

A STUDY OF ANTICOAGULANT ACTIVITY OF *Eupatorium triplinerve* (AYAPANA) For THERAPEUTIC APPLICATIONS: AN *INSILICO & INVITRO* APPROACH

Submitted in partial fulfilment of the requirements for the award of
Bachelor of Technology degree in Biotechnology

by

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DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF BIO AND CHEMICAL ENGINEERING

SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

Accredited with Grade “A” by NAAC

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Deepalakshmi.P (37230011)** who carried out the project entitled "A Study of Anticoagulant activity of *Eupatorium triplinerve* (Ayapana) for therapeutic applications: An *Insilico* & *Invitro* approach" under our supervision from February 9 2021 to March 10 2021

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I **Deepalakshmi.P(37230011)** hereby declare that the Project Report entitled “**A Study of Anticoagulant activity of *Eupatorium triplinerve* (Ayapana) for therapeutic applications: An Insilico& Invitro approach**” done by me under the guidance of **Dr. S Usha Nandhini** and **Dr.P.L.SUJATHA** at Tamil Nadu Veterinary Science University(TANUVAS)is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

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Abstract

Eupatorium triplinerve (Ayapana) is a medicinal plant largely used in folk medicine as anticoagulant and antiseptic agent. Considering that the anticoagulant activity could be beneficial for various cardiovascular diseases, this study aims to investigate the anticoagulant activities of Ayapana leaves by *invitro* and *insilico* analysis. Methanolic extract was prepared from the shade dried leaves of ayapana and five concentrations of extracts (1,3, 10, 30, 100 µg per ml) were made. Canine blood was used an invitro model to investigate the anticoagulant activities by performing activated partial thromboplastin time (APTT), prothrombin time (PT) and whole blood clot lysis assay for monitoring its thrombolytic activity. The prepared crude extract was given for GC-MS analysis to identify the potential compounds present. Next, the binding affinity of phytoconstituents from Methanolic extract of *Eupatorium triplinerve* (MEET) was assessed with various targets involved in the pathogenesis of Blood coagulation pathway. The ligands and targets were retrieved from PubChem and Protein Data Bank respectively and docked using Biovia Discover studio. The pharmacokinetic (Drug likeliness, ADME, Toxicity) and pharmacodynamic profile of the lead compounds were predicted using PreAdmet software and PASS online server. It is found that the MEET prolonged the APTT clotting time by 52.74 ± 2.88 secs and exhibited highest thrombolytic activity of 37.17% at the concentration 100µg/ml. Among the compounds docked, linolenic acid interacted very effectively with Coagulation Factor VII and showed highest dock score of 137.20 with a relative energy of -8.25. Overall, palmitic acid, linolenic acid and phytol showed highest interaction with all the targets selected when compared to other phytochemicals. From the *insilico* studies it can be concluded that palmitic acid, linolenic acid and phytol may have significant role in preventing the disorders involving blood coagulation pathway which was also positively supported by invitro model study. From this preliminary study, these compounds can be separated and studied for its therapeutic potential as a lead compound in future.

LIST OF ABBREVIATIONS

µg- Microgram
ADMET- Absorption, Distribution, Metabolism, Elimination, Toxicity
ADP- Adenosine Diphosphate
APTT- Activated partial thromboplastin time
BBB- Blood brain barrier
CaCl₂- Calcium chloride
COX- Cyclooxygenase
CVD- Cardiovascular diseases
DTI- Direct thrombin inhibitor
FTIR- Fourier transform infrared
GC- MS- GAS CHROMATOGRAPHY-MASS SPECTROSCOPY
GMP- Guanosine monophosphate
GP- Glycoprotein
HIA- Human intestinal absorption
HPAEC- High performance anion exchange chromatography
HPGPC- High performance gel permeation chromatography
I.U- International Unit
INR- International normalized ratio
LMWH- Low molecular weight heparin
M- Molar
M/Z- Mass-to-charge ratio
MEET- Methanolic extract of *Eupatorium triplinerve*
ml- Millilitre
MM- MILLIMETER
NIH- National Institute of Health
NIST- National Institute of Standards and Technology
NMR- Nuclear magnetic resonance
PAD- Pulsed amperometric detection

PASS- Prediction of Activity Spectra for Substances
PD- Pharmacodynamics
PDB- Protein Data Bank-
PK- Pharmacokinetics
PRP- Platelet rich plasma
Rpm- Revolution per minute
TF- Tissue factor
TxA2- Thromboxane A2
UFH- Unfractionated heparin
VKA- Vitamin K antagonists
VWF- Von Willebrand factor
WHO-World Health Organization

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CHAPTER 1- INTRODUCTION

1.1 AN OVERVIEW

Plants are rich sources of metabolites possessing enormous pharmaceutical and therapeutic applications. Their role has been vital in curing normal sickness to deadly infections since ancient times. The World Health Organization (WHO) reported that around 80 % of the population in developing countries depend on traditionally used medicinal plants for their primary health care (Rakotoarivelo *et al.*,2015). Though the custom of modern drug usage is more than medicinal plants, there is emerging interest in medicinal plants owing to its less toxicity and side effects. So far, there are many conventional drugs that have come from many of the indigenous parts. The phytochemical-pharmacological research work has recently yielded effective solutions to certain diseases which the synthetic drug industry has failed to afford (Refaz *et al.*,2017). Few effective drugs which were derived from plants include quinine (from cinchona bark), aspirin (from willow bark), morphine (from the opium poppy) and digoxin (Vickers, A *et al.*, 2001).

1.2 INDIAN SYSTEMS OF MEDICINE

Traditional medicines refer to health practices, approaches, belief, spiritual therapies incorporating animal, plant and mineral based medicines, manual techniques and exercises, implied individually or in combination to treat, diagnose and prevent illnesses or maintain well-being (Fokunang C N, *et al.*,2011). Medicinal plants based traditional systems of medicines are playing vital role in providing health care to huge section of population, especially in developing countries. Indian Systems of medicine are one among the globally well-known traditional systems of medicine having six recognized systems of medicine which is Siddha, Ayurveda, Unani and yoga, Naturopathy and Homeopathy. There are large number of healers in the folklore stream apart from these systems which are not categorized into any of these categories (Ravishankar and Shukla, 2007).

1.2.1 Ayurvedic medicine

Ayurveda, one of the traditional Indian medicinal systems remains the most ancient yet living traditions with philosophical and experimental basis. It follows a holistic approach to health and personalized medicine. There is growing interest in public for ayurvedic and other complementary medicine mainly due to the possible side effects in synthetic drugs, high cost of new drugs, lack of treatment facilities for several chronic diseases, microbial resistance, and emerging diseases. Though ayurvedic treatment is highly effective, many of the important Ayurvedic drugs are still not fully explored for their mode of action, pharmacology, pharmacovigilance and pharmacokinetic properties. Fundamental research needs to be done in the fields of Ayurvedic physiology, pathology, pharmacology (fundamental and clinical), and pharmaceuticals. (Chauhan, 2015). Approximately 90% of ayurvedic preparations are plant based. Ayurvedic plants have a well-built

reaction on the body than either food or spices. It is also proven that Polyherbal combinations are lastingly effective than single herbs. Most of the classical preparations of Ayurveda are polyherbal, involving a combination of 3 to 30 plant. The constituents are combined and formulated in such a way that the formula isreproducible and also balanced. The most commonly propagated ayurvedic herbs/plants are turmeric (*Curcuma longa*), ginger (*Zingiber officinale*), *Aloe vera*, tulsi (*Tulsiocimumtenuiflorum*), moringa (*Moringa oleifera*), Guduchi/Amrut (*Tinospora cordifolia*), Pippali(*Piper longum*) etc (Nishteswar K, et al, 2014). There are many ayurvedic plants which are not fully explored for its scientific properties and Ayapana is one among them. Ayapan is the common Ayurvedic name of *Eupatorium triplinerve*. The ayurvedic literatures suggest that the leaves contain a highly aromatic chemical known as coumarin which has blood thinning and anti-coagulant properties.

1.2.1.1 *Eupatorium triplinerve* (Ayapana)

Eupatorium triplinerve commonly called as Ayapana is a tropical American shrub that is commonly known as water hemp. It belongs to the family Asteraceae. This plant has long and slender leaves which are often used to make herbal medicinal extracts. The flowers are pale pink and the thin, hairless stem is reddish in color [Gurib-Fakim and Brendler, 2004]. Apart from being a common ornamental plant, it also possesses many medicinal benefits. It is used in herbal medicine as an anti-tumor remedy with a popular hemostatic activity against various hemorrhages and also found to be useful for treating yellow fever (Begum *et al.*, 2010). It is practically used for healing wounds and preventing formations of ulcer, to manage fever, cough and cold naturally. The whole part of the plants is made into a decoction to relieve malarial fever, whereas the fresh leaves of the plant are used to treat oral health problems like gingivitis and stomatitis and insect bites. The plant yields many chemicals such as coumarin, cineol, alpha-phellendrene, alpha terneol, ayapanin, ayapin borneol, sabinene, umbelliferone and many others. (Ghani, 2003) reported the presence of Ayapanin, ayapin and coumarin having pronounced haemostatic properties.

1.3 CARDIOVASCULAR DISEASES

Cardiovascular diseases (CVDs) are the number 1 cause of death globally. The World Health Organization (WHO) reports suggest that CVDs take an estimated 17.9 million lives each year (Ruan *et al.*, 2018). CVDs are a group of heart and blood vessels disorder that includes cerebrovascular disease, coronary heart disease, rheumatic heart disease and other conditions. Four out of 5CVD deaths are due to heart attacks and strokes, and one third of these deaths occur prematurely in people under 70 years of age. By 2030 more than 22.2 million people will die annually from CVDs (Namara K, *et al*, 2019). In many of the cardiovascular diseases, it is recommended to use anticoagulants for thrombotic event prevention, including stroke prevention in atrial fibrillation, treatment and

secondary prevention of acute coronary syndrome. For many years, the anticoagulant therapy generally included parenterally administered unfractionated heparin (UFH) or orally administered vitamin K antagonists (VKAs), usually warfarin. In the past 20 years, Low-molecular weight heparins (LMWHs) have come into use and much more recently, the parenteral direct thrombin inhibitors (DTIs) like fondaparinux have become available for clinical use (De Caterina *et al*, 2009). Many modern anticoagulant drugs have been in more usage in the community, but in most cases, they have more adverse side effects, especially those made from chemical materials. Best example includes the bleeding complications of warfarin administration (Ekim *et al.*, 2018) Therefore, medicinal plants can be a best alternative choice to avoid harmful side effects.

1.3.1 Common Anticoagulant drugs

Anticoagulant drugs eliminate the risk of blood clots by help preventing or breaking the dangerous blood clots that can potentially block the blood circulation. It works by interfering with the proteins(factors) involved in the coagulation process. Different anticoagulants interfere with different coagulation factors to prevent clot formation (Schattauer *et al.*, 2014).

Table 1.1: Anticoagulant drugs and their potential targets

CATEGORY	AVAILABLE DRUGS	TARGET
Antithrombotic		
Vitamin K Antagonist	Warfarin	Factor II, VII, IX and X (vitamin k dependent clotting factors) and anticoagulant proteins C, S and Z
Factor Xa inhibitor	Fondaparinux Rivaroxiban Apixaban	Factor Xa
Direct Thrombin Inhibitor	Bivalirudin Dabigatran Argatroban	Thrombin
Fibrinolytics	Alteplase Reteplase Tenecteplase	Plasminogen Fibrinogen alpha chain Plasminogen activator inhibitor, Annexin A2, tetranectin, etc.
Heparin	Unfractionated heparin (UFH) Low molecular weight heparin (LMWH)- Dalteparin and Enoxaparin.	Factor Xa and Thrombin Factor Xa Indirect effect on Antithrombin III

Antiplatelet			
Thromboxane pathway blockers	A2	Aspirin Valdecoxib Ridogrel	COX-1 and COX-2
ADP receptor antagonists		Clopidogrel (Plavix), Ticlopidine (Ticlid)	P2Y1 and P2Y12
GPIIB/IIIA antagonists		Abciximab (ReoPro), Eptifibatide (Integrilin).	GPIIB/IIIA (fibrinogen receptor)
Phosphodiesterase inhibitors		Cilostazol (Pletal)	Phosphodiesterase (type III)
Thrombin receptor inhibitors		Atopaxar (E5555) Vorapaxar (SCH-530348)	PAR1 and PAR4
Adenosine reuptake inhibitors		Dipyridamole (Persantine) Aggrenox (aspirin/dipyridamole)	Adenosine deaminase and cyclic guanosine monophosphate GMP phosphodiesterase (type V)

1.4 HEMOSTASIS

Hemostasis is the physiological process that stops bleeding at the site of an injury while maintaining normal blood flow elsewhere in the circulation. Blood loss is stopped by formation of a hemostatic plug (Gale, 2011).

1.4.1 Primary hemostasis

Primary haemostasis results from complex interactions between platelets, vessel wall and adhesive proteins leading to the formation of initial 'platelet plug'. The endothelial cells lining the vascular wall exhibit the antithrombotic properties due to multiple factors viz: negatively charged heparin-like glycosaaminoglycans, neutral phospholipids, synthesis and secretion of platelet inhibitors, coagulation inhibitors and fibrinolysis activators. In contrast, subendothelial layer is highly thrombogenic and contains collagen, Von Willebrand factor (vWF) and other proteins like laminin, thrombospondin and vitronectin that are involved in platelet adhesion. Any vascular insult results in arteriolar vasospasm, mediated by reflex neurogenic mechanisms and release of local mediators like endothelin and platelet-derived thromboxane A₂ (TxA₂). (Periayah et al, 2017)

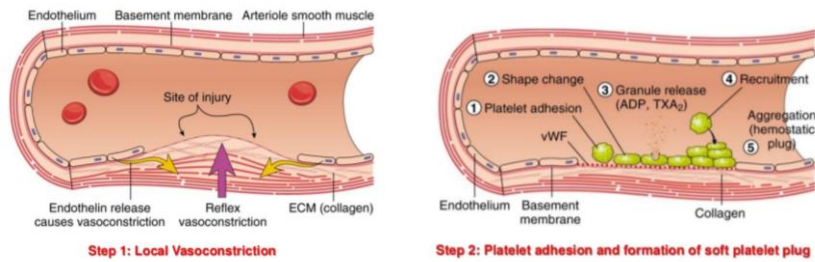


Fig.1.1: Steps involved in platelet plug formation

1.4.2 Secondary hemostasis

Secondary hemostasis is defined as the formation of insoluble, cross-linked fibrin by activated coagulation factors, specifically thrombin. Fibrin stabilizes the primary platelet plug, particularly in larger blood vessels where the platelet plug is insufficient alone to stop hemorrhage. The blood coagulation system consists of various cascade of enzyme activation events where the proenzymes and procofactors are activated by serine proteases via limited proteolysis. The eventual outcome is the blood clot formation by polymerization of fibrin and activation of platelets. This is a normal hemostasis process initiated to seal up vascular damage as a protection to prevent excessive blood loss following injury (Smith *et al*, 2018). The blood clotting is triggered by two important mechanisms, termed the Extrinsic or Tissue factor (TF) pathway and Intrinsic or contact pathway. (Palta S, *et al.*, 2014).

1.4.2.1 Extrinsic or Tissue Factor (Tf) pathway

The extrinsic pathway is the shorter pathway of secondary hemostasis. Once the damage to the vessel is done, the endothelial cells release tissue factor which goes on to activate factor VII to factor VIIa. Factor VIIa goes on to activate factor X into factor Xa. This is the point where both extrinsic and intrinsic pathways become one. The extrinsic pathway is clinically measured as the prothrombin time (PT) (Mackman *et al.*, 2007)

1.4.2.2 Intrinsic or Contact Pathway

Sequential activation of FXII, FXI, and FIX leads to formation of the intrinsic Xase, a complex of FVIIIa (activated factor VIII) and FIXa (activated factor IX), that also catalyzes the generation of FXa. FXa in complex with its cofactor FVa forms the prothrombinase complex that catalyzes the cleavage of prothrombin (FII) to thrombin (FIIa) (Wheeler *et al.*, 2016)

1.4.2.3 Common pathway

Thrombin as the terminal coagulation protease catalyzes the formation of insoluble fibrin through cleavage of soluble fibrinogen monomers and activation of the transglutaminase FXIII (Figure). In addition, thrombin is a potent activator of platelets through cleavage of cell surface protease-activated receptors (Steven and Grover, 2019)

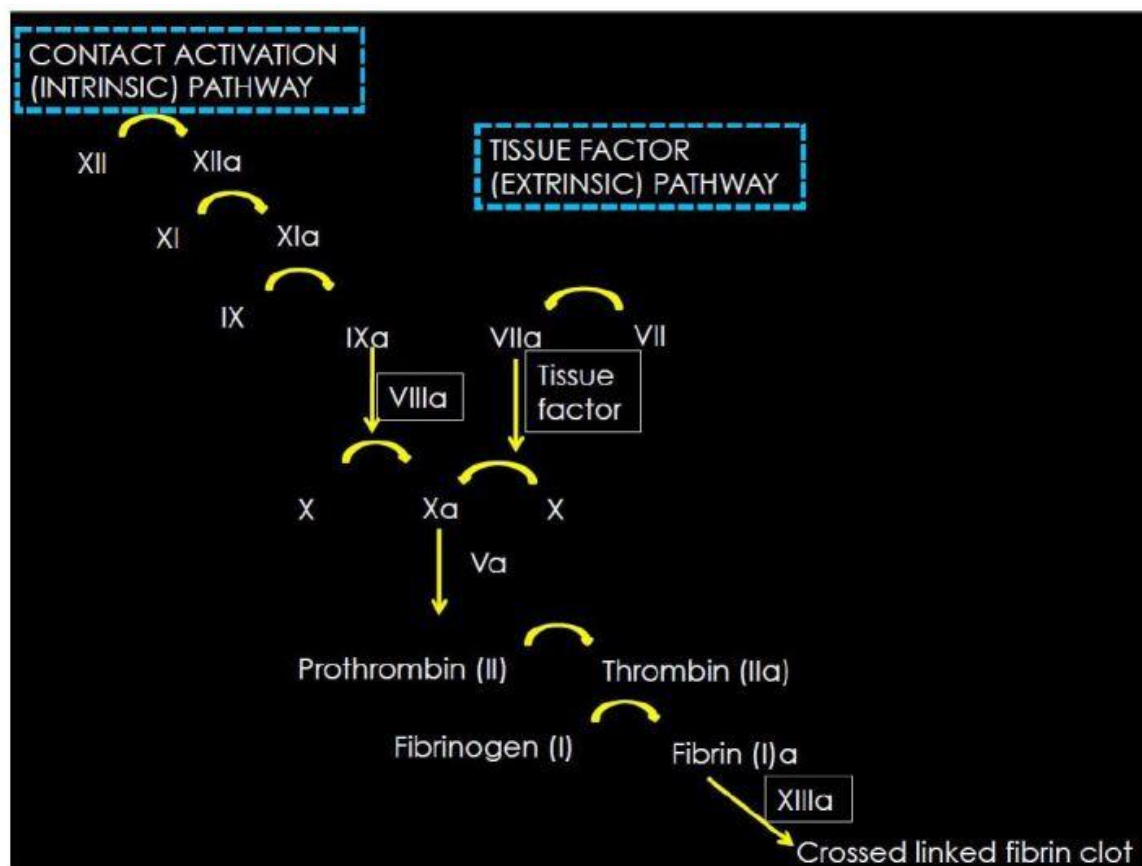


Fig1.2: Human coagulation cascade

1.5 INSILICO STUDIES

In-silico termed as “something that is performed on the computer or through the computer simulation”. In 1989 the expression “*in silico*” was first used in public during the workshop of “Cellular Automata: Theory and Applications” in Los Alamos, New Mexico. Computational research can speed up the rate of discovery of drugs and reduces the expensive lab work and clinical trials. One of the best ways to achieve this is drug designing using molecular docking by bioinformatic softwares.

1.5.1 Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSBPDB)

The Protein Data Bank (PDB) is the single global archive for experimentally determined, atomic-level three-dimensional structures of biological macromolecules (proteins, DNA, RNA). The PDB archive is managed by the Worldwide Protein Data Bank organization which currently includes three founding regional data centers, located in the US, Europe and Japan. The RCSB PDB website (rcsb.org) offers multiple tools for structure query, browsing, analysis and molecular visualization. Users can perform simple searches using the top menu bar search including PDB ID, name, sequence and ligand SMILES; or build complex search combinations of parameters and criteria with the Advanced

Search interface (Rose *et al.*, 2017). The three-dimensional structures of the biological macromolecules data available with PDB are determined by experimental methods such as X-ray crystallography, Nuclear magnetic resonance (NMR) spectroscopy, electron microscopy (Parasuraman, 2011)

1.5.2 Pubchem

PubChem is a public database for data and biological activity on chemical substances. Since its launch in 2004 as a part of the U.S. National Institutes of Health's (NIH) Molecular Libraries Roadmap Initiatives, PubChem has evolved quickly to become a main chemical information tool that provides science groups in many fields, including cheminformatics, chemical biology, therapeutic chemistry, and drug discovery (Kim, *et al.*, 2016).

1.5.3 Pharmacokinetic study

In drug discovery and designing of a new drug, several intensive tests are required to be carried out on drug(s) candidate compounds some of which include; toxicity to the body and oral bioavailability among others (Lee *et al.*, 2002)

A critical piece in drug discovery and development is conducting DMPK (Drug Metabolism and Pharmacokinetics) studies, often referred to as ADMET (Absorption, Distribution, Metabolism, Elimination, Toxicity) studies. (Roman *et al.*, 2019)

1.5.4 Pharmacodynamic study

Pharmacodynamics is the study of the relationship between the concentration of drugs at the site of action and the biochemical and physiological effect. PK/PD analysis combines PK and PD model components to describe the dose–concentration–response time course. PK/PD models are especially useful for biopharmaceuticals since dose- and time-dependent effects on PK and responses are common (Meibohm and Derendorf, 1997)

1.5.5 Molecular interaction

The molecular docking approach can be used to model the interaction between a small molecule and a protein at the atomic level, which allow us to characterize the behavior of small molecules in the binding site of target proteins as well as to elucidate fundamental biochemical processes. The docking process involves two basic steps: prediction of the ligand conformation as well as its position and orientation within these sites (usually referred to as pose) and assessment of the binding affinity (Meng *et al.*, 2011). It can be characterized as an issue of optimization that would define a ligand's "best-fit" orientation and the protein is flexible, a "hand-in-glove" analogy is more acceptable than "lock-and-key". Molecular docking can be thought of as a problem of "lock and key", where one is interested in finding the correct relative orientation of the "key" which will open up the "lock". The ligand and the protein adjust their conformation to achieve an overall "best-fit" and this kind of conformational adjustments resulting in overall binding is referred to as "induced-fit" (Wei *et al.*, 2004).

CHAPTER 2- LITERATURE SURVEY

(Wonhwa Lee, 2013) had studied the anticoagulant effects of piperlonguminine isolated from *P.longum* fruits by in vitro and in vivo analysis. The anticoagulant effects were examined by monitoring the activated-partial-thromboplastin-time (aPTT), prothrombin time (PT), and the activities of thrombin and activated factor X (FXa). It was noted that aPTT and PT were significantly prolonged by piperloguminine (PL) which marked its inhibitory effect on extrinsic and intrinsic human coagulation pathway. Tail bleeding times were determined to confirm their invitro results which were also significantly prolonged by PL. Furthermore, PL inhibited the activities of thrombin, FXa and TNF- α induced PAI-1 production.

(Alikhani Pour, 2017) worked on Cheminformatics-Based Anticoagulant Study of Traditionally Used Medicinal Plants. 15 native medicinal plants of Iran with potential anticoagulant properties were selected from available databases such as Google scholar and PubMed. The selected plants were evaluated by in vitro prothrombin time and activated partial thromboplastin test. The final results suggested that among the selected 15 medicinal plants, *Terminalia bellirica*(P=0.0019), *Astragalus arbusculinus*(P=0.0021) and *Origanum vulagare* (P=0.0014) showed more prominent anticoagulant effect.

(Kemal,2015) investigated the anticoagulant activity of some *Artemisia dracunculus* (tarragon) leaf extracts by determining the presence of coumarins, wherein the extracts also indicated the major amounts of coumarin content. The results of anticoagulant study by Prothrombin time (PT) and the international normalized ratio (INR)showed that the best anticoagulant activity was found in the methanolic extract at concentration of 5%.

(Weironcai, 2015) from the purified polysaccharides of *Gentiana scabra* Bunge roots had studied the invitro anticoagulant by APTT, PT and TT test. The three water soluble polysaccharide fractions (GSP-1,2 &3) were obtained by using hot water extraction, ethanol precipitation and freeze-drying method. Chemical characterizations of the crude extracts were determined by high performance gel permeation chromatography (HPGPC), high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) and

Fourier transform infrared (FT-IR) spectrophotometer. The monosaccharide composition analysis revealed the presence of rhamnose, arabinose, galactose, glucose and galacturonic acid. Overall, the results of anticoagulant tests concluded that GSP-3 exhibited the highest anticoagulant activity among the GSP fractions isolated.

(Hao Chen, 2013) showed the Effect of *Toona microcarpaharms* leaf extract on the coagulation system by preparing an ethyl acetate extract from the air-dried leaves of *Toona microcarpaharms*. They investigated the in vitro anticoagulant activity by APTT, PT and TT assays and antiplatelet aggregation activity by using adenosine diphosphate (ADP), thrombin and collagen as agonist. The invitro studies determined that *Toona microcarpaHarms* leaf extract (TMHE) prolonged APTT, PT, and TT clotting times in a dose-dependent manner and significantly inhibited platelet aggregation induced by thrombin, but not collagen or ADP. The invitro results were proved by Clotting time and bleeding time assays in which TMHE significantly prolonged clotting and bleeding times in mice (in vivo). Their results demonstrated the potent anticoagulant activity of THME via the inhibitory effect against thrombin.

(Do Thi Viet Huong, 2020) studied the anti-diabetic compounds which were identified and isolated from the methanol leaf extract of *Eupatorium triplinerve* and evaluated for their α -Glucosidase Inhibitory Activity. They provided an efficient approach towards rapid identification of coumarins in *E.triplinerve* by NMR and HPLC analysis and the Coumarins were confirmed as the main constituents in the leaf extracts by proton NMR profiling and quantitative RP-HPLC-PDA analysis. In their inhibitory activity test against yeast α -glucosidase of compounds, methoxycinnamic acid showed the highest inhibitory activity of 81%.

(Sugumar, 2015) determined the presence of phytochemical constituents and antimicrobial activity of different extract of leaves of *Eupatorium triplinerve* Vahl. Their study concluded that the different extracts from *Eupatorium triplinerve* leaves contained a broad spectrum of secondary metabolites and also exhibited antimicrobial activity against all the tested microorganisms. Ethyl acetate extract was found to be more effective against all the solvents followed by Hexane,

Chloroform, Ethyl acetate and Methanol extracts with *Staphylococcus aureus* (19.4mm-1000µg/mg) to be the most sensitive organism.

(Bose, 2007) evaluated the hepatoprotective and antioxidant effect of the methanol extract of *Eupatorium ayapana* (MEEA) leaves in Wistar albino rats. The MEEA produced significant ($p < 0.05$) hepatoprotective effect by decreasing the activity of serum enzymes, bilirubin, uric acid, and lipid peroxidation and significantly ($p < 0.05$) increased the levels of SOD, CAT, GSH and protein in a dose dependent manner. From these results, it was suggested that MEEA possess potent hepatoprotective and antioxidant properties and is considered to be a therapeutic agent in the treatment of various diseases, as dyspepsia.

(Dongfang, 2020) carried out a virtual high-throughput screening of 500,064 compounds within Enamine database to obtain the most potent inhibitors of FXIIa. Human coagulation factor XIIa (FXIIa) is an important component involved in the initiation of the intrinsic pathway. So, 18 compounds with significant binding energy (from -65.195 to -15.726 kcal/mol) were selected, wherein the representative inhibitors were selected based on their ADMET properties. Three compounds (Z1225120358, Z432246974, and Z146790068) exhibited excellent binding affinity and drug ability. The results of activated factor XIIa assay, when tested with five compounds Z1225120358, Z432246974, Z45287215, Z30974175, and Z146790068, suggested that other four compounds exhibited higher FXIIa inhibitory effect than Z432246974. Collectively, compounds Z1225120358, Z45287215, Z30974175, and Z146790068 were anticipated to be promising drug candidates for inhibition of FXIIa.

(Khalid, 2018) worked on the antiplatelet and anticoagulant actions of 1,2,4 triazole hydrazone and sulphonamide novel derivative by synthesis, characterization and molecular docking evaluation. Six new 1,2,4-triazole derivatives were synthesized and the test compounds when checked for their anticoagulant potential showed that hydrazone derivatives ZE-4b and ZE-4c were more potent as compared to sulphonamide derivatives activity by increasing PRT and BT time. Molecular docking studies against targets of blood aggregation and coagulation pathways using the software autodock revealed that the test compounds possessed high affinity for COX-1, GP-IIb/IIIa and F-X receptors. Thus in silico, in vitro and in vivo investigations of ZE-4b, ZE-4c, ZE-5a and ZE-5b proved their antiplatelet and anticoagulant potential.

CHAPTER 3 -AIM & SCOPE

3.1 AIM

To study the anticoagulant activity of *Eupatorium triplinerve* (Ayapana) through invitro model study and insilico molecular docking approaches.

3.2 OBJECTIVES

- Collection and processing of plant samples
- Preparation of crude methanolic extraction by cold maceration
- Identification of phytochemicals by GC-MS Analysis
- Prediction of drug likeliness, toxicity, ADME (Pharmacokinetics) and biological activity of drugs (Pharmacodynamics) using PreADMET & PASS online server
- APTT, PT & Thrombolytic Assay- Invitro screening method to identify the efficacy of Ayapana as an anti-coagulant.
- Molecular docking of plant ligands with proteins involved in blood clotting mechanism(target)

3.3 SCOPE OF THE PROJECT

To look into the anticoagulant property of *Eupatorium triplinerve* by analysing its interaction with our blood coagulation cascades through molecular docking and invitro model study to confirm with same the predictions made. This project paves way as a preliminary study for the discovery and development of an anticoagulant drug.

CHAPTER 4- MATERIALS & METHODS

4.1 PLANT SAMPLE COLLECTION

The plant *Eupatorium triplinerve* (Ayapana) was collected from State Forest Research institute, Kolapakkam, Chennai.

4.1.1 Drying & Storage

Fresh and healthy leaves(300g) were separated and rinsed thoroughly in distilled water and shade dried for 7-10 days. The dried leaves(46g) were finely grinded in an electric blender and stored in an air tight container before use.

4.1.2 Crude extract preparation

The powdered sample was extracted with 460ml methanol (1:10 dilution) and macerated at room temperature for 48 hours with intermittent shaking. After 48 hours, the methanolic extract was first filtered in a muslin cloth and followed by a second filtration using Whatman filter paper. The crude Methanolic extract of *Eupatorium triplinerve* (MEET) was subjected to dryness and the yield was 3g.

4.1.3 Preparation of working standard solution of MEET

The stock solution was prepared by diluting 3 grams of MEET in 3ml of methanol (1000mg/ml). The stock solution was further diluted in physiological saline to give the following concentrations: 1, 3, 10, 30 and 100 µg/ml. The above prepared plant concentrations were only used in all assays performed.

4.2 COLLECTION OF BLOOD SAMPLE

The blood samples were collected from healthy dog volunteers after getting approval from the pet owners.

4.3 CLOT LYSIS ASSAY

- 5ml of Venous blood from each volunteer(n=3) was collected in separate serum tubes and transferred to pre weighed sterile microcentrifuge tubes(1ml/tube) and incubated at 37°C for 45 minutes.
- After clot formation, serum was completely removed without disturbing the clot.
- The tubes were weighed to determine the clot weight (clot weight =weight of clot containing tube – weight of tube alone).
- After prior labelling of micro centrifuge tubes, 100µl of plant samples (1, 3, 10, 30 and 100 µg/ml) were added to the pre-weighed clot. Physiological saline was also added to one of the tubes containing clot and this serves as a negative thrombolytic control.
- The tubes were incubated at 37°C for 90 minutes and observed for clot lysis. After incubation, the fluid was completely removed and tubes were again weighed to observe the difference in weight after clot disruption.
- Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis.
$$\% \text{ of clot lysis} = (\text{wt of released clot} / \text{clot wt}) \times 100$$

4.4. COAGULATION ASSAYS- APTT & PT ASSAY

4.4.1 Preparation of blood sample

Dog plasma was obtained from the blood of healthy volunteers without a history of bleeding or thrombosis (n=3). The blood was collected in 3.2% sodium citrate (nine parts blood to one part citrate) and centrifuged at 3000 rpm for 15 min and the supernatant plasma was collected without disturbing the buffy coat.

4.4.2 Activated Partial Thromboplastin time (APTT) Test:

100 µl of Platelet rich plasma (PRP) was mixed with 20 µL of each concentration of plant extract and incubated at 37°C for 3 min. After the plant/APTT mixture had been subjected to incubation at 37°C for 3 min, 100 µl of APTT assay reagent (Agappe Diagnostics) was added and incubated for 1 minute. Finally, 100 µl of prewarmed 0.020 M CaCl₂ solution (50 µL) was added to the incubated ones to initiate the reaction. Then the time taken for the mixture to clot was recorded on an automated coagulometer. Saline and heparin are used as negative and positive thrombolytic control and their clotting time was recorded.

4.4.3 Prothrombin time (PT) test:

100 µl of PRP was mixed with 20 µl of each concentration of the plant extract and incubated at 37°C for 5 minutes. Lastly, 200 µl of PT assay reagent (Agappe diagnostics) preincubated at 37°C, was added to the mixture, and the PT clotting time was recorded. Heparin and saline were used as positive and negative thrombolytic control

4.5 ANALYSIS OF THE CRUDE EXTRACT USING GAS CHROMATOGRAPHY-MASS SPECTROSCOPY (GC-MS)

The methanolic crude extract was subjected to GC-MS using Agilent Technologies 7890B Network Gc system for gas chromatography. 1.0µl of sample was injected in the column Stationary phase DB-5ms (5% Phenyl 95% Polydimethylsiloxane) of dimension :30m X 0.25mm I.D. Analysis was performed under injector temperature of 280°C where helium is the carrier gas, while the flow rate was set at 1.3ml/min. The column temperature program was set to hold at 40°C for 1min, followed by an increase of 10°C per min till 260°C after which the temperature increased by 10°C per min till 300°C and the temperature was held for 3min. The ionization temperature was set at 230°C. The entire column run time was clocked to be 33.3mins. The compounds are separated by gas chromatography and identified using mass spectrometry of range 40m/z to 650 m/z. The weight and structure were determined by the analysis of mass spectral peaks with that of the known compounds present in the National Institute of Standards and Technology (NIST14) library.

4.6 INSILICO STUDY

4.6.1 Retrieval of target

The protein targets which are involved in the coagulation pathway of mammalian blood was identified with the help of databases UniProtKB, NCBI database and from literary databases. The possible targets include the proteins involved in the primary hemostasis (formation of primary platelet plug) and the coagulations factors and coenzymes involved in Contact and Tissue Factor (TF) pathway. The 3D structures of the listed protein targets in the table were downloaded from the online Protein Database Protein Data Bank (PDB).

- The PDB database was opened and a keyword was given for the identified protein 3D Structure
- The available PDB text file of the identified protein was downloaded from the PDB database
- The 3D structure of identified proteins was viewed in BIOVIA Discovery Studio Visualizer.

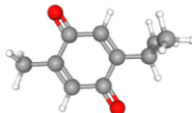
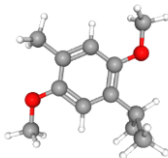
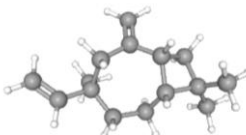
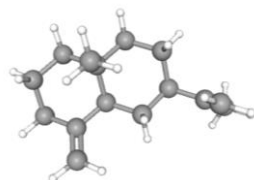
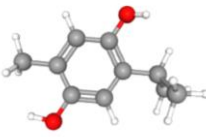
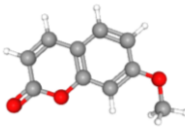
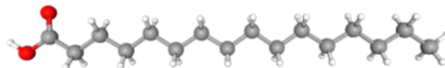
Table 4.1 Proteins involved in the mammalian blood coagulation cascade

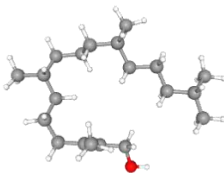
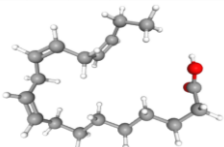
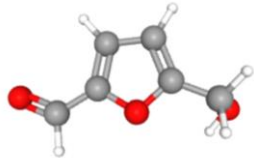
S.NO.	Name of The Target	PDB ID
1.	Coagulation Factor Xa	1FAX
2.	Human Coagulation Factor IXA	1RFN
3.	Human Plasma Coagulation FXI	5EOD
4.	Crystal Structure of P2Y12 Receptor	4PXZ
5.	Human Cox-1 Crystal Structure	6Y3C
6.	Human Coagulation Factor XIIA (FXIIA)	6L63
7.	Coagulation Factor VII Zymogen	1JBU
8.	Crystal Structure of Human Factor VIII	3CDZ
9.	Crystal Structure of Tissue Factor- Factor VIIA Complex	1J9C
10.	Structure of Human Factor VIIA	1QFK
11.	Unliganded Wild-Type Human Thrombin	3U69
12.	Crystal Structure of Native Heparin Cofactor I	1JMJ
13.	Structure of Glycoprotein 1b	1GWB
14.	Crystal Structure of Antithrombin-III	2B4X
15.	Integrin AlphaIIb-Beta3 Transmembrane Complex	2K9J
16.	Crystal Structure of Platelet Factor 4	1F9Q
17.	Crystal Structure of Human Plasminogen Catalytic Domain	1DDJ
18.	Full Length Human Plasma Kallikrein	601G

4.6.2 Retrieval of ligands

The phytochemical compounds identified in the crude extract using Gas chromatography-Mass spectroscopy (GC-MS) are selected as ligands for molecular docking studies. The chemical compounds listed in the table is retrieved in the .SDF format from the Pubchem database based on Lipinski's rule.

Table 4.2: Phytochemical compounds of MEET

S.NO.	Ligand name	PUBCHEM ID	Structure
1.	Thymoquinone	10281	
2.	Benzene,1-4-dimethoxy-2-methyl-5-isopropyl-	6427071	
3.	Bicyclo [5.2.0] nonane, 2-methylene-4,8,8-trimethyl-4-vinyl-	564746	
4.	Beta selinene	564746	
5.	Thymohydroquinone	95779	
6.	7-methoxy coumarin	10748	
7.	Palmitic acid	985	

8.	Phytol	5280435	
9.	Linolenic acid	5280934	
10.	5-hydroxy methyl furfural	237332	

4.6.3 Prediction of Drug Likelihood, Adme & Toxicity using PreADMET:

The identified phytochemicals are screened for ideal drug-likeness, ADME and toxicity properties by PreADMET server. PreADMET is a web-based application for predicting ADME data and building drug-library using in silico method. The SMILES notations retrieved from Pubchem are loaded onto Online SMILES translator and translated into MolFile(.mol). The contents of MolFile are pasted in the text area of PreADMET server for pharmacokinetic analysis of the lead compounds selected.

4.6.4 Prediction of Biological activity using Prediction of Activity spectra for Substances (Pass)

The smiles notation of the phytochemical compounds identified is given as a query input through the online tool “PASS prediction” access directly through the website <http://195.178.207.233/PASS/index.html>. The activity of the query molecule is predicted by comparing the structure of the molecule with the structure of the known bioactive molecules present within a database using the Bayesian approach. The tool predicts based on the Pa: Pi (active, inactive ratio) at various prediction threshold of Pa>30%, Pa>50% and Pa>70%. The prediction estimate of the biological activity is said to be accurate to about 95% (Parasuraman, S., 2011).

4.6.5 Molecular interaction study

The study of molecular interaction between targets and ligands was predicted using a docking software, discovery studio. It is a comprehensive software suite for analyzing and modeling molecular structures, sequences, and other data of relevance to lifescience researchers. The molecules which qualified the pharmacokinetics and pharmacodynamics prediction for ideal lead compounds are selected and docked against the selected protein targets of blood coagulation system.

- Import of the protein molecules as .PDB text file
- The bound ligands if any was deleted.

- Preparation of proteins
- Defining binding sites from receptor cavity for the prepared protein
- Import of ligand molecule
- Preparation of ligand molecules
- Selection of parameters
- Interaction of Ligand molecule with different binding site of each target molecule
- Calculation of energy and dock score
- Analysis of the data obtained.

CHAPTER 5: RESULTS& DISCUSSIONS

5.1 PREPARATION OF PLANT SAMPLE

The plant sample obtained from State forest research institute, Chennai was washed meticulously to remove topsoil, foreign organisms and other non-suitable materials for extraction. In order to assess their anticoagulant property, only the leaves were separated and shade dried for further study process.



Fig 5.1 Drying procedure of test sample a) Fresh plant sample
b) Separated healthy leaves c) Dried leaves d) Grounded dried leaves

5.2 PREPARATION OF CRUDE EXTRACT

The coarsely powdered leaves were macerated with methanol (menstruum) at room temperature for three days. It was kept closed and stirred periodically to ensure complete extraction. At the end of extraction, the extracted compounds (micelle) were separated from insoluble substances using muslin cloth for first filtration and Whatman Grade 1 filter paper for second filtration. Subsequently the extracted compounds were separated from methanol (menstruum) by evaporation as methyl alcohol readily evaporates at room temperature.

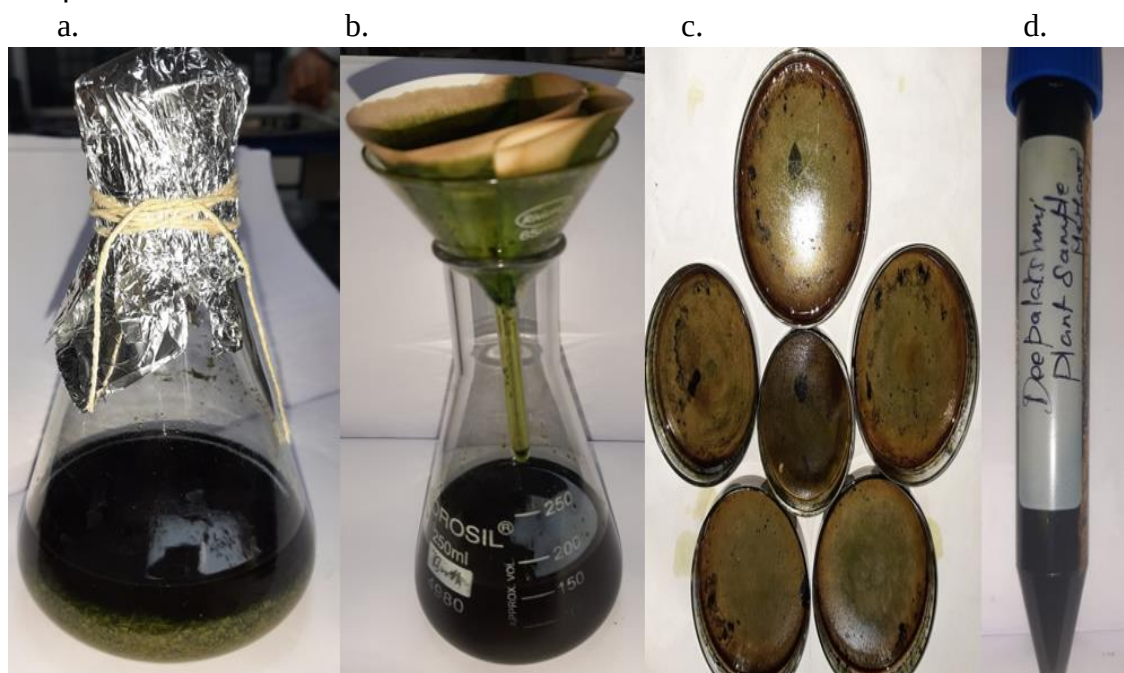


Fig 5.2 Methanolic extraction of plant sample a) Maceration of dried leaves with methanol. b) Filtration of methanol extracted compounds c) Drying at room temperature d) Collected crude extract

5.3 PREPARATION OF WORKING STANDARD SOLUTION OF MEET

The final yield of 3 grams after evaporation was dissolved in 3ml of methanol(3g/ml). From the prepared concentrate, the desired working standard solution of 1 μ g/ml, 3 μ g/ml, 10 μ g/ml, 30 μ g/ml and 100 μ g/ml was prepared by diluting with physiological saline.

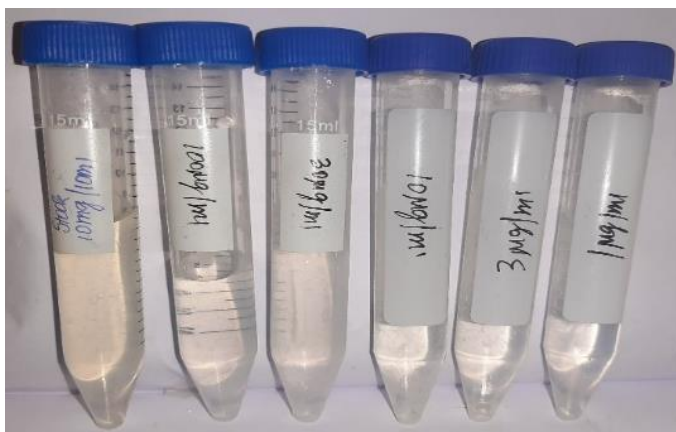


Fig 5.3 Working solution of test sample

5.4 COLLECTION OF CANINE BLOOD SAMPLE

The blood from healthy donors were collected in glass tubes containing no additives. The blood was centrifuged immediately within 15 minutes of sample collection.

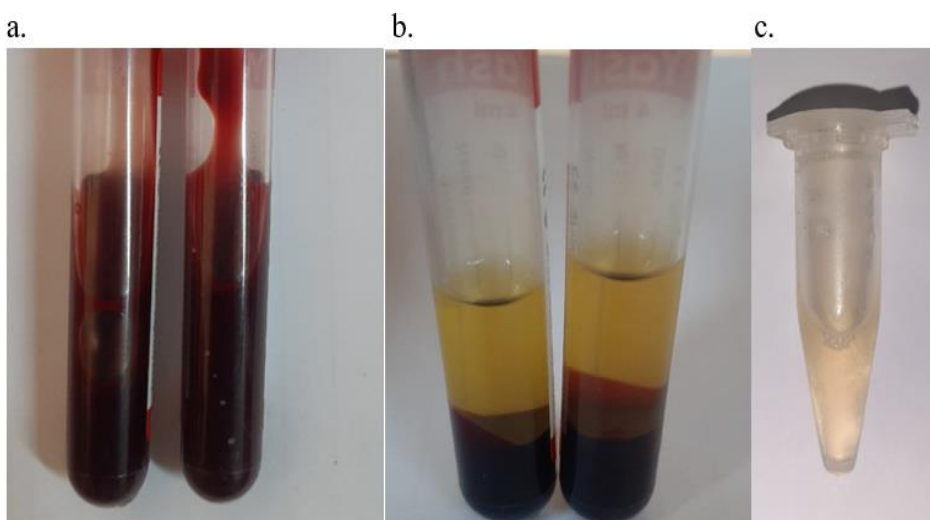


fig 5.4 Separation of Canine Platelet rich plasma (PRP) a) collected blood sample b) Separation of plasma after centrifugation. c) collected plasma

5.5 ACTIVATED PARTIAL THROMBOPLASTIN TIME (APTT) TEST

For the invitro anticoagulant assay, MEET prolonged the APTT clotting time in a dose-dependent manner. It prolonged the clotting time from 26.31 ± 4.003 s to 52.74 ± 3.88 s at a concentration of 100 $\mu\text{g/ml}$. The positive control heparin prolonged the clotting time more than 82.7 secs at concentration of 100 $\mu\text{g/ml}$ (50 I.U) The resulting data was expressed as mean $\pm$ S.D along with the types of sample tested and quantity of reagents used.

Table 5.1: Results of APTT clotting time prolongation by MEET

Sample	Sample to be tested		PRP	APTT Reagent	CaCl ₂ (0.20M)	Clotting time
	(μl)	Conc. ($\mu\text{g/ml}$)	(μl)	(μl)	(μl)	(secs)
TS-1	20	1	100	100	100	29.48 ± 4.003
TS-2	20	3	100	100	100	31.38 ± 2.11
TS-3	20	10	100	100	100	36.48 ± 3.024
TS-4	20	30	100	100	100	39.92 ± 2.960
TS-5	20	100	100	100	100	52.74 ± 3.88
PC (Heparin)	20	100	100	100	100	82.7 ± 1.82
NC (Saline)	20	-	100	100	100	26.31 ± 2.21

The dose-dependent APTT clotting time prolongation is represented graphically in fig. The X- Axis represents the concentration (in micrograms) of the of sample tested and Y-axis denotes the clotting time of canine blood.

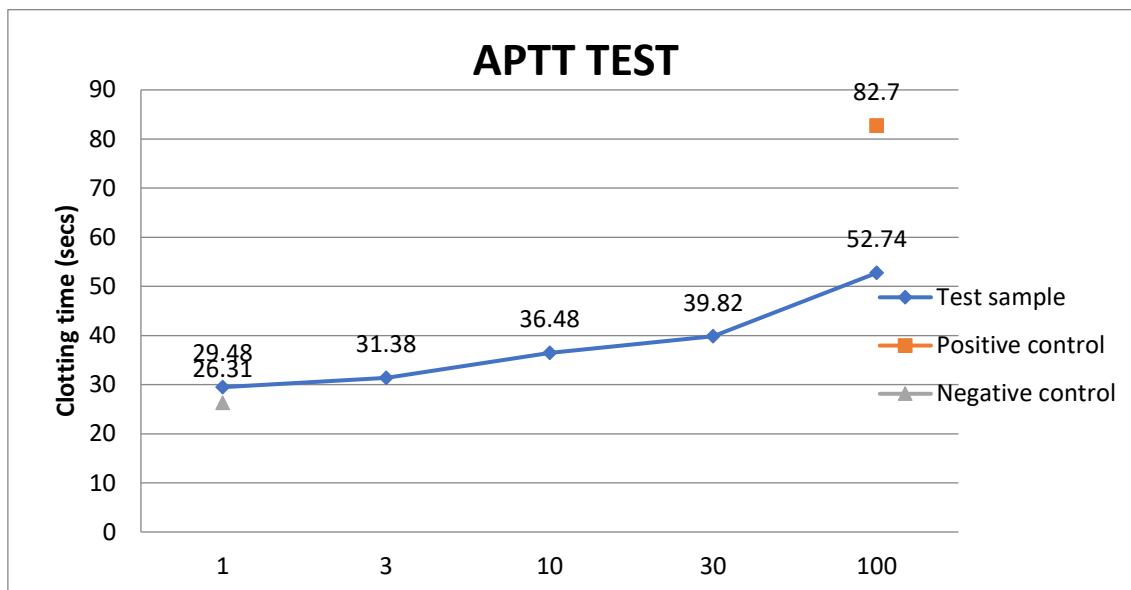


Fig 5.5: Graphical representation of APTT prolongation by MEET

5.6 WHOLE BLOOD (WB) CLOT LYSIS ASSAY

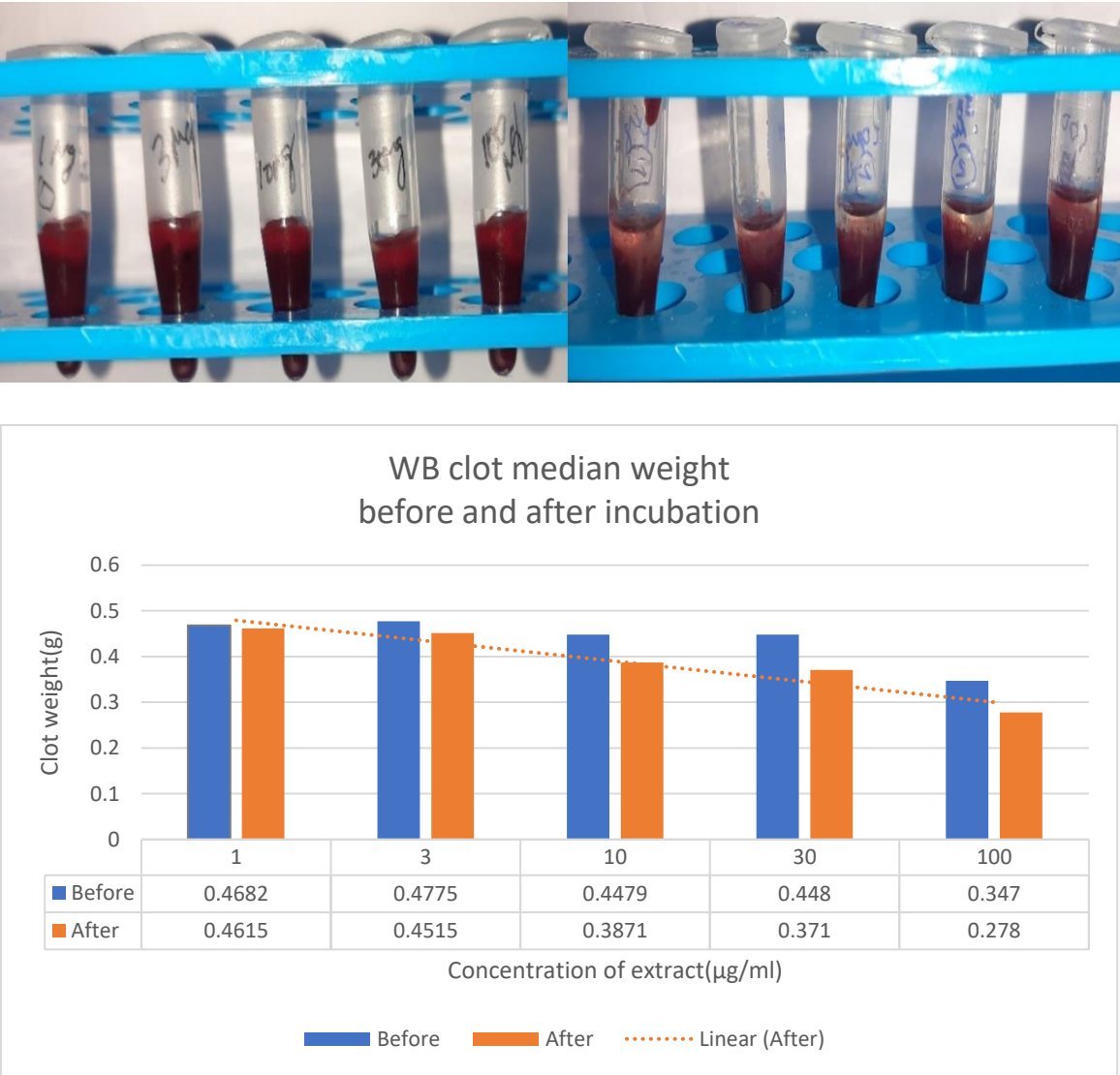
The clot lysis assay was performed thrice by collecting venous blood from healthy volunteers. The mean clot lysis percentage was calculated by the difference in the clot weight before and after addition of test sample divided by the weight the clot before the addition of test sample. The results of their inhibitory effect on thrombin at different concentration was tabulated and the data was expressed as mean $\pm$ S.D.

Table 5.2: Results of thrombolytic activity of MEET

	Whole blood of Canine	Sample		Clot lysis
	(ml)	(ml)	Conc. ($\mu\text{g/ml}$)	(%)
TS-1	0.5	0.5	1 μg	3.56 $\pm$ 2.14
TS-2	0.5	0.5	3 μg	5.44 $\pm$ 3.63
TS-3	0.5	0.5	10 μg	13.59 $\pm$ 2.78
TS-4	0.5	0.5	30 μg	14.83 $\pm$ 1.49
TS-5	0.5	0.5	100 μg	37.17 $\pm$ 3.62
NC (Saline)	0.5	0.5	---	1.81 $\pm$ 2.87

Visual representation of clot lysis is shown in fig. When 500μl of water was added to the control clot, negligible clot lysis was observed. Whereas, tubes to which different dilutions of MEET was added, significant clot lysis was observed by the dose-dependent reduction of clot weight. Highest clot lysis % of 37.17 was observed at the MEET concentration of 100μg/ml.

Fig 5.6: Visual representation of inhibitory effect of MEET on thrombin (before and after)



The mean clot lysis % of MEET with all different concentration is graphically represented in. The was found to differ significantly when compared to water. The

X-axis refers to different concentration of MEET in $\mu\text{g/ml}$. The Y-axis denotes the whole clot lysis percentage.

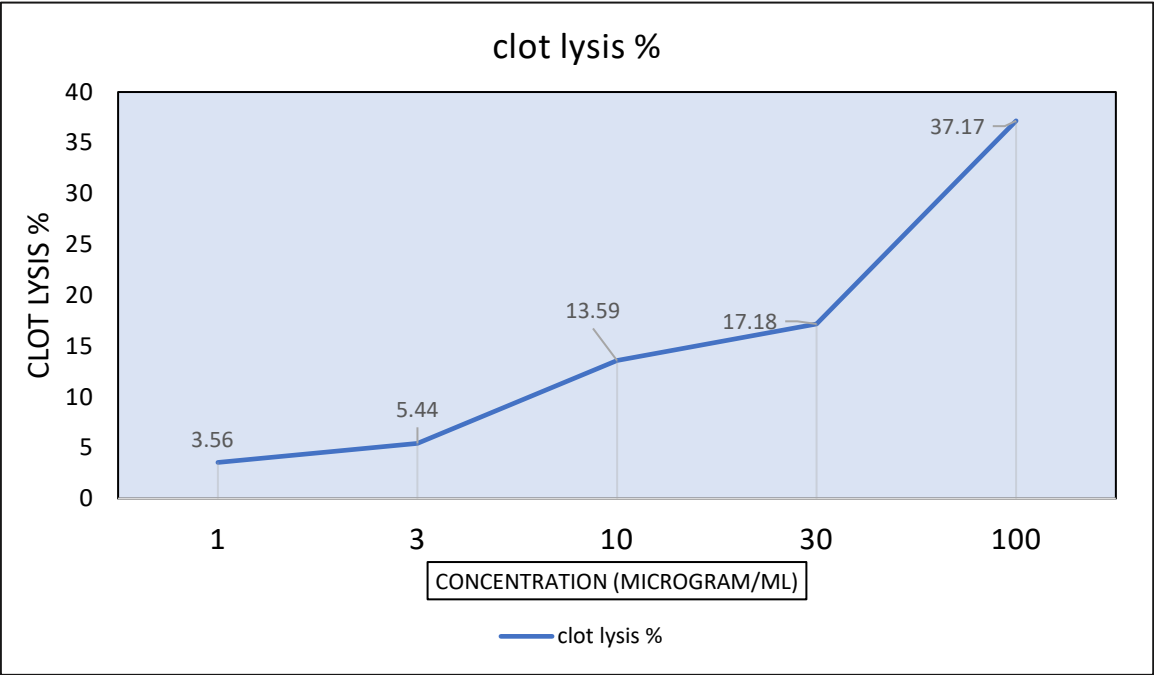


Fig 5.7: Graphical representation of thrombin inhibition of MEET

5.7 ANALYSIS OF THE CRUDE EXTRACT USING GAS CHROMATOGRAPHY-MASS SPECTROSCOPY (GC-MS)

GC-MS analysis of the plant extract led to the identification of 10 components of which the prevalent compounds were Benzopyran-2-one, 7-methoxy (100%) and Benzene, 1-4-dimethoxy-2-methyl-5-isopropyl-(63.12 %). The major peaks exhibited in GC analysis were identified and listed along with the name of the compound, retention time and area percentage of the compounds found in the methanolic extract.

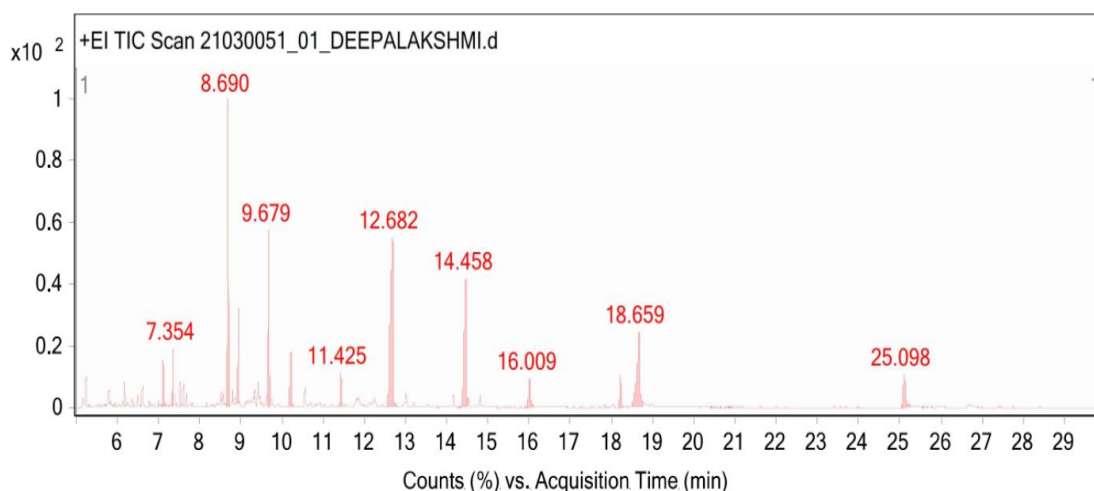


Fig 5.8: GC-MS spectrum for methanol extract

The compounds were identified with cross referencing of spectral signals obtained to that with the NIST14 spectral library for chemical compounds.

Table 5.3: Compounds identified in the GC-MS analysis of MEET

Name	R. Time	Area	Area %
5-Hydroxymethylfurfural	7.114	461909685.09	10.38
Thymoquinone	7.354	424460937.03	9.54
Benzene, 1-4-dimethoxy-2-methyl-5-isopropyl-	8.69	2807698043.49	63.12
Bicyclo [5.2.0] nonane, 2-methylene-4,8,8-trimethyl-4-vinyl-	8.938	708135865.57	15.92
Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methyethenyl)- [4aR-(4a. alpha.,7. alpha.,8a. beta.)]-	9.679	1682563026.53	37.83
p-cymene-2,5-diol	10.214	8579845864.4	19.29
2H-1-Benzopyran-2-one,7-methoxy-	12.682	4448141287.09	100
n-Hexadecanoic acid	16.009	157879768.05	9.56
Phytol	18.205	168891888.92	10.23
9,12,15-Octadecatreinoic acid, (Z, Z, Z)-	18.659	2021103927.1	45.44

5.8 EVALUATION OF DRUG LIKELINESS, ADME & TOXICITY USING PREADMET SOFTWARE

5.8.1 Drug Likelihood

The drug likelihood properties of the compounds were predicted by Lipinski's rule ("Rule of five") which was published by Christopher A. Lipinski et al. It is largely used as filter for selecting leads. The compounds are more-likely to have poor absorption or permeation when there more than 5 hydrogen bond donors (The sum of Ohs and NHs), 10 hydrogen bond acceptors (The sum of Os and NS), molecular weight greater than 500 and the Calculated LogPvalue greater than 4.5 (CLogP). The compounds following about 90% of the above subset is included in the defined range with better solubility and permeability. Out of the ten compounds screened, all the screened lead compounds qualified lipinski's rule of five by following the desired range of subset.

5.8.2 ADME studies

The PreAdmet software predicts 19 ADME properties including plasma protein binding, Blood brain barrier penetration (BBB), Skin permeability, Human intestinal absorption (HIA) and Caco₂ permeability. The compounds are predicted to have better ADME profile if the Blood Brain Barrier (BBB) Penetration (represented as $BB = \frac{[Brain]}{[Blood]}$) is more than 2.0 referring to high absorption to Central Nervous system (CNS) and low absorption to BB less than 0.1; Plasma Protein binding of a drug influences not only the drug's action but also its disposition and efficacy. The chemicals may strongly bind if the PPB percentage is more than 90 and weakly bound if it is less than 90; skin permeability which is crucial for transdermal delivery of drugs is given as $\log K_p$, K_p (cm/hr); Human Intestinal Absorption (HIA) which predicted by summing of bioavailability and absorption evaluated from ratio of excretion or cumulative excretion in urine, bile and feces. The compounds are predicted to be well absorbed if the HIA % is between 70-100%, moderately absorbed in the range 20- 70% and poorly absorbed below 20%; Caco₂ cell model and MDCK cell model is used a reliable invitro model for the prediction of oral drug absorption. From the experimental values, the compounds are predicted to have low permeability if the P_{caco2} (nm/sec) is less than 4, middle permeability for P_{caco2} value in the range of 4- 70 and high permeability for more than 70 P_{caco2} ; CYP (involved in drug metabolism) inhibition for multiple isoforms. The ADME predictions of thymoquinone, palmitic acid, phytol, linolenic acid and bicyclo [5.2.0] nonane, 2-methylene-4,8,8-trimethyl-4-vinyl-was tabulated. The results predicted that all the compounds pass across the Blood Brain Barrier and their plasma protein binding (PPB) ability was strongly bound. Human Intestinal absorption which is an important parameter for identifying potential drug candidate, showed nearly 100% absorption for all the compounds evaluated.

Table 5.4: Prediction of ADME properties of Biyclo, Thymoquinone, Phytol, Palimitic & Linolenic Acid

ID	THY	PALMITIC ACID	PHYTOL	LINOLENIC ACID	BICYCLO
----	-----	------------------	--------	-------------------	---------

BBB	1.78938	8.21885	8.21885	6.16921	11.9103
Buffer_solubility_mg_L	357.209	136.24	136.24	2711.95	13.9227
Caco2	23.037	26.0735	26.0735	27.9738	23.4925
CYP_2C19_inhibition	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Non
CYP_2C9_inhibition	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor
CYP_2D6_inhibition	Non	Non	Non	Non	Non
CYP_2D6_substrate	Non	Non	Non	Non	Non
CYP_3A4_inhibition	Non	Inhibitor	Inhibitor	Inhibitor	Non
CYP_3A4_substrate	Substrate	Non	Non	Non	Substrate
HIA	99.28634	98.29711	98.29711	98.27361	100
MDCK	61.0892	70.5083*	70.5083*	74.7897	46.5129*
Pgp_inhibition	Non	Inhibitor	Inhibitor	Inhibitor	Inhibitor
Plasma_Protein_Binding	100	100	100	100	100
Pure_water_solubility_mg_L	61.7082	1.70214	1.70214	7.66782	7.17653
Skin_Permability	-1.78115	-0.58154	-0.58154	-0.53827	-0.857
SKlogD_value	2.5149	5.26919	5.26919	5.21491	4.59533
SKlogP_value	2.5149	6.51719	6.51719	6.46291	4.59533
SKlogS_buffer	-2.66246	-3.27466	-3.27466	-2.01144	-4.16666
SKlogS_pure	-3.42504	-5.17797	-5.17797	-4.56005	-4.45447

The ADME predictions of Beta selinene, thymohydroquinone, 5-Hydroxy furfural Benzene,1-4-Dimethoxy-2-Methyl-5-Isopropyl- and 7-methoxy coumarin was tabulated. The results predicted that all the compounds pass across the Blood Brain Barrier and their plasma protein binding (PPB) ability was strongly bound, except 5-hydroxy furfural and 7-methoxy coumarin which showed weak binding efficacy.

Table 5.5: Prediction of ADME Properties of Beta Selinene, Coumarin, Benzene, Thymohydroquinone & 5-Hydroxy Furfural

ID	Bet	Thy.Hyd	5-hydroxy	Benzene	Coumarin
BBB	13.4992	4.97726	0.52231	1.30947	1.82065
Buffer_solubility_mg_L	0.641423	770.942	48.3988	128.013	915.578
Caco2	23.4924	20.394	6.41666	57.833	40.683
CYP_2C19_inhibition	Non	Inhibitor	Inhibitor	Inhibitor	Inhibitor
CYP_2C9_inhibition	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Inhibitor
CYP_2D6_inhibition	Non	Non	Non	Non	Non
CYP_2D6_substrate	Non	Non	Non	Non	Non
CYP_3A4_inhibition	Inhibitor	Inhibitor	Inhibitor	Inhibitor	Non

CYP_3A4_substrate	Substrate	Non	Non	Substrate	Non
HIA	100	89.19143	88.68205	100	99.1351
MDCK	57.2175*	37.7191	152.574	50.4576	59.0427
Pgp_inhibition	Inhibitor	Non	Non	Non	Non
Plasma_Protein_Binding	100	100	49.46599	100	69.84908
Pure_water_solubility_mg_L	4.21652	401.127	12840	96.6524	365.574
Skin_Permability	-0.64245	-1.61884	-2.9279	-1.38889	-2.35347
SKlogD_value	4.93112	3.25102	0.50086	3.52836	1.81946
SKlogP_value	4.93112	3.25102	0.50086	3.52836	1.81946
SKlogS_buffer	-5.50324	-2.33366	-3.41592	-3.18116	-2.28424
SKlogS_pure	-4.68543	-2.6174	-0.99219	-3.3032	-2.68296

5.8.3 TOXICITY RESULTS

The toxicity profile of the ten identified compounds were predicted by PreAdmet predictive software to analyze, simulate and visualize the toxicity of chemicals.

The toxicity of lead compounds is mainly predicted by Rodent Carcinogenicity. Rodent Carcinogenicity test predicts the toxicity that causes cancer in body by exposing the compounds to mice or rat. The negative results indicate clear evidence of carcinogenic activity and positive results indicate no evidence of carcinogenic activity. The toxicity results of all the ligands were tabulated. The results predicted that the phytochemicals show no evidence of carcinogenic activity in rat, whereas the compounds were predicted to be carcinogenic in mouse except linolenic acid and thymoquinone.

Table 5.6: Toxicity prediction of compounds identified in MEET

Ligands	carcino_mouse	Carcino_rat
Thymoquinone	positive	positive
Palmitic acid	Negative	Positive
Phytol	Negative	Positive
Linolenic acid	Positive	Positive
Bicyclo bicyclo [5.2.0] nonane, 2-methylene-4,8,8-trimethyl-4-vinyl-	Negative	Positive
Beta selinene	Negative	Positive
thymohydroquinone	Negative	Positive
5-Hydroxy furfural	Negative	Positive
Benzene,1-4-Dimethoxy-2-Methyl-5-Isopropyl-	Negative	Positive

7-methoxy coumarin	Negative	Positive
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5.9 PREDICTION OF BIOLOGICAL ACTIVITY USING PREDICTION OF ACTIVITY SPECTRA FOR SUBSTANCES (PASS)

Prediction of Activity Spectra for Substances (PASS) is the openly available online server used to predict the pharmacological effects and biochemical mechanisms of a compound with an average accuracy of more than 95% (Filimonov et al., 2014). The Smiles notation of the identified compound is given as a query input in the PASS server. The prediction depends on the Pa: Pi (active, inactive ratio) at prediction threshold of Pa>30%, Pa>50% and 70% (Parasuraman, 2011). The Pa value of greater than 0.7 represents the greater probability of exhibiting the activity of a substance in an experiment, while Pa > 0.5 gives quite reliable prediction and the Pa > 0.3 threshold describes that a compound is less likely to have the desired activity. The SMILES notation was retrieved from the PubChem database to perform PASS analysis done for the 10 compounds identified in the methanolic extract

The SMILES notation for the compound 5-Hydroxy methyl furfural was C1=C(OC(=C1)C=O)CO. Based on the report 44 possible activities at Pa>70% were predicted. (Sarkar et al. 2021, refno.15)

Table 5.7: Biological activity of 5-hydroxy methyl furfural

☒ All ☐ Pa>Pi ☐ Pa>0,3 ☐ Pa>0,7

0,885	0,012	CDP-glycerol glycerophosphotransferase inhibitor
0,865	0,006	Anaphylatoxin receptor antagonist
0,859	0,008	Sugar-phosphatase inhibitor
0,867	0,020	Membrane integrity agonist
0,832	0,004	Aspartate-phenylpyruvate transaminase inhibitor
0,817	0,005	UDP-N-acetylglucosamine 4-epimerase inhibitor
0,805	0,017	Alkenylglycerophosphocholine hydrolase inhibitor
0,749	0,018	G-protein-coupled receptor kinase inhibitor
0,595	0,030	Limulus clotting factor B inhibitor

The SMILES notation for the compound Thymoquinone was CC1=CC(=O)C(=CC1=O)C(C)C. Based on the report 49 possible activities at Pa>70% were predicted with a large probability for fibrinolytic activity.

Table 5.8: Biological activity of thymoquinone

☒ All ☐ Pa>Pi ☐ Pa>0,3 ☐ Pa>0,7

0,921	0,004	Ubiquinol-cytochrome-c reductase inhibitor
0,890	0,002	Carminative
0,881	0,005	Mucomembranous protector
0,873	0,003	Alkane 1-monooxygenase inhibitor
0,873	0,010	Testosterone 17beta-dehydrogenase (NADP+) inhibitor
0,860	0,021	CYP2C12 substrate
0,779	0,005	Fibrinolytic
0,670	0,013	Vasoprotector
0,642	0,014	Cholesterol antagonist
0,635	0,022	Limulus clotting factor B inhibitor
0,510	0,028	Limulus clotting factor C inhibitor
0,528	0,044	Platelet adhesion inhibitor

The SMILES notation for the compound Benzene,1-4-Dimethoxy-2-Methyl-5-Isopropyl- was CC1=CC(=C(C=C1OC)C(C)C)OC. Based on the report 31 possible activities at $P_a > 70\%$ were predicted with a large probability for fibrinolytic activity.

Table 5.9: Biological activity of Benzene,1-4-dimethoxy-2-methyl-5-isopropyl-

☒ All
 ☐ $P_a > P_i$
☐ $P_a > 0,3$
☐ $P_a > 0,7$

0,914	0,002	Carminative
0,911	0,004	Ubiquinol-cytochrome-c reductase inhibitor
0,875	0,014	Aspulvinonedimethylallyltransferase inhibitor
0,856	0,004	Fibrinolytic
0,849	0,005	Membrane permeability inhibitor
0,853	0,009	Mucomembranous protector
0,633	0,015	Cholesterol antagonist
0,633	0,016	Vasoprotector
0,618	0,019	Vasodilator, peripheral
0,544	0,034	Platelet adhesion inhibitor

The SMILES notation for the compound Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4-vinyl- was CC1(CC2C1CCC(CC2=C)(C)C=C)C. Based on the report 10 possible activities at $P_a > 70\%$ were predicted.

Table 5.10: Biological activity of bicyclo [5.2.0] nonane, 2-methylene-4,8,8-trimethyl-4-vinyl-

☒ All ☐ Pa>Pi ☐ Pa>0,3 ☐ Pa>0,7

0,899	0,005	Antineoplastic
0,899	0,004	Apoptosis agonist
0,867	0,005	Antiinflammatory
0,832	0,002	Nitric oxide antagonist
0,822	0,002	Transcription factor stimulant
0,559	0,010	Caspase 8 stimulant

The SMILES notation for the compound Beta selinene was CC(=C)C1CCC2(CCCC(=C)C2C1)C. Based on the report 21 possible activities at Pa>70% were predicted.

Table 5.11 Biological activity of beta selinene

☒ All ☐ Pa>Pi ☐ Pa>0,3 ☐ Pa>0,7

0,935	0,003	Antieczematic
0,936	0,004	Antineoplastic
0,894	0,004	Dermatologic
0,870	0,001	Retinol dehydrogenase inhibitor
0,833	0,006	Apoptosis agonist
0,833	0,008	CYP2C substrate
0,764	0,009	Antiinflammatory
0,598	0,007	Caspase 8 stimulant
0,536	0,011	Menopausal disorders treatment

The SMILES notation for the compound thymoquinone was CC1=CC(=C(C=C1O)C(C)C)O. Based on the report 68 possible activities at Pa>70% were predicted.

Table 5.12: Biological activity of thymohydroquinone

☒ All
 ☐ Pa>Pi
 ☐ Pa>0,3
 ☐ Pa>0,7

0,934	0,003	Ubiquinol-cytochrome-c reductase inhibitor
0,932	0,003	Antiseptic
0,901	0,001	SULT1A3 substrate
0,897	0,001	SULT1A2 substrate
0,888	0,003	Alkane 1-monooxygenase inhibitor
0,889	0,005	Mucomembranous protector
0,880	0,003	Fibrinolytic
0,729	0,012	Membrane integrity antagonist
0,709	0,008	Cholesterol antagonist
0,669	0,013	Vasoprotector
0,652	0,009	Cholestanetriol 26-monooxygenase inhibitor
0,596	0,006	Vitamin-K-epoxide reductase (warfarin-insensitive) inhibitor
0,614	0,026	Limulus clotting factor B inhibitor
0,591	0,023	Vasodilator, peripheral
0,569	0,022	Platelet adhesion inhibitor
0,570	0,036	Platelet aggregation stimulant

The SMILES notation for the compound 7-methoxy coumarin was COC1=CC2=C(C=C1)C=CC(=O)O2. Based on the report 66 possible activities at Pa>70% were predicted.

Table 5.13: Biological activity of 7-methoxy coumarin

☒ All
 ☐ Pa>Pi
 ☐ Pa>0,3
 ☐ Pa>0,7

0,962	0,003	CYP2C12 substrate
0,948	0,001	4-Nitrophenol 2-monooxygenase inhibitor
0,944	0,001	CYP2A11 substrate
0,942	0,003	Aspulvinonedimethylallyltransferase inhibitor
0,900	0,011	Membrane integrity agonist
0,880	0,004	CYP2A6 substrate(COUMARIN AFFINITY)
0,870	0,002	CYP2F1 substrate

0,829	0,005	Caspase 3 stimulant
0,791	0,001	CYP2A5 inhibitor
0,723	0,006	Cholestanetriol 26-monooxygenase inhibitor
0,648	0,046	Fibrinolytic
0,609	0,014	Vasodilator, coronary
0,610	0,019	Vasoprotector

The SMILES notation for the compound palmitic acid was CCCCCCCCCCCCCCCC(=O)O. Based on the report 425 possible activities at $Pa > 70\%$ were predicted which depicts a very high probability for new biological activity.

Table 5.14: Biological activity of palmitic acid

☒ All ☐ $Pa > Pi$ ☐ $Pa > 0,3$ ☐ $Pa > 0,7$

0,973	0,001	Acylcarnitine hydrolase inhibitor
0,966	0,001	Alkylacetylgllycerophosphatase inhibitor
0,963	0,002	Alkenylglycerophosphocholine hydrolase inhibitor
0,962	0,002	CYP2J substrate
0,834	0,004	Vasoprotector
0,808	0,005	Lipid metabolism regulator
0,780	0,005	Fibrinolytic
0,754	0,007	Limulus clotting factor B inhibitor
0,728	0,003	Thromboxane B2 antagonist
0,723	0,007	Cholesterol antagonist
0,719	0,004	Vitamin-K-epoxide reductase (warfarin-insensitive) inhibitor
0,665	0,006	Fibrinogen receptor antagonist
0,642	0,016	Vasodilator, peripheral
0,621	0,013	Antithrombotic

The SMILES notation for the compound phytol was CC(C)CCCC(C)CCCC(C)CCCC(=CCO)C. Based on the report 36 possible activities at $Pa > 70\%$ were predicted.

Table 5.15: Biological activity of phytol

☒ All ☐ $Pa > Pi$ ☐ $Pa > 0,3$ ☐ $Pa > 0,7$

0,911	0,002	Prenyl-diphosphatase inhibitor
0,907	0,001	Retinol dehydrogenase inhibitor
0,905	0,005	Ubiquinol-cytochrome-c reductase inhibitor

0,893	0,007	Phobic disorders treatment
0,690	0,007	Platelet aggregation stimulant
0,674	0,016	Limulus clotting factor B inhibitor
0,662	0,004	Vitamin-K-epoxide reductase (warfarin-insensitive) inhibitor
0,545	0,022	Antithrombotic

The SMILES notation for the compound Linolenic acid was CCC=CCC=CCC=CCCCCCCCC(=O)O. Based on the report 202 possible activities at $P_a > 70\%$ were predicted which depicts a very high probability for new biological activity.

Table 5.16: biological activity of linolenic acid

☒ All
 ☐ $P_a > P_i$
☐ $P_a > 0,3$
☐ $P_a > 0,7$

0,971	0,001	CYP2J substrate
0,970	0,001	CYP2J2 substrate
0,963	0,002	Antieczematic
0,952	0,002	Linoleate diol synthase inhibitor
0,919	0,002	Lipoprotein lipase inhibitor
0,887	0,004	Lipid metabolism regulator
0,852	0,004	Cholesterol antagonist
0,814	0,005	Vasoprotector
0,810	0,002	Platelet aggregation stimulant
0,790	0,001	Thromboxane synthase stimulant
0,744	0,002	Platelet adhesion inhibitor
0,745	0,005	Antithrombotic
0,706	0,004	Vitamin-K-epoxide reductase (warfarin-insensitive) inhibitor
0,694	0,010	Vasodilator, peripheral
0,691	0,026	Fibrinolytic
0,659	0,007	Fibrinogen receptor antagonist
0,619	0,005	Thromboxane B2 antagonist

5.10 RESULTS OF MOLECULAR INTERACTIONS BETWEEN SELECTED TARGETS & LIGANDS

A lead compound is predicted to have good binding affinity with the target (protein) based on the parameters of dock score, relative energy and most importantly hydrogen bond distance. A high dock score with a very low relative denotes that the compound consumes only less energy to bind with the potential drug targets. The hydrogen bond is one of the strongest intermolecular attractions, but weaker than a covalent or an ionic bond. So, if the hydrogen bond distance between the interacting amino acid residue is more, the bonds can be broken easily which fails to qualify its criteria as a lead compound. The typical hydrogen bond length for a stronger interaction is predicted to be 1.5- 3 Å.

Molecular docking of lead compounds was performed with all the potential targets involved in coagulation cascade. Only the best interacted ligand with each target was tabulated.

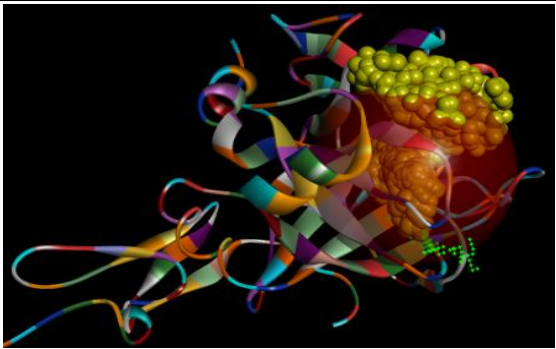
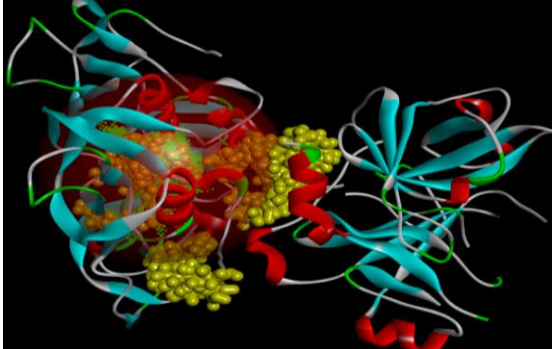
Table: 5.17 Results of top molecular interactions

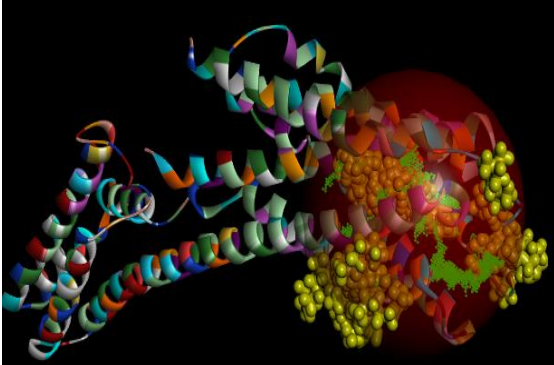
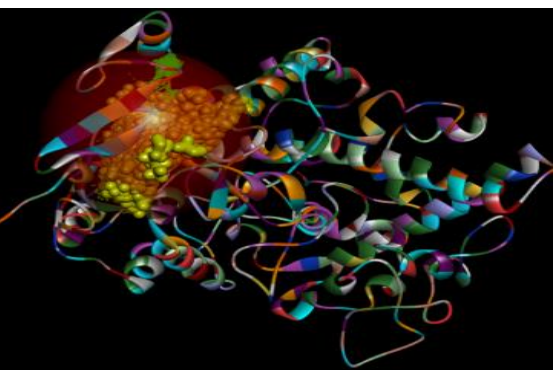
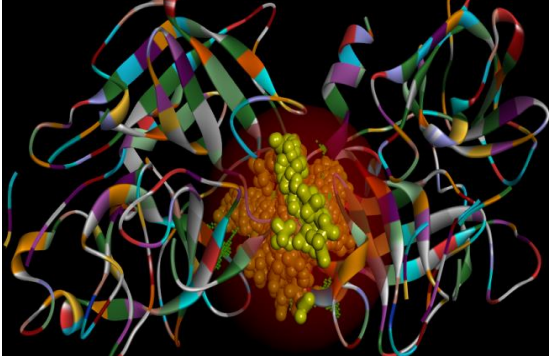
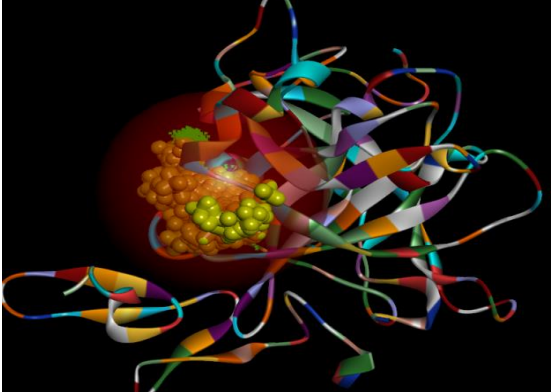
TARGET (PDB ID)	LIGAND	DOCK SCORE	RELATIVE ENERGY	NO. OF H BONDS	H BOND LENGTH (Å)
1FAX	Linolenic acid	128.34	-3.14	234	2.22
1RFN	Palmitic acid	119.47	-8.28	277	1.78
5EOD	Linolenic acid	117.86	-10.07	62	3.34
4PXZ	Phytol	125.23	-5.244	14	2.99
6Y3C	Phytol	121.56	-8.82	153	1.69
6L63	Linolenic acid	129	-16.08	164	2.66
1JBU	Linolenic acid	137.20	-8.25	214	2.71
3CDZ	Linolenic acid	108.09	-11.46	10	2.47
1QFK	Linolenic acid	130.804	-8.44	210	2.31
3U69	Phytol	118.82	-4.65	139	1.33
1JMJ	Linolenic acid	126.08	-5.23	112	1.60
1GWB	Linolenic acid	115.78	-3.100	190	2.73
2B4X	Linolenic acid	124.77	-3.95	214	2.10
1J9C	Linolenic acid	131.07	-3.12	246	2.92
2K9J	Phytol	110.54	-7.38	66	2.48

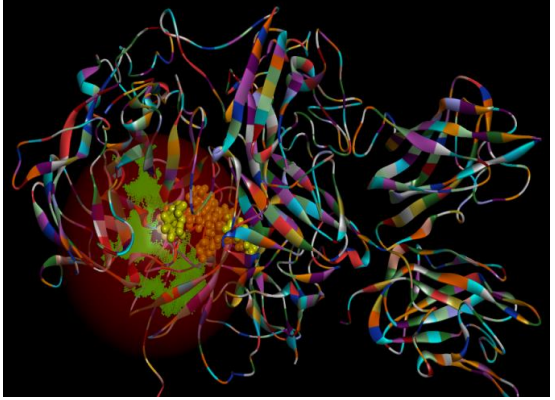
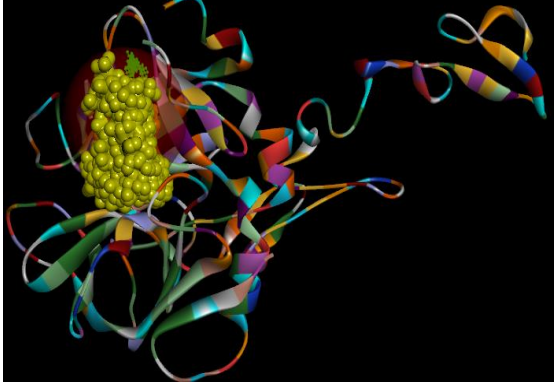
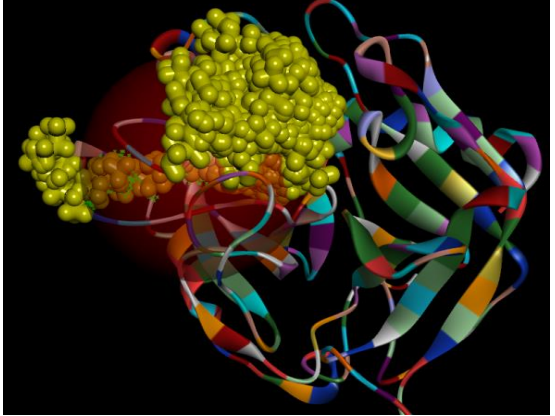
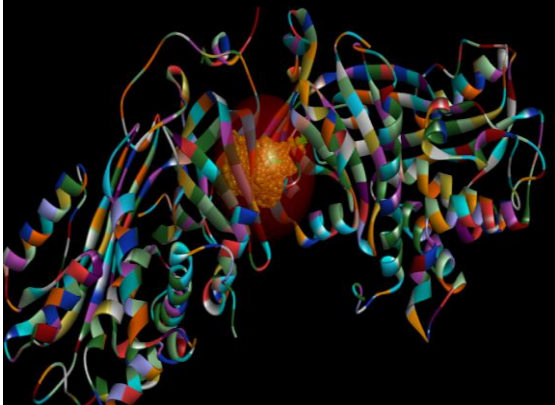
1F9Q	Linolenic acid	124.99	-8.28	194	2.94
1DDJ	Linolenic acid	123.82	-2.44	181	2.41
6O1G	Linolenic acid	115.12	-15.51	175	2.45

Among the compounds docked, linolenic acid interacted very effectively with Coagulation Factor VII and showed highest dock score of 137.20 with a relative energy of -8.25. Overall, palmitic acid, linolenic acid and phytol showed highest interaction with all the targets selected when compared to other phytochemicals. From the *insilico* studies it can be concluded that palmitic acid, linolenic acid and phytol may have significant role in preventing the disorders involving blood coagulation pathway which was also positively supported by invitro model study.

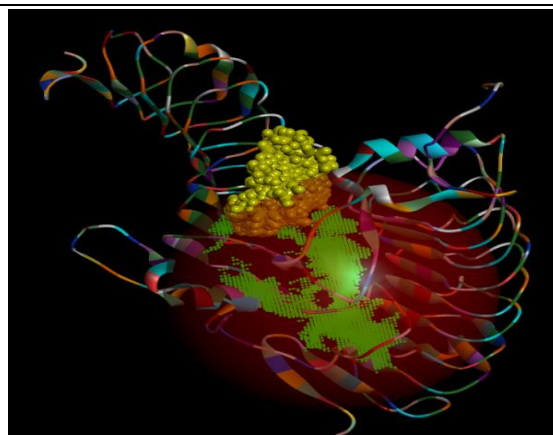
Table: 5.18: 3D images of best interacted targets and ligands

Compounds involved in interaction (target-ligand)	3D view of Molecular interaction
Coagulation Factor Xa- Linolenic acid	
Human Coagulation Factor IXa - Palmitic acid	
Human Plasma Coagulation FXI- Linolenic acid.	

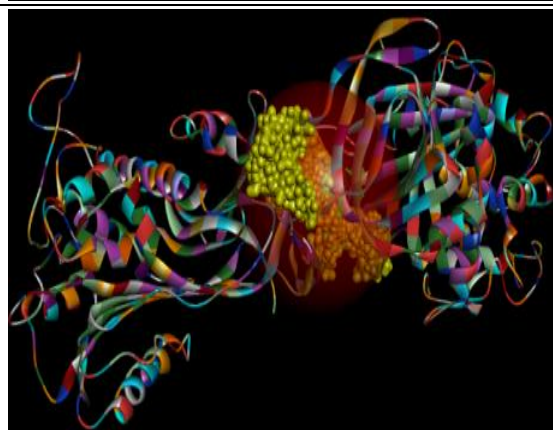
Crystal structure of P2Y12 receptor-Phytol	
Human Cox-1 Crystal Structure-Phytol	
Human Coagulation Factor XIIA (FXIIA)- Linolenic acid	
Coagulation Factor VII Zymogen-Linolenic acid	

Human coagulation factor VIII	
Structure of Human Factor VIIA - Linolenic acid	
Unliganded Wild-Type Human Thrombin- Phytol	
Crystal Structure of Native Heparin Cofactor I- Linolenic acid	

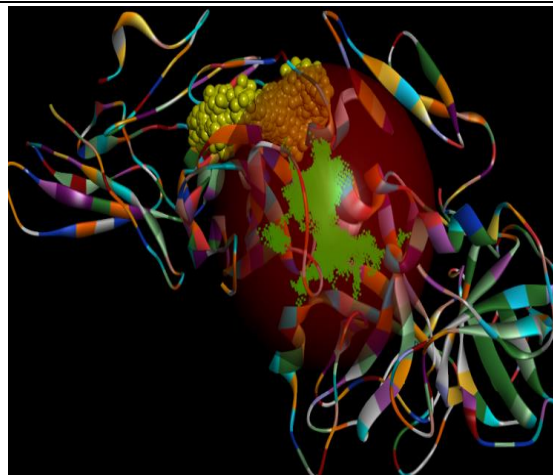
Structure of Glycoprotein 1b-
Linolenic acid



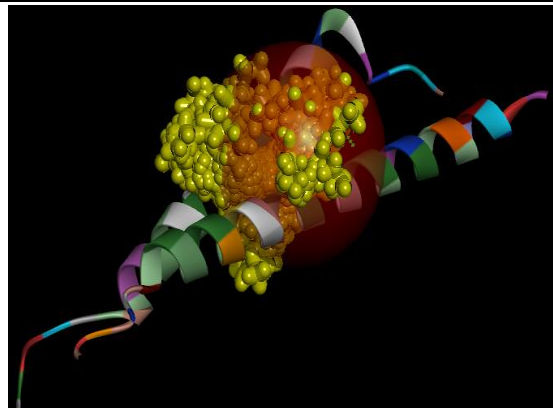
Crystal Structure of Antithrombin-III-
Linolenic acid



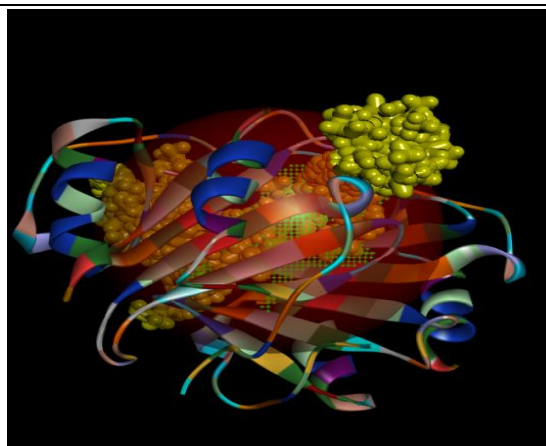
Crystal Structure of tissue factor-factor
VIIa complex



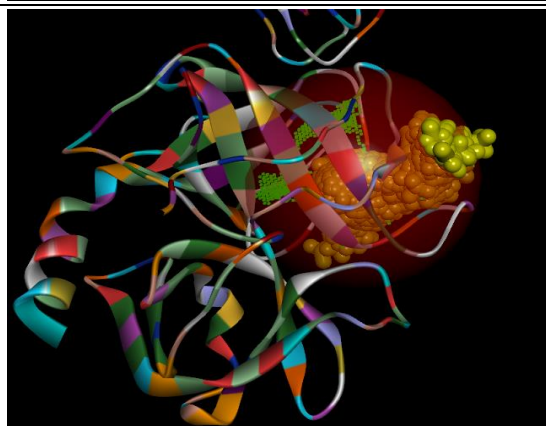
Integrin AlphaIIb-Beta3
Transmembrane Complex



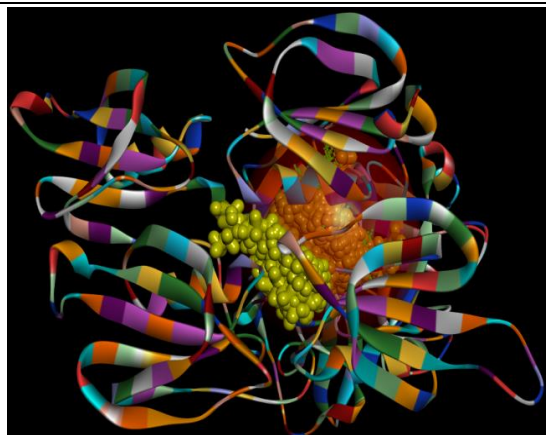
Crystal Structure of Platelet Factor 4-
Linolenic acid



Crystal Structure of Human
Plasminogen Catalytic Domain-
Linolenic acid



Full Length Human Plasma
Kallikrein- Linolenic acid



REFERENCES

1. Rakotoarivelo NH, Rakotoarivony F, Ramarosandratana AV, et al. Medicinal plants used to treat the most frequent diseases encountered in Ambalabe rural community, Eastern Madagascar. *J Ethnobiol Ethnomed*. 2015; 11:68.
2. Refaz Ahmad Dar, Mohd Shahnawaz, Parvaiz Hassan Qazi. General overview of medicinal plants: A review; *The Journal of Phytopharmacology* 2017; 6(6): 349-351
3. Vickers, A., Zollman, C., & Lee, R. (2001). Herbal medicine. *The Western journal of medicine*, 175(2), 125–128.
4. Fokunang, C N et al. "Traditional medicine: past, present and future research and development prospects and integration in the National Health System of Cameroon." *African journal of traditional, complementary, and alternative medicines: AJTCAM* vol. 8,3 (2011): 284-95.
5. Ravishankar, B, and V J Shukla. "Indian systems of medicine: a brief profile." *African journal of traditional, complementary, and alternative medicines: AJTCAM* vol. 4,3 319-37. 16 Feb. 2007.
6. Chauhan A, Semwal DK, Mishra SP, Semwal RB. Ayurvedic research and methodology: Present status and future strategies. *Ayu*. 2015;36(4):364-369.
7. Nishteswar, K. "Credential evidences of Ayurvedic cardio-vascular herbs." *Ayu* vol. 35,2 (2014): 111-2.
8. Brendler, Thomas & Gurib-Fakim, Ameenah. (2004). Medicinal and aromatic plants of Indian Ocean Islands: Madagascar, Comoros, Seychelles and Mascarenes.
9. BEGUM, JARIPA & Bhuiyan, Mohammad. (2010). Chemical composition and antimicrobial activity of essential oil from *Eupatorium triplinerve* Vahl. aerial parts. *Asian Jr. of Microbiol. Biotech. Env. Sc.* 12. 543-547.
10. A. Ghani, "Medicinal Plants of Bangladesh: Chemical Constituents & Uses," 2nd Edition, Asiatic Society of Bangladesh, Dhaka, 2003, pp. 1-16.
11. Mc Namara K, Alzubaidi H, Jackson JK. Cardiovascular disease as a leading cause of death: how are pharmacists getting involved? *Integr Pharm Res Pract*. 2019; 8:1-11. Published 2019 Feb 4.
12. De Caterina R. The current role of anticoagulants in cardiovascular medicine. *J Cardiovasc Med (Hagerstown)*. 2009 Aug;10(8):595-604. PMID: 19571765.
13. Ruan, Y., Guo, Y., Zheng, Y. et al. Cardiovascular disease (CVD) and associated risk factors among older adults in six low-and middle-income countries: results from SAGE Wave 1. *BMC Public Health* **18**, 778 (2018).
14. Ekim M, Ekim H (2018) Management of the Bleeding Complications in Patients on Warfarin Therapy. *J Liver Clin Res* 5(2): 1050.

15. De Caterina R, Husted S, Wallentin L, Andreotti F, Arnesen H, Bachmann F, Baigent C, Huber K, Jespersen J, Kristensen SD, Lip GY, Morais J, Rasmussen LH, Siegbahn A, Verheugt FW, Weitz JI; European Society of Cardiology Working Group on Thrombosis Task Force on Anticoagulants in Heart Disease. *Thromb Haemost.* 2013 Apr;109(4):569-79 PMID: 23447024.
16. Smith SA, Travers RJ, Morrissey JH. How it all starts: Initiation of the clotting cascade. *Crit Rev Biochem Mol Biol.* 2015;50(4):326-336. doi:10.3109/10409238.2015.1050550
17. Gale, Andrew J. "Continuing education course #2: current understanding of hemostasis." *Toxicologic pathology* vol. 39,1 (2011): 273-80.
18. Periyah MH, Halim AS, Mat Saad AZ. Mechanism Action of Platelets and Crucial Blood Coagulation Pathways in Hemostasis. *Int J Hematol Oncol Stem Cell Res.* 2017;11(4):319-327.
19. Palta S, Saroa R, Palta A. Overview of the coagulation system. *Indian J Anaesth.* 2014;58(5):515-523.
20. Nigel Mackman, Rachel E. Tilley, and Nigel S. Key Role of the Extrinsic Pathway of Blood Coagulation in Hemostasis and Thrombosis, 7 Jun 2007; *Arteriosclerosis, Thrombosis, and Vascular Biology*, 2007; 27:1687–1693
21. Wheeler AP, Gailani D. The Intrinsic Pathway of Coagulation as a Target for Antithrombotic Therapy. *Hematol Oncol Clin North Am.* 2016;30(5):1099-1114.
22. Steven P. Grover, Nigel Mackman. Intrinsic Pathway of Coagulation and Thrombosis Insights from Animal Models. *Arterioscler Thromb Vasc Biol.* 2019; 39:331-338.
23. Rose PW, Prlić A, Altunkaya A, et al. The RCSB protein data bank: integrative view of protein, gene and 3D structural information. *Nucleic Acids Res.* 2017;45(D1): D271-D281.
24. Parasuraman S. Prediction of activity spectra for substances. *J Pharmacol Pharmacother.* 2011;2(1):52-53.
25. Kim S, Thiessen PA, Bolton EE, Chen J, Fu G, Gindulyte A, Han L, He J, He S, Shoemaker BA, Wang J, Yu B, Zhang J, Bryant SH. PubChem Substance and Compound databases. *Nucleic Acids Res.* 2016 Jan 4;44(D1):D1202-13.
26. Lee, Sung & Lee, I.H. & Kim, Hee joong & Chang, G.S. & Chung, J.E. & No, Kyoung Tai. (2002). The PreADME Approach: Web-based program for rapid prediction of physico-chemical, drug absorption and drug-like properties. *euro QSAR 2002 - Designing Drugs and Crop Protectants: Processes Problems and Solutions.* 418-420.
27. Roman Diana Larisa, Roman Marin, Som Claudia, Schmutz Mélanie, Hernandez Edgar, Wick Peter, Casalini Tommaso, Perale Giuseppe, Ostafe Vasile, Isvoran Adriana; Computational Assessment of the Pharmacological Profiles of Degradation Products of Chitosan; *JOURNAL=Frontiers in Bioengineering and Biotechnology.* VOLUME=7: YEAR=2019.

28. Meibohm B, Derendorf H. Basic concepts of pharmacokinetic/pharmacodynamic (PK/PD) modelling. *Int J Clin Pharmacol Ther.* 1997 Oct;35(10):401-13.
29. Meng XY, Zhang HX, Mezei M, Cui M. Molecular docking: a powerful approach for structure-based drug discovery. *Curr Comput Aided Drug Des.* 2011;7(2):146-157.
30. Bingqing Q. Wei, Larry H. Weaver, Anna M. Ferrari, Brian W. Matthews, Brian K. Shoichet. Testing a Flexible-receptor Docking Algorithm in a Model Binding Site, *Journal of Molecular Biology*, Volume 337, Issue 5, 2004, Pages 1161-1182.
31. Lee, W., Yoo, H., Ku, S. K., Kim, J. A., & Bae, J. S. (2013). Anticoagulant activities of piperlonguminine in vitro and in vivo. *BMB reports*, 46(10), 484–489.
32. Alikhani Pour, Mahdi et al. "Cheminformatics-Based Anticoagulant Study of Traditionally Used Medicinal Plants." *Iranian biomedical journal* vol. 21,6 (2017): 400-5.
33. Durić K, Kovac Besovic EE, Niksic H, Muratovic S, Sofic E. Anticoagulant activity of some *Artemisia dracunculus* leaf extracts. *Bosn J Basic Med Sci.* 2015 May 13;15(2):9-14.
34. Weirong, Cai & Xu, Huiling & Xie, Liangliang & Sun, Jian & Sun, Taotao & Wu, Xiaoyan & Fu, Qinbao. (2015). Purification, characterization and in vitro anticoagulant activity of polysaccharides from *Gentiana scabra* Bunge roots. *Carbohydrate Polymers*. 140. 10.1016/j.carbpol.2015.12.054.
35. Chen H, Jin M, Wang YF, Wang YQ, Meng L, Li R, Wang JP, Gao L, Kong Y, Wei JF. Effect of *Toona microcarpa* Harm's leaf extract on the coagulation system. *Biomed Res Int.* 2014; 2014:615363. doi: 10.1155/2014/615363. Epub 2014 Apr 10
36. Cheriyan BV Sr, Kadhirvelu P Sr, Nadipelly J Jr, Shanmugasundaram J, Sayeli V Sr, Subramanian V Sr. Anti-nociceptive Effect of 7-methoxy Coumarin from *Eupatorium Triplinerve* vahl (Asteraceae). *Pharmacogn Mag.* 2017;13(49):81-84.
37. Huong, Do & Giang, Phan & Vu, Minh Trang. (2020). Coumarins and Polar Constituents from *Eupatorium triplinerve* and Evaluation of Their α -Glucosidase Inhibitory Activity. *Journal of Chemistry.* 2020. 1-8. 10.1155/2020/8945063.
38. Sugumar N, Karthikeyan S, Gowdhami T. Phytochemical analysis and antimicrobial activity of *Eupatorium triplinerve* Vahl. *Int J Appl Res* 2015;1(12):108-112.
39. Bose, Partha & Gupta, Maynk & Mazumder, Kishor & Kumar, R.S. & Sivakumar, T. (2007). Hepatoprotective and antioxidant effects of *Eupatorium ayapana* against carbon tetrachloride induced hepatotoxicity in rats. *Iranian Journal of Pharmacology and Therapeutics.* 6. 27-33.
40. Dongfang Xu, Guangpu Xue, Bangya Peng, Zanjie Feng, Hongling Lu, Lihu Gong, "High-Throughput Docking and Molecular Dynamics Simulations towards the Identification of Potential Inhibitors against Human Coagulation Factor XIIa", *Computational and Mathematical Methods in Medicine*, vol. 2020, Article ID 2852051, 13 pages, 2020.

41. Khalid W, Badshah A, Khan AU, Nadeem H, Ahmed S. Synthesis, characterization, molecular docking evaluation, antiplatelet and anticoagulant actions of 1,2,4 triazole hydrazone and sulphonamide novel derivatives. Chem Cent J. 2018 Feb 7;12(1):11. doi: 10.1186/s13065-018-0378-5. PMID: 29411174; PMCID: PMC5801135