, skin, hair, eye color changes, drowsiness, sedation, diarrhea, tremors, salivation, respiration, and any abnormal behavior. The main purpose of acute toxicity study was to evaluate the short-term toxicity associated with single exposure of test material and help in selection of dosages for sub-acute toxicity study.

4.15.1 Study protocol

Table 4.1 : Acute toxicity study- Experimental groups (n=6 per group)

SL NO	GROUP NAME	TREATMENT (single dose on first day of study)
1	**Control**	200 µl of 1% propylene glycol (v/v) as vehicle
2	**Treated**	2000 mg/kg bodyweight of *CGMR* dissolved in 200 µl of 1% propylene glycol v/v (vehicle).

4.16 Sub acute toxicity

Sub-acute oral toxicity of *CGMR* was evaluated in accordance with Organization of Economic Cooperation and Development (OECD) guideline 407 (Yang *et al*. 2019). Twenty-four female Swiss albino mice (8 weeks old) weighing 25 to 30 g were randomly assigned into four groups of six animals each (n=6). The animals were grouped such that the average body weight variation of each group was within ± 20% of the mean body weight. Group I received 1% propylene glycol (v/v) in distilled water as vehicle. Groups II, III and IV were administered 100, 200, and 400 mg/ kg body weight of *CGMR* (dissolved in 1% propylene glycol v/v) respectively. All doses were administered orally through gavage in a constant volume (200 µl) by varying the extract concentration in 1% propylene glycol (vehicle), once daily for 28 days. During experimental period food intake, water consumption, and changes in body weight were recorded every week. All animals were individually observed twice daily for mortality and morbidity. One week prior to scheduled necropsy (week 4), animals were observed for signs of toxicity such as changes in skin color, eye color, pupil size, salivation, grooming pattern, abnormal gait, posture, repetitive circling. After 28 days surviving animals were fasted overnight, and sacrificed on the next day. Blood was collected directly from heart puncture in both heparin-coated and non-heparin vials for the estimation of hematological parameters and biochemical assays respectively. Different organs (liver, kidney, heart, brain, ovaries) were collected for histolopathological analysis.

4.16.1 Study protocol

Table 4.2: Sub-acute toxicity- Experimental groups (n=6 per group)

GROUP No	GROUP NAME	TREATMENTS (Daily dose)
1	**Control**	1% propylene glycol (v/v) in distilled water as vehicle
2	**Low dose**	100mg/kg b. wt of *CGMR* dissolved in 1% propylene glycol (v/v)
3	**Moderate dose**	200mg/kg b. wt of *CGMR* dissolved in 1% propylene glycol (v/v)
4	**High dose**	400mg/kg b. wt of *CGMR* dissolved in 1% propylene glycol (v/v)

4.16.2 Food intake and water consumption

Sufficient quantities of weighed food pellets were kept in each cage to avoid scarcity. The leftover food pellets were collected from each cage on every fourth day, and weighed. Water level in feeder bottles (300 ml) was measured on every fourth day and refilled to maximum level to provide *ad libitum* water supply. The food intake and water consumed for each week was calculated (Schweisthal, Cole, and Mercer 1982).

4.16.3 Body and organ weight

The weight of each mouse was recorded on the first day and in weekly interval throughout the experimental period and average body weight was calculated (Schweisthal, Cole, and Mercer 1982). Major organs like liver, kidney, heart, brain, and ovary were dissected out and washed thoroughly in ice-cold saline (0.9%) to remove blood contaminants. The weights of the organs were taken and relative organ weight in relation to bodyweight was calculated for each organ.

4.16.4 Hematological parameters

Blood collected through cardiac puncture was taken in a heparin-coated tube was used for the estimation of hematological parameters such as hemoglobin, total RBC, platelet count, WBC count and differential count using fully automated Mindray BC 20s analyzer.

4.16.5 Biochemical parameters

The blood collected through cardiac puncture was kept at room temperature for 30 minutes for clotting and serum was separated by centrifugation at 5000 rpm for 10 minutes for further analysis. Liver function markers Serum Glutamate Oxaloacetate Transaminase-SGOT (IFCC method), Serum Glutamate Pyruvate Transaminase-SGPT (IFCC method), total protein, albumin, and bilirubin were determined using commercially available kits (Agappe diagnostics, India). Kidney function tests like serum creatinine and urea were measured using kits available from Agappe diagnostics.

4.16.6 Histopathology

The liver, kidney, heart, brain, ovaries were dissected out and washed thoroughly in ice-cold saline (0.9%). A small portion of the tissue was fixed with 10% neutral buffered formalin. Sections of paraffin-embedded tissues were taken as thin 4µm and stained with hematoxylin-eosin and observed under light microscope 200X magnification (Knoblaugh, Hohl, and La Perle 2018).

4.17 Determination of LD_{50} of venom

The LD_{50} of *DRSV* was calculated by Reed and Munch method according to WHO guidelines(Meier and Theakston 1986). Briefly female Swiss albino mice weighing 20-25 g each were randomly grouped into 4 groups (n=6). The lyophilized venom sample (100 mg) was dissolved in 4ml of 0.9% NaCl (25 mg/ml). A calculated amount of venom (5, 10, 15, and 20 µg) in increasing dosage was administered intraperitoneal (i.p) to animals of each

group respectively. The cumulative survival/death of the animal was recorded over a period of 24 hr from the time of venom administration.

4.17.1 Study protocol

Table 4.3: Determination of LD_{50} - Experimental groups (n=6 per group)

GROUP NO.	VENOM DOSE (µg)	TREATMENTS (single dose, i.p)
1	**Low dose**	5µg of venom injected i.p
2	**Moderate dose**	10µg of venom injected i.p
3	**High dose**	15µg of venom injected i.p
4	**Very high dose**	20µg of venom injected i.p

4.18 Neutralization of venom

The *in vivo* neutralization of venom was performed according to a standard method as described by Theakston and Reid (Theakston and Reid 1983). Briefly, Swiss albino mice were administered with different doses of CGMR (i.p), immediately after administration of LD_{50} quantity of venom intraperitoneally (i.p). The extracts were prepared in a constant volume of 200 µl propylene glycol, while venom was administered by dissolving LD_{50} quantity in 0.9% NaCl. The positive control group received 0.6 mg of antivenom (Bharat Serums and Vaccines limited, Ambernath), while the vehicle group received 200 µl of propylene glycol. The *in vitro* neutralization of venom was performed by administering (i.p) pre-incubated mixture of venom and extract at 37^{o}C. In both methods the 24 hour survivability of the animal was considered as the endpoint.

4.18.1 Study protocol

Table 4.4 : *In vivo* neutralization study - experimental groups (n=6 per group)

SL NO.	GROUPS	TREATMENTS (i.p)
1	**Control**	No treatment
2	**Vehicle**	Saline + 1% propylene glycol
3	**Venom**	Venom LD_{50}
4	**Low dose**	Venom LD_{50}+100mg/kg b.wt of *CGMR*
5	**Moderate dose**	Venom LD_{50}+200mg/kg b.wt of *CGMR*
6	**High dose**	Venom LD_{50}+400mg/kg b.wt of *CGMR*
7	**Antivenom (positive control)**	Venom LD_{50}+ Antivenom

4.18.2 Study protocol

Table 4.5 : *In vitro* neutralization study - experimental groups (n=6 per group)

SL NO.	GROUPS	TREATMENTS (i.p)
1	**Control**	No treatment
2	**Vehicle**	Saline + 1% propylene glycol
3	**Venom**	Venom LD_{50}
4	**Low dose**	Venom LD_{50} + 200mg/kg b.wt of CGMR (pre-incubated)
5	**High dose**	Venom LD_{50} + 400mg/kg b.wt of CGMR (pre-incubated)
6	**Antivenom**	Venom LD_{50}+Antivenom (pre-incubated)

4.19 Prophylactic effect of the extract

Swiss albino mice were grouped into 5 groups of 6 animals each. Group 1 (control) consisted of mice not administered with CGMR and not treated with venom. Group 2 (vehicle control) consisted of mice administered with 1% propylene glycol. Group 3 (Venom group) consisted of mice injected with 0.9% NaCl (i.p), each day, for a period of 14 days and then challenged with LD_{50} dose of DRSV on final day. Group 4 (CGMR-DRSV low dose group) consisted of mice injected with 100 mg/kg b.wt of CGMR (i.p), each day, for a period of 14 days and then challenged with LD_{50} dose of DRSV on final day. Group 4 (CGMR-DRSV high dose group) consisted of mice injected with 200 mg/kg b.wt of CGMR (i.p), each day, for a period of 14 days and then challenged with LD50 dose of DRSV on final day (Fung *et al*. 2009).

4.19.1 Study protocol

Table 4.6: Prophylactic study - experimental groups (n=6 per group)

SL NO.	GROUPS	TREATMENTS (i.p)
1	**Control**	No treatment
2	**Vehicle**	Saline + 1% propylene glycol (10 days)
3	**Venom**	Saline administered (10 days) + LD_{50} venom injected on final day
4	**Low dose**	200mg/kg b.wt of *CGMR* (10days) + LD_{50} venom injected on final day
5	**High dose**	400mg/kg b.wt of *CGMR* (10 days) + LD_{50} venom injected on final day

4.19.2 Clotting time test

Mice were anesthetized using chloroform and stabilized on a horizontal platform. The tail was pulled into a fixed size opening set as a template. About 2cm of terminal tail edge was cut along the surface of template using a surgical blade no. 21. The procedure ensures uniform cut in all experimental animals. The first drop of blood was cleaned away using

surgical spirit and the second drop was taken into capillary tube for assessing the clotting time. The time was recorded during the start of the experiment and during each 1min interval of capillary break (Parasuraman, Raveendran, and Kesavan 2010). The formation was fibrin threads were observed following each break of capillary tube and corresponding time was noted. The experiment was concluded after the appearance of the first fibrin thread.

4.19.3 Bleeding time test

In the same experimental set up the blood obtained after incision was applied on Whatman filter paper No.1 (EISCO, Ambala, India) every 30 sec, without disturbing the clot. The experiment was concluded until complete cessation of bleeding through hemostasis. The terminal edge of the tail was sterilized using surgical spirit at the end of the experiment (Kung *et al*. 1998).

4.19.4 Histopathological analysis- Time based study

Table 4.7 : Time based study- Experimental groups (n=6)

	TREATMENT
Group I	LD_{50} dose of venom injected and sacrificed immediately
Group II	Pretreated with 100mg/kg body weight of *CGMR* (i.p) for 10 days + LD_{50} of venom injected and sacrificed immediately
Group III	LD_{50} dose of venom injected and sacrificed at the end of 12 hrs
Group IV	Pretreated with 100mg/kg body weight of *CGMR* (i.p) for 10 days + LD_{50} of venom injected and sacrificed at the end of 12 hrs

The liver, kidney, heart, brain, ovaries were dissected out and washed thoroughly in ice-cold saline (0.9%). A small portion of the tissue was fixed with 10% neutral buffered formalin. Sections of paraffin-embedded tissues were taken as thin 4µm and stained with hematoxylin-eosin and observed under light microscope 40x magnification.

4.20 Statistical analysis

(1) The values are expressed as mean ± SD. The statistical significance was compared between untreated animals and experimental groups by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests (For food intake, water consumption, body weight, and organ weight analysis of subacute toxicity study) using GraphPad instat 3 software (GraphPad Software, inc. La Jolla, USA).

(2) The values are expressed as mean ± SD. The statistical significance was compared between experimental groups by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests (For total phenol content, total flavonoid content and PLA2 inhibition study) using GraphPad instat 3 software (GraphPad Software, inc. La Jolla, USA).

(3) For *in vivo* study- The mortality rate of each treatment group was calculated and the percent survival was calculated. Probit analysis was performed to arrive at a statistically significant value.

CHAPTER 5

RESULTS

5.1 Extraction of *CGMR*

The percentage yield of crude extract, MF (methanol fraction) and HF (Hexane fraction) was 2.823g, 1.28g, and 0.91g respectively. The percentage yield of extract was calculated using the following formula.

% yield= residue weight (g) / initial weight of the plant material (g)

5.2 Phytochemical screening

Table 5.1: Preliminary phytochemical screening of *CGMR*

Sl. No	Test	Result
1	Alkaloids	Present
2	Flavonoids	Present
3	Phenols	Present
4	Tannins	Present
5	Phlobotannins	Absent
6	Anthraquinones	Present
7	Resins	Absent
8	Coumarins	Present
9	Emodins	Absent
10	Saponins	Present
11	Leucoanthocyanins	Absent
12	Glycosides	Absent
13	Carbohydrates	Absent
14	Protein and free amino acids	Present
15	Terpenoid	Absent

Table 5.1 shows the qualitative analysis of phytochemical present in *CGMR*. The results indicate the presence of diverse pharmacologically important compounds.

5.3 Total phenol and flavonoid content

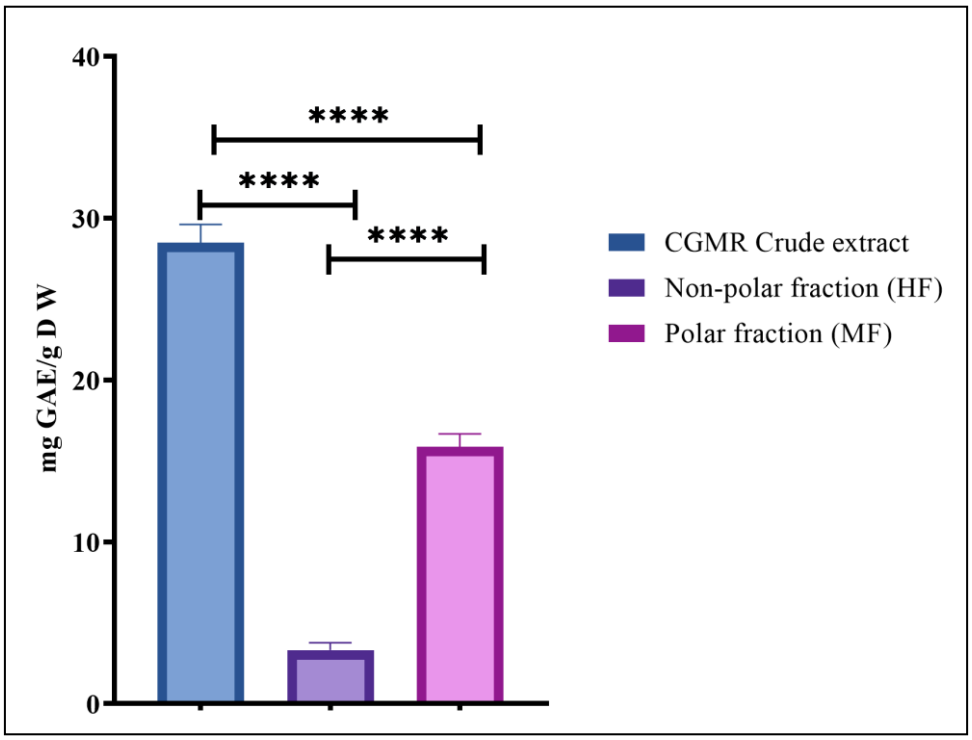


Figure 5.1A : Determination of total phenols content (TPC) in crude extract, methanol fraction (MF) and hexane fraction (HF). TPC expressed as -mg GAE/g DW= mg of gallic acid equivalents per gm dry weight of root extract. (Statistical comparison: Tukey-Kramer multiple comparison; ****P<.00001; ***$P < .001$;**$P < .01$; *$P < .05$ compared to all groups).

The total phenol content (TPC) in crude *CGMR*, HF and MF was estimated as 28.72±1.343, 3.52±1.012, and 15.92±0.9126 mg GAE/g DW respectively. **Figure 5.1A** shows higher total phenol content in CGMR crude extract, when compared to HF (*P=.0001*) and MF (*P=.0001*) fractions. In the polar methanolic fraction (MF), the total phenols were determined to be higher than non-polar hexane fraction (HF) (*P=.0001*). The hexane fraction contained least amount of total phenol.

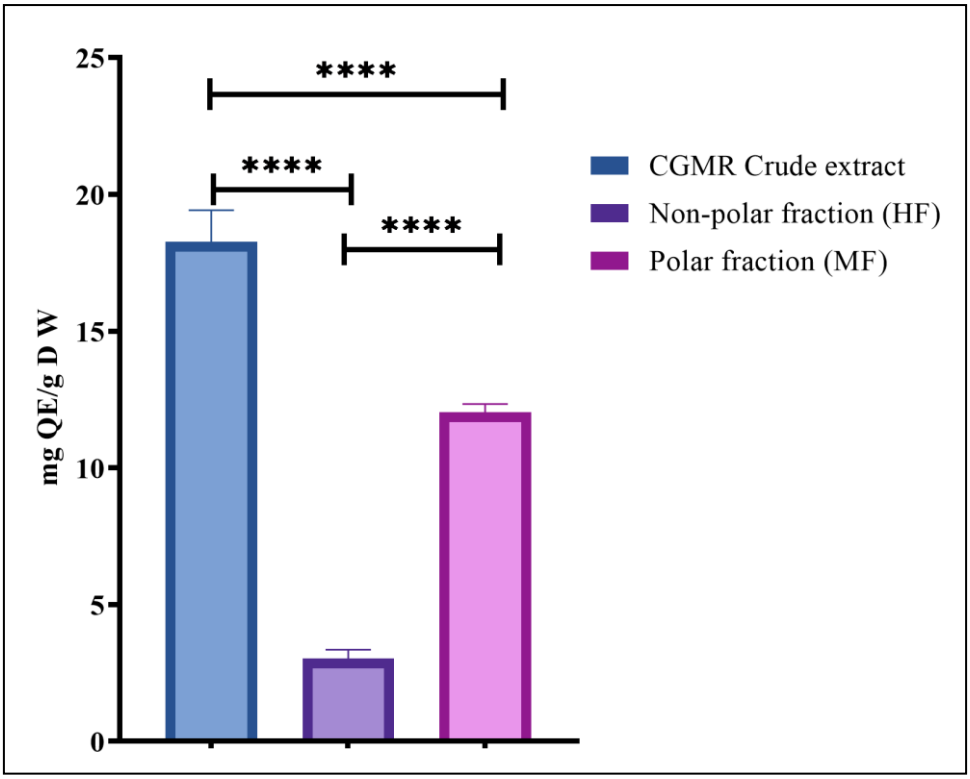


Figure 5.1B: Determination of total flavonoids content (TFC) in crude extract, methanol fraction (MF) and hexane fraction (HF). TFC expressed as- mg Quercetin equivalent/ g DW= mg of quercetin equivalent per gm dry weight of root extract. (Statistical comparison: Tukey-Kramer multiple comparison; ****$P<.00001$; ***$P<.001$;**$P<.01$; *$P<.05$ compared to all groups).

The total flavonoid content (TFC) in crude CGMR, HF and MF was estimated as 18.22±1.213, 3.12±1.242, and 12.92±0.826 mg Quercetin equivalent/ g DW respectively. **Figure 5.1B** shows higher TFC in CGMR crude extract, when compared to HF ($P=.0001$) and MF ($P=.0001$) fractions. In the polar methanolic fraction (MF), the TFC were determined to be higher than non-polar hexane fraction (HF) ($P=.0001$). The hexane fraction contained least amount of total phenol.

5.4 *In vitro* PLA2 assay

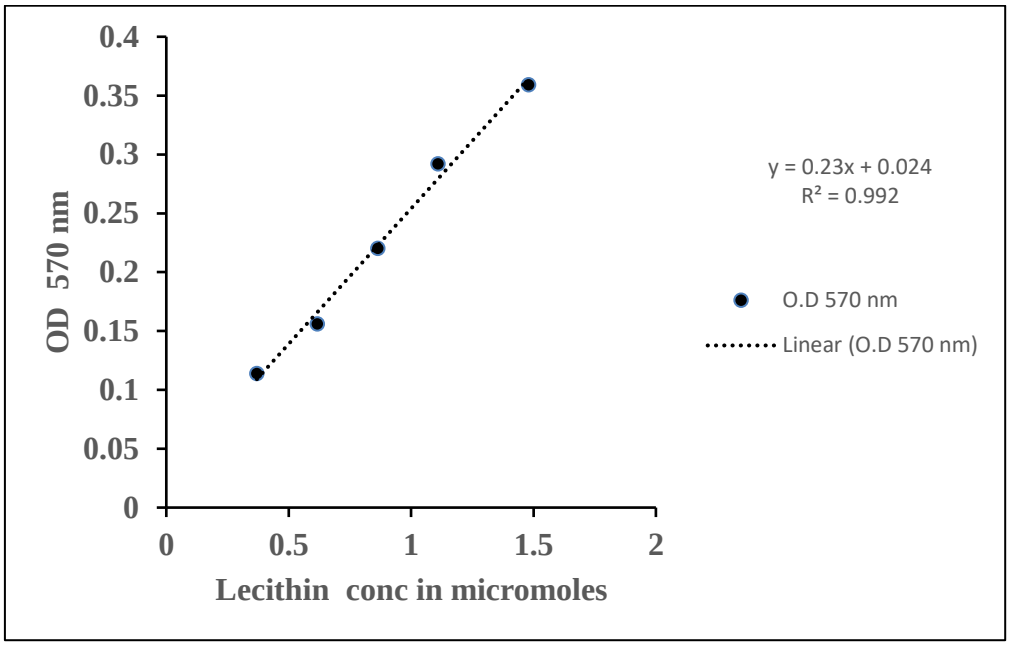


Figure 5.2A : Standard curve of Lecithin- X axis = micromoles of Lecithin (Phosphatidyl choline) ; Yaxis= absorbance at 570 nm.

Figure 5.2A shows the standard curve obtained for lecithin in increasing concentrations 0.24, 0.37, 0.617, 0.864, 1.11, and 1.48 µmol. The O.D measured at 570 nm was proportional the hydroxamic acid derivative formed in the reaction mixture. An R^2 value of 0.992 indicates that the data points fits well with the regression line. Using the standard curve further enzyme experiments was performed. Firstly, the activity of PLA2 enzyme was determined by checking the amount of hydroxamic derivative formed per minute (uninhibited reaction). The activity of the enzyme and was found to be 686.246 units/mg of enzyme. Later the PLA2 enzyme concentration (0.05 µl) and lecithin substrate concentration was kept constant all through the experiment, while extract was taken in increasing concentration to determine the enzyme activity of inhibited reaction. The difference between the activity of PLA2 enzyme in uninhibited and inhibited reaction was calculated to estimate the percentage inhibition of the extract.

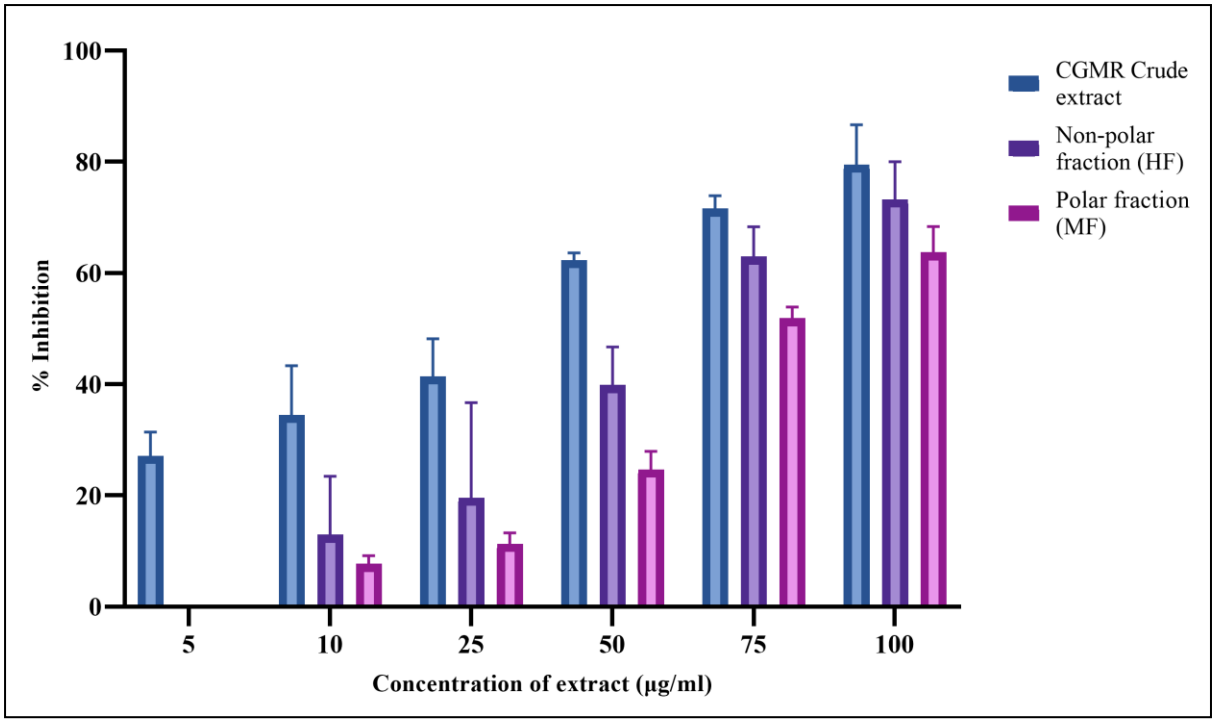


Figure 5.2B: Inhibition of PLA2 activity- Comparison of percentage inhibition of *CGMR* crude extract, non-polar (HF) and polar (MF) fractions (µg/ml). (X axis- % inhibition, Y axis- Concentration of extract in µg/ml)

The data from **Figure 5.2B** indicates that crude *CGMR* significantly inhibited PLA2 activity. The non-polar fraction and polar fraction show activity at low concentration (5µg/ml). The non-polar and polar fractions were obtained by dissolving the crude extract in hexane: water: methanol mixture (2:1:2). The phyto-chemicals were separated based on polarity. PLA2 activity was ascertained against hexane fraction (HF) containing mostly non-polar phyto-chemicals and against methanol fraction (MF) containing mostly polar constituents.

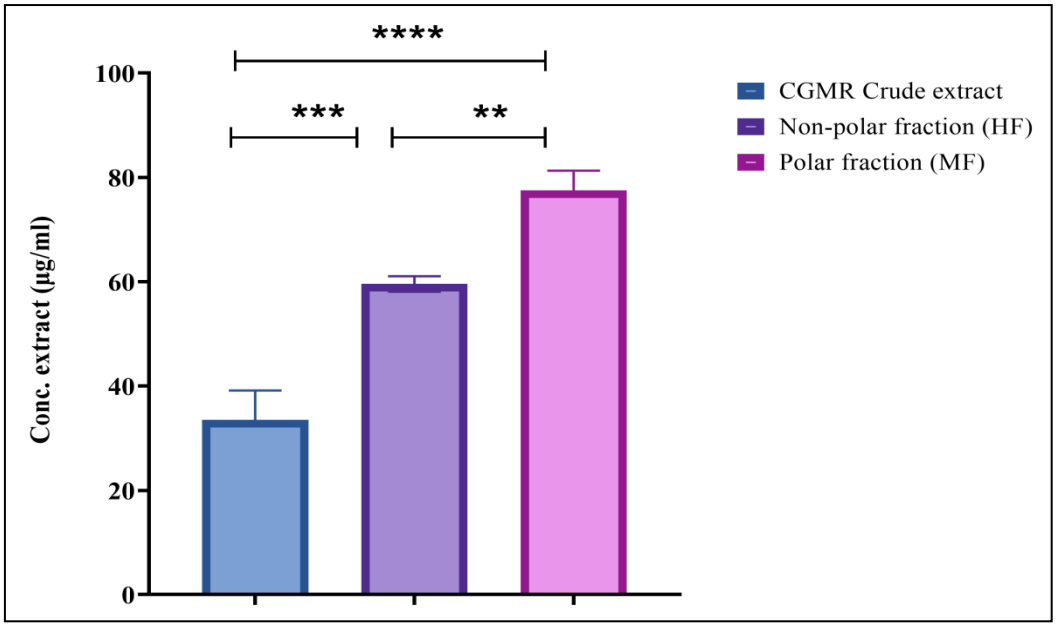


Figure 5.2C: Inhibition of PLA2 activity- Comparison of IC_{50} values of *CGMR* crude extract, non-polar (HF) and polar (MF) fractions (µg/ml). (Statistical comparison: Tukey-Kramer multiple comparison; ****$P<.00001$; ***$P<.001$;**$P<.01$; *$P<.05$ compared to all groups).

The data from **Figure 5.2C** shows that crude extract has highest inhibition against PLA2 activity (IC_{50}=33.531±5.63 µg/ml) compared to non-polar ($P=.0005$) and polar fractions ($P=.0001$). The non-polar fraction inhibited PLA2 activity (IC_{50}=59.586±1.491µg/ml) significantly compared to polar fraction (IC_{50}=77.505±3.772µg/ml) ($P=.0037$).

This result suggests that the phenols and flavonoids present abundantly in polar fraction have insignificant role in PLA2 inhibition. While the phenol and flavonoid deficient non-polar fraction has a distinct phytochemical profile which may also include PLA2 inhibitors. In the past several studies have contemplated the inhibitory role of polyphenols and flavonoids. However the phenolic and flavonoid composition of *CGMR* is distinct and doesn't contain any significant PLA2 inhibitory phenols or flavonoids. A more detail account of this result is discussed under the discussion **section 6.2**.

5.5 MS analysis

5.5.1 GC/MS analysis

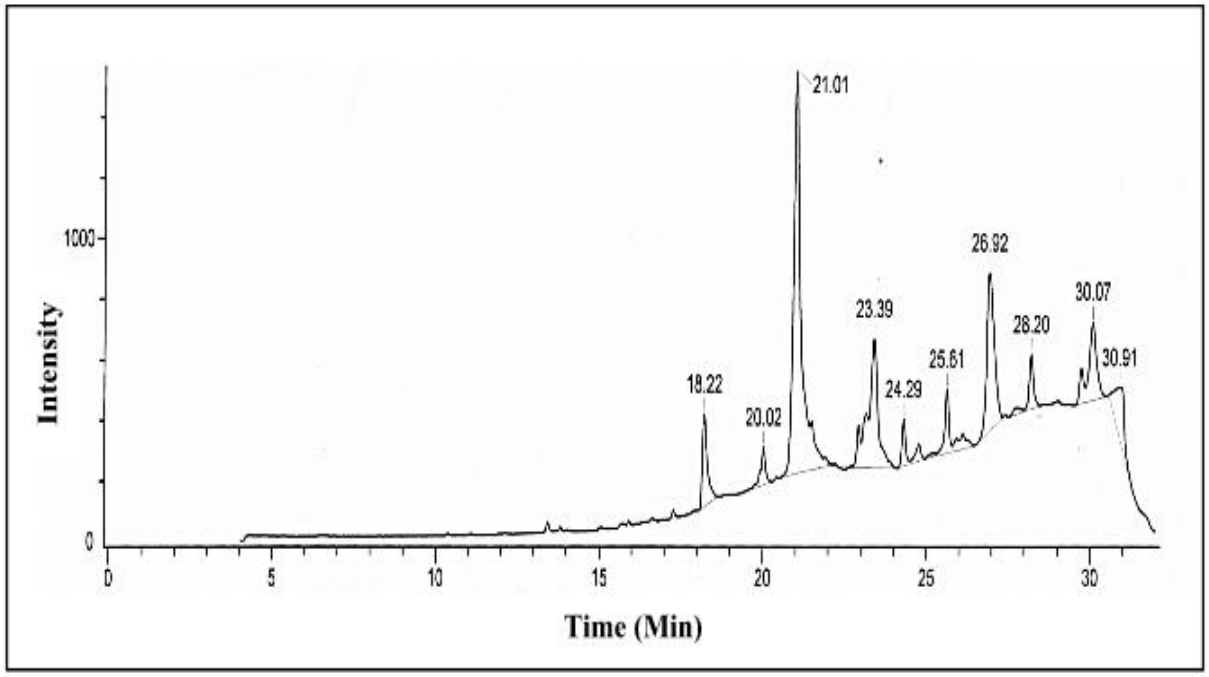


Figure 5.3 : Gas chromatogram of CGMR. X-axis= time; Y axis =intensity, each peak is labeled with peak time.

The methanolic root extract of *CGMR* when subjected to gas chromatography (**Figure 5.3**), documented the presence of pharmacologically important compounds (**Table 5.2**) like ar-tumerone (Relative Area=0.28%), n-hexadecanoic acid (4.37%), oleic acid (1.72%) and allyl octadecyl ester oxalic acid (2.2 %). 9, 12 Octadecadienoic acid (29.38%) or linoleic acid (18:2) was noted to be the most abundant bioactive compound in the extract. The structures of the compounds are shown in **Figure 5.4**. Among these compounds the antivenom activity of ar-tumerone, n-hexadecanoic acid, oleic acid and 9, 12 Octadecadienoic acid has been previously reported (**Table 5.3**).

Table 5.2 : List of compounds identified in *CGMR* extract through GCMS.

Sl NO.	Compound name	Rt (m)	Peak time	Area (%)	Mol. Wt	Chemical formula
1.	Ar-Tumerone	13.61	13.44	0.28	216	$C_{15}H_{20}O$
2.	n-Hexadecanoic acid	18.58	18.22	4.37	256	$C_{16}H_{32}O_2$
3.	Oleic acid	20.21	20.02	1.72	282	$C_{18}H_{34}O_2$
4.	9,12 Octadecadienoic acid	21.42	21.01	29.38	280	$C_{18}H_{32}O_2$
5.	Oxirane, tetradecyl	21.85	21.48	3.71	240	$C_{16}H_{32}O$
6.	Z-10 Pentadecen-1-ol	22.47	21.92	1.15	226	$C_{15}H_{30}O$
7.	2-Piperidinone	23.01	22.93	16.71	233	$C_9H_{16}BrNO$
8.	Sulfurous Acid, Octadecyl pentyl ester	23.96	23.39	9.41	404	$C_{23}H_{48}O_3S$
9.	Oxalic acid , allyl octadecyl ester	25.78	25.61	2.22	382	$C_{23}H_{42}O_4$
10.	1-Decanol, 2-Hexyl	27.29	26.92	12.1	242	$C_{16}H_{34}O$

The peak area, molecular formulae and molecular weight of the compounds were matched with a repository of compounds present in the library of National Institute of Standards and Technology [NIST] for identification purpose. The fragmentation pattern of few selected compounds is shown in **Figure 5.5**.

Table 5.3 : Antivenom activity of compounds that are previously reported

Sl No.	Compound name	Antivenom activity
1.	Ar-Tumerone	neutralizes hemorrhagic activity of *Bothrops jararaca* venom (L. A. F. Ferreira et al. 1992)
2.	n-Hexadecanoic acid	Competitive inhibitor of *Apis Mellifera* venom PLA2 (Aparna *et al*. 2012)
3.	Oleic acid	Inhibition of L- amino acid oxidase, ATPase inhibitor (Sivaraman *et al*. 2020)
4.	9,12 Octadecadienoic acid	Neutralization of Deinagkistrodon acutus venom (Xiong *et al*. 2018) (Subasri *et al*. 2016)

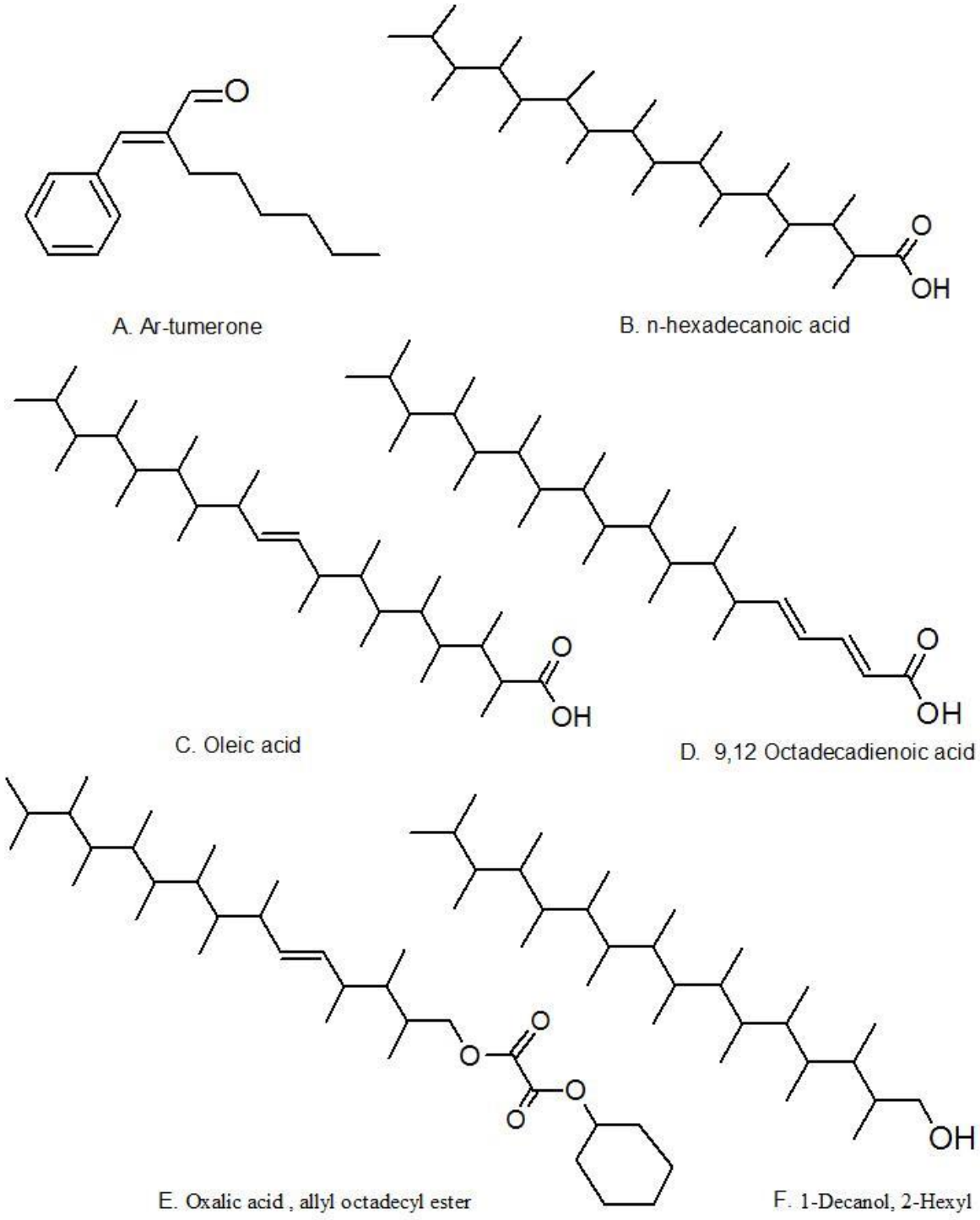


Figure 5.4 : Chemical structures of few selected compounds obtained in GC/MS analysis. (A) Ar-tumerone, (B) n-hexadecanoic acid, (C) Oleic acid, (D) 9,12 Octadecadienoic acid, (E) Oxalic acid, allyl octadecyl ester, (F) 1-Decanol,2-hexyl

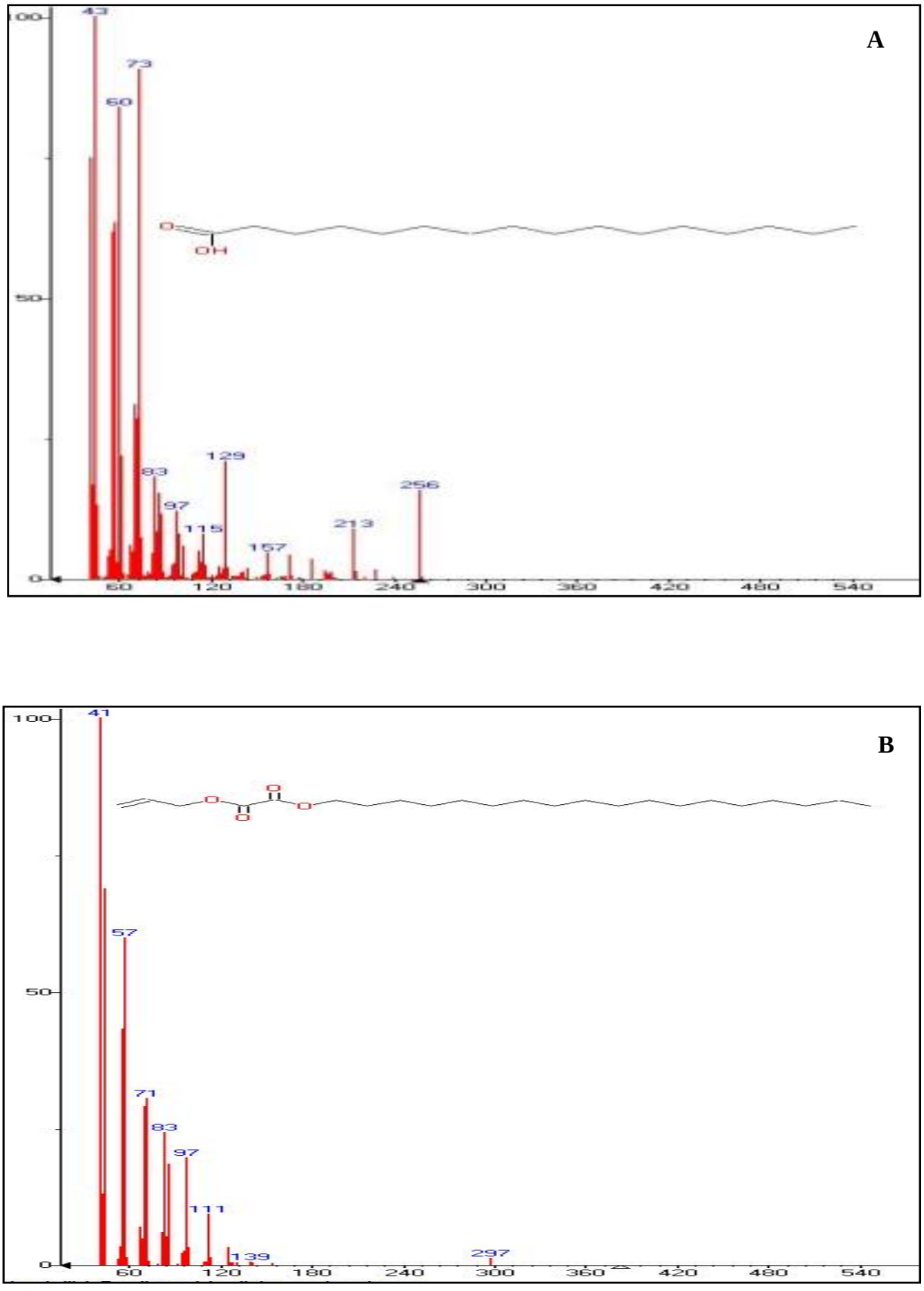


Figure 5.5 : (A) Fragmentation pattern of n-hexadecanoic acid and (B) Oxalic acid , allyl octadecyl ester Key- Y axis = abundance, X axis = m/z

Figure 5.5A and **B** shows the fragmentation patterns of n-hexadecanoic acid and allyl octadecyl ester oxalic acid. After the separation of compound from the gas chromatography column the mass spectrometer detector captures the positively charged molecules and converts it into a signal that is read out as bars in the graph. In the graph abundance is plotted against mass by charge ratio and hence it also measures mass of the compound.

5.5.2 LC/MS analysis

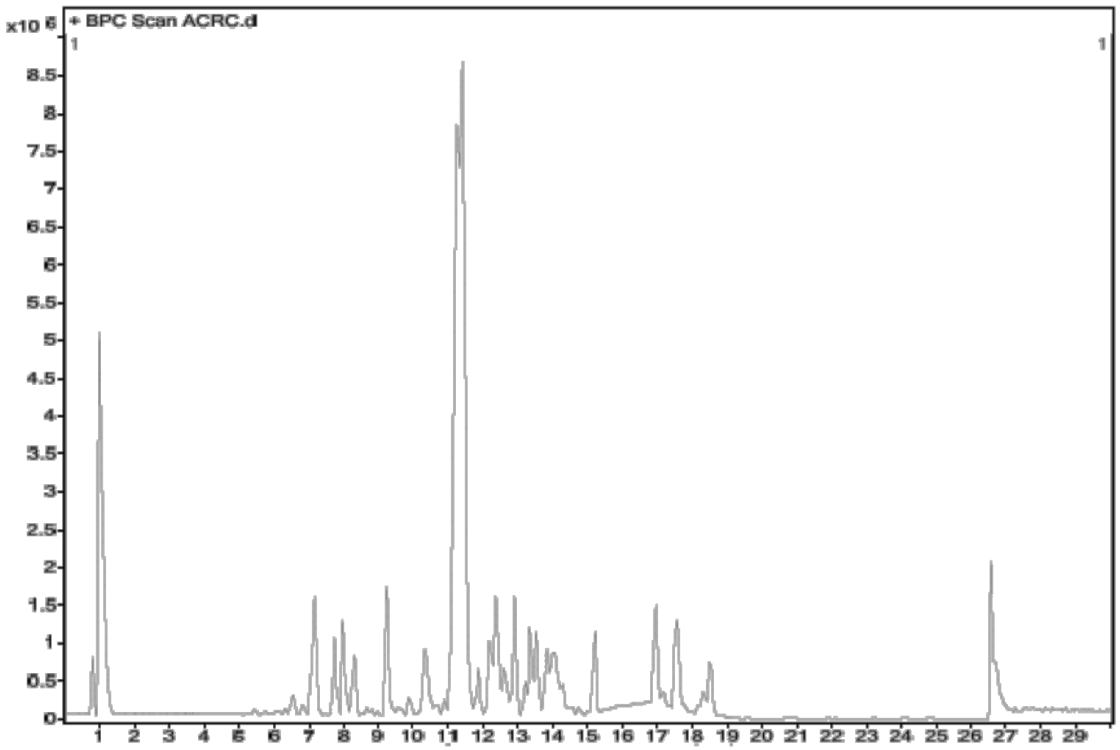


Figure 5.6 : Chromatogram (LC/MS) of *CGMR*; X axis = time, Y axis = intensity

The LC/MS analysis of *CGMR* (**Figure 5.6**) revealed the presence of 40 diverse compounds in the extract (**Table 5.4**). Many of these compounds were identified to possess important pharmacological actions. Anti-inflammatory compounds like cosmosiin, fluoxymesterone, and capsaicin supports the anti-inflammatory effect exhibited by the extract (Mandal 2017). Presence of antioxidant compounds like hemmatomic acid, fraxidin methyl ether and piperine substantiates the free radical scavenging property of the extract. Few of these compounds like

piperine, resrpine and cosmosiin have been shown to exhibit antivenom property (**Table 5.5**). But none of the compounds docked efficiently with PLA2 macromolecule, indicating that their antivenom action is by other mechanism.

Table 5.4 : List of compounds identified in *CGMR* extract through LC/MS.

SL No	Compound name	RT (m)	Mol Wt (g)	Chemical Formula
1	Lactulose	1.004	342	$C_{12}H_{22}O_{11}$
2	D-Pantetheine 4'-phosphate	1.004	358	$C_{11}H_{23}N_2O_7P$
3	Triparanol	1.038	143	$C_7H_{13}NO_2$
4	isoamyl nitrite	1.047	117	$C_5H_{11}NO_2$
5	Scopoline	1.077	155	$C_8H_{13}NO_2$
6	Cosmosiin	7.06	432	$C_{21}H_{20}O_{10}$
7	Phenylpyruvic acid	7.179	164	$C_9H_8O_3$
8	Fluoxymesterone	7.741	336	$C_{20}H_{29}FO_3$
9	Desoxycorticosterone acetate	7.741	372	$C_{23}H_{32}O_4$
10	Etretinate	7.743	354	$C_{23}H_{30}O_3$
11	9a-Fluoro-Bhydroxyandrosterone	7.745	324	$C_{19}H_{29}FO_3$
12	Haematommic Acid, Ethyl Ester	7.776	224	$C_{11}H_{12}O_5$
13	Betaxolol	7.988	307	$C_{18}H_{29}NO_3$
14	Capsaicin	8.08	305	$C_{18}H_{27}NO_3$
15	Fraxidin Methyl Ether	8.311	236	$C_{12}H_{12}O_5$
16	Isopimpinellin	9.899	246	$C_{13}H_{10}O_5$
17	Hydroxyprogesterone	10.331	330	$C_{21}H_{30}O_3$
18	Normorphine	10.401	271	$C_{16}H_{17}NO_3$
19	Galanthamine	11.136	287	$C_{17}H_{21}NO_3$
20	8S-hydroxy-2-Decene-4,6-diynoic acid	11.34	178	$C_{10}H_{10}O_3$
21	Norcodeine	11.349	285	$C_{17}H_{19}NO_3$
22	Piperine	11.661	285	$C_{17}H_{19}NO_3$
23	12-epi-LTB4	11.876	336	$C_{20}H_{32}O_4$
24	10,12,15-octadecatrienoic acid	11.877	278	$C_{18}H_{30}O_2$
25	Methyl Reserpate	11.88	414	$C_{23}H_{30}N_2O_5$
26	Terbinafine metabolite	12.188	313	$C_{19}H_{23}NO_3$
27	2-Methoxyxanthone	12.332	226	$C_{14}H_{10}O_3$
28	Nalorphine	12.371	311	$C_{19}H_{21}NO_3$
29	Pentazocine trans acid	12.594	315	$C_{19}H_{25}NO_3$
30	Capsaicin	12.785	305	$C_{18}H_{27}NO_3$
31	N-Desethyloxybutynin	13.196	329	$C_{20}H_{27}NO_3$
32	His- Ala- Ile	13.233	339	$C_{15}H_{25}N_5O_4$

33	3alpha-Hydroxy-4,4-Bisnor-8,11,13-Podocarpatriene	13.522	216	C_{15} H_{20} O
34	Estradiol Diacetate	13.531	356	C_{22} H_{28} O_4
35	p-Hydroxypropoxyphene	13.985	355	C_{22} H_{29} N O_3
36	Dibucaine	14.025	343	C_{20} H_{29} N_3 O_2
37	Nafronyl	15.216	383	C_{24} H_{33} N O_3
38	Reserpine	16.901	608	C_{33} H_{40} N_2 O_9
39	Oleamide	16.936	281	C_{18} H_{35} N O
40	Khayanthone	17.177	570	C_{32} H_{42} O_9

Table 5.5 : Antivenom activity of compounds that are previously reported.

Sl no	Compound name	Antivenom activity reported
1	Cosmosiin	Antiplatelet aggregation activity (Chaves *et al*. 2011)
2	Capsaicin	Reduction of hemorrhage induced by *Bothrops jararaca* venom (Gonçalves and Mariano 2000)
3	Piperine	Inhibition of lethality, necrosis, defibrinogenation and hemorrhage induced by Russells viper venom (Shenoy *et al*. 2013)
4	Methyl Reserpate	Traditionally used for venom neutralization (Boopathi 2019)
5	Dibucaine	Inhibits PLA2 activity at higher concentration (Scherphof and Westenberg 1975)
6	Reserpine	Traditionally used for venom neutralization (Boopathi 2019)

5.6 Active site determination

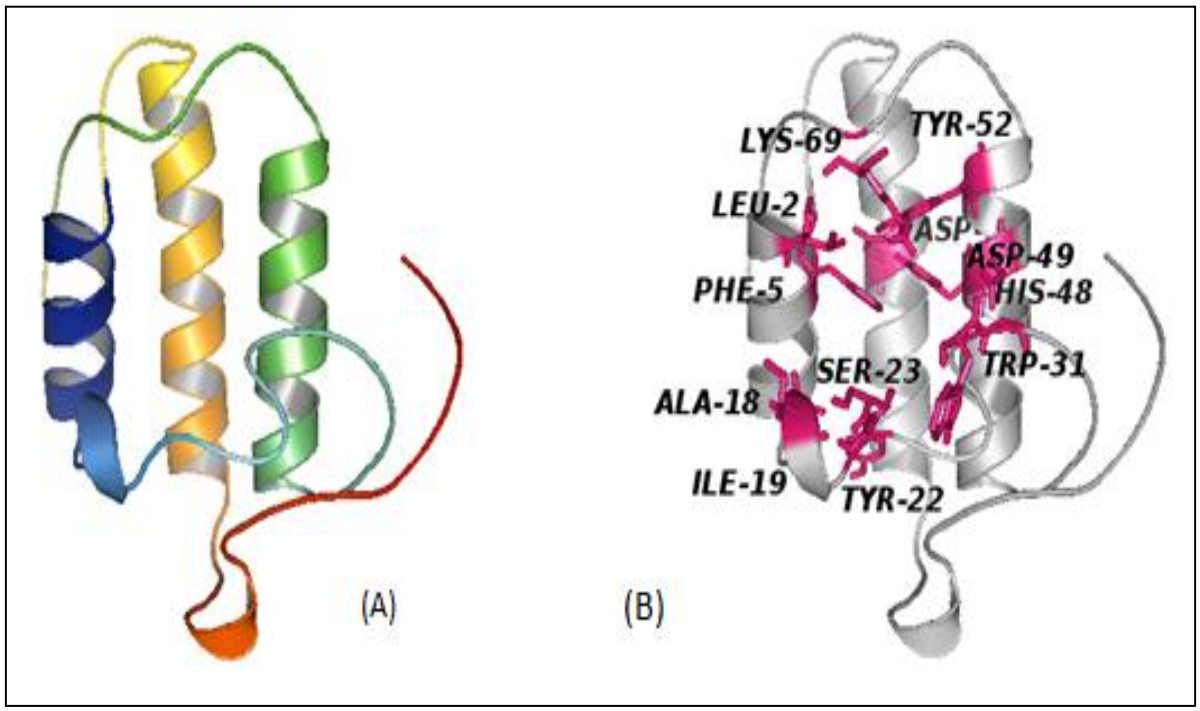


Figure 5.7 : (A) Depiction of active site groove in *Daboia russelii* snake venom Phospholipase A2 (svPLA2) (PDBID- 3CBI), (A chain). (B) The active site residues are depicted in magenta.

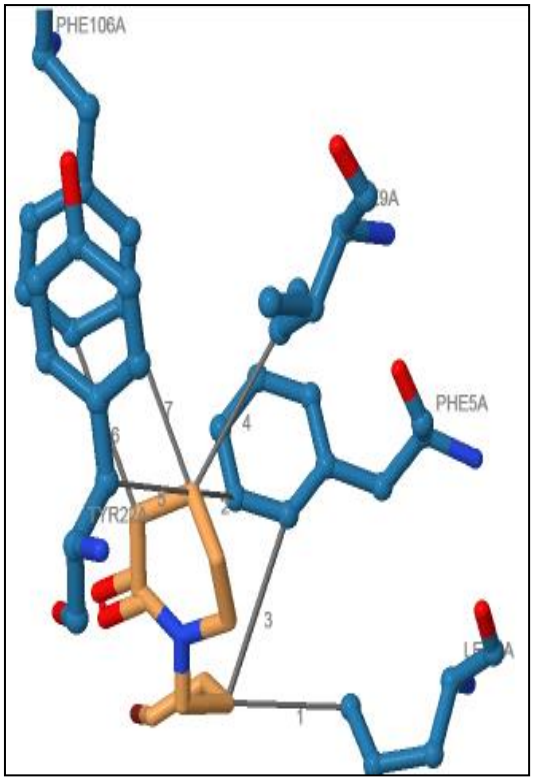
Phospholipase A2 [PLA2] in this inset contains only the A chain (121 amino acids) and the heteroatom ajmaline is removed. The **Figure 5.7** shows the active site groove of PLA2 enzyme. The metapocket webserver (v2.0) predicted the active site residues using six different programs and provided a consensus sequence of ligand binding residues. *Leu2, Gly30, His48, Ile19, Trp31, Asp99, Lys69, Tyr52, Ser23, Tyr22, Asp49, Phe5, Ala18* residues formed the active site of the enzyme. His48 and Asp49 are involved in catalysis of the enzyme. Trp31 is present at the entrance of the active site (Deka et al. 2017).

5.7 Docking

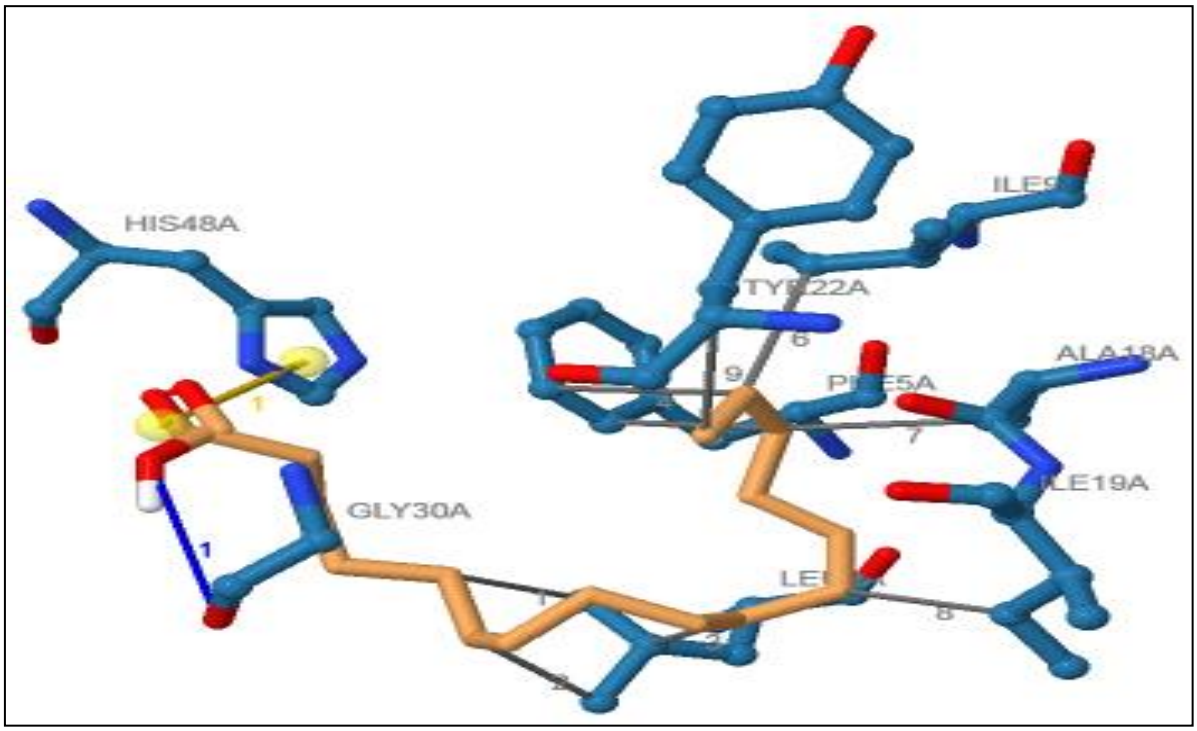
Table 5.6 : Interaction of ligands with Active Site Residues of PLA2 enzyme.

												Others		
Compound name	Leu 2	Phe 5	Ala 18	Ile 19	Tyr 22	Gly 30	His 48	Asp 49	Tyr 52	Lys 69	Asp 99			
Arachidonic acid	****	**	*				H	H				Ile 9*	Phe 106*	
Ar-Tumerone	**	*	*	*	*							Ile9 *		
n-Hexadecanoic acid	***	**	*	*	*	H	H					Ile9 *		
Oleic acid	***	*	*		*		H		*			Ile9 *	Asp 28 H	
9,12 Octadecadienoic acid	**	**			*		H					Tyr 28 H	Phe 106 *	
Oxirane, tetradecyl	***	**	*	*	*							Ile9 *		
2-Piperidinone	*	**			*							Ile9 *	Phe106 **	
Sulfurous Acid, Octadecyl pentyl ester	****	**	*	*	*							Ile9 *	Leu10 *	Leu17 **
Oxalic acid , allyl octadecyl ester	***	**	*	*			HH					Lys 7 *	Phe106 **	
1-Decanol, 2-Hexyl	**	***	*		*	H						Ile9 *	Phe106 *	

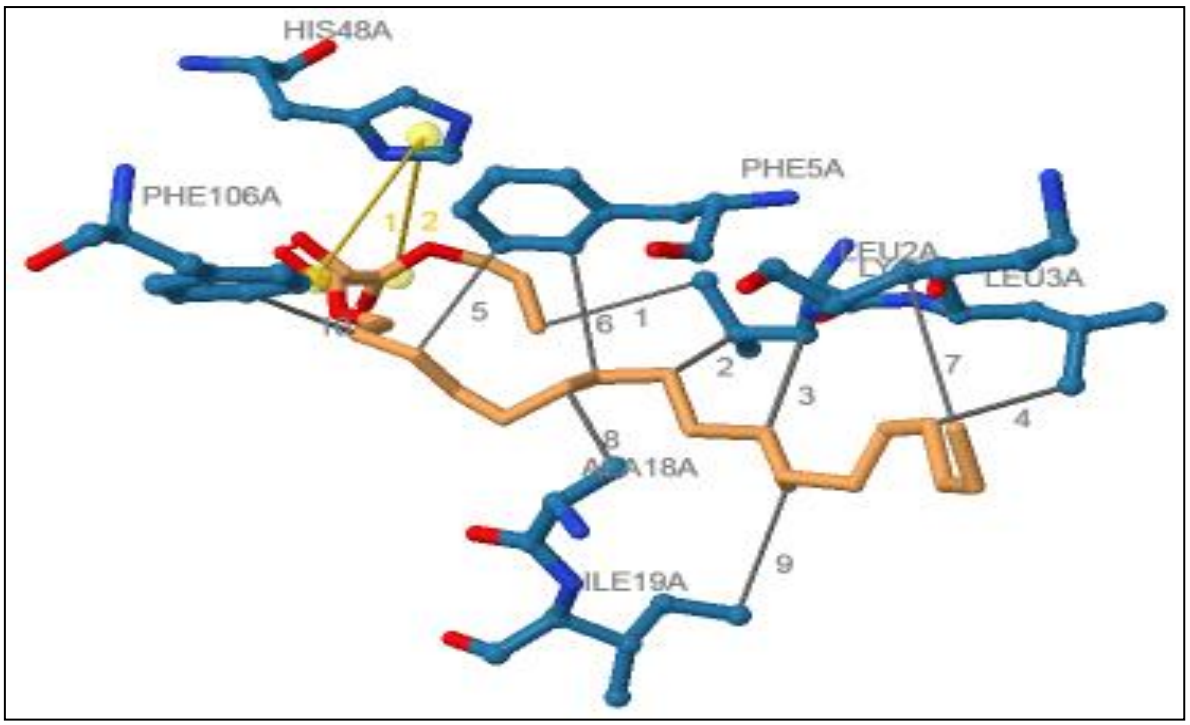
Table 5.6 : n-Hexadecanoic acid, Oleic acid, 9, 12 Octadecadienoic acid and allyl octadecyl ester oxalic acid interact with catalytic residue His48. Compounds also interacted with amino acid residues which are not present within the active site of PLA2 enzyme. Those interactions are detailed in right panel (others). Key: H=Hydrogen Bond, *=Hydrophobic Interaction.



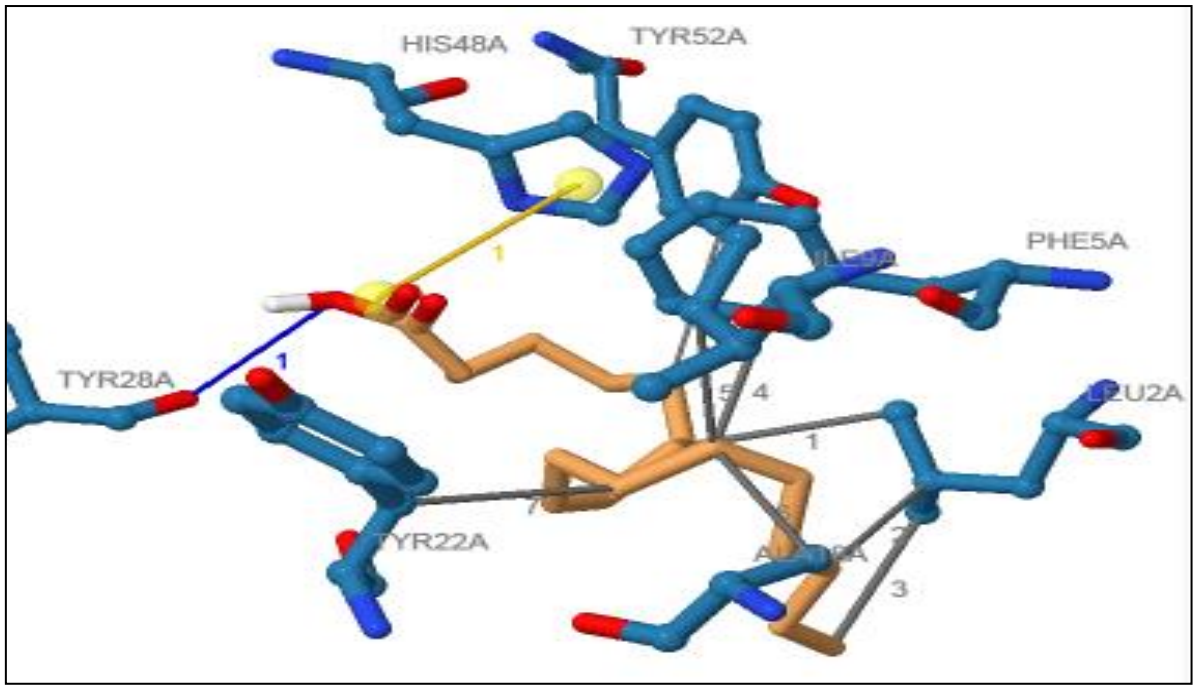
A. Ar-tumerone



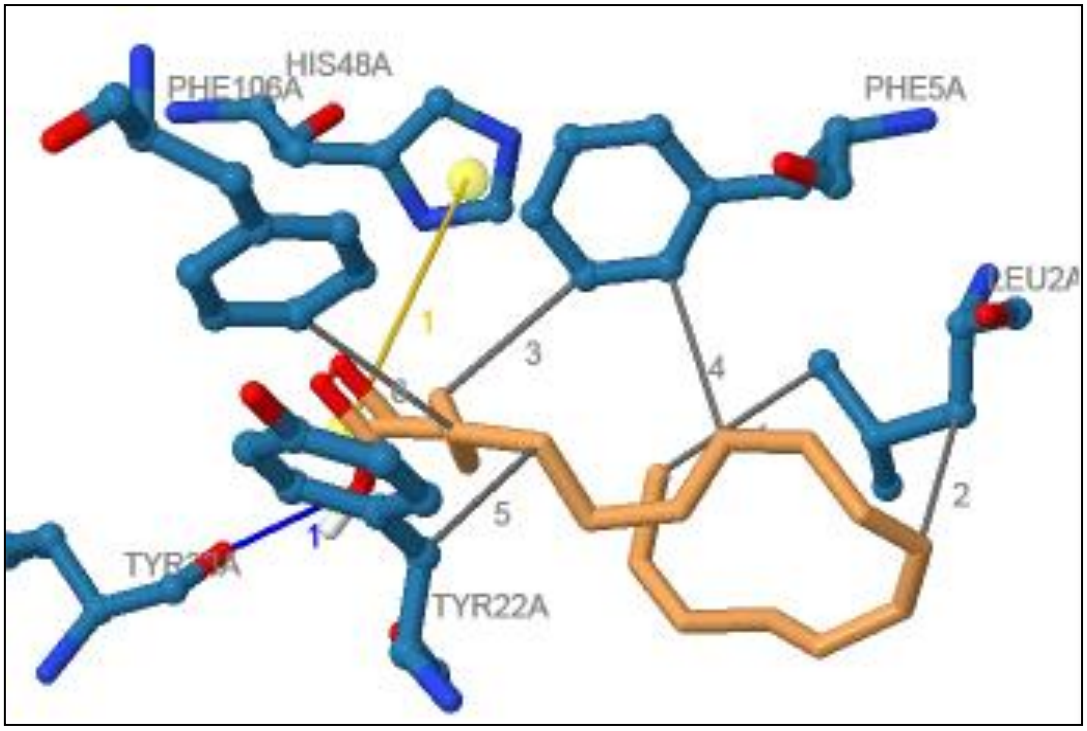
B. n-hexadecanoic acid



C. Oxalic acid, allyl octadecyl ester



D. Oleic acid



E. 9, 12 Octadecadienoic acid

Figure 5.8 : Interactions of Ligand with active site residues of PLA2 enzyme (A) Artumerone (B) n-Hexadecanoic (C) Oxalic acid, allyl octadecyl ester (D) Oleic acid (E) 9,12 Octadecadienoic acid. Salt bridge interaction with His48 (catalytic residue of PLA2 enzyme) is depicted.

Figure 5.8 and **Table 5.6** indicate the protein ligand interactions. n-Hexadecanoic acid (K_i=1.58 X 10^{-5} M; IC_{50} = 43.26 X 10^{-5} M) a known competitive inhibitor of PLA2, interacted with His48 through H bond, and other predicted active site residue of PLA2 enzyme. Interestingly 3 other phyto-chemicals, Oleic acid (18:1), allyl octadecyl ester Oxalic acid and 9, 12 Octadecadienoic acid (18:2) also interacted with His48 through H bonds. The interactions are shown in **Figure 5.8** and respective residues are tabulated in **Table 5.8**. Moreover all three compounds and n-Hexadecanoic acid, showed similar type of chemical interactions with active site residues of PLA2.

Though Ar-tumerone is a known for its antivenom property but the binding analysis with PLA2 indicate that it does not bind strongly (Binding energy= -5.5kcal). While saturated fatty acid n-hexadecanoic acid (Binding energy = - 8.9kcal) binds strongly to the binding residues, in comparison with Arachidonic acid (Binding energy= -9.1kcal). Also allyl octadecyl oxalic acid with a binding energy of (-8.5kcal) could be a potent inhibitor of PLA2 activity. In addition, it interacts with His48 with 2H bonds. Oleic acid (-8.1kcal) and 9, 12 octadecadienoic acid (-7.8k cal) could be researched further as potent inhibitors of PLA2.

5.8 Toxicity study

5.8.1 Acute toxicity

Table 5.7 : Acute oral toxicity changes of CGMR in Swiss albino mice

PARAMETER	CONTROL	CGMR treated
Body weight	Normal	No change
Feed and water intake	Normal	No change
Rate of respiration	Normal	Normal
Change in skin, eye, hair and fur	No effect	No effect
Drowsiness	Not observed	Not observed
Tremors, convulsions	Not observed	Not observed
Salivation	No effect	No effect
Sedation	No effect	No effect
Diarrhea	Not observed	Not observed
Grooming pattern	Normal	No change
General physics	Normal	No change
Death or alive	alive	alive

In the 14-day acute toxicity evaluation, oral administration of *CGMR* limit dose (2000 mg/kg b.wt) did not result in mortality, and no sign of pain, suffering, morbidity or distress were observed in the treated animals within 4h of continuous observation and even after 24 hours. The morphological, physiological and behavioral characteristics appeared normal in treated animals and are illustrated in **Table 5.7**. Food intake, water consumption, relative organ weights and body weight changes were normal. The control group animals receiving vehicle (200µl of 1% propylene glycol v/v) were normal and did not show any signs of toxicity during the 14 days study period. A rapid heartbeat was observed in the first hour after

administration and became normal which may be due to the stress of receiving extract. Since all observed parameters appeared normal and no mortality or suffering was recorded in treated animals, the *CGMR* extract at doses below 2000mg/kg bodyweight was safe and the LD50 value was considered to be higher than 2000mg/kg bodyweight. *CGMR* oral doses of $1/5^{th}$, $1/10^{th}$, and $1/20^{th}$ of limit dose viz, 100, 200 and 400 mg / kg body weight were therefore selected to evaluate sub-acute toxicity associated with the extract.

5.8.2 Sub-acute toxicity evaluation

During 28 days of treatment with CGMR, no mortality was recorded in mice at doses of 100 (low), 200 (moderate) and (high) 400 mg / kg b.wt. There were no indications of pain, suffering or morbidity in treated animals and no obvious toxic signs, such as morphological changes, functional changes or behavioral changes were observed. The control group receiving vehicles (200µl of 1% propylene glycol v/v) were normal during the 28 days study period.

5.8.2.1 Effect of CGMR on food intake

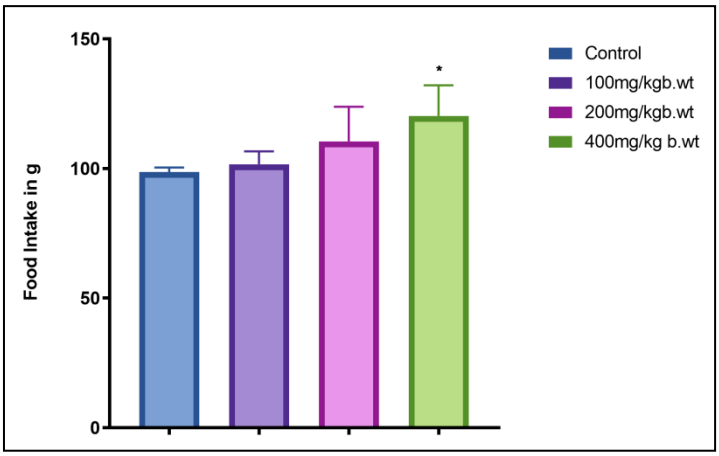


Figure 5.9A : Effect of *CGMR* consumption on food intake X axis= treatment groups; Y axis = total food intake in gm.; Statistical comparison: Dunnet's t-test multiple comparison; ****$P<.00001$; ***$P<.001$;**$P<.01$; *$P<.05$ compared control groups.

Figure 5.9A shows that the total food intake during the course of the experiment (28days) is increased in case of high dose (400 mg/kg b.wt) treated group when compared statistically with control (*P=.0165*). However no significant change was observed in low dose and moderate dose group when compared to control groups. The total food intake during the period of study for control, low dose, moderate dose and high dose group animals were calculated as 98.6±1.756, 101.675±4.951, 110.45±13.395, and 120.3±11.796 grams respectively.

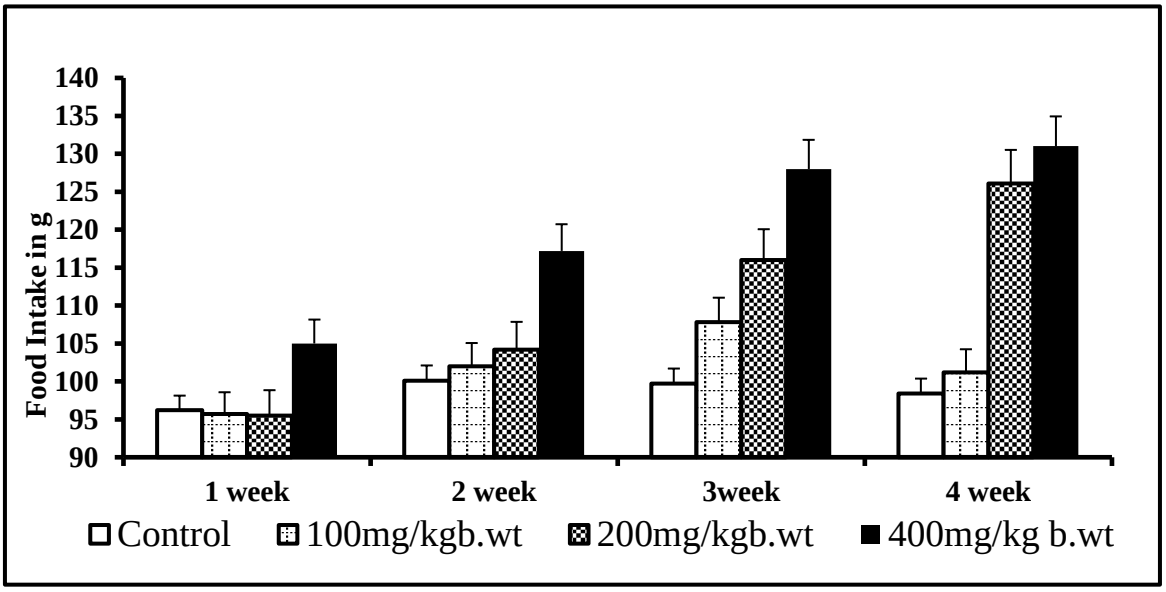


Figure 5.9B : Weekly trend indicating the effect of *CGMR* on food intake. A remarkable increase in food intake is seen from first week evidently in high dose group.

Data from **Figure 5.9B** shows the weekly trend in food consumption pattern. The amount of food taken by *CGMR* treated groups, measured at a 7-day interval over the 28 day treatment period showed a dose dependent increase, when compared with control group. Animals treated with higher dose of *CGMR* showed remarkable increase from the first week of treatment and same trend continued till the last week of experiment. With respect to lower dose and moderate dose the relative amount of food intake increased but was not statistically significant.

5.8.2.2 Effect of *CGMR* on body weight

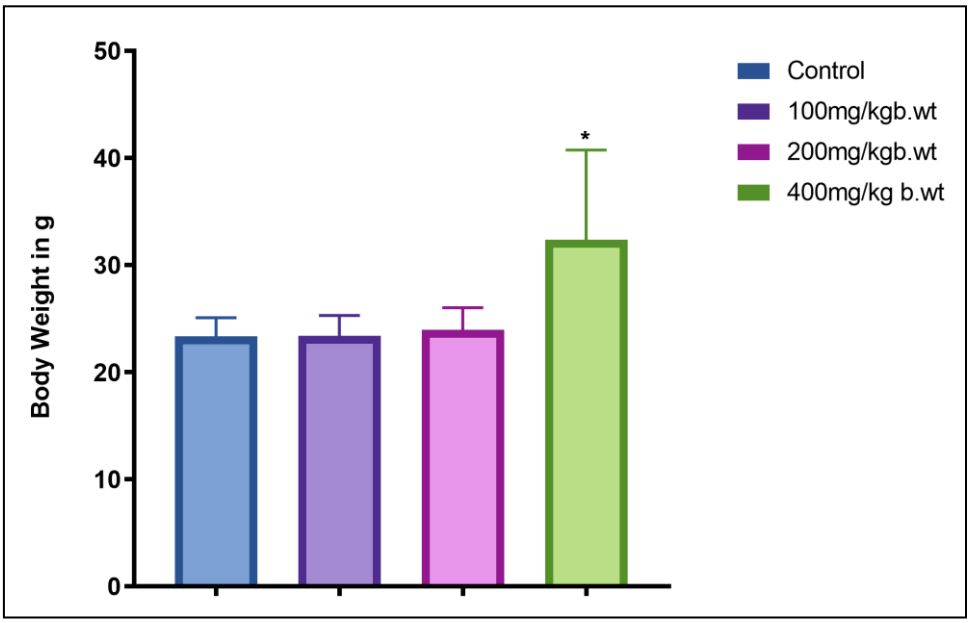


Figure 5.10A : Effect of CGMR consumption on total body weight. Values are expressed as mean ± S.D. ($n = 6$); Statistical comparison: Dunnet's t-test multiple comparison; ****$P<.00001$; ***$P < .001$;**$P < .01$; *$P < .05$ compared control groups.

Figure 5.10A shows that the total body weight during the course of the experiment (28days) is increased in case of high dose group when statistically compared with control ($P=.0386$). However no significant change was observed in low dose and moderate dose group when compared to control groups.

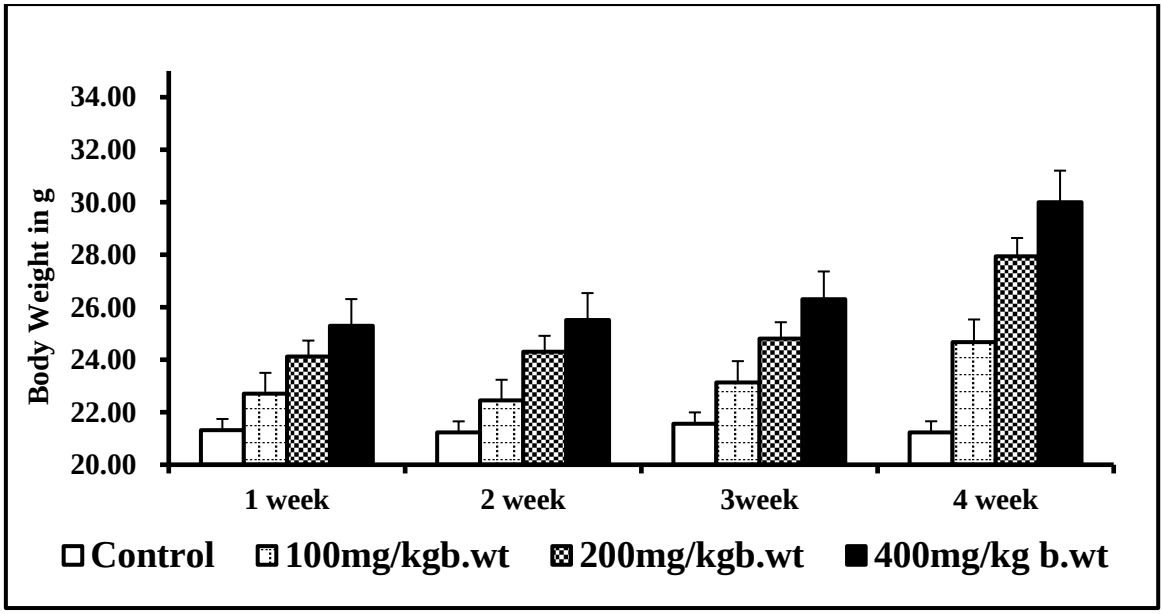


Figure 5.10B : Weekly trend indicating the effect of *CGMR* on bodyweight- The bodyweight significantly increased in high dose group animals from 3 rd week of treatment with *CGMR*.

Data from **Figure 5.10B** shows the weekly trend in food consumption pattern. The amount of food taken by *CGMR* treated groups, measured at a 7-day interval over the 28 day treatment period showed a dose dependent increase, when statistically compared with control group. The bodyweight in high dose group increased from 3rd week of treatment and continued till the 4th week. A slight increase in bodyweight was noted in low and moderate dose (*p=0.14*) groups during the entire course of study.

5.8.2.3 Effect of *CGMR* on water consumption

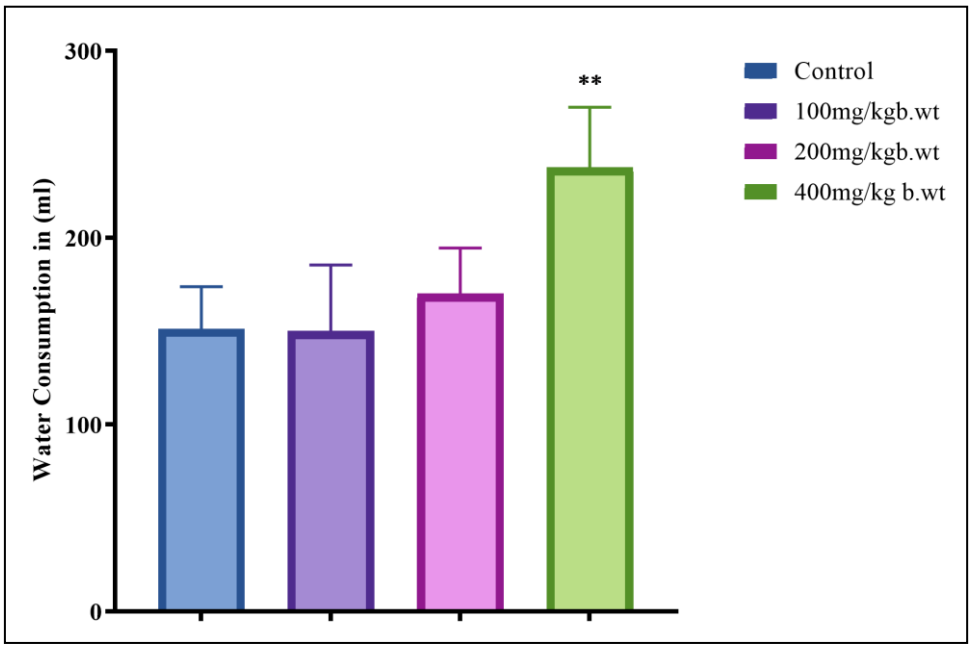


Figure 5.11A: Effect of CGMR on total water consumption (28 days). Statistical comparison: Dunnet's t-test multiple comparison; ****$P<.00001$; ***$P<.001$;**$P<.01$; *$P<.05$ compared control groups.

Data from **Figure 5.11A** shows that the administration of CGMR for 28 days results in significant increase in water consumption with respect to high dose group animals when compared with control group animals ($P= .0036$). While in the case of low and moderate dose group animals, the total water consumption pattern was found to be equivalent and comparable to control group animals.

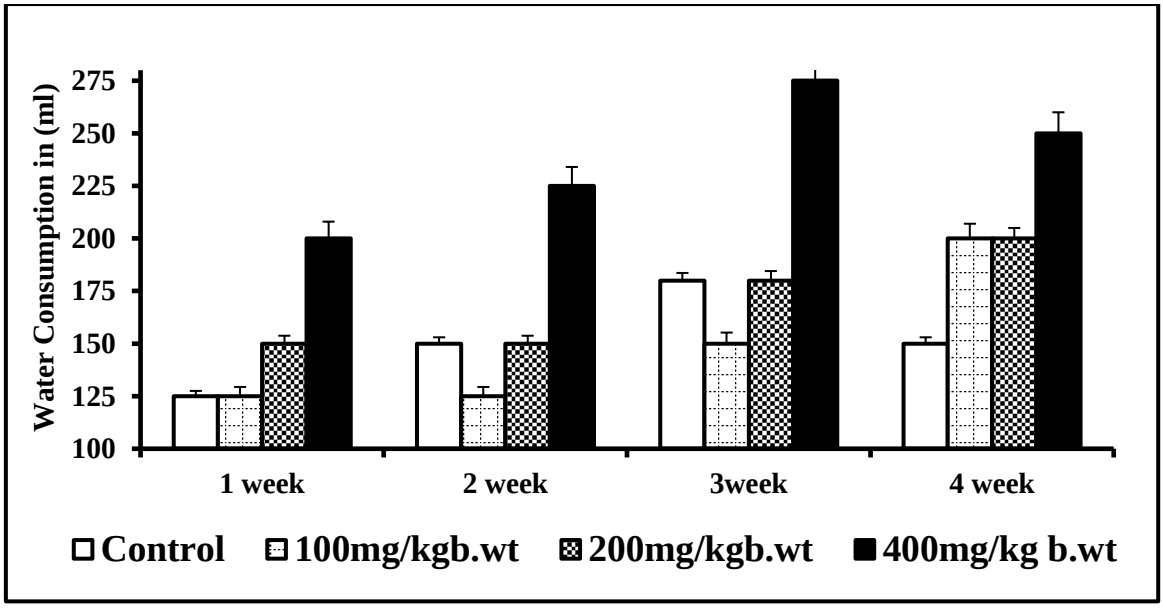


Figure 5.11B: Weekly trend indicating the effect of CGMR on water consumption for 28 days

Figure 5.11B shows the weekly water consumption pattern of treated and control animals. It is clear from the graph that the water consumption was higher by the high dose group from the 1st week of treatment. But that trend is not seen in case of low and moderate group animals compared to control group.

5.8.2.4 Relative organ weight (ROW)

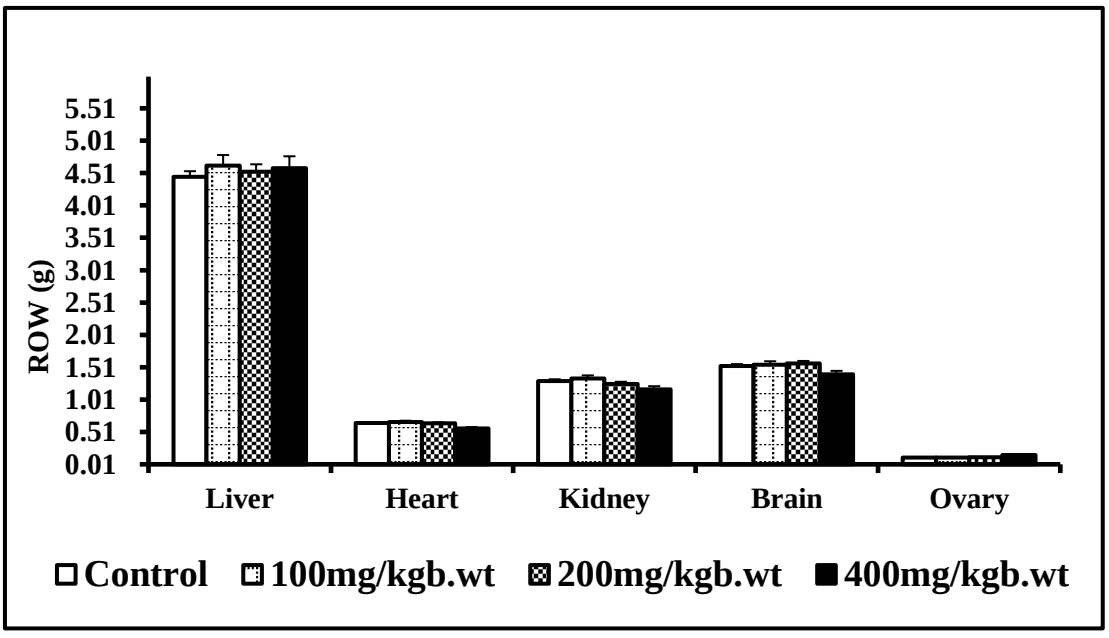


Figure 5.12 : Effect of *CGMR* on relative organ weights- Liver, Heart, Kidney, Brain and Ovary. Statistical comparison: Dunnet's t-test multiple comparison; ****$P<.00001$; ***$P < .001$;**$P < .01$; *$P < .05$ compared control groups.

Data from **Figure 5.12** indicates that there exist no significant changes with respect to relative organ weights when control and treated groups are statistically compared. Though the food intake, water consumed and bodyweight was found to be significantly increased in high dose group, remarkable changes in relative organ weight were not observed. The relative organ weight of liver, heart, brain, kidney and ovary isolated from all groups were within normal limits and were comparable with control group.

5.8.2.5 Hematological analysis

Table 5.8 : Effect of *CGMR* extract on hematological in mice treated for 28 days

Parameters	**Control**	**100 mg/kg b.wt**	**200 mg/kg b.wt**	**400 mg/kg b.wt**
Hb (g/dl)	11.9±0.1	12.7±0.4	13.13±1.789	12.96±0.808
RBC (10^6µL)	7.26±0.026	7.67±0.161	8.04±0.713	7.67±0.467
MCV (fL)	54±2	53±2	55±2	56±1
MCH (pg)	16	17	17	18
MCHC (%)	29.666±1.514	31.666±1.154	30.333±0.577	30±1.732
PCV	39.33±1.154	40.66±0.577	46.33±1.154	44.33±1.154
Platelet count (10^5mm$^{3)}$	7.8±0.888	7.09±1.110	7.5±0.624	4.833±0.750**
Total count (10^3µL)	8433.33±351.1	9633.33±378.59	9233.33±378.59	9766.67±288.67
Neutrophils (%)	22.66±2.51	20.66±2.51	19±4	17.333±3.055
Lymphocytes (%)	73.666±2.51	77.333±3.05	75.666±4.72	79.666±3.51
Eosinophils (%)	4±0.511	2.666±0.577	3.333±0.577	3.666±0.577

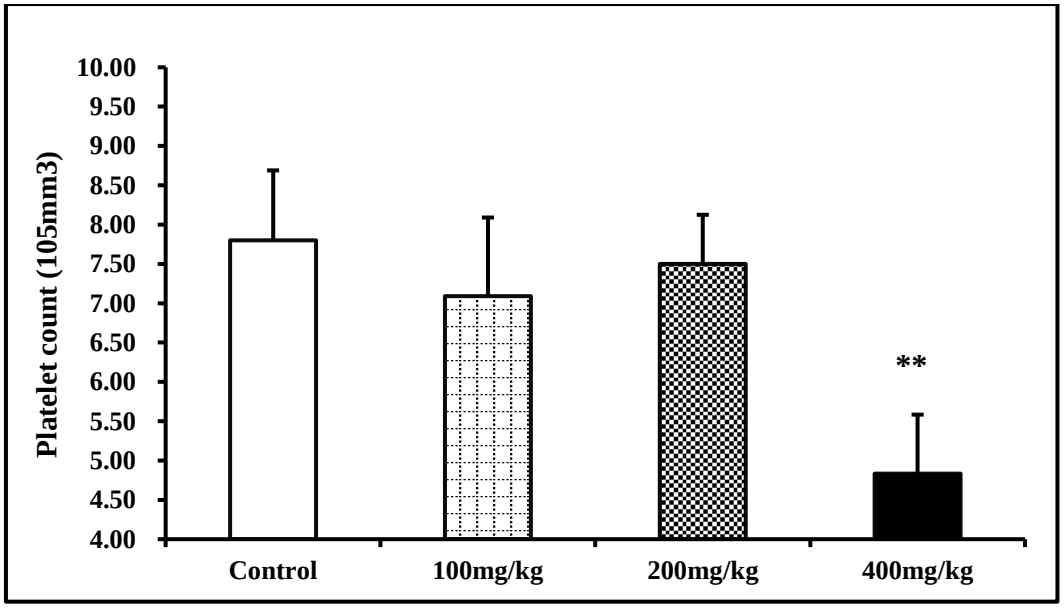


Figure 5.13: Effect of *CGMR* administration (28 days) on total platelet count. Statistical comparison: Dunnet's t-test multiple comparison; ****$P<.00001$; ***$P<.001$;**$P<.01$; *$P<.05$ compared control groups.

The data shown in **Figure 5.13** and **Table 5.8** indicates that the oral administration of *CGMR* at a concentration of 100, and 200 mg/kg body weight did not alter the hematological parameters of mice; results are summarized in the (**Table 5.8**). In this study hematological parameters including hemoglobin, RBC count, PCV, platelet count, total leukocyte count and differential leukocyte count were analyzed. In case of animals receiving high dose (400 mg/kg b.wt) statistically significant decline in platelet count ($10^5 mm^{3)}$) ($P=.0048$) was recorded. All other hematological parameters in *CGMR* treated animals were found to be within normal limits. No toxicological significant differences between treated and untreated animals were recorded.

5.8.2.6 Biochemical analysis

Table 5.9 : Effect of *CGMR* extract on biochemical parameters (Liver panel) in mice treated for 28 days

Parameters	**Control**	**100 mg/kg b.wt**	**200 mg/kg b.wt**	**400 mg/kg b.wt**
Bilirubin (Total) (mg/dl)	0.3±0.054	0.3±0.051	0.333±0.057	0.366±0.577
Bilirubin (Direct) (mg/dl)	0.1±0.043	0.1±0.052	0.1±0.051	0.1±0.054
Bilirubin (Indirect) (mg/dl)	0.2±0.047	0.2±0.032	0.233±0.057	0.233±0.057
SGOT (IU/L)	253±24.04	228±84.85	225±26.87	270±11.31
SGPT (IU/L)	97±9.89	79±18.38	91±4.24	106±9.89
Alkaline Phosphatase[ALP] (IU/L)	230±22.62	252±2.82	231±1.41	252±19.79
Total protein (g/dl)	7.6±0.28	6.7±0.42	7.1±0.14	8.3±0.14
Albumin (g/dl)	3.6±0.21	3.2±0.28	3.5±0.14	4.2±0.18
Globulin (g/dl)	4.2±0.28	3.5±0.14	3.6±0.17	4.1±0.14

Values were

Table 5.10 : Effect of *CGMR* extract on blood urea and creatinine (Kidney panel) in mice treated for 28 days

Parameters	**Control**	**100 mg/kg b.wt**	**200 mg/kg b.wt**	**400 mg/kg b.wt**
Urea (mg/dl)	39.5±4.94	42±2.82	40.5±0.70	39.5±0.70
Creatinine (mg/dl)	0.5±0.18	0.45±0.07	0.4±0.15	0.5±0.17

Data from **Table 5.9** and **Table 5.10** indicates that CGMR administration for 28 day period did not show any significant changes with respect to low dose and moderate dose groups. Parameters related to hepatic function including SGPT, SGOT, ALP, total bilirubin and total protein were not altered (**Table 5.9**). The renal function markers such as blood urea and creatinine were found to be within normal range. But in case of high dose group statistically

non-significant increase in SGOT levels, and SGPT levels was recorded, which indicates hepatotoxic and cardiotoxic nature of extract. However tests conducted for evaluating kidney function (urea and creatinine) were found to be normal.

5.8.2.7 Histopathological studies

The liver, kidney, brain, heart, and ovaries from both treated and control mice were necropsied, on the last day of the treatment, and were examined for any histopathological aberrations. The tissue sections of the control group (1%propylene glycol v/v) animals had normal cellular architecture with no histological variations. There were no obvious histopathological variation seen in any of the tissue sections, with respect to low dose and moderate dose treated animals, when compared with control groups. However, in case of high dose group, notable histopathological changes in liver and heart tissue were observed.

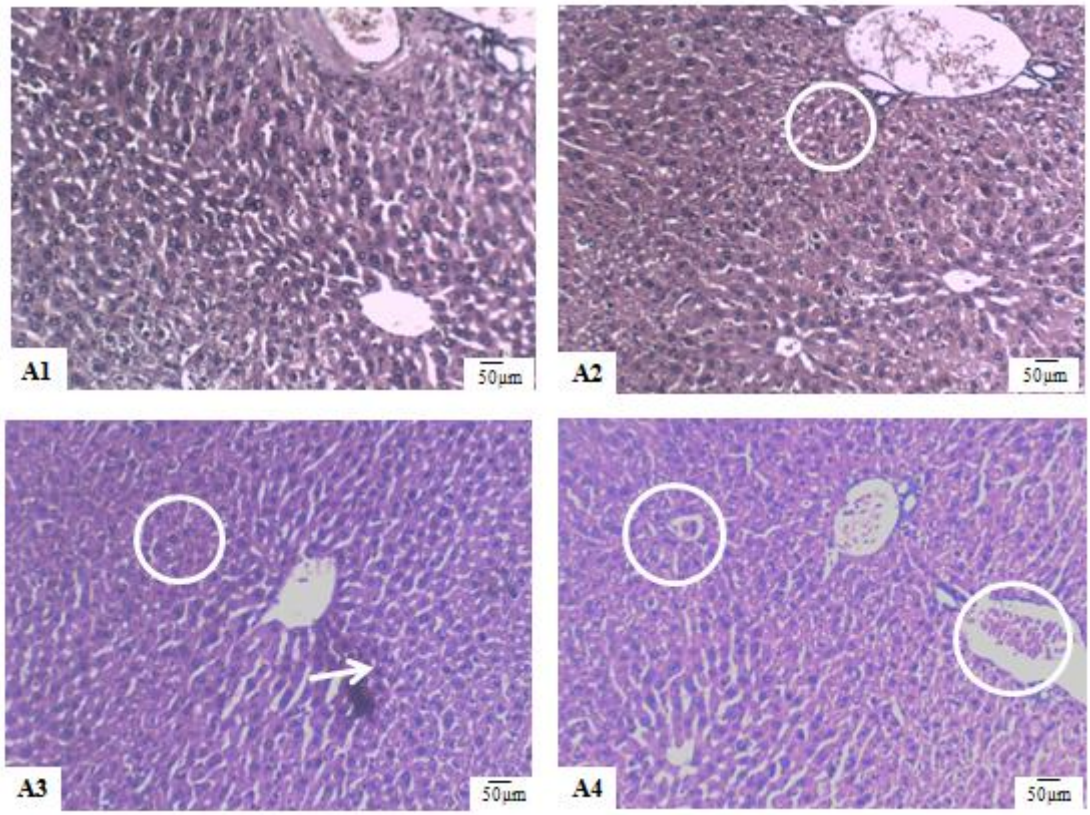


Figure 5.14: Histopathological analysis - Liver panel - photomicrograph of liver sections stained with eosin and hematoxylin from (A1) control (x40) (A2, A3 and A4) treated group 400 mg/kg b.wt (x40)

Multiple sections of liver tissue showed periportal necrosis (indicated by white circles in **Figure 5.14A2** and **A3**) with minimal inflammatory cell infiltrate along with porto-porto bridging necrosis (indicated by white arrow in (**Figure 5.14A3**). Also central venous congestion and dilatation was seen at places (denoted by white circles in (**Figure 5.13A4**).

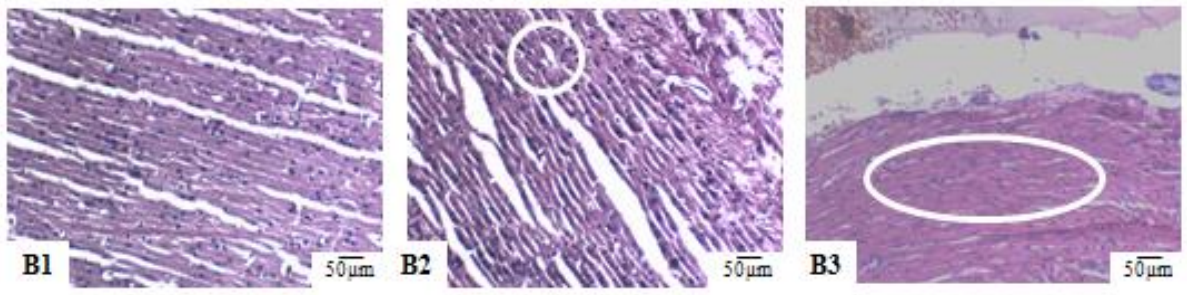


Figure 5.14 A: Histopathological analysis– Heart panel- photomicrograph of heart sections stained with eosin and hematoxylin from (B1) control (x40) (B2 and B3) treated group 400 mg/kg b.wt (x40)

The treated group (400mg/kg b.wt) heart sections showed nuclear pyknosis **(Figure 5.14A-B2-section)** at areas along with waviness of cardiomyocytes and mild increase in eosinophilia of the cytoplasm (**Figure 5.14A-B3-section**) when compared to control group heart section **(Figure 5.14A-B1 section)**.

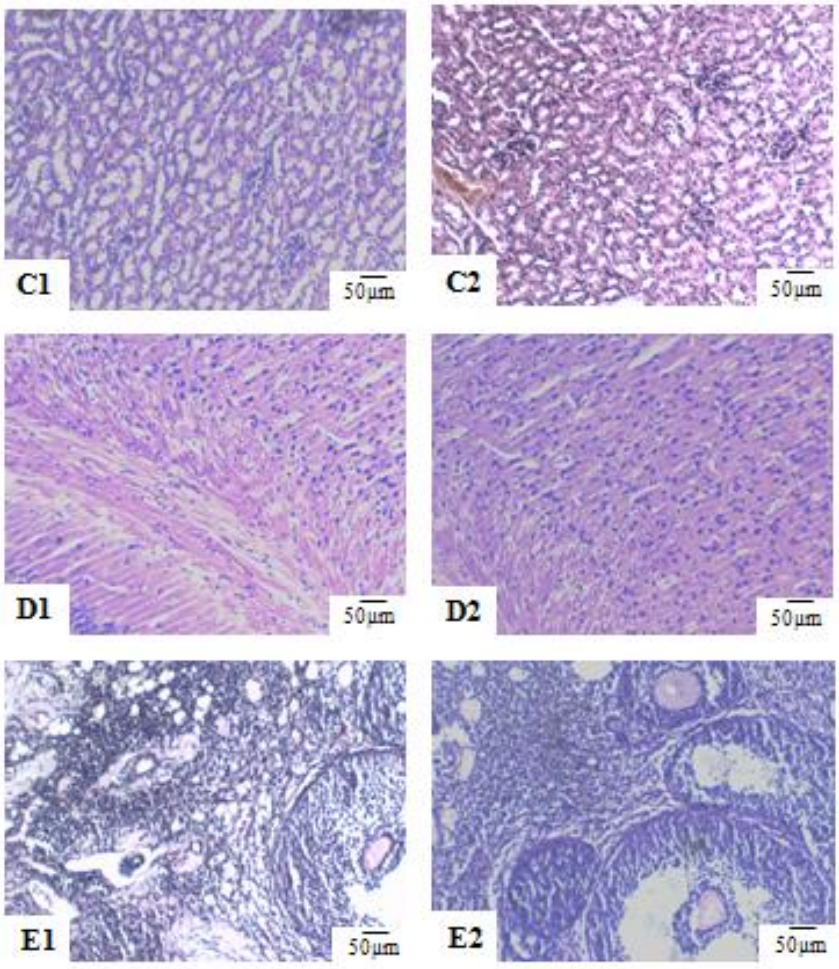


Figure 5.14 B: Histopathological analysis- shows photomicrograph of kidney sections stained with eosin and hematoxylin from (C1) control (x40) (C2) treated group 400mg/kg b.wt (x40). Brain panel- (D1) control (x40) (D2) treated group 400mg/kg b.wt (x40). Ovary panel-(E1) control (x40) (E2) treated group 400mg/kg b.wt (x40).

The histopathological examination of kidneys brain and ovaries showed normal pathology and no histological aberration were observed for both control and high dose treated groups (**Figure 5.14B**).

5.9 Determination of LD_{50} of *Daboia russelii* snake venom (*DRSV*)

Table 5.11 : Determination of LD_{50} dose of *Daboia russelii* snake venom

Dose of *DRSV* in µg	No of mice	Weight of mice (g)	No. of survival after 24hrs	No of Dead after 24hrs	% Dead	LogC	Probit
5	6	28-30	5	1	16.66667	0.69897	4.01
10	6	28-30	4	2	33.33333	1	4.56
15	6	28-30	2	4	66.66667	1.176091	5.41
20	6	28-30	1	5	83.33333	1.30103	5.95

Abbreviation DRSV**:** *Daboia russelii* snake venom

Table 5.11 shows the results of the LD50 determination. It is clearly evident that the number of dead increase proportionally over a 24 hr period, as the dose of venom increased.

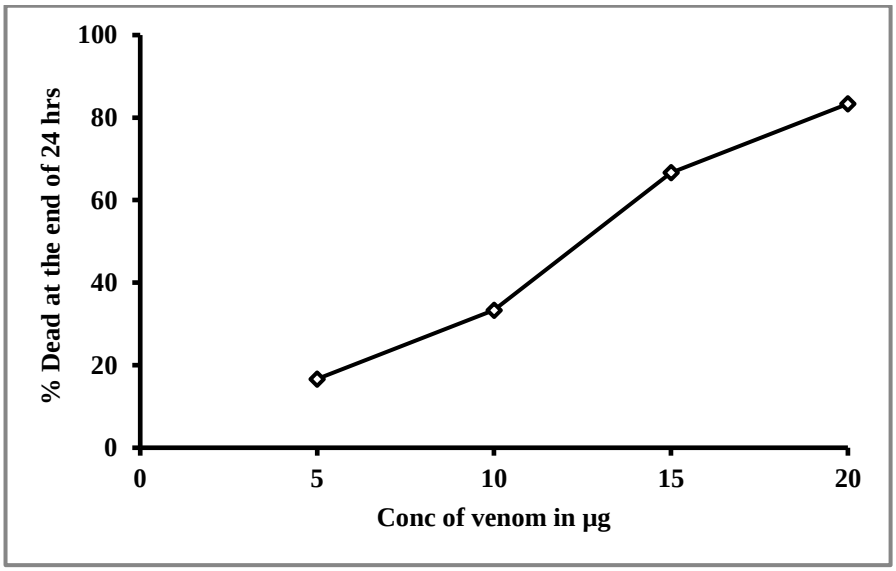


Figure 5.15: Determination of LD_{50} dose of *Daboia russelii* snake venom

From the data shown in **Figure5.15** and **Table 5.11** the LD_{50} dose of DRSV was determined as 11.27 µg/dose or 0.375 µg/g body weight. The results were also cross checked with venom supplier. The 50% death rate was analyzed to be between 10-15 µg doses. Accurate measurement was done using probit analysis.

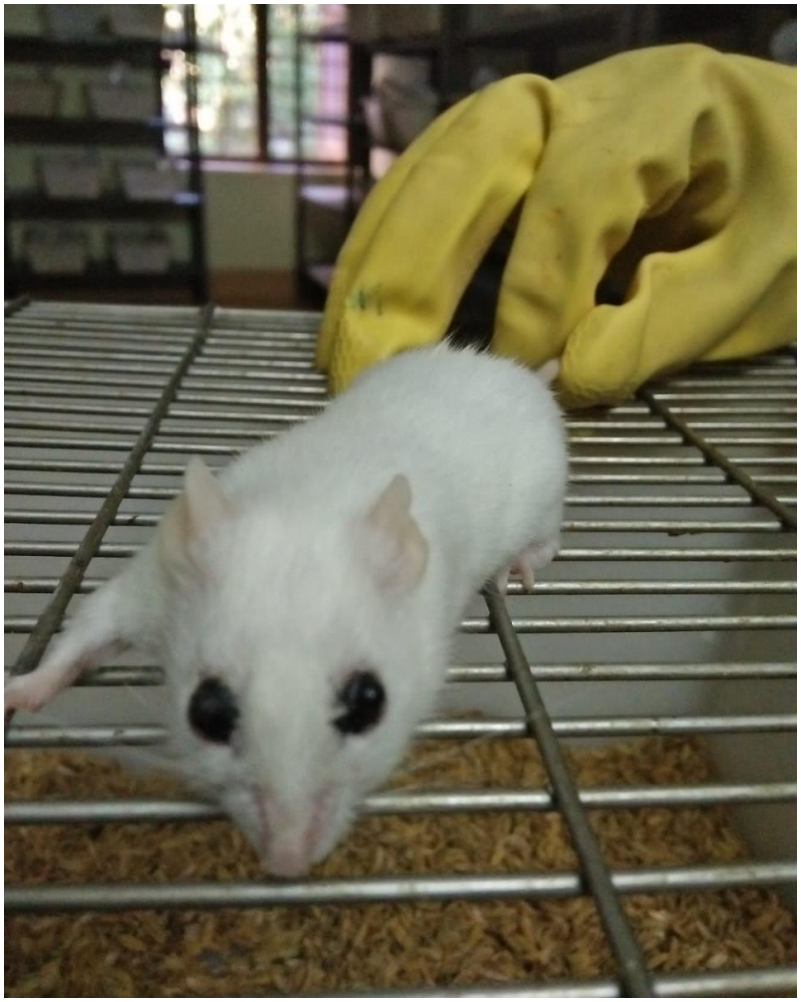


Figure 5.15A: Photograph of envenomed animal (LD50 dose = 11.27µg/dose)

The inset **Figure 5.15A** shows the picture of envenomed animal, after receiving LD50 dose. Immediately after receiving the dose gasping and convulsions were observed in few animals. In some there was no movement and animal laid flat. In other few animals bleeding through mouth and severe convulsions were observed. A very common observation that was observed in all animals was the slow blackening of the pupil and bulging of eyes (within a few minutes after administration).

5.10 *In vivo* neutralization

Table 5.12 : *In vivo* neutralization of venom *DSRV* by *CGMR*

Dosage	No of mice	Weight of mice (g)	Mortality (no. of dead / total mice)	% Survival	% corrected	Probit
Saline+ Propylene glycol	6	28-30	0/6	100	4.15	3.26
Venom LD50	6	28-30	3/6	50	50	5.00
Venom LD50 + 100mg/kg b.wt of *CGMR*	6	28-30	3/6	50	50	5.00
Venom LD50 + 200mg/kg b.wt of *CGMR*	6	28-30	3/6	50	50	5.00
Venom LD50 + 400mg/kg b.wt of *CGMR*	6	28-30	2/6	66.67	62.52	5.75
Venom LD50 + Antivenom	6	28-30	6/6	100	95.85	6.75

The LD_{50} dose of venom was significantly neutralized by animals treated with and 400 mg/kg b.wt of *CGMR* extract. Further the extract neutralized venom in concentration dependent manner (**Table 5.12**) (**Figure 5.16**). The death rate in LD_{50} venom group and group receiving 100mg/kg.bwt remained same. However death was delayed in 100mg/kgb.wt group. Also no mortality or morbid condition was recorded in group receiving saline + 1%propylene glycol. Following venom administration (P.O) asphyxia and restlessness were observed. After a couple of minutes dilatation and blackening of pupils were observed in all cases. After noting this observation extract was administered (P.O). The 24 hr survival rate was taken as endpoint and probit values were calculated. The experiment was performed by keeping Venom LD_{50} + antivenom group as positive control. On the whole the *in vivo* neutralization experiment confirms that extract delays the death in envenomed animals.

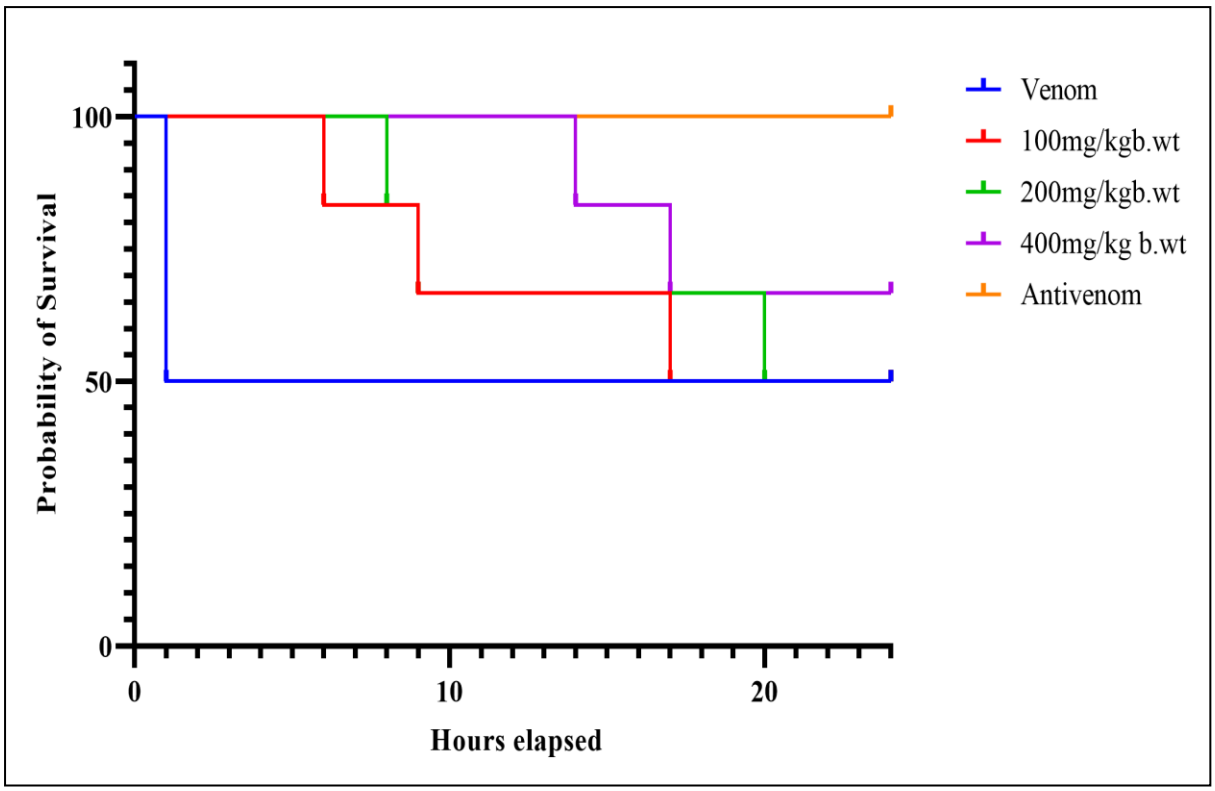


Figure 5.16: Kaplan-Meier curves of *in vivo* neutralization study (24hr). Each tick on X axis represents 1hr interval, decent in the graph denotes a death while plateaus indicate surviving animals. Y axis = probability of survival in percentage.

The time line indicated in **Figure 5.16** shows that extract possess significant neutralization ability even at very low dose. For instance, death recorded in LD_{50} group is at 1.5hr, 6hr and 11hr. While in 100 mg/kg b.wt group deaths were recorded at 5hr, 8hr and 15 hr following administration. Contrary to this in high dose group (400 mg/kgb.wt) first death was recorded in after 13hrs of administration which validates the neutralization effect of extract.

5.11 *In vitro* neutralization

Table 5.13 : *In vitro* neutralization of *DRSV* by *CGMR*

Neutralization *In vitro*	No of mice	Wt of mice (g)	Mortality (no. of dead / total mice)	% Survival	% corrected	Probit
Saline + propylene glycol	6	28-30	0/6	100	4.15	3.25
LD50 venom	6	28-30	3/6	50	50	5.00
Venom LD50 + 200mg/kg b.wt of *CGMR*	6	28-30	1/6	83.33	79.18	5.95
Venom LD50 + 400mg/kg b.wt of *CGMR*	6	28-30	0/6	100	95.83	6.75
Venom LD50 + Antivenom	6	28-30	0/6	100	95.83	6.75

The LD_{50} dose of venom was significantly neutralized by pre-incubation with 200 mg/kg b.wt and 400 mg/kg b.wt of *CGMR* extract. Further the extract neutralized venom in concentration dependent manner as seen in case of *in vivo* neutralization. The survival rate of the animals improved remarkably compared to *in vivo* study. In fact no death was recorded in group receiving venom incubated with 400 mg/kg.bwt. In case of 200 mg/kg b.wt group, first death was reported at 10 hrs following administration which is higher than 8hrs reported for *in vivo* study (**Table5.13**). The findings of this study indicate the presence of inhibitors in extract that may be acting on venom components during incubation.

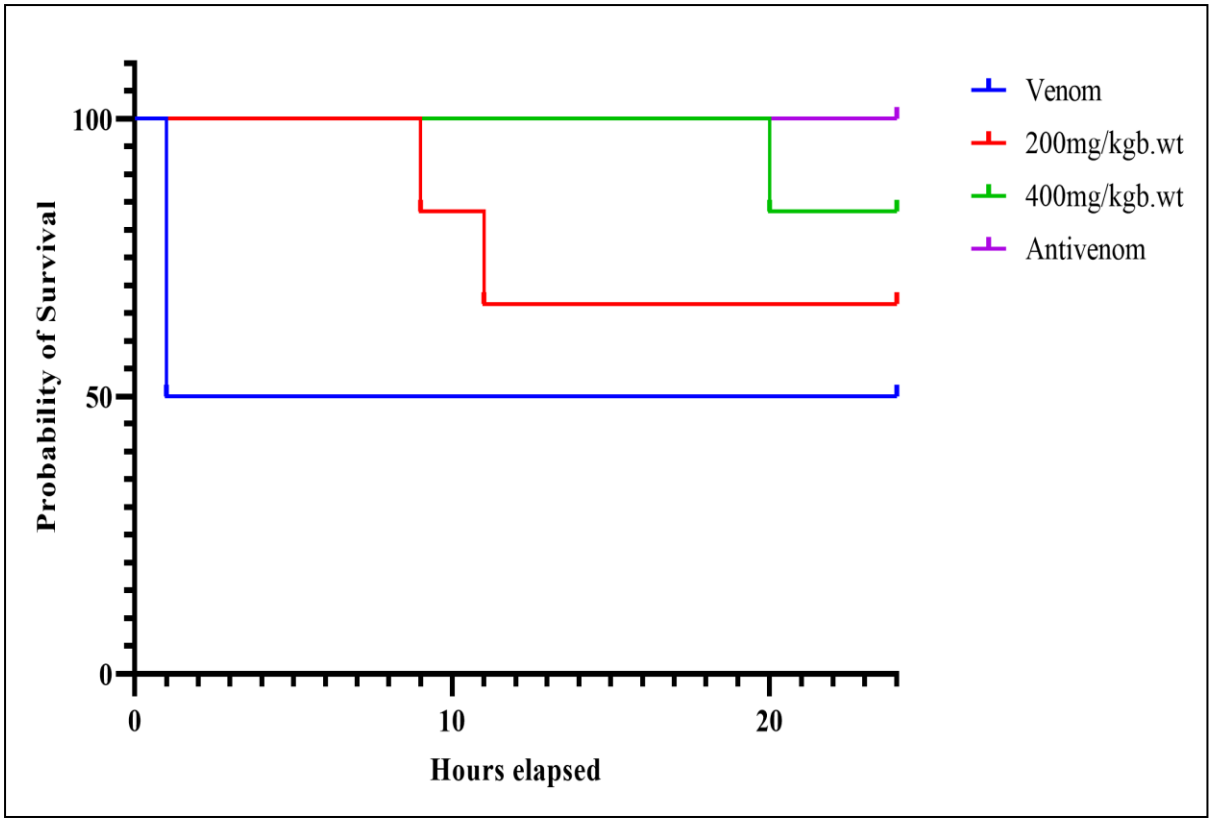


Figure 5.17 : Kaplan-Meier curves of *in vitro* neutralization study (24hr). Each tick on X axis represents 1hr interval, decent in the graph denotes a death while plateaus indicate surviving animals.

The *in vitro* neutralization time line indicated in **Figure 5.17** shows that the neutralization of venom was better compared to *in vivo* study. The first death noted in case of 400mg/kg b.wt is at 19 hrs from envenomation. In addition the number of deaths also decreased in high dose group compared to *in vivo* study.

5.12 Protective effect

Table 5.14 : Protection effect of *CGMR* against *DRSV*

Protection effect	No of mice	Weight of mice (g)	Mortality (no. of dead / total mice)	% Survival	% corrected	Probit
Control	6	28-30	0/6	100	4.15	3.25
Saline + propylene glycol	6	28-30	0/6	100	50	5.00
LD_{50} venom	6	28-30	3/6	50	50	5.00
200mg/kg b.wt of CGMR + Venom LD50	6	28-30	3/6	50	50	5.00
400mg/kg b.wt of *CGMR* + Venom LD50	6	28-30	1/6	83.33	79.18	5.95

Data from **Table 5.14** suggests that the death rate decreased as the concentration of pretreatment with extract increased. Physical observation of pre-treated animals after venom challenge showed normal signs. Dilatation of pupils, asphyxia and lethargy seen during envenomation was not commonly seen in pretreated animals. The survival rate also increased in pretreated animals suggesting that the *CGMR* extract has a prophylactic action against *Daboia russelii* venom.

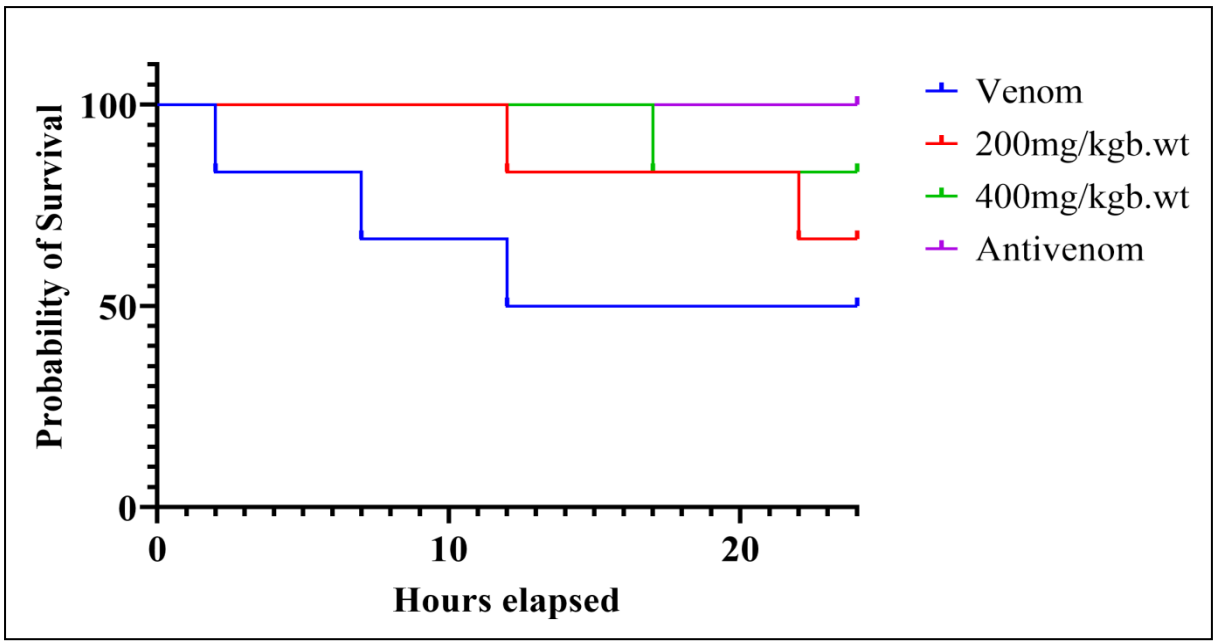


Figure 5.18 : Kaplan-Meier curves of protective action of extract against venom. Each tick on X axis represents 1hr interval, decent in the graph denotes a death while plateaus indicate surviving animals.

Animals pretreated with *CGMR* showed more tolerance to venom insult, as it is evident from (**Figure 5.18**). The histopathological observation of organ tissue proves the protection effect of the extract.

5.12.1 Bleeding time BT and Clotting time test CT

Table 5.15 : Determination of Bleeding and Clotting time

Sl No	Test	Control	Vehicle Control	Venom (LD50) (1hr)	Venom (LD50) (12 hr)	*CGMR*-400 mg/ kg b.wt	*CGMR*-400 mg/ kg b.wt +Venom (0hr)	*CGMR*-400 mg/ kg b.wt +Venom (12hr)
1	BT	1.50±0.85	1.40±0.75	3.5±1.2	4.1±0.9	1.45±0.82	2.8±0.65	1.8±0.58
2	CT	4.8±0.88	0.51±0.66	>20	>20*	5.12±0.77	>20*.	18±0.32

Clotting time increased following venom administration in all groups. In groups receiving only venom the clotting time exceeded 20 min. This result is suggestive of consumptive coagulopathy changes induced by the venom. Surprisingly the clotting time increased immediately after venom administration suggesting the fast action of venom components on coagulation cascade. In 12 hour group CT was increased. But in *CGMR* pretreated animals the increase was limited and the condition improved after 12 hrs of venom administration.

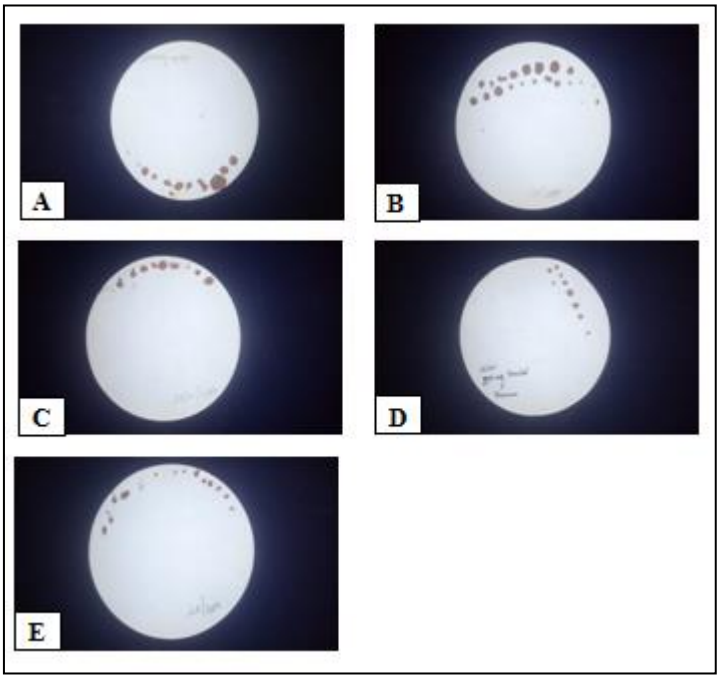


Figure 5.19: Determination of bleeding time using whatmann filter paper.

Key- A. 0hr after venom administration, B. 12hr after venom administration, and C. 0hr after venom administration in 400mg / kgb.wt *CGMR* treated mice, D. 12hr after venom administration in 200mg/kgb.wt *CGMR* treated mice, E. Control

Estimation of bleeding time in venom treated and low dose group animals (200mg/kgb.wt) gave a clear indication of protection rendered by the extract, especially after 12hr of venom administration (**Table 5.19**) (**Figure 5.19**). The bleeding time is remarkably increased in both venom treated groups. The bleeding time estimated after 1hr of venom administration

(3.5±1.2) is increased to 6.1±0.9 after 12 hrs. This indicates the lethal action of venom on blood components, especially the clotting factors. Usually in case of *DRSV* envenomation coagulopathy is seen due to deficiency of clotting factors and bleeding time is drastically increased. But in *CGMR* pretreated group slight improvement in bleeding time is noted suggesting the protective action of extract. The results are more evident after 12 hr of venom administration (1.8±0.58).

5.12.2 Histopathological analysis

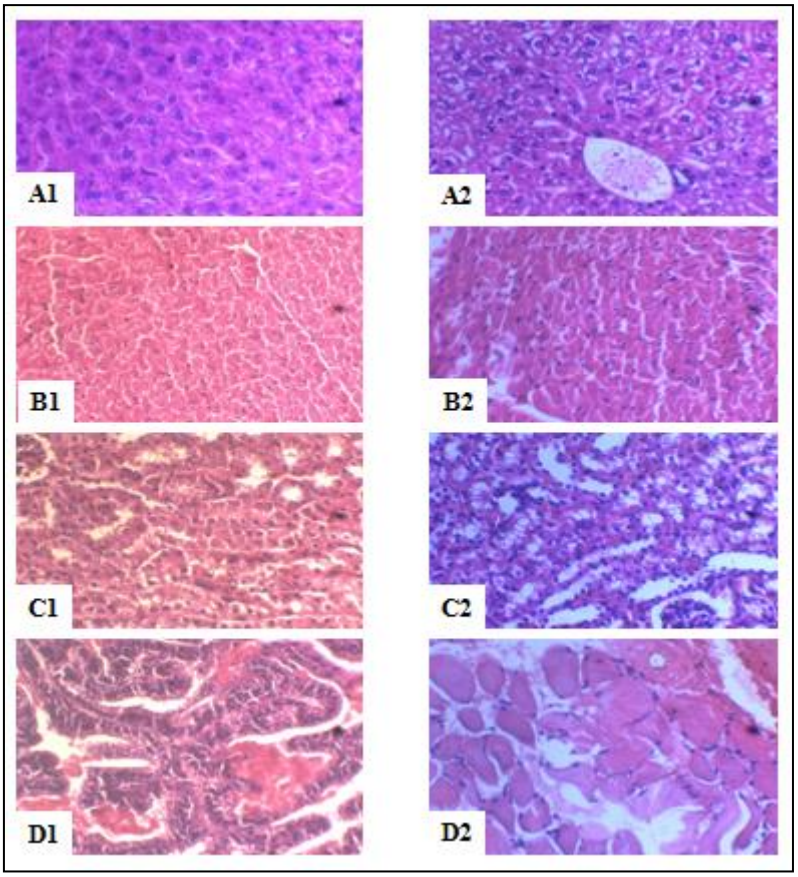


Figure 5.20A: Histopathological analysis – time based study (0hr) - (A1) Liver- LD50 venom treated (x40) (A2) Liver -Pretreated with CGMR 400mg/ kg b.wt and injected with LD50 venom. (B1) Heart- LD50 venom treated (x40) (B2) Heart -Pretreated with CGMR 400mg/ kg b.wt and injected with LD50 venom (x40). (C1) Kidney- LD50 venom treated (x40) (C2) Kidney -Pretreated with CGMR 400mg/ kg b.wt and injected with LD50 venom.

(D1) Diaphragm- LD50 venom treated (x40) (D2) Diaphragm -Pretreated with CGMR 400mg/ kg b.wt and injected with LD50 venom.

Time based study- After venom injection the organs were necropsied from the animal to assess the immediate changes that are occurring due to venom insult. Gross necrosis was seen in liver tissue and kidney compared to extract pretreated liver tissue (**Figure 5.20A)**. Diaphragm was damaged beyond the scope of evaluation, with hemorrhages and necrosis all around the section. But in *CGMR* pre treated mice the deleterious effect on diaphragm is circumvented and the tissue along with vasculature is intact.

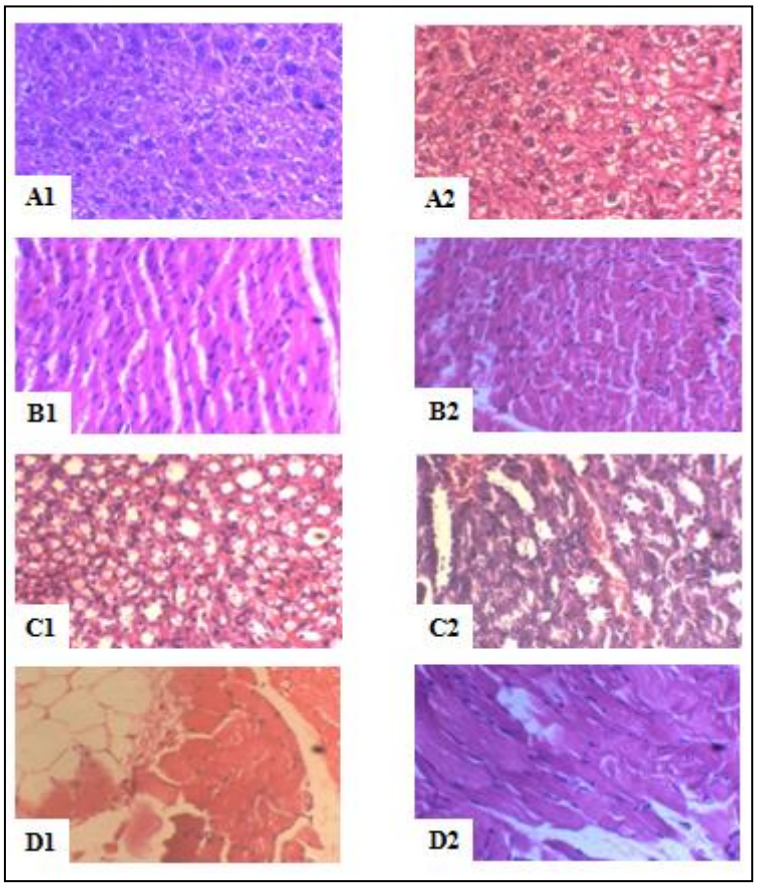


Figure 5.20B: Histopathology analysis – time based study (12hr) - (A1) Liver- LD50 venom treated (x40) and sacrificed after 12 hrs (A2) Liver -Pretreated with CGMR 400mg/ kg b.wt and injected with LD50 venom and sacrificed after 12 hrs (x40). (B1) Heart- LD50 venom treated and sacrificed after 12 hrs (x40) (B2) Heart -Pretreated with CGMR 400mg/ kg b.wt

and injected with LD50 venom and sacrificed after 12 hrs (x40). (C1) Kidney- LD50 venom treated and sacrificed after 12 hrs (x40) (C2) Kidney -Pretreated with CGMR 400mg/ kg b.wt and injected with LD50 venom and sacrificed after 12 hrs (x40). (D1) Diaphragm- LD50 venom treated and sacrificed after 12 hrs (x40) (D2) Diaphragm -Pretreated with CGMR 400mg/ kg b.wt and injected with LD50 venom and sacrificed after 12 hrs (x40).

Time based study (**Figure 5.20B**) - 12 hrs after venom injection the organs were necropsied from the animal to assess the changes that are occurring due to venom insult. 12 hours after venom injection the organs were necropsied from animals to assess the changes that are occurring due to venom insult. Gross necrosis is observed in liver and diaphragm while in *CGMR* pretreated group the extent of damage is limited.

5.13: Comparison of survival curves of *in vivo*, *in vitro* and protective effect of *CGMR* against *DRSV*

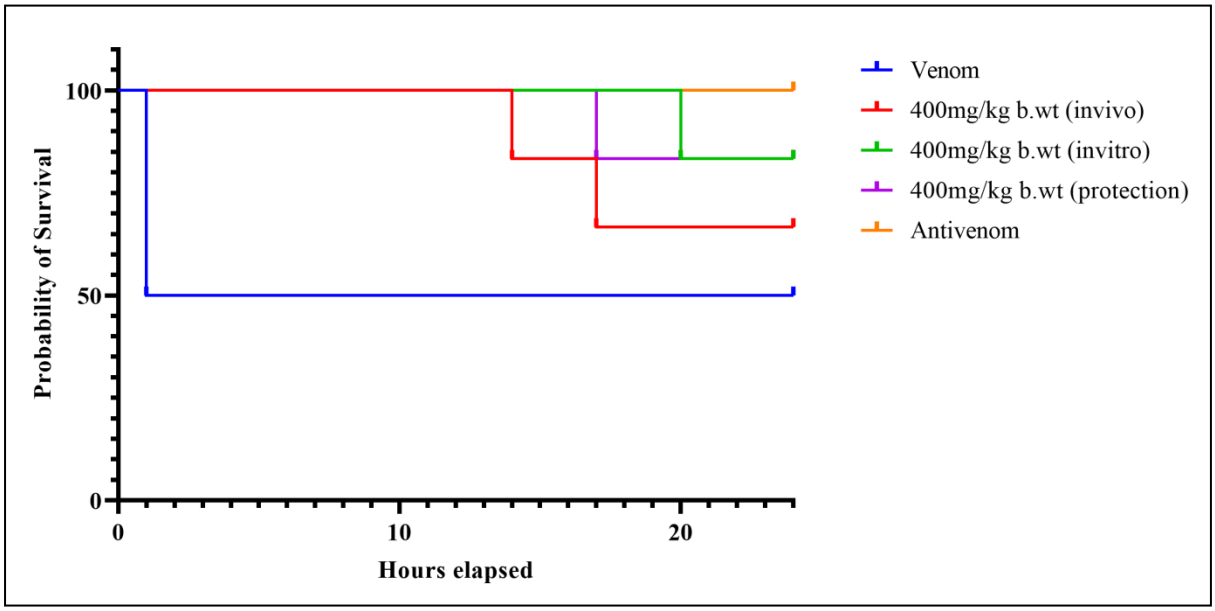


Figure 5.21 : Kaplan Meier survival curve of 400mg/ kg b.wt from *in vivo*, *in vitro* and protective action of *CGMR* experiments.

In **Figure 5.21** the effect of 400 mg/kg b.wt of the *CGMR* extract on *in vivo* neutralization, *in vitro* neutralization and protective action experiments is shown. The effect of the extract to ameliorate the properties of venom is better in case of *in vitro* experiment. No deaths or morbidity was observed in group receiving venom + 400mg/kg b.wt of the extract (pre-incubated). This result reemphasizes the action of PLA2 inhibitors present in the extract that inhibit PLA2 integral venom components

CHAPTER 6
DISCUSSION

6.1 Extraction of plant material

Calotropis gigantea is a perennial shrub, locally found in dry land areas of India (V. Singh 2012). It is commonly known as “milkweed” or “shallow wort” (Bhardwaj and Misra 2018). The roots of the plant, in particular, are regarded to possess high medicinal value. Previous studies have investigated innumerable pharmacologically active substances such as cardiac glycosides (calactin, calotropin and calotoxin), steroids and anti-inflammatory compounds present in the root extract. Many phytochemical studies have found methanol as a good solvent for herbal extraction, especially if the study intends to identify and isolate plant secondary metabolites (Truong *et al*. 2019). *Calotropis gigantea* methanolic leaf and latex extracts have also demonstrated exceptional venom neutralizing ability in many other studies (Chacko *et al*. 2012).

6.2 *In vitro* inhibition of PLA2 activity

This study ascertained the inhibitory activity of crude *CGMR* on PLA2 activity (major toxic component of venom) through in-vitro inhibition assay. The *in vitro* PLA2 inhibition was highest in crude extract, followed by non-polar fraction, and polar fraction. The increased percent inhibition seen in non-polar fraction may be due to PLA2 inhibitors which are not identified so far. On the other hand the CGMR extract is known for its anti-inflammatory and steroidal properties (Adak and Gupta 2006). In the past many compounds possessing anti-inflammatory properties have been investigated for PLA2 inhibition(Meyer *et al*. 2005). The low percentage inhibition obtained in polar extract may be due to lack of concentration of metabolite. In addition the polar extract in this study has shown very high content of phenols and flavonoids which are previously proven to posses anti-PLA2 activity (Lindahl and Tagesson 1997). Flavonoids like rutin and polyphenols compounds like rosmarinic acid and aristolochic acid have been demonstrated to posses PLA2 inhibition activity (S. L. da Silva et

al. 2009). So, comprehensive characterization of both the fractions is inevitable for better understanding of their bioactivity. In addition, it is possible that PLA2 inhibitors of polar and nonpolar nature might have additive effect in the crude extract.

6.3 Phytochemical study and docking analysis

The phytochemicals present in root extract has not been profiled, so far. For the first time, in this study a detailed phytochemical analysis is performed using GC/MS, LC/MS and routine phytochemical tests. However, the phytochemical constituents present in *C.gigantea* leaves (24 compounds) and latex (22 compounds) have been profiled (Sharma, Kumari, and Sharma 2016). Shalini.et,al have documented that ~60% of the compounds were common in both leaf and latex extract while the remainder compounds were unique to either of the extracts studied. In contrast to the above finding our GC/MS data of root extract revealed a distant phytochemical profile which predominantly consists of unsaturated and saturated long chain fatty acids. Most of which were exclusively confined to root. The study confirms the variation between the chemical constituents of leaf, latex and root which shows their different potential of therapeutic activities.

The GC/MS analysis of *CGMR* indicated the presence of 10 compounds such as ar-turmerone, n-hexadeanoic acid and 2-piperidinone. Among these compounds ar turmerone, oleic acid, n-hexadeanoic acid and 9, 12 Octadecadienoic acid have been shown previously to possess significant anti-venom activity (Table 5.4). Ar-turmerone, extracted from *Curcuma longa*, has been shown to inhibit hemorrhagic activity induced by *Bothrops jararaca* venom. In the same study it has been reported that ar-turmerone fraction significantly lowered the lethal effect induced by *Crotalus durissus terrificus* venom (L. A. F. Ferreira *et al*. 1992). n-Hexadecanoic acid ($Ki=1.58 \times 10^{-5}$)was found to be competitive inhibitor of PLA2 isolated from bee venom (Aparna *et al*. 2012). Further GC/MS data indicates the presence n-

Hexadecanoic acid (4.3%), a known competitive inhibitor (Ki=1.58 x 10^{-5}) of PLA2. The concentration of this metabolite according to GC/MS data is about 4% which is sufficiently high to show its effect. This is significantly low if we compare it with 9, 12 octadecadienoic acid concentration (29.2%). However in other studies it has been postulated that just 2% of metabolite is a significant quantity for pharmacological action. The concentration of Coumarins for instance is mere 2% in plants belonging to Fabaceae and Rubiaceae, but coumarins are sourced from these plants for cosmetic use (Wink 2015). Docking analysis has revealed the possible inhibitory role of other phyto-chemicals like allyl octadecyl ester oxalic acid, oleic acid, 9, 12, Octadecadienoic acid.

PLA2 is regarded as the single most toxic component of the venom and its inhibition certainly ensures neutralization of venom and its components (Manjunatha Kini 2003). An *in silico* study has predicted that n-hexadecanoic acid binds to catalytically important residues on PLA2 viz, His48, Asp49 and Gly30 significantly (docking score= -3.41, glide energy =-45.95kcal/mol). In the same study, 9, 12-octadecadecenoic acid is predicted to bind PLA2 at Gly30 and Asp49 residue (Docking score= -6.46, glide score= -43.58)(Subasri *et al*. 2016). Further molecular dynamics simulations have been performed to show that n-hexadecanoic acid and 9, 12 Octadecadienoic acid bind strongly to target PLA2. Another study has postulated the antivenom activity of 9,12 Octadecadienoic acid against *Deinagkistrodon acutus* venom(Xiong *et al*. 2018). Phospholipase A2 inhibitors are potential anti-inflammatory agents as they block the release of Arachidonic acid (Meyer *et al*. 2005). Few unsaturated long chain fatty acids such as oleic acid (18:1), palmitoleic (16:1), linoleic acid (18:2), arachidonic acid (20:4) and linolenic (18:3), are proven to be inhibiting human platelet PLA2 activity by 50%. Further these long chain fatty acids were shown to inhibit PLA2 activity non-competitively ($5X10^{-7}$) M. Also methylated long chain unsaturated fatty acids failed to inhibit PLA2 activity while demethylation restored the inhibition (Ballou and

Cheung 1985). The GC/MS profile of the extract indicates the presence of long chain fatty acids and hence these compounds may be involved in inhibition of venom PLA2 activity. This assumption is supported by two important findings of this study. The results of protein-ligand docking performed in this study are in line with this assumption. As many unsaturated fatty acids present in the extract were shown to bind strongly to PLA2 at the active site. Also the docking study has purported the involvement of allyl octadecyl ester oxalic acid, another unsaturated fatty acid present in the extract, as a potent PLA2 inhibitor. That may be potentially inhibiting PLA2 activity along with other known PLA2 inhibitors present in the extract.

In addition, the findings of this study support the above assumption wherein, increased PLA2 inhibition was observed in non-polar fraction of the extract and increased neutralization of venom is seen when non-polar fraction was administered. The degree of inhibition of PLA2 and neutralization of venom is found lesser in polar fraction compared to non-polar fraction. Comparatively high PLA2 inhibition and neutralization noted in non-polar fraction may be due to synergistic action of all the long chain unsaturated fatty acids. However the polar fraction also shows significant inhibition of PLA2 (*in vitro*) and remarkably neutralizes venom in *in vivo* study. Therefore presence of compounds exhibiting antivenom property in the polar extract cannot be neglected. Their effect may be diminished either due to the concentration or dilution with other polar compounds or due to their comparison with synergistic action of fatty acids present in non- polar fraction.

The LC/MS analysis of CGMR indicated the presence of 40 diverse compounds in the extract (**Table- 5.5**). However none of the compounds bound to PLA2 in docking study. On the other hand the *in vitro* study provides evidence of some PLA2 inhibitors in the polar fraction of the extract (IC50=120μg/ml). May the concentration of these inhibitors in the extract is not

significant enough and therefore very low inhibition is seen even at 100µg/ml of extract concentration. However other pharmacological benefits that accrue from these compounds cannot be neglected. Hence a list of their known pharmacological actions is given in Table 6.1 Many of these compounds are identified to possess important pharmacological actions. Anti-inflammatory compounds like cosmosiin, fluoxymesterone, and capsaicin supports the anti-inflammatory effect exhibited by the extract (Mandal 2017). Presence of antioxidant compounds like hemmatomic acid, fraxidin methyl ether and piperine substantiates the free radical scavenging property of the extract (Biswasroy *et al*. 2020). Steroids like Fluoxymesterone, Desoxycorticosterone acetate, 9a-Fluoro-Bhydroxyandrosterone and Hydroxyprogesterone may be responsible for extracts steroid like activity. Cardenolides like calactin, uzarigenin and calotropin possessing steroidal ring system have been isolated from this plant (Chan *et al*. 2017). These cardenolides molecules are glycosides and shown to cause cytotoxicity and hence used in cancer treatment (El-Seedi *et al*. 2019). Traditionally, they are used as heart tonics to treat arhythmmias. (You *et al*. 2013) (Wen et al.2016). Surprisingly, our GC/MS data do not show the presence of any cardenolides. Reserpine and cosmosiin are known to posses' antivenom activity but their mechanism of action is not well understood. Cosmosiin is an anti-inflammatory agent possessing anti platelet aggregation activity. The venom neutralizing potential has been investigated in a study (Chaves *et al*. 2011). It may be countering the venom action by modulating the platelet action. Historically blood thinners and anticoagulants have been used to treat snakebite victim. However their use in snakebite treatment is contraindicated due to increased comorbidities that occur post treatment (Levine *et al*. 2014). Reserpine has been used as a medication for snake bite treatment; however the mechanism of its action remains unclear. A study has investigated its antivenom property but there is no details of its mode of action(Sivaraman *et al*. 2020) (Boopathi 2019). The antivenom property of piperine and its derivatives have been

investigated in the past (Shenoy *et al*. 2013). But the mechanism of its action is not elucidated, so far. The inhibition of PLA2 activity seen in this study by polar fraction may be due their combined and non-specific action.

Table 6.1: Pharmacological actions of compounds obtained from LC/MS analysis of CGMR

SL No	Compound name	Compound type	Pharmacological action
1	Lactulose	Carbohydrate	Management of constipation (Kot and Pettit-Young 1992)
2	D-Pantetheine 4'-phosphate	Vitamin	Carrier of acyl residues in metabolic reactions (Czumaj *et al*. 2020)
3	Triparanol	Stilbenoid	Anticancer (Bi *et al*. 2012), Lowers
4	isoamyl nitrite	Alkyl nitrite	Vasodilator, cyanide antidote and anti-hypertensive (Kielbasa and Fung 2000)
5	Cosmosiin	Flavonoid	Anti-inflammatory, Antiplatelet aggregation activity (Chaves *et al*. 2011)
6	Phenylpyruvic acid	Keto acid	Inhibition of macrophage migration inhibition factor (Carpy, Haasbroeck, and Oliver 2004)
7	Fluoxymesterone	Steroid	Suppression of testosterone and spermatogenesis (Jones *et al*. 1977)
8	Desoxycorticosterone acetate	Steroid	Addisons disease treatment (Mccullagh and Ryan1940)
9	Etretinate	Retinoid	Treatment of psoriasis and dyskeratoses (Ward *et al*. 1983)
10	9a-Fluoro-Bhydroxyandrosterone	Steroid	Psoriasis treatment (Imaizumi *et al*. 1975)
11	Haematommic Acid, Ethyl Ester		Antioxidant (Whang *et al*. 2005a)
12	Betaxolol		Glaucoma treatment (Buckley, Goa, and Clissold 1990)
13	Fraxidin Methyl Ether	Coumarin	Antioxidant (Whang *et al*. 2005b)
14	Isopimpinellin	Coumarin	Anti-HCV activity (Widyawaruyanti *et al*. 2021)
15	Hydroxyprogesterone	Steroid	Prevention of preterm delivery (Facchinetti and Vaccaro 2009)
16	Normorphine	opioid	Analgesic (Lasagna and De Kornfeld

			1958)
17	Galanthamine	alkaloid	Alzheimers, and Dementia treatment (Scott and Goa 2000)
18	Norcodeine	opioid	Sedative property (Fraser, Isbell, and Horn 1960)
19	Piperine	alkaloid	Inhibition of lethality, necrosis, defibrinogenation and hemorrhage induced by Russells viper venom (Shenoy *et al*. 2013)
20	Methyl Reserpate	alkaloid	Present in Rouwolfia serpentina n
21	Nalorphine	opioid	Analgesic (Paul *et al*. 1991)
22	p-Hydroxypropoxyphene		Treatment of pain (Launay-Vacher *et al*. 2005)
23	Nafronyl		Vasodilator used in treatment of claudication (Goldsmith and Wellington 2005)
24	Reserpine		Antivenom
25	Oleamide	Fatty acid	Used as hypnotic (Mendelson and Basile 2001)

6.4 In silico analysis

Docking analysis indicated that inhibitors of PLA2 are present in the extract. According to obtained GCMS data, n-Hexadecanoic acid ($Ki=1.58 \times 10^{-5}$) a known competitive inhibitor of PLA2 is found in the extract. It interacted with catalytic residue (His48) of PLA2. Interestingly, three other phyto-chemicals found in the extract, oleic acid, 9, 12 Octadecadienoic acid and allyl octadecyl ester oxalic acid also formed interactions with His48 of PLA2. Moreover all three compounds including n-Hexadecanoic acid, showed similar type of chemical interactions with active site residues of PLA2. Allyl octadecyl ester oxalic acid, in particular seems to be a potent inhibitor of PLA2, as it form two H bonds with His48. Further the bond lengths (2.38Å and 2.24Å) are within the prescribed bond lengths for strong binding (L. Ferreira *et al*. 2015). Using the PLIP visualization tool the bond lengths can be adjusted to 4Å that provides more non-specific interactions. When bond lengths were set to 4Å allyl octadecyl ester oxalic acid interacted with more crucial residues like Tyr119

and ASP40. Tyr119 is a gateway to the active site groove and its blockage stops the ligand from entering the active site groove (Fernandes *et al*. 2015). Few other known inhibitors like rosmarinic acic , aristolochic acid and caffeic acid inhibit PLA2 through this mechanism. Asp49 is involved in catalysis, and its blockade is known to relieve myonecrosis (Mora-Obando *et al*. 2014). Together the studies indicate the presence of a potent inhibitor in non-polar fraction of the extract.

Further the Lipinski's rule of five which is generally applied for evaluating druggability of the compound is not performed in this study. As the rule holds good for only oral drugs and not for non-oral drugs (Choy and Prausnitz 2011). From literature survey it was predictable that polar fraction with more phenols and flavonoids may have higher antivenom potential compared to phenol and flavonoid deficient non-polar fraction. Several studies have investigated the anti-PLA2 potential of phenols and flavonoids studies (E. Arnold et al. 2015) (Lindahl and Tagesson 1997). Surprisingly in this study the non-polar fraction inhibits PLA2 enzyme effectively. So it can be concluded that PLA2 inhibition by CGMR is because of saturated and unsaturated fatty acids like n-hexadecanoic acid and oleic acid but not due to phenols or flavonoids.

All the findings are supportive and reassert the use of *C.gigantea* root extract as phyto-antidote for snake bite treatment. However, the study has provided only a snapshot of phyto-chemicals present in the extract and their possible roles in venom neutralization. The toxicity of the extract, adverse reaction and efficacy of the drug in comparison to polyvalent anti snake venom is not elucidated. Hence in-vivo studies have to be conducted to collect more reaffirming results.

6.5 Toxicity study

The roots of this plant, in particular, are regarded as highly medicinal and harmful as well, by local population. Despite its toxic nature, the root extract is traditionally used as a natural contraceptive, expectorant, purgative and laxative by local healers, making its usage controversial. In addition, the toxicological properties of the root extract have not been comprehensively studied so far, which would guarantee the traditional claim. In acute toxicity study, oral administration of limit dose (2000 mg/kg b.wt) does not result in any mortality and no signs of toxicity are recorded in mice throughout the 14 days experimental period. The behavioral, morphological and physiological parameters are found to be normal and comparable with control group animals. In the 28 day sub-acute study, all the endpoints recorded such as mortality, morphology and behavior changes, food intake, water consumption, bodyweight gain, relative organ weight, hematological analysis, biochemical analysis, and histopathological examination were observed to be normal in all animals, treated with low dose (100mg/kg.bwt) and moderate dose (200 mg/kg b.wt). But in high dose group (400 mg/kg b.wt), statistically significant increase in food intake, water consumption, and bodyweight gain was recorded. Contrary to our finding other studies have documented a decrease in bodyweight following *CGMR* administration. The high dose group animals also showed significant decline in platelet count which indicates antithrombotic effect of the extract. The SGOT levels increased compared to control group, but the increase was not statistically significant. The histopathological examination of liver and heart tissue section indicated necrosis, suggesting that the extract is hepatotoxic and cardiotoxic at high dose. The study for the first time has investigated the toxicological properties of *CGMR* in mice, and found that the extract is safe for oral use below 400 mg/kg b.wt.

The oral administration of *CGMR* at a concentration of 100, 200 and 400 mg/ kg body weight did not alter the morphological characteristics or general behavior of animals, and there was

no mortality recorded in any of the groups. All the treated animals were found to be healthy with no signs of lethargy, weakness, heavy breathing and self-isolation for 28 days, especially at higher doses. However a slight aggressive behavior was observed in animals treated with higher dose and restraining the animal while administering became difficult as the experiment progressed, when compared with control group or lower and moderate dose group animals.

6.6 Neutralization of venom

In vivo experiment conducted in this study confirmed the venom neutralization ability of the extract. The low dose of the extract (100mg/kgb.wt) delayed the death significantly compared to venom treated group. As the concentration of the extract increased the survival rate improved. Neutralization of venom experiment was conducted to check the effect of plant secondary metabolites that inhibit venom components. The results obtained from docking and *in vitro* studies gave hints about the presence of inhibitors in *CGMR* extract. So pre-incubation of venom with extract at 37 ºC for 1hr before administration could provide ample time for inhibitors of PLA2 to act. The result obtained in *in vitro* neutralization was better than the *in vivo* neutralization suggesting that PLA2 inhibition by fatty acids (n-hexadecanoic acid, oleic acid) present in CGMR extract play a crucial role in neutralization of venom.

The results of this experiment guarantees the traditional claim made by few tribal healers, who apply crushed *C.gigantea* root extract on the snake bite site. Snake venom is a fast acting toxin and oral consumption of medication may delay the action of drug against venom components(Bhutani, Basu, and Majumdar 2021). In Sundargarh district of Orissa the extract is mixed with cow's milk and taken orally (Dey and De 2011). Also certain plant compounds are used as prophylactics to prevent snake bite. May be the orally consumed Calotropis gigantea has a long acting effect and prevents the person from snake bite. In Nigeria for

instance Velvet beans (*Mucana pruriens*) seed is taken as a oral prophylactic for snake bite treatment(Fung *et al*. 2009).

Several plant extracts have been demonstrated to neutralize snake venom, in particular viper venom (Chandrashekara *et al*. 2009) (Dhananjaya *et al*. 2011) . The main focus of these studies is to evaluate the anti-mytotoxic, anti-hemorrhagic, and anti-neurotoxic properties of the plant extract. But in this study we have seen the neutralization ability and focused more on the biochemical changes that take place at different time points after envenomation. Together these studies confirm the antivenom potential of *Calotropis gigantea* root extract against hemorrhagic venom components of *Daboia russelii*. The study confirms that the extract apart from neutralizing also has significant protective function. Finally, the research investigations conducted in this study have validated the traditional claim and support the application of *Calotropis root* extract to treat snakebite victims.

Summary

Man's connection with nature is one of give and take. Our reliance on nature, both directly and indirectly, is inevitable. Our long-standing reliance on nature to extract medicinal benefit from plant items extends back centuries. Modern improvements in medication discovery and treatment procedures, on the other hand, have eroded traditional knowledge of plant-based remedies. However, in recent years, these therapies have been explored in order to harness their pharmacological worth, particularly in the treatment of long-standing diseases such as snakebites. Tribes with little interaction with the outside world have retained these age-old medical traditions, which must be explored to offer scientific evidence in order to accomplish their pharmacological potential.

Snake bites are a long-standing health hazard that has resulted in substantial mortality all across the world. The lacks of available first aid, lack of awareness, and limitations of standard antivenom therapy have prompted researchers to look into newer therapeutic options. Traditional medications are currently being investigated as alternatives, and several plants with antivenom potential are being investigated. Many Indian tribes, such as the *Bagata, Meena*, and *Damor*, have used the root extract as antivenom, however there is no scientific evidence to support this practise.In view of the foregoing, the purpose of the study investigate the antivenom potential of *Calotropis gigantea* root extract, which is frequently used as an antidote for snake bites.

In this study, the *Calotropis gigantea plant* was identified with the help of its botanical characteristics and the roots were isolated. A methanolic extract of the roots was prepared using conventional scientific procedures and the polar and non-polar phytochemicals were separated by fractionation. The PLA2 inhibition was determined in crude, non-polar and polar fractions using *in vitro* methods. The crude extract showed a highest inhibition of PLA2 activity. And non-polar fraction had higher inhibition compared to polar fraction. This

indicated the presence of few inhibitors in the non-polar fraction. The phytochemicals present in the extract was profiled using high performance chromatographic techniques, viz GC/MS and LC/MS. The compounds were virtually docked with PLA2 to study the protein ligand binding interaction. n-hexadecanoic acid a known competitive inhibitor was identified in the extract and the study found a novel PLA2 inhibitor, allyl octadecyl oxalic acid present in the extract. However the *in vitro* and *In silico* analysis do not reflect the real setting. Therefore *in vivo* experiments were performed for better acceptability of the research data.

The extract at a low dose of 100mkg/kg b.wt was able to delay the death of the animal in the *in vivo* neutralization experiment. The 400mg/kg b.et group significantly neutralized the venom when compared to antivenom group. The results of this experiment indicate the remarkable antivenom potential present in the extract. The *in vitro* neutralization experiment provided proof for the antivenom mechanism of action of the extract. As pre-incubation provided ample time for the inhibitors of snake venom components present in the extract to act. The extract completely neutralized the venom in case of *in vitro* neutralization experiment at a high dose. No deaths were recorded in high dose group when compared to antivenom group. The protection effect of the extract against snake venom was significant. Together the studies have provided considerable proof for the traditional claim.

Conclusions

- The sub-acute toxicity study results confirm the anti-platelet activity of the extract. The histopathology analyses confirm the mild toxicity associated with liver and heart at higher dose (above 400mg/kg b.wt) and therefore the extract should be used cautiously.

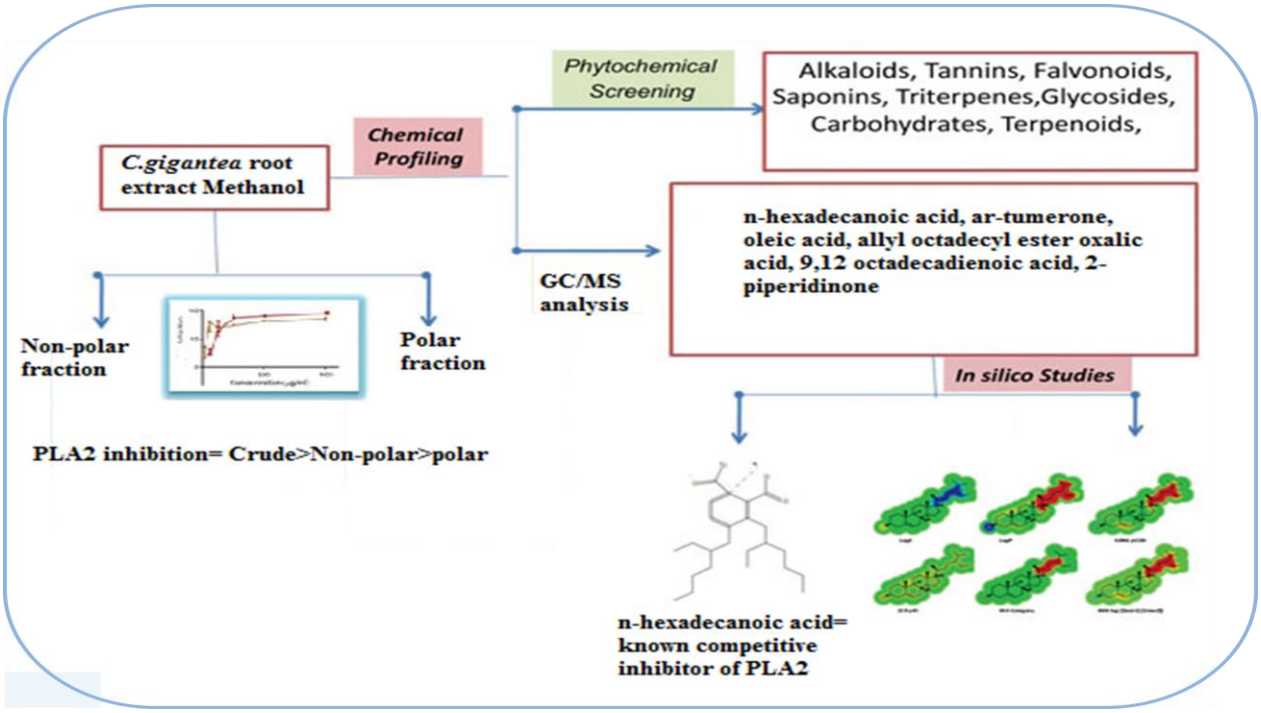
- The competitive inhibitors of PLA2 like n-hexadecanioc acid may contribute to venom neutralization. Further allyl octadecyl oxalic acid ester may be researched as a potent PLA2 inhibitor.

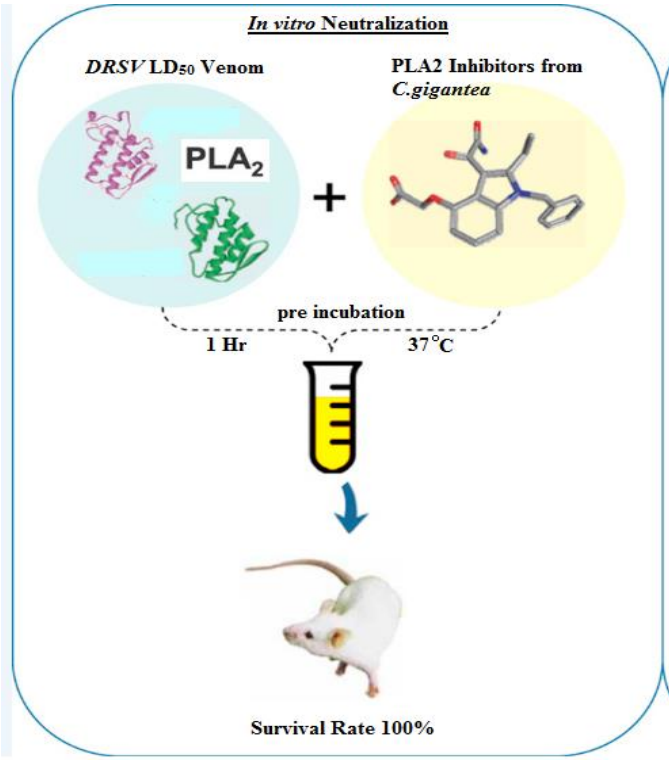
- The extract significantly neutralized venom action even at a low dose and also showed better neutralization with *in vitro* studies which emphasizes the presence of inhibitory compounds in the extract against snake venom PLA2

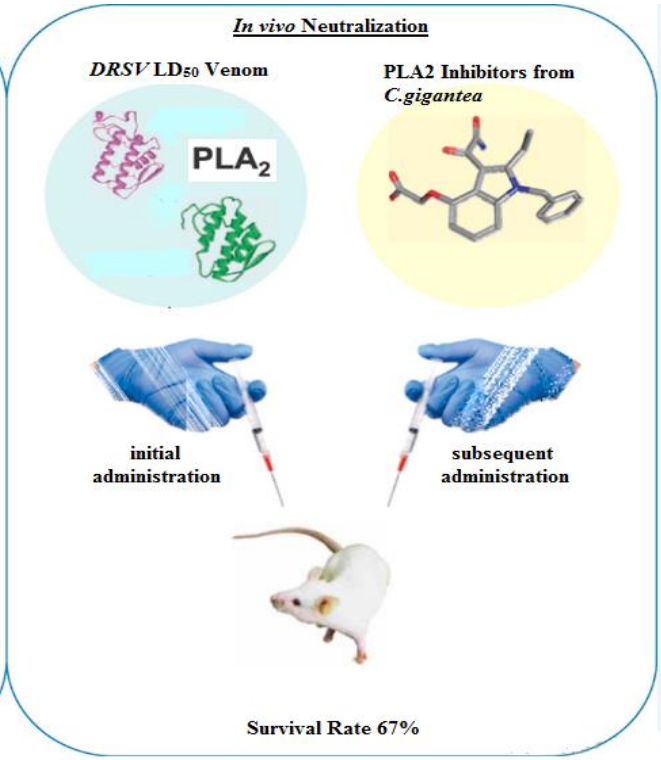
- The extract enhanced the coagulation ability of incoagulable blood (venom treated blood) and therefore confirms the possible prophylactic role of the extract against venom insult.

- The above findings guarantee the traditional claim of the extract which is used as antidote for snake bite.

Graphical abstract







Future perspectives of the study

The *in vitro* experiments conducted in this study confirm that the non-polar fraction of the extract (hexane fraction) possess PLA2 inhibition activity. The *in silico* experiments conducted in this study has identified the phytochemicals responsible for PLA2 inhibition. Although a known competitive inhibitor n-hexadecanoic acid was found in the extract, *in silico* studies have supported the possible role of allyl octadecyl oxalic acid ester as a possible PLA2 inhibitor. Further the *in silico* interaction studied here suggest that allyl octadecyl oxalic acid ester is a more potent inhibitor of PLA2 than n-hexadecanoic acid, as it forms two important H bonds with the active site His48. In future this compound can be tested and confirmed for its possible utility as a potent PLA2 inhibitor.

The *CGMR* extract has high medicinal properties and is traditionally used to treat arthritis, snakebites, bronchitis and eczema, however the toxicological properties of the extract was not studied so far. The toxicity findings of this study confirm that the extract is mild toxic above 400mg/kgb.wt. Slight cardiotoxicity and hepatoxicity was observed. In addition anti –platelet activity of the extract was evidently observed. Therefore in future the extract should be cautiously used with proper treatment advice.

Despite its wide use in traditional and folkloric medicine there was no comprehensive analysis of phytochemicals of the extract. Studying the phytochemical profile of the extract explores the mechanism of action of the extract. The GC/MS and LC/MS analysis data in this studied has identified about 50 pharmacologically important compounds present in the extract. This data is a goldmine for ascertaining the role and action of extract in different pathophysiology.

The drawbacks of antivenom administration and the clinical consequences associated with its use have made its use controversial. Current researchers are drawn towards alternative ways of neutralizing the snake venom. In this regard numerous plant extracts have been tested and demonstrated to exhibit antivenom property. *Calotropis gigantea* root extract though traditionally used for snakebite has not been evaluated for its antivenom property so far. The findings of *in vivo* neutralization and *in vitro* neutralization confirm the antivenom activity of *CGMR*. Further at a high dose 400mg/kg b.wt significant neutralization was observed in comparison to antivenom treated group. The extract exhibited antivenom property (delaying the death of envenomed animal) even at a low dose of 100mg/Kg.bwt. In future the antivenom property of this extract may be evaluated further and possibly may be used as a replacement for antivenom administration as the side effects observed with natural treatments is insignificant.

The extract has demonstrated the prophylactic action against venom challenge significantly. This is evident from the bleeding time, clotting time and histopathological analysis performed as a time based study in this work. The biochemical changes that the extract induced in the treated animals can provide insights into its mechanism of neutralization of venom. This aspect can be studied in future for developing novel pharmacological antivenom compounds.

Limitations of the study

The study used *in silico* methods to propose allyl octadecyl oxalic acid ester as a novel and effective inhibitor of PLA2 activity. It cannot be considered as a conclusive result. Molecular dynamics simulation study [MDS] in combination with docking analysis would be better accepted. Even so, *in silico* analyses are only simulation-based research that may or may not reflect the molecule's true biological function.

The study has failed to analyze whether the extract is acting against myotoxic, neurotoxic or hemotoxic property of the venom. The polar and non-polar fraction may be acting in different modes to neutralize the venom. This finding would have made the study more interesting and added more future perspectives.

The results of study have confirmed the presence of PLA2 inhibitors in the non-polar fraction. But further analysis and scientific deliberation is required to see the activities of these compounds in the *in vivo* experiment.

The assessment of biochemical changes in protection of *CGMR* against venom action is elusive and could be included with better hematological markers like activated thromboplastin time and determination of clotting factors. However inclusion of these parameters was difficult during the actual experiment due to the fast action of venom components and mixed action of venom components on each animal.

Though the results of this study are corroborative the actual use of the extract as an anti-venom is possible only after clinical trials and other adverse reaction experiments. It is premature to conclude and state that the extract could be a possible replacement for anti-venom. Nevertheless the study has attempted to provide scientific data and ample proof for its anti-venom property and reasserted its traditional value.

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Annexure I: Plagiarism report

BLDE (DEEMED TO BE UNIVERSITY)

Annexure -I

PLAGIARISM VERFICATION CERTIFICATE

1. **Name of the Student:** Vikram P **Reg No:** 13PHD003
2. **Title of the Thesis :**
   "Evaluation of Daboia russelii Venom Induced Biochemical Changes in Calotropis gigantea (L). R.Br Treated Mice"
3. **Department:**Biochemistry
4. **Name of the Guide & Designation:** Dr. Basavaraj Devaranavadgi, MD, Professor and Head, Department of Biochemistry, Shri B.M Patil Medical College, Hospital and Research Center, BLDEU (Deemed to be University), Vijayapur, Karnataka
5. **Name of the Co Guide & Designation:** Dr. Achuthan Raghava Menon, Associate Professor, Department of Biochemistry, Amala Cancer Research Center, Amala nagar, Thrissur, Kerala

The above thesis was verified for similarity detection. The report is as follows:

Software used: Ouriginal **Date:** 23/07/21

Similarity Index (%): 6% **Total word Count:** 20430

The report is attached for the review by the Student and Guide.

The plagiarism report of the above thesis has been reviewed by the undersigned. The similarity index is below accepted norms.

The similarity index is above accepted norms, because of following reasons:

……

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……

The thesis may be considered for submission to the University. The software report is attached.

Dr. B B Devaranavadgi
Signature of the Guide
Name & Designation
Professor & HOD
Dept. of Biochemistry
BLDE (Deemed to be University)
Shri B.M.Patil Medical College,
Vijayapur-586103.

Dr. Achuthan C. Raghavamenon, Ph.D,
Signature of Co-Guide
Name & Designation
Associate Professor
Dept. of Biochemistry
Amala Cancer Research Centre
Amala Nagar, Thrissur-680555, Kerala

Signature of Student

Verified by (Signature)
Name & Designation
Librarian
B.L.D.E. Deemed to be University
Shri B. M. Patil Medical College,
Vijayapur.

Annexure II: Animal ethical committee certificate

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Amala Cancer Research Centre

(A Society Registered T. C Act, XII of 1955 sl. No. 56 of 1984)

MANAGING DIRECTOR : FR. FRANCIS KURISSERY, C M I
RESEARCH DIRECTOR : DR. RAMADASAN KUTTAN, Ph.D

AMALANAGAR - 680 555, THRISSUR
KERALA, INDIA

Ref :

Date: 04.11.2015

ANIMAL ETHICAL COMMITTEE CERTIFICATE

This is to certify that the institutional animal ethical committee approves the PhD thesis work entitled **"Evaluation of *Dabolia russelli* venom induced biochemical changes in *Calotropis gigantea* (L) Br. treated mice"** submitted by Mr. Vikram P.

Dr. Jos Padikkala
Secretary, IAEC
Amala Cancer Research Centre

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Amala Cancer Research Centre

Annexure III: Institutional Animal Ethical Clearance

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Institutional Animal Ethical Committee

(Reg. No. 149/PO/Rc/S/1999/CPCSEA)

Amala Cancer Research Centre

RESEARCH DIRECTOR &
CHAIRMAN, IAEC : DR. RAMADASAN KUTTAN, Ph.D.

AMALANAGAR - 680 555, THRISSUR
KERALA, INDIA

Ref :

Date:

Approval No: ACRC/IAEC/18(2)-P7

18.12.2018

CERTIFICATE

This to certify that the project entitled "Evaluation of *Daboia russelli* venom induced biochemical changes in *Calotropis gigantea* root extract treated mice" has been approved by the Institutional Animal Ethical Committee.

Signature with date

18.12.2018

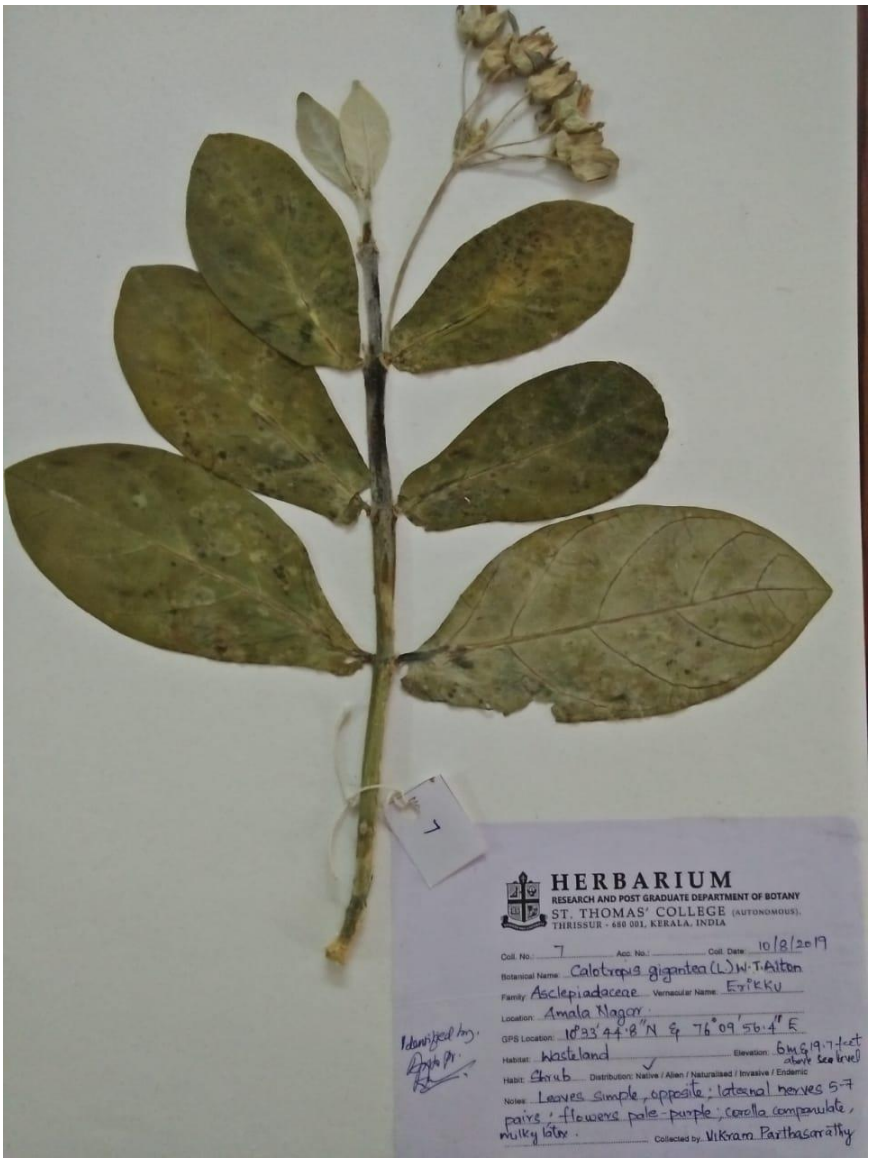
Name of Chairman/ Member Secretary IAEC
Dr. Ramadasan Kuttan Ph.D.

18/12/18

Name of CPCSEA Nominee
Prof. (Dr.) E Vijayan Ph.D.

(Kindly make sure that minutes of the meeting duly signed by all the participants are maintained by office)

Annexure IV: Herbarium specimen of *Calotropis gigantea*



Annexure V: Presentations and awards

- **Second prize**, **Oral Paper Presentation** – *In silico* and *In vitro* approaches to evaluate anti-snake venom potential of *Calotropis gigantea*, ACBICON-2018, South zone, Manipal

- **Poster presentation** at – Evaluation of *Daboia russelii* venom induced biochemical changes in *Calotropis gigantea* treated mice. IABS -2019, NIIST, Thiruvananthapuram.

- **Poster presentation** - Identification of *Daboia russelii* snake venom Phospholipase A2 [PLA2] inhibitors present in methanolic root extract of Calotropis gigantea. BLDE 2020, Research day meet.

Annexure VI: Publications

- **Vikram Parthasarathy**, Achuthan Raghava Menon, Basavraj Devaranavadgi, *Identification of anticancer targets of calactin, calotropin and calotoxin using reverse screening strategy*. Current Drug Discovery Technologies. Curr Drug Discov Technol - Bentham Science. Dec7 2020. online ahead of print - https://pubmed.ncbi.nlm.nih.gov/33292137/

- **Vikram Parthasarathy**, Achuthan Raghava Menon, Basavraj Devaranavadgi, *in silico approaches to demonstrate uzarigenin and calotropagenin as potential carbonic anhydrase II (CAII) inhibitors*. IJPR, Jun 2020, Sup (1) ; 428-33

- **Vikram Parthasarathy**, Achuthan Raghava Menon, Basavraj Devaranavadgi, *Identification of Daboia russelii snake venom Phospholipase A2 [PLA2] inhibitors present in methanolic root extract of Calotropis gigantea*. IJPR, Jun 2020. Sup (1); 1191-95

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MANIPAL 25

Association of Clinical Biochemists of India - South Zone Conference 2018

Theme: Recent trends in Biochemistry Education, Diagnostics & Research

Certificate of Participation

This is to certify that

Vikram Parthasarathy

has presented a Scientific Paper (Oral / Poster)

titled IN-SILICO & IN-VITRO APPROACHES TO DEMONSTRATE ANTI-SNAKE VENOM POTENTIAL OF CALOTROPIS ROOT

in "Association of Clinical Biochemists of India - South Zone Conference"

organized by Department of Biochemistry, Kasturba Medical College, Manipal, MAHE held between 7 & 8 December 2018

Karnataka Medical Council has granted **FOUR credit hours** for delegates. Vide letter no KMC/CME/517/2018 Dated: 12-11-2018

Dr Krishnananda Prabhu R V
Organizing Secretary
Professor & Head, Department of Biochemistry, KMC Manipal

Dr Pragna Rao
Dean
Kasturba Medical College, Manipal

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Association of Clinical Biochemists of India - South Zone Conference 2018

Theme: Recent trends in Biochemistry Education, Diagnostics & Research

Certificate of Appreciation

This is to certify that

Dr. Vikram Parthasathy

has won Second Prize in Scientific Paper (Oral / Poster) Presentation

titled IN-SILICO & IN-VITRO APPROACHES TO DEMONSTRATE ANTI-SNAKE VENOM POTENTIAL OF CALOTROPIS ROOT

in "Association of Clinical Biochemists of India - South Zone Conference"

organized by Department of Biochemistry, Kasturba Medical College, Manipal, MAHE

held between 7 & 8 December 2018

Dr Krishnananda Prabhu R V
Organizing Secretary
Professor & Head, Department of Biochemistry
KMC Manipal

Dr Pragna Rao
Dean
Kasturba Medical College, Manipal
Manipal Academy of Higher Education, Manipal

8th Annual Meeting of Indian Academy of Biomedical Sciences and Conference on Deliberation on Translation of Basic Scientific Insights into Affordable Healthcare Products

February 25-27, 2019

Certificate

This is to certify that Dr. / Mr. / Ms. Vikram Parthasarathy *has participated / delivered an Oral / Poster presentation entitled* "In silico and in vitro approaches to demonstrate anti-snake ..... gigantea roots."

in the 8th Annual Conference of Indian Academy of Biomedical Sciences held at CSIR-NIIST, Thiruvananthapuram, Kerala.

Dr. Hariom Sharma
President, IABS 2019

Dr. K.G. Raghu
Organising Secretary, IABS 2019

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Shri B. M. Patil Medical College, Hospital & Research Centre, Vijayapura, Karnataka, India

Research Day Celebration

Certificate of Merit

This is to certify that **Dr. Vikram Parthasarathy,** Department of Biochemistry, BLDE(DU)'s Shri B. M. Patil Medical College,Hospital and Research Centre, Vijayapura has been awarded **'RESEARCH DAY CERTIFICATE OF MERIT'** in Postgraduate/**Ph.D. Scholars**/Faculty Category for his exceptional presentation during Research Day Celebration of BLDE (Deemed to be University) on July 20, 2020.

Prof. Dr. Aravind V. Patil
Dean, FoM & Principal

July 20, 2020

Dr. Akram Naikwadi
Dean, R & D

In silico and *in vitro* approaches to demonstrate anti-snake venom potential of *Calotropis gigantea* roots

Vikram Parthasarathy [1], Basavaraj Devaranavadgi[1], Achuthan Raghava Menon [2]

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2. Department of Biochemistry, Amala Cancer Research Center, Trichur, Kerala.

Background

- In India, snake bite fatalities are mainly attributed to 'big four' species. King cobra (*Naja naja*), Russell's viper (*Daboia russelli*), Common krait (*Bungarus caeruleus*), and Saw scaled viper (*Echis carinatus*).
- Snake venom is composite that contains neurotoxins, disintegrins, hemotoxins, proteases, phospholipase A2 [PLA2] enzymes and DNases. The toxicity is mainly attributed to PLA2 enzymes or their protein complexes per se.
- Though polyvalent anti-snake venom is widely used for treatment, indigenous tribes apply Calotropis gigantea (Asclepiadaceae) root paste locally on bite sites.
- Pertinent to above traditional practice, in this study we have investigated the anti snake venom potential possessed by *C.gigantea* roots.

MATERIALS & METHODS

- *C.gigantea* root was extracted in methanol.
- In vitro inhibition of PLA2 activity by extract was assayed.
- GC-MS analysis of C.gigantea extract to ascertain phyto-chemicals was performed.
- Docking analysis- Phyto-chemicals (ligands) were virtually screened for their binding ability with PLA2 (macromolecule) using Autodock v. 4.2

RESULTS

- The methanolic crude extract (IC_{50}=50 µg/ml), non-polar fraction (IC_{50}=75 µg/ml) and polar fraction (IC_{50}= 120 µg/ml) of the root extract effectively inhibited PLA2 activity *in vitro*.
- Non-polar fraction inhibited PLA2 with more efficacy than polar fraction.
- The GC-MS profile of crude methanolic extract indicated the presence n-Hexadecanoic acid (K_i=1.58 x 10^{-5}) a known competitive inhibitor of PLA2.
- Docking analysis revealed that n-Hexadecanoic acid (ligand) formed salt bridge with catalytic residue (*His48*) of PLA2 (PDBID-3CBI, macromolecule).
- Interestingly, three other phyto-chemicals found in the extract, oleic acid, 9, 12 Octadecadienoic acid and allyl octadecyl ester oxalic acid also formed salt bridges with His48 of PLA2.
- Moreover all three compounds including n-Hexadecanoic acid, showed similar type of chemical interactions with active site residues of PLA2.

Discussion

- In this study, we ascertained the inhibitory effect of crude methanolic extract of *C.gigantea* root on PLA2 activity (most toxic component of venom) through in-vitro inhibition assay.
- Further GC/MS data indicates the presence n-hexadecanoic acid (4.3%), a known competitive inhibitor of PLA2.
- Docking analysis has revealed the possible inhibitory role of other phyto-chemicals like allyl octadecyl ester oxalic acid, oleic acid, 9, 12, octadecadienoic acid. .
- Docking analysis revealed that n-Hexadecanoic acid (ligand) formed salt bridge with catalytic residue (*His48*) of PLA2 (PDBID-3CBI, macromolecule).
- All the findings are supportive and reassert the use of *C.gigantea* root extract as phyto-antidote for snake bite treatment.

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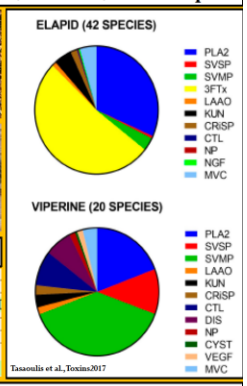
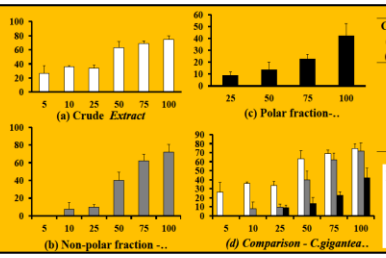
"Big 4 Snakes"- 1. Cobra , 2. Russells Viper , 3. Krait , 4. Saw Viper



Multiple Sequence Alignment of PLA2

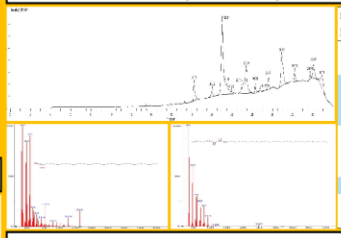
In vitro inhibition of PLA2



Conc. Of extract (µg/ml)	Crude extract	Non-polar fraction	Polar fraction
5	26.22±10.92	-	-
10	35.66±1.60	7.69±7.42	-
25	33.57±4.73	9.79±2.97	8.74±2.97
50	62.94±9.04	39.69±9.64	13.46±6.68
75	69.06±3.71	62.24±7.42	22.90±3.71
100	74.48±5.17	71.68±8.9	42.31±10.38

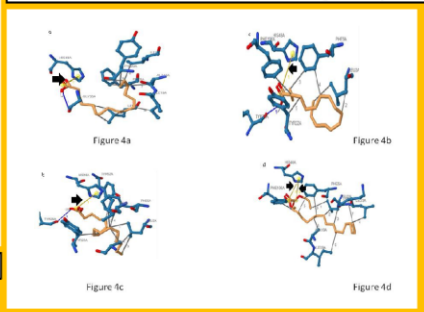
Keys-

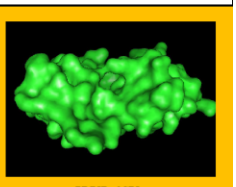
GC-MS Analysis of Phyto-chemicals in *C.gigantea* roots



SL No.	Compound name	RT	A %
1.	Ar-Tumerone	13.61	0.28
2.	n-Hexadecanoic acid	18.58	4.37
3.	Oleic acid	20.21	1.72
4.	9,12 Octadecadienoic acid	21.42	29.38
5.	Oxirane, tetradecyl	21.85	3.71
6.	Z-10 Pentadecen-1-ol	22.47	1.15
7.	2-Piperidinone	23.01	16.71
8.	Sulfurous Acid, Octadecyl pentyl ester	23.96	9.41
9.	Oxalic acid, allyl octadecyl ester	25.78	2.22
10.	1-Decanol, 2-Hexyl	27.29	12.1

Docking Analysis





PDBID- 3CBI

Compound name	Leu 2	Phe 5	Ala 18	Ile19	Tyr 22	Gly 30	His 48
Ar-Tumerone	**	*	*	*	*		
n-Hexadecanoic acid	***	**	*	*	*	H	S
Oleic acid	***	*	*		*		S
9,12 Octadecadienoic acid	**	**			*		S
Oxirane, tetradecyl	***	**	*	*	*		
2-Piperidinone	*	**			*		
Sulfurous Acid, Octadecyl pentyl ester	****	**	*	*	*		
Oxalic acid , allyl octadecyl ester	***	**	*	*			S S
1-Decanol, 2-Hexyl	**	***	*		*	H	

Curr Drug Discov Technol. 2020 Dec 7. doi: 10.2174/1570163817666201207143958.
Online ahead of print.

Target Fishing of Calactin, Calotropin and Calotoxin Using Reverse Pharmacophore Screening and Consensus Inverse Docking Approach

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Affiliations + expand

PMID: 33292137 DOI: 10.2174/1570163817666201207143958

Abstract

Background: The anticancer properties of natural products calactin, calotropin and calotoxin are well established. However the mechanisms of their action are unclear and the molecular targets pertinent to them are not detailed. In this study, potential anti-cancer targets of these compounds have been identified using reverse screening approaches that may provide valuable insights into anti cancer drug development.

Objective: To identify the potential anticancer targets of calactin, calotropin and calotoxin using reverse screening strategy.

Methods: The ligands were screened for potential targets based on their shape similarity and pharmacophore model matching. The overlapping targets obtained from both methods were verified using reverse docking approach and validated by docking analysis. MM/PBSA calculation was performed to predict binding affinities between ligand and confirmed targets.

Results: Interleukin-2 inducible T cell kinase [ITK] was confirmed as a potential target of calactin (Ki= -10.3 kcal/mol), calotropin (Ki= -8.7 kcal/mol) and calotoxin (Ki= -10.2 kcal/mol). The ligands interacted with hinge region residues such as Met438 and Asp500 which occupy the highly conserved ATP binding site. Binding energies of calactin (ΔEbind = -29.18 kJ/mol), calotropin (-28.57 kJ/mol) and calotoxin (-21.21 kJ/mol) with ITK were higher than (more negative) positive control sunitinib (-15.03 kJ/mol) and standard staurosporine (-21.09 kJ/mol). Besides this, Interstitial collagenase [MMP1] was confirmed as potential target of calotoxin (Ki= -8.2 kcal/mol).However the binding energy (ΔEbind = -11.89 kJ/mol) was lower compared to positive control batimastat (-21.07 kJ/mol).

Conclusion: The results of this study confirmed ITK as a potential target for calactin, calotropin and calotoxin. These compounds can therefore be used as lead molecules for the development of novel ITK inhibitors, which may have immense therapeutic applications as immune-suppressants and as anticancer drugs.

Keywords: Calactin; Calotoxin; Calotropin; ITK inhibitors; Interleukin-2 inducible T cell kinase [ITK]; Reverse docking; anticancer; immune-suppressants.

ISSN 0975-2366
DOI: https://doi.org/10.31838/ijpr/2020.SP1.178

Research Article

Identification of Daboia russelii snake venom Phospholipase A2 [PLA2] inhibitors present in methanolic root extract of Calotropis gigantea

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Received: 04.04.20, Revised: 03.05.20, Accepted: 02.06.20

ABSTRACT

Background: Calotropis gigantea roots are widely used as folklore medicine to treat snake bite victims. However the exact neutralization mechanism of the extract is not clear. In this study, snake venom phospholipase A2 [PLA2] inhibitors were identified in the root extract. The toxicity of the snake venom is mainly attributed to phospholipase A2 [PLA2] enzymes or their protein complexes per se. Therefore findings of this study provide valuable insights into neutralization mechanism of the extract.

Methods: In this study the phyto-chemicals present in C.gigantea root methanolic extract were profiled using high resolution GC-MS. The phyto-chemicals were specifically docked into crystal structure of Daboia russelii phospholipase A2 [PLA2] to identify possible inhibitors.

Results: The GC-MS profile of crude methanolic root extract indicated the presence of phyto-chemicals like n-Hexadecanoic acid, Oleic acid, 9, 12 Octadecadienoic acid and allyl octadecyl ester oxalic acid. Docking analysis revealed that n-Hexadecanoic acid interacted with catalytic residue (His48) of PLA2 (PDBID-3CBI). Previous studies have shown n-Hexadecanoic acid (K_i=1.58 x 10^{-5}) as a potent competitive inhibitor of PLA2. Interestingly, allyl octadecyl ester oxalic acid also formed similar interaction with His48 of PLA2.

Conclusion: n-Hexadecanoic acid and allyl octadecyl ester oxalic acid were identified as potent inhibitors of Daboia russelii snake venom PLA2. This result provides valuable insights about anti-snake venom potential of Calotropis Gigantea root extract.

Keywords: n-Hexadecanoic acid; allyl octadecyl ester oxalic acid; PLA2, Calotropis Gigantea

INTRODUCTION

In India, snake bite fatalities are mainly attributed to 'big four' species. King cobra (Naja naja), Russell's viper (Daboia russelii), Common krait (Bungarus caeruleus), and Saw scaled viper (Echis carinatus) [1]. Though polyvalent anti-snake venom is widely used for treatment, indigenous tribes apply C.gigantea root paste locally on bite sites [2]. Pertinent to above traditional practice, in this study we have investigated Daboia russelii snake venom PLA2 inhibitors present in C.gigantea root extract.

Daboia russelii snake venom is a milieu of neurotoxins, hemorrahgins, 3-finger toxins [3FT], disintegrins, hemotoxins, phospholipase A2 [PLA2], proteases and DNases [3]. However, PLA2 enzymes or their protein complexes are known to be single most toxic component of venom. For example; all known pre-synaptic neurotoxins from snake venom are PLA2 enzymes per se or contain PLA2 as an integral part [4]. PLA2 is present in mammalian tissues, insects, snake venom, bee venom and frog venom [5]. PLA2 (E.C- 3.1.1.4) catalytically hydrolyzes the Sn-2 acyl bond of phospholipids to release arachidonic acid and lysophospholipids. Oxidation of Arachidonic acid by cyclooxygenases generates active inflammatory mediators called eicosanoids-prostaglandins (PG), thromboxanes (TX) and leukotrienes (LT) [6].

Calotropis gigantea (Asclepiadaceae) also called milky weed, is a commonly found shrub across Indian sub continent [7]. Traditionally, leaves, roots and latex of C. gigantea plant are used as phyto-antidote to treat snake bite. However the roots, in particular are extensively used by Indian tribes [8].The traditional practice has been well substantiated by invivo neutralization study wherein 400 mg/Kg b.wt of C.gigantea leaf extract administered to Balb/c mice, substantially neutralized $2LD_{50}$ and $3LD_{50}$ dose of Viper russelli venom [9]. But, the mechanism of neutralization

is not clear and the role of phyto-chemicals in venom neutralization is not well studied.

Despite the widespread use of C. gigantea plant as phyto-antidote for snake bite by tribal people, there are no comprehensive studies that have investigated the venom neutralizing ability of C. gigantea. Though, some studies have investigated the venom neutralizing ability of leaf and latex, a detailed study on root extract of the plant which is extensively used by tribes has been neglected. Comparative analysis of phytochemical constituents in leaf, latex and root extract would provide knowledge about anti-venom property.

METHODS AND MATERIALS

Plant collection

C.gigantea plant was identified and collected from medicinal garden located within Amala medical college campus, Thrissur. The sampling site is located at $10^0$33'44.8"N and $76^0$09'56.4''E Southern India. The average elevation is 6m and 19.7 feet above sea level. A herbarium specimen of plant bearing voucher number KFRI-1770 was deposited at Kerala Forest Research Institute.

Extract preparation

Fresh roots of C.gigantea were obtained after thorough washing with tap water followed by distilled water wash. The roots were shade dried and powdered using mechanical grinder. The powdered root was extracted in methanol. 10 grams of root powder was soaked in 100ml methanol and allowed to stir overnight at a speed of 120 rpm for 24 hrs at room temperature. The mixture was centrifuged at 2000 rpm for 15 min to get a clear supernatant. This procedure was repeated; supernatant was collected, and evaporated to dryness [10]. The dried extract was collected, weighed and reconstituted in adequate volume of methanol.

GC/MS analysis

GC-MS analysis of the methanol extract of C. gigantea root was performed at SAIF, IITB facility, using Thermo Scientific Triple Quadrupole GC-MS (Trace 1300 GC, Tsq 8000 triple quadrupole MS) equipped with TG 5 MS (30 m X 0.25 mm, 0.25 μm) column [11]. Helium was used as the carrier gas at a flow rate of 1ml/min. using an injection volume of 1.0 μL. Injector temperature was kept at 250°C and ion source temperature was 230°C. The oven temperature was maintained at 50°C isothermal at 280°C.

Molecular docking

The phyto-chemicals were docked with PLA2 enzyme of snake venom (Daboia russelli- PDBID-3CBI) using molecular docking software-Autodock Vina version 4.1, to obtain 8 different binding modes of the ligand with PLA2 [12]. The binding energy and predicted Ki was calculated for each ligand. The intermolecular interaction between ligand and PLA2 was analyzed using PLIP software [13].

RESULTS

GC/MS analysis

The methanolic root extract of C.gigantea when subjected to gas chromatography (Figure 1), documented the presence of pharmacologically important compounds (Table 1) like Ar-Tumerone (Relative Area=0.28%), n-hexadecanoic acid (4.37%), oleic acid (1.72%) and allyl octadecyl ester oxalic acid (2.2 %). 9, 12 octadecadienoic acid (29.38%) or linoleic acid (18:2) was noted to be the most abundant bioactive compound in the extract. The identification of compound was based on peak area, molecular weight, molecular formula and the compound structures and matched with National Institute of Standards and Technology [NIST] library data. A brief overview about their biological activity is provided in (Table 2).

Docking studies

The active site residues of PLA2 (D.russelli - PDBID- 3CBI) and the mechanism of actions are detailed clearly (5) (6) . His48 of the enzyme was involved in catalysis. Leu2, Gly30, His48, Ile19, Trp31, Asp99, Lys69, Tyr52, Ser23, Tyr22, Asp49, Phe5, Ala18 residues formed the active site of the enzyme. In this study phyto-chemicals identified in GC/MS data were downloaded as SDF files from zinc database and later converted to PDB files. The phyto-chemicals (ligands) were locally docked to the active site of macromolecule PLA2 (D.russelli - PDBID- 3CBI) using Autodock vina software (version 4.1). The ligand – macromolecule interaction was visualized in pymol molecular graphics system 1.7.x. Finally the molecular interaction between ligand and macromolecule obtained from web based PLIP software was tabulated for each compound (Table 3).

n-Hexadecanoic acid (K_i=1.58 X 10^{-5} M ; IC_{50} = 43.26 X10^{-5} M) a known competitive inhibitor of PLA2 interacted with His48 (catalytic residue of PLA2 enzyme) H bond [14]. Interestingly allyl octadecyl ester oxalic acid also interacted with His48. Moreover both compounds n-Hexadecanoic acid, and allyl octadecyl ester oxalic acid showed similar type of chemical interactions with active site residues of PLA2 (Figure 2) (Table 4).

DISCUSSION

The phytochemical constituents present in C.gigantea leaves (24 compounds) and latex (22

compounds) have been profiled [15]. However, the root extract in-spite of its probable importance in snake bite treatment has not been profiled. Previous studies have documented that ~60% of the compounds were common in both leaf and latex extract while the remainder compounds were unique to either of the extracts studied. In contrast to the above finding GCMS data of root extract revealed a distant phytochemical profile which predominantly consists of unsaturated long chain fatty acids. Most of which were exclusively confined to root. The study confirms the variation between the chemical constituents of leaf, latex and root which shows their different potential of therapeutic activities.

In this study, we ascertained the inhibitory effect of crude methanolic extract of C.gigantea root on PLA2 activity (most toxic component of venom) through docking analysis. Further GC/MS data indicates the presence n-hexadecanoic acid (4.3%), a known competitive inhibitor (K_i=1.58 x 10^{-5}) of PLA2 [14]. Docking analysis has revealed the possible inhibitory role of other phyto-chemicals like allyl octadecyl ester oxalic acid. All the findings are supportive and reassert the use of C.gigantea root extract as phyto-antidote for snake bite treatment. However, the study has provided only a snapshot of phyto-chemicals present in the extract and their possible roles in venom neutralization. The toxicity of the extract, adverse reaction and efficacy of the drug in comparison to polyvalent anti snake venom is not elucidated. Hence in-vivo studies have to be conducted to collect more reaffirming results.

CONCLUSION

The study has identified potential inhibitors of D.russelii snake venom PLA2 present in C.gigantea root extract. Both, n-hexadecanoic acid and allyl octadecyl ester oxalic acid can be used as viable drug candidates in PLA2 inhibition studies. The antivenom potential of the extract may be due to the antagonistic role of these phyto-chemicals against PLA2 present in venom. Together the study results provide ample proof and reassert the traditional use of the extract to treat snake bite victims

ACKNOWLEDGEMENT

We are thankful to Sophisticated Analytical Instrumentation Facility (SAIF), IIT Bombay (IIT-B) for analyzing and interpreting GC/MS data.

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FIGURES

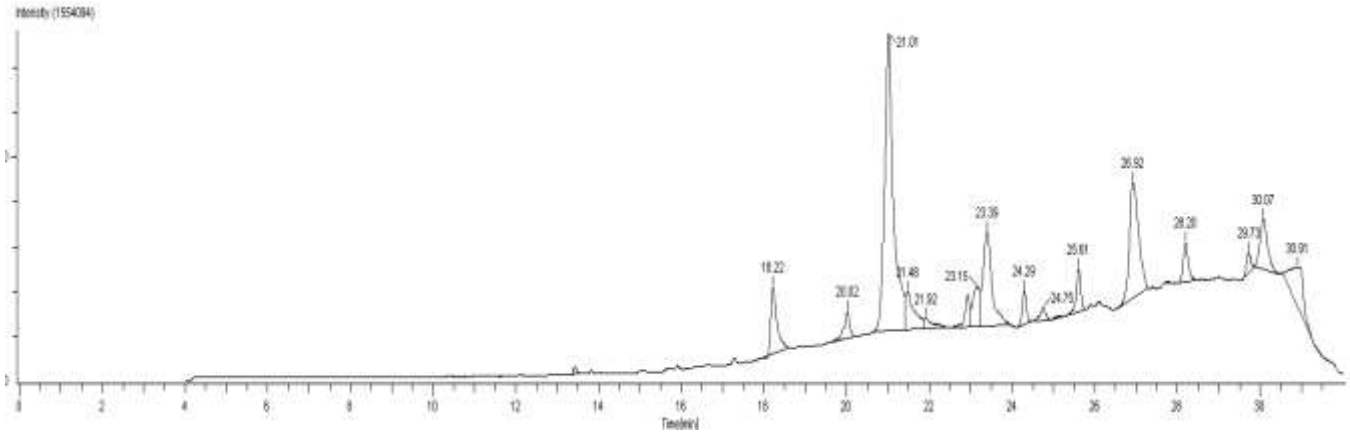

Fig.1: Gas chromatogram of C.gigantea methanolic root extract

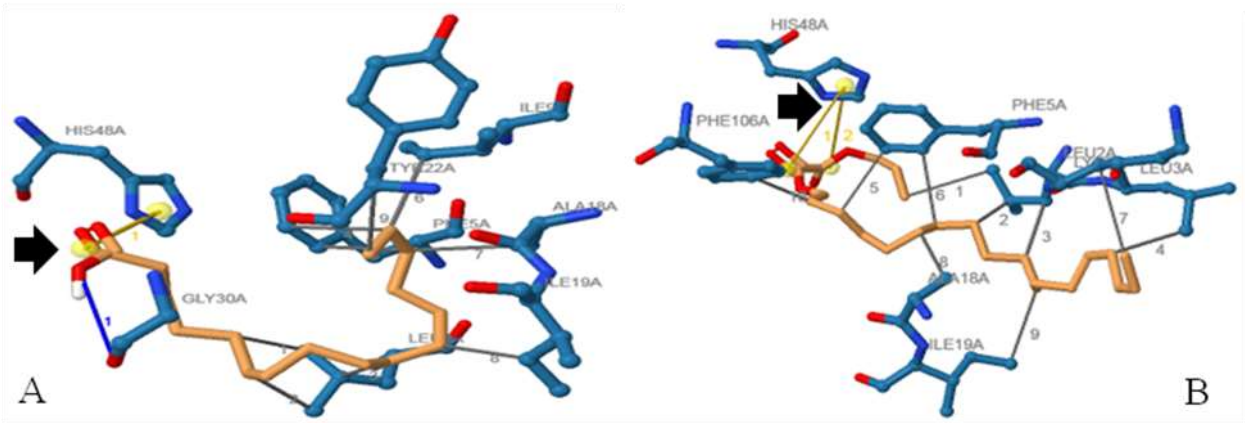

Fig.2: Interactions of Ligand with active site residues of PLA2 enzyme (a) n-Hexadecanoic acid (b) Allyl octadecyl ester oxalic acid. Interaction with His48 (catalytic residue of PLA2 enzyme) is depicted in black arrows

TABLES

Table 1: GC-MS analysis of methanolic extract of C.gigantea root

SL No.	Compound name	Retention Time(min)	Peak Time (min)	Relative Area %	Mol. weight	Chemical Formula
1.	Ar-Tumerone	13.61	13.44	0.28	216	$C_{15}H_{20}O$
2.	n-Hexadecanoic acid	18.58	18.22	4.37	256	$C_{16}H_{32}O_2$
3.	Oleic acid	20.21	20.02	1.72	282	$C_{18}H_{34}O_2$
4.	9,12 Octadecadienoic acid	21.42	21.01	29.38	280	$C_{18}H_{32}O_2$
5.	Oxirane, tetradecyl	21.85	21.48	3.71	240	$C_{16}H_{32}O$
6.	Z-10 Pentadecen-1-ol	22.47	21.92	1.15	226	$C_{15}H_{30}O$
7.	2-Piperidinone	23.01	22.93	16.71	233	$C_9H_{16}BrNO$
8.	Sulfurous Acid, Octadecyl pentyl ester	23.96	23.39	9.41	404	$C_{23}H_{48}O_3S$
9.	Oxalic acid , allyl octadecyl ester	25.78	25.61	2.22	382	$C_{23}H_{42}O_4$
10.	1-Decanol, 2-Hexyl	27.29	26.92	12.1	242	$C_{16}H_{34}O$

Table 2: Biological activity of Bio-active compounds extracted from C.gigantea roots

Compound name	Biological Activity
Ar-Tumerone	Wound healing, regenerative ability
n-Hexadecanoic acid	Competitive inhibitor of PLA2, Anti-inflammatory,
Oleic acid	Cancer treatment, wound healing, immune modulation
9,12 Octadecadienoic acid	Hypocholesterolemic, Anti-eczemic, Anti-Histamine
Oxirane, tetradecyl	Synthetic waxes, fabric softener, cosmetics
Z-10 Pentadecen-1-ol	Increase zinc availability
2-Piperidinone	Component of scent gland secretions of garter snakes
Sulfurous Acid, Octadecyl pentyl ester	Deoxygenating selenoxides
Oxalic acid , allyl octadecyl ester	Acts as UV light stabilizer
1-Decanol, 2-Hexyl	Causes skin irritation, respiratory discomfort

Table 3: Interactions of ligands with active site residues

								Other
Compound name	Leu 2	Phe 5	Ala 18	Ile19	Tyr 22	Gly 30	His 48	
Ar-Tumerone	**							Ile9 *
n-Hexadecanoic acid	***	**	*	*	*	H	H	Ile9 *
Oleic acid	***							Ile9 *
9,12 Octadecadienoic acid	*	**						Tyr 28
Oxirane, tetradecyl				*	*			Ile9 *
2-Piperidinone					*			Ile9 *
Sulfurous Acid, Octadecyl pentyl ester		**			*			Ile9*
Oxalic acid , allyl octadecyl ester	***	**	*	*			H	Lys 7*
1-Decanol, 2-Hexyl			*		*			Ile9 *

Table- 3 (A) n-Hexadecanoic acid, (B) Allyl octadecyl ester oxalic acid interact with catalytic residue His48 through H bonds. Compounds also interacted with amino acid residues which are not present within active site of PLA2 enzyme. Those interactions are detailed in right panel (others). Key: H=Hydrogen, *=Hydrophobic interaction

ISSN 0975-2366
DOI: https://doi.org/10.31838/ijpr/2020.SP1.054

Research Article

Insilico approaches to demonstrate uzarigenin and calotropagenin as potential carbonic anhydrase II (CAII) inhibitors

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Received: 25.04.20, Revised: 13.05.20, Accepted: 06.06.20

ABSTRACT
Background: Inhibition studies on carbonic anhydrase II (CAII) (EC 4.2.1.1) activity are gaining attention due to their immense therapeutic application in the treatment of cancer, and obesity. Clinically used CAII inhibitors (CAIs), such as acetazolamide, and brinzolamide produce undesirable side effects like depression, and nausea. So non-toxic and natural CAI are being researched with special interest. Steroids such as bile acids, steroidal sulfamates, and sex hormones have previously been shown to significantly inhibit CAII activity. In the current insilico study, cardiotonic steroids (uzarigenin and calotropagenin) have been investigated as possible CAII inhibitors.
Objective: To evaluate uzarigenin and calotropagenin as potential inhibitors of carbonic anhydrase II (CAII) activity, using insilico methods
Methods and materials: Reverse pharmacophore screening and inverse docking of ligands was performed to identify potential targets. The results were validated by docking study. The binding affinity and interactions of docked ligands viz, uzarigenin, calotropagenin, acetazolamide (standard) and cholic acid (positive control) with CAII macromolecule, was comparatively analyzed. MMPBSA calculation of protein ligand complex were computed to determine the strength of binding. ADMET analysis was conducted to ascertain drug like properties of ligands.
Results and conclusion: Uzarigenin (Ki= -7.6 kcal/mol) and calotropagenin (Ki= -7.9 kcal/mol), by virtue of their interaction with catalytically important residues (Phe130, Ile91, Gln92), good fit score (2.82, and 2.93 respectively), and significant binding energy (ΔE_{bind}=-21.18 and -23.57kJ/mol respectively) in MMPBSA calculation can be further investigated as lead CAII inhibitors.

Keywords: Carbonic anhydrase II, cardenolides, reverse pharmacophore, uzarigenin, calotropogenin

INTRODUCTION

Carbonic Anhydrase II (CAII) (EC 4.2.1.1) is an important pharmacological target for diuretics, anticancer, antiglaucoma and antiobesity drugs [1] [2] [3]. CAII catalyzes the hydration of CO_2 and also facilitates bicarbonate reabsorption in renal tubules. Structurally, CAII consists of a hydrophobic core, which includes active site residues 190-210. Further, through mutagenesis studies, it has been shown that Thr198, Thr199, His64, Phe130, Val121, Cys205, and Leu197 are catalytically important [4]. The existing sulphonamide drugs like acetazolamide, and brinzolamide are potent CAII inhibitors, however adverse reactions like nausea, drowsiness, acidosis, renal calculi and vomiting are reported, following their use [3]. Therefore small molecule drugs, which are natural, non-toxic and capable of inhibiting CAII activity, are being researched with special interest. Recent research has identified molecules possessing steroid ring (Cycloparaphenylene ring system) as potential inhibitors of CAII [5]. Several studies conducted in the past have reported that bile acids with a steroid ring significantly inhibited CAII activity. Cholic acid (Ki=48.9μM), hyocholic acid (Ki=38.9μM), and deoxycholic acid (Ki=51μM) were identified as potent CAII inhibitors [5]. Unfortunately, not much research is done to establish the CAII inhibitory activity of large

numbers of other plant and animal-derived steroids. For instance, the CAII inhibitory role of cardiotonic steroid compounds like cardenolides and bufadenolides have not been comprehensively studied so far. Since steroid molecules available in nature is exhaustive, testing and validating all the steroid like molecules using wet lab experiments is time consuming [6]. Instead, insilico tools like reverse pharmacophore mapping, and docking that enable easy screening and identification of potential inhibitors can be employed [7] [8]. So in this study the potential use of two low molecular weight cardenolides (uzarigenin and calotropagenin) (Figure 1) as CAII inhibitors was investigated using Insilico methods.

Calotropis gigantea and other milkweed plants belonging to Apocyanacea family are a rich source of naturally occurring cardenolides [9]. The root extract of Calotropis gigantea in particular contains cardenolides such as calotropin, uzarigenin, calotropagenin, frugoside and afroside [10]. Traditionally, cardenolides like calotropin, were used to treat cardiac arrhythmia and congestive heart failure [11]. However, recent research has shown multiple therapeutic uses of these compounds as anticancer agents, emetics, and natural diuretics [9]. Uzarigenin for instance is traditionally used as a cardiotonic steroid, anti diahorreal and as diuretic [12][13]. Calotropagenin is known for its anticancer property [14]. The findings of the current study would provide more valuable insights into the therapeutic aspect of these compounds.

MATERIALS AND METHODS

Target prediction

Reverse pharmacophore screening helps in the identification of potential macromolecules (targets) to which a ligand (query) can bind. This method has enabled the development of in-silico approaches to study drug repositioning, replacement, adverse effects, and toxicity [15]. The reverse screening strategy adopted in this study is presented as graphical abstract in Figure 2. PharmMapper, an online web server was employed in this study. Basically, it identifies key pharmacophore features in the ligand and matches it with other pharmacophores in its database to identify new targets. The output file contained the top 300 potential targets, ranked according to the fit score [16]. The top 10 targets obtained were included for further analysis. Targets that bound to both cardenolides or the overlapping hits were identified by using Microsoft Excel's COUNTIF function.

Reverse docking and MMPBSA calculation

Reverse docking of ligands was performed using ACID-Auto in silico consensus inverse docking tool to confirm targets obtained through reverse pharmacophore screening[17]. It also calculates vander waals energy (E_{vdw}), electrostatic energy (E_{ele}), ΔE_{MM} (E_{vdw} + E_{ele}), polar solvation energy (G_{polar}), non-polar solvation energy ($G_{non\text{-}polar}$) and binding energy (ΔE_{bind}) of protein ligand complex[18].

Macromolecule and ligand preparation

The crystal structure of Carbonic Anhydrase II (CAII) (PDB ID-1OKL)- structure of Homo sapiens CAII bound to Zinc, Mercury and Dansylamide, was downloaded from the Protein Data Bank [19]. Heteroatoms were removed in Argus lab tool and energy minimization was done using Swiss PDB Viewer [20]. The structures of uzarigenin and calotropagenin cardenolides, naturally occurring in Calotropis gigantea root, were obtained from an article published previously [10]. The structures were downloaded from the PubChem database in SDF format (Figure 1) [21]. The structures were checked for torsion count, amide bonds if present were considered non-rotatable, non-polar hydrogen were merged, and energy minimization was done using the mmff94 force field [22].

Active site prediction

The active site of the macromolecule was predicted using metapocket 2.0 online server. It utilizes a multi computational consensus approach by employing 8 different algorithms for active site prediction viz. LIGSITE, PASS, Q-siteFinder, SURFNET, Fpocket, GHECOM, ConCavity and POCASA. Based on z-score, the top three pocket sites in each predictor are obtained and clustered based on spatial similarity [23].

Docking

The 3D structures of ligands viz, uzarigenin, calotropagenin, acetazolamide (standard) and cholic acid (positive control) were docked with Carbonic Anhydrase (CAII) (PDB ID-1OKL) using molecular docking software-Autodock Vina version 4.1, to obtain 9 different binding modes of the ligand with CAII [24]. The predicted binding affinity was obtained for each ligand. The docked pose having RMSD<2 and least binding affinity (more negative) was included for analyzing intermolecular interaction using PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC and PLIP software [25].

Molinspiration

The bioactivity score and drug-likeness of cardenolides were determined using Molinspiration chemoinformatics tool. It computes cLog P, molecular weight, hydrogen bond donors and hydrogen bond acceptor for queried compounds [26]. Also compounds activity as a enzyme inhibitor is calculated as bioactivity score.

RESULTS AND DISCUSSION

Target prediction

Three common pharmacophore features were noted in both the cardenolides viz, 2 Hydrophobic and one acceptor feature. Carbonic anhydrase II ranked among top 10 targets with respect to both cardenolides and hence was selected for further analysis (Table 1). The normalized score for uzarigenin (0.9623) and calotropagenin(0.9771) indicated CAII as a potential target.

Reverse docking and MMPBSA calculation

The ligands were reverse docked in ACID webserver and UNIPROT ID but not PDBID was used for searching potential targets . The reverse docking results confirmed CAII as a potential target of both ligands (Table 1). $E_{vdw,}$ $E_{ele,}$ and $G_{non-polar}$ correlate negatively whereas G_{polar} correlates positively with binding energy. Cholic acid (positive control) showed high binding affinity in the range of -42.09 kJ/mol, high dock score (-9.34) and considerably high ΔE_{MM} (-134.91 kJ/mol) (Table-2). A high ΔE_{MM} value obtained for positive control and standard indicates the importance of vander waals and electrostatic interactions in stabilizing protein –ligand complex. Uzarigenin (-21.18 kJ/mol), calotropagenin (-23.57 kJ/mol) showed good binding energy (more negative). However the binding energies were less compared to positive control cholic acid (-42.09 kJ/mol) and acetazolamide (-58.69 kJ/mol). This indicates that both ligands have good binding potential but their affinities are weak compared to standard. So further optimization of functional groups in the ligands could be a possible option for designing novel CAII inhibitors.

Active site prediction

Top 3 binding sites obtained in Metapocket 2.0 were cross-checked with existing data through a literature search. Accordingly, His94, His96, His119, Val121,Val142, Trp208, Thr198, Leu197, Gln92, Leu140, Phe130, Ile91, Asn62, Ala65, Asn67, Thr199,Val134, His64, Asn243,Tyr7, Pro200, Pro201,Trp5, Phe20 were considered as potential binding residues. The active site and potential binding residues were visualized in Pymol and illustrated in (Figure 3).

Docking

The reverse screening results were confirmed by site-specific, flexible docking using AutoDock Vina V 4.2 available on the PyRx platform. Uzarigenin, calotropagenin, acetazolamide (standard) and cholic acid (positive control) were docked with Carbonic Anhydrase (CAII) (PDB ID-1OKL) Highest binding affinity was obtained with respect to acetazolamide (-9.3 kcal/mol), followed by cholic acid (-8.1 kcal/mol), calotropagenin (-7.9 kcal/mol), and uzarigenin (-7.6 kcal/mol) (Table 3). Binding mode analysis indicated that both cardenolides interacted with active site residues like Phe130, Ile91, and Gln92 (Figure 4).

Validation

The docking simulation was validated by redocking dansylamide to Carbonic anhydrase (CAII) crystal structure (PDB ID-1OKL) containing an inbuilt dansylamide. The interaction and the bond lengths between docked and crystal structure were the same, with a deviation of 0.12A. The RMSD values of heavy atoms in dansylamide were in the range of 0.3-0.14A

Bioactivity prediction

Both uzarigenin and calotropagenin, had lower than stipulated molecular mass of 500 Daltons. However all other parameters like the number of hydrogen donors (nOHNH), acceptors (nON) and miLogP was within Lipinski's limit (Table 4). Interestingly both cardenolides showed significant bioactivity score for nuclear receptor ligand and enzyme inhibition (Table 5). Hence, it was concluded that uzargenin and calotropagenin have drug-like action and also have significant enzyme inhibitory score.

CONCLUSION

All the insilico methods adopted in this study, reverse pharmacophore mapping, reverse docking, MMPBSA calculation, docking, and ADMET analysis point out that uzarigenin and calotropagenin are good lead candidates for designing CAII inhibitors. However their binding affinities were determined to be weaker compared to standard and therefore further optimization of functional groups may be required. Also the study needs to be replicated in invitro, and invivo models to validate and confirm the present findings.

AKNOWLEDGEMENT

We are thankful to Dr. Ramadasan Kuttan, ex-research director of Amala Cancer Research Center (ACRC), Thrissur for providing opportunity to conduct research at ACRC research facility, and for his constant support and guidance pertaining to all aspects of this study.

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Table 1:Target prediction using reverse pharmacophore and reverse docking

	Reverse Pharmacophore (Normalized Fit Score)		Reverse docking (Dock score)	
Targets	Uzarigenin	Calotropagenin	Uzarigenin	Calotropagenin
Carbonic Anhydrase II (1OKL)	0.9623	0.9771	-8.72	-8.88
TREM2 (1Q8M)	0.9333	0.9512	NIL	-8.10
Pregesterone receptor (2OVM)	0.9353	0.9402	-5.34	-7.23
Liver Carboxylesterase 1 (1YA8)	0.9412	0.8972	-6.43	NIL
Prothrombin (1NO9)	0.8901	0.9012	NIL	-7.74
Caspase-7(1SHJ)	0.8888	0.9432	-5.86	-6.43

Table 2:MMPBS calculation

Ligand	ΔE_{MM}	ΔG_{Sol}	ΔE_{Bind}	Dock score
Uzarigenin	-51.72	23.69	-21.18	-8.72
Calotropagenin	-69.04	37.02	-23.57	-8.88
Acetazolamide	-165.72	149.33	-58.69	-11.45
Cholic acid	-134.91	113.23	-42.09	-9.34

Table 3:Binding affinity prediction of docked ligands

Ligands	Binding affinity (kcal/mol)	H bonds	Hydrophobic interaction
Uzarigenin	-7.6	Glu69 (2.11)	Glu69, Ile91, Asp129, Phe130
Calotropagenin	-7.9	Asn62(2.43),Asp72(2.32)	Ile91, Gln92,Phe130,Phe130
Acetazolamide	-9.3	Thr199(2.03), Thr200(3.31)	Phe130, His94, His96, His119
Cholic acid	-8.1	Gln92	Ile91,Phe130,Phe130, His94*, His96*, His119*

Table 4:Druglikeness prediction of ligands

Cardenolide	Molecular weight	nON	nOHNH	miLogP
Uzarigenin	374.52	4	2	2.47
Calotropagenin	404.50	6	3	0.83

Table 5:Bioactivity prediction of ligands

Cardenolides	GPCR Ligand	Ion channel inhibitor	Kinase inhibitor	Nuclear receptor ligand	Protease inhibitor	Enzyme inhibitor
Uzarigenin	0.14	0.10	-0.39	0.53	-0.03	0.80
Calotropagenin	0.05	-0.06	-0.43	0.50	0.13	0.82

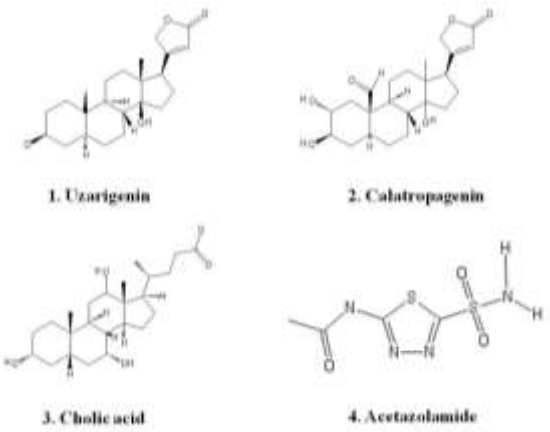

Fig.1:Structures of ligands, acetazolamide (standard) and cholic acid (positive control). Structures drawn using Pubchem sketcher web server V2.4

Fig.2- Work flow of reverse screening strategy adopted in this study

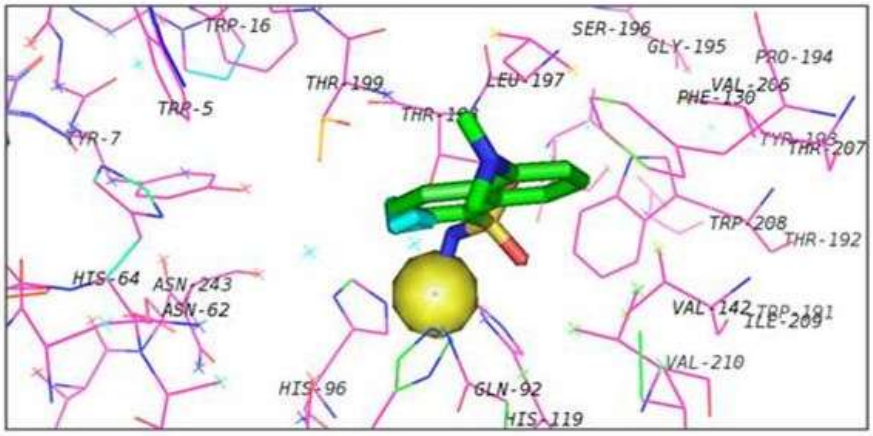

Fig.3:Indicating active site residues of CAII. Molecule shown in green is inbuilt ligand dansylamide.

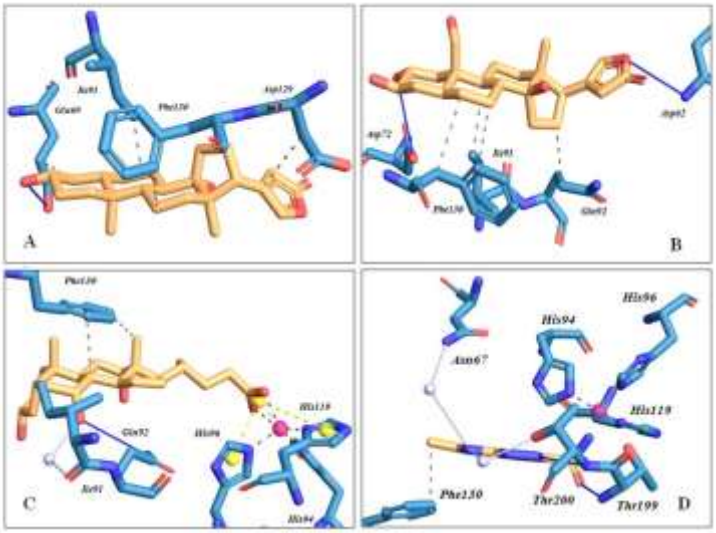

Fig.4:Binding interaction analysis of (A) uzarigenin (B)calotropagenin (C) cholic acid (D) acetazolamide with CAII macromolecule (PDB ID-1OKL)