Procedure to enhance cellular viability of blood and its components to improve its shelf life

A Major Project dissertation submitted in partial fulfilment of the requirement for the degree of

Master of Technology
In
Bioinformatics

Submitted by

Ishita Goyal

(DTU/13/M.TECH/356)

Delhi Technological University, Delhi, India

Under the supervision of

Dr. Vimal Kishor Singh

Department of Biotechnology
Delhi Technological University
(Formerly Delhi College of Engineering)
Shahbad Daulatpur, Main Bawana Road, Delhi-42

CERTIFICATE

This is to certify that the M. Tech. Major dissertation entitled **"Procedure to enhance cellular viability of blood and its components to improve its shelf life"**, submitted by **Ishita Goyal (DTU/13/M.Tech/356)** in partial fulfilment of the requirement for the award of the degree of Master of Engineering, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by her under my guidance.

The information and data enclosed in this dissertation is original and has not been submitted elsewhere for honouring of any other degree.

Date: July 21, 2015

Dr. Vimal Kishor Singh
(Project Mentor)
Department of Bio-Technology
Delhi Technological University

Prof D. Kumar
Head of Department
Department of Bio-Technology
Delhi Technological University

DECLARATION

I, **Ishita Goyal (DTU/13/M.TECH/356)** declare that M. Tech. dissertation entitled **"Procedure to enhance cellular viability of blood and its components to improve its shelf life"**, submitted in partial fulfilment of the requirement for the award of the degree of Master of Technology, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of my own work carried out under the guidance of **Dr.Vimal Kishor Singh**.

The information and data enclosed in this dissertation is original and has not been submitted elsewhere for honouring of any other degree

Date: July 21, 2015 Signature:

Place: Delhi (ISHITA GOYAL)

ACKNOWLEDGEMENT

I am privileged to express my deep sense of gratitude and profound regards to my guide and project mentor **Dr. Vimal Kishor Singh** for his apt guidance and noble supervision during the hours when this work was materialized. I also thank him for helping me improve upon my mistakes all through the project work and inspiring me towards inculcating a scientific temperament and
Keeping my interest alive in the subject as well as for being approachable at all times.

I am also grateful to my seniors Mr. Abhishek Saini, Mr. Neeraj Kumar and Ms. Manisha Kalsan, Stem cell Research Laboratory, Department of Biotechnology, DTU, for extending full help for the completion of this work and their day-to-day support and conversation. I would like to thank my lab mates Ms. Sweety and Ms. Bhagyeshwari Chouhan for the help and support.

I would like to extend my sincere thanks to Mr. Arpit Singh and Mr. Ashish Chahal for constant encouragement and volunteering for donation of blood for this project work, without which this thesis would not have seen the light of the day.

I would also like to extend my gratitude towards Mr. Sunil T, staff in Environmental Department and Mr. Sandeep Mishra,Chemistry Department.
Lastly, I would like to thank faculty and staff of department of Biotechnology, especially Mr. Chhail Bihari Singh and Mr. Jitendra Singh, for their constant support and for allowing us to work overtime in the laboratory.

Ishita Goyal
2K13/BT/06

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

2,3 DPG	2,3 Diphosphoglycerate
AAS	Atomic absorption spectroscopy
ACD	Acidified citrate dextrose
AHD	Alkalin Haematin Detergent
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
CBC	Complete blood count
CPD	Citrate phosphate dextrose
CPDA	Citrate phosphate dextrose-adenine
GC-MS	Gas chromatography – Mass spectrometry
Hb	Haemoglobin
IL	Interleukin
MTT	3-*(4,5-Dimethylthiazol-2-Yl)*-2,5-Diphenyltetrazolium Bromide
NIST	National Institute of Standards and technology
PGE 1	Prostaglandin E1
PS	Phosphotidylserine
PPP	Platelet poor plasma
PRP	Platelet rich plasma
R_f	Retention factor
RBC	Red Blood Cells
SEM	Scanning electron microscope
TMP	Thrombopoietin mimetic peptide
TPO	Thrombopoietin
TPOR	Thrombopoietin receptor

Procedure to enhance cellular viability of blood and its components to improve its shelf life

Ishita Goyal

Delhi Technological University, Delhi, India

1. ABSTRACT

Transfusion of blood is important in medical regimen, so for a smooth process a decent inventory should be maintained. Scientific community has given a huge attention towards storage of blood and its components but still there is scope for further research. RBCs and Platelets are the two components of blood majorly used in transfusion, so keeping this in mind efforts have been made to improve the quality of cells under storage conditions. Studies have suggested that *Carica papaya* leaf extract stabilizes erythrocytes membrane and have the potential to increase RBC and platelet count. Knowing the medicinal value of *Carica papaya* and its association with diseases like dengue made it the best neutraceutical for the purpose. Promising components were obtained in the GC-MS analysis of *Carica papaya* leaf extracted in different solvent system namely, Hexane, Acetone, Ethanol and Water, which were further used in different concentrations in blood and platelet to access the quality of cells under storage condition. A few components such as vitamins, tannins, flavonoids, alkaloids, antifungal agents, anti bacterial, oleic acid etc were identified, which could help in blood and platelet for quality improvement. Another approach to increase the quality of platelet during storage condition can be done by adding a specific growth factor. Platelets are generated from megakaryocytic cells in bone marrow using a number of growth factors, but HPCs can easily be directed into megakaryocytic lineage using a single growth factor, Thrombopoietin. So to improve cellular viability of platelet by preventing programmed cell death i.e. apoptosis, an *in-silico* mimetic for Thrombopoietin was designed. Through the screening of combinatorial library of peptide mimetic, efficient mimetic is reported with the results of docking and physicochemical properties. Mimetic is found to be showing good docking result with an E_{total} of -409.66. Resulting peptide mimetic is found stable and has nature similar to TPO. Further Ex-vivo study of designed mimetic with TPOR can confirm its role in increasing the shelf life of platelet.

***KEYWORDS*:** Blood, RBC, Platelet, *Carica papaya*, Shelf life, Storage lesions, TPO, TPOR, Mimetic and Docking.

2. INTRODUCTION

Blood transfusion is common to the entire medical regimen now-a-days. For both clinical as well as medical purposes storing blood and its components is very important. Patients under gone surgeries or traumatic accidents, or diseases like leukemia, thalassemia needs to replenish the loss of blood and therefore an external source for supple is needed. Blood banks are the warehouses for blood; blood from a healthy donor is stored here for the patients. So a question arises whether old, donated blood heightens the risks in patients. The answer to this question seems to be an indubitable "Yes". Storage lesions, namely metabolic, structural and morphological changes, play role in decreasing the shelf life of blood. So over the time various efforts have been made to increase the shelf life of blood switching from glass bottles to plastic bags, addition of various anticoagulants such as CPD, ACD, CPDA in combination with additive solutions like SAG, SAGMAN, ADSOL containing saline, dextrose, adenine, mannitol and many more experimental combinations are reported. During World War I human blood was stored in citrate solution for the first time. Even with the most updated storage method blood is stored for only six weeks. So major challenges faced nowadays is increasing the shelf life of blood, making the process of storage cost effective without comprising the quality. Storing blood and its components for a longer period of time will benefit both the patients and the blood banks. Patients will receive more viable cells, less RBC breakdown whereas Blood banks will also be benefited from decreased shipping, longer inventory and reduced wastage.

As time passes stored blood's quality deteriorates; RBCs lyses takes place, thus releasing free haemoglobin in the blood and therefore haemoglobin containing iron molecules become free and evoke the immune responses when transfused in patients and might cause diseases like multiple organ failure, transfusion associated acute lung injury, anaphylactic reactions, fluid overload, acute intravascular haemolysis, bacterial contamination or septic shock and sometimes it may even cause death in recipient because of old haemolysed blood. The quality of stored RBCs can be measured by morphology, haemolysis, cell count, pH, and haematocrit.

Platelets are the smallest cells in blood ranging from 1-2 μm, cells are enucleated, discoid in shape and play an important role in maintaining homeostasis, healing wounds, stopping bleeding. Size of Platelet varies from $1.5 – 4 \times 10^6$ cells/μl in blood. Platelets are generated from megakaryocytes from their precursor cells in bone marrow along with various growth

factors (Machlus and Italiano, 2013). Transfusing plasma to control massive bleeding is flourishing in clinical practices. In 1950s the idea of storing platelets in plasma free media came into existence with artificial preservatives such as salt, acetate, phosphate buffer solution, glucose that can be used along with plasma. Platelets have a shorter life span, in human body, after every 10 days platelet replenishes which thus creates a problem in platelet availability and inventory. The biggest problem which is faced in storing platelet is controlling the bacterial growth since platelet cannot be given either too high or too low temperature. The ideal temperature for storing platelet is 22 °C in a closed system (Ringwald *et al*., 2006). Research suggests three fundamental quality parameters for accessing the platelet shelf life namely platelet count, pH value, and absence of bacteria. All the deleterious changes taking place in platelets during storage are referred as 'platelet storage lesion' and it can be defined as the progressive detrimental changes in platelet structure and function that appears from the minute blood is withdrawn from a donor till they are transfused to a recipient. Since platelet has small life span so increasing the inventory by a day or two could be a great achievement. Currently in blood banks platelets are stored at 22 °C, with gentle agitation. Storage/Preservation time is limited to 5 days in case of platelet due to platelet storage lesions along with the risk of bacterial contamination. (Chandra T. *et al*., 2006)

Motivation to conduct this study came from the fact that efforts are being made to store blood components since a long time. Scientific community has given a huge attention towards this area but still there is scope to improve and conducting extensive research can give fruitful results. The aim is to study the effects of *Carica papaya* leaf extract in different concentrations in red blood cells, and platelet to access and probably improve the quality of blood cells.

Carica papaya, a neutraceutical plant, belongs to the family of Caricaceae, have a wide range of pharmacological properties. Every part of the plant has its own medicinal property. Papaya is an evergreen plant and is said to be the powerhouse of nutrients. Papaya is reported to be a rich source of antioxidants, minerals, vitamins, fibre and other inorganic compounds. Remedial use of the plant involves apportioning of roots, seeds, leaves, stems, and barks. Papaya leaves in particular are used for the treatment of various diseases like dengue, malaria. Papaya leaf extracts have phenolic compounds, Antimicrobial compounds (Anjum V. *et al,* 2013). The leaves of the papaya plant contain chemical compounds of karpain; it's a

substance which kills microorganism. Fresh papayas leaves have antiseptic properties, whereas brown, stored dried leaves are used as blood purifier. (Amazu L. *et al.*, 2010).

The purpose of this work is to establish a relationship among the in-vitro damages on blood cells (mainly RBC and platelet) during storage, the biochemistry of the cell heading to such damage and effect of *Carica papaya* leaf extract in controlling these damages.

Another approach to increase the quality of platelet during storage condition can be done by adding a specific growth factor. Platelets are generated from megakaryocytic cells in bone marrow using a number of growth factors, but HPCs can easily be directed into megakaryocytic lineage using a single growth factor, TPO. (Christian *et al.*, 2010)

a. TPO levels increase when platelet count is low, to stimulate additional production.
b. TPO is absorbed by transfused platelets, which may decrease available TPO levels.

Keeping this in mind many researchers attempt to mimic similar experiment in research settings. So another focus is on designing an *in-silico* mimetic for TPO, which in further *Ex-vivo* study could confirm its role to improve the quality of platelets (Xia *et al*, 2000).

3. REVIEW OF LITERATURE

3.1 Blood

A wide number of life threatening diseases need blood transfusion. Blood has various components, namely Red blood cells, Platelets, White blood cells, Plasma, and each of them functions separately. Red blood cell transfusion saves lives by increasing RBC mass in those where due to traumatic or surgical hemorrhage oxygen carrying capacity reduces, or in condition with defective hemoglobin (hemaglobinopathies, thalassemias), decrease in bone marrow production in condition called aplastic anemia, reduced RBC survival in conditions like hemolytic anemias (Satpathy, G. R., *et al*, 2004; Scott K.L. *et al*, 2005). Platelet transfusion is in burgeoning use in condition of massive bleeding, maintain proper homeostasis. White blood cells are the immunity providers and Plasma gives fluidity, salts, minerals etc to these cells to function properly, thus helping maintain body temperature and blood pressure. Preservation of blood is needed to ensure a readily available blood supply for transfusion (Xia *et al*, 2000). .

Even with the most updated storage method blood is stored for six or seven weeks. The currently used additive solutions for blood storage are SAGM, Nutricel (AS-3), ADSOL (AS-1), and Optisol (AS-5) where the storage duration is 42 days (Hogman C.F., 1998; Ghasemzadeh, A. *et al*, 2013). These solutions don't preserve blood, cell viability and function completely.

3.1.1 Components of blood

Table 1: Components of Blood and their characteristics. [Hess J.R., 2000]

Property	Whole Blood	Red Blood Cells	Platelets	Plasma	Cryoprecipitated AHF
Colour	Red	Red	Colourless	Yellowish	White
Morphology & shape	Mixture of all blood cells	Discoid shape	Smallest cells Discoid shape 1-2 µm	Liquid	Liquid
Shelf life	21 / 35 Days*	Up to 42 Days*	5 Days	1 Year	1 Year
Storage condition	Refrigerated	Refrigerated	Room temperature with constant agitation to prevent clumping	Frozen	Frozen
Use of cell type	Trauma Surgery	Trauma Surgery Anemia Any blood loss Blood disorders, such as sickle cell	Cancer treatments Organ transplants Surgery	Burn patients Shock Bleeding disorders	Coagulation abnormality Diseases like Hemophilia Von Willebrand, Rich source of Fibrinogen

Table 2: Blood Components and Storage conditions (Hess J.R. 2005**)**

Parameter	Whole blood	Red Cell Concentrate	Platelet Concentrates	
			Pooled	**Plateletpheresis**
Quantity	510 ml containing 450 ml donor blood with 63 ml CPDA-1 anticoagulant.	150–200 ml red cells from 1 unit whole blood.	50–60 ml of plasma containing 1.5 – 4.5 x 10^6 cells/Ul	Volume 150–300 ml, Platelet content 150–500 x 10^9,
Description	haemoglobin 12g/ml hematocrit 35% - 45%	haemoglobin 20g/100 ml hematocrit 55% - 75%	Pooled Platelets are prepared from 4 - 6 donors into one pack to contain at least 240 x 10^9 platelets.	Platelets are prepared from a single donor.
storage	at +2°C and +6°C	at +2°C and +6°C	Up to 72 hours at 20°C to 24°C (with agitation)	Up to 72 hours at 20°C to 24°C (with agitation)
Changes after storage	Changes may occur from red blood cell metabolism.	Changes occur in cell viability, pH, loss of Hb, ion imbalance, morphology, glucose, ATP depletion.	Longer storage increases risk of bacterial contamination	Longer storage increases risk of bacterial contamination

3.2 Red blood cells storage lesions

Table 3: Changes that occur in stored red blood cells (Hess J.R. 2009)

S. No.	Changes	Description
1.	Storage effect on red blood cell metabolism	For the formation of ATP, red blood cell is dependent on anaerobic glycolytic pathway, which plays a role in maintain its shape and viability. To remain cells viable during storage the metabolic cycle of cells should continue.
2.	Effect on 2, 3 DPG and O2 release	2, 3 DPG binds to de-oxygenated Hemoglobin which forms a complex with low O_2 affinity. In preserved blood, 2, 3-DPG is exhausted, thus, resulting in a shift of O_2 dissociation curve to the left. Depletion of 2, 3 DPG affects O_2 release by Hb
3.	Cell shape	Cellular integrity of cells cannot be maintained in vitro (failure of Na+/K+ATPase). Some RBC becomes spherical which increases cell rigidity. If stored blood transfused after 28 days, 10 to 20% RBC destroyed within 24hrs.
4.	Effect on pH	During storage period due to accumulation of lactic acid pH falls gradually. Extreme pH increases the electrostatic repulsions which causes protein unfolding and denaturation. During storage pH changes will affect the protein aggregation rates and chemical degradations.
5.	Electrolytes	During blood storage there is a leakage of K+ from cells into the plasma. In kidney diseases even small amount of K+ fluctuation can be dangerous. For neonatal exchange and top up transfusion, blood less than 5 days old is recommended due to high k+ content of stored blood.
6.	Oxidative damage	Red blood cells are susceptible to oxidative

		damage, because; 1) of high oxygen tension. 2) It has no capacity to repair its damaged components. 3) Membrane components are more responsive to lipid per-oxidation. 4) The hemoglobin is susceptible to autooxidation. Oxidative stress induces generation of free radicals which can react with cellular macromolecules leading to protein oxidation and lipid peroxidation. Lipid peroxidation can lead to cells membrane damage and protein oxidation can lead to cytoskeleton and cytosolic protein damage (Kurata, M, 1993).
7.	Effect of temperature	Optimum storage temperature for red blood cells and whole blood is between 2° to 6° with elevation to 10°C is acceptable. Delay in refrigeration increases the loss of 2, 3 DPG during this period. Platelets and granulocytes retain their function better when stored at room temperature.
8.	Effect on cellular elements	White blood cells lose their bactericidal and phagocytic property within 4 to 6 hrs of collection and after 24 hours of storage they become nonfunctional. After 3 weeks of storage few lymphocytes may remains viable and in whole blood, platelets lose their function within 48 hrs at 4°C.
9.	Anticoagulant solutions	Different anticoagulants have different storage time. With trisodium citrate rapid deterioration take place and after 1 week only 50%cells are viable. Blood with ACD and CPD can stored for 28 days with 24 hrs survival 77%. In CPDA blood can store for 35 days, addition of adenine maintains high ATP level and improved the storage of blood.

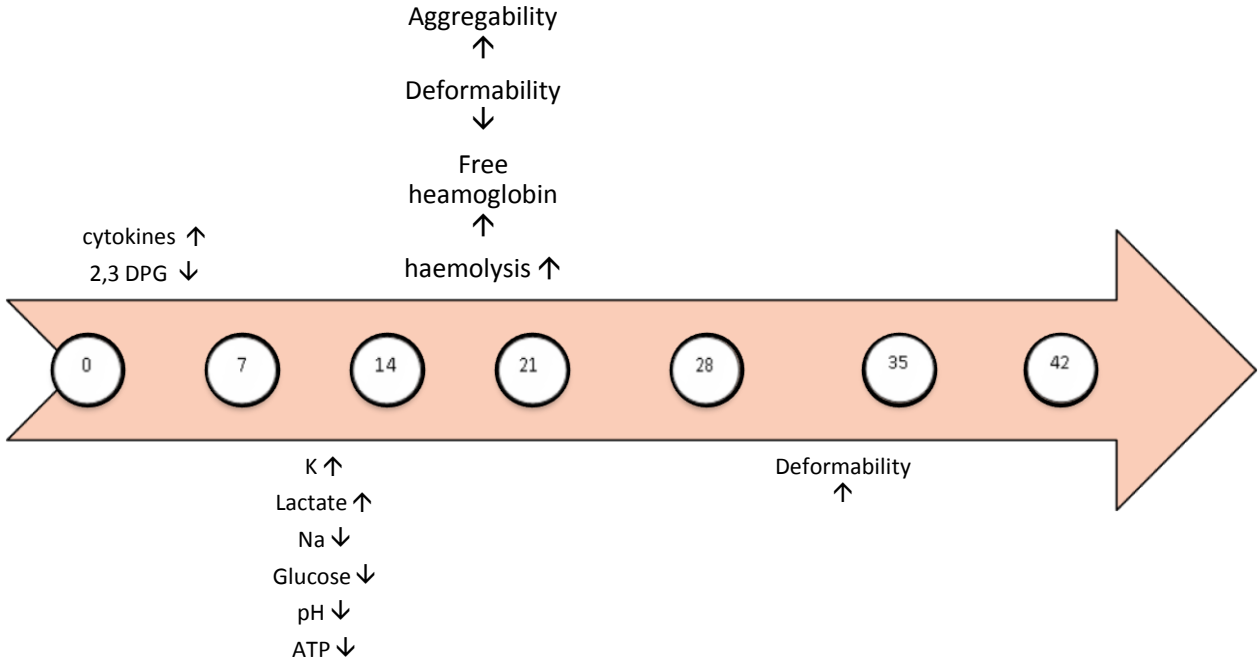


Figure 1: 'Observed storage lesion timeline in RBCs (in days) [Cohen B. *et al,* 2013]

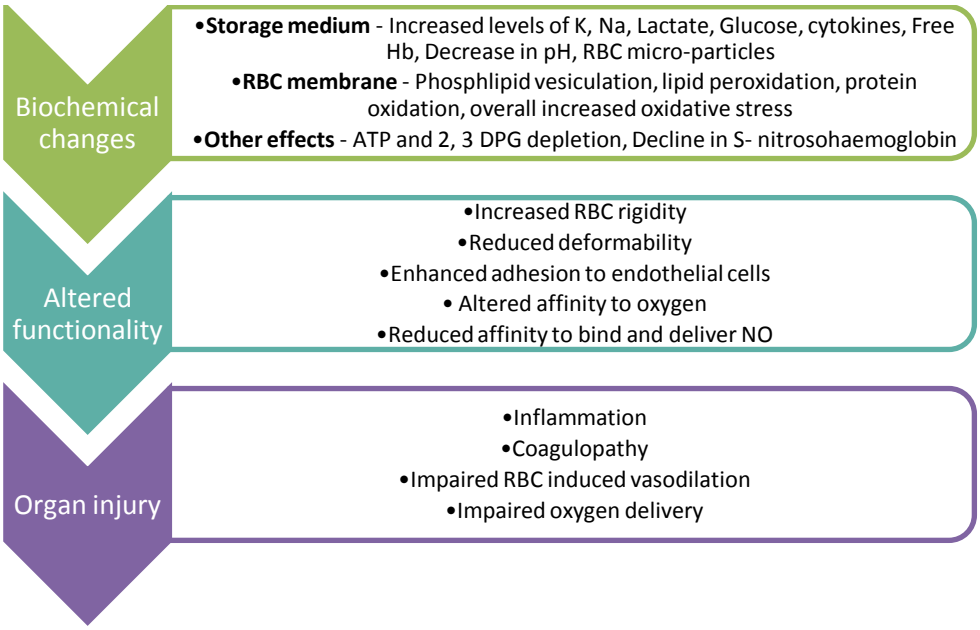


Figure 2: Effects on cells and storage medium of blood preservation

[Cohen B. *et al,* 2013]

3.2.1 Parameters to study Red Blood Cell storage

- Cell viability
- Change in pH
- RBC shape modification
- Alterations in haemoglobin content
- Change in the concentration of ions (Na2+ K+ & Ca2+) in extra and intracellular medium.

These parameters can be accessed using methods mentioned below.

1. Cell viability

Various reagents are used for cell viability assay, which are based on cell functions, such as cell membrane permeability, co-enzyme production, cell adherence, ATP production, enzyme activity, and nucleotide uptake activity. Other methods like Crystal Violet method, Tritium-Labelled Thymidine Uptake method, Colony Formation method and MTT methods are used for live cells counting. [14] The flow cytometric method using acetoxymethyl ester of calcein (calcein-AM) and ATP measurement using firefly luciferase are commonly used methods for viable red blood cell estimation. Haematocrit measure is also used to check RBC viability. Haematocrit is usually measured by an automatic machine that gives CBC, but it can be calculated manually as well by centrifuging whole blood. When whole blood tube is centrifuged a pack of red blood cells settle at the bottom, so the portion of cells settled at the bottom of the tube to total volume of blood can be measured easily which forms the packed cell volume and when converted to percentage it is called haematocrit (Riss, T. L. *et al, 2004*).

2. Hemoglobin estimation

Techniques for measuring Hb:

- Photometric techniques
- Visual comparative Techniques

Drabkin’s method

Drabkin’s method is a quantitative analysis, which does a colorimetric determination of hemoglobin concentration in blood at 540 nm. Techniques for blood hemoglobin determination were based on estimation of carbon monoxide, oxygen capacity, or iron

content. These assays are unreliable because of heterogeneous nature of hemoglobin. A colorimetric cyanmethemoglobin method was used where total hemoglobin is converted to cyano derivative at alkaline pH. The cyanoderivative absorbance is determined at 540 nm.

Drabkin's reagent reacts with all forms of hemoglobin except sulfhemoglobin that normally present in small concentrations in blood. The procedure is based on the oxidation of haemoglobin and its derivatives; potassium ferricyanide (oxidizing agent) converts the ferrous state of Hb to ferric state– methaemoglobin to methemoglobin, this then combines with potassium cyanide to form cyanmethemoglobin, which has absorption at 540 nm. The haemoglobin concentration in solutions is proportional to the color intensity measured at 540 nm.

Alkaline Hematin Method

To measure hemoglobin photometrically, Hb is converted to a colored compound which can be measured easily. A reagent is used that contain chemicals which react with Hb to form a colored compound. Alkaline Hematin D reagent containing Sodium hydroxide (NaOH), Triton X-100 (or equivalent) and Distilled water is used.

The red blood cells are hemolyzed by the detergent and Hb is oxidized to alkaline hematin D-575 by NaOH, which is a stable colour compound and whose concentration equal to the concentration of Hb. When Alkaline Hematin D reagent is added to blood sample, the haemoglobin present is converted to alkaline haematin, a stable colour compound. Then the absorbance of alkaline haematin formed is measured at 575±5 nm (Chakravarthy K *et al*, 2012).

3. Electrolytes estimation

The changes in the concentration of ions (Na2+ K+ & Ca2+) in extra and intracellular medium were assessed by atomic absorption spectroscopy. Atomic absorption spectroscopy (AAS) is a technique used for the determination of metals, ions present in the sample. In AAS, an aqueous sample containing metal analyte is aspirated into flame, causing vaporization of free metal ions in the sample and evaporation of the solvent. This process is known as atomization. Every atom in the sample has its own pattern of wavelengths at which it absorbs energy. Absorbance which is obtained from the process is directly proportional to the concentration of analyte used. The concentration is determined from a calibration curve of standards with known concentration.

4. Glucose concentration

During storage, blood sample contains low glucose and high lactate concentration because of continued uptake and metabolism of glucose by RBCs and WBCs. Changes in concentration can occur after blood sample collection, depending on glycolytic rate, temperature and pH. It can be measured using simple glucometer test.

5. Cell count

Red blood cells were counted by haemocytometer or automated hematology system. Automated hematology system based on principles involved in the RF/DC detection method, Hydro Dynamic Focusing (DC Detection) and flow cytometry method (which employs a semiconductor laser). In RF/DC detection method the size of blood cells is calculated by the changes in direct-current resistance, and the blood cell interior density is calculated by the changes in radio-frequency resistance. In Hydro Dynamic Focusing method blood cells passes through the aperture in a straight line, which avoids abnormal blood cell pulse. Flow Cytometry method is based on light scattering. Information about cell size and material properties can be obtained by detecting the scattered light.

For RBC count in haemocytometer, blood in centrifuged and the suspended blood in pipette out and is diluted in PBC then a using haemocytometer and inverted microscope with focal lens of 10X and 40X cell count is done.

6. Morphological changes

To investigate the morphology of blood cells, drawn blood were studied with scanning electron microscopy (SEM). The Blood cells were fixed with 2% glutaraldehyde solution. The cells were fixed, dehydrated, dried and metalcoated. Red blood cells, leucocytes and platelets were identified and photographed using a light microscope. Observation with a SEM shows that erythrocytes were biconcave in shape with smooth surfaces. It was studied that most blood cells can be identified by their unique topographic characters using SEM (Yang S.C. 2012).

3.3 PLATELET

3.3.1 General

Platelets are generated from megakaryocytic cells in Bone Marrow using a number of growth factors. Opposed to RBCs, Hematopoietic progenitor cells can be easily directed into megakaryocytic lineage differentiation pathways by setting its path via TPO only. Keeping this in mind many researchers attempt to mimic similar experiment in research settings. Further we will discuss about TPO and its mimetics. (Machlus and Italiano, 2013)

The idea of storing platelets started in 1950s. Various attempts to store platelets have been made ever since then,

- Tullis' salt solution including acetate and gelatin
- Phosphate buffered salt solution containing glucose
- Some plasma was also used.
- PRP in a modified Tyrode's medium at room temperature.
- citrate and dextrose in combination with Plasma-Lyte A

It has been reported that platelet sample containing 15% plasma (residual) show similar in vitro platelet quality to those with 100% plasma after 72 hours. (ohtto H. *et al* 2011)

Similar to other living cells survival of platelets depends on biochemical balance maintenance amongst different substances, glucose and hydrogen ions in particular.

The progressive changes that occur in platelet while storage, from the time fresh blood is drawn to the time it is transfused in recipients are detrimental. The changes are called as platelet storage lesions. In vivo after every 10 days platelets are replenished and in vitro shelf life of platelet is 5 days, so even an improvement of 1 – 2 days would be a great success for platelet inventory. To study platelet storage lesions various laboratory tests have been suggested, ranging from simple pH to the complicated tests of platelet function. (Akhoon, B. a., Gupta *et al* 2010)

The optimum temperature to store liquid platelet is 22 – 24 C with constant agitation. It has been reported that glycolysis at this temperature results in an increased lactate production and subsequent fall in pH. Thus, Platelet morphology begins to change around pH 6.8 and loss of viability at pH 6. (Shrivastava M. 2009)

The chief reason for the fall in pH is considered to be lactate production by glycolysis, so the need of glucose in storage is debatable. Platelets use the anaerobic pathway of glycolysis for energy production at room temperature only upto some extent, roughly upto 15 %, majority requirement is fulfilled via oxidative pathway of TCA cycle. (Mittal K. *et al*, 2015)

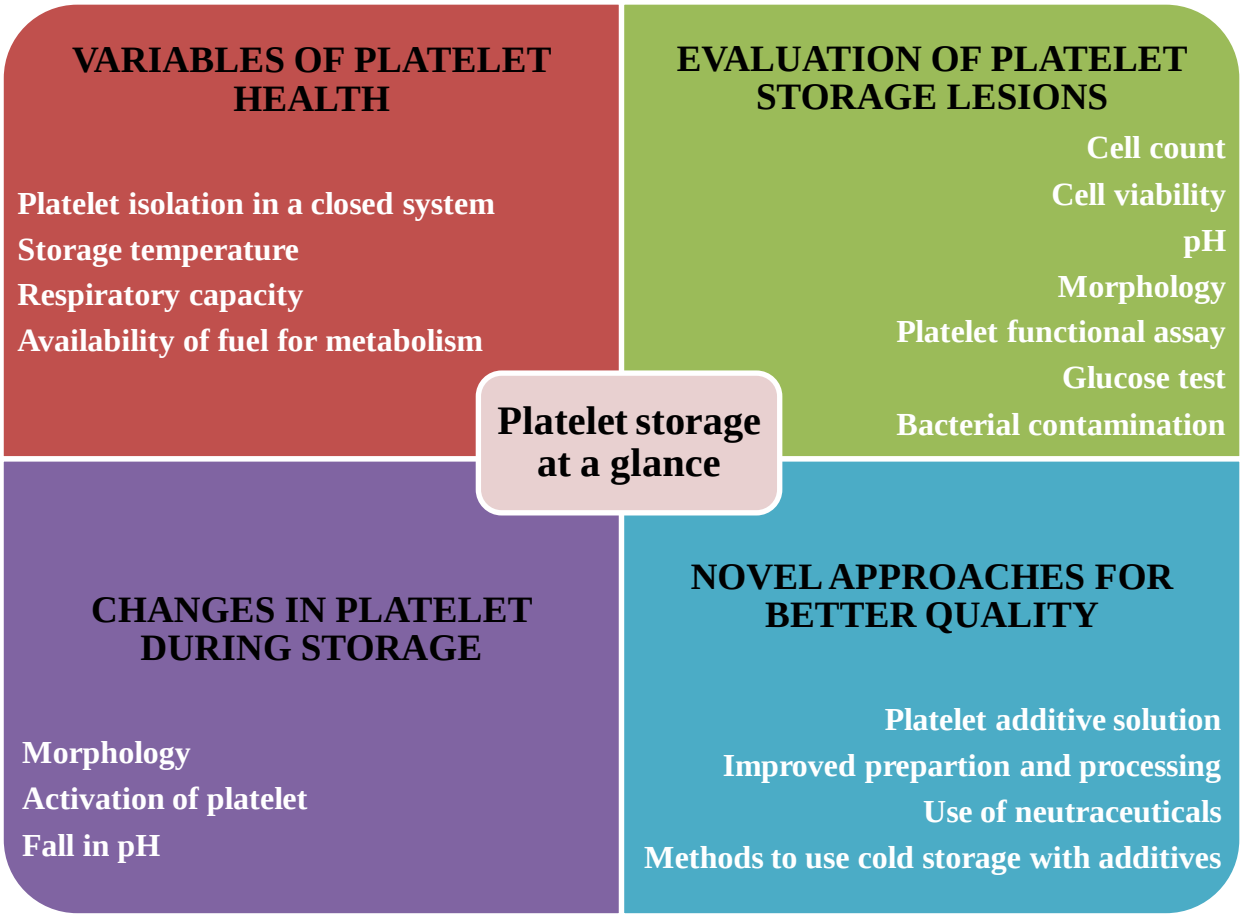


Fig. 3 Platelet storage at a glance (Mittal K. *et al*, 2015)

3.3.2 Parameters to study Platelet storage

- Cell count
- Cell viability
- Change in pH
- Platelet morphology
- Platelet functional assay
- Change in Glucose concentration.
- Bacterial contamination

These parameters can be accessed using methods mentioned below.

1. Cell count: Platelets are counted by haemocytometer or automated hematology system. Automated hematology system based on principles involved in the RF/DC detection method, Hydro Dynamic Focusing (DC Detection) and flow cytometry method (which employs a semiconductor laser In RF/DC detection method the size of blood cells is calculated by the changes in direct-current resistance, and the blood cell interior density is calculated by the changes in radio-frequency resistance. In Hydro Dynamic Focusing method blood cells passes through the aperture in a straight line, which avoids abnormal blood cell pulse. Flow Cytometry method is based on light scattering. Information about cell size and material properties can be obtained by detecting the scattered light.

For Platelet count using haemocytometer,

a. Take 950 µl, 1% ammonium oxalate.
b. Add 50Ul whole blood sample.
c. Keep aside for 5 minutes at room temperature.
d. Platelets are counted in the same square as RBC on an inverted microscope with 10X and 40X lens.

2. Cell viability: Various reagents used for cell viability detection are based on various cell functions such as cell membrane permeability, co-enzyme production, cell adherence, ATP production, enzyme activity, and nucleotide uptake activity. Various other methods like Crystal Violet method, Tritium-Labelled Thymidine Uptake method, Colony Formation method and MTT methods are used for live cells counting. The MTT colorimetric assay is based on turning of the yellow color of MTT to a soluble blue formazen by mitochondrial enzymes by living cells. The amount of blue formazan appeared is directly proportional to the number of living cells, present during MTT exposure. Hence this assay can be used to measure the cell viability.

3. Change in pH: The chief reason for the fall in pH is considered to be lactate production by glycolysis. It has been scientifically published that glycolysis at 22 - 24 ° C results in an increased lactate production and subsequent fall in pH. Thus, Platelet morphology begins to change around pH 6.8 and loss of viability at pH 6.

4. **Platelet morphology**: To investigate the morphology of cells, drawn platelet sample was studied with SEM. The cells were fixed with 2% glutaraldehyde solution. The cells were fixed, dehydrated, dried and metal coated. Platelets were identified and photographed using a light microscope. SEM results show that platelets are discoid in shape with uneven surfaces. It was studied that most blood cells can be identified by their unique topographic characters using SEM (Yang S.C. 2012).

5. **Platelet functional assay**: Various tests can be done to access functionality of platelets. One test is to measure both platelet adhesion and aggregation. Another test is platelet factor 3 tests which determines the clotting time of blood, using kaolin/ silica for surface activation and $CaCl_2$ for clotting blood. 100 µl of PRP with 100 µl of PPP in a test tube held at 37° C in a water bath. 200 µl surface activator was added and the stop watch was started. Incubate for 20 min with occasional shaking and add 200 µl of CaCl2 and clotting time was recorded with a second stop watch.

6. **Change in Glucose concentration**: During storage, sample contains low glucose and high lactate concentration because of continued uptake and metabolism of glucose by blood cells. Changes in concentration can occur after blood sample collection, depending on glycolytic rate, temperature and pH. A simple glucometer test can be done to measure the amount of glucose present.

7. **Bacterial contamination**: As Platelets cannot be stored at cold temperature due to the activity of macrophages, they are stored at 22 -24 C, which when not stored extra cautiously can cause bacterial contamination thus, making them unfit for transfusion.

Studies have suggested that *Carica papaya* leaf extract stabilizes erythrocytes membrane and have the potential to increase RBC and platelet count.

3.4 *Carica papaya* leaf extract

Carica papaya, a neutraceutical plant, belongs to the family of Caricaceae, have a wide range of pharmacological properties. Papaya is an evergreen plant and is said to be the powerhouse of nutrients. It is a soft tissue plant, herbaceous and fast growing. Common names include papaya, papayer (French), mamao, melonenbaum (German), papaw or pawpaw, lechosa (Spanish), mamoeiro (Portuguese), malakol (Thailand) and mugua (Chinese). It is used locally in the treatment of urinary tract infections. The juice of papaya when extracted and dried is used as medication for digestion problems, chewing gum, meat tenderizers and toothpaste. It is used to treat digestive problems and intestinal worms as well as sinusitis, warts, cutaneous tubercules, eczema and hardness of the skin (Pérez-Gutiérrez *et al*, 2011).

Papaya is reported to be a rich source of antioxidants, minerals, vitamins, fibre and other inorganic compounds. Remedial use of the plant involves apportioning of roots, seeds, leaves, stems, and barks. Papaya leaves in particular are used for the treatment of various diseases like dengue, malaria. Papaya leaf extracts have phenolic compounds, Antimicrobial compounds. The leaves of the papaya plant contain chemical compounds of karpain, it's a substance which kills microorganism. Fresh papayas leaves have antiseptic properties, whereas brown, stored dried leaves are used as blood purifier.

Properties	**Component of Papaya involved**
Abortifacient activity, hypoglycaemic, fever, asthma	roots and immature fruits
Post testicular anti fertility drug activity	Seeds
Anthelmintic activity	latex of plant
Wound healing properties	extracts of seeds and fruit pulp
Treatment of dengue, jaundice, malaria, immunomodulatory and antiviral activity, gastric problems	Leaves

Table 4 Remedial properties of *Carica papaya*(Anjum, V *et al*, 2013**)**

However, the knowledge of the phytochemical components in the *Carica papaya is* still limited. To investigate the role of the compounds on biological system, the study of active components from the crude plant extracts is necessary. GC-MS is commonly used in the phytochemical research (Iyappan, G. *et al*, 2014). Chemical composition of papaya leaves extracts with hexane, acetone, ethanol and water can be studied using GC-MS and the compounds found was matched with the databases such as WILEY and NIST library. Analysis shows different peaks out of which some active compounds are present.

3.4.1 Extraction

Extraction is the crucial step in the analysis of plant components and is required for further characterization. The basic steps are:

1. Pre-washing
2. Drying of plant leaves
3. Ground into fine powder which improves the yield of extraction and also increasing the sample surface contact area with the solvent system.

Measures must be taken to avoid elimination of active components. Solvent is selected based upon the nature of target bioactive compounds. Different solvents can be used to extract the bioactive components eg. For the extraction of hydrophilic compounds polar solvents such as ethanol, methanol or ethyl-acetate are used. Target compounds ranges from polar to non-polar to thermally labile; hence a suitable extraction methods with apt solvent should be considered. Methods such as soxhlet extraction, sonification, heating under reflux, maceration etc are commonly used for plant extraction.

The other modern techniques include solid-phase extraction, supercritical-fluid extraction, microwave-assisted extraction, pressurized-liquid extraction and surfactant-mediated techniques, which reduce sample degradation, organic solvent consumption and increases efficiency of extraction (Sasidharan, S *et al*, 2011; Trusheva, B. *et al*, 2007).

3.4.2 Identification and characterization

Many bioactive compounds/phytochemicals with different polarities are present in plants; their separation is a bigger challenge for characterization and identification of bioactive compounds. The numbers of different separation techniques are used for isolation of these bioactive compounds. Various types of chromatography are used like column

chromatography, Sephadex chromatography, flash chromatography, TLC, and HPLC. Besides that, some non-chromatographic techniques like phytochemical screening assay, FTIR, immunoassay, which uses MAb, can also be used for the identification of bioactive compounds.

3.4.3 Thin-layer chromatography (TLC)

A simple, inexpensive procedure that might give an idea of the number of components present in a mixture sample. It is also used to identity an unknown compound in a given sample by comparing the R_f of compounds with known compounds. For proper visualization a spray or any other colouring reagent or UV light is used for screening, which changes colour of bands according to the phytochemicals present in extract. TLC can be used as a confirmatory test to identify purity of isolated compounds.

3.4.4 Phytochemical screening assay

Phytochemicals are present in plants and are also referred as secondary metabolic compounds. These are simple and inexpensive procedures that give an idea of various phytochemicals present in a mixture.

Experimental studies suggest that *C. Papaya* leaf extracts can stabilize erythrocyte membrane (Ranasinghe, P *et al*, 2012)and may increase red blood cell and platelet counts (Dharmarathna S.L.C.A *et al*, 2013)

A recent trial shows that platelet count is increased by papaya leaves juice after 40-48 hours of dose administration (Subenthiran, S. *et al*, 2013). From this we can conclude that *C. papaya* leaf extract have active components that can improve quality of preserved blood and the cells in it.

The aim to conduct this study is to understand the effects of *Carica papaya* leaf extract in different concentrations in whole blood, and platelet to access and probably improve the quality of blood cells.

Another approach to increase the quality of platelet during storage condition can be done by adding a specific growth factor. Platelets are generated from megakaryocytic cells in bone marrow using a number of growth factors, but HPCs can easily be directed into megakaryocytic lineage using a single growth factor, Thrombopoietin. (Christian *et al.*, 2010)

a. TPO levels increase when platelet count is low, to stimulate additional production.
b. TPO is absorbed by transfused platelets, which may decrease available TPO levels (Xia *et al*, 2000).

3.5 TPO mimetic

TPO is primarily synthesized in human liver parenchymal cells and kidney. It's a 353 amino acid protein which on removal of signal peptide of 21 amino acid forms a mature molecule. Mature molecule consists of two domains: receptor binding and carbohydrate rich region. Levels of TPO increases with decline in platelet mass and remain elevated throughout thrombocytopenia. Structure-function studies have demonstrated that while the first 153 amino acids of the c-Mpl ligand are all that are required for its thrombopoietic effect in vitro, this truncated molecule has a decreased circulatory half-life compared to the native protein. Presumably, the glycosylated second half of the molecule confers stability and prolongs the circulatory half-life. (Kuter D., 1995)

Platelets are generated from megakaryocytic cells in Bone Marrow using a number of growth factors like TPO, IL - 2, 3, 6, 11. Opposed to RBCs, Hematopoietic progenitor cells can be easily directed into megakaryocytic lineage differentiation pathways by setting its path via TPO only. (Machlus and Italiano, 2013)

It specifically interacts with c-MPL receptor and thus, a cascade of cellular signal gets triggered, which involves,

- JAK2 and TYK2 pathways
- Mitogen-activated protein kinase pathway
- Phosphatidylinositol 3-kinase pathway
- Nuclear factor kappa B pathway (Kuter D., 1995)

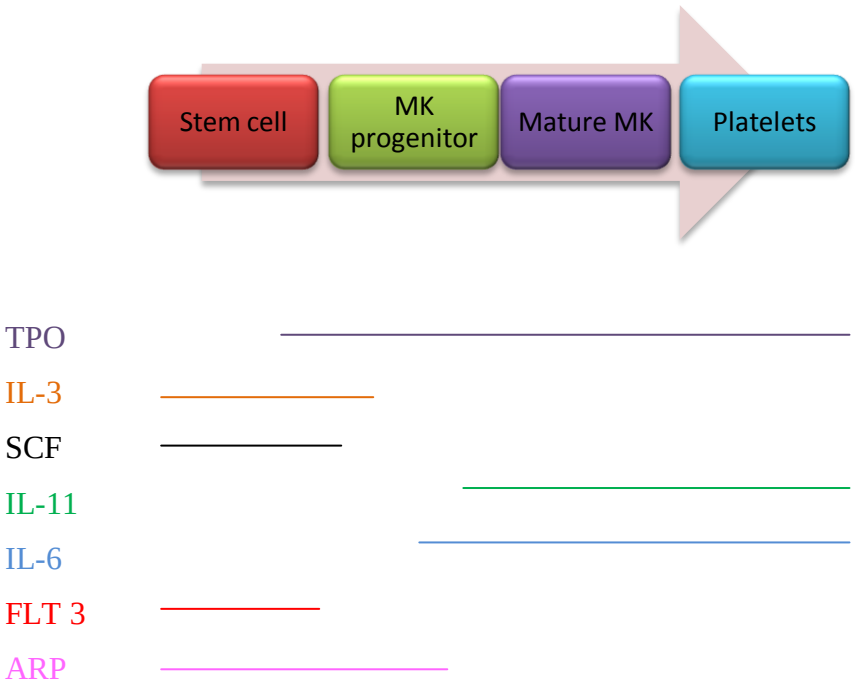


Fig 4: Regulation of megakaryocytopoiesis by various hematopoietic growth factors.

A study was conducted in mice which showed that TPO deficient mice show a reduction of 90% in bone marrow megakaryocytes and circulating platelets quantity and administration of TPO to normal mice increases platelet count almost 200%, 500% increase in megakaryocytes and 1000% increase in bone marrow colony forming unit megakaryocytes. (Chen *et al*, 1995; Harker *et al*, 1996).

TPO plays an important role in hematopoiesis, any change in the hormone or to its receptor may trigger any congenital or acquired disease state of thrombocytopenia, thrombocytosis or aplastic anaemia.

TPO is a 95 kDa glycoprotein and possess 2 domain structures, namely, cytokine domain at N terminal followed by carbohydrate domain at C terminal. The cytokine domain comprises of first 153 amino acids up till Arg 153. The carbohydrate domain starts from Arg 154 till 332nd amino acids. The carbohydrate domain shows no biological activity but still assists TPO to be soluble. It is also proteotypically cleaved from the protein before it binds to its receptor.

TPO receptor is a 635 amino acid protein with an extracellular domain of 485 amino acid starting from 26th amino acid up to 491st amino acid. TPO interacts with c-MPL in the extracellular domain only. c- MPL has two extracellular subunits, namely subunit I from 26 – 277 amino acids and subunit II from 253 – 491. Subunit I shows greater interaction with the ligand. (Dennis P.H. Hsieh *et al*, 2000).

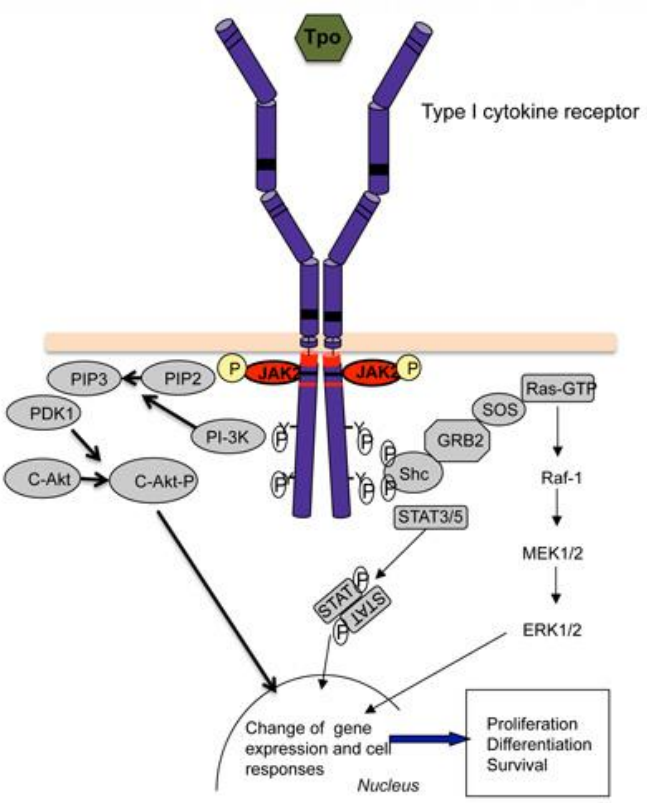


Fig 5: TPO binding to its receptor TPOR and its activation (ludwig *et al*)

TPOR is a type I Trans membrane proteins that is bound to JAK2 and remain inactive in the absence of ligand, TPO. Binding of TPO to the TPOR leads to a conformational change of trans-membrane domains that is transmitted to the cytosolic domain, leading to activation of the pre-bound JAK2 kinases. They phosphorylate each other and the tyrosines on the cytosolic tail of the receptor, which then attracts signalling proteins to the receptor. These proteins become substrates of JAK2. The three main pathways activated by cytokine receptors are the STAT (Signal Transducer and Activator of Transcription) pathway, the ras-MAP-kinase pathway and the phosphatydylinositol-3'-kinase (PI-3'K) pathway. Signals are decoded at the level of chromatin, where gene expression is regulated leading to effects on cell survival, proliferation and differentiation.

The role of thrombopoietin as a physiologic regulator of platelet production has been supported by the inverse relationship between the platelet count and serum thrombopoietin level in animal models of thrombocytopenia induced by antiplatelet-antiserum or by chemotherapy or radiation treatment, as well as in cancer patients following myeloablative chemotherapy and bone-marrow transplantation. The role of TPO appears to be the suppression of apoptosis in the MK lineage. Apoptosis or programmed cell death is an essential process involved in removing damaged cells (Kroemer *et al.*, 1995). With blood tissues being the most proliferative organ, apoptosis plays a major role in regulating hematopoiesis (Koury, 1992). pH is an important parameter for activation of apoptosis. (Hansen *et al.*, 2006)

Initially, recombinant form of human TPO was developed as first generation thrombopoietic agent but their development was terminated because cross reactivity was observed with endogenous TPO. Now second generation thrombopoiesis stimulating, these have distinguished pharmacological properties and show no homology to Thrombopoietin. Romiplostim and Eltrombopag are two agents which have completed phase III trials and they have been authorized for marketing for the use of disease purpose. There are very few peptides mimetic available for TPO so the efforts are being made for the same.

Table: The Thrombopoietins (Kuter D., 1995)

Endogenous thrombopoietin (TPO)
Recombinant human thrombopoietin (rhTPO)
Pegylated recombinant human growth and development factor (PEG-rHuMGDF)
Promegapoietin (TPO/IL3 fusion protein)
Thrombopoietin peptide mimetics (peptides, pegylated peptides, peptide-immunoglobulin constructs)
Thrombopoietin non-peptide, small molecule mimetics

4. OBJECTIVES

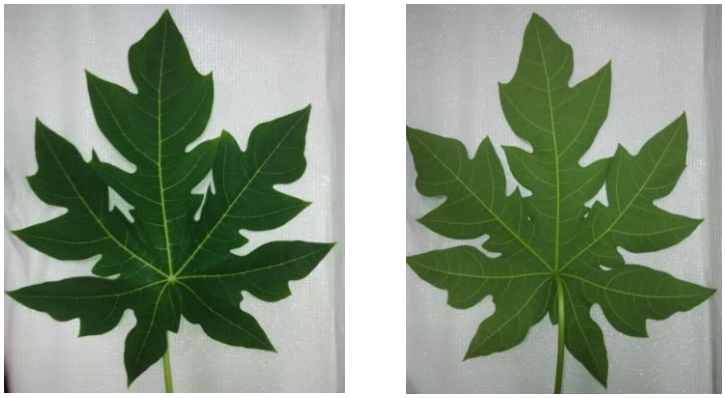
a. To identify bioactive components in *Carica papaya* leaf extracted in different solvent systems.
b. To improve red blood cell shelf life using *Carica papaya* leaf extracts.
c. To access the quality of platelets in storage condition.
    i. Using *Carica papaya* leaf extracts
    ii. Designing an in-silico mimetic for Thrombopoietin to improve cellular viability of platelet.

5. METHODOLOGY

5.1 *Carica Papaya* extracts preparation

5.1.1 Material requirements

5.1.1.1 **Papaya leaves** 50 gm



Dorsal view **Ventral view**

Fig 6: Macroscopic characters of C. papaya leaves

5.1.1.2 Soxhlet extraction

250 ml of hexane, acetone, 60% ethanol, 40% ethanol and water

Fig 7: Soxhlet extraction apparatus

5.1.1.3 **Shimadzu GC-MS QP2010 plus** system

Fig 8: GC-MS Instrument

5.1.1.4 **TLC** solvent system: Chloroform: methanol = 9:1, n-hexane: acetone = 8.5:1.5 and benzene: ethyl acetate = 1:1.

5.1.2 Methods

5.1.2.1 Collection and Identification of plant materials

The *Carica papaya* leaves were handpicked from Delhi Technological University. They were washed and sun dried for 2 weeks to remove the residual moisture. The dried plant material was then ground into fine powder removing the stalk and woody part using mortar and pestle in the laboratory and stored in an air tight container away from moisture to use for further study.

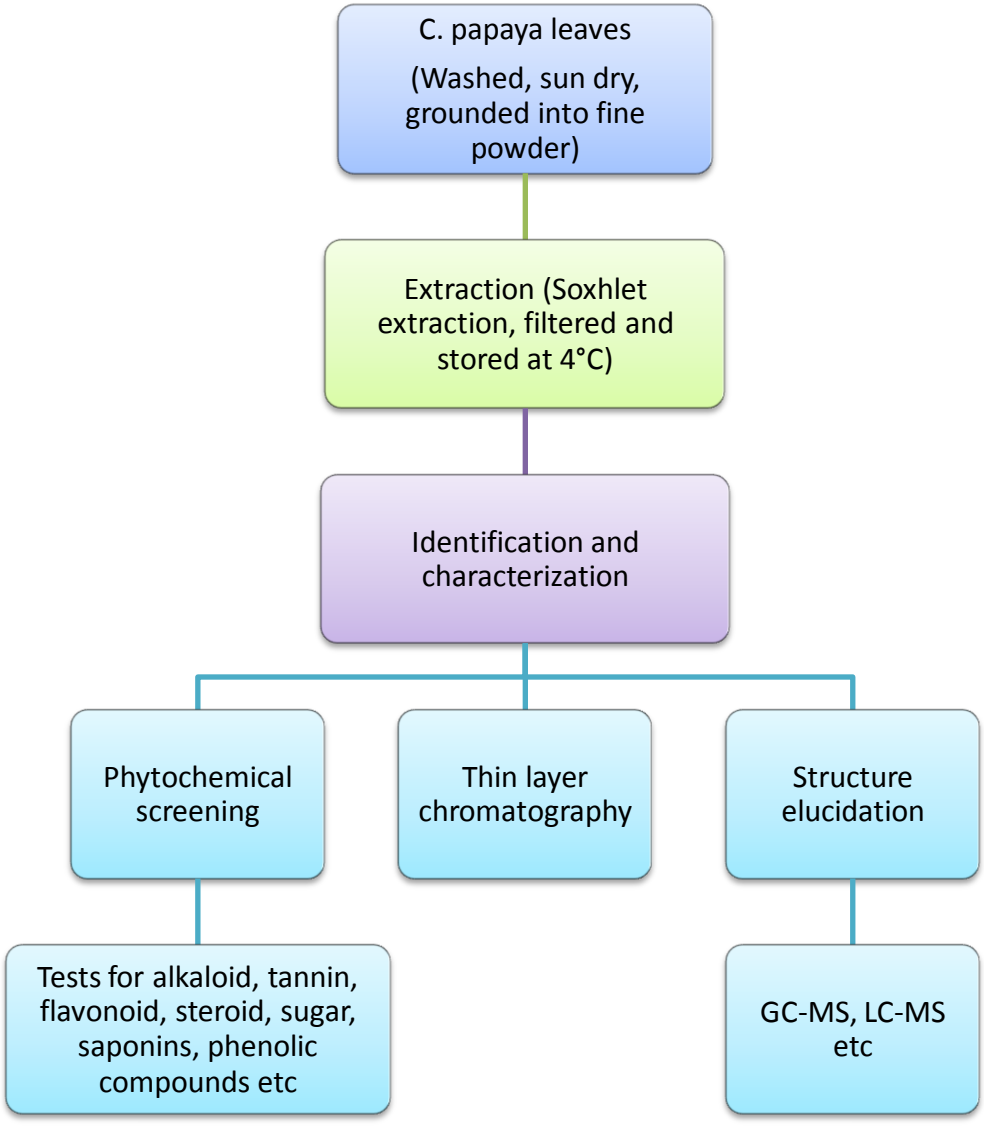


Figure 9: A brief summary of extraction, identification and characterization of compounds from papaya leaf extracted in different solvent systems.

5.1.2.2 Extraction Papaya leaves extraction was carried out by sequential Soxhlet extraction with different solvents of increasing polarity. Fifty grams of powdered leaves were sequentially extracted in a Soxhlet extractor using 250 ml of hexane, acetone, 60% ethanol, 40% ethanol and water. The extraction time was about 5-8 hrs for each solvent. At the end of extraction the ethanol and water extracts were concentrated by using vacuum evaporator for GC-MS. The resulting extract was than filtered and the filtrate was stored at 4°C.

S. No.	Solvents (250ml)	Temp. (°C)
1.	Hexane	68
2.	Acetone	56
3.	60% Ethanol	80
4.	40% Ethanol	80
5.	Water	100

5.1.2.3 GC-MS analysis

GC-MS analysis of the C. Papaya leaves extracts were performed using a Shimadzu GC-MS QP2010 plus system and GC-MS equipped with Omega wax capillary column (30 meter) and a Flame Ionization Detector (FID) was used for detection. Helium was used as carrier gas at a constant flow of 1.21 ml/min and an injection volume was 2-5 micro litres. The column temperature is 60º C, with an increase of 5 °C /min, to 250 °C then 10 °C /min to 280 °C. Mass spectra were taken at a scan interval of 0.2s and fragments from 50 to 1000 Da.

5.1.2.4 Identification of phytochemical constituents

The phytocomponents in the hexane, acetone, 60% ethanol, 40% ethanol and H_2O extracts of the C. Papaya leaves were identified based on the retention time. Mass spectrums were interpreted using WILEY and NIST library having large number of patterns. The name, structure and molecular weight of the components of the extracts were identified.

5.1.2.5 Phytochemical screening assay

After obtaining the plant extract, phytochemical screening can be performed with different tests to get an idea about the type of phytochemicals present in the plant extracts.

1. Test for flavonoids:

NaOH test: - Treat 3ml of the extracts with 1ml of 10% w/v NaOH, followed by addition of dilute HCl. A yellow solution with NaOH, turns colorless with dilute HCl indicate the presence of flavonoids (Onwukaeme, D. N. *et al*, 2007).

2. Test for cardiac glycosides:

Kellar – Kiliani test: - Add 2ml of each extracts with 1ml of glacial acetic acid, 1ml concentrated sulphuric acid and 1ml ferric chloride. Green-blue coloration of the extracts indicates the presence of cardiac glycosides (Parekh *et al*, 2007).

3. Test for tannins:

$FeCl_3$ test: - 1ml of the extracts was diluted with 2ml of distilled water and 2–3 drop of 5% ferric chloride solution was added. The green – black or blue coloration of the sample indicated tannin (Parekh *et al*, 2007).

4. Test for saponins:

Foam test: - Treat 0.5ml of each extracts with 5ml of distilled water and shake properly. Persistence of frothing indicates the presence of saponins (Parekh *et al*, 2007).

5. Test for phenolic compounds:

Lead acetate test: Treat 0.5ml of each extracts with a few drops of 10% lead acetate solution. Formation of white precipitate indicates the presence of phenolic compounds.

5.1.2.6 TLC Analysis of the Fractions:

Each of the five extracts was checked by Thin Layer Chromatography (TLC) on silica gel plates. For each extract, three different solvent mixtures were used. These were chloroform: methanol=9:1, n-hexane: acetone=8.5:1.5 and benzene: ethyl acetate=1:1. After saturation with mobile phase for 30 min, iodine vapours were used to detect the band spots on the TLC plates. The movement of the analytes was expressed by its retention factor (R_f) were calculated in each solvent systems for different samples.

$$\mathrm{Rf} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

5.2 BLOOD QUALITY ASSESSMENT

5.2.1 Materials

A. Blood from volunteers.

B. Hemoglobin estimation

Table 5 AHD reagent preparation (pH 11.8)

S. No.	Chemicals	Amount
1	Sodium hydroxide	4 gm
2	Triton-x 100	25 gm
3	Distilled water	1000 ml

C. pH estimation

pH strips and pH meter.

D. Cell counting Sysmex XE-2100

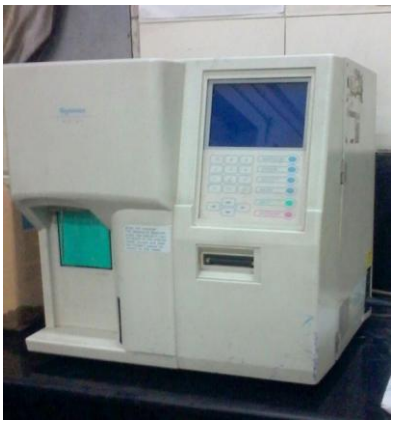


Fig 10: Sysmex XE-2100 automated hematology system for blood cells count

E. Cell morphology: Inverted microscope and SEM

5.2.2 Method

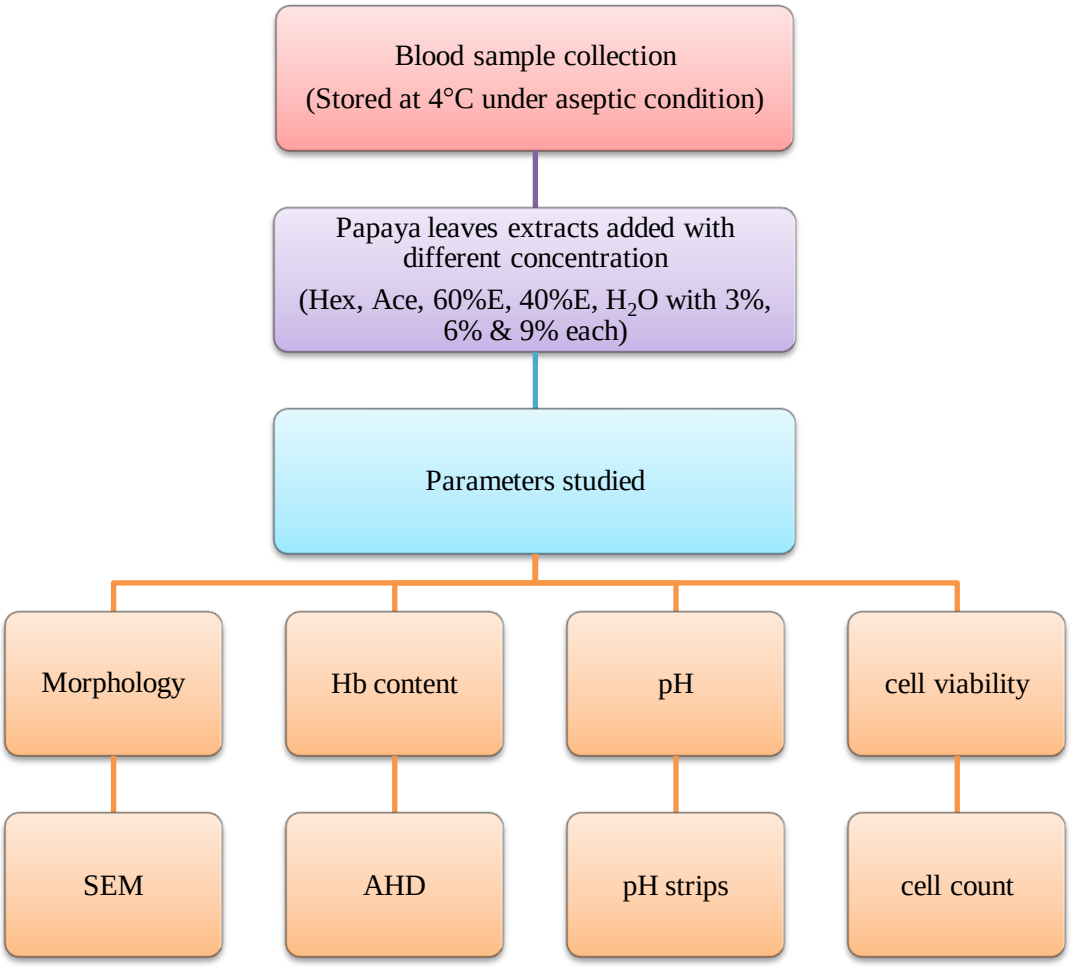


Figure 11: Methodology for blood's quality assessment at a glance

5.2.2.1 Blood sample preparation

1. Blood sample from the donor was collected into vacutainer containing CPDA anticoagulant and divided into aliquots from week 0 to week 6 or week 7 i.e. day 0, day 7, day 14, day 21, day 28, day 35, day 42 or day 49. Label properly with name, date.
2. Aseptic condition were maintained throughout the process and transfer of sample was done only in laminar hood using autoclaved tips, pipettes and eppendorffs and gloves were worn and clean hand with alcohol or spirit.

5.2.2.2 Addition of extracts

The extracts obtained after extraction contain organic solvents hexane, acetone and ethanol which affects the blood cells if we directly add the extracts into blood. So to remove these solvents the extracts were converted to crude sample by evaporating the solvent of sample. These crude samples than dissolve in phosphate buffered saline (PBS) to add into blood sample. The resulting extracts were than filtered using 0.2µ syringe filter and the filtrate were added with different concentration (Hex, Ace, 60%E, 40%E, H_2O with 3%, 6% & 9% each) into blood under aseptic condition.

5.2.2.3 Parameters studied:-

5.2.2.3.1Haemoglobin Estimation

AHD standard

1. 100ul of blood sample was taken.
2. Dilutions of 1:10, 1:100, and 1:1000, 1:10000 were made.
3. Then 2ml of AHD reagent was added and the absorbance was taken at 75±5nm.

AHD method

1. Collected sample from donor in a vacutainer containing CPDA anticoagulant and divided into aliquots from week 0 to week 6 and labelled properly with date and name.
2. 100µl of sample was taken and centrifuged at 5000 rpm for 5 minutes after centrifugation the pellet and plasma were separated.
3. Washed pellet with PBS and then volume was made by adding 100 µl of PBS in washed pellets.
4. AHD was performed on both the pellets and supernatant.
5. Then 20µl of cell suspension was taken and 2ml of AHD reagent was added. The absorbance was taken at 580nm and same process was followed for supernatant.
6. Pellet and supernatant were used as duplicates.

5.2.2.3.2 Cell counting: Red blood cells are count by automated hematology system Sysmex XE-2100.

5.2.2.3.3 Morphology of RBC

To investigate the morphology of blood cells, drawn blood was studied with scanning electron microscopy (SEM). It was observed that most blood cells can be identified by their unique topographic characters using SEM. The Blood cells were fixed with 2.5% glutaraldehyde solution then dehydrated with 35-70% ethanol, dried and metal coated. Observation with a SEM shows that erythrocytes were biconcave in shape with smooth surfaces and the shape and surface of RBC is changed from biconcave to ecthionocytes to spherocytes to eliptocytes during storage.

5.3 PLATELET QUALITY ASSESSMENT

5.3.1 Materials

A. **Blood** from volunteers.

B. **Buffers** used in Platelet Isolation

ACD buffer (pH 7.4)	Platelet wash buffer (pH 7.4)	HEP buffer (pH 7.4)	Tyrode's buffer (pH 7.4)
• 39mM citric acid • 135 mM dextrose • 75 mM sodium citrarte	• 1% (w/v) dextrose • 1mM EDTA • 150 mMNaCl • 10mM sodium citrate	• 5 mM EDTA • 3.8 mM HEPES • 2.7 mMKCl • 140 mMNaCl	• 134 mMNaCl • 12mM NaHCO3 • 2.9 mMKCl • 0.34 mM Na2HPO4 • 1mM MgCl2 • 10mM HEPES • 1 mM CaCl2 • 5 mM glucose

C. **pH estimation** pH strips and pH meter.

D. **Cell morphology** Inverted microscope and SEM

E. **Cell viability**

a. MTT solution:-5 mg/ml MTT in PBS.

b. MTT solvent:-DMSO

F. **Platelet functional assay:** Platelet function was assessed by platelet factor 3 with silica and CaCl2.

G. **Glucose test**: Glucometer

H. **Bacterial contamination**: Luria Broth Agar, Nutrient broth, Spectrophotometer.

5.3.2 Method

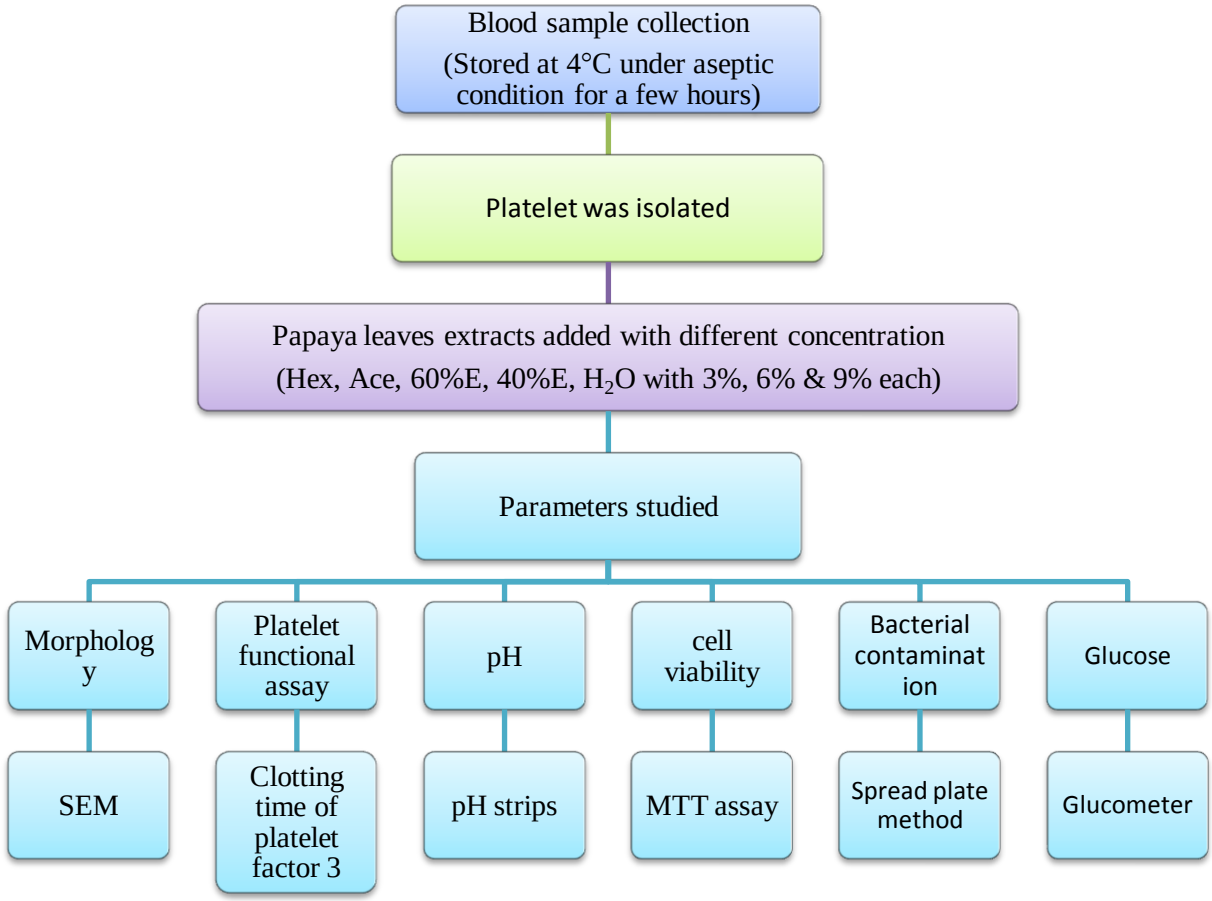


Fig 12. Platelet quality assessment methodology at a glance

5.3.2.1 Blood sample preparation

Blood sample from the donor were contained into vacutainer containing ACD anticoagulant maintaining aseptic conditions through the process and transfer of sample is done only in laminar hood using autoclaved tips, pipettes and eppendorffs and wear gloves and clean hand with alcohol or spirit.

5.3.2.2 Procedure for Platelet Isolation

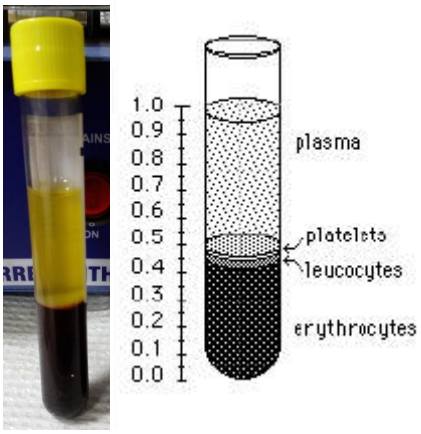


Fig. 13 Platelet rich plasma separation after centrifugation

a. Whole blood was centrifuged at room temperature at 200 X g for 20 min.
b. After spin three distinct layers were observed, namely, bottom layer will be Red blood cells; Middle layer will be buffy coat, top layer platelet rich plasma.
c. 2/3rd of the PRP was transferred in another tube.
d. HEP buffer was added in 1:1 ratio (v/v). PGE 1 was added (1 µl final concentration) to prevent platelet activation.
e. Gentle mixing was done.
f. Spinning was done for 15 – 20 min at room temperature at 100 X g without any brake.
g. The supernatant was transferred into a new tube.
h. Pellet platelet was centrifuged at room temperature at 800 X g for 15 – 20 min.
i. Supernatant was discarded.
j. Platelet wash buffer was used to rinse the pellet platelet by gently adding wash buffer and slowly removing it with a pipette.
k. Slowly suspend the pellet in tyrode's buffer and freshly prepared BSA (3mg/ml)
l. To prevent platelet activation, PGE 1 (1 µl) was added. Minimizing the agitation.

5.3.2.3 Addition of extracts

The extracts obtained after extraction contain organic solvents hexane, acetone and ethanol which affects the blood cells if we directly add the extracts into platelet. So to remove these solvents the extracts were converted to crude sample by evaporating the solvent of sample. These crude samples than dissolve in phosphate buffered saline (PBS) to add into platelet sample. The resulting extracts were than filtered using 0.2µ syringe filter and the filtrate were added with different concentration (Hex, Ace, 60%E, 40%E, H_2O with 3%, 6% & 9% each) into blood under aseptic condition.

5.3.2.4 Parameters studied:

5.3.2.4.1 Platelet functional assay

- 100 µl of platelet rich plasma with 100 µl of platelet poor normal plasma was added in a test tube held at 37° C in a water bath.
- 200 µl silica was added and the stop watch was started.
- 20 min incubation with occasional shaking and 200 µl of CaCl2 was added and clotting time was recorded with a second stop watch.

5.3.2.4.2 **Glucose determination** was done by glucometer.

5.3.2.4.3 **Bacterial contamination** by spread plate method.

- 20 grams of premix LB agar powder was added.
- dd H2O was added to make up the volume of 500 ml. (pH 7.5)
- Autoclave was done for 20 min; 15 psi.
- Thin layer of agar was poured into each plate.
- Each plate was allowed to cool until it is solid.
- Plates can be stored at 4 ° Celsius.
- 35 µl sample was spread on nutrient agar plate and incubated at 37 ° Celsius for 48 hours.
- It was checked for growth.

5.3.2.4.4 MTT assay for cell viability

Method followed:

- $10^5 – 10^6$ cells/ ml were maintained per sample.
- Incubation was done for 6 to 24 hours.
- 20 µL MTT Reagent (MTT stock solution) was added.
- Incubation was done for 4 hours at 37 ° C.
- 200 µL Detergent Reagent (DMSO) was added.
- Left at room temperature in the dark for 1 hours.
- Absorbance was recorded at 570 nm, after diluting the sample with PBS.
- A graph was plotted between days and absorbance.

5.3.2.4.5 Morphology of Platelet

To investigate the morphology of cells, stored platelets were studied with SEM. It was observed that most blood cells can be identified by their unique topographic characters using SEM. The cells were fixed with 2.5% glutaraldehyde solution then dehydrated with 35-70% ethanol, dried and metal coated. Observation with a SEM shows that platelets have three major shape changes namely, platelet with smooth contours, dendritic, balloon.

- **Platelets with smooth contours** — discs and spheres retaining normal size.
- **Dendrites** — Platelets that have developed pseudopodia or dendritic processes; and long tubular forms.
- **Balloons** — Platelets that have undergone swelling after losing the capacity to maintain an osmotic gradient across their membrane, clumps of multiple Platelets and could not be counted individually. (Jain A. *et al*, 2015)

5.4 TPO mimetic

5.4.1 Materials

Table 6 Various tools, servers, databases and software used for the study of the TPO mimetic

Pep fold	Tool for prediction of *de novo* 3D structure of oligo peptides.	Pep fold was used for predicting 3D structure of the peptide sequences.
Phyre2	Tool for prediction of *de novo* 3D structure of proteins.	Phyre2 was used for predicting 3D structure of the protein sequences.
HEX 8.0.0	Protein – protein docking server.	Peptide sequences were docked with receptor.
Protein peptide calculator	Identifies various properties of a peptide sequence.	Shortlisted peptide sequences were run through the Protein peptide calculator for determining physicochemical properties.
Cello predictor	Determines the sub-cellular localization of target protein	Shortlisted peptides were run through this tool for predicting the subcellular localization.
Pymol	For 3D view of proteins and analysis of PDB structure.	TPO-TPOR & TMP-TPOR complexes and other 3D structure viewed and analyzed.
BLAST	For searching similar protein or nucleotide sequences.	BLAST was used to determine homology of TPOR with the database.
Protein Data Bank (PDB)	PDB consists of 3D structures of bio molecules and their complexes	3D structures of the protein were retrieved from PDB and used for structure and interaction analysis

5.4.2 Project pipeline

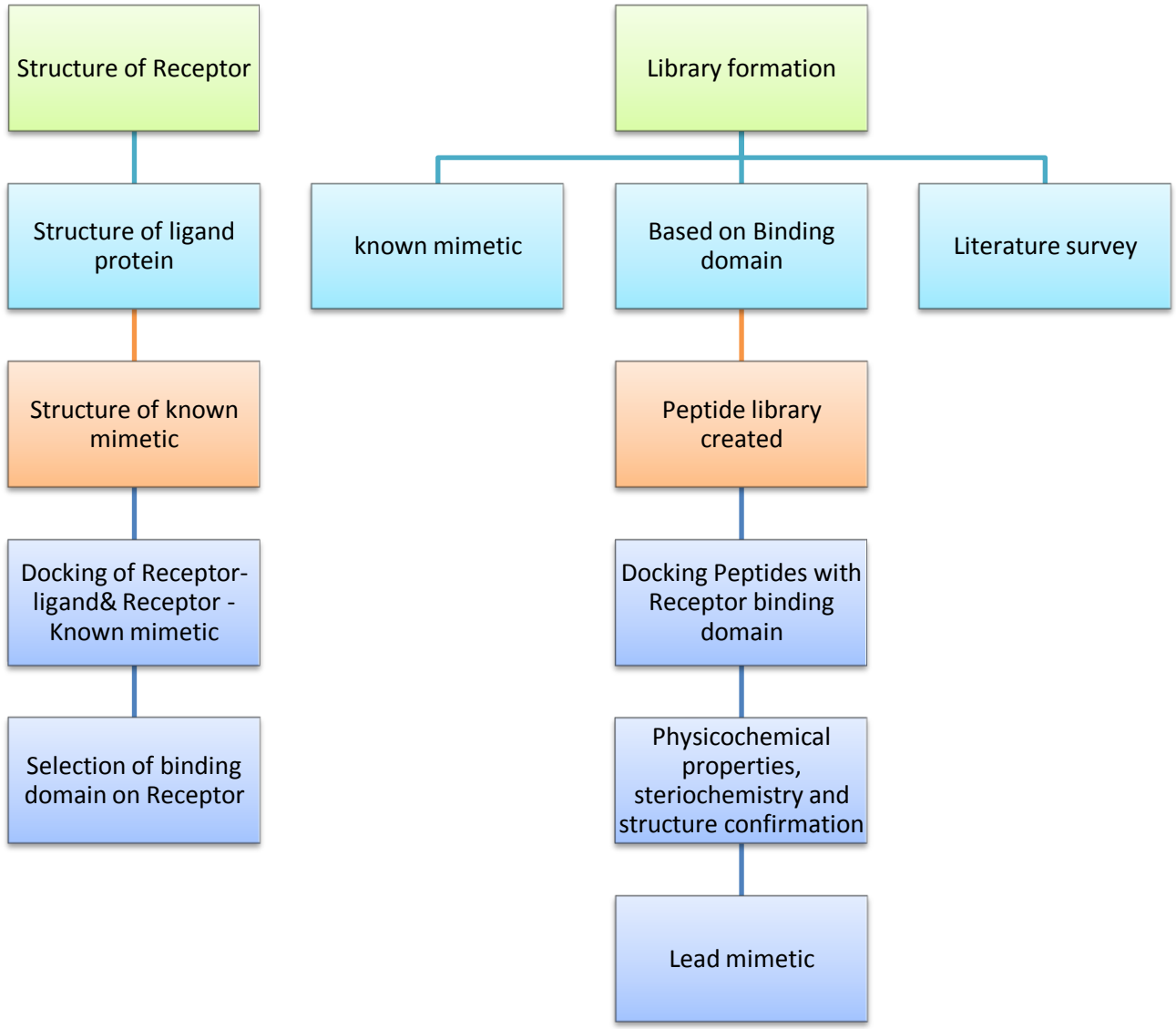


Fig.14: Project pipeline for designing mimetic

5.4.3 Method

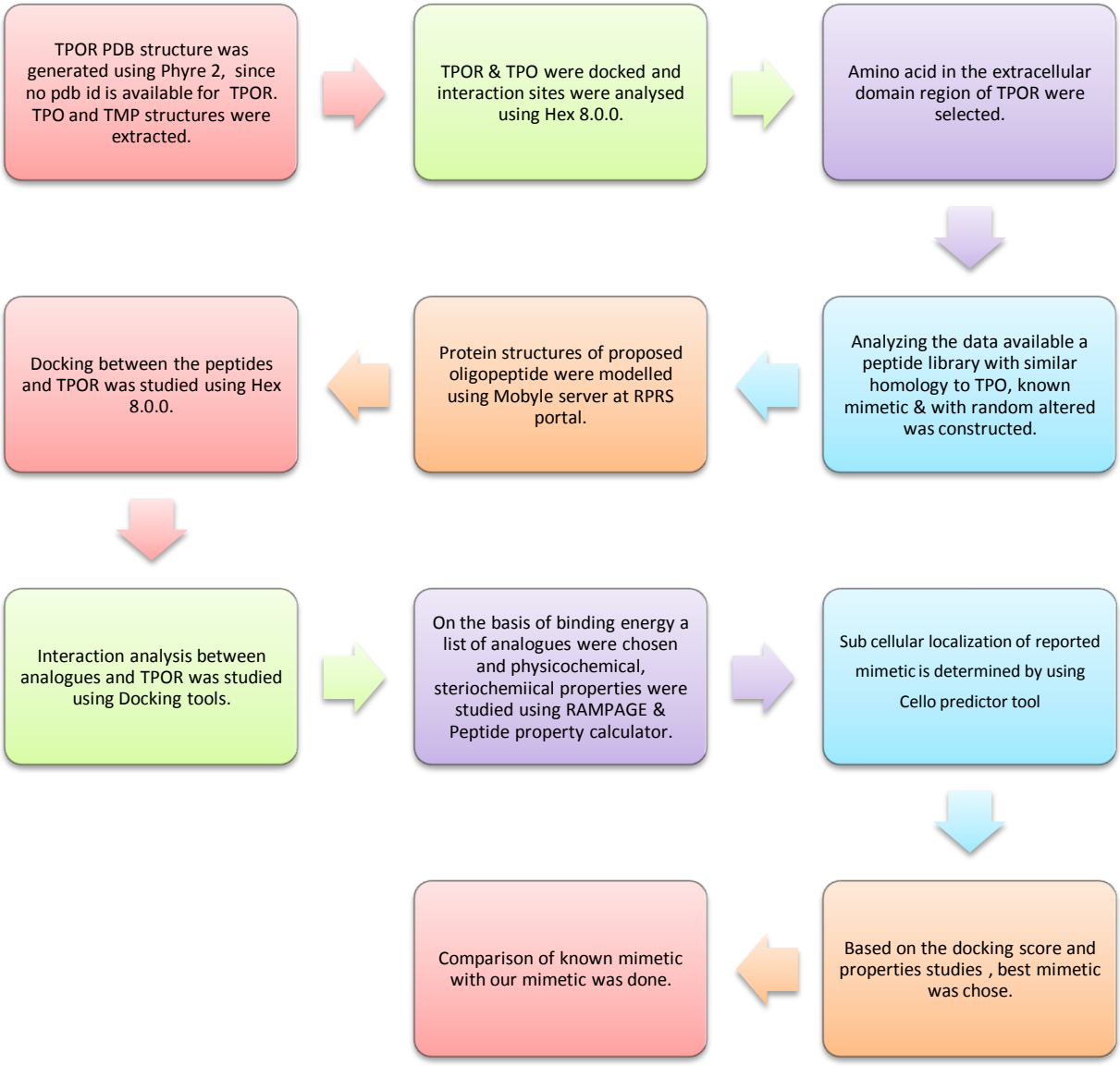


Fig. 15 Methodology of designing TPO mimetic at a glance

1. Library formation

In this study a predicted peptide library is formed on the basis of interaction studies between TPOR, TPO and TMP (known mimetic).

TMP binds to TPOR and activates it. Interaction site between TPOR and mimetic determined by docking is used for library formation. Random changes of amino acid at different location are done for making the various combinations of oligopeptide library. Library was also designed based on TPO – TPOR interaction residues and literature survey.

2. Receptor protein preparation

TPO binds to TPOR on extra cellular domain region (ECD). The 3D structure of TPOR was not available on RSCB protein data bank. So from NCBI protein sequence was retrieved and homology search was done. BLAST result showed a maximum similarity of 24% with EPO which was not enough for the predicting structure based on homology so using structure prediction tool phyre 2, which is based on *ab-initio* search and fold recognition, is used. Binding site (Extracellular region) of TPOR protein is selected. Native TPOR protein was prepared for docking analysis using HEX 8.0.0. TPOR protein chains were identified for finding any duplication of any chain, if present one of the chains will be removed. Hydrogen was added to the protein molecule then protein structure is selected for energy minimization and followed by optimization for docking analysis.

3. TMP, Known mimetic preparation

Known mimetic, TMP, is reported to mimic the role of TPO for activation and dimerization of TPOR. 3D structure of the known mimetic was retrieved and saved in PDB format. For docking analysis, known mimetic was prepared by extracting water. PDB file is imported to Maestro workspace and prepared.

4. Interaction analysis of TPOR and TMP

The binding site of TPOR and TMP is determined using HEX 8.0.0 docking module. TMP sequence was retrieved from literature survey and its PDB file is retrieved from mobyle server. Resulting PDB file from HEX 8.0.0 was exported to Maestro server and the binding site of TPOR and determined and which binding sites were used to predict peptide sequences.

5. Ligand preparation of peptide sequences

In Peptide Library, first the 3D structure was predicted by Pep fold peptide structure prediction server. Different peptides imported in workspace of Pep fold and saving the project name and sequence and format allowed to run. Pep fold returns in PDB file of target peptides. PDB file of ligands were prepared using Prep wizard removing water molecules and unwanted ligands.

6. Interaction analysis of TPOR and combinatorial library of peptides-

For peptide library (peptide sequences) binding energy is analyzed by using the docking tool HEX 8.0.0. This required prepared receptor and ligand in PDB format. Receptor and ligand PDBs were imported in the workspace of HEX and grid for domain of interaction are selected and allowed to run. HEX results in a number of best PDB of interacting receptor and ligand and log files. This gives the binding energy E_{total} score.

7. Physiochemical and biological activity prediction-

Lead mimetic sorted out with better binding efficiency, then known mimetic. Selected mimics are further analyzed for physicochemical properties. Shortlisted peptide mimics sequence biological properties and physiochemical properties determined by using Peptide protein calculator, RAMPAGE and Cello server for determine peptide position localization in humans.

6. RESULT

6.1 Carica papaya

6.1.1 Papaya leaves extracts

S. No.	Solvent	Colour of extract
1.	Hexane	Dark green
2.	Acetone	Dark green
3.	60% Ethanol	Brown
4.	40% Ethanol	Yellowish Brown
5.	Water	Dark brown

Fig. 16 Papaya leaf extracts

6.1.2 Phytochemical screening assay

Table7 Phytochemical screening assay of each extracts

S. No.	Phytoconstituents	Tests	Hexane	Acetone	60% Ethanol	40% Ethanol	Water
1.	Flavonoids	NaOH test	+	+	+	+	-
2.	Cardiac glycosides	Kellar-Kiliani test	+	+	-	-	-
3.	Tannins	$FeCl_3$ test	-	-	+	+	+
4.	Phenolic componuds	Lead acetate test	+	+	+	+	+
5.	Saponins	Froth forming test	-	+	+	+	+

6.1.3 TLC Analysis of the Fractions:

Table 8 The retention factor (Rf) values of each extracts of papaya leaves in different solvent system

1. N-hexane: acetone=8.5:1.5

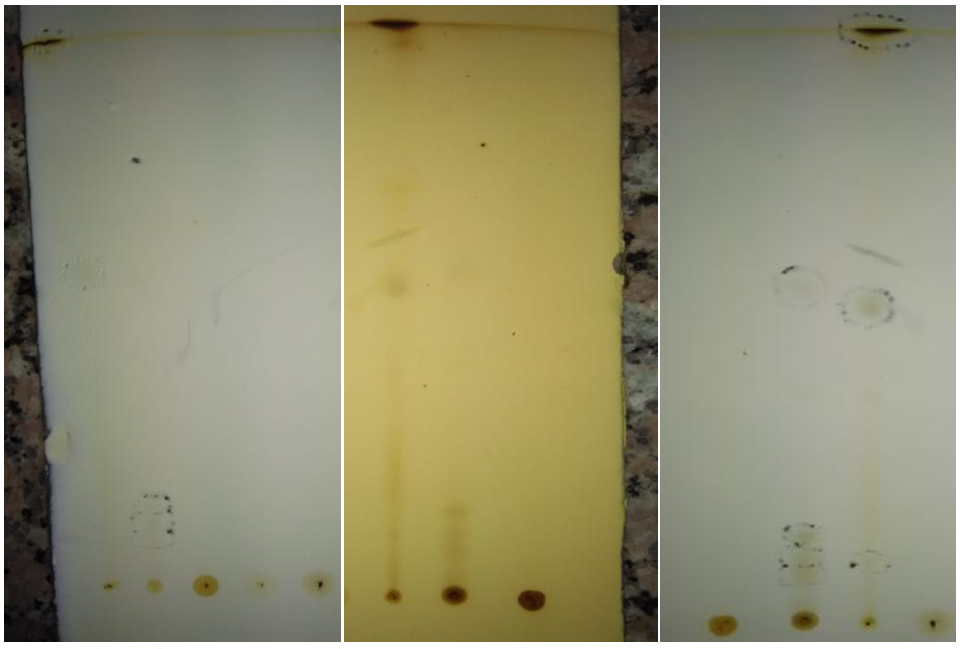
S. No.	Extract	No of spots	rf values
1.	Hexane	5	0.104, 0.174, 0.383, 0.539, 0.991
2.	Acetone	5	0.087, 0.113, 0.156, 0.383, 0.565
3.	60% Ethanol	0	-
4.	40% Ethanol	0	-
5.	Water	0	-

2. Chloroform: methanol= 9:1

S. No.	Extract	No of spots	rf values
1.	Hexane	2	0.14, 0.96
2.	Acetone	3	0.06, 0.45, 0. 94
3.	60% Ethanol	1	0.21
4.	40% Ethanol	1	0. 91
5.	Water	1	0. 91

3. Benzene: ethyl acetate=1:1

S. No.	Extract	No of spots	rf values
1.	Hexane	3	0.069, 0.915, 0.977
2.	Acetone	4	0.085, 0.154, 0.908, 0.977
3.	60% Ethanol	0	-
4.	40% Ethanol	1	0.985
5.	Water	0	-



Chloroform: methanol (9:1) n-hexane: acetone (8.5:1.5) Benzene: ethyl acetate (1:1)

Fig. 17 TLC analysis of papaya leaf extracts with different solvents

6.1.4 **GC-MS analysis**

a) Hexane extract

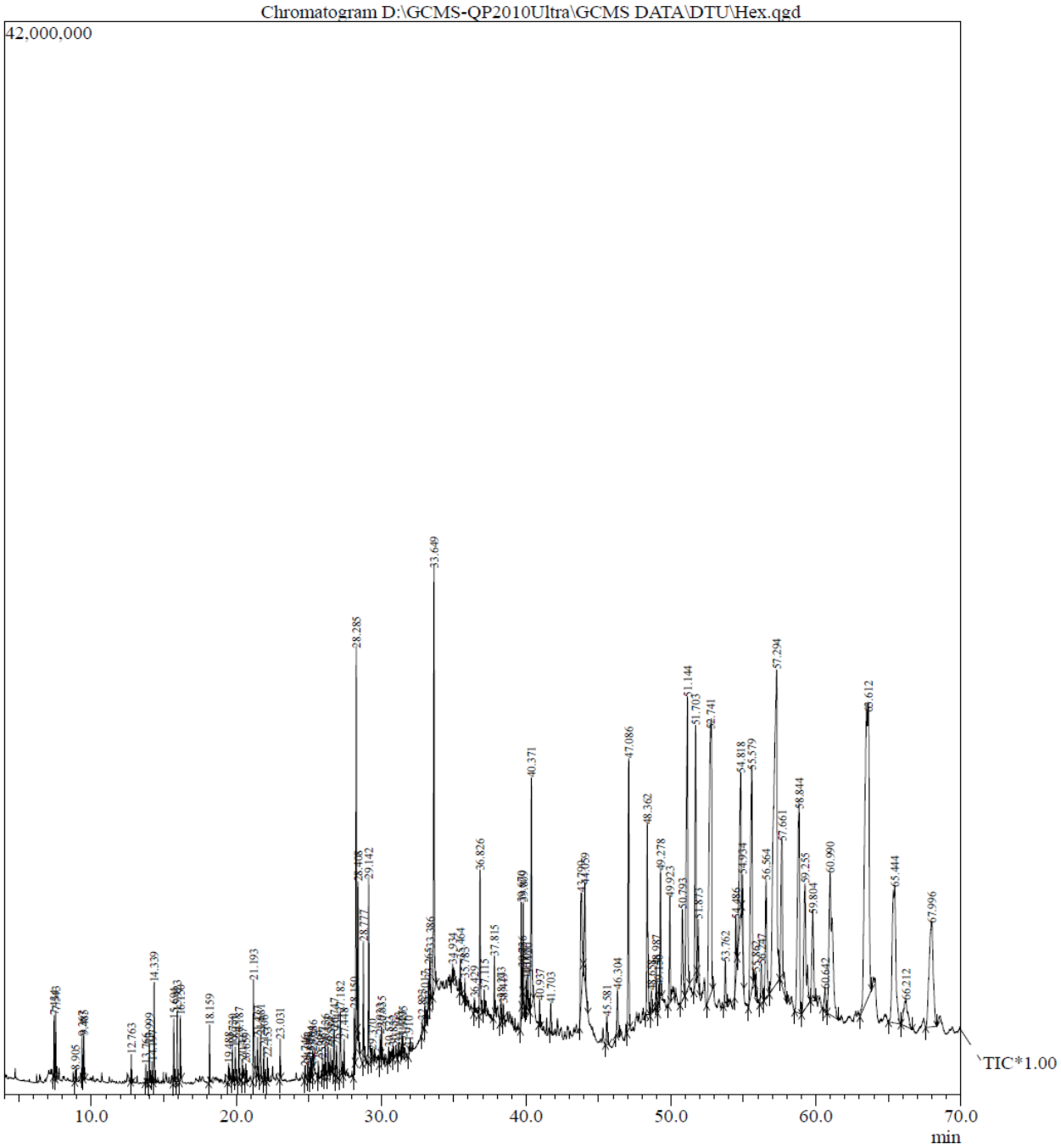


Fig. 18 Graph Showing the GC-MS analysis of hexane extract

Table 9 Phytocomponents identified in hexane extract of papaya leaves by GC-MS (Iyappan *et al, 2014;* Bhaskar *et al***,** 2013**)**

S. No.	RT	Compound		Mol. formula	Mol. Wt.	Peak area %	Function
1.	28.777	2,6,10 trimethyl,14-ethylene-14-pentadecne	Phytol	$C_{20}H_{38}$	278	1.75	Anticancer, antioxidant and anti-inflammatory, diuretic.
2.	33.386	9,12,15 Octadecatrienoic acid	Linolenic acid	$C_{18}H_{30}O_2$	278.42	0.34	Anti-inflammatory, Hypocholesterolemic, Cancer preventive, Hepatoprotective, Nematicide, Insectifuge Antihistaminic,Antiarthritic,Anticoronary, Antieczemic Antiacne, 5-Alpha reductase.
3.	28.150	3,7,11,15Tetramethyl-2-hexadecanoic acid	Terpene alcohol	$C_{20}H_{40}O$	296.53	0.32	Flavor and Lubricanting agent.
4.	30.035	Hexadecanoic acid, methyl ester	Palmitic acid	$C_{17}H_{34}O_2$	270	0.15	Antioxidant, Pesticide, Flavor, 5-Alpha Reductase-inhibitor, Antifibrinolytic, Hemolytic, Lubricant, Nematicide,Antialopecic
5.	47.086	Supraene	Squalene	$C_{30}H_{50}$	410	2.46	
6.	9.367	2-Isopropyl-5-methyl-1-heptanol	-	$C_{11}H_{24}O$	172	0.33	Antifungal compounds
7.	59.255	9, 19-cyclolanost-24-en-3-ol, (3.beta.)-	Cycloartenol	$C_{30}H_{50}O$	426	2.56	Antilisterial activities (antibacterial activity)
8.	63.612	1-Heptacosanol	Fatty alcohol	$C_{27}H_{56}O$	396	11.0	Decrease aggregation of platelets, cholesterol

							reduction, help reduce blood clots, extra synthese, producer of phytochemical reference material
9.	51.144	Gamma.-Tocopherol	-	$C_{28}H_{48}O_2$	416	3.80	One of the naturally occurring forms of Vitamin E. Most abundant in soybean and corn oils.
10.	54.818	Ergost-5-en-3-ol, (3.beta.,24r)-	campesterol	$C_{28}H_{48}O$	400	2.22	A phytosterol, inhibit the intestinal absorption of cholesterol.
11.	57.294	Stigmast-5-en-3-ol, (3.beta.)	beta-Sitosterol	$C_{29}H_{50}O$	414	3.44	Anti-inflammatory, immunomodulator and used in the treatment of prostatic adenoma

b) Acetone extract

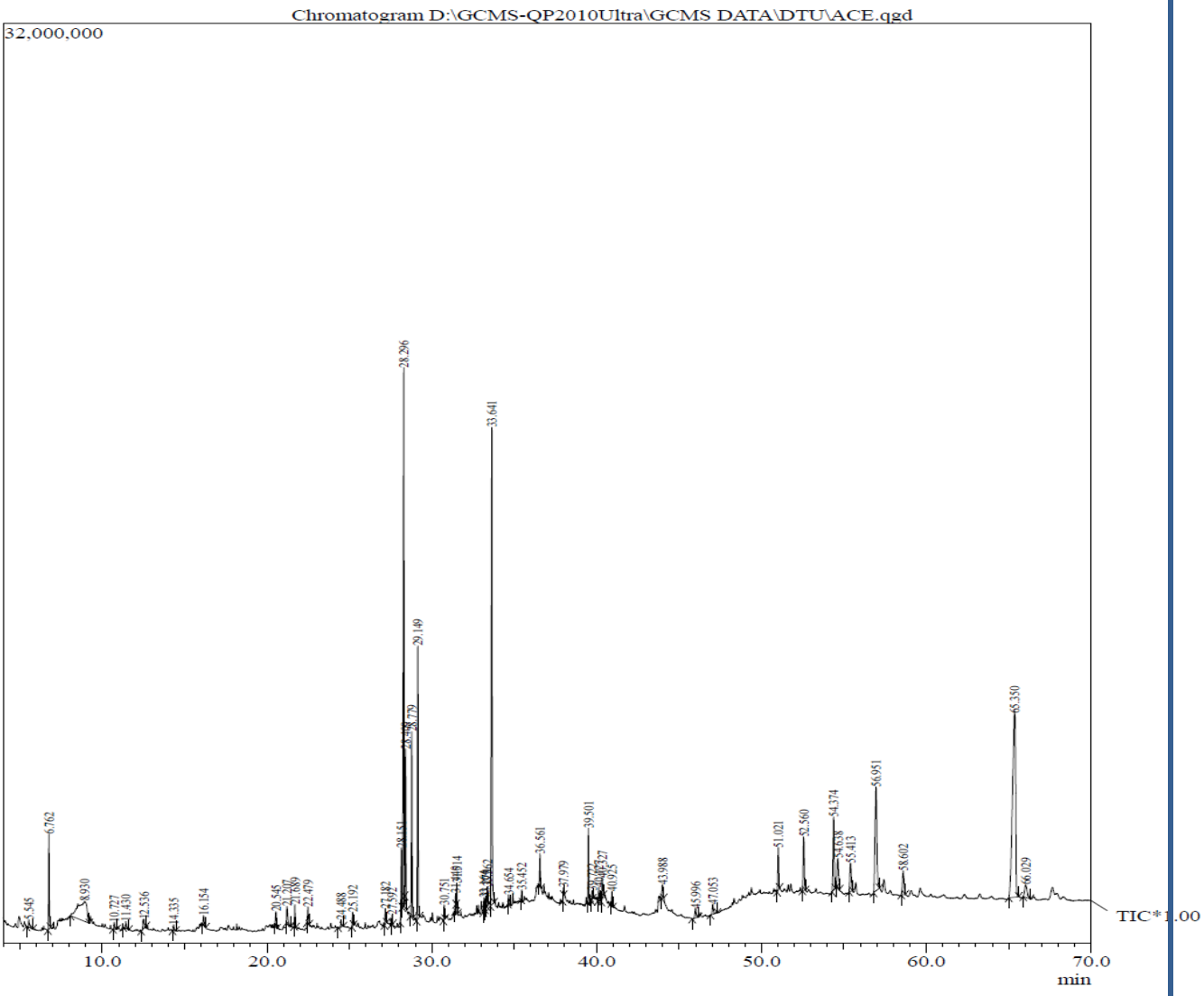


Fig. 19 Graph Showing the GC-MS analysis of acetone extract

Table 10 Phytocomponents identified in acetone extract of papaya leaves by GC-MS (Iyappan *et al, 2014*; Cravatt *et al*, 1996**)**

S. No.	RT	Compound		Mol. Formula	Mol. Wt.	Peak area %	Function
1.	5.545	Cyclohexanone	-	$C_6H_{10}O$	98	0.22	Forms peroxide with H_2O_2, and react vigorously with

							oxidizing materials i.e. nitric acid.
2.	6.762	2-Propanol, 1,1,1-trichloro-2-methyl-	Chlorobutanol	$C_4H_7Cl_3O$	176	1.97	Antimicrobial
3.	8.930	1,2,3-propanetriol	Glycerol	$C_3H_8O_3$	92	5.76	Used to aid in casting gradient gels and as a protein stabilizer and storage buffer component.
4.	10.727	Benzyl isocyanate	-	C_8H_7NO	133	0.19	Antiestrogen
5.	22.479	Dodecanoic acid	Lauric acid	$C_{12}H_{24}O_2$	200	0.46	Intermediates of Liquid Crystals, increases total serum cholesterol more than other fatty acids
6.	28.296	2,6,10-trimethyl,14-ethylene-14-pentadecne	Neophytadiene	$C_{20}H_{38}$	278	24.48	Enzyme inhibitor
7.	33.164	2-hexadecen-1-ol,3,7,11,15-tetramethyl	Terpene alcohol	$C_{20}H_{40}O$	296	17.7	Flavor and Lubricanting agent
8.	33.362	9-octadecenoic acid (z)-, methyl ester	oleic acid	$C_{19}H_{36}O_2$	296	0.48	Cell membrane , healthy skin, cholesterol metabolism and prostaglandin production
9.	37.979	9-octadecenamide	Oleamide	$C_{18}H_{35}NO$	281	0.18	Therapeutic agent for the treatment of pain and sleep disorders
10.	52.560	Vitamin E	Methylated Phenols	$C_{29}H_{50}O_2$	430	1.98	Antioxidant activity, anti-atherogenic, antithrombotic, anticoagulant, neuroprotective, antiviral, immune-modulatory, cell membrane-stabilizing and antiproliferative actions
11.	15.236	Hexadecanoic acid, methyl ester	Palmitic acid	$C_{17}H_{34}O_2$	270	0.48	Antioxidant, Pesticide, Flavor, 5-Alpha-Reductase-inhibitor, Antifibrinolytic,

							Hemolytic, Lubricant, Nematicide, Antialopecic
12.	19.136	Dehydroabietic acid	-	$C_{21}H_{30}O_2$	314	1.00	Used in rosin-type nucleating agent for polypropylene (PP) and commonly in the synthesis of surfactants, antioxidants and chiral catalysts.
13.	27.830	Gamma.-Tocopherol 27.830	-	$C_{28}H_{48}O_2$	416	1.98	Naturally occurring forms of Vitamin E. Most abundant in soybean and corn oils.
14.	30.969	Stigmasterol	Sterol	$C_{29}H_{48}0$	412	0.84	Used as a precursor in the synthesis of progesterone.
15.	32.109	Stigmast-5-en-3-ol, (3.beta.)-	beta-Sitosterol	$C_{29}H_{50}O$	414	3.24	Anti-inflammatory, immunomodulator and used in the treatment of prostatic adenoma.

c) 60% Ethanol extract

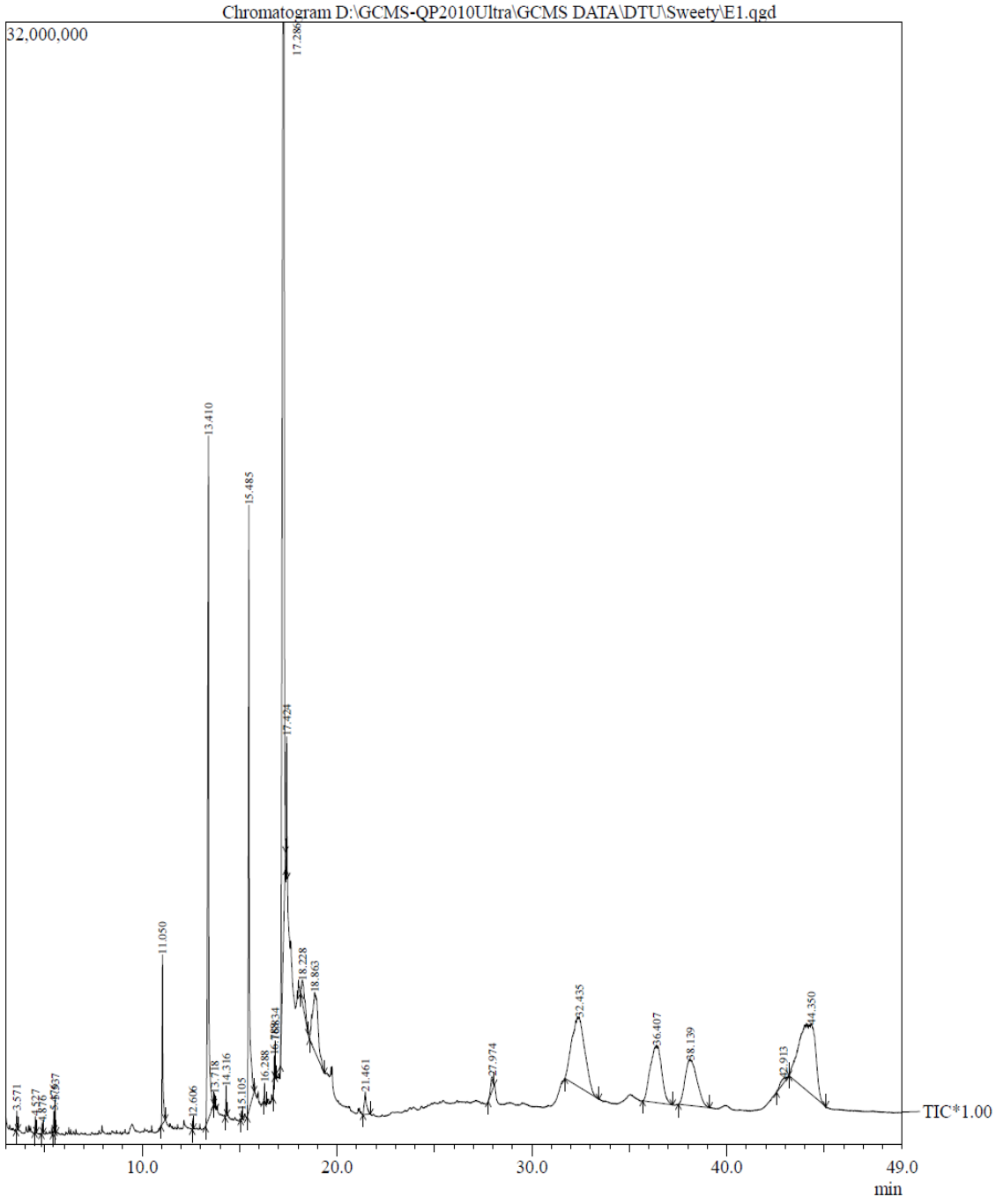


Fig. 20 Graph Showing the GC-MS analysis of 60% Ethanol extracts

Table 11 Phytocomponents identified in 60% Ethanol extract of papaya leaves by GC-MS (Bhaskar *et al*, 2013; Cravatt *et al*, 1996**)**

S. No.	RT	Compound		Mol. Formula	Mol. Wt.	Peak area %	Function
1.	4.527	1,3,5-triazine-2,4,6-triamine	Melamine	$C_3H_6N_6$	126	0.12	Forms synthetic resins with formaldehyde
2.	4.876	Cyclopropylmethanol	Cyclopropyl carbinol	C_4H_8O	72	0.08	Used to increase selectivity of pyridyl-cinnoline phosphodiesterase, inhibitors against phosphodiesterase 3 (PDE3). Also used in the synthesis of benzodiazapin-2-ones
3.	5.537	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	-	$C_6H_8O_4$	144	0.22	Strong antioxidant activity
4.	11.050	Dodecanoic acid	Lauric acid	$C_{12}H_{24}O_2$	200	1.82	Intermediates of Liquid Crystals, increases total serum cholesterol more than other fatty acids
5.	13.410	Tetradecanoic acid	myristic acid	$C_{14}H_{28}O_2$	228	10.19	Cell membrane , acts as a lipid anchor in biomembranes, healthy skin, cholesterol metabolism and prostaglandin production
6.	13.718	Hexadecanoic acid, ethyl ester	Palmitic acid	$C_{18}H_{36}O_2$	284	0.08	Antioxidant, Pesticide, Flavor, 5-Alpha-Reductase-inhibitor, Antifibrinolytic, Hemolytic, Lubricant, Nematicide, Antialopecic
7.	15.105	Hexadecanoic acid, methyl ester		$C_{17}H_{34}O_2$	270	0.05	
8.	15.485	Pentadecanoic acid	-	$C_{15}H_{30}O_2$	242	7.92	potential anxiolytic, antinociceptive and antimicrobial properties
9.	16.834	9-octadecenoic acid, methyl ester	oleic acid	$C_{19}H_{34}O_2$	294	0.18	Cell membrane , healthy skin, cholesterol metabolism and prostaglandin production

d) 40% Ethanol extract

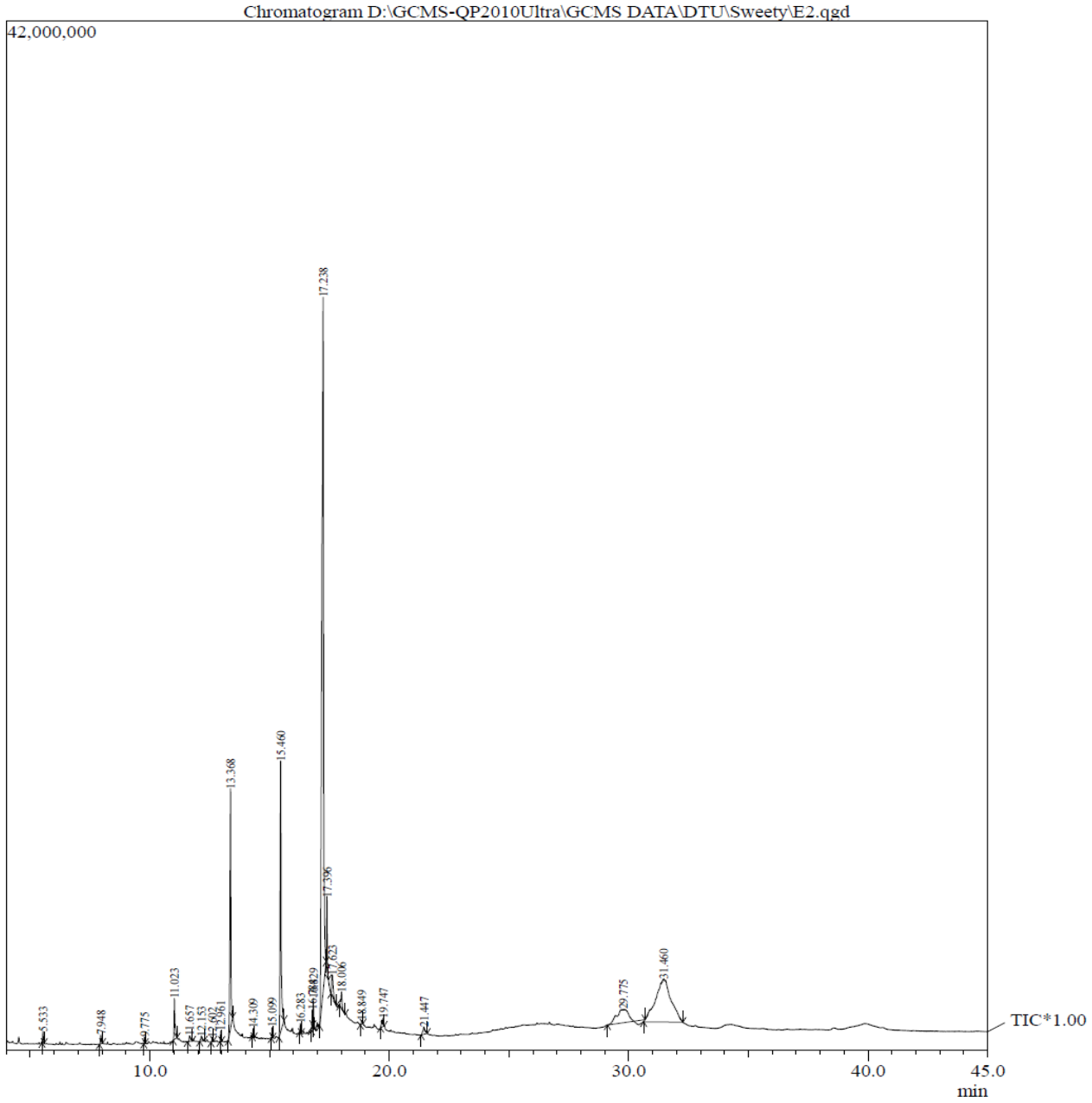


Fig. 21 Graph Showing the GC-MS analysis of 40% Ethanol extract

Table 12 Phytocomponents identified in 40% Ethanol extract of papaya leaves by GC-MS (Iyappan *et al, 2014*; Cravatt *et al*, 1996; Bhaskar *et al***,** 2013**)**

S. No.	RT	Compound		Mol. Formula	Mol. Wt.	Peak area %	Function
1.	7.948	2-methoxy-4-vinylphenol	Phenolic	$C_9H_{10}O_2$	150	0.30	Antimicrobial, Antioxidant, Anti-inflammatory and Analgesic
2.	13.368	Tetradecanoic acid	myristic acid	$C_{14}H_{28}O_2$	228	8.12	Cell membrane, acts as a lipid anchor in biomembranes, healthy skin, cholesterol metabolism and prostaglandin production
3.	15.099	Hexadecanoic acid, methyl ester	Palmitic acid	$C_{17}H_{34}O_2$	270	0.19	Antioxidant, Pesticide, Flavor, 5-Alpha-Reductase-inhibitor, Antifibrinolytic, Hemolytic, Lubricant, Nematicide, Antialopecic
4.	16.829	9-octadecenoic acid (z)-, methyl ester	oleic acid	$C_{19}H_{36}O_2$	296	0.50	Cell membrane, healthy skin, cholesterol metabolism and prostaglandin production
5.	18.849	Hexadecanoic acid	Palmitic acid	$C_{16}H_{32}O_2$	256	0.10	Antioxidant, Pesticide, Flavor, 5-Alpha-Reductase-inhibitor, Antifibrinolytic, Hemolytic, Lubricant, Nematicide, Antialopecic
6.	19.747	9-octadecenamide	Oleamide	$C_{18}H_{35}NO$	281	0.47	Therapeutic agent for the treatment of pain and sleep disorders

e) Water extract

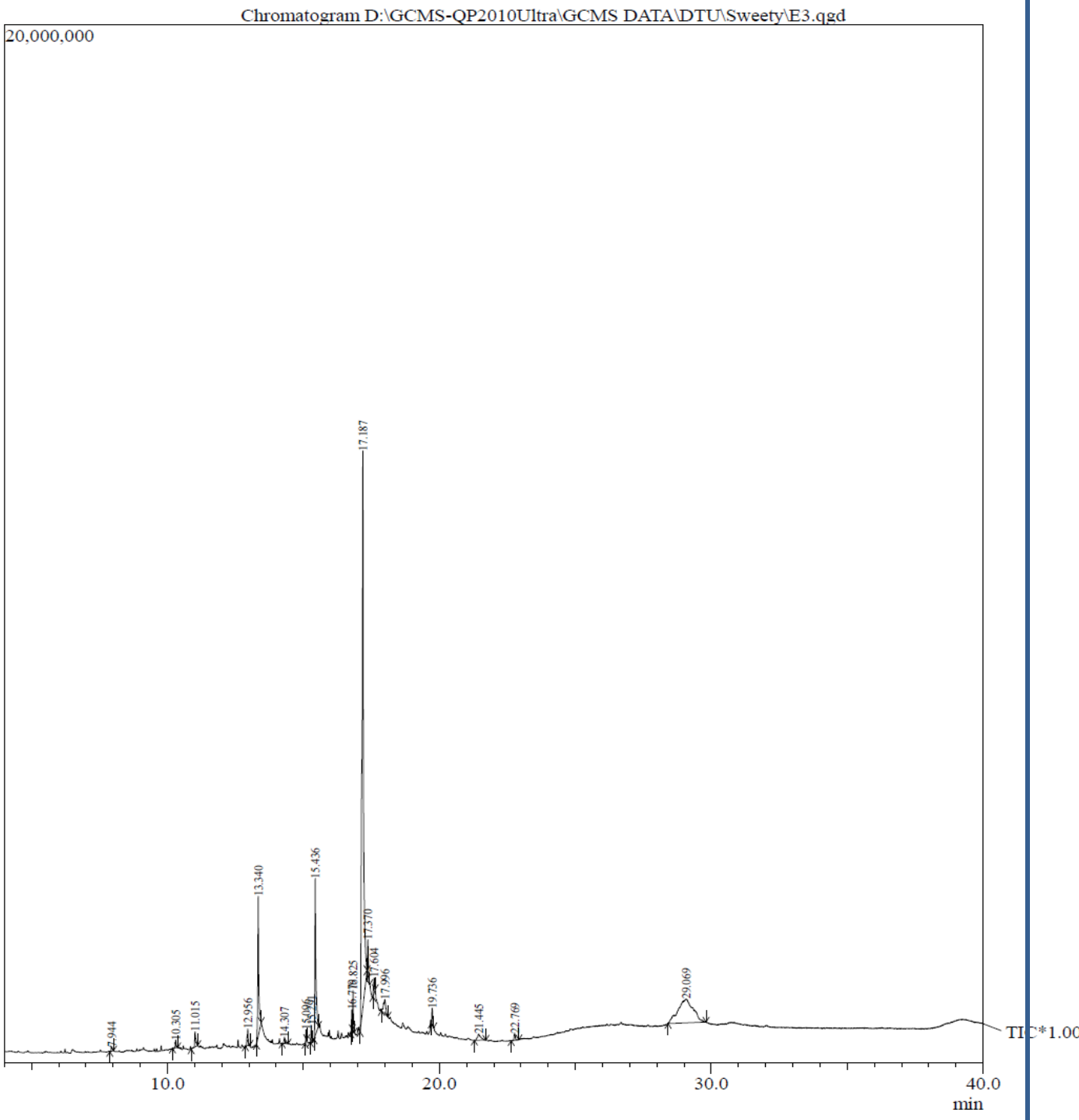


Fig 22: Graph Showing the GC-MS analysis of water extract

Table 13 Phytocomponents identified in water extract of papaya leaves by GC-MS (Iyappan *et al, 2014*; Cravatt *et al*, 1996; Bhaskar *et al***,** 2013**)**

S. No.	RT	Compound		Mol. Formula	Mol. Wt.	Peak area %	Function
1.	7.944	2-methoxy-4-vinylphenol	Phenolic	$C_9H_{10}O_2$	150	0.30	Antimicrobial, Antioxidant, Anti-inflammatory, and Analgesic
2.	11.015	Dodecanoic acid	Lauric acid	$C_{12}H_{24}O_2$	200	1.11	Intermediates of Liquid Crystals and increases total serum cholesterol more than other fatty acids.
3.	13.340	Tetradecanoic acid	myristic acid	$C_{14}H_{28}O_2$	228	8.31	Cell membrane, acts as a lipid anchor in biomembranes, healthy skin, cholesterol metabolism and prostaglandin production
4.	15.096	Hexadecanoic acid, methyl ester	Palmitic acid	$C_{17}H_{34}O_2$	270	0.35	Antioxidant, Pesticide, Flavor, 5-Alpha-Reductase-inhibitor, Antifibrinolytic, Hemolytic, Lubricant, Nematicide, Antialopecic
5.	16.825	9-octadecenoic acid (z)-, methyl ester	oleic acid	$C_{19}H_{36}O_2$	296	1.30	Cell membrane, healthy skin, cholesterol metabolism and prostaglandin production
6.	15.436	Pentadecanoic acid	-	$C_{15}H_{30}O_2$	242	9.39	potential anxiolytic, antinociceptive and antimicrobial properties
7.	19.736	9-octadecenamide	Oleamide	$C_{18}H35NO$	281	0.82	Therapeutic agent for the treatment of pain and sleep disorders

6.2 Blood

6.2.1 Haemoglobin Estimation

Table 14 AHD standard

Dilution	absorbance
1	1.602
0.1	0.145
0.01	0.027
0.001	0.007
0.0001	0

Table 10: AHD standard readings

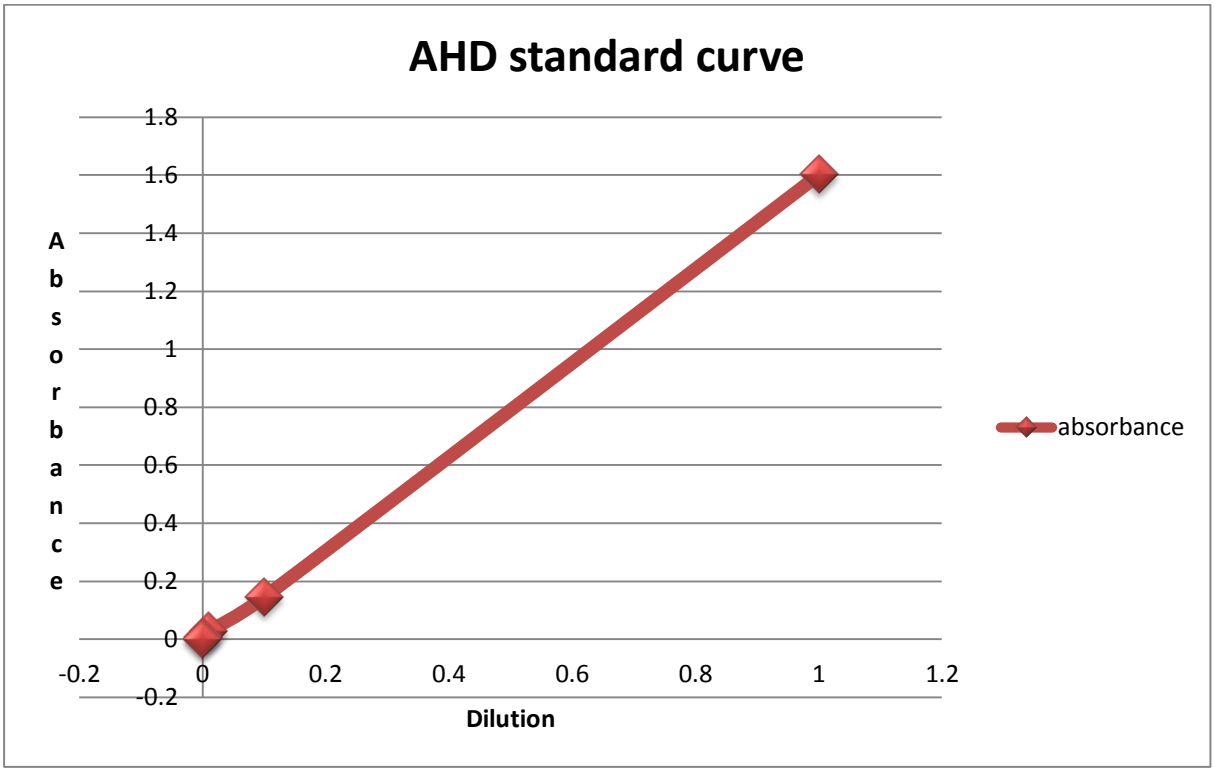


Fig. 23 AHD standard curve

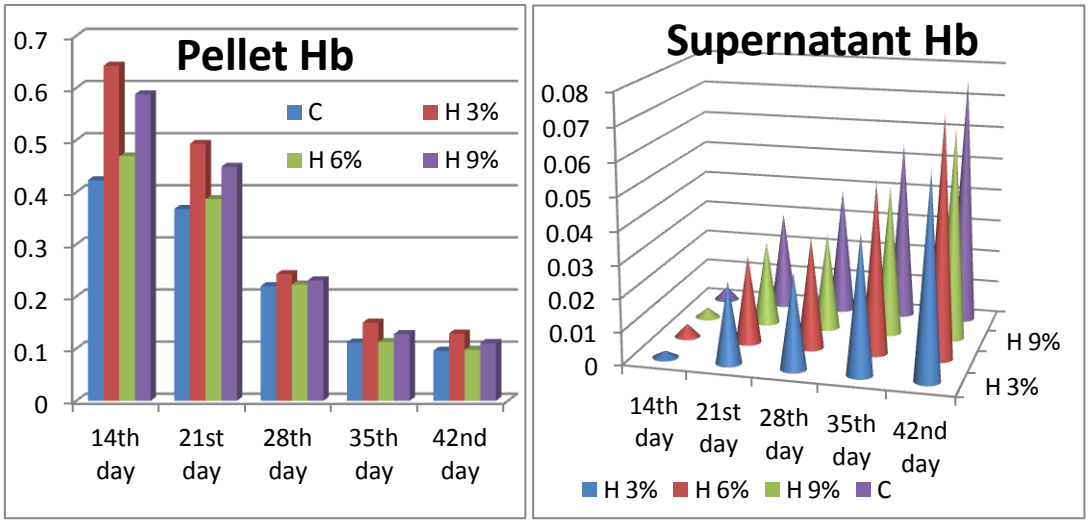
Table15 AHD control

Controls	Pellet	Supernatant
Positive control (Fresh blood+CPDA)	0.687	0.001
Negative control (100% Lysis)	0.027	0.531

Absorbance of each extracts by AHD method

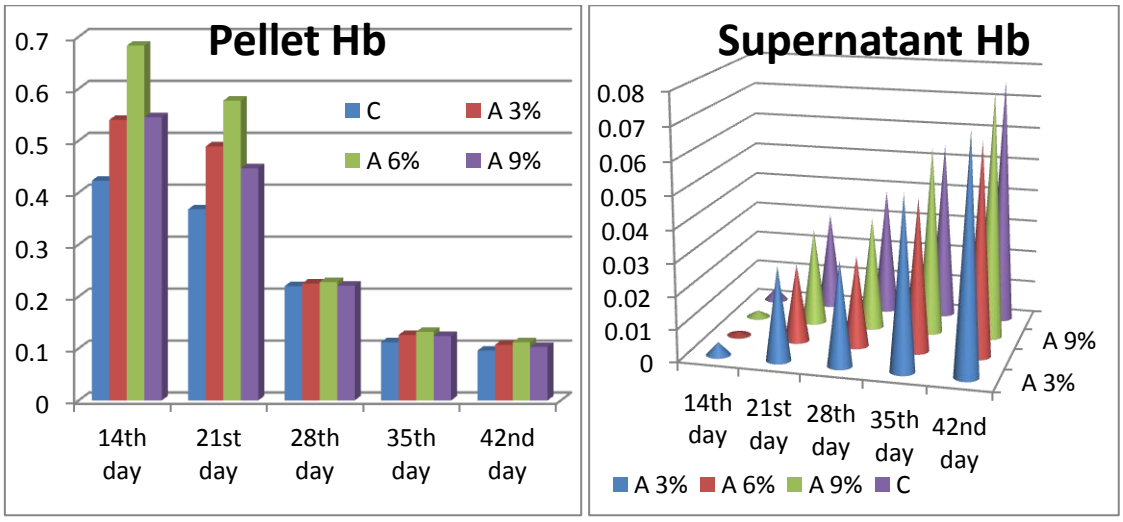
Sample	14 days (9 June)		21 days (16 June)		28 days (23 June)		35 days (30 June)		42 days (6 July)	
	S	P	S	P	S	P	S	P	S	P
HEXANE										
3%	0.002	0.643	0.025	0.493	0.029	0.243	0.042	0.150	0.061	0.129
6%	0.004	0.469	0.027	0.387	0.034	0.223	0.052	0.113	0.073	0.098
9%	0.003	0.588	0.026	0.449	0.030	0.231	0.046	0.128	0.065	0.111
ACETONE										
3%	0.004	0.540	0.029	0.489	0.032	0.225	0.053	0.126	0.072	0.107
6%	0.001	0.683	0.024	0.577	0.028	0.228	0.047	0.132	0.065	0.112
9%	0.002	0.545	0.030	0.447	0.035	0.221	0.058	0.124	0.076	0.103
60% ETHANOL										
3%	0.002	0.504	0.028	0.395	0.033	0.227	0.049	0.125	0.068	0.109
6%	0.005	0.411	0.039	0.335	0.042	0.197	0.058	0.110	0.078	0.087
9%	0.004	0.418	0.036	0.348	0.041	0.220	0.055	0.112	0.075	0.095
40% ETHANOL										
3%	0.003	0.432	0.026	0.389	0.038	0.228	0.052	0.117	0.076	0.098
6%	0.001	0.455	0.023	0.405	0.035	0.221	0.049	0.114	0.063	0.093
9%	0.005	0.403	0.035	0.344	0.043	0.213	0.057	0.103	0.079	0.081
WATER										
3%	0.004	0.536	0.030	0.498	0.039	0.229	0.054	0.121	0.072	0.101
6%	0.003	0.625	0.029	0.597	0.037	0.285	0.051	0.138	0.069	0.115
9%	0.005	0.417	0.031	0.361	0.040	0.200	0.056	0.108	0.077	0.088
FRESH										
3%	0.003	0.521	0.025	0.465	0.032	0.225	0.049	0.135	0.070	0.117
6%	0.001	0.601	0.022	0.557	0.027	0.227	0.053	0.179	0.075	0.153
9%	0.003	0.515	0.029	0.452	0.035	0.224	0.049	0.124	0.072	0.103
CONTROL	0.004	0.423	0.030	0.68	0.039	0.220	0.055	0.112	0.076	0.096

Hexane



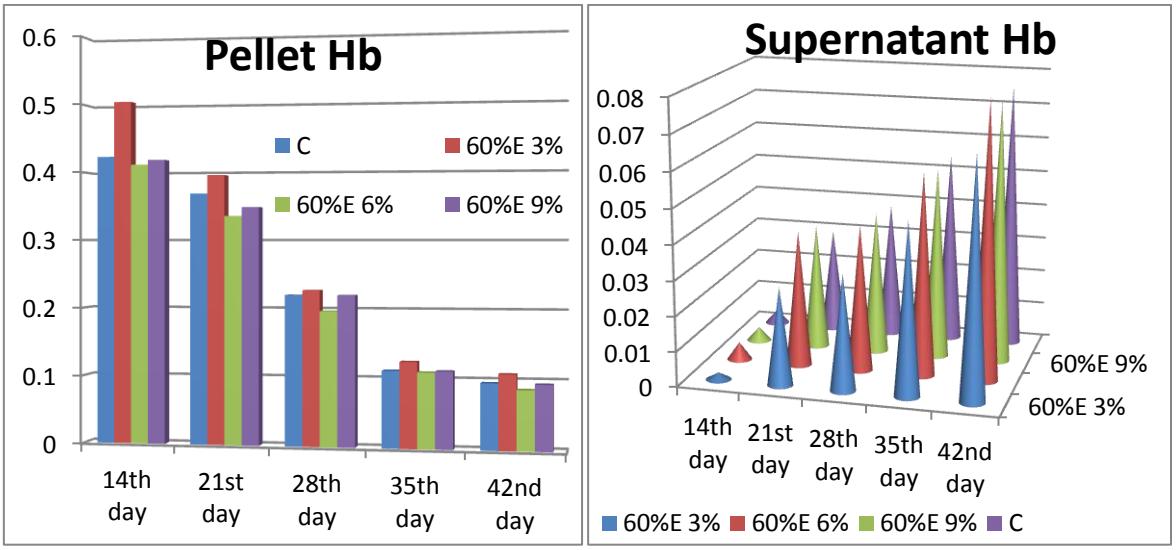
The graph compares the amount of Hb in pellet and supernatant of blood sample stored in papaya leaf extracted in hexane solvent.In pellet the absorbance of 3%, 6% & 9% hexane (H) extracts increases while in supernatant the absorbance of each hexane extracts decreases as compared to control. This shows the haemolysis of RBCs in extracts is less than control during storage period.

Acetone



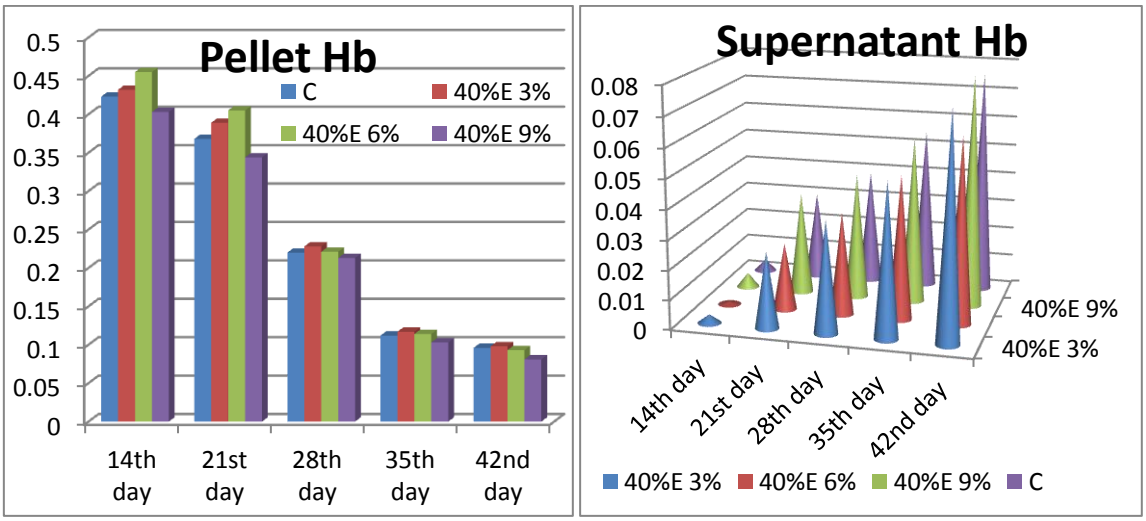
The graph compares the amount of Hb in pellet and supernatant of blood sample stored in papaya leaf extracted in acetone solvent In pellet the absorbance of 3%, 6% & 9% acetone (A) extract increases while in supernatant the absorbance of each acetone extracts decreases as compared to control. This shows the haemolysis of RBCs in extracts is less than control during storage period.

60% Ethanol



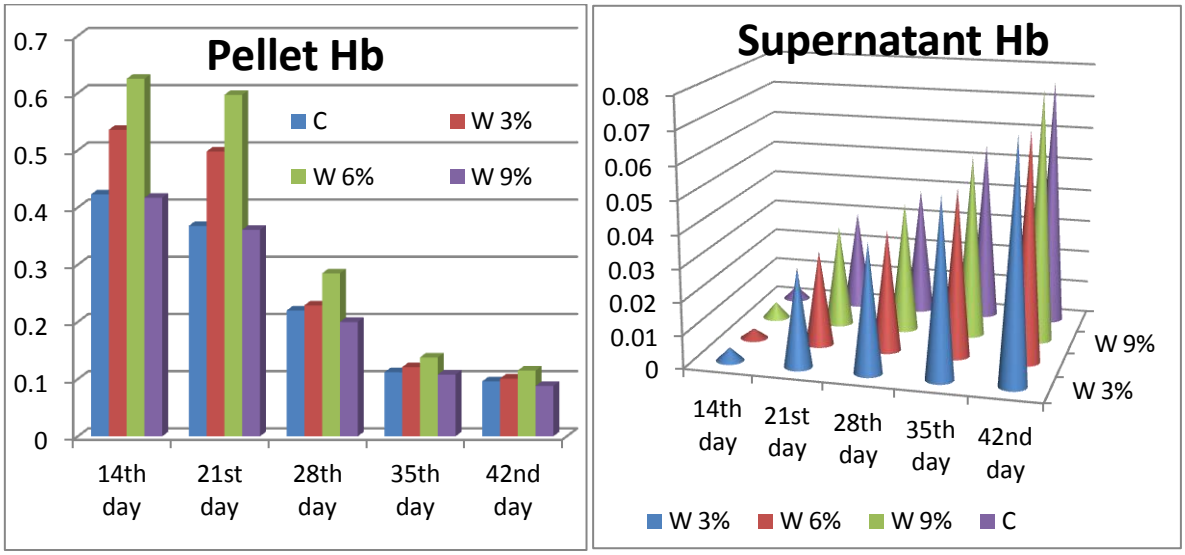
The graph compares the amount of Hb in pellet and supernatant of blood sample stored in papaya leaf extracted in 60% Ethanol solvent In pellet the absorbance of 3% of 60% Ethanol (E) increases and 6% & 9% of 60% E extract decreases while in supernatant the absorbance of 3% of 60% E decreases and 6% & 9% of 60% E extract increases as compared to control. This shows the haemolysis of RBCs in 3% of 60% E extract is less than control.

40% Ethanol



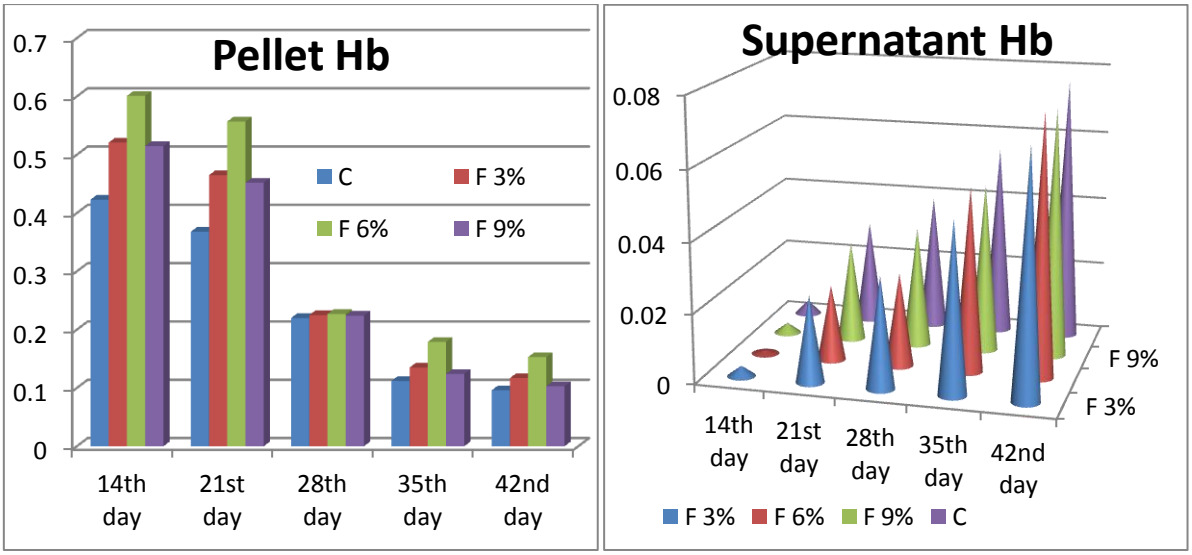
The graph compares the amount of Hb in pellet and supernatant of blood sample stored in papaya leaf extracted in 40% Ethanol solvent. In pellet the absorbance of 6% of 40% Ethanol (E) increases and 3% & 9% of 40% E extracts decreases while in supernatant the absorbance of 6% of 40% E decreases and 3% & 9% of 40% E extract increases as compared to control. This shows the haemolysis of RBCs in 6% of 60% E extract is less than control.

Water



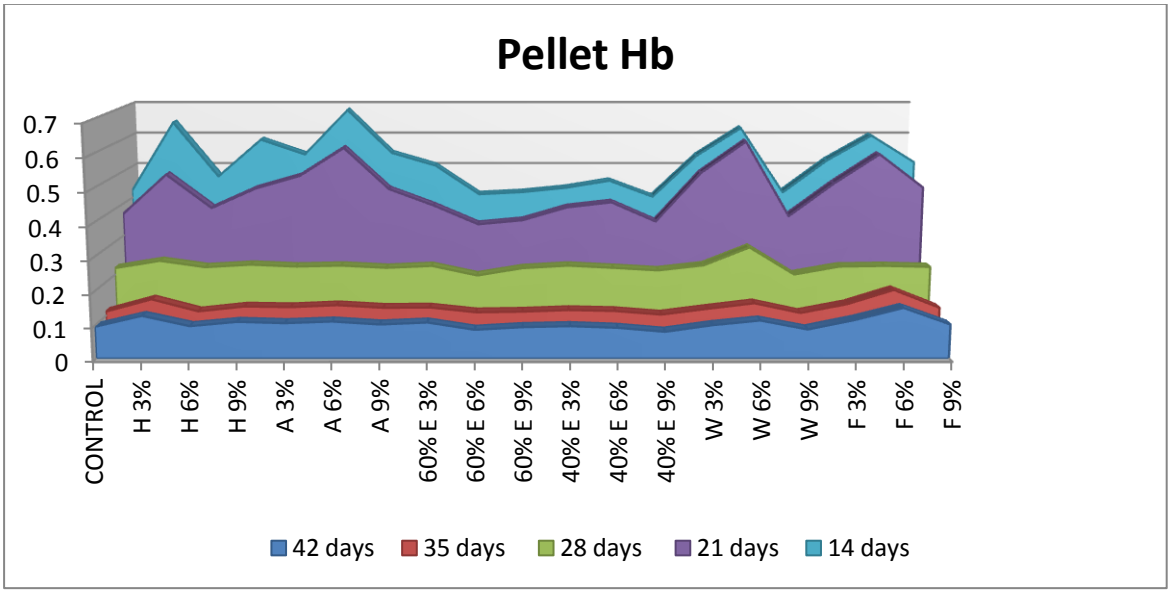
The graph compares the amount of Hb in pellet and supernatant of blood sample stored in papaya leaf extracted in Water solvent. In pellet the absorbance of 3% & 6% of water (H_2O) increases and 9% of H_2O extract decreases while in supernatant the absorbance of 3% & 6% of H_2O decreases and 9% of H_2O increases as compared to control. This shows the haemolysis of RBCs in 3% and 6% of H_2O extracts is less than control.

Fresh leaves

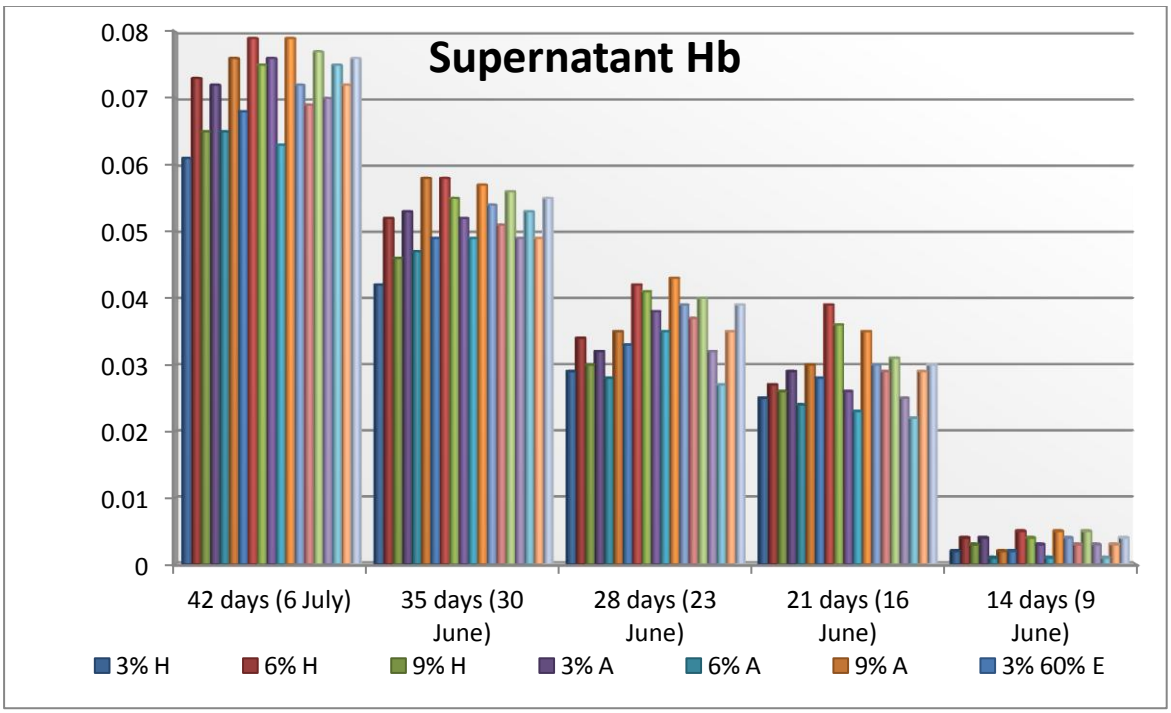


The graph compares the amount of Hb in pellet and supernatant of blood sample stored in fresh papaya leaf. In pellet the absorbance of 3%, 6% & 9% fresh (F) leaves extracts increases while in supernatant the absorbance of each fresh extracts decreases as compared to control. This shows the haemolysis of RBCs in extracts is less than control during storage period.

Fig.24 Comparative analysis of Haemoglobin of each extract with control.



The graph compares the amount of Hb in pellet of blood sample stored in papaya leaf extracted in Hexane (H), Acetone (A), 60% Ethanol (60% E), 40% Ethanol (40% E), Water (W) solvent and fresh leaves. Highest haemoglobin content of pellet shown by fresh leaves extract and the least is shown by 9% of 40% E extract. The haemoglobin content of hexane, acetone, fresh leaves, 60% E 3%, 40% E 3% & 6% and H_2O 3% & 6% extract is more with respect to control.



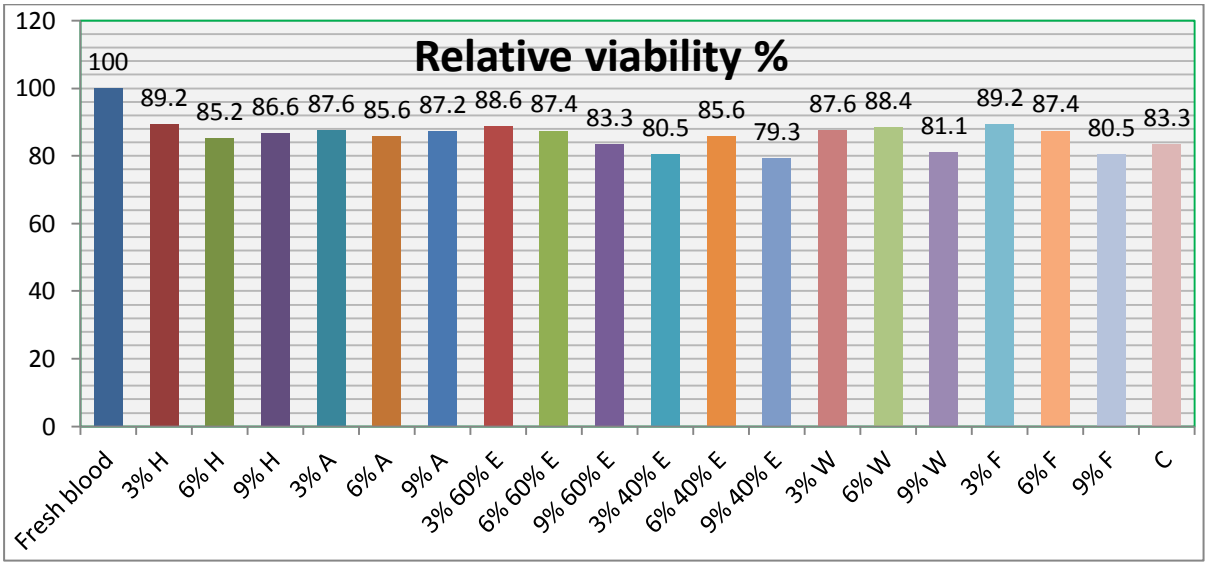
The graph compares the amount of Hb in supernatant of blood sample stored in papaya leaf extracted in Hexane (H), Acetone (A), 60% Ethanol (60% E), 40% Ethanol (40% E), Water (W) solvent and fresh leaves. The absorbance of supernatant of 9% of 40% E is highest which shows the haemoglobin release in supernatant is more and the least is shown by fresh leaves extract. The haemoglobin content of supernatant of hexane, acetone, fresh leaves, 60% E 3%, 40% E 3% & 6% and H_2O 3% & 6% extract is less with respect to control means haemolysis of RBCs in these extracts is less than control.

Fig. 25 Comparative analysis of each extract during storage period in pellet and supernatant separately.

6.2.2 Cell count results

S.No	Sample	Cell count ($x10^6$ cells/µl)	Relative viability (%)
1.	Fresh blood (day 0)	4.92	100
Blood with extracts (day 40)			
2.	HEXANE		
a)	3%	4.39	89.2
b)	6%	4.19	85.2
c)	9%	4.26	86.6
3.	ACETONE		
a)	3%	4.31	87.6
b)	6%	4.21	85.6
c)	9%	4.29	87.2
4.	60% ETHANOL		
a)	3%	4.36	88.6
b)	6%	4.30	87.4
c)	9%	4.10	83.3
5.	40% ETHANOL		
a)	3%	3.96	80.5
b)	6%	4.21	85.6
c)	9%	3.90	79.3
6.	WATER		
a)	3%	4.39	87.6
b)	6%	4.35	88.4
c)	9%	3.99	81.1
7.	FRESH		
a)	3%	4.39	89.2
b)	6%	4.30	87.4
c)	9%	3.96	80.5
8.	CONTROL	4.10	83.3

Table 16 Cell count and cell viability of RBCs



The graph shows the cell viability percentage of RBCs stored in papaya leaf extract in solvent systems namely, hexane (H), acetone (A), fresh leaves (F), 60% Ethanol (60% E), 40% Ethanol (40% E) and Water (W) and control w.r.t fresh blood. All concentrations of H, A, 60% E, along with 6% 40% E Water, 6% W, 3% F, 6% F have a positive effect on the viability of cells, whereas 3% 40% E, 9% 40% E, 9% W, 9% F have cell viability less than Control.

Fig.26 Relative viability of RBCs stored in papaya leaf extract in various solvent systems after 40 days

6.2.3 pH

Table 12: change in pH during storage period

Days	pH of Blood with CPDA and Extracts	Control
Day 0	7.4	7.2
Day 7	7.2	7.0
Day 14	7.1	7.0
Day 21	7.0	6.9
Day 35	6.9	6.8
Day 42	6.9	6.8

Table 17 Change in pH during storage period

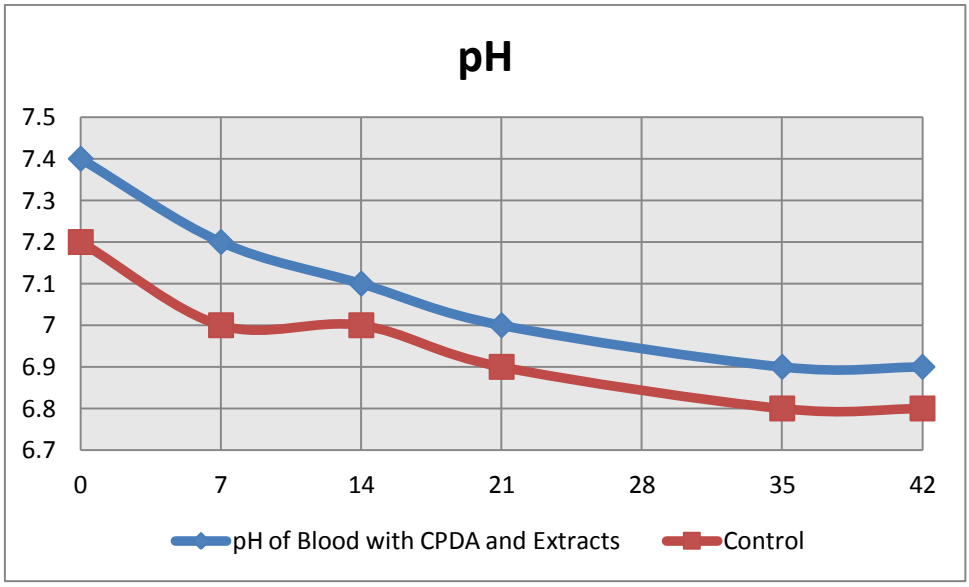
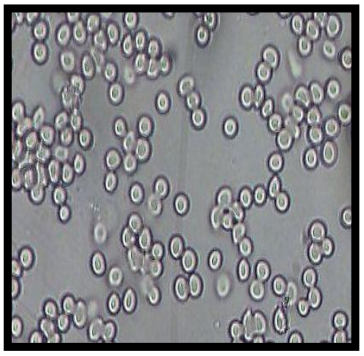
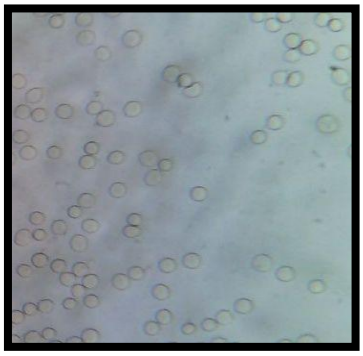
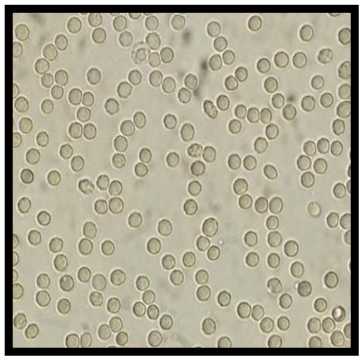
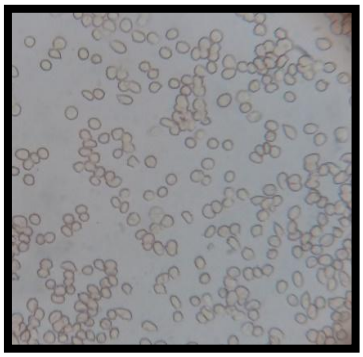
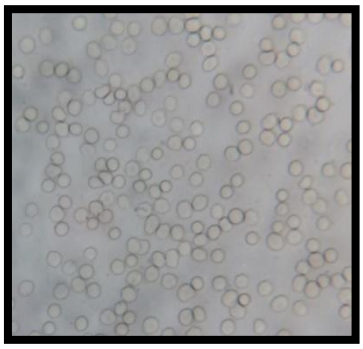


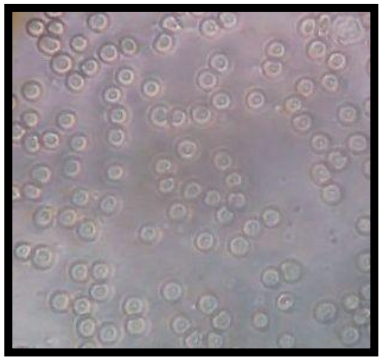
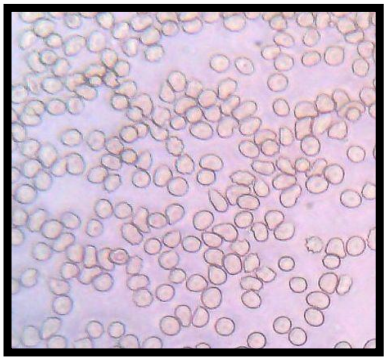
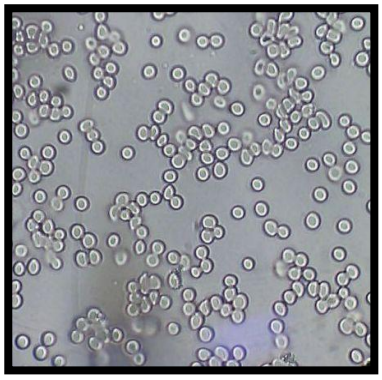
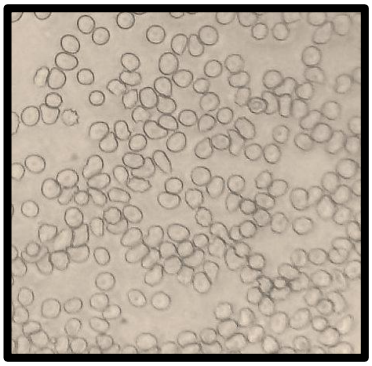
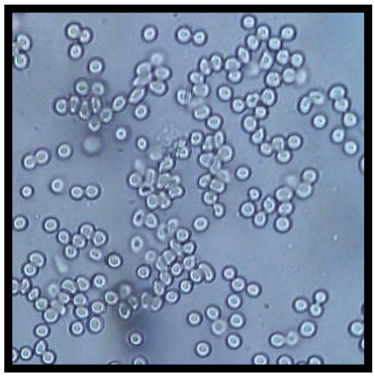
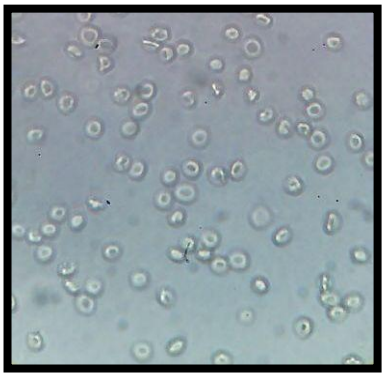
Fig. 27 pH of blood with CPDA and extracts vs. control

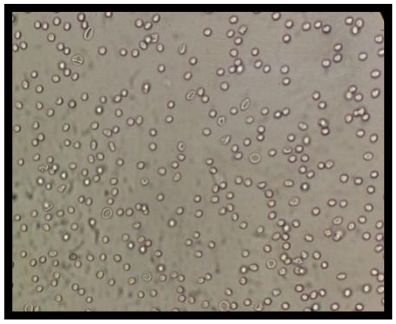
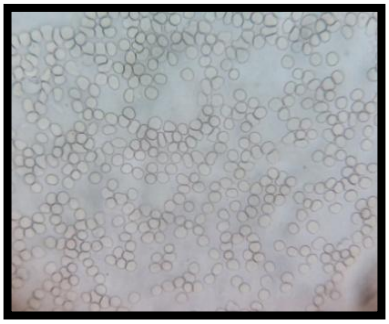
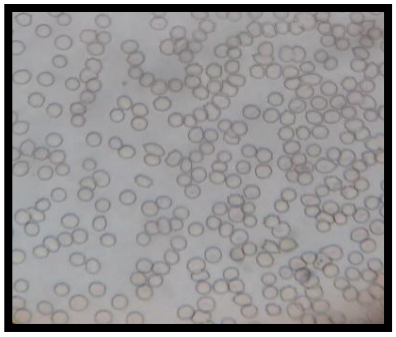
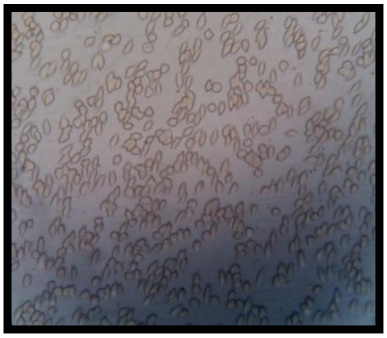
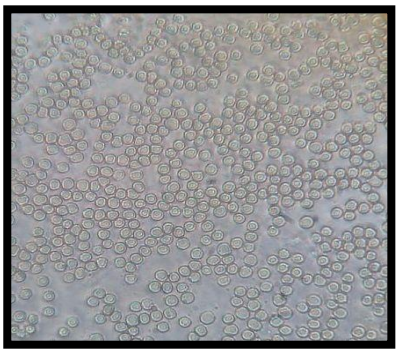
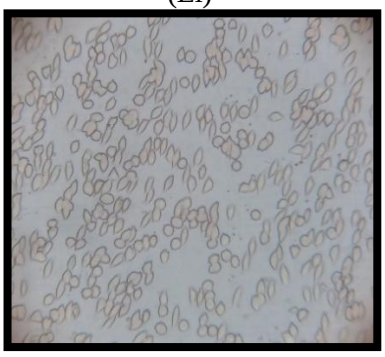
The graph shows change in pH from day 0 to day 42, pH change shows that the acidity of stored blood is increased day by day but the presence of CPDA + extracts in blood slows down the rate of acidity in comparison to CPDA alone in blood.

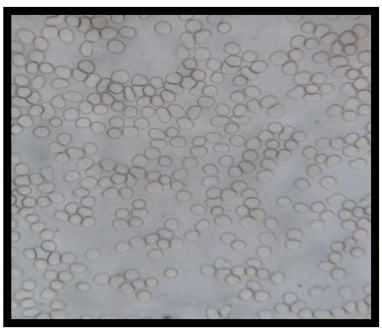
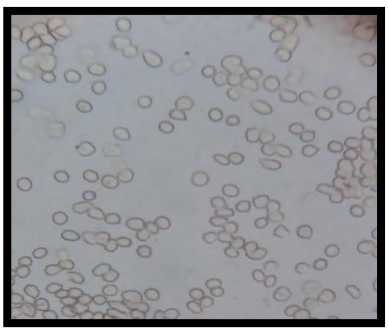
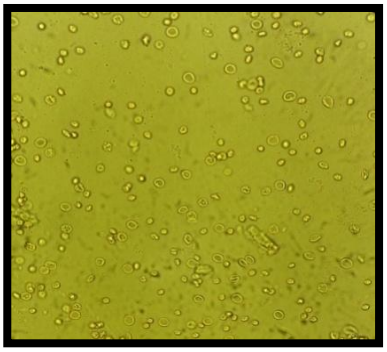
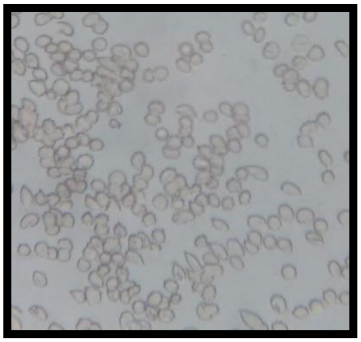
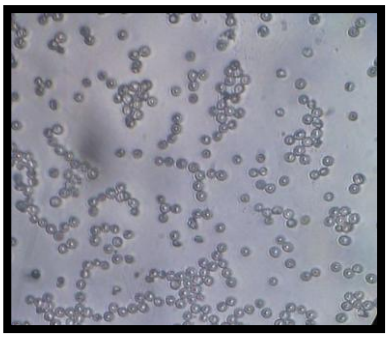
6.2.4 Cell morphology

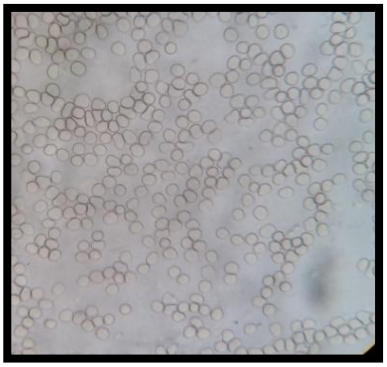
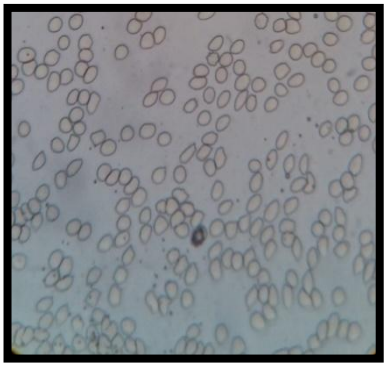
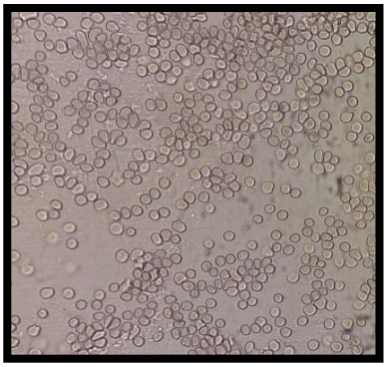
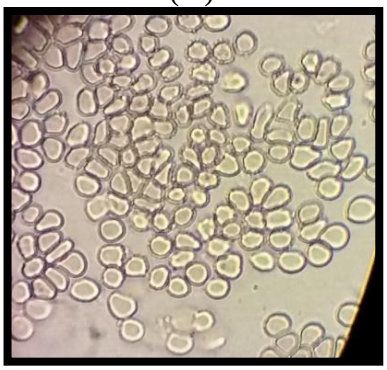
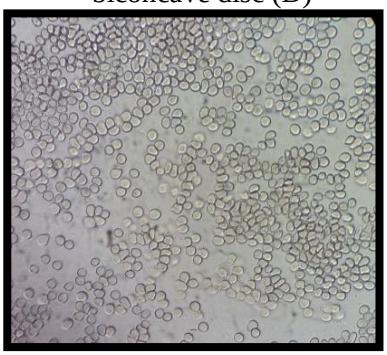
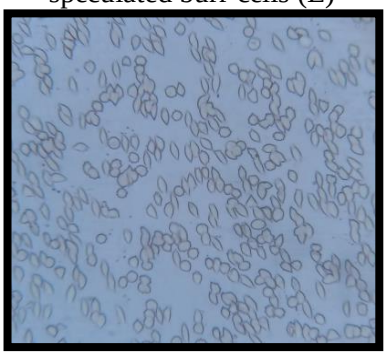
a) Microscopic images of RBCs on inverted microscope with 40X lens.

Sample	Day 14	Day 28
	HEXANE	
3%		
	Discocytes – biconcave disc (D)	Spherocytes (S)
6%		
	Discocytes – biconcave disc (D)	Spherocytes (S)
9%		
	Discocytes – biconcave disc (D)	Spherocytes (S)

Sample	Day 14	Day 28
	ACETONE	
3%		
	Discocytes – biconcave disc (D)	Spherocytes (S)
6%		
	Discocytes – biconcave disc (D)	Spherocytes (S)
9%		
	Discocytes – biconcave disc (D)	Discocytes – biconcave disc (D) and Spherocytes (S)

Sample	Day 14	Day 28
	60 % ETHANOL	
3%		
	Discocytes – biconcave disc (D) and Spherocytes (S)	Spherocytes (S)
6%		
	Spherocytes (S)	Elliptocytes or dumbbell shaped (El)
9%		
	Spherocytes (S)	Elliptocytes or dumbbell shaped (El)

Sample	Day 14	Day 28
	40 % ETHANOL	
3%		
	Spherocytes (S)	Spherocytes (S) and Echinocytes – speculated burr cells (E)
6%		
	Discocytes – biconcave disc (D) and Spherocytes (S)	Spherocytes (S) and few Elliptocytes or dumbbell shaped (El)
9%		
	Spherocytes (S)	Elliptocytes or dumbbell shaped (El)

Sample	Day 14	Day 28
	WATER	
3%		
	Spherocytes (S)	Spherocytes (S) and few Elliptocytes or dumbbell shaped (El)
6%		
	Spherocytes (S) and few Discocytes – biconcave disc (D)	Spherocytes (S) and Echinocytes – speculated burr cells (E)
9%		
	Spherocytes (S)	Elliptocytes or dumbbell shaped (El)

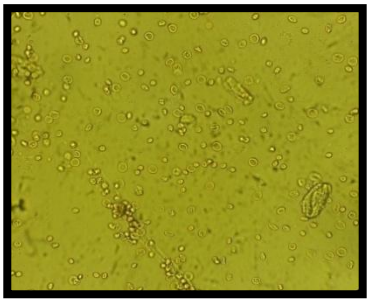
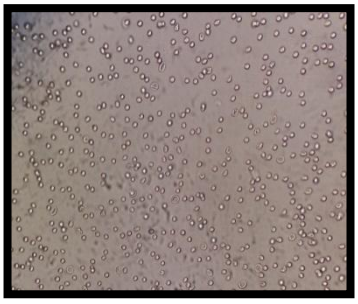
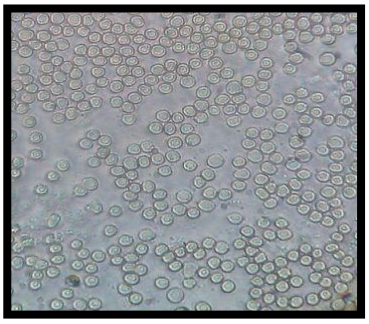
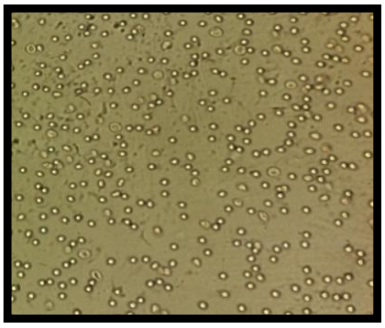
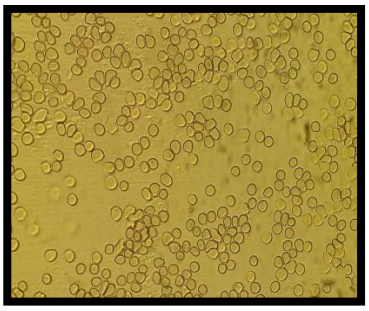
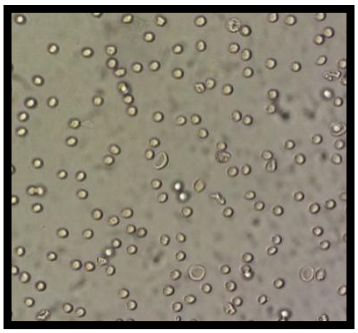
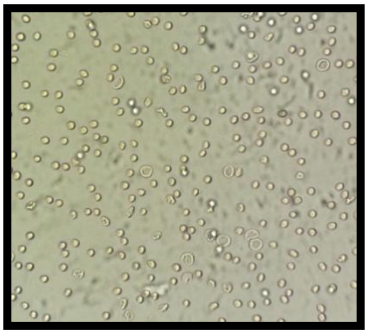
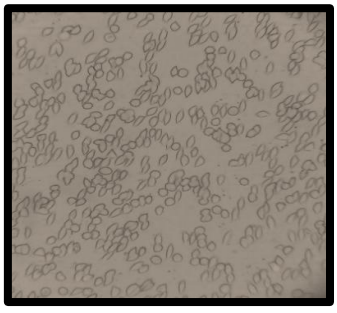
Sample	Day 14	Day 28
	FRESH	
3%		
	Discocytes – biconcave disc (D)	Spherocytes (S)
6%		
	Discocytes – biconcave disc (D)	Spherocytes (S)
9%		
	Discocytes – biconcave disc (D)	Spherocytes (S)
CONTROL		
	Spherocytes (S)	Elliptocytes or dumbbell shaped (El)

Fig. 28 Morphology of RBCs over the period of time

Morphology changes from discoid or biconcave shape to Echinocytes to spherocytes and finally to Elliptocytes but changes in shape are different in all extract. Morphological studies explained about the cell death.

b) SEM

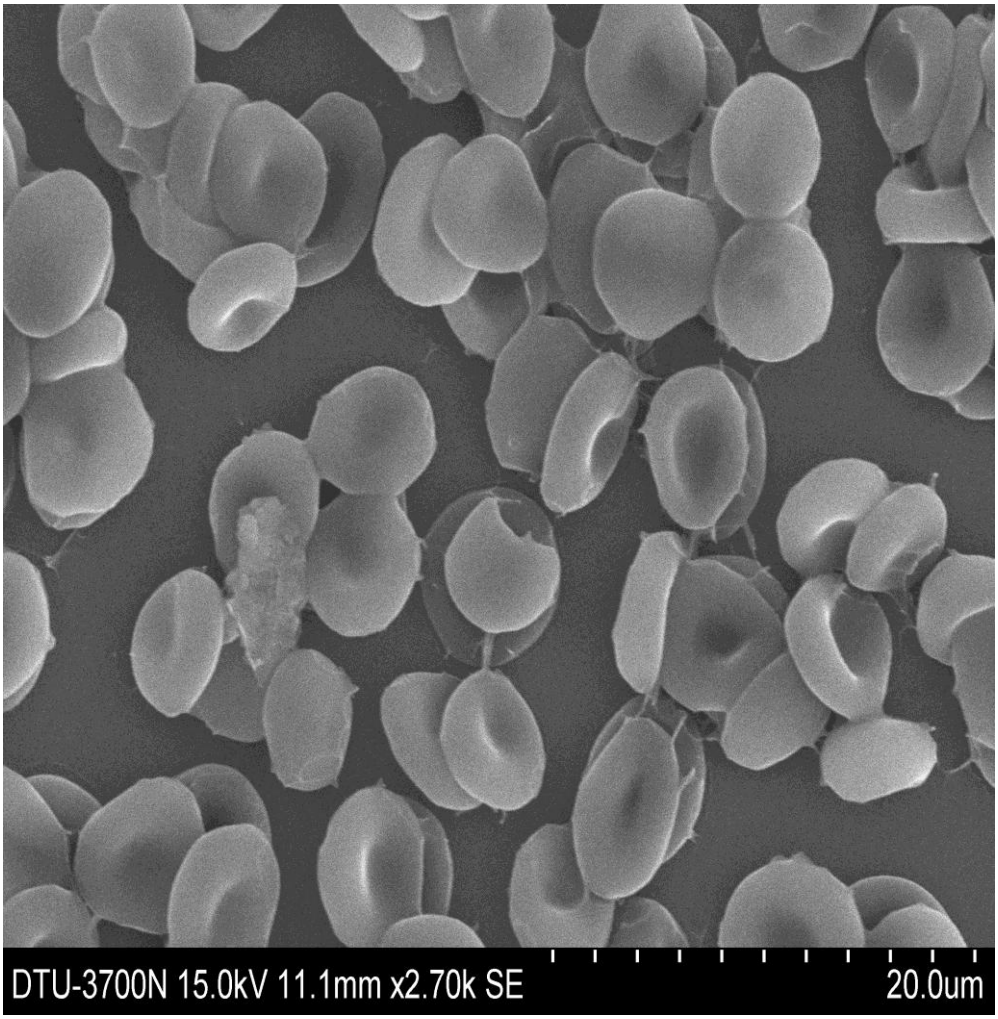
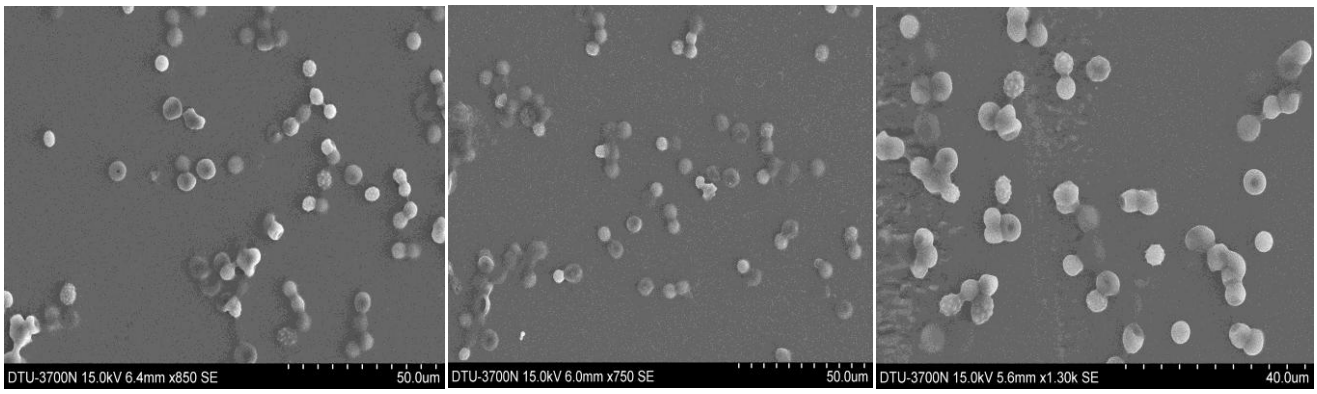


Fig. 29 SEM image of Fresh Blood Sample

Discocytes – biconcave disc (D)

Morphology after 6 weeks

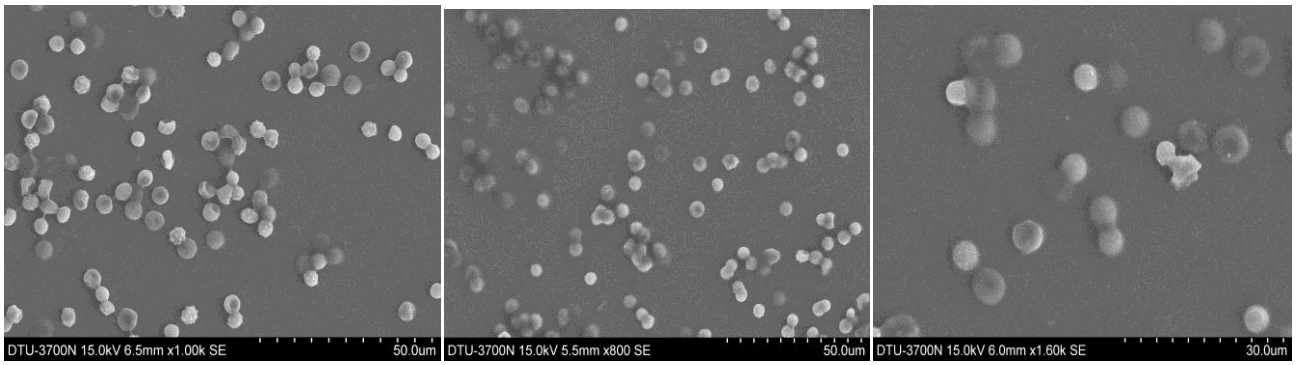
Hexane extract sample



3% 6% 9%

Discocytes – biconcave disc (D)**,** spherocytes (S) & Echinocytes – speculated burr cells (E)

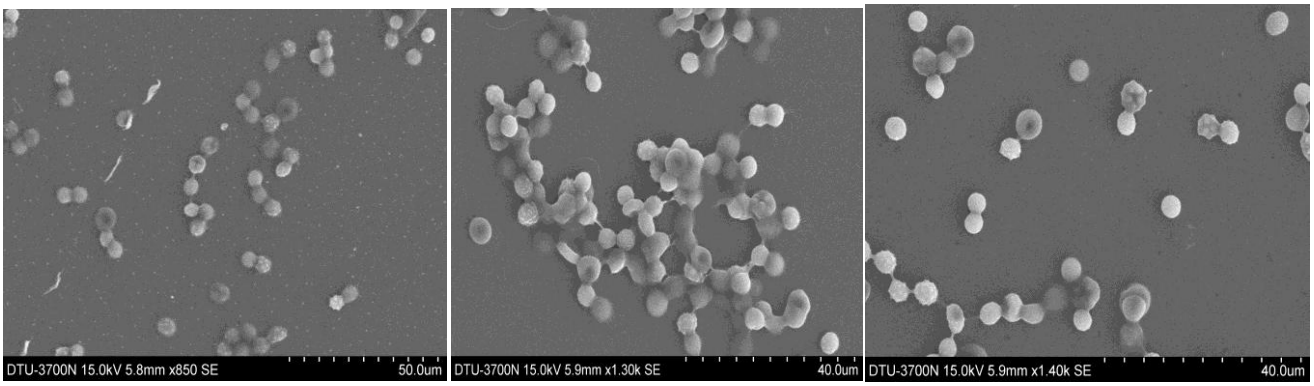
Acetone extract sample



3% 6% 9%

Discocytes – biconcave disc (D)**,** spherocytes (S) & Echinocytes – speculated burr cells (E)

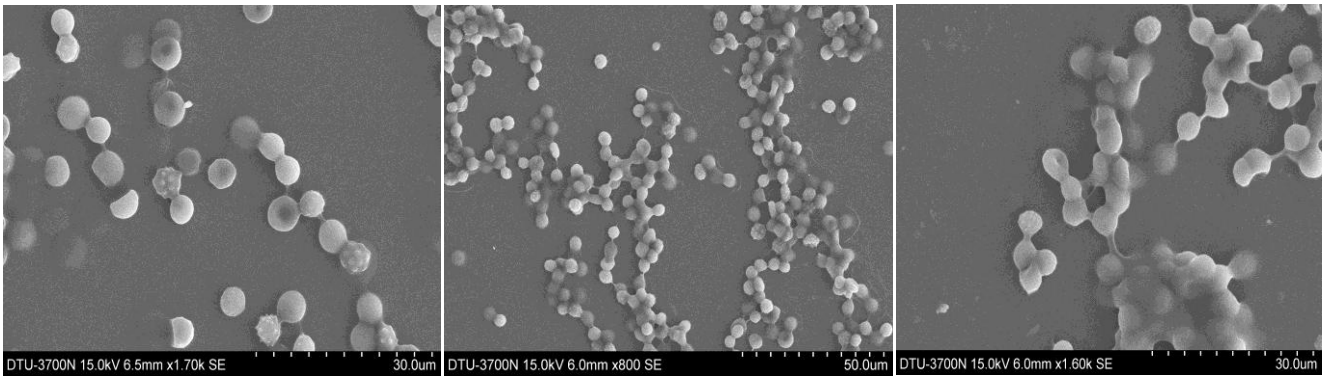
60% Ethanol extract sample



3% 6% 9%

Spherocytes (S) & Echinocytes – speculated burr cells (E)

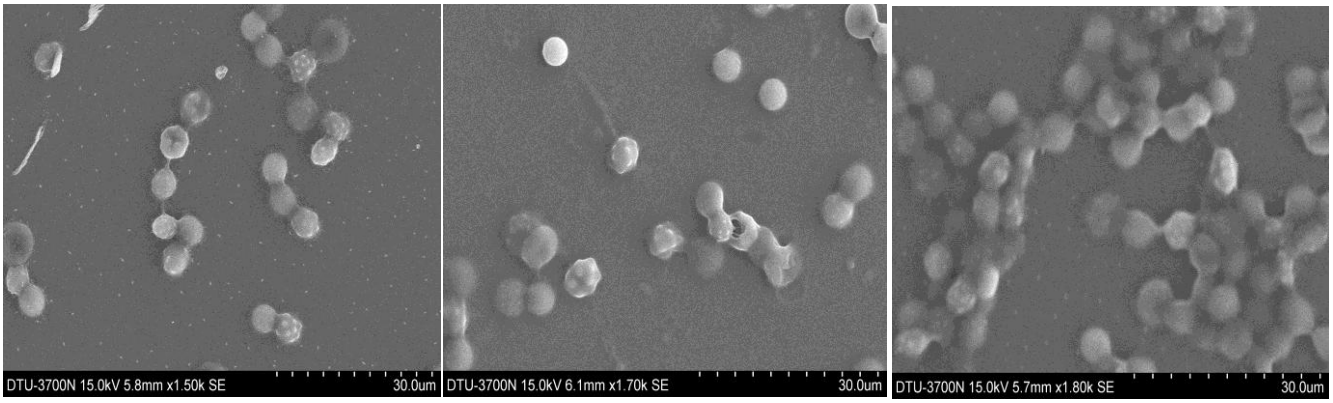
40% Ethanol extract sample



3% 6% 9%

Spherocytes (S) & Echinocytes – speculated burr cells (E)

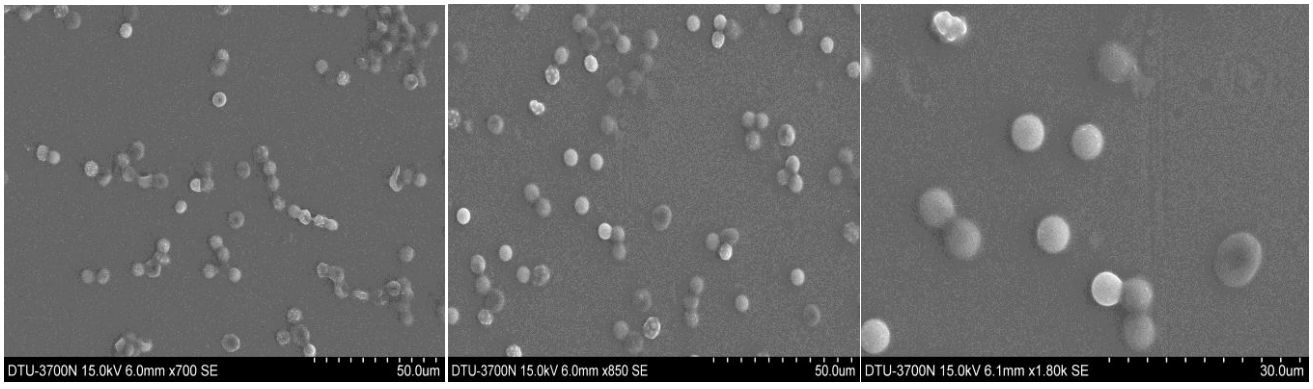
Water extract sample



3% 6% 9%

Echinocytes – speculated burr cells (E)

Fresh leaves extract sample



3% 6% 9%

Discocytes – biconcave disc (D)**,** spherocytes (S) & few Echinocytes – speculated burr cells (E)

Fig 30 SEM images of RBCs

6.3 PLATELET

6.3.1 pH

Sample	Day 1	Day 4	Day 7
Control	7.5	7.3	7.2
3% H	7.5	7.5	7.5
6% H	7.5	7.5	7.5
9% H	7.5	7.5	7.5
3% A	7.5	7.4	7.4
6% A	7.5	7.5	7.4
9% A	7.5	7.4	7.4
3% 60% E	7.5	7.3	7.2
6% 60% E	7.5	7.3	7.2
9% 60% E	7.5	7.4	7.2
3% 40% E	7.5	7.3	7.1
6% 40% E	7.5	7.3	7.1
9% 40% E	7.5	7.3	7.1
3% H_2O	7.5	7.4	7.1
6% H_2O	7.5	7.4	7.1
9% H_2O	7.5	7.4	7.1
3% F	7.5	7.4	7.1
6% F	7.5	7.4	7.1
9% F	7.5	7.4	7.1

Table 18 Change in pH of Platelet during storage period

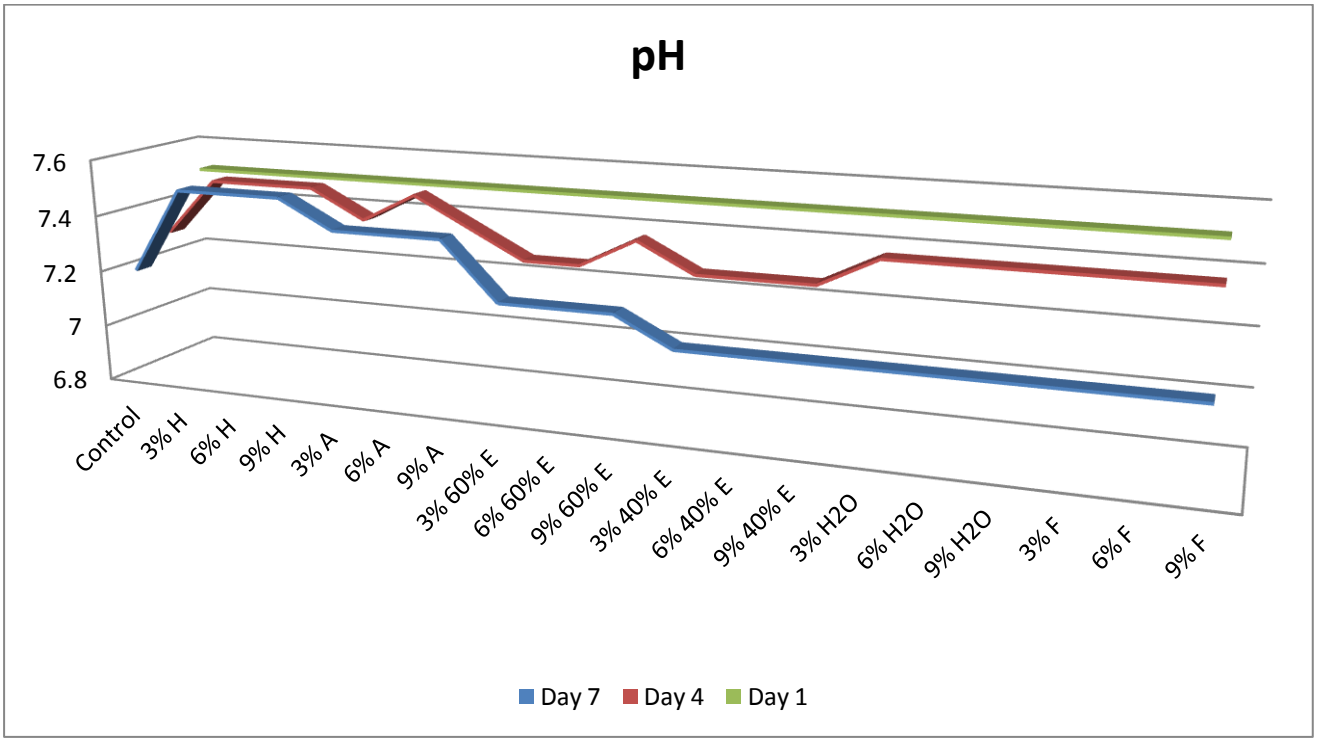


Fig. 31 Comparative analysis of pH amongst platelet samples stored in papaya leaf extracts of different solvent systems namely, Hexane (H), Acetone (A), 60% Ethanol (60% E), 40% Ethanol (40% E), Water (H_2O), Fresh (F) and Control. The graph is plotted against platelet stored in various extracts and pH on day 1, 4, and 7.

6.3.2 Clotting factor time

Sample	Day 1 (sec)	Day 4 (sec)	Day 7 (sec)
Control	82	293	420
3% H	82	147	203
6% H	82	151	225
9% H	82	145	202
3% A	82	149	199
6% A	82	156	207
9% A	82	155	210
3% 60% E	82	165	225
6% 60% E	82	169	203
9% 60% E	82	161	220
3% 40% E	82	180	243
6% 40% E	82	164	236
9% 40% E	82	178	240
3% H_2O	82	165	230
6% H_2O	82	168	230
9% H_2O	82	168	232
3% F	82	170	232
6% F	82	169	240
9% F	82	173	246

Table 19 Clotting factor time

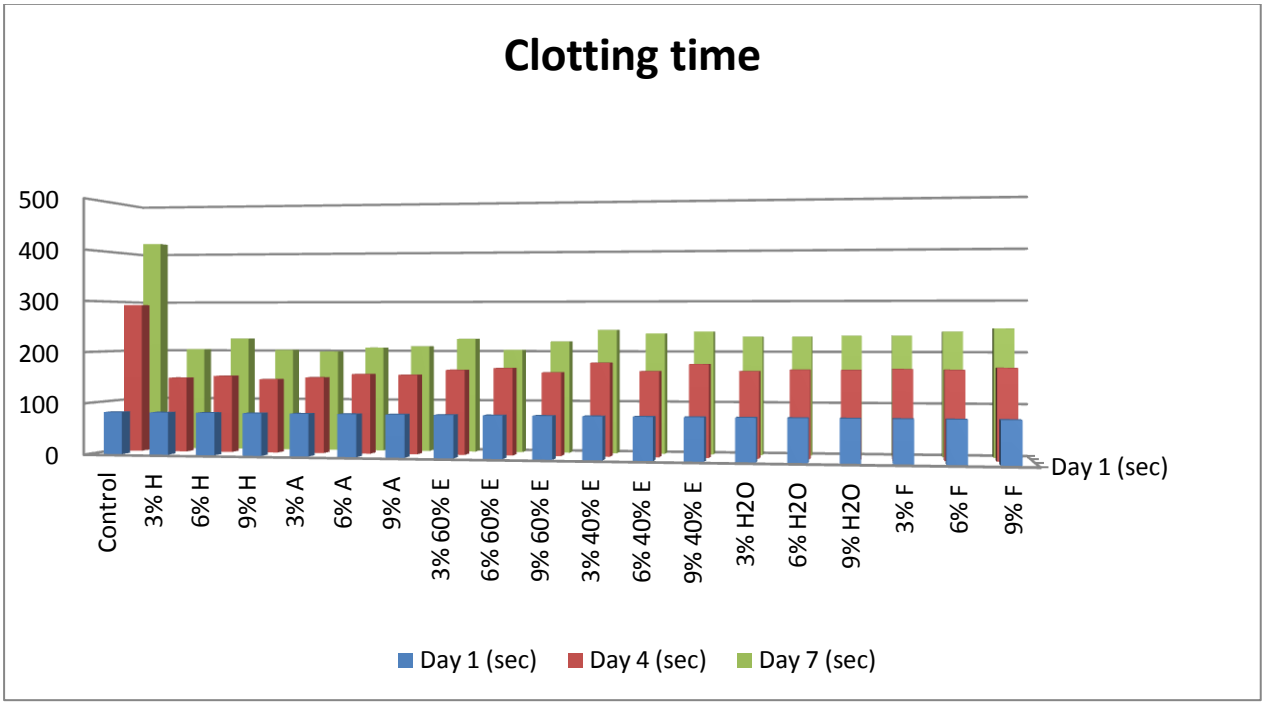


Fig. 32 Comparative analysis of Clotting time of Platelet stored in papaya leaf extract in different solvent systems namely, Hexane (H), Acetone (A), 60% Ethanol (60% E), 40% Ethanol (40% E), Water (H_2O), Fresh (F) and Control. The graph is plotted against platelet stored in various extracts and time (sec) on day 1, 4, and 7.

6.3.3 Glucose

Sample	Day 1	Day 4	Day 7
Control	294	217	201
3% H	294	267	248
6% H	294	210	197
9% H	294	285	263
3% A	294	252	237
6% A	294	216	213
9% A	294	247	225
3% 60% E	294	275	252
6% 60% E	294	245	226
9% 60% E	294	252	239
3% 40% E	294	238	217
6% 40% E	294	215	198
9% 40% E	294	212	193
3% H_2O	294	230	215
6% H_2O	294	226	204
9% H_2O	294	224	201
3% F	294	258	241
6% F	294	245	227
9% F	294	156	135

Table 20 Glucose concentration

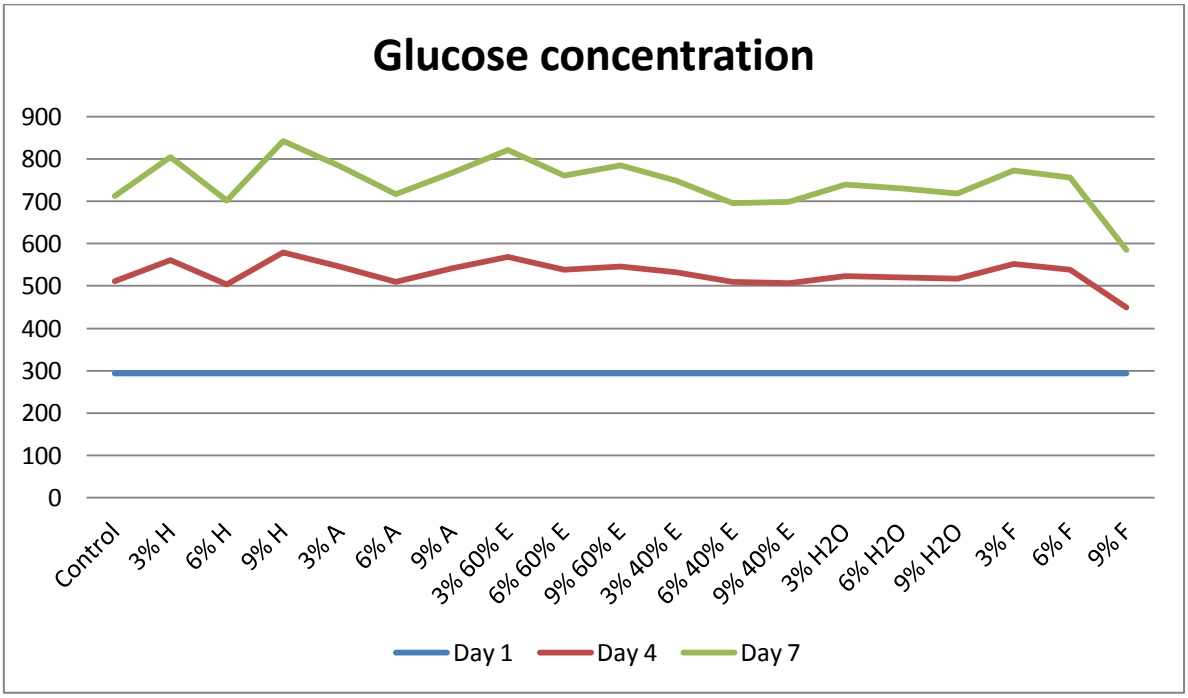


Fig. 33 Comparative analysis of glucose concentration of Platelet stored in papaya leaf extract in different solvent systems namely, Hexane (H), Acetone (A), 60% Ethanol (60% E), 40% Ethanol (40% E), Water (H_2O), Fresh (F) and Control. The graph is plotted against platelet stored in various extracts and glucose concentration on day 1, 4, and 7.

6.3.4 MTT assay

Sample	Day 4	Day 7	Relative viability %
Control	0.002	0.001	50
3% H	0.009	0.006	67
6% H	0.006	0.004	67
9% H	0.008	0.006	75
3% A	0.021	0.015	71
6% A	0.015	0.008	53
9% A	0.004	0.002	50
3% 60% E	0.004	0.002	50
6% 60% E	0.015	0.007	47
9% 60% E	0.021	0.011	52
3% 40% E	0.02	0.004	20
6% 40% E	0.003	0.001	33
9% 40% E	0.003	0.001	33
3% H_2O	0.006	0.002	33
6% H_2O	0.003	0.002	67
9% H_2O	0.003	0.002	67
3% F	0.002	0.001	50
6% F	0.004	0.003	75
9% F	0.005	0.001	20

Table 21 MTT assay for cell viability

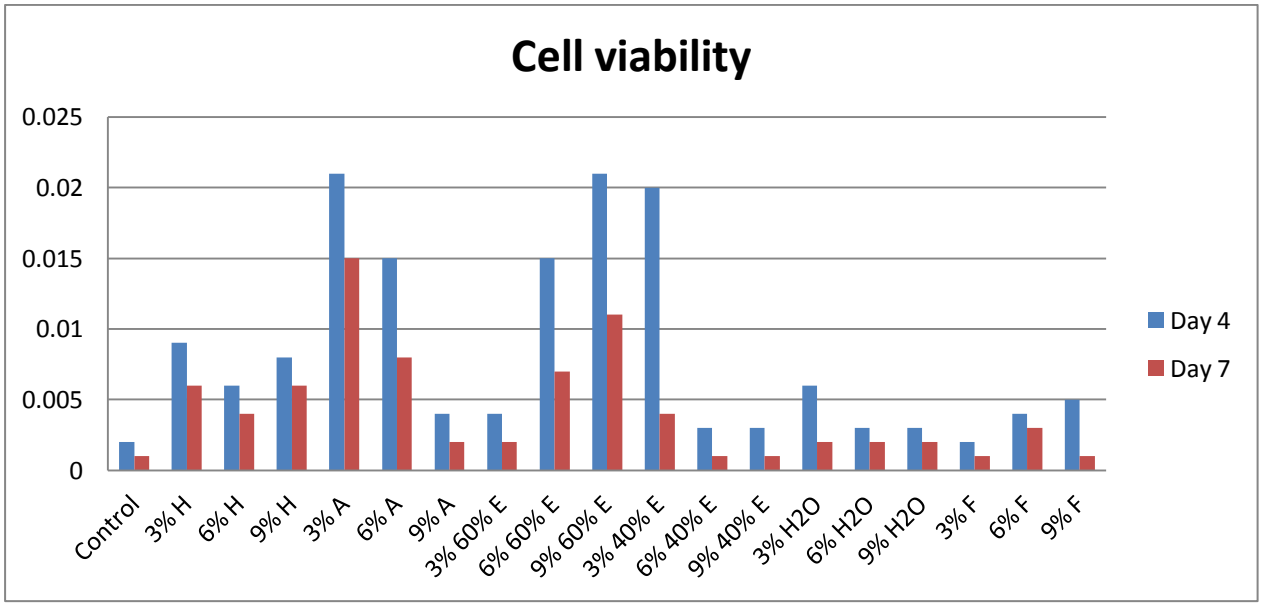


Fig. 34 Comparative analysis of cell viability of Platelet stored in papaya leaf extract in different solvent systems namely, Hexane (H), Acetone (A), 60% Ethanol (60% E), 40% Ethanol (40% E), Water (H_2O), Fresh (F) and Control on day 4 and 7. The graph is plotted against platelet stored in various extracts and absorbance on day 4, and 7.

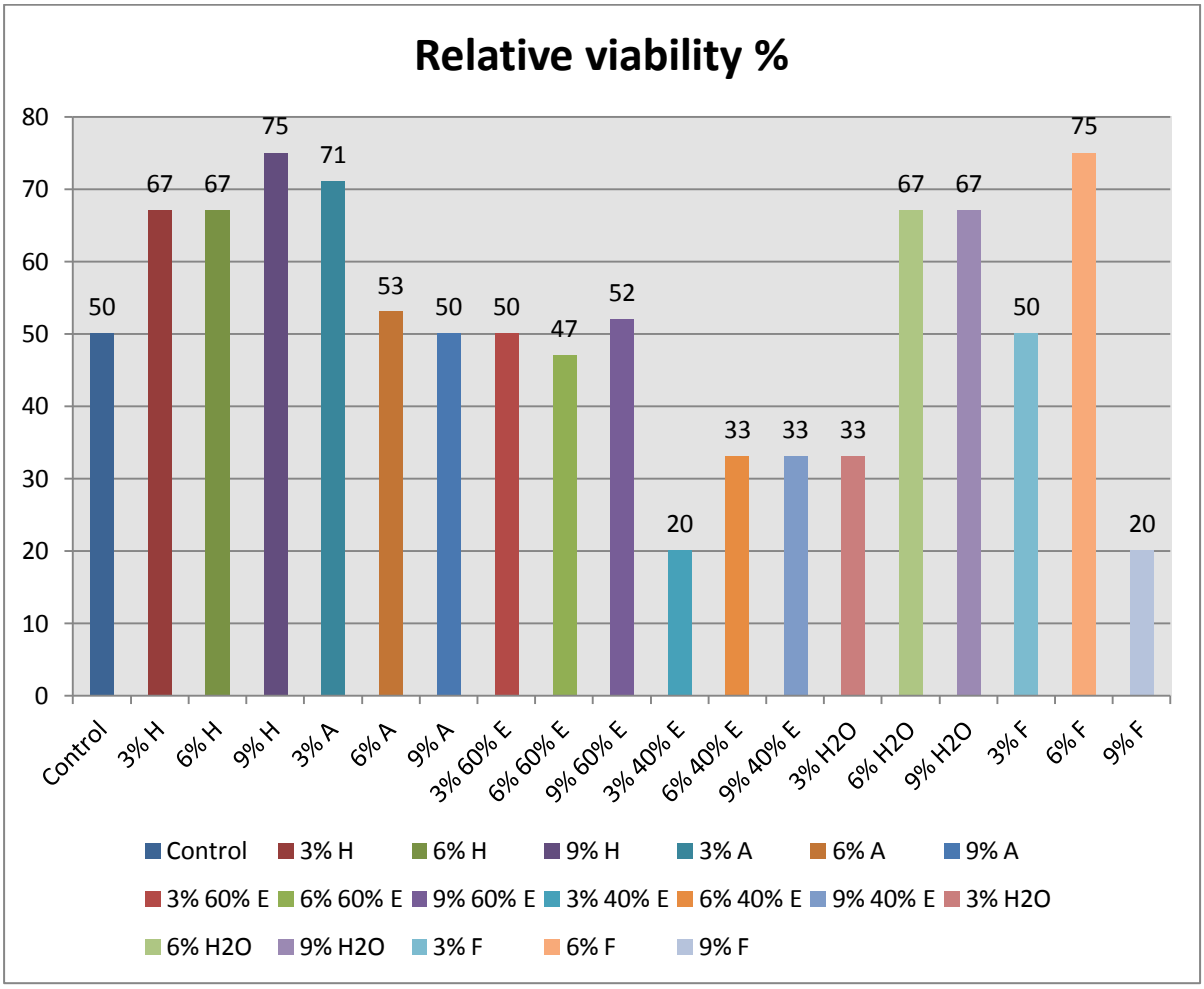


Fig. 35 Relative viability of Platelet stored in papaya leaf extract in different solvent systems namely, Hexane (H), Acetone (A), 60% Ethanol (60% E), 40% Ethanol (40% E), Water (H_2O), Fresh (F) and Control. The graph is plotted against platelet stored in various extracts and relative viability w.r.t day 4 and 7.

6.3.5 Bacterial contamination

1. Using nutrient broth (Day 4)

Sample	Absorbance
Control	0.664
Control + Antibiotic	0.130
3% H	0.114
6% H	0.236
9% H	0.087
3% A	0.119
6% A	0.138
9% A	0.082
3% 60% E	0.352
6% 60% E	0.467
9% 60% E	0.513
3% 40% E	0.478
6% 40% E	0.224
9% 40% E	0.358
3% H_2O	0.363
6% H_2O	0.370
9% H_2O	0.362
3% F	0.300
6% F	0.347
9% F	0.470

Table 22 Bacterial contamination using nutrient broth

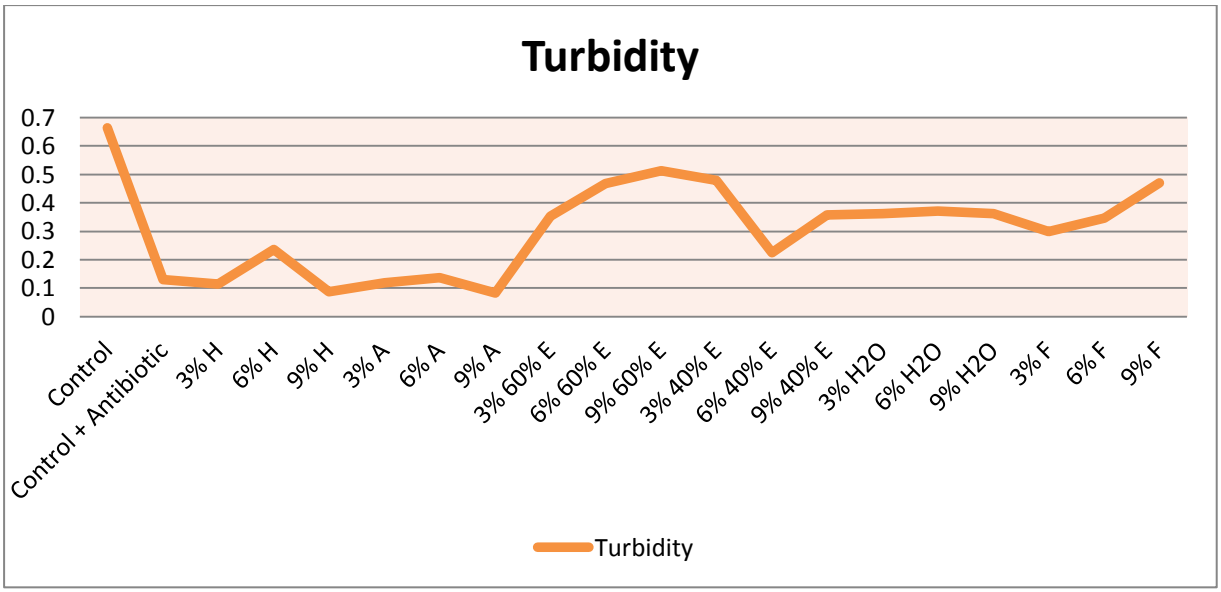
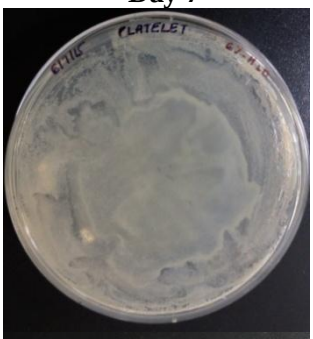
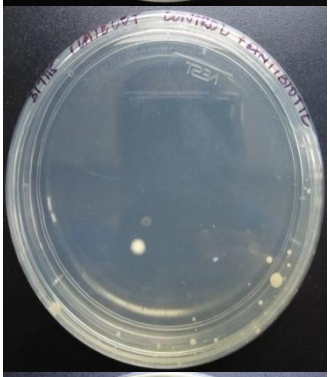
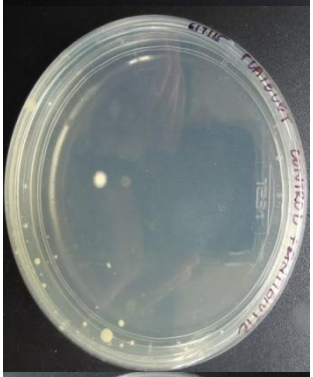
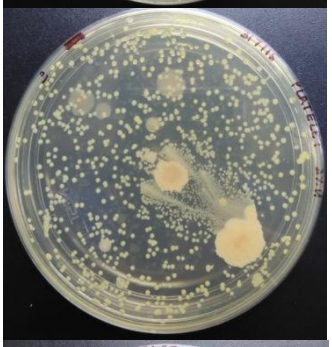
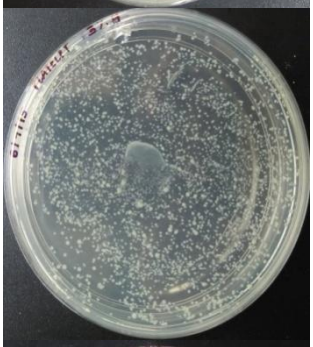
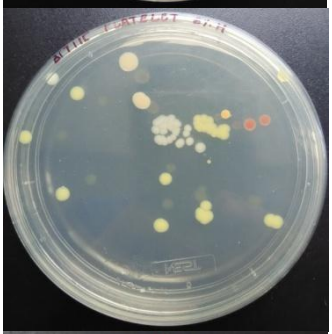
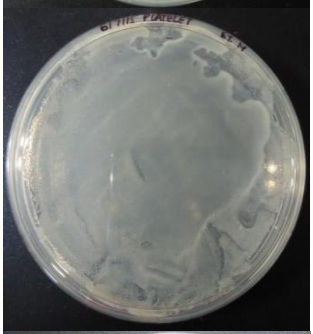
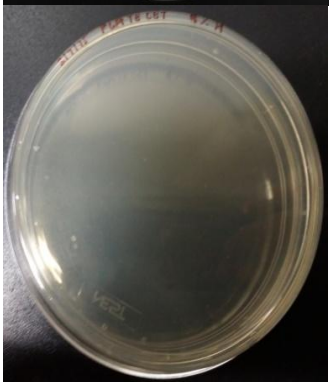
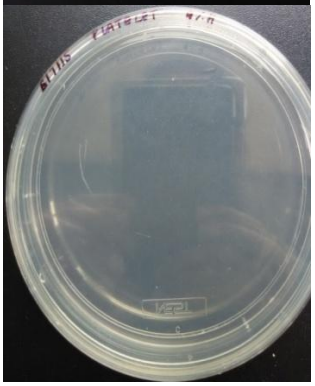


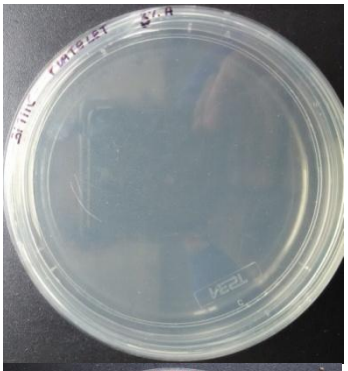
Fig. 36 The graph is plotted against platelet stored in various extracts and absorbance on day 4 for bacterial contamination. Absorbance was taken at 560 nm. Turbidity was checked in nutrient broth after addition of the platelet sample stored in papaya leaf extract in different solvent systems namely, Hexane (H), Acetone (A), 60% Ethanol (60% E), 40% Ethanol (40% E), Water (H_2O), Fresh (F), Control and control + antibiotic on day 4.

2. Bacterial contamination

Sample	Presence		Morphology	
	Day 4	Day 7	Day 4	Day 7
Control	+	+		
Control & Antibiotic	+	+		
3% H	+	+		
6% H	+	+		
9% H	-	-		

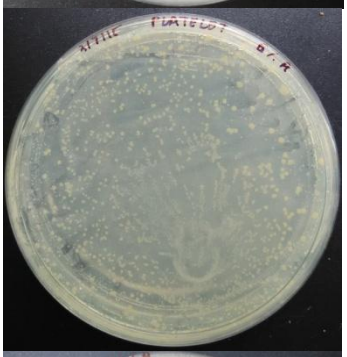
3% A

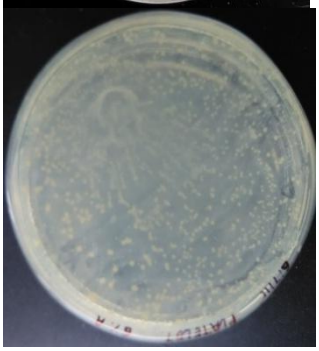
\- +



6% A

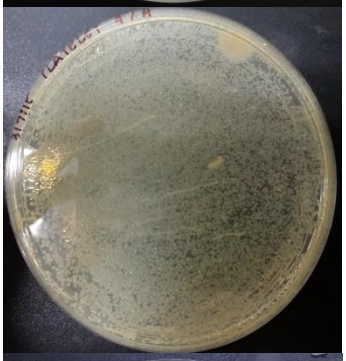
\+ +

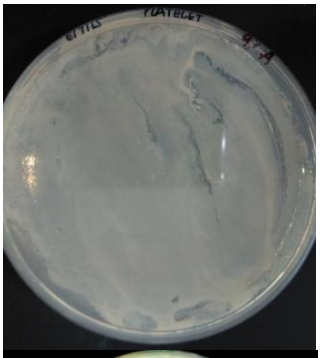




9% A

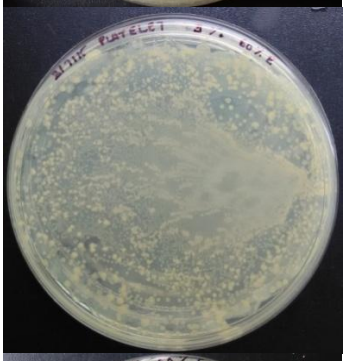
\+ +

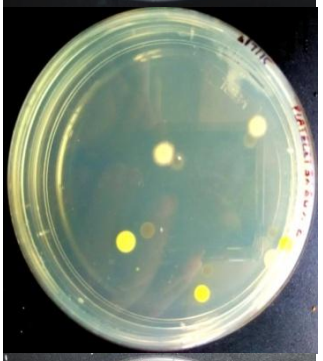




3% 60% E

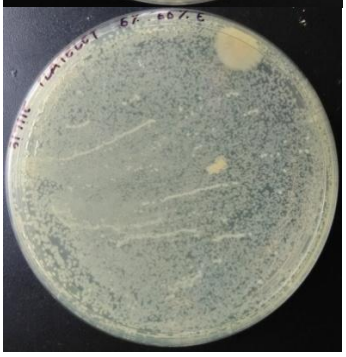
\+ +

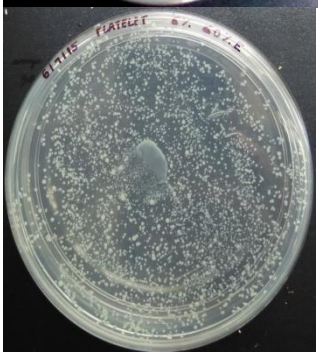


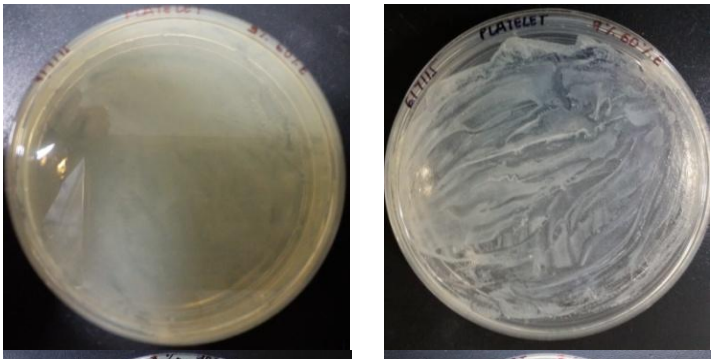
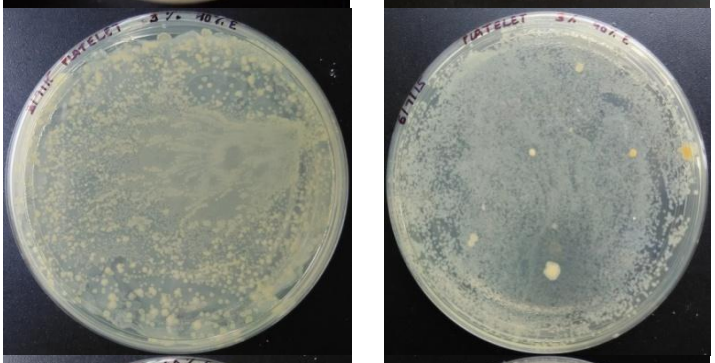
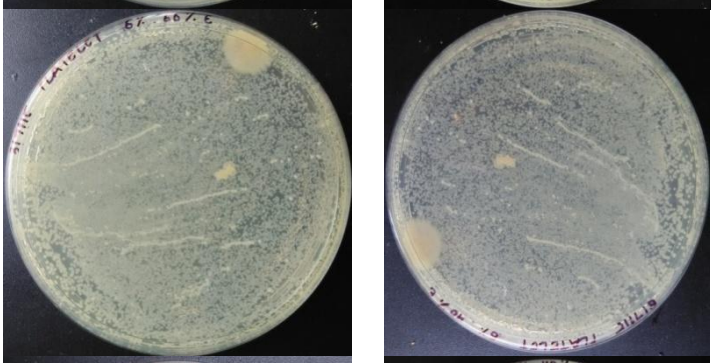
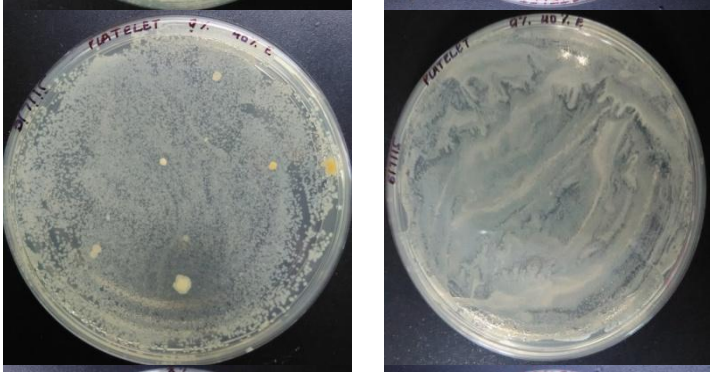
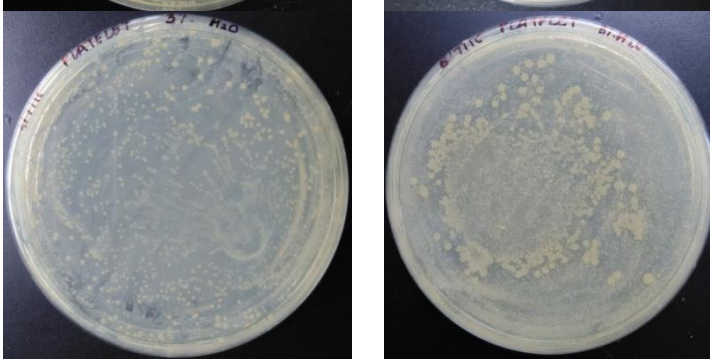


6% 60% E

\+ +





9% 60% E	-	+	
3% 40% E	+	+	
6% 40% E	+	+	
9% 40% E	+	+	
3% H_2O	+	+	

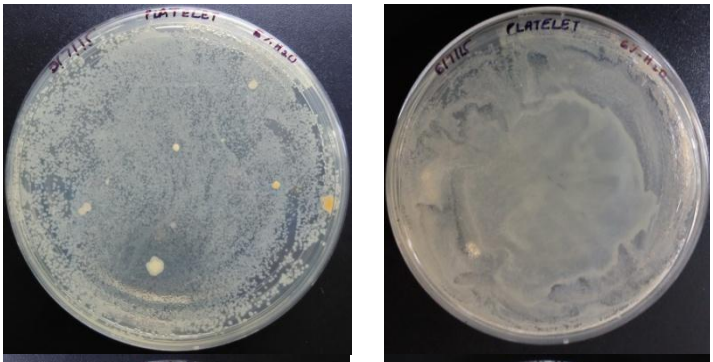
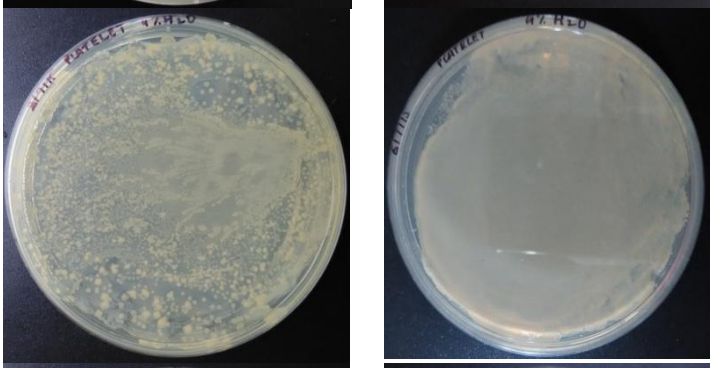
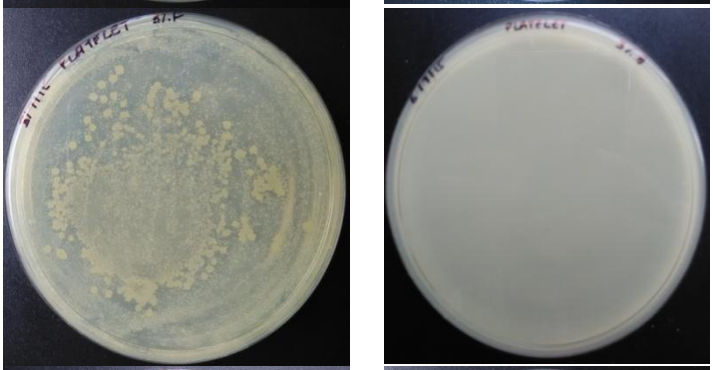
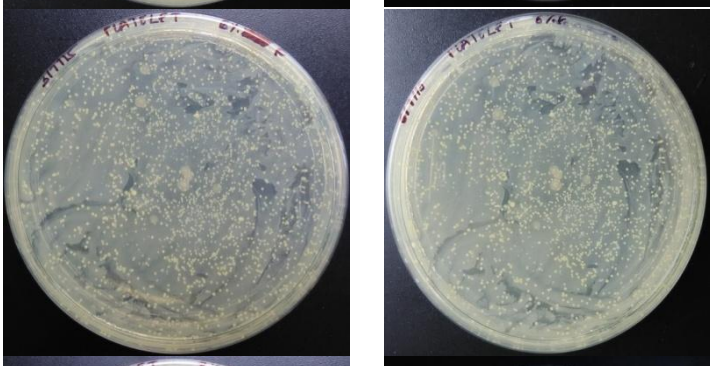
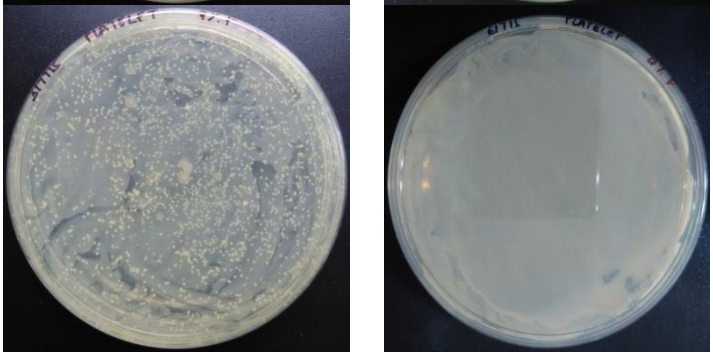
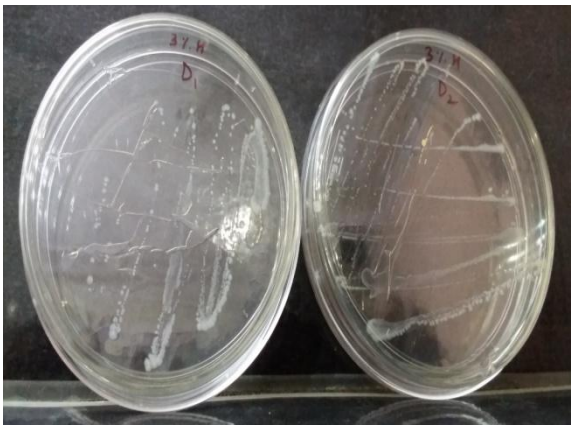
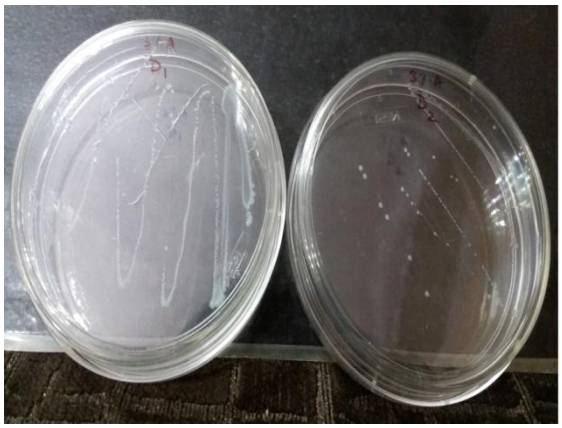
6% H_2O	+	+	
9% H_2O	+	+	
3% F	+	+	
6% F	+	+	
9% F	+	+	

Fig. 37 To check bacterial contamination in platelet samples

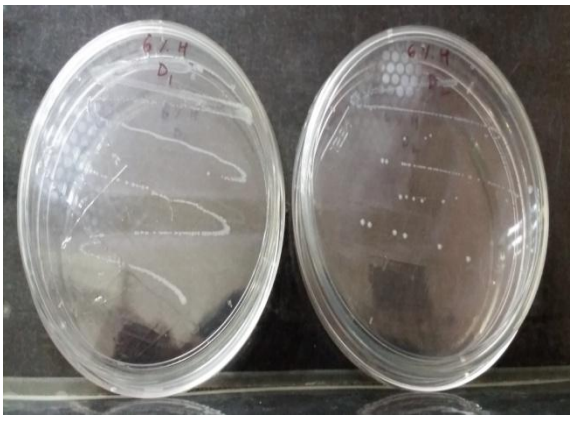
3. Confirmatory test for Bacterial contamination

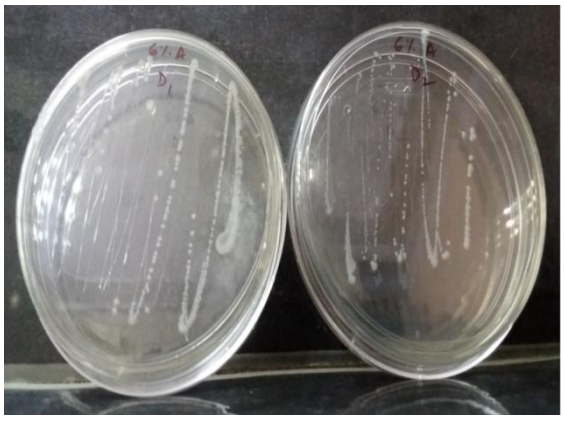
Hexane Acetone



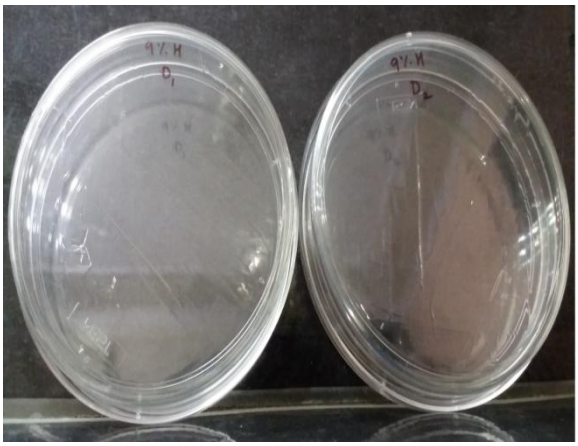


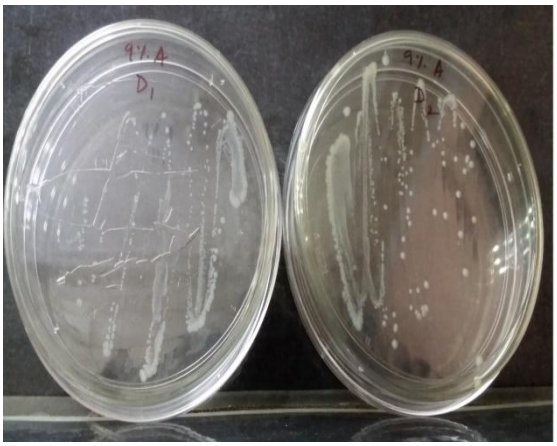
3%





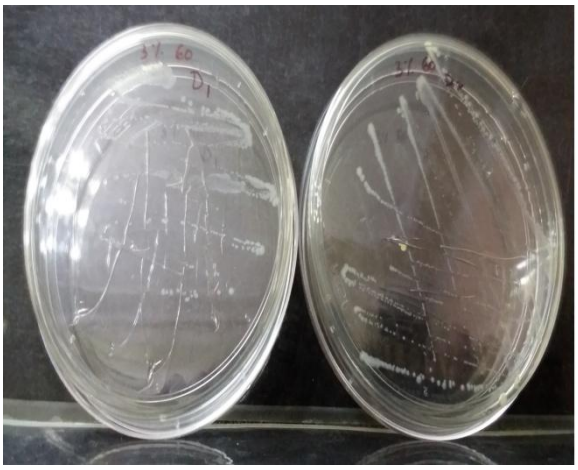
6%



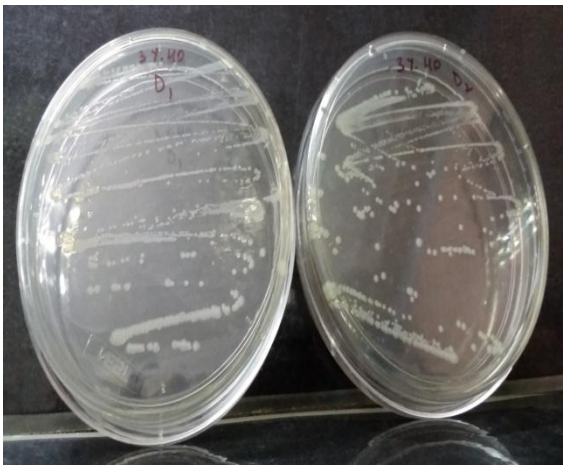


9%

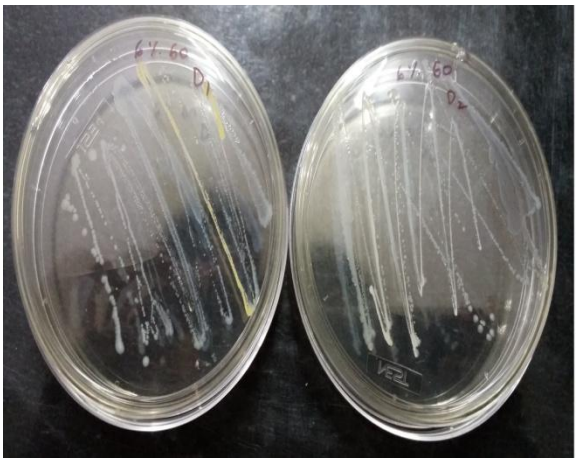
60% Ethanol

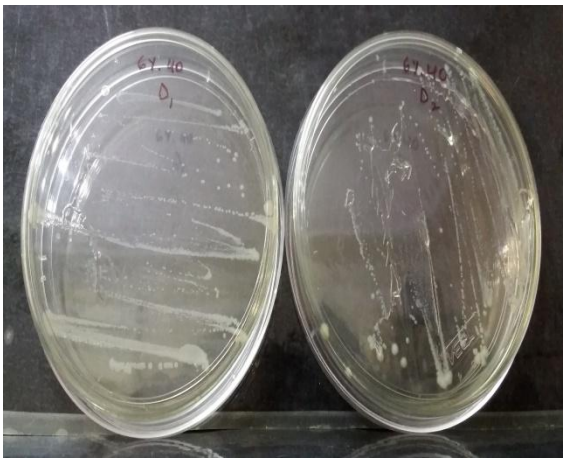


40% Ethanol

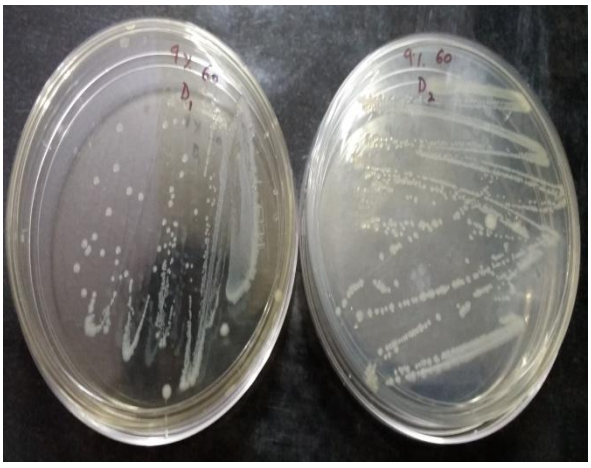


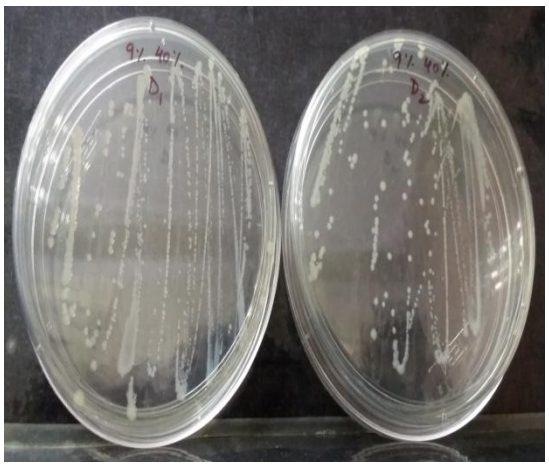
3%





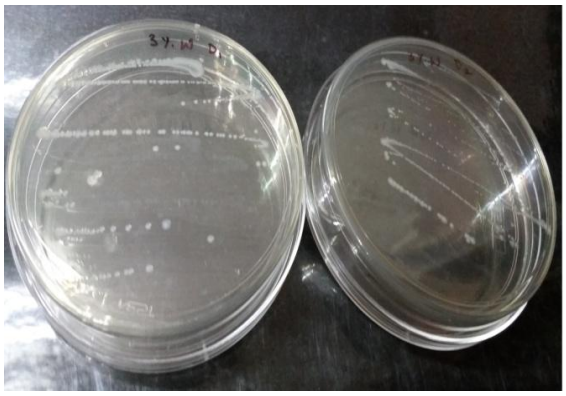
6%



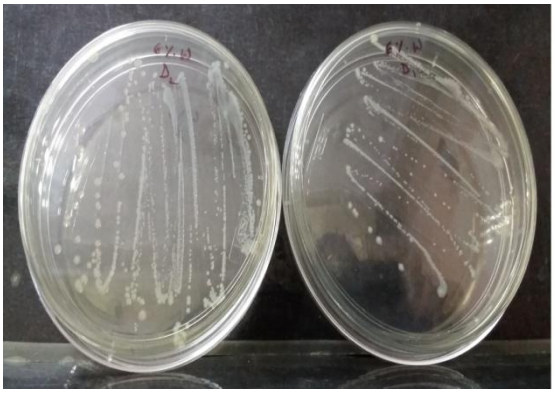


9%

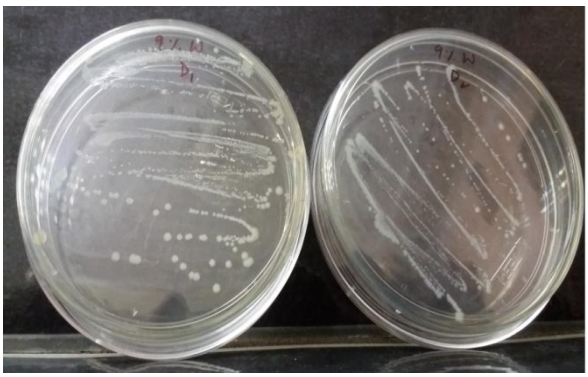
Water



3%

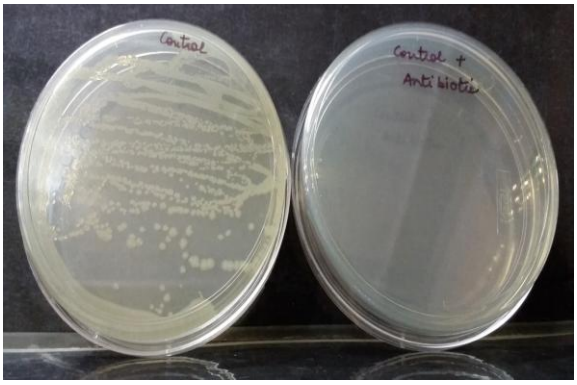


6%



9%

Control

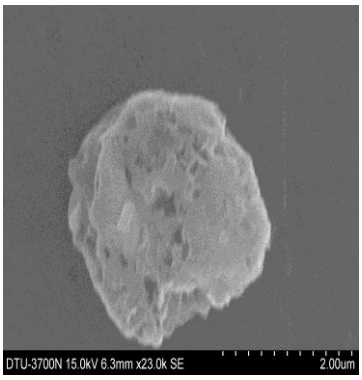


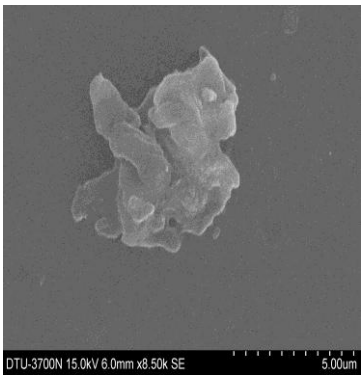
Control vs. Control + Antibiotic

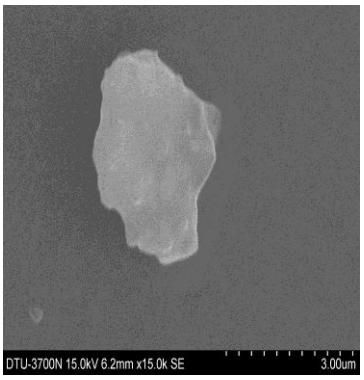
Fig.38 Confirmatory test for Bacterial contamination. These plates contain Luria Broth agar along with papaya leaf extracts in different solvent systems namely, Hexane, Acetone, 60% Ethanol, 40% Ethanol, Water in different concentrations i.e. 3%, 6%, 9% and the presence or absence of bacterial growth was checked by streaking the plates with *E.coli* DH5α strain. It's a confirmatory test for the experiment of bacterial contamination performed on stored platelets. 9% Hexane showed maximum anti-bacterial activity with no microbial growth.

6.3.6 Morphology after 5 days

Hexane extract sample

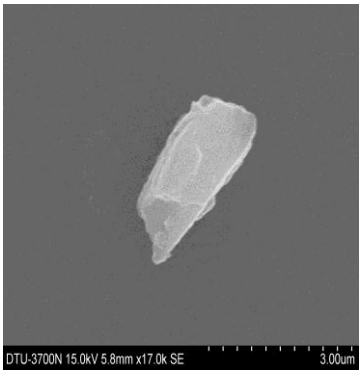


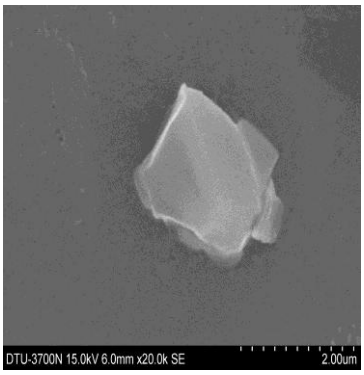


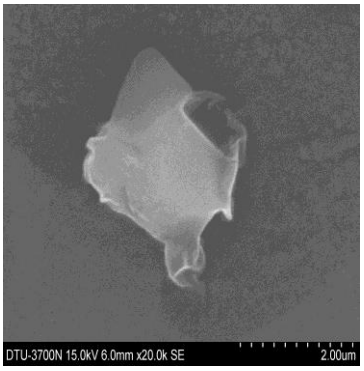


3 %	6%	9%
Balloon	Dendrites	Platelet with smooth contours

Acetone extract sample

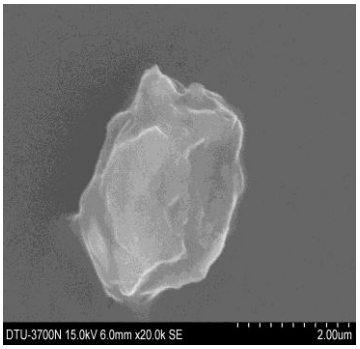


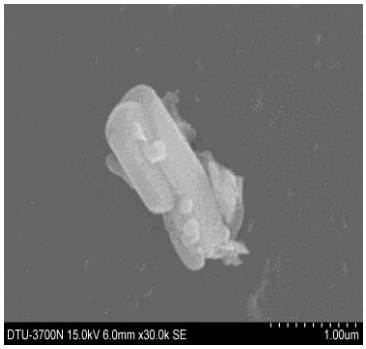


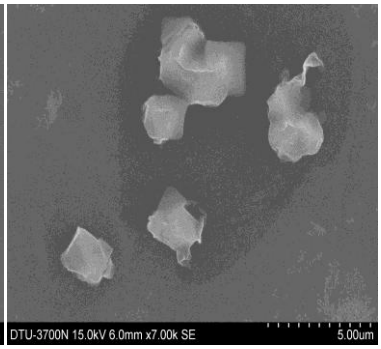


3%	6%	9%
Platelet with smooth contours	Platelet with smooth contours	Platelet with smooth contours

60% Ethanol extract sample

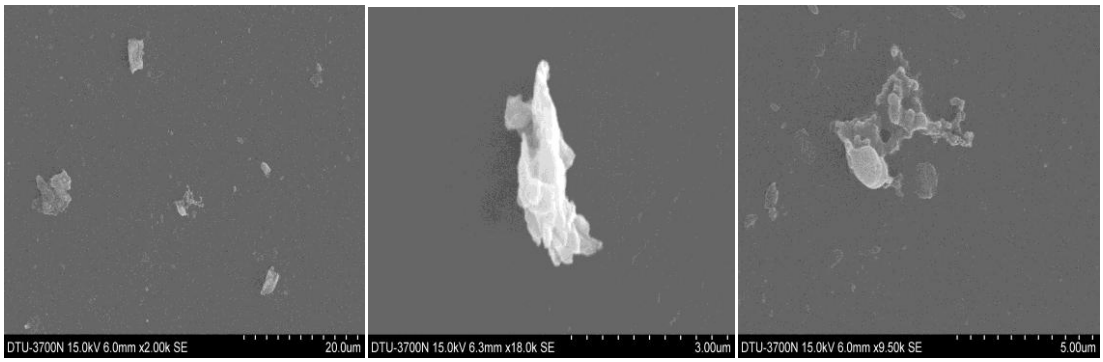






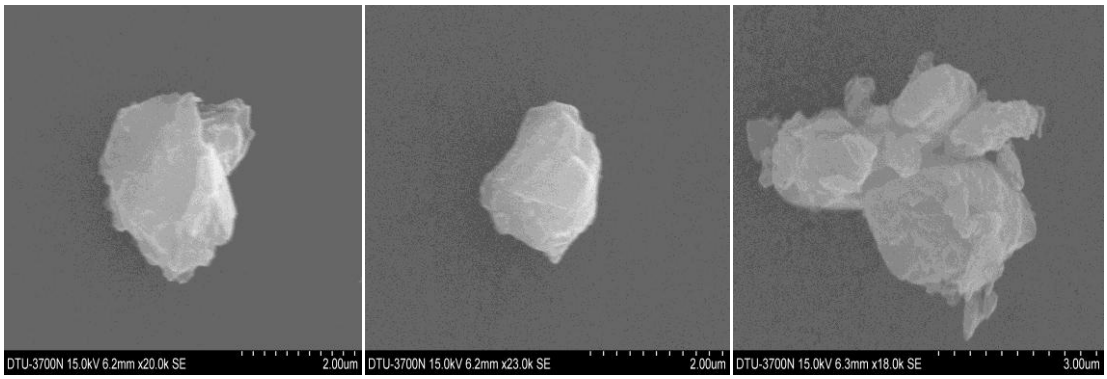
3%	6%	9%
Balloon	Balloon	Dendritic

40% Ethanol extract sample



3%	6%	9%
Dendritic	Dendritic	Dendritic

Water extract sample



3%	6%	9%
Balloon	Balloon	Ballon

Fig: Morphology study of platelets at Day 5 using SEM

Fig. 39 Morphology study of platelets at Day 5 using SEM Platelets with smooth contours — discs and spheres retaining normal size. Dendrites — Platelets that have developed pseudopodia or dendritic processes. Balloons — Platelets that have undergone swelling after losing the capacity to maintain an osmotic gradient across their membrane, clumps of multiple Platelets and could not be counted individually. (Jain A. *et al*, 2015)

6.4 TPO results

Library formation- Peptide mimetic predication

Peptide sequences were predicted on the basis of binding site of TPO and TMP on TPOR. First protein is prepared and docking is allowed. Binding sites were identified and random sequences from the binding sites and based on literature survey were predicted.

Receptor Preparation:

TPOR BLAST result

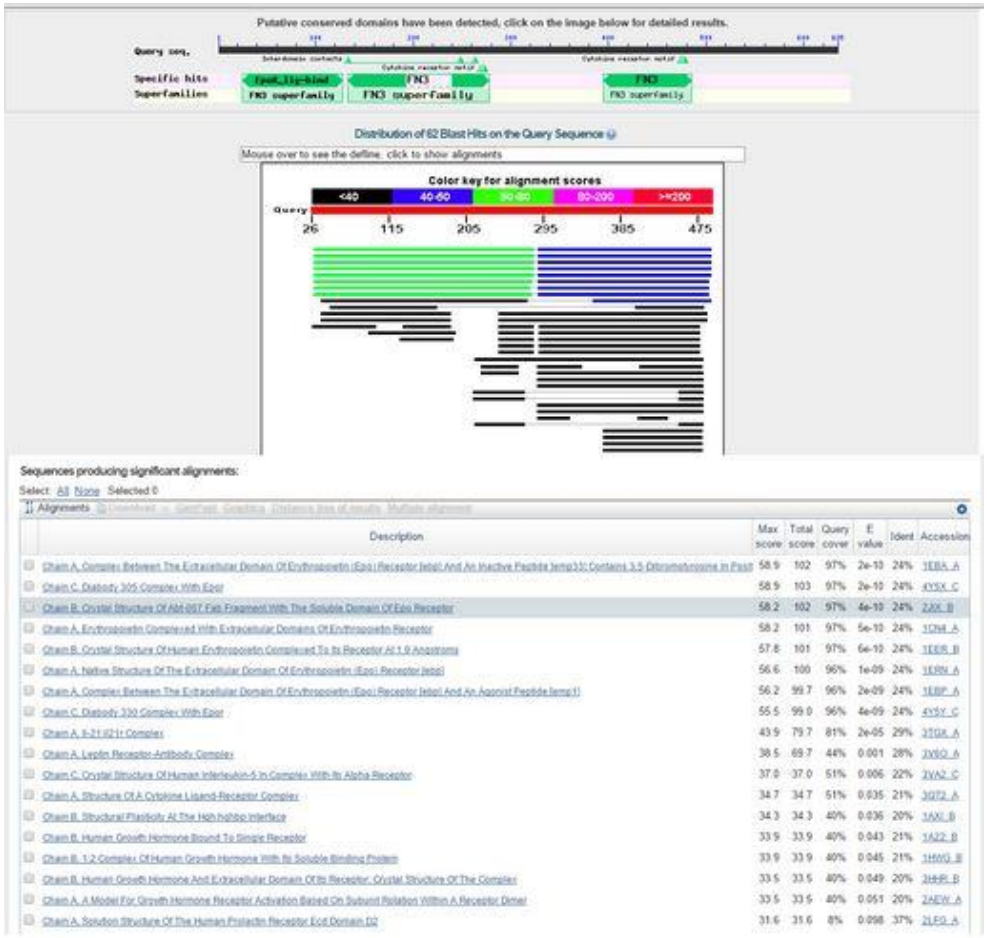


Fig. 40 TPOR BLAST result from NCBI, the result shows a maximum of 24% identity with EPO.

TPOR 3D structure Analysis- TPO-TPOR complex was imported to Maestro and it was found that TPOR binding to TPO with two different sites. TPOR is having two similar chains in pairs A, B and C, D chain binding to TPO. Receptor ligand binding critically depends on the orientation of interaction. Both pairs of chains of TPOR have similar amino acid sequences so for docking analysis duplicated chain C & D is removed from the receptor protein. Protein is prepared using Prep wizard by importing receptor file and minimizing and optimizing the receptor protein.

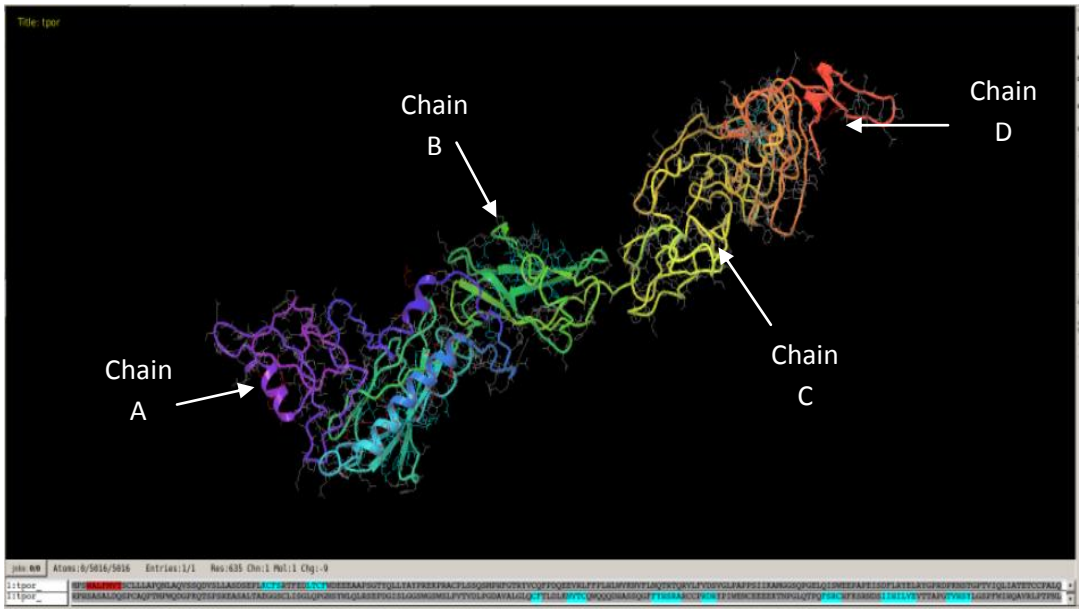


Fig. 41 3D sturucture of TPOR

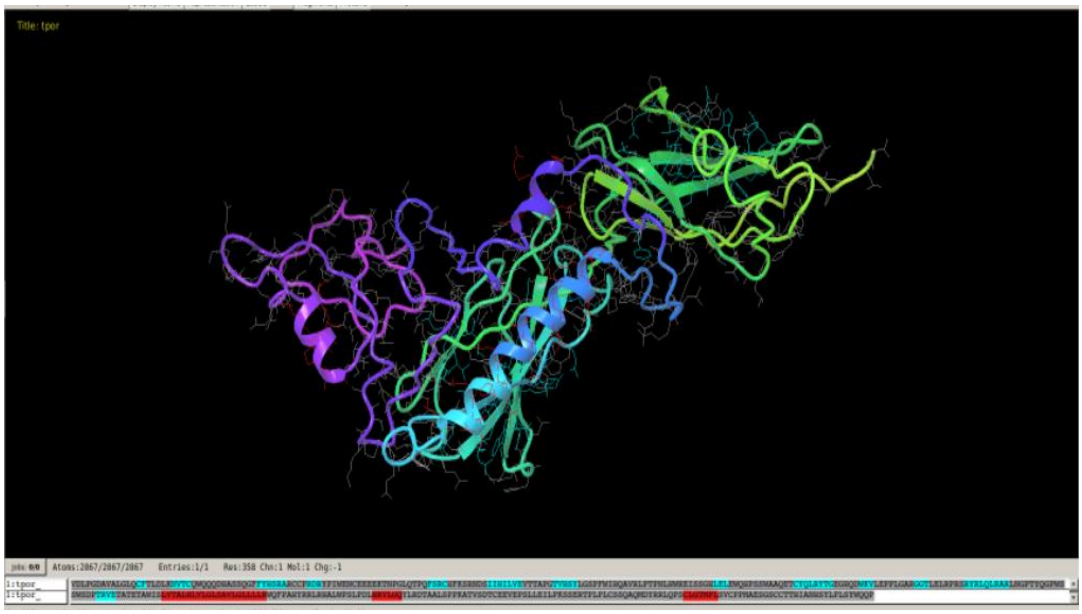


Fig.42 3D structure of TPOR, representing chains A & B. (after removing duplicate chains C & D and water molecules)

Binding Site Identification:

PDB complex of TPO and TPOR was retrieved from Hex 8.0.0. The complex is analyzed in Maestro work space for identifying the interacting residues responsible for binding. TPOR interact with TPO with two different sites 1 and 2. First site of TPOR hydrophobic in nature mainly due to Phe141 which are responsible for non polar interaction, its side chain also consisted of hydrophilic amino acids also involved in the interaction with the ligand.

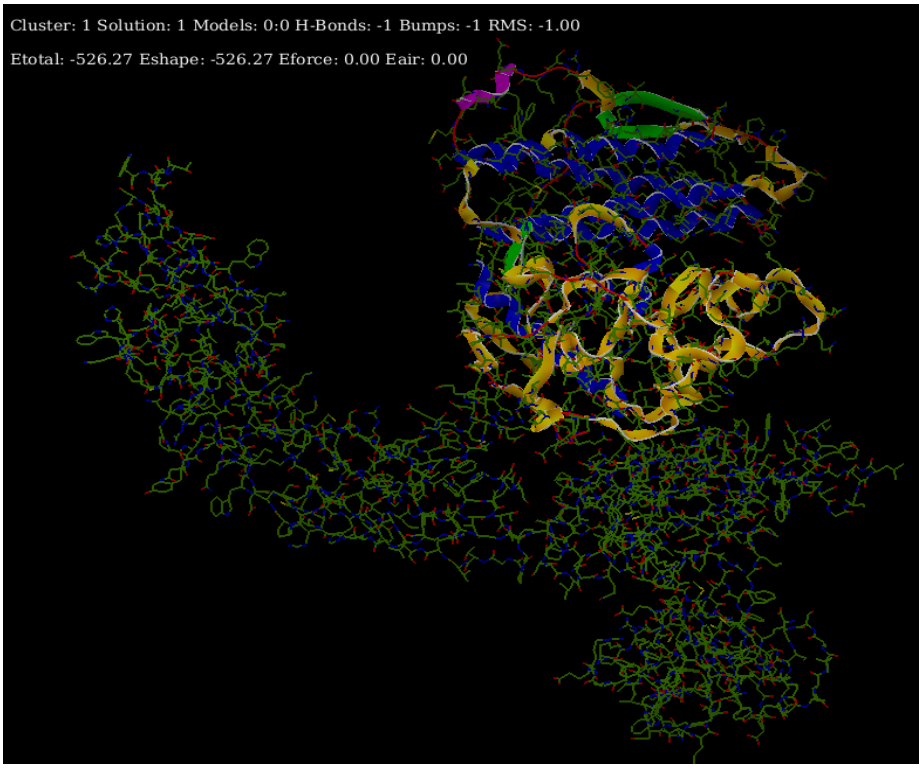


Fig.43 Interacting TPO-TPOR and showing binding sites

Identified residues which are interacting were – V44, D45, F46, S47, L48, E50, A126, L129, Q132, H133, R140, F141, and L144.

Interaction analysis of TPOR and TMP

Prepared receptor protein TPOR and known mimetic TMP were docked using HEX 8.0.0 server. PDB files of receptor and ligand imported and interaction domain and number out coming result 10 were selected. Best resulting PDB of ligand and receptor analyzed to determining binding sites. Random combination of these amino acids made and peptide sequences predicted.

2. Ligand preparation of peptide sequences

3D structures of peptides are predicted by Pepfold. It is de-novo peptide structure prediction tool. Peptide sequences were pasted on the workspace and allowed to run which gives 3D structure in PDB format. PDB files imported to Maestro and Prepwizard used to prepare structures.

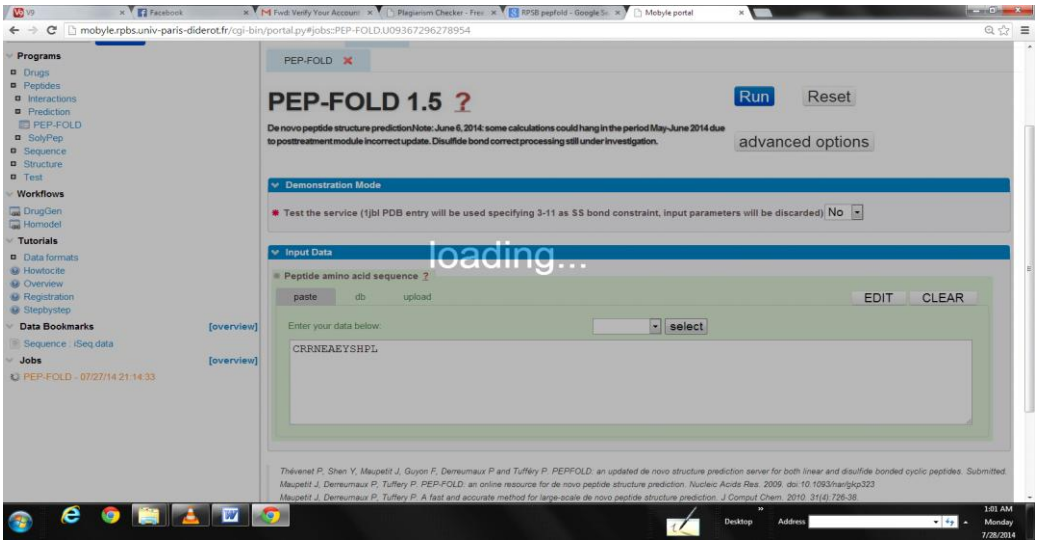


Fig.44 Web server Interface of Pepfold: To generate 3D structure of peptides

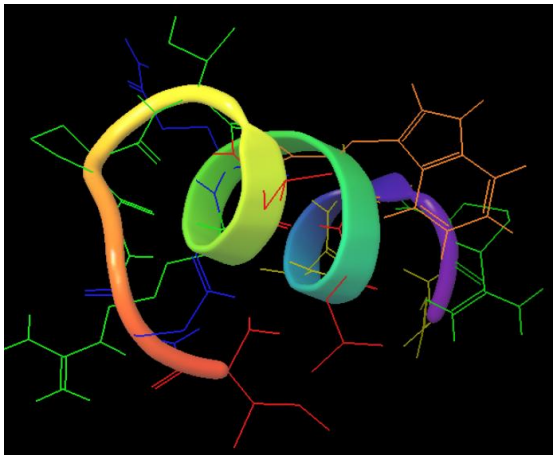


Fig.45 3Dstucture for TMP retrieved from Pep fold.

3. Interaction analysis of TPOR and combinatorial library of peptides-

Combinatorial library of peptides interaction with TPOR was analyzed using HEX 8.0.0 software. Initially receptor and ligand are prepared for removing water and unwanted lignads then PDBs of receptor and ligand peptides were uploaded to the software. Parameters of interaction analysis calculation device number of results and domain of interaction is selected and then run the programme. Hex returns the E_{total} in log file and best PDBs for interaction of receptor and ligand. (Table:)

S.no.	Oligopeptides	E_{total}
Known mimetic		
0.	IEGPTLRQWLAARA (TMP)	-159.25
Unknown mimetic		
1.	**LLDLLFIVLAARLLVLLFFQLL**	**-409.66**
2.	LLELLFIVLAARLLVLLFFQLL	-333.85
3.	LLSLIVLAARLLVLLFFQLLLF	-299
4.	LLSLLFIVLAADELVLLFFQLL	-333.66
5.	SPAPPACQVHPLPTPVLLPAVDFSLG	-356.03
6.	LLSLLFIVLAARLLVLLFFQLL	-340.91
7.	LLSLLFIVLAAHLLVLLFFQLH	-333.51
8.	SPAPPACQVHPLPTPVLLPAVDF	-300.07
9.	LLSLLFIVLAAHLLVLLFFQLH	-315.22
10.	LLSLLFIVLEAHLLVLLDFQLH	-330.20
11.	SPACQVHLTDFGEWKQMIRQ	-300.44
12.	EDKRKHTQRRNH	-252.85
13.	THRRKKRDGWEQNH	-252.85
14.	WQDRKPELLRKLRNH	-194.78
15.	SPAPPACQVHPLPTPVLLPA	-256.49
16.	SPAPPACQVHPLPTPVL	-138.59

17.	CRNEAESHYCYYNNASHL	-209.83
18.	NQDRKPELLRKLRNH	-225.85
19.	LLSLLFRVLAADELVLLKFQLL	-276.24
20.	LLSLLFIVADRLLVLLFFQLL	-233.35
21.	ILVGTLIVLIPVLIVLVFLYWQ	-226.99

Table 23 E_{total} score of TPOR and peptide library using HEX 8.0.0

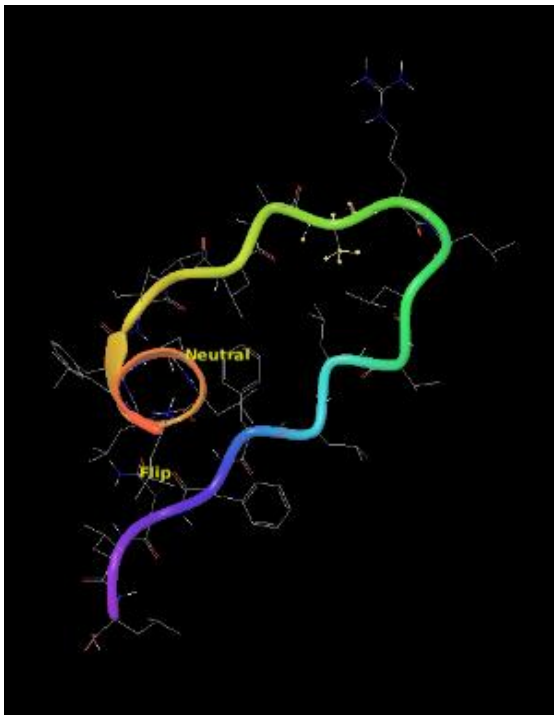


Fig. 46 Peptide 1 retrieved from pep fold and processed in maestro

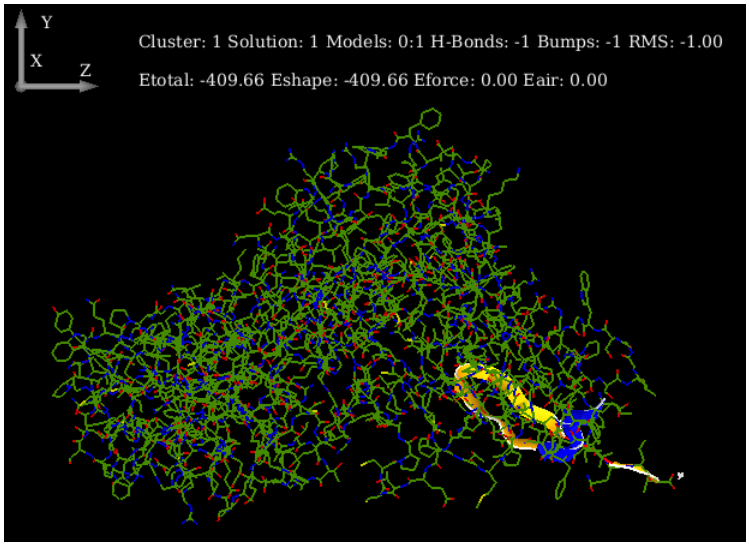


Fig.47 Peptide 1 interacting to its target with TPOR

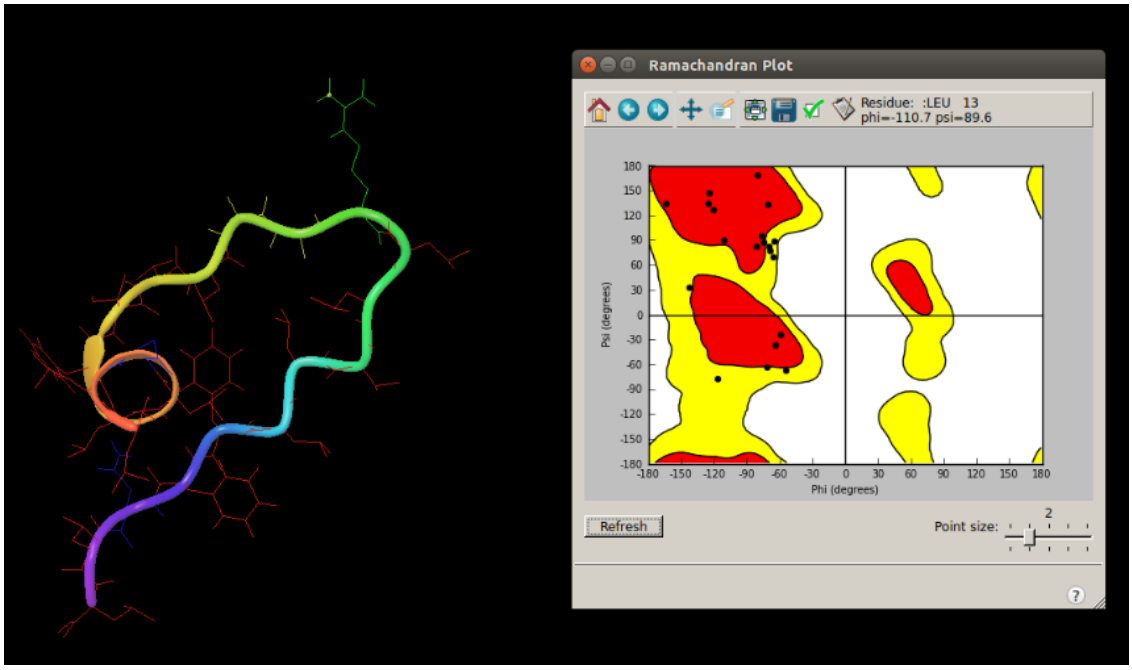
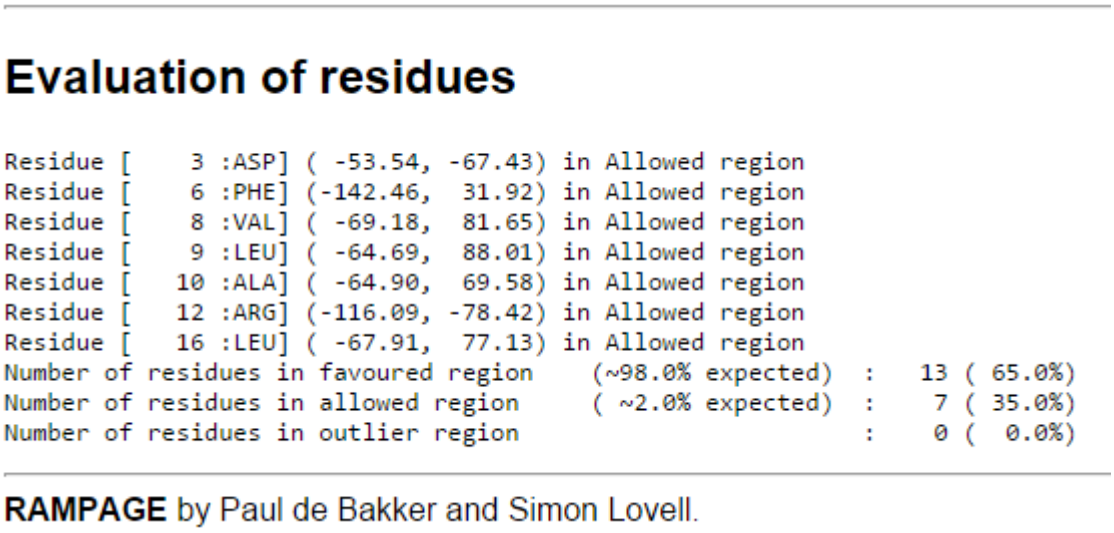


Fig.48 Ramachandran plot generated by maestro for Peptide 1



Evaluation of residues

```
Residue [    3 :ASP] ( -53.54, -67.43) in Allowed region
Residue [    6 :PHE] (-142.46,  31.92) in Allowed region
Residue [    8 :VAL] ( -69.18,  81.65) in Allowed region
Residue [    9 :LEU] ( -64.69,  88.01) in Allowed region
Residue [   10 :ALA] ( -64.90,  69.58) in Allowed region
Residue [   12 :ARG] (-116.09, -78.42) in Allowed region
Residue [   16 :LEU] ( -67.91,  77.13) in Allowed region
Number of residues in favoured region    (~98.0% expected)  :   13 ( 65.0%)
Number of residues in allowed region     ( ~2.0% expected)  :    7 ( 35.0%)
Number of residues in outlier region                        :    0 (  0.0%)
```

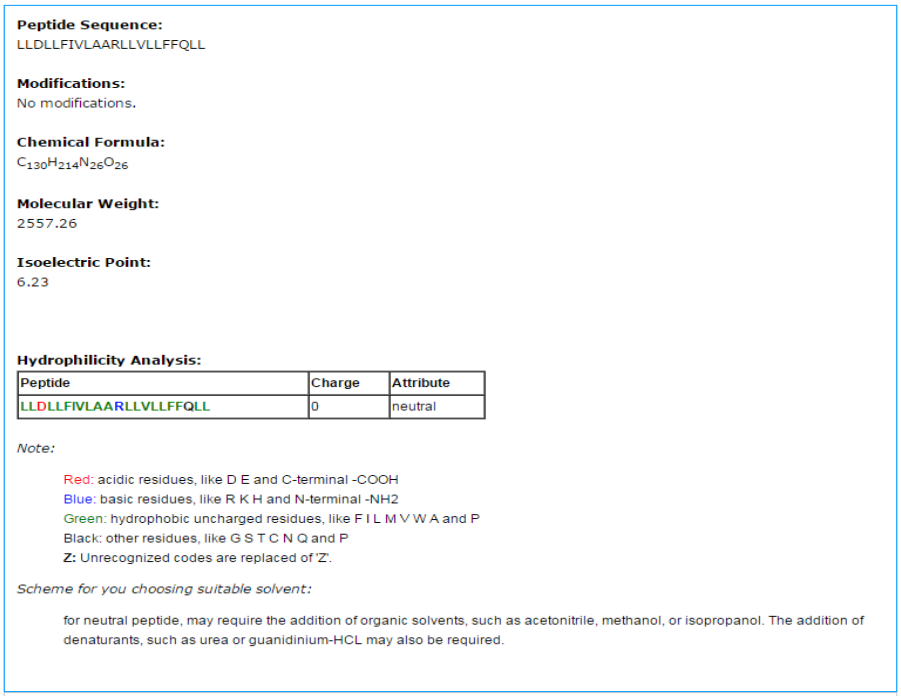
RAMPAGE by Paul de Bakker and Simon Lovell.

Fig. 49 Details of Ramachandran plot generated by RAMPAGE

Physiochemical and biological activity prediction-

Physicochemical property of peptide

Peptide mimetic found better on the basis of good binding energy value were further evaluated for physicochemical properties using Protein peptide calculator. The software results in finding the molecular weight, isoelectric pH, nature and charge.



Peptide Sequence:
LLDLLFIVLAARLLVLLFFQLL

Modifications:
No modifications.

Chemical Formula:
$C_{130}H_{214}N_{26}O_{26}$

Molecular Weight:
2557.26

Isoelectric Point:
6.23

Hydrophilicity Analysis:

Peptide	Charge	Attribute
LLDLLFIVLAARLLVLLFFQLL	0	neutral

Note:

Red: acidic residues, like D E and C-terminal -COOH
Blue: basic residues, like R K H and N-terminal -NH2
Green: hydrophobic uncharged residues, like F I L M V W A and P
Black: other residues, like G S T C N Q and P
Z: Unrecognized codes are replaced of 'Z'.

Scheme for you choosing suitable solvent:

for neutral peptide, may require the addition of organic solvents, such as acetonitrile, methanol, or isopropanol. The addition of denaturants, such as urea or guanidinium-HCL may also be required.

Fig.50 peptide 1 parameters identification using protein peptide calculator

S.no.	Oligopeptides	E_{total}	Isoelectric point	Nature	Charge
Known mimetic					
0.	IEGPTLRQWLAARA (TMP)	-159.25	10.45	basic	1
Unknown mimetic					
1.	**LLDLLFIVLAARLLVLLFFQLL**	**-409.66**	**6.23**	**neutral**	**0**

2.	LLELLFIVLAARLLVLLFFQLL	-333.85	6.34	neutral	0
3.	LLSLIVLAARLLVLLFFQLLLF	-299	10.55	basic	1
4.	LLSLLFIVLAADELVLLFFQLL	-333.66	3.55	acidic	-2
5.	SPAPPACQVHPLPTPVLLPAVDFSLG	-356.03	7.37	basic	1
6.	LLSLLFIVLAARLLVLLFFQLL	-340.91	10.55	basic	1
7.	LLSLLFIVLAAHLLVLLFFQLH	-333.51	7.72	basic	2
8.	SPAPPACQVHPLPTPVLLPAVDF	-300.07	5.38	neutral	0
9.	LLSLLFIVLAAHLLVLLFFQLH	-315.22	7.72	basic	2
10.	LLSLLFIVLEAHLLVLLDFQLH	-330.20	5.30	neutral	0
11.	SPACQVHLTDFGEWKQMIRQ	-300.44	7.37	basic	1
12.	EDKRKHTQRRNH	-252.85			
13.	THRRKKRDGWEQNH	-252.85			
14.	WQDRKPELLRKLRNH	-194.78			
15.	SPAPPACQVHPLPTPVLLPA	-256.49			
16.	SPAPPACQVHPLPTPVL	-138.59			
17.	CRNEAESHYCYYNNASHL	-209.83			
18.	NQDRKPELLRKLRNH	-225.85			
19.	LLSLLFRVLAADELVLLKFQLL	-276.24			
20.	LLSLLFIVADRLLVLLFFQLL	-233.35			
21.	ILVGTLIVLIPVLIVLVFLYWQ	-226.99			

Table 24 E_{total} score of TPOR and peptide library using HEX 8.0.0, including pI, nature and charge of each peptide.

Cello predictor

Subcellular localization of shortlisted peptides is detemined by Cello predictor. itdetrmines the localalization of peptides after secratorypatheway.

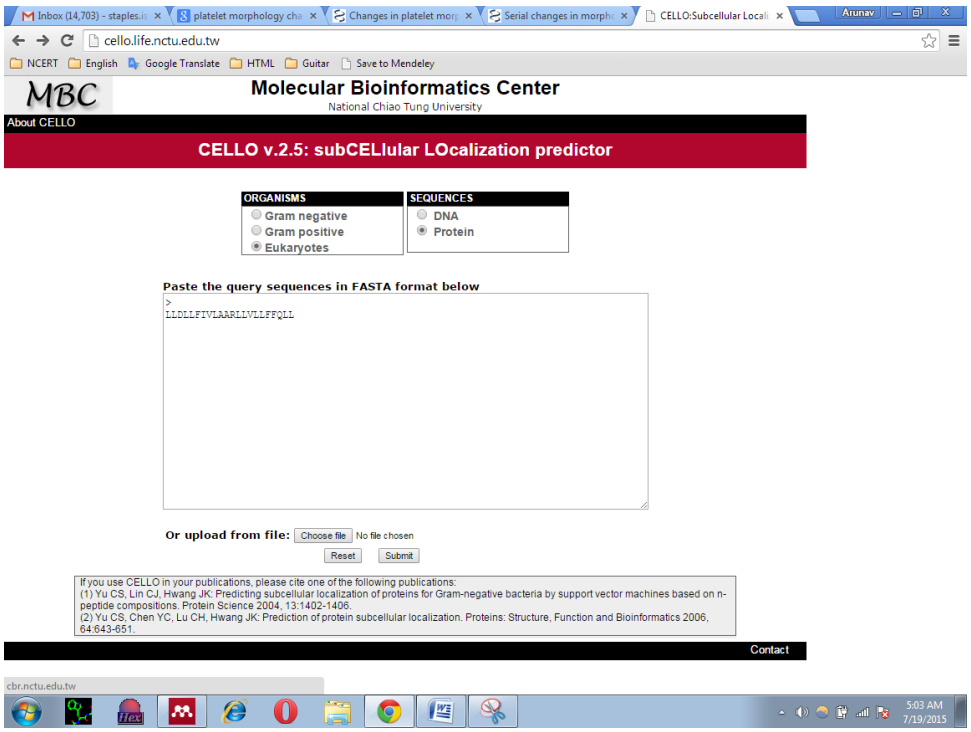


Fig.51 Cello home page

CELLO RESULTS

SeqID: unknow

Analysis Report:

SVM	LOCALIZATION	RELIABILITY
Amino Acid Comp.	Nuclear	0.345
N-peptide Comp.	Extracellular	0.594
Partitioned seq. Comp.	Nuclear	0.418
Physico-chemical Comp.	Chloroplast	0.385
Neighboring seq. Comp.	Extracellular	0.437

CELLO Prediction:

	Extracellular	1.304 *
	Nuclear	1.141 *
	Mitochondrial	0.851
	Chloroplast	0.748
	Cytoplasmic	0.485
	PlasmaMembrane	0.340
	Peroxisomal	0.033
	ER	0.027
	Golgi	0.019
	Vacuole	0.018
	Cytoskeletal	0.018
	Lysosomal	0.017

****

Table 25 Cello predictor results for peptide 1

7. CONCLUSION

In this study it has been demonstrated that currently used anticoagulants for the storage of blood and its components have a very limited life span and there is still scope to improve the quality of blood cells in storage and increase the shelf life from 42 days in case of RBC and 5 days in case of platelet. It will be advantageous to prolong the shelf life of blood cells for situations like disaster where stockpiling is necessary, or for the individuals with rare blood type, it can also act as an insurance/ reserve for irregular supply. So keeping this thought in mind an effort was made to access the effect of *Carica papaya* leaf extract (in different solvent systems) on blood cells already preserved in anticoagulant CPDA against control, remarkable results were observed. TLC profiling of *Carica papaya* leaves confirmed the presence of phytochemical components and then by phytochemical analysis presence of compounds like alkaloids, glycosides, saponins, tannins, flavonoids, phenolic compounds was confirmed. Different R_f values of the compounds in different solvent system provide information about their polarity. GC-MS results further confirmed that Papaya leaf extracts contain compounds which show medicinal properties like antimicrobial, antioxidant, anti-inflammatory, analgesic, anti-atherogenic, antithrombotic, anticoagulant, neuroprotective, antiviral, immune-modulatory, cell membrane-stabilizing and antiproliferative activities. These medicinal properties of extracts help in maintaining cellular viability of blood cells during storage.

So in case of Red blood cells we can conclude that almost all the extracts showed positive results but hexane, acetone and fresh leaves extracts showed better activity than other extracts and helped maintaining cellular viability, morphology, pH and reduced haemolysis.

In case of Platelet more specific conclusion can be made and we can say that leaf extract in solvent system hexane showed better results. Platelet storage lesions in hexane were controlled as compared to control and other extracts. Microbial contamination was absolutely zero. Discs prepared from these stored platelets showed no microbial growth. No growth in liquid broth was confirmed by spectrophotometer.

In case of designing a TPO mimetic which can activate the TPOR cytokine protein and consequent signalling for thrombopoiesis have been designed using computational analysis. TMP is reported to mimic TPO and activate TPOR in the absence of TPO but they are less efficient than natural endogenous hormone; hence study required to find more potent mimetic. Here in this study, combinatorial library of peptide was designed and study found out peptide with better binding efficiency and determined the physiochemical properties

which prove that the peptide is stable and effective. In future further *Ex-vivo* study of designed mimetic with TPOR can confirm its role in increasing the shelf life of platelet by acting as an anti-apoptotic agent.

8. DISCUSSION & FUTURE PERSPECTIVE

The study suggests a significant improvement in cellular viability, pH, morphology, Hb content in red blood cells stored in anticoagulant CPDA and papaya leaf extracts in different solvent systems namely, Hexane, Acetone, Ethanol, and water compared to blood stored in anticoagulant alone. Hexane, acetone and fresh leaf extracts showed better activity in comparison to other extracts in red blood cells, where as in case of Platelet Hexane showed maximum activity in overcoming platelet storage lesions such as pH, Morphology, cell viability, clotting factor time, Glucose and controlling bacterial contamination. The results of Phytochemical analysis and GC-MS of the extracts confirmed the presence of glycosides, saponins, tannins, flavonoids, phenolic etc. Literature survey confirmed that the metabolites present in the extracts possess medicinal value such as antimicrobial, antioxidant, anti-inflammatory, analgesic, anti-atherogenic, antithrombotic, anticoagulant, neuroprotective, antiviral, immune-modulatory, cell membrane-stabilizing and antiproliferative activities. Metabolites helped in overcoming the storage lesions both in blood and platelet. Increase in the amount of free Hb, damage caused to cells due to acidic pH thus, affecting the viability of cells while storage is controlled by the metabolites present in the leaf extracts. They also improved the morphology of stored blood cells by stabilizing the membrane of cells.

In stored Platelet, platelet storage lesions such as pH, glucose, platelet functional assay i.e. platelet factor 3 test determining the clotting factor time, cell viability along with bacterial contamination were overcome in papaya leaf extract with 9% Hexane solvent. Since the biggest problem faced by blood banks in storage of platelets is microbial growth because of the storage temperature (which is 22 – 24° C) we can say Hexane at higher concentration (9% in comparison to 3% and 6%) has maximum antimicrobial activity. It was observed that when 9% hexane extract was added to media plates no contamination was seen, thus the metabolites present in this extract can be considered for further analysis. The activity shown by the extract could be possibly due to tannis for its antimicrobial activity along with alkaloids and flavonoids.

Since multiple components are present in papaya having the potential to act as bioactive component for improving blood cell's quality; separation, purification and detection of the effect of each component on blood cells individually is required to confirm the bioactive components affecting blood cells in a positive manner.

Another study included in this thesis work was Designing an in-silico mimetic for Thrombopoietin to improve cellular viability of platelet by preventing programmed cell death i.e. apoptosis, combinatorial library of peptide sequence derived from binding sites of TPOR with TPO and known mimetic and literature survey are created. Library was tested for interaction analysis with TPOR extra cellular domain region for binding and regulation of thrombopoiesis. For the interaction study, first in the receptor, TPOR, extra cellular domain region was prepared by structure prediction method via phyre 2 and then by removing water structure was prepared. Similar procedure was followed for TPO since PDB structures for both the proteins is not available. TPO-TPOR active site is analyzed using PDB structure of the complex obtained from docking results by HEX 8.0.0 in maestro server. TPOR found to interact with two regions in TPO forming the receptor dimer. Hydrophobic amino acids namely, His133, Lys14, Lys138, Phe141 are present in receptor binding site. Peptide mimetic 1 has E_{total} value of -409.66 which is better than E_{total} value of known mimetic TMP, -158.90. Lead Peptide mimetic was further analyzed for physicochemical properties and stability. Peptide mimetic showed higher hydrophobicity which is similar to natural TPO variant and is necessary to the membranes. Lead peptide hydrophilic and hydrophobic nature may have important role in binding to TPOR as we had seen polar and non polar both interaction involved in binding of TPO-TPOR complex. Cello predictor results showed that peptide localization by secretary pathway is to nuclear and mitochondria where it can bind to its receptor and activate the signalling further for platelet formation. In future further *Ex-vivo* study of designed mimetic with TPOR can confirm its role in increasing the shelf life of platelet by acting as an anti-apoptotic agent. Mimetic in future possesses important role which can be used for ex vivo platelet generation and in many other applications in field of regenerative medicine.

9. REFERENCES

Abe, M., Suzuki, K. I., Sakata, C., Sugasawa, K., Hirayama, F., Koga, Y., … Itoh, H. (2011). Pharmacological profile of AS1670542, a novel orally-active human thrombopoietin receptor agonist. *European Journal of Pharmacology*, *650*(1), 58–63. http://doi.org/10.1016/j.ejphar.2010.09.072

Aiswarya, G., Gupta, R., & Kambhoja, S. (n.d.). Research Journal of Pharmaceutical , Biological and Chemical Sciences Isolation of 28-pentyl-3-galloyl-betulinate and11-hydroxy friedelane from the. *Adsorption Journal Of The International Adsorption Society*, *1*(3).

Akhoon, B. a., Gupta, S. K., Verma, V., Dhaliwal, G., Srivastava, M., Gupta, S. K., & Ahmad, R. F. (2010). In silico designing and optimization of anti-breast cancer antibody mimetic oligopeptide targeting HER-2 in women. *Journal of Molecular Graphics and Modelling*, *28*(7), 664–669. http://doi.org/10.1016/j.jmgm.2010.01.002

Albertoni, G., Andrade, S. S., Araújo, P. R. B., Carvalho, F. O., Girão, M. J. B. C., & Barreto, J. a. (2011). Evaluation of two detection methods of microorganisms in platelet concentrates. *Transfusion Medicine*, *21*(6), 408–416. http://doi.org/10.1111/j.1365-3148.2011.01105.x

Alexander, W. S., Roberts, a W., Maurer, a B., Nicola, N. a, Dunn, a R., & Metcalf, D. (1996). Studies of the c-Mpl thrombopoietin receptor through gene disruption and activation. *Stem Cells*, *14 Suppl 1*, 124–132. http://doi.org/10.1002/stem.5530140716

Alhumaidan, H., & Sweeney, J. (2012). Current status of additive solutions for platelets. *Journal of Clinical Apheresis*, *27*(2), 93–98. http://doi.org/10.1002/jca.21207

Amazu, L., Azikiwe, C., Njoku, C., Osuala, F., Nwosu, P., Ajugwo, A., & Enye, J. (2010). Antiinflammatory activity of the methanolic extract of the seeds of Carica papaya in experimental animals. *Asian Pacific Journal of Tropical Medicine*, *3*(11), 884–886. http://doi.org/10.1016/S1995-7645(10)60212-X

Anjum, V., Ansari, S. H., Naquvi, K. J., & Arora, P. (2013). Physico-Chemical and Phytochemical Evaluation of Carica Papaya Linn. Unripe Fruits. *International Research Journal of Pharmacy*, *4*(8), 101–106. http://doi.org/10.7897/2230-8407.04817

Anjum, V., Ansari, S. H., Naquvi, K. J., Arora, P., & Ahmad, A. (2013). Development of quality standards of Carica Papaya Linn . leaves, *5*(July 2012), 370–376.

Bethel, M., Barnes, C. L. T., Taylor, A. F., Cheng, Y.-H., Chitteti, B. R., Horowitz, M. C., … Kacena, M. a. (2015). A Novel Role for Thrombopoietin in Regulating Osteoclast Development in Humans and Mice. *Journal of Cellular Physiology*, *230*(9), 2142–2151. http://doi.org/10.1002/jcp.24943

Bhaskar, A., & Upgade, A. (2013, September 6). CHARACTERIZATION AND MEDICINAL IMPORTANCE OF PHYTOCONSTITUENTS OF C. PAPAYA FROM DOWN SOUTH INDIAN REGION USING GAS CHROMATOGRAPHY AND MASS

SPECTROSCOPY. *Asian Journal of Pharmaceutical and Clinical Research*. Retrieved from http://innovareacademics.in/journals/index.php/ajpcr/article/view/366

Building, T. (2010). Electron microscopy procedures manual, (July), 1–25.

Cao, Z., Bell, J. B., Mohanty, J. G., Nagababu, E., & Rifkind, J. M. (2009). Nitrite enhances RBC hypoxic ATP synthesis and the release of ATP into the vasculature: a new mechanism for nitrite-induced vasodilation. *American Journal of Physiology. Heart and Circulatory Physiology*, *297*(4), H1494–H1503. http://doi.org/10.1152/ajpheart.01233.2008

Cerdán-Calero, M., Sendra, J. M., & Sentandreu, E. (2012). Gas chromatography coupled to mass spectrometry analysis of volatiles, sugars, organic acids and aminoacids in Valencia Late orange juice and reliability of the Automated Mass Spectral Deconvolution and Identification System for their automatic identification and quantification. *Journal of Chromatography A*, *1241*, 84–95. http://doi.org/10.1016/j.chroma.2012.04.014

Chandra, T., Gupta, A., & Kumar, A. (2014a). Extended shelf life of random donor platelets stored for 7 days in platelet additive solution at different temperatures. *Biomedical Journal*, *37*(4), 211. http://doi.org/10.4103/2319-4170.117896

Chandra, T., Gupta, A., & Kumar, A. (2014b). Extended shelf life of random donor platelets stored for 7 days in platelet additive solution at different temperatures. *Biomedical Journal*, *37*(4), 211. http://doi.org/10.4103/2319-4170.117896

Dasouki, M., Saadi, I., & Ahmed, S. O. (2015). THPO–MPL pathway and bone marrow failure. *Hematology/Oncology and Stem Cell Therapy*, *8*(1), 6–9. http://doi.org/10.1016/j.hemonc.2014.11.005

Gupta, A., Chandra, T., & Kumar, A. (2011). Retracted: Platelet storage lesion: current proteomics approach. *Platelets*, *22*(2), 9537104. http://doi.org/10.3109/09537104.2010.547958

Chavan, N., & Patil, P. D. (2012). Potential testing of fatty acids from Aegiceras corniculatum (L.) Blanco. *International Journal of Pharmacy and Pharmaceutical Sciences*, *4*(4), 569–571. Retrieved from http://www.researchgate.net/publication/257657375_Potential_testing_of_fatty_acids_from_Aegiceras_corniculatum_(L.)_Blanco

Chen, W. M., Yu, B., Zhang, Q., & Xu, P. (2010). Identification of the residues in the extracellular domain of thrombopoietin receptor involved in the binding of thrombopoietin and a nuclear distribution protein (human NUDC). *Journal of Biological Chemistry*, *285*(34), 26697–26709. http://doi.org/10.1074/jbc.M110.120956

Christian H., Rüster B., Seifried E., Henschler R. (2010). Platelet precursor cells can be generated from cultured human CD34+ progenitor cells but display recirculation into hematopoietic tissue upon transfusion in mice. Transfus. Med. Hemother. 37, 185–190. 10.1159/000316975

Chu, H., Puchulu-Campanella, E., Galan, J. a., Tao, W. a., Low, P. S., & Hoffman, J. F. (2012). Identification of cytoskeletal elements enclosing the ATP pools that fuel human red blood cell membrane cation pumps. *Proceedings of the National Academy of Sciences*, *109*(31), 12794–12799. http://doi.org/10.1073/pnas.1209014109

Cohen, B., Matot, I., & Hemmings, H. C. (2013). Aged erythrocytes: A fine wine or sour grapes? *British Journal of Anaesthesia*, *111*(SUPPL.1), 62–70. http://doi.org/10.1093/bja/aet405

Corporation, P. (n.d.). NAD / NADH-Glo ™ Assay NAD / NADH-Glo ™ Assay.

Craddock, C. G. (2014). B L 00 D, 257–271.

Cravatt, B. F., Lerner, R. A., & Boger, D. L. (1996). Structure Determination of an Endogenous Sleep-Inducing Lipid, cis -9-Octadecenamide (Oleamide): A Synthetic Approach to the Chemical Analysis of Trace Quantities of a Natural Product. *Journal of the American Chemical Society*, *118*(3), 580–590. http://doi.org/10.1021/ja9532345

Das, D. (2009). Red Blood Cell Stabilization: Effect of Hydroxyethyl Starch on RBC Viability, Functionality and Oxidative State During Different Freeze Thaw Conditions. *Cell*, 1–55.

Dasouki, M., Saadi, I., & Ahmed, S. O. (2015). THPO–MPL pathway and bone marrow failure. *Hematology/Oncology and Stem Cell Therapy*, *8*(1), 6–9. http://doi.org/10.1016/j.hemonc.2014.11.005

Deane, C. M., Kroemer, R. T., & Richards, W. G. (1997). A structural model of the human thrombopoietin receptor complex. *Journal of Molecular Graphics and Modelling*, *15*(3), 170–178. http://doi.org/10.1016/S1093-3263(97)00102-2

Delmer, a, & Zittoun, R. (1991). *Use of hematopoietic growth factors in oncology*. *Bulletin du cancer* (Vol. 78).

Design, P., & Applications, B. (2009). *Peptide and Protein Design for Biopharmaceutical Peptide and Protein Design for Biopharmaceutical Applications*.

Deveaux, B. S., Filipe, A., Lemarchandel, V., Ghysdael, J., Romeo, P., & Mignotte, V. (2015). and Ets Proteins in the Coregulation of Megakaryocyte-Specific Genes, *87*(11), 4678–4685.

Devine, D. V., & Serrano, K. (2010). The Platelet Storage Lesion. *Clinics in Laboratory Medicine*, *30*(2), 475–487. http://doi.org/10.1016/j.cll.2010.02.002

Dharmarathna, S. L. C. A., Wickramasinghe, S., Waduge, R. N., Rajapakse, R. P. V. J., & Kularatne, S. A. M. (2013a). Does Carica papaya leaf-extract increase the platelet count? An experimental study in a murine model. *Asian Pacific Journal of Tropical Biomedicine*, *3*(9), 720–724. http://doi.org/10.1016/S2221-1691(13)60145-8

Dharmarathna, S. L. C. A., Wickramasinghe, S., Waduge, R. N., Rajapakse, R. P. V. J., & Kularatne, S. A. M. (2013b). Does Carica papaya leaf-extract increase the platelet count? An experimental study in a murine model. *Asian Pacific Journal of Tropical Biomedicine*, *3*(9), 720–4. http://doi.org/10.1016/S2221-1691(13)60145-8

Drachman, J. G., & Kaushansky, K. (1997). Dissecting the thrombopoietin receptor: functional elements of the Mpl cytoplasmic domain. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(6), 2350–2355. http://doi.org/10.1073/pnas.94.6.2350

Facts, Q. (2005). ATP Determination Kit (A22066) Quick Facts. *Microscope*, 1–3.

Feese, M. D., Tamada, T., Kato, Y., Maeda, Y., Hirose, M., Matsukura, Y., … Kuroki, R. (2004). Structure of the receptor-binding domain of human thrombopoietin determined by complexation with a neutralizing antibody fragment. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(7), 1816–1821. http://doi.org/10.1073/pnas.0308530100

Filip, J., & Sibley, A. (2015). The Effect of Platelet on Adenine, *45*(6).

Formation, P. (2011). NIH Public Access. *Medicine*, *47*(3), 220–226. http://doi.org/10.1053/j.seminhematol.2010.03.005.PLATELET

Frederickson, S., Renshaw, M. W., Lin, B., Smith, L. M., Calveley, P., Springhorn, J. P., … Bowdish, K. S. (2006). A rationally designed agonist antibody fragment that functionally mimics thrombopoietin. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(39), 14307–14312. http://doi.org/10.1073/pnas.0602658103

Fukushima-Shintani, M., Suzuki, K. I., Iwatsuki, Y., Abe, M., Sugasawa, K., Hirayama, F., … Nakahata, T. (2009). AKR-501 (YM477) a novel orally-active thrombopoietin receptor agonist. *European Journal of Haematology*, *82*(4), 247–254. http://doi.org/10.1111/j.1600-0609.2008.01198.x

Ghasemzadeh, A., & Jaafar, H. Z. E. (2013). Profiling of phenolic compounds and their antioxidant and anticancer activities in pandan (Pandanus amaryllifolius Roxb.) extracts from different locations of Malaysia. *BMC Complementary and Alternative Medicine*, *13*(1), 341. http://doi.org/10.1186/1472-6882-13-341

Gujjeti, R. P., & Mamidala, E. (2013). Phytochemical Analysis and TLC Profile of Madhuca indica Inner Bark Plant Extract, *4*(10), 1507–1510.

Gupta, A., Chandra, T., & Kumar, A. (2011). Retracted: Platelet storage lesion: current proteomics approach. *Platelets*, *22*(2), 9537104. http://doi.org/10.3109/09537104.2010.547958

Hess, J. R. (2009). Red cell storage: when is better not good enough? *Blood Transfusion = Trasfusione Del Sangue*, *7*(3), 172–3. http://doi.org/10.2450/2009.0110-09

Hess, J. R. (2009). Red cell storage: When is better not good enough? *Blood Transfusion*, *7*(3), 172–173. http://doi.org/10.2450/2009.0110-09

Hess, J. R., Kagen, L. R., Van Der Meer, P. F., Simon, T., Cardigan, R., Greenwalt, T. J., … Hogman, C. (2005). Interlaboratory comparison of red-cell ATP, 2,3-diphosphoglycerate and haemolysis measurements. *Vox Sanguinis*, *89*(1), 44–48. http://doi.org/10.1111/j.1423-0410.2005.00635.x

Hess, J. R., Rugg, N., Knapp, A. D., Gormas, J. F., Silberstein, E. B., & Greenwalt, T. J. (2000). L 1012, *40*(August), 1012–1016.

Högman, C. F. (1998). Preparation and Preservation of Red Cells. *Vox Sanguinis*, *74*(S2), 177–187. http://doi.org/10.1111/j.1423-0410.1998.tb05419.x

Hou, J., & Zhan, H. (1998). Expression of active thrombopoietin and identification of its key residues responsible for receptor binding. *Cytokine*, *10*(5), 319–330. http://doi.org/10.1006/cyto.1997.0299

Hsieh, D. P. H., Huxtable, S., Ng, K. F., Chen, H. M., Tsang, P. W. K., Wang, J., & Xu, P. (2000). Determination of interactions between human thrombopoietin and its receptor MPL by yeast two-hybrid system and affinity biosensor. *International Journal of Biochemistry and Cell Biology*, *32*(5), 481–488. http://doi.org/10.1016/S1357-2725(99)00132-6

Iyappan, G., Daniel, D., & Poovanalingam, T. (2014). " ASCERTAINING THE PHYTOCOMPONENTS IN THE CRUDE ETHANOLIC EXTRACTS OF CARICA PAPAYA SEEDS BY GC- MS ," *3*(9), 942–949.

Jagerschmidt, A., Fleury, V., Anger-leroy, M., Thomas, C., Agnel, M., & Brien, D. P. O. (1998). Functionally Important Residues, *734*, 729–734. http://doi.org/10.1002/prot.22374

Jain, A., Marwaha, N., Sharma, R. R., Kaur, J., Thakur, M., & Dhawan, H. K. Serial changes in morphology and biochemical markers in platelet preparations with storage. *Asian Journal of Transfusion Science*, *9*(1), 41–7. http://doi.org/10.4103/0973-6247.150949

Johnson, L. N., Winter, K. M., Reid, S., Hartkopf-Theis, T., & Marks, D. C. (2011). Cryopreservation of buffy-coat-derived platelet concentrates in dimethyl sulfoxide and platelet additive solution. *Cryobiology*, *62*(2), 100–106. http://doi.org/10.1016/j.cryobiol.2011.01.003

Johnson, L., Reade, M. C., Hyland, R. a., Tan, S., & Marks, D. C. (2014). In vitro comparison of cryopreserved and liquid platelets: potential clinical implications. *Transfusion*, n/a–n/a. http://doi.org/10.1111/trf.12915

Julianti, T., De Mieri, M., Zimmermann, S., Ebrahimi, S. N., Kaiser, M., Neuburger, M., … Hamburger, M. (2014). HPLC-based activity profiling for antiplasmodial compounds in the traditional Indonesian medicinal plant Carica papaya L. *Journal of Ethnopharmacology*, *155*(1), 426–434. http://doi.org/10.1016/j.jep.2014.05.050

Kalyan Chakravarthy, V., Naveen Chandra, D., Santhoshi Prasanna, B., Jaya Mastan Rao, T., & Ranga Rao, D. (2012). Haemoglobin estimation by non-cyanide methods. *Journal of Clinical and Diagnostic Research*, *6*(6), 955–958.

Karnicki, K., Johnson, C., St. Cyr, J., Ericson, D., & Rao, G. (2003). Platelet storage solution improves the in vitro function of preserved platelet concentrate. *Vox Sanguinis*, *85*(4), 262–266. http://doi.org/10.1111/j.0042-9007.2003.00362.x

Kato, T., Matsumoto, a, Ogami, K., Tahara, T., Morita, H., & Miyazaki, H. (1998). Native thrombopoietin: structure and function. *Stem Cells*, *16*(5), 322–328. http://doi.org/10.1002/stem.160322

Kaufman, R. M. (2006). Platelets: testing, dosing and the storage lesion--recent advances. *Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program*, 492–496. http://doi.org/10.1182/asheducation-2006.1.492

Kaushansky, K. (2009). Molecular mechanisms of thrombopoietin signaling. *Journal of Thrombosis and Haemostasis*, *7*(SUPPL. 1), 235–238. http://doi.org/10.1111/j.1538-7836.2009.03419.x

Kaushansky, K., Broudy, V. C., Lin, N., Jorgensen, M. J., McCarty, J., Fox, N., … Lofton-Day, C. (1995). Thrombopoietin, the Mp1 ligand, is essential for full megakaryocyte development. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(8), 3234–3238. http://doi.org/10.1073/pnas.92.8.3234

Kaushansky, K., & Drachman, J. G. (2002). The molecular and cellular biology of thrombopoietin: the primary regulator of platelet production. *Oncogene*, *21*(21), 3359–3367. http://doi.org/10.1038/sj.onc.1205323

Kelebek, H., Selli, S., Gubbuk, H., & Gunes, E. (2015). Comparative evaluation of volatiles, phenolics, sugars, organic acids and antioxidant properties of Sel-42 and Tainung papaya varieties. *Food Chemistry*, *173*, 912–919. http://doi.org/10.1016/j.foodchem.2014.10.116

Keong, C. K., Nadarajah, V. D. V, & Lee, T. J. (2007). Development of a purification method of pure primary lymphocytes for cell viability assays. *Malaysian Journal of Medical Sciences*, *14*(1), 38–45.

Kurata, M., Suzuki, M., & Agar, N. S. (1993). Antioxidant systems and erythrocyte life-span in mammals. *Comparative Biochemistry and Physiology. B, Comparative Biochemistry*, *106*(3), 477–87. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8281748

Kuter, D. J. (2009). Thrombopoietin and thrombopoietin mimetics in the treatment of thrombocytopenia. *Annual Review of Medicine*, *60*, 193–206. http://doi.org/10.1146/annurev.med.60.042307.181154

Kuter, D. J. (2015). Review article New thrombopoietic growth factors, *109*(11), 4607–4617. http://doi.org/10.1182/blood-2006-10-019315.

Lee, E.-J., Be, C. L., Vinson, A. R., Riches, A. G., Fehr, F., Gardiner, J., … Haylock, D. (2015). Immobilisation of a thrombopoietin peptidic mimic by self-assembled monolayers for culture of CD34+ cells. *Biomaterials*, *37*, 82–93. http://doi.org/10.1016/j.biomaterials.2014.10.029

Li, C., & Zheng, L. (2014). The pharmacology and clinical application of thrombopoietin receptor agonists. *International Journal of Hematology*, *100*(6), 529–539. http://doi.org/10.1007/s12185-014-1660-5

Li, J., Xia, Y., & Kuter, D. J. (1999). Interaction of thrombopoietin with the platelet c-mpl receptor in plasma: Binding, internalization, stability and pharmacokinetics. *British Journal of Haematology*, *106*(2), 345–356. http://doi.org/10.1046/j.1365-2141.1999.01571.x

Liebman, H. a., & Pullarkat, V. (2011). Diagnosis and Management of Immune Thrombocytopenia in the Era of Thrombopoietin Mimetics. *Hematology*, *2011*(1), 384–390. http://doi.org/10.1182/asheducation-2011.1.384

Liem-Moolenaar, M., Cerneus, D., Molloy, C. J., End, D., Brown, K. H., de Kam, M. L., … Burggraaf, J. (2008). Pharmacodynamics and pharmacokinetics of the novel thrombopoietin mimetic peptide RWJ-800088 in humans. *Clinical Pharmacology and Therapeutics*, *84*(4), 481–487. http://doi.org/10.1038/clpt.2008.96

Liu, C.-F., Liu, C.-F., Feige, U., Feige, U., Cheetham, J., Cheetham, J., … Us1998000105348P, 1998-10-23. (1999). Dimeric Thrombopoietin Peptide Mimetics binding to MP1 Receptor and having Thrombopoietic activity, 1–5. Retrieved from file:///H|/projekte/literatur/modelling/files/WO00024770A3_amg_tpo_2000.pdf

Macalood, J. S., Vicente, H. J., Boniao, R. D., Gorospe, J. G., & Roa, E. C. (2013). Chemical Analysis of *Carica papaya* L. Crude Latex. *American Journal of Plant Sciences*, *04*(10), 1941–1948. http://doi.org/10.4236/ajps.2013.410240

Machlus K. R., Italiano J. E. (2013). The incredible journey: from megakaryocyte development to platelet formation. J. Cell Biol. 201, 785–796. 10.1083/jcb.201304054

Mann, M. K., & Cairns, B. E. (2006). Growth Factors and Cytokines. *Handbook of Pharmaceutical Biotechnology*, 1173–1196. http://doi.org/10.1002/9780470117118.ch08a

Manuscript, A. (2012). NIH Public Access. *Changes*, *29*(6), 997–1003. http://doi.org/10.1016/j.biotechadv.2011.08.021.Secreted

Meinander, K. (2014). *PSEUDOPEPTIDES AND PEPTIDOMIMETICS MODULATING THE PROTEOLYTIC ACTIVITY OF KALLIKREIN-RELATED PEPTIDASE 3*.

Mensink, R. P., Zock, P. L., Kester, A. D. M., & Katan, M. B. (2003). Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. *The American Journal of Clinical Nutrition*, *77*(5), 1146–55. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12716665

Mignotte, V., Vigon, I., Boucher de Crèvecoeur, E., Roméo, P. H., Lemarchandel, V., & Chrétien, S. (1994). Structure and transcription of the human c-mpl gene (MPL). *Genomics*. http://doi.org/10.1006/geno.1994.1120

Moulis, G., Bagheri, H., Sailler, L., Jonville-Bera, A.-P., Weber, E., Guy, C., … Montastruc, J.-L. (2014). Are adverse drug reaction patterns different between romiplostim and eltrombopag? 2009–2013 French PharmacoVigilance assessment. *European Journal of Internal Medicine*, *25*(8), 777–780. http://doi.org/10.1016/j.ejim.2014.09.006

Nicola, N. a. (2000). Hematopoietic Growth Factors. *Cytokine*. http://doi.org/10.1006/rwcy.2000.02009.THE

Nwaehujor, C. O., Ode, J. O., Ekwere, M. R., & Udegbunam, R. I. (2014). Anti-fertility effects of fractions from Carica papaya (Pawpaw) Linn. methanol root extract in male Wistar rats. *Arabian Journal of Chemistry*. http://doi.org/10.1016/j.arabjc.2014.10.018

Ohgami, K. (n.d.).Development of Thrombopoietin: Its Structure and Functio, *10*, 113–117.

Ohto, H., & Nollet, K. E. (2011). Overview on platelet preservation: Better controls over storage lesion. *Transfusion and Apheresis Science*, *44*(3), 321–325. http://doi.org/10.1016/j.transci.2011.03.008

Onwukaeme, D. N., Ikuegbvweha, T. B., & Asonye, C. C. (2007). Evaluation of Phytochemical Constituents, Antibacterial Activities and Effect of Exudate of *Pycanthus Angolensis* Weld Warb (Myristicaceae) on Corneal Ulcers in Rabbits. *Tropical Journal of Pharmaceutical Research*, *6*(2), 725–730. http://doi.org/10.4314/tjpr.v6i2.14652

Otsuki, N., Dang, N. H., Kumagai, E., Kondo, A., Iwata, S., & Morimoto, C. (2010). Aqueous extract of Carica papaya leaves exhibits anti-tumor activity and immunomodulatory effects. *Journal of Ethnopharmacology*, *127*(3), 760–767. http://doi.org/10.1016/j.jep.2009.11.024

Owoyele, B. V., Adebukola, O. M., Funmilayo, A. a., & Soladoye, A. O. (2008). Anti-inflammatory activities of ethanolic extract of Carica papaya leaves. *Inflammopharmacology*, *16*(4), 168–173. http://doi.org/10.1007/s10787-008-7008-0

Papaya Leaves in Dengue Fever : Is there Scientific Evidence ? Reversible Corneal Clouding in Neonatal Hyperglycemia. (2014). http://doi.org/10.1136/bcr-2013-200016.2.

Parekh, J., & Chanda, S. V. (2007). In vitro Antimicrobial Activity and Phytochemical Analysis of Some Indian Medicinal Plants. *Turk J Biol*, *31*(10), 53–58. Retrieved from http://journals.tubitak.gov.tr/biology/issues/biy-07-31-1/biy-31-1-9-0610-4.pdf

Pérez-Gutiérrez, S., Zavala-Sánchez, M. A., González-Chávez, M. M., Cárdenas-Ortega, N. C., & Ramos-López, M. A. (2011). Bioactivity of Carica papaya (Caricaceae) against Spodoptera frugiperda (Lepidoptera: Noctuidae). *Molecules (Basel, Switzerland)*, *16*(9), 7502–9. http://doi.org/10.3390/molecules16097502

Peter, J. K., Kumar, Y., Pandey, P., & Masih, H. (2014). Antibacterial Activity of Seed and Leaf Extract of Carica Papaya var . Pusa dwarf Linn, *9*(2), 29–37.

Ranasinghe, P., Ranasinghe, P., Abeysekera, W. P. K. M., Premakumara, G. A. S., Perera, Y. S., Gurugama, P., & Gunatilake, S. B. (2012). In vitro erythrocyte membrane stabilization properties of Carica papaya L. leaf extracts. *Pharmacognosy Research*, *4*(4), 196–202. http://doi.org/10.4103/0974-8490.102261

Ringwald, J., Zimmermann, R., & Eckstein, R. (2006). The new generation of platelet additive solution for storage at 22??C: Development and current experience. *Transfusion Medicine Reviews*, *20*(2), 158–164. http://doi.org/10.1016/j.tmrv.2005.11.003

Rios, R., Sangro, B., Herrero, I., Quiroga, J., & Prieto, J. (2005). The role of thrombopoietin in the thrombocytopenia of patients with liver cirrhosis. *American Journal of Gastroenterology*, *100*(6), 1311–1316. http://doi.org/10.1111/j.1572-0241.2005.41543.x

Riss, T. L., Niles, A. L., & Minor, L. (2004). Cell Viability Assays Assay Guidance Manual. *Assay Guidance Manual*, 1–23.

Samtani, M. N., Perez-Ruixo, J. J., Brown, K. H., Cerneus, D., & Molloy, C. J. (2009). Pharmacokinetic and pharmacodynamic modeling of pegylated thrombopoietin mimetic peptide (PEG-TPOm) after single intravenous dose administration in healthy subjects. *Journal of Clinical Pharmacology*, *49*(3), 336–350. http://doi.org/10.1177/0091270008329559

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K. M., & Latha, L. Y. (2011a). EXTRACTION , ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM PLANTS ' EXTRACTS Institute for Research in Molecular Medicine (INFORM), Universiti Sains Malaysia , Minden 11800 ,. *Afr J Tradit Complement AlternMed.*, *8*(1), 1–10.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K. M., & Latha, L. Y. (2011b). EXTRACTION , ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS FROM PLANTS ' EXTRACTS Institute for Research in Molecular Medicine (INFORM), Universiti Sains Malaysia , Minden 11800 ,. *Afr J Tradit Complement AlternMed.*, *8*(1), 1–10.

Satpathy, G. R., Török, Z., Bali, R., Dwyre, D. M., Little, E., Walker, N. J., … Tsvetkova, N. M. (2004). Loading red blood cells with trehalose: a step towards biostabilization. *Cryobiology*, *49*(2), 123–36. http://doi.org/10.1016/j.cryobiol.2004.06.001

Sciences, B. (2009). In-vitro sensitivity pattern on some urinary tract isolates to Carica papaya extracts. *Bayero Journal of Pure and Applied Sciences*, *2*(2), 75–78.

Scott, K. L., Lecak, J., & Acker, J. P. (2005). Biopreservation of Red Blood Cells: Past, Present, and Future. *Transfusion Medicine Reviews*, *19*(2), 127–142. http://doi.org/10.1016/j.tmrv.2004.11.004

Shrivastava, M. (2009). The platelet storage lesion. *Transfusion and Apheresis Science : Official Journal of the World Apheresis Association : Official Journal of the European Society for Haemapheresis*, *41*(2), 105–113. http://doi.org/10.1016/j.transci.2009.07.002

Singh, V. K., Kumar, N., Kalsan, M., & Saini, A. (2015). In silico designing and optimization of EPO mimetic using combinatorial library, *4*(1).

Singh, V. K., Saini, A., Tsuji, K., Sharma, P. B., & Chandra, R. (2014). Manufacturing blood ex vivo: a futuristic approach to deal with the supply and safety concerns. *Frontiers in Cell and Developmental Biology*, *2*(June), 1–18. http://doi.org/10.3389/fcell.2014.00026

Slichter, S. J., Corson, J., Jones, M. K., Christoffel, T., Pellham, E., Bailey, S. L., & Bolgiano, D. (2014). Exploratory studies of extended storage of apheresis platelets in a platelet additive solution (PAS). *Blood*, *123*(2), 271–280. http://doi.org/10.1182/blood-2013-05-501247

Sommer, M. J., Rutman, M. G., Wask-rotter, E., Wagoner, H., & Fritsche, E. T. (n.d.). Determination of Calcium in Serum Samples by AAS Using a Fuel Lean, 1–6.

Subenthiran, S., Choon, T. C., Cheong, K. C., Thayan, R., Teck, M. B., Muniandy, P. K., … Ismail, Z. (2013). Carica papaya Leaves Juice Significantly Accelerates the Rate of Increase in Platelet Count among Patients with Dengue Fever and Dengue Haemorrhagic Fever. *Evidence-Based Complementary and Alternative Medicine : eCAM*, *2013*, 616737. http://doi.org/10.1155/2013/616737

Takatoku, M., Kametaka, M., Shimizu, R., Miura, Y., & Komatsu, N. (1997). Identification of functional domains of the human thrombopoietin receptor required for growth and differentiation of megakaryocytic cells. *The Journal of Biological Chemistry*, *272*(11), 7259–7263. http://doi.org/10.1074/jbc.272.11.7259

Talukdar, D., & Choudhury, D. (2010). Phytochemical screening and TLC profiling of plant extracts of Cyathea gigantea (Wall . Ex . Hook .) Haltt . and Cyathea brunoniana . Wall . ex . Hook . (Cl . & Bak .). *Asian University of Science & Technology*, *5*(1), 70–74.

Technical, P., & Sheet, I. (2012). Photometric Detection of Nicotinamide Adenine Dinucleotides, (July).

Teofili, L., Giona, F., Martini, M., Torti, L., Cenci, T., Foà, R., … Larocca, L. M. (2010). Thrombopoietin receptor activation, thrombopoietin mimetic drugs, and hereditary thrombocytosis: Remarks on bone marrow fibrosis. *Journal of Clinical Oncology*, *28*(19), 317–318. http://doi.org/10.1200/JCO.2010.29.0387

Thon, J. N., Schubert, P., & Devine, D. V. (2008). Platelet Storage Lesion: A New Understanding From a Proteomic Perspective. *Transfusion Medicine Reviews*, *22*(4), 268–279. http://doi.org/10.1016/j.tmrv.2008.05.004

Trusheva, B., Trunkova, D., & Bankova, V. (2007). Different extraction methods of biologically active components from propolis: a preliminary study. *Chemistry Central Journal*, *1*(1), 13. http://doi.org/10.1186/1752-153X-1-13

UNIVERSITI PUTRA MALAYSIA ANTIFUNGAL PEPTIDE MODELING , FOLDING AND MIMETIC DESIGN SHOEIB MORADI. (2009).

Vadhan-Raj, S. (2010). Clinical Findings With the First Generation of Thrombopoietic Agents. *Seminars in Hematology*, *47*(3), 249–257. http://doi.org/10.1053/j.seminhematol.2010.03.004

Volume, B. (2010). Escherichia coli, klebsiella pneumoniae and proteus vulgaris isolates, *3*(1), 195–198.

Wang, S., Shen, M., Xu, Y., Chen, F., Chen, M., Chen, S., … Wang, J. (2013). Rational and efficient preparation of a chimeric protein containing a tandem dimer of thrombopoietin

mimetic peptide fused to human growth hormone in Escherichia coli. *Applied Microbiology and Biotechnology*, *97*(7), 2885–2894. http://doi.org/10.1007/s00253-012-4553-7

Wolf, H. U., Diagnostics, M., Zander, R., & Lang, W. (2010). Development of the AHD 575 method, 1–21.

Xia, Y., Li, J., Bertino, A., & Kuter, D. J. (2000). Blood component s, *40*(August), 976–987.

Yamane, N., Takahashi, K., Tanaka, Y., Kato, K., Takayama, M., Ohyabu, N., … Takemoto, H. (2008). Discovery of novel non-peptide thrombopoietin mimetic compounds that induce megakaryocytopoiesis. *Bioscience Reports*, *28*(5), 275–285. http://doi.org/10.1042/BSR20080086

Yang, S. C. Scanning electron microscopy of normal human peripheral blood cells. *Taiwan Yi Xue Hui Za Zhi. Journal of the Formosan Medical Association*, *88*(11-12), 1128–32. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2636250

Yang, S. C. (2015). Scanning electron microscopy of normal human peripheral blood cells. *Taiwan Yi Xue Hui Za Zhi. Journal of the Formosan Medical Association*, *88*(11-12), 1128–1132.

Zhang, J. G., Carter, C. J., Culibrk, B., Devine, D. V., Levin, E., Scammell, K., … Gyongyossy-Issa, M. I. C. (2008). Buffy-coat platelet variables and metabolism during storage in additive solutions or plasma. *Transfusion*, *48*(5), 847–856. http://doi.org/10.1111/j.1537-2995.2008.01645.x

Zimrin, a. B., & Hess, J. R. (2009). Current issues relating to the transfusion of stored red blood cells. *Vox Sanguinis*, *96*(2), 93–103. http://doi.org/10.1111/j.1423-0410.2008.01117.x

10. APPENDIX

GC-MS specifications

```
====== Analytical Line 1 ======

[AOC-20i+s]
# of Rinses with Presolvent                    :5
# of Rinses with Solvent(post)                 :10
# of Rinses with Sample                        :2
Plunger Speed(Suction)                         :High
Viscosity Comp. Time                           :0.2 sec
Plunger Speed(Injection)                       :High
Syringe Insertion Speed                        :High
Injection Mode                                 :Normal
Pumping Times                                  :5
Inj. Port Dwell Time                           :0.0 sec
Terminal Air Gap                               :No
Plunger Washing Speed                          :High
Washing Volume                                 :6uL
Syringe Suction Position                       :0.0 mm
Syringe Injection Position                     :0.0 mm
Solvent Selection                              :All A,B,C

[GC-2010]
Column Oven Temp.          :60.0 °C
Injection Temp.            :260.00 °C
Injection Mode             :Split
Flow Control Mode          :Linear Velocity
Pressure                   :73.3 kPa
Total Flow                 :16.3 mL/min
Column Flow                :1.21 mL/min
Linear Velocity            :40.1 cm/sec
Purge Flow                 :3.0 mL/min
Split Ratio                :10.0
High Pressure Injection    :OFF
Carrier Gas Saver          :OFF
Splitter Hold              :OFF
Oven Temp. Program
  Rate                     Temperature(°C)          Hold Time(min)
   -                       60.0                     2.00
  5.00                     250.0                    6.00
  10.00                    280.0                    21.00

< Ready Check Heat Unit >
     Column Oven           : Yes
     SPL1                  : Yes
     MS                    : Yes
< Ready Check Detector(FTD) >
< Ready Check Baseline Drift >
< Ready Check Injection Flow >
     SPL1 Carrier          : Yes
     SPL1 Purge            : Yes
< Ready Check APC Flow >
< Ready Check Detector APC Flow >
External Wait              :No
Equilibrium Time           :0.5 min
```

[GC Program]

[GCMS-QP2010 Ultra]

IonSourceTemp	:230.00 °C
Interface Temp.	:270.00 °C
Solvent Cut Time	:3.50 min
Detector Gain Mode	:Relative
Detector Gain	:+0.00 kV
Threshold	:1000

[MS Table]

--Group 1 - Event 1--

Start Time	:4.00min
End Time	:69.98min
ACQ Mode	:Scan
Event Time	:0.20sec
Scan Speed	:2500
Start m/z	:40.00
End m/z	:500.00
Sample Inlet Unit	:GC

[MS Program]

Use MS Program	:OFF

Softwares used

Maestro server

Maestro is Schrödinger application which involves combination of various molecular modelling tools. It can be run on graphical interface or command bas on windows and Linux depends on the tools.

Prepwizard

Prepwizard tool is used to preparation of receptor proteins. This tool removes the water molecules and unwanted ligands attached to the receptor protein. It differentiates the different chains of the protein and minimization and optimization of the protein.

Pepfold

Pepfold tool denovo 3D structure prediction tool for peptide sequence. This gives PDB structure prediction in PDB format. It is tool of Mobyler server include the Pepfold another various peptide sequences analysis tools.

HEX

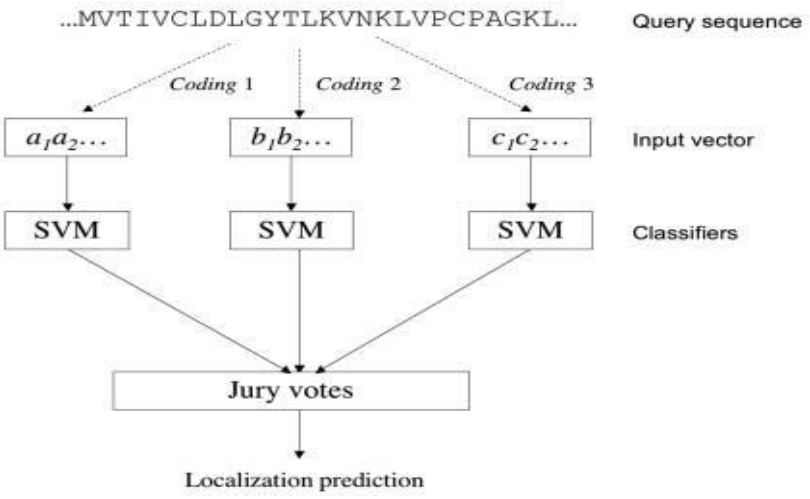
HEX server is protein docking analysis module. Receptor and ligand only in PDB format can be imported in the module. Receptor and ligands imported to the tool first prepared for removing water and unwanted molecules then interaction analysis executed. HEX results in PDB of ligand and receptor protein and with the log files reporting the E_{total} values for interaction. Default parameters and interaction domain have been set which can be changed according to the need.

Protein peptide calculator

Protein peptide calculator is designed to identify various properties of the target protein sequences. It determines the pI, molecular weight, stability prediction, hydrophilicity and hydrophobicity of the protein.

Cello

This is prediction tool for determining the sub cellular localization of target protein in the cell. It is support vector machine system which identifies physicochemical property and then predicts the location of target protein. Architecture is shown below-



Pymol

Pymol is freely available tool for the 3D view of PDB structure of proteins. It supports various known format for 3D structures. This tool discriminates the different chains and domain of protein structure for sharp view.