Methods to improve quality of stored blood

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

Master of Technology

In

Biomedical Engineering

Submitted by

SWEETY

(DTU/13/M.Tech/390)
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-110042, INDIA

CERTIFICATE

This is to certify that the M. Tech. Minor dissertation entitled **"Methods to improve quality of stored blood"**, submitted by **SWEETY (DTU/13/M.Tech/390)** 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: 20/07/2015

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, **Sweety (DTU/13/MTECH/390)** declare that M. Tech. dissertation entitled **"Methods to improve quality of stored blood"** 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: 20/07/ 2015 Signature:

Place: Delhi (SWEETY)

ACKNOWLEDGEMENT

I would like to express my profound gratitude and deep regards to various people who have helped and supported me for the building up of this project. I would like to extend my regards and express my greatest appreciation to my guide and project mentor **Dr. Vimal Kishor Singh** for his belief in my abilities and his exemplary guidance for the project. I am very grateful to him for giving me this opportunity to carry out such a novel and valuable work.

I am also grateful to my seniors, Mr. Abhishek Saini, Mr. Neeraj Kumar and Ms. Manisha Kalsan for the successful completion of my project.

Lastly, I would like to thank faculty and staff of department of Biotechnology, especially Mr. Chhail Bihari Singh and Mr. Jitendra Singh, for providing me a healthy environment to work on my project.

Sweety
2K13/BME/06

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

2, 3- DPG	2, 3- diphosphoglycerate
ACD	Acid citrate dextrose
AHD	Alkaline haematin method
ATP	Adenosine triphosphate
CPDA	Citrate phosphate dextrose- adenine
Hb	haemoglobin
NO	Nitric oxide
RBC	Red blood cell
SNO-Hb	S-nitrosohemoglobin
TRALI	Transfusion related acute lung injury
NIST	National Institute of Standard and Technology
SAGM	saline-adenine-glucose-mannitol
AAS	Atomic absorption spectroscopy
SEM	Scanning electron microscopy
GC-MS	Gas chromatography/mass spectrometry
TLC	Thin-layer chromatography

METHODS TO IMPROVE QUALITY OF STORED BLOOD

Sweety

Delhi Technological University, Delhi, India

1. ABSTRACT

Blood transfusion is common to the entire medical regimen now-a-days. Storing blood and blood components is very important in clinical and medical purposes. Patients with major operations or traumatic accidents need to replenish the blood loss and thus need a supply from an external source. During storage, various metabolic, structural and morphological changes called the storage lesions plays role in reducing the life of the blood. So, over the time various efforts have been made to increase the shelf life of blood switching from glass bottles to plastic bags and addition of various anticoagulants have also been reported. Even with the most updated storage method blood can no longer be stored for more than six weeks. So major challenges faced nowadays are increasing the shelf life, making the process of storage cost effective without comprising the quality. *Carica papaya* (*C. Papaya*) is used in the agricultural industry and many traditional medicines worldwide. For evaluation of blood storage parameters, we stored blood with anticoagulant CPDA along with papaya leaf extracts, in different solvents such as hexane, acetone, ethanol and water, for 6 weeks under aseptic conditions. The effect of extracts on the blood storage parameters were assessed by cell viability, change in pH, RBC shape modification by SEM, and alterations in haemoglobin content using Alkaline Hematin method (AHD). To investigate the role of *C. Papaya* leaf extracts on blood quality we evaluate the chemical compositions of the hexane, acetone, ethanol and water papaya leaf extracts using GC-MS and the compounds found were matched with the WILEY and National Institute of Standard and Technology (NIST) library. Analysis showed different peaks in which some active compounds are also present. So, it is important to identify those compounds in the extracts which can be used to enhance the shelf life of blood.

Keywords- Stored blood, RBC, AHD, shelf life and *Carica papaya*

2. INTRODUCTION

Blood is a highly specialized tissue composed of various components, namely Red blood cells, Platelets, White blood cells, Plasma, and each of them functions separately. The most abundant cells in blood are red blood cells. They are produced continuously in our bone marrow by the process known as erthropoiesis. Erythropoiesis includes hematopoietic progenitor cells, which are first differentiated into erythroid progenitors and then converted to functional red blood cells in a regulated manner. This dynamic process is regulated through a number of growth factors which maintain the regular homeostasis of the red blood cells. The entire process of erythropoiesis occur in three stages: (a) Erythropoietic commitment of hematopoietic stem cells which includes development of erythroid committed blast cells from multipotent hematopoietic progenitors; (b) Division and differentiation of these erythroid progenitor cells and (c) Terminal differentiation including morphological changes i.e. enucleation, to produce reticulocytes and then ultimately maturation of red blood cells.

Preservation of blood is needed to ensure a readily available blood supply for transfusion. Blood transfusion is the main source for critical conditions where the patient suffering from life threatening diseases like leukaemia, thalassemia and haemophilia and in case of critical injuries or trauma to prevent patient from this condition and save their life. So for fulfilment of blood requirements every country has their own blood bank which stores blood. We know that the shelf life of RBCs in human body is 120 days but during storage the shelf life reduces from 120 days to 42 to 49 days and this major change in shelf life occurs due to some biochemical changes and these changes are called as storage lesions. This includes decrease in pH of blood due to increase the concentration of lactic acid which is produced by glycolytic pathway, deformability in the shape of RBCs, decrease in cell viability, loss of membrane and formation of micro particles. Other changes include the change in the concentration of ions in extra and intracellular medium like sodium, potassium and calcium ions. These changes cause the lysis of RBC which releases free haemoglobin in stored blood and we know that haemoglobin contain iron molecules which evoke immune responses when transfused in patients and can also cause diseases like multiple organ failure, transfusion associated acute lung injury, anaphylactic reactions, fluid overload, acute intravascular haemolysis, bacterial contamination and septic shock and sometimes it even causes death in recipient of old haemolysed blood.

To overcome these situations various alternate methods have been developed to get blood from non donor derived sources. Researchers have developed different artificial molecules that mimic the functionalities of RBCs. Haemoglobin based oxygen carriers or perflorocarbon molecules have been mainly used but they have been reported to be inefficient as compared to oxygen carrying capacity of RBCs and perflorocarbon molecules have limited functional applications.

Carica papaya (*C. Papapa*) is a plant with medicinal properties, especially used in the agricultural industry. Papaya leaves are used for the treatment of diabetes mellitus, inflammation, dengue fever and for dressing septic wounds [32]. The therapeutic effects of papaya leaf extracts are due to some bioactive compounds present which show antiasthamatic, antifungal and antioxidants properties. These antioxidants compounds reduce lipid peroxidation, exhibit immune modulatory effects and antitumor activity [36].

Some studies suggest that *C. Papaya* leaf extracts can stabilize the erythrocyte membrane [46] and may increase red blood cells and platelet counts [44]. This shows that the *C. papaya* leaves extract have some active components which can improve the quality of stored blood. To examine the potential role of papaya to enhance the stored blood quality, we analyzed the papaya leaf extracts in different solvents with increasing polarity by GC-MS. The purpose of this study is to identify the active components of *C. Papaya* and use them to enhance the shelf life of stored blood. By storing blood and its components for a longer period of time, both the patients and blood banks will be benefitted. Patients will receive more viable cells experiencing less RBC breakdown. Blood banks will also be benefited from decreased shipping, longer inventory and reduced wastage.

3. REVIEW OF LITERATURE

3.1 Bio-preservation of Blood

Bio-preservation is the process of maintaining the functionality and integrity of cells outside the native environment for extended storage period [46]. Different biopreservation protocols and storage methods have been developed for cells, whole blood, and blood products. Further improvement of the existing methods requires matching the increasing demand of the medical technologies.

A wide variety of injuries and medical conditions where the patient suffering from life threatening diseases like leukaemia, thalassemia and haemophilia and in case of critical injuries or trauma require transfusion of red blood cells [44]. Red blood cell transfusions save lives by increasing RBC mass that have low oxygen–carrying capacity due to increased RBC loss (traumatic/surgical hemorrhage), defective haemoglobin (hemaglobinopathies and thalassemias), decreased bone marrow production (aplastic anemias), and decreased RBC survival (hemolytic anemias) [46]. Thus, there is an urgent need for safe blood and blood products. Therefore, biopreservation of blood is needed to ensure a readily available blood supply for transfusion.

Even with the most updated storage method blood can no longer be stored for more than six weeks. The currently additive solutions for blood storage are saline-adenine-glucose-mannitol (SAGM), Nutricel (AS-3), ADSOL (AS-1), and Optisol (AS-5) where the storage duration is 42 days [19, 25]. These solutions do not fully preserve blood viability and function. At storage temperature cellular metabolism is not completely suppressed and hence the preservation time decreases. So major challenges faced nowadays is increasing the shelf life, making the process of storage cost effective without comprising the quality.

3.2 Changes that occur in stored whole blood

- **Storage effect on red cell metabolism**: For the formation of ATP, RBCs are dependent on anaerobic glycolytic pathway, which plays a role in maintaining its shape and viability. To keep the cells viable during storage the metabolic cycle of cells must continue.
- **Effect on 2, 3 DPG and O_2 release**: 2, 3 DPG binds to de-oxy Hb forming a complex with low O_2 affinity. In stored blood, 2, 3-DPG is depleted, resulting in a

shift of O_2 dissociation curve to the left. Depletion of 2, 3 DPG affects O_2 release by Hb.

- Cellular integrity of cells cannot be maintained in vitro (failure of Na^+/K^+ ATPase). Some RBCs become spherical which increases cell rigidity. If stored blood is transfused after 28 days, 10 to 20% RBCs get destroyed within 24hrs [26].
- **Effect on pH**: During storage period there is a gradual fall in pH due to accumulation of lactic acid. 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.
- **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 transfusions, blood less old than 5 days is recommended due to high K^+ content of stored blood.
- **Oxidative damage**: Red blood cells are susceptible to oxidative damage [31], because;

  1) It is exposed to high oxygen tension.

  2) It has no capacity to repair its damaged components.

  3) Its membrane components are susceptible to lipid peroxidation.

  4) The hemoglobin is susceptible to autooxidation.

  Oxidative stress induces generation of free radicals which can react with cellular macromolecules which may lead to protein oxidation and lipid peroxidation. Lipid peroxidation can lead to cell membrane's damage and protein oxidation which can lead to cytoskeleton and cytosolic protein damage.
- **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. Refrigeration and freezing minimizes the proliferation of bacteria that can enter the blood unit during venepuncture.
- **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 remain viable and in whole blood, platelets lose their function within 48 hrs at 4°C.

- **Anticoagulant solutions:** Different anticoagulants have different storage time. With trisodium citrate rapid deterioration takes place and after 1 week only 50% cells are viable. Blood with ACD and CPD can be stored for 28 days with 24 hrs survival 77%. In CPDA blood can be stored for 35 days. Addition of adenine maintains high ATP level and improved the storage of blood.

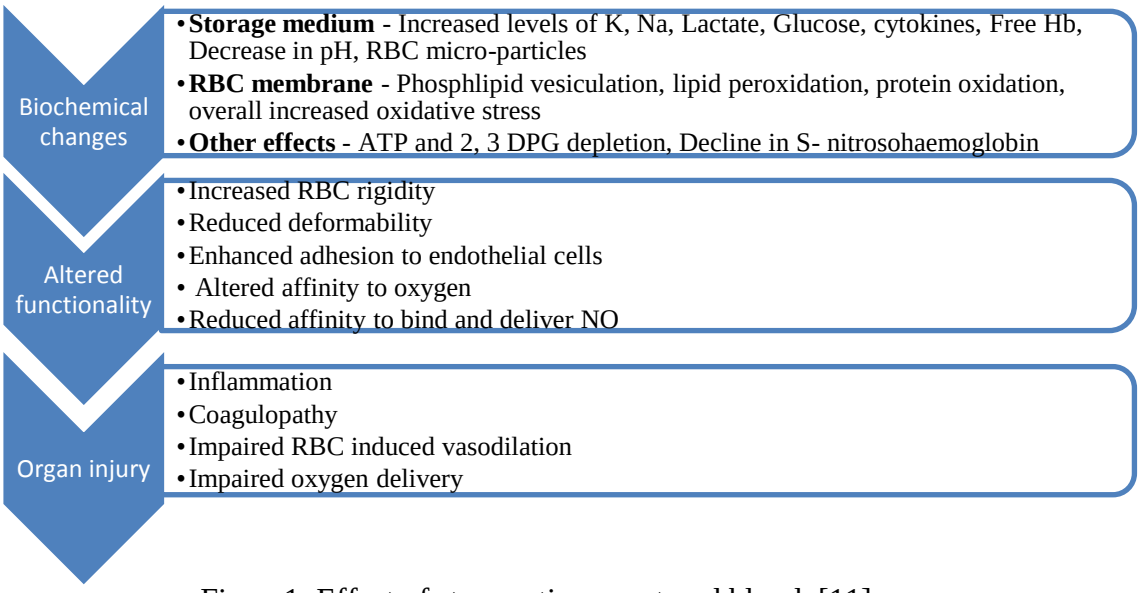


Figure1: Effect of storage time on stored blood. [11]

3.3 Parameters to study:

The blood storage parameters like cell viability, change in pH, RBC shape modification, alterations in haemoglobin content and change in the concentration of ions (Na^+, K^+ & Ca^{2+}) in extra and intracellular medium were assessed during storage period.

3.3.1 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 [42]. The flow cytometric method using acetoxymethyl ester of calcein (calcein-AM) and ATP measurement using firefly luciferase are commonly used methods for viable cells estimation.

3.3.2 Hemoglobin estimation

Techniques for measuring hemoglobin:

- Photometric techniques
- Visual comparative Techniques

Drabkin's method

Drabkin's Reagent is used for quantitative, 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 were 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 hemoglobin and its derivatives; potassium ferricyanide (oxidizing agent) converts the ferrous iron of hemoglobin to ferric state– methaemoglobin to methemoglobin. This combines with potassium cyanide to form cyanmethemoglobin, which has absorption at 540 nm. The hemoglobin concentration in solutions is proportional to the color intensity measured at 540 nm [21].

Alkaline Hematin Method

To measure hemoglobin photometrically, Hb should convert to a colour compound to be measured easily. A reagent should be used that contain chemicals which react with Hb to form a colored compound. The Alkaline Hematin D reagent can be used. This reagent contains Sodium hydroxide (NaOH), Triton X-100 (or equivalent) and distilled water. 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 is equal to the concentration of Hb. When Alkaline Hematin D reagent is added to blood sample, the hemoglobin present is converted to alkaline haematin, a stable coloured compound. Then, the absorbance of alkaline hematin formed is measured at 575±5 nm [29].

3.3.3 Electrolytes estimation

The changes in the concentration of ions (Na^+, K^+ & Ca^{2+}) in extra and intracellular medium were assessed by atomic absorption spectroscopy. Atomic absorption spectroscopy (AAS) is a technique used for the determination of a large number of metals present in the sample. In AAS, an aqueous sample which contains the metal analyte is aspirated into flame, causing vaporization of the free metal atoms in the sample and evaporation of solvent. This process is known as atomization. Every

atom in the sample has its own pattern of wavelengths at which it absorbs energy. Absorbance obtained is directly proportional to the concentration of analyte. The concentration is determined from a calibration curve of standards with known concentrations.

3.3.4 **Morphological changes**

To investigate the morphology of blood cells, drawn blood samples were studied with scanning electron microscopy (SEM). The Blood cells were fixed with 2.5% 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 [54].

3.3.5 **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. Various methods are used to avoid this loss; many require separation of cellular components. The removal of blood cells allows plasma glucose measurement, which reflects the composition of extracellular fluids independent of hematocrit.

3.3.6 **Blood Cell count**

Red blood cells were counted by haemocytometer or automated hematology system. Automated hematology system, in principle, is based on methods: RF/DC detection method, Hydro Dynamic Focusing and flow cytometry method. The RF/DC detection method detects the density of blood cells interior by changes in radio frequency resistance and the size of blood cells by measuring the changes in direct current resistance. In Hydro Dynamic Focusing method, blood cells passes through aperture in a line which prevents abnormal blood cell pulses generation. Flow Cytometry method is based on light scattering. By measuring the intensity of scattered light, information about cell size and material properties is obtained.

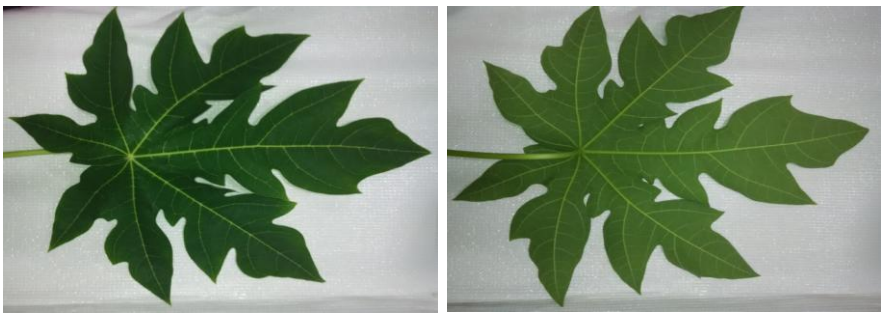
For red blood cell count in haemocytometer, centrifuged blood suspended pellets were diluted with PBS and red blood cell numbers were counted on inverted microscope with 10X and 40X lens. To investigate the morphology of blood cells, drawn blood samples were studied with scanning electron microscopy (SEM).

3.4 *Carica papaya* is a member of the family Caricaecae. It is a common papaya and extensively grown over the world. The plant is soft tissued, 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 [45]. 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.

Carica papaya is a well known fruit with medicinal properties in extracts and different parts of the papaya species. It shows abortifacient activity in roots and immature fruits post testicular anti fertility drug activity in seeds, anthelmintic activity in latex of plant and wound healing properties in extracts of seeds and fruit pulp. Also it is used to relieve the symptoms of fever, asthma, treatment in the gastric problems, cardiovascular risks and amoebic dysentery reduction etc [40].

Carica papaya leaves are used in treatment of dengue, jaundice, malaria, immunomodulatory and antiviral activity. Papaya leaves are mainly rich in phenolic compounds, flavonoids, cynogenetic compounds and alkaloids. Both leaves and fruits of papaya contains carotenoids namely β- carotene, anthraquinones glycoside, lycopene and shows medicinal properties like abortifacient, anti-inflammatory, wound healing, hypoglycaemic, antifertility, hepatoprotective and recently studies are going on its antihypertensive and antitumor activities [5].

However, the knowledge of the phytocomponents present in the papaya leaves is very less. To investigate the role of the compounds on biological system, the study of active components from the crude plant extracts is necessary. Mainly Gas chromatography/mass spectrometry (GC-MS) is commonly used in the phytochemical research. [27]



Dorsal view Ventral view

Figure 2: Macroscopic characters of *C. papaya* leaves

3.4.1 Extraction

In the analysis of medicinal plants, extraction is a crucial step and it is required to extract the desired chemical constituents from the plant extracts for further characterization. The basic steps are pre-washing, drying of plant leaves, grinding into fine powder which improves the yield of extraction and also increasing the sample surface contact area with the solvent system. Proper actions should be taken to assure that active components are not lost or destroyed during the process of plant extract preparation. The selection of solvent depends on the nature of targeted bioactive compounds present. Different solvents are available to extract the bioactive components. For extraction of hydrophilic compounds polar solvents are used like ethanol, methanol or ethyl-acetate. The target compounds can be polar to non-polar and thermally labile; hence the suitable extraction methods must be considered. Various methods like soxhlet extraction, sonification, heating under reflux, maceration and others 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 increase efficiency of extraction. [43, 51]

Figure 3: Soxhlet extraction apparatus

3.4.2 Identification and characterization

The plant extract contains various types of bioactive compounds/phytochemicals with different polarities; their separation remains a big challenge for characterization and identification of bioactive compounds. A number of different separation techniques are used for isolation of these bioactive compounds like column chromatography, Sephadex chromatography, flash chromatography, TLC, and HPLC. Besides that, some non-chromatographic techniques like phytochemical screening assay, FTIR, immunoassay, which use monoclonal antibodies (MAbs), can also be used for the identification of bioactive compounds.

3.4.3 Thin-layer chromatography (TLC)

TLC is a simple and inexpensive procedure that gives an idea of how many components are present in a mixture. It is also used to identity an unknown compound in a mixture by comparing the R_f of an unknown compound with a known compound. For visualization the spray of phytochemical screening reagents are used, which changes colour of bands according to the phytochemicals present in extract; or by viewing the TLC plate under UV light. This can also be used for confirmation of identity and purity of isolated compounds.

3.4.4 Phytochemical screening assay

Phytochemicals are secondary metabolic compounds present in plants. Phytochemical screening assay is simple and inexpensive procedure that gives an idea of various phytochemicals present in a mixture. 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.

Some studies suggest that *C. Papaya* leaf extracts can stabilize the erythrocyte membrane [41] and may increase red blood cell and platelet counts [15]. A recent trial showed that platelet count increases after 40-48 hours of papaya leaves juice dose [48]. This shows that the *C. papaya* leaves extract have some active components which can improve the quality of stored blood. To examine the potential role of papaya to enhance the stored blood quality, we analyzed the papaya leaf extracts of different solvents with increasing polarity by GC-MS. The purpose of this study is to identify the active components of *C. Papaya* and used them to enhance the shelf life of stored blood.

4. OBJECTIVE

- To identify bioactive components in papaya leaf extracts to improve blood shelf life.
- To study quality parameters of stored blood in papaya leaf extracts

5. METHODOLOGY

5.1 Material requirements

A. Papaya leaves 50 gm

B. Soxhlet extraction

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

C. Shimadzu GC-MS QP2010 plus system

D. TLC solvent system:-

Chloroform: methanol=9:1, n-hexane: acetone=8.5:1.5 and benzene: ethyl acetate=1:1.

E. Blood from volunteers.

F. Hemoglobin estimation

Table 1: AHD reagent preparation (pH 11.8)

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

G. pH estimation

pH strips and pH meter.

H. Cell counting

Sysmex XE-2100

I. Cell morphology

Inverted microscope and SEM

5.2 Methods

5.2.1 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.

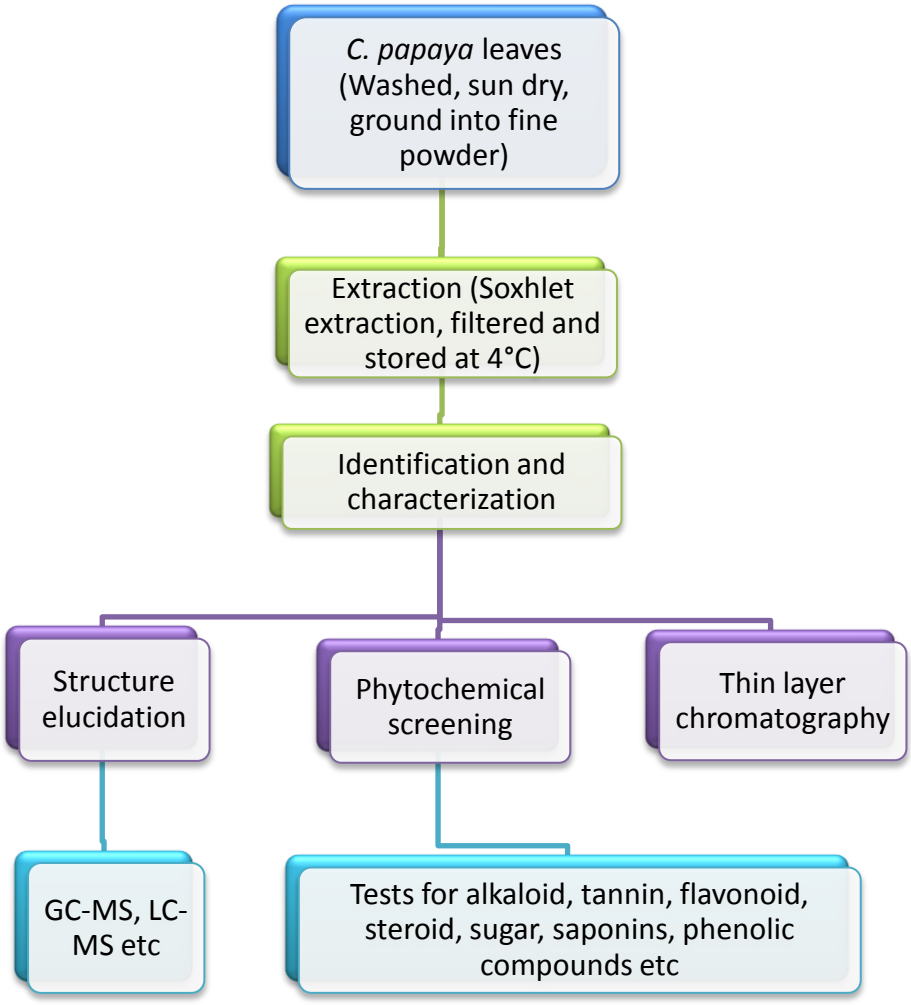


Figure 4: A brief summary of extraction, identification and characterization of bioactive compounds from papaya leaf extracts

5.2.2 Extraction Papaya leaves extract was prepared 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 then 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

5.2.3 GC-MS analysis

GC-MS analysis of the *C. Papaya* leaves extracts was 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.

Shimadzu GC-MS QP2010 plus system

5.2.4 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 spectra were interpreted using WILEY and National Institute of Standard and Technology (NIST) library having large number of patterns. The name, structure and molecular weight of the components of the extracts were identified.

5.2.5 Phytochemical screening assay

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

1. **Test for flavonoids:**

   NaOH test: - 3ml of the extracts were treated with 1ml of 10% w/v NaOH, followed by addition of dilute HCl. A yellow solution with NaOH, turns colorless with dilute HCl indicating the presence of flavonoids [35].

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 [39].

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 colouration of the sample indicated tannin [39].

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 [39].

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.

5.2.6 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) which was calculated for each solvent system for different samples.

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

5.2.7 Blood sample preparation

1. Collect blood sample from the donor 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. Labelled properly with name, date.
2. Maintained aseptic condition throughout the process and transfer of sample was done only in laminar hood using autoclaved tips, pipettes and eppendorffs and gloves were worn and hand were wiped with alcohol or spirit.

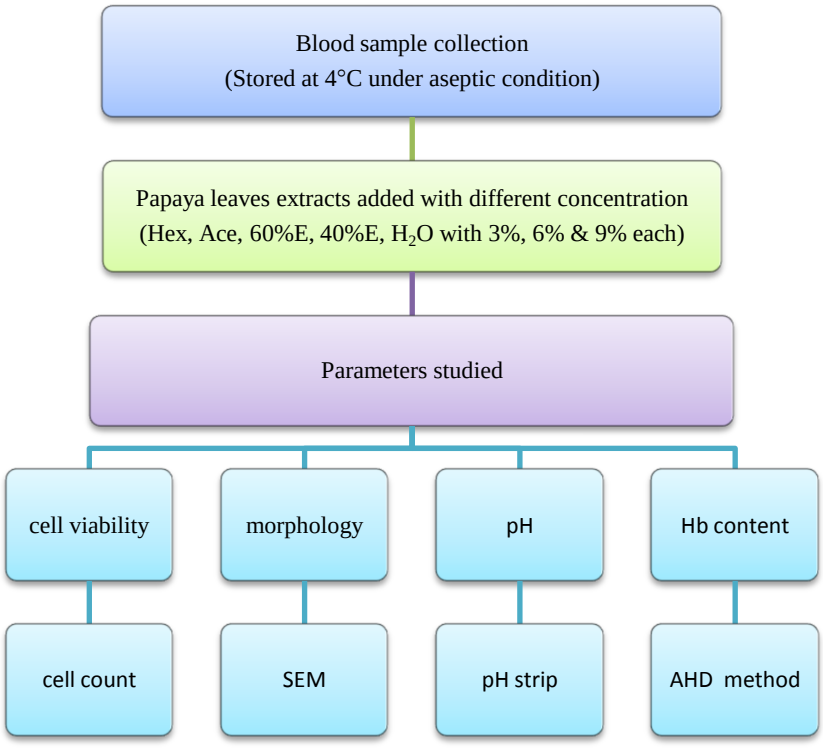


Figure 5: Methodology at a glance

5.2.8 Addition of extracts

Papaya leaves extraction was carried out by sequential Soxhlet extraction with different solvent of increasing polarity using 250 ml of hexane, acetone, 60% ethanol, 40% ethanol and water. The extracts obtained after extraction contained organic solvents hexane, acetone and ethanol which could affect the blood cells if we directly add the extracts into blood. To remove these solvents the extracts were converted to crude sample by evaporating the solvent of sample. These crude samples were then dissolved in phosphate buffered saline (PBS) to be added into blood samples. The resulting extracts were then filtered using 0.2µ syringe filter and the filtrates were added with different concentration (Hex, Ace, 60%E, 40%E, H_2O with 3%, 6% & 9% each) into blood under aseptic conditions.

5.2.9 Parameters studied:-

5.2.9.1 Haemoglobin 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 575±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.

5.2.9.2 Cell counting

Red blood cells are count by automated hematology system Sysmex XE-2100.

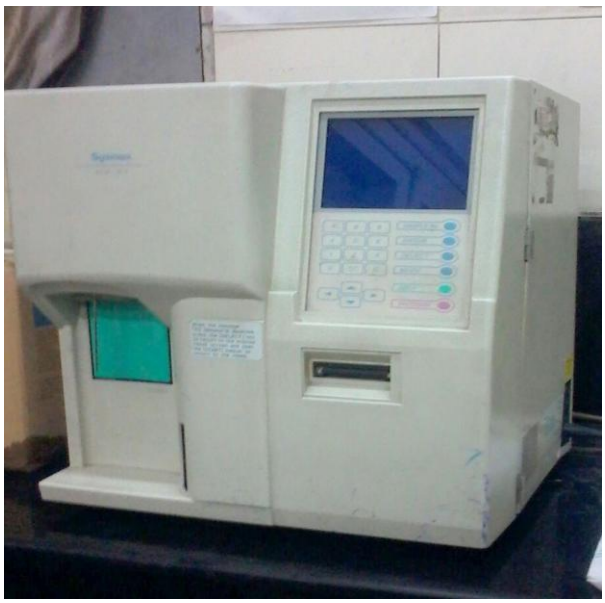


Figure 6: Sysmex XE-2100 automated hematology system for blood cells count

5.2.9.3 Morphology of RBC

To investigate the morphology of blood cells, drawn blood were studied with scanning electron microscopy (SEM). It was studied 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 during storage.

6 RESULTS

6.1 Papaya leaf extracts

Table 2: Colors of papaya leaf extracts

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

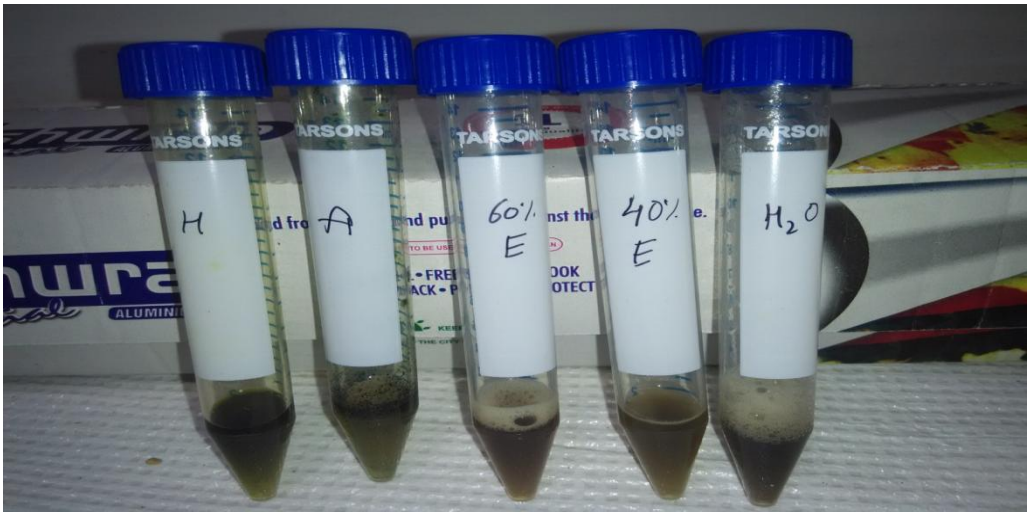


Figure 7: Papaya leaf extracts

6.2 Phytochemical screening assay

Table 3: 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	-	+	+	+	+

6.3 TLC Analysis of the Fractions:

Table 4: The retention factor (R_f) values of each extracts of papaya leaves in different solvent system

1. N-hexane: acetone=8.5:1.5

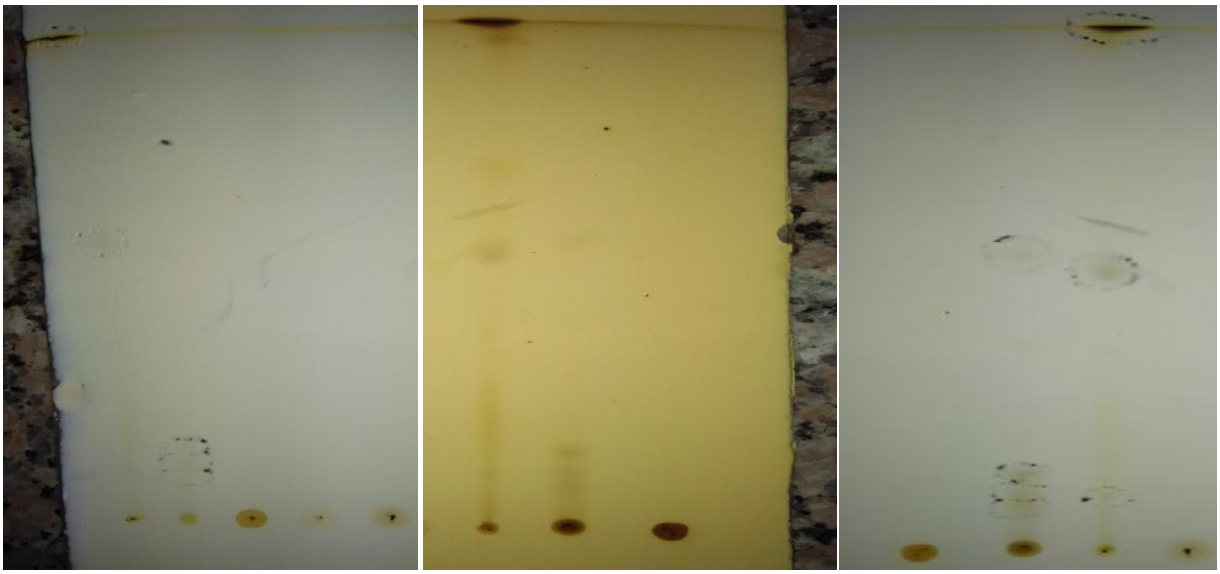
S. No.	Extract	No of spots	rf values
1.	Hexane	5	0.104, 0.174, 0.383, 0.539, 0.991
2.	Acetone	5	0.087, 0.113, 0.156, 0.383, 0.565
3.	60%Ethanol	0	-
4.	40% Ethanol	0	-
5.	Water	0	-

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)

Figure 8: TLC analysis of papaya leaf extracts with different solvents

6.4 GC-MS analysis

a) Hexane extract

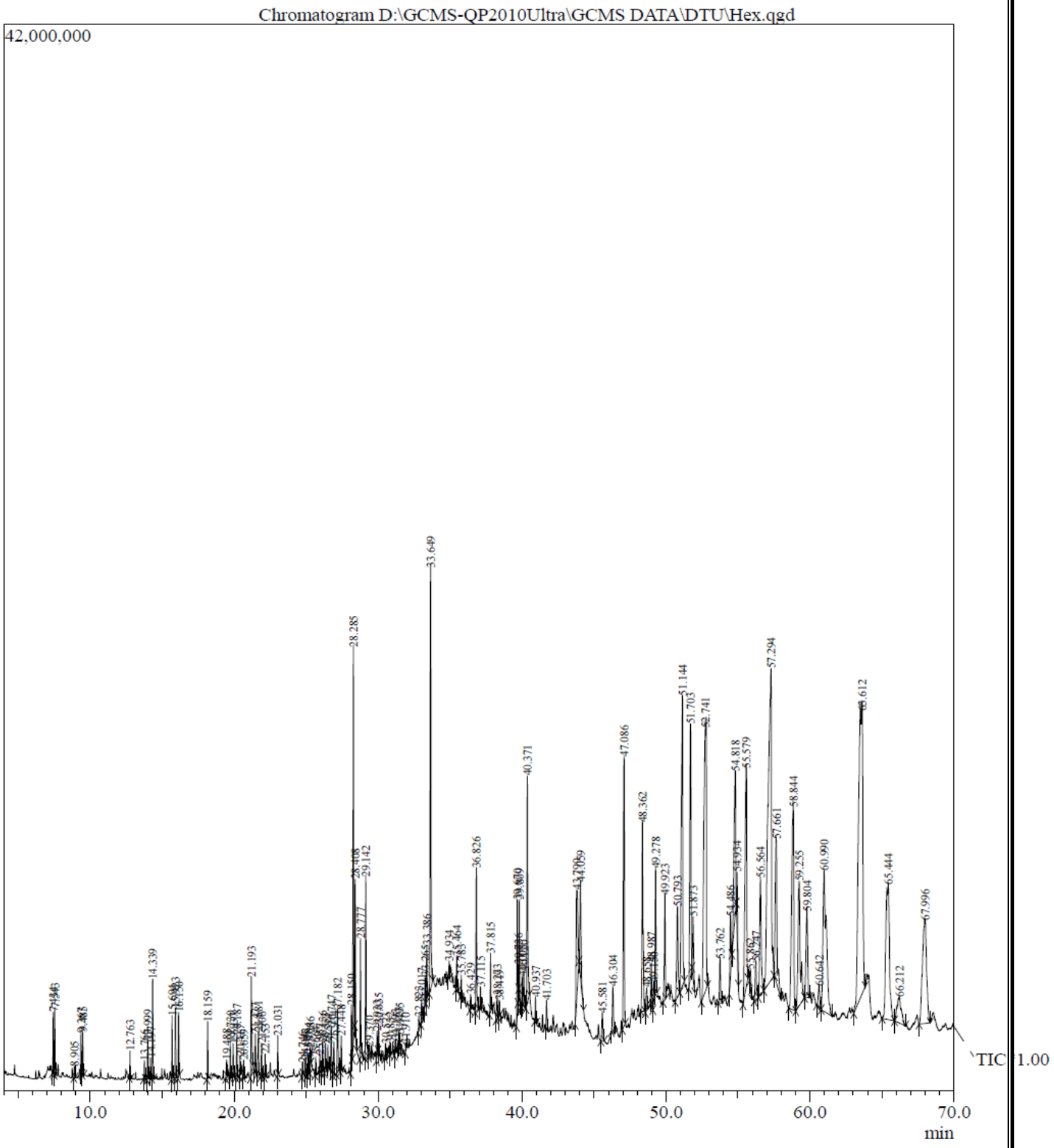


Figure 9: Graph Showing the GC-MS analysis of hexane extract

Table 5: Phytocomponents identified in hexane extract of papaya leaves by GC-MS

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 [27].
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 [27].
3.	28.150	3,7,11,15Tetra methyl-2-hexadecanoic acid	Terpene alcohol	$C_{20}H_{40}O$	296.53	0.32	Flavor and Lubricanting agent [27].
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 [6].
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

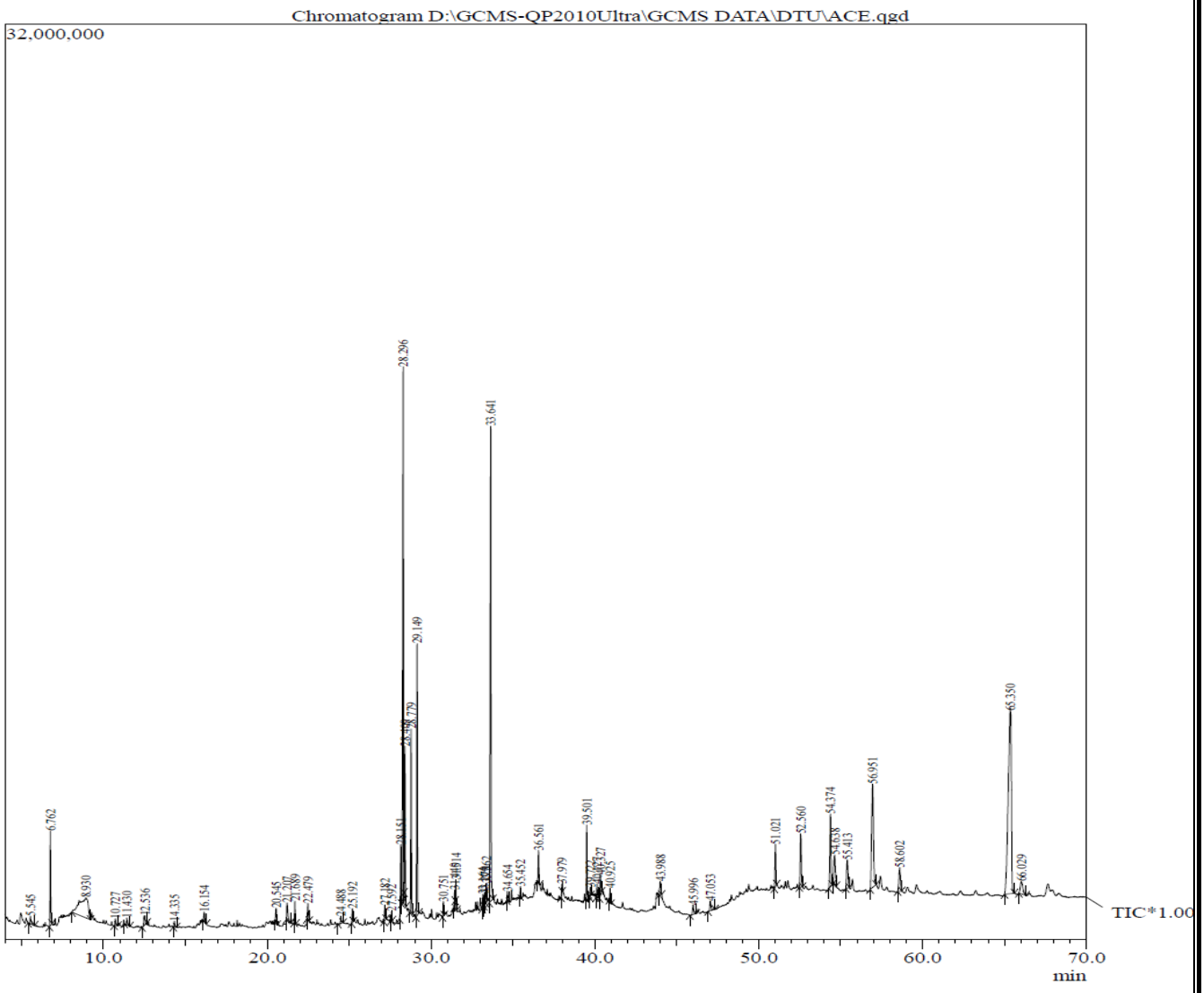


Figure 10: Graph Showing the GC-MS analysis of acetone extract

Table 6: Phytocomponents identified in acetone extract of papaya leaves by GC-MS

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 [27].
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	17.70	Flavor and Lubricanting agent [27].
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 [13].
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 [27].
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 [27].
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

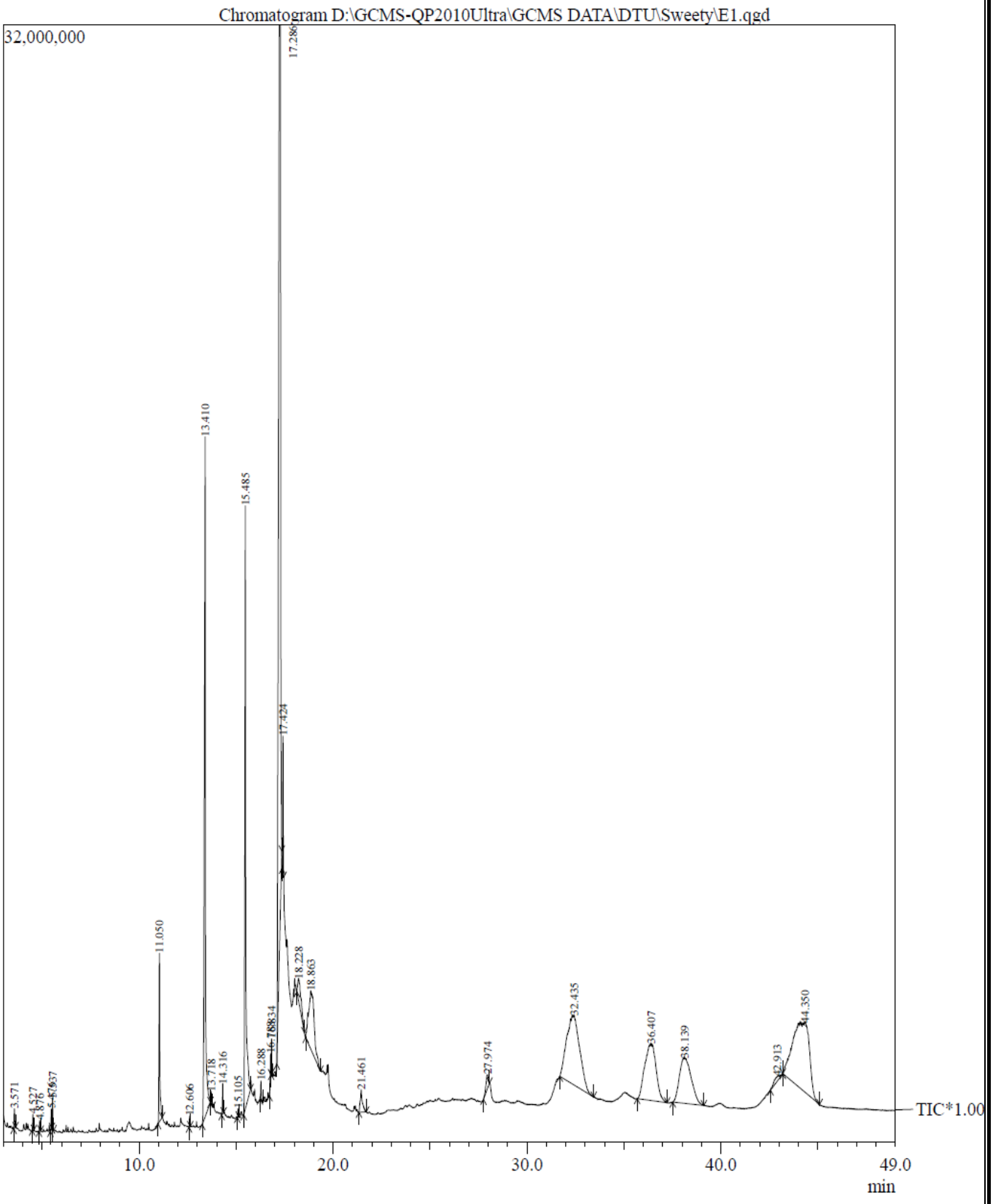


Figure 11: Graph Showing the GC-MS analysis of 60% Ethanol extract

Table 7: Phytocomponents identified in 60% Ethanol extract of papaya leaves by GC-MS

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 (DDMP)	-	$C_6H_8O_4$	144	0.22	Strong antioxidant activity [6].
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 [13].

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 [6].
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 [13].

d) 40% Ethanol extract

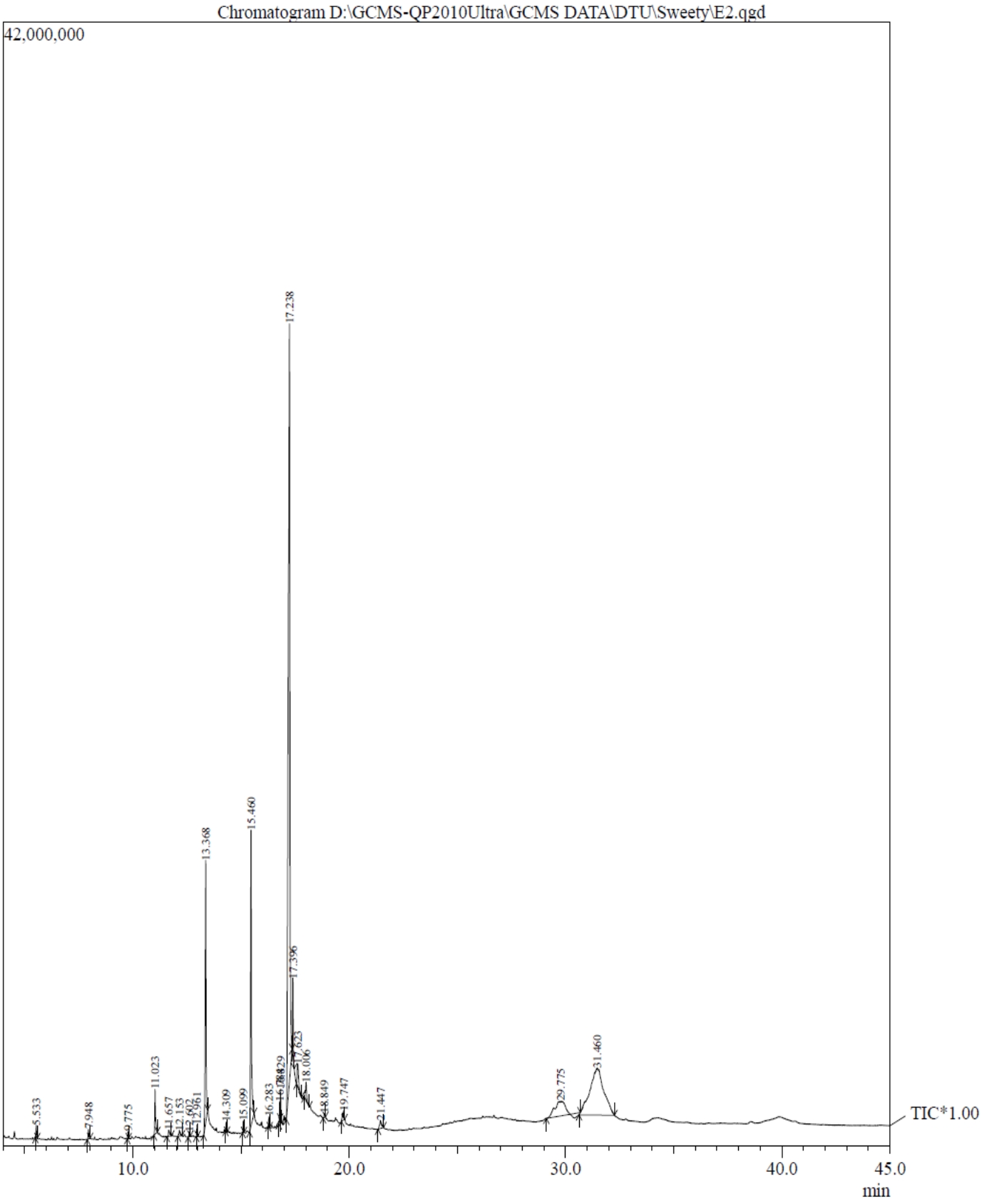


Figure 12: Graph Showing the GC-MS analysis of 40% Ethanol extract

Table 8: Phytocomponents identified in 40% Ethanol extract of papaya leaves by GC-MS

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 [27].
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 [13].
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 [6].
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 [13].
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

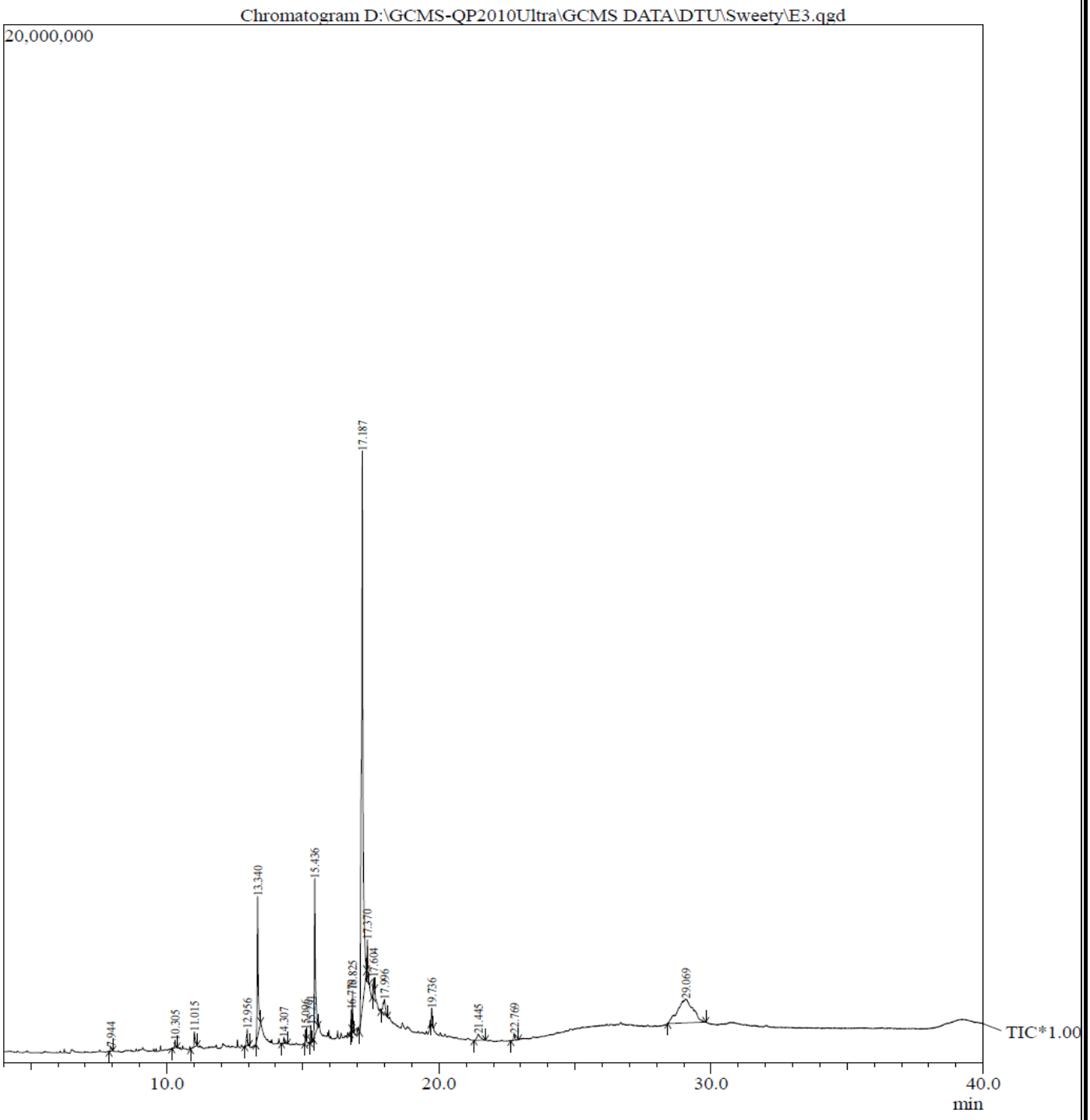


Figure 13: Graph Showing the GC-MS analysis of water extract

Table 9: Phytocomponents identified in water extract of papaya leaves by GC-MS

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 [27].
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 [13].
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 [6].
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 [13].

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.

6.5 Haemoglobin Estimation

Table 10: AHD standard readings

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

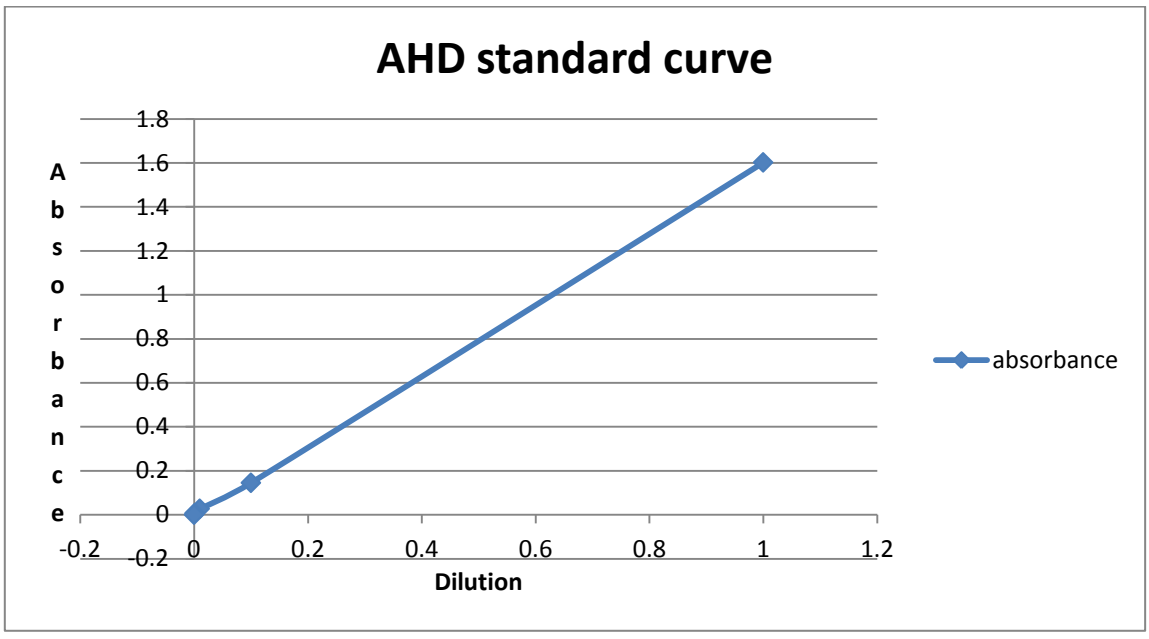


Figure 14: AHD standard curve

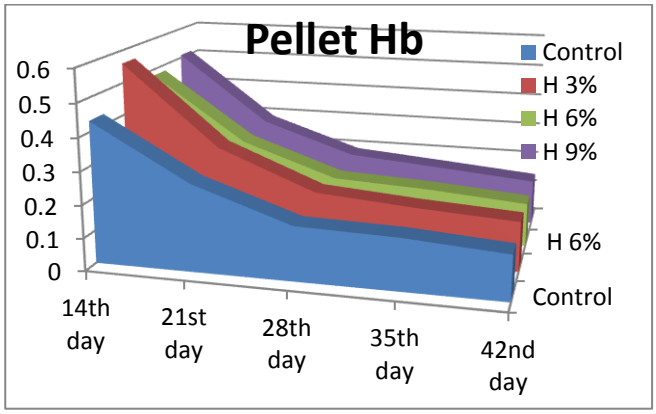
Table 11: AHD readings

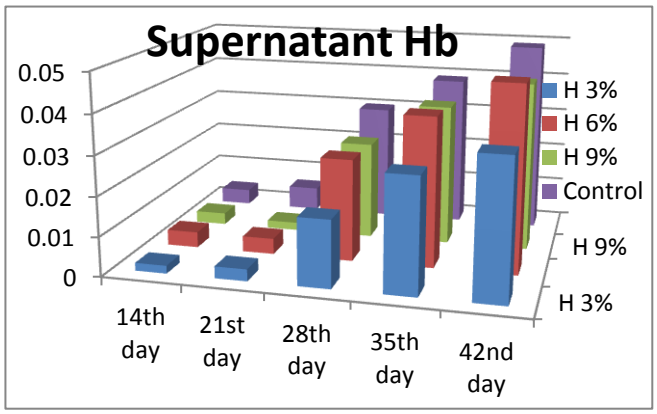
Controls	Pellet	Supernatant
Positive control (Fresh blood+CPDA)	0.585	0.001
Negative control (100% Lysis)	0.015	0.487

Absorbance of each extracts by AHD method

Extracts	14^{th} day		21^{st} day		28^{th} day		35^{th} day		42^{nd} day	
	Pellet	SN	Pellet	SN	Pellet	SN	Pellet	SN	Pellet	SN
H 3%	0.560	0.002	0.314	0.003	0.195	0.017	0.171	0.029	0.153	0.035
H 6%	0.480	0.004	0.276	0.004	0.174	0.026	0.159	0.038	0.135	0.047
H 9%	0.509	0.003	0.290	0.002	0.192	0.025	0.167	0.036	0.141	0.043
A 3%	0.511	0.001	0.359	0.003	0.234	0.020	0.197	0.030	0.172	0.038
A 6%	0.506	0.003	0.317	0.004	0.187	0.024	0.173	0.035	0.157	0.042
A 9%	0.499	0.003	0.267	0.005	0.183	0.028	0.170	0.037	0.151	0.047
60% E 3%	0.519	0.002	0.322	0.004	0.200	0.023	0.183	0.031	0.164	0.040
60% E 6%	0.434	0.004	0.203	0.005	0.166	0.029	0.154	0.038	0.133	0.051
60% E 9%	0.303	0.005	0.194	0.006	0.160	0.031	0.152	0.039	0.131	0.053
40% E 3%	0.429	0.004	0.268	0.005	0.161	0.033	0.146	0.041	0.097	0.055
40% E 6%	0.436	0.002	0.301	0.004	0.204	0.022	0.168	0.028	0.147	0.037
40% E 9%	0.426	0.005	0.265	0.006	0.165	0.035	0.157	0.039	0.136	0.045
H_2O 3%	0.513	0.001	0.329	0.003	0.216	0.023	0.206	0.032	0.188	0.041
H_2O 6%	0.501	0.002	0.310	0.005	0.198	0.025	0.174	0.035	0.153	0.041
H_2O 9%	0.431	0.004	0.262	0.007	0.166	0.029	0.115	0.039	0.123	0.051
F 3%	0.561	0.002	0.331	0.004	0.212	0.024	0.210	0.027	0.192	0.034
F 6%	0.504	0.002	0.305	0.005	0.203	0.025	0.189	0.028	0.168	0.036
F 9%	0.501	0.003	0.275	0.006	0.197	0.029	0.181	0.031	0.165	0.037
Control	0.433	0.004	0.267	0.006	0.166	0.030	0.158	0.039	0.137	0.049

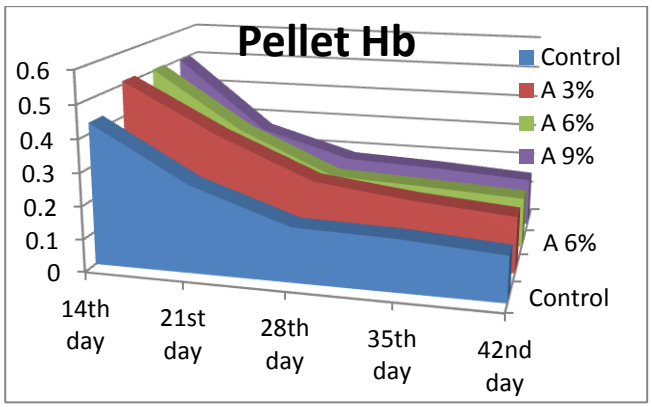
Hexane

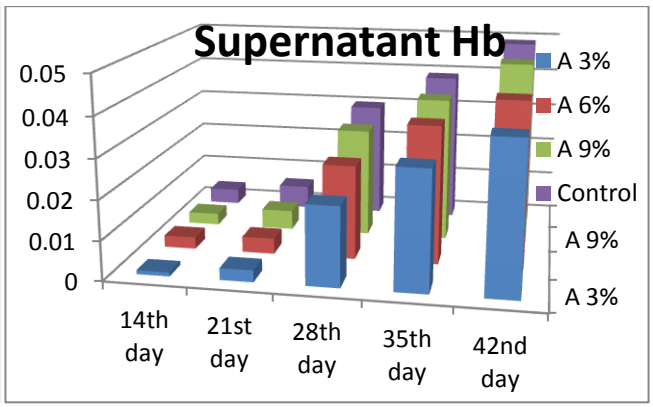




In pellet the absorbance of 3%, 6% & 9% hexane (H) extracts increase while in supernatant the absorbance of each hexane extracts decrease as compared to control. This shows the haemolysis of RBCs in extracts is less than control during storage period.

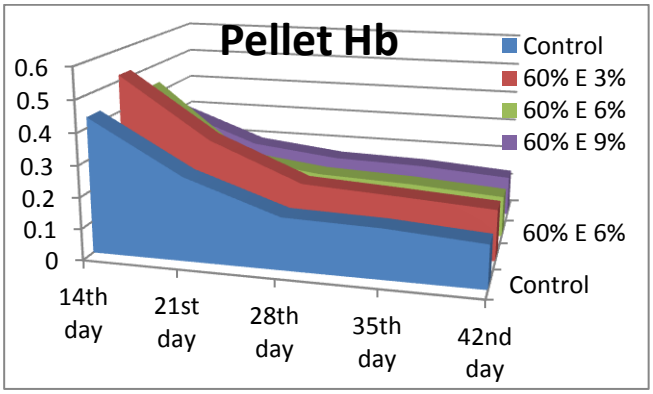
Acetone

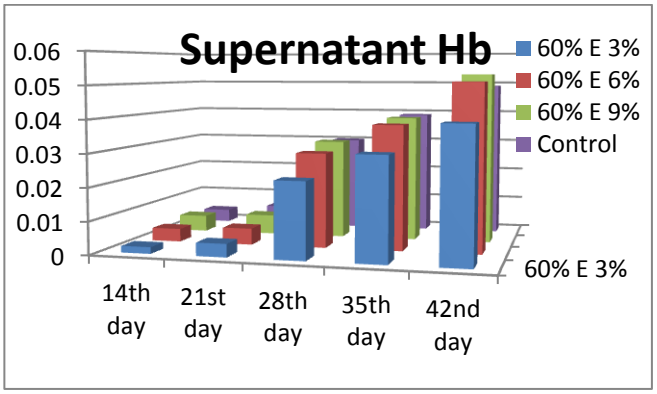




In pellet the absorbance of 3%, 6% & 9% acetone (A) extract increase while in supernatant the absorbance of each acetone extracts decrease as compared to control. This shows the haemolysis of RBCs in extracts is less than control during storage period.

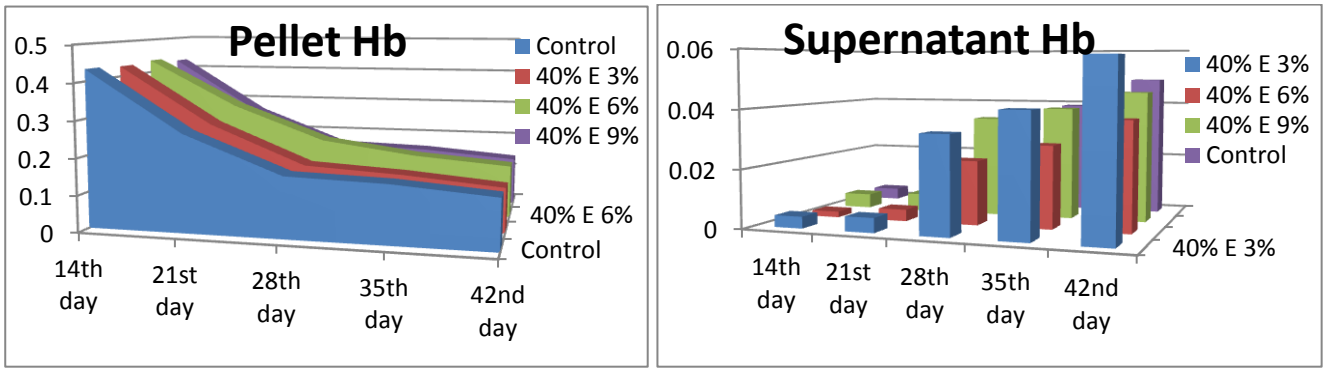
60% Ethanol





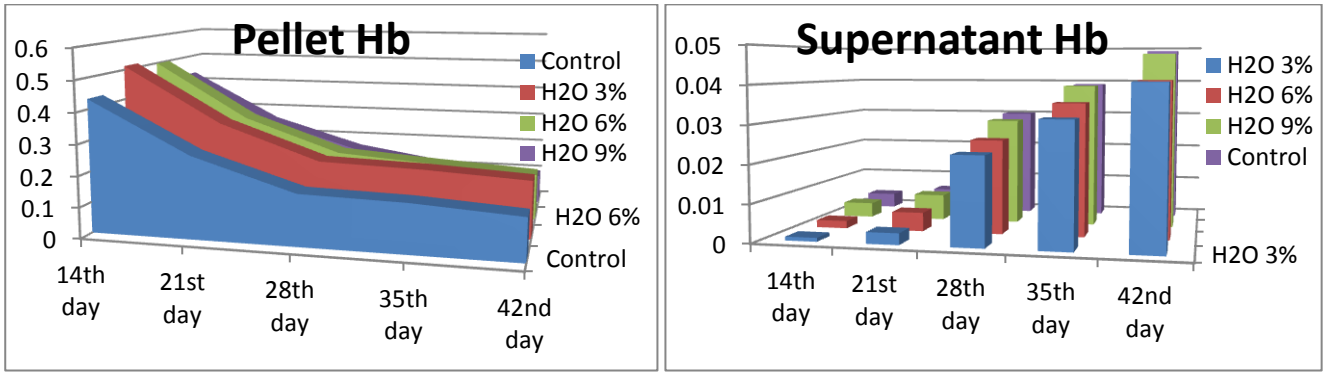
In pellet the absorbance of 3% of 60% Ethanol (E) increases and 6% & 9% of 60% E extract decrease while in supernatant the absorbance of 3% of 60% E decrease and 6% & 9% of 60% E extract increase as compared to control. This shows the haemolysis of RBCs in 3% of 60% E extract is less than control.

40% Ethanol



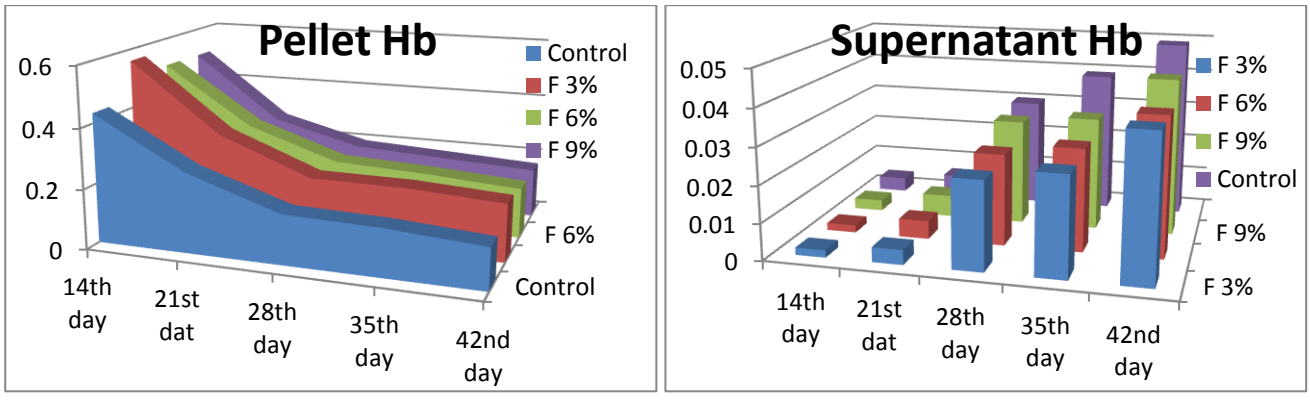
In pellet the absorbance of 6% of 40% Ethanol (E) increases and 3% & 9% of 40% E extracts decrease while in supernatant the absorbance of 6% of 40% E decreases and 3% & 9% of 40% E extract increase as compared to control. This shows the haemolysis of RBCs in 6% of 60% E extract is less than control.

Water



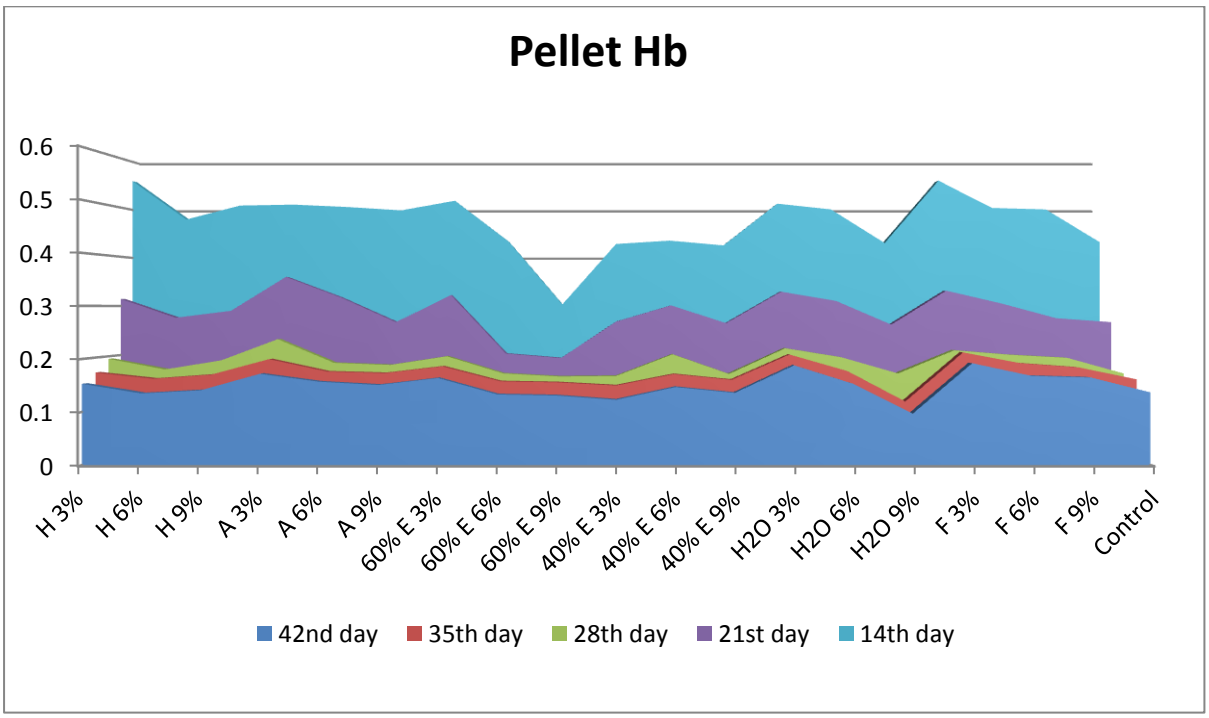
In pellet the absorbance of 3% & 6% of water (H_2O) increases and 9% of H_2O extract decreases while in supernatant the absorbance of 3% & 6% of H_2O decrease and 9% of H_2O increases as compared to control. This shows the haemolysis of RBCs in 3% and 6% of H_2O extracts is less than control.

Fresh leaves

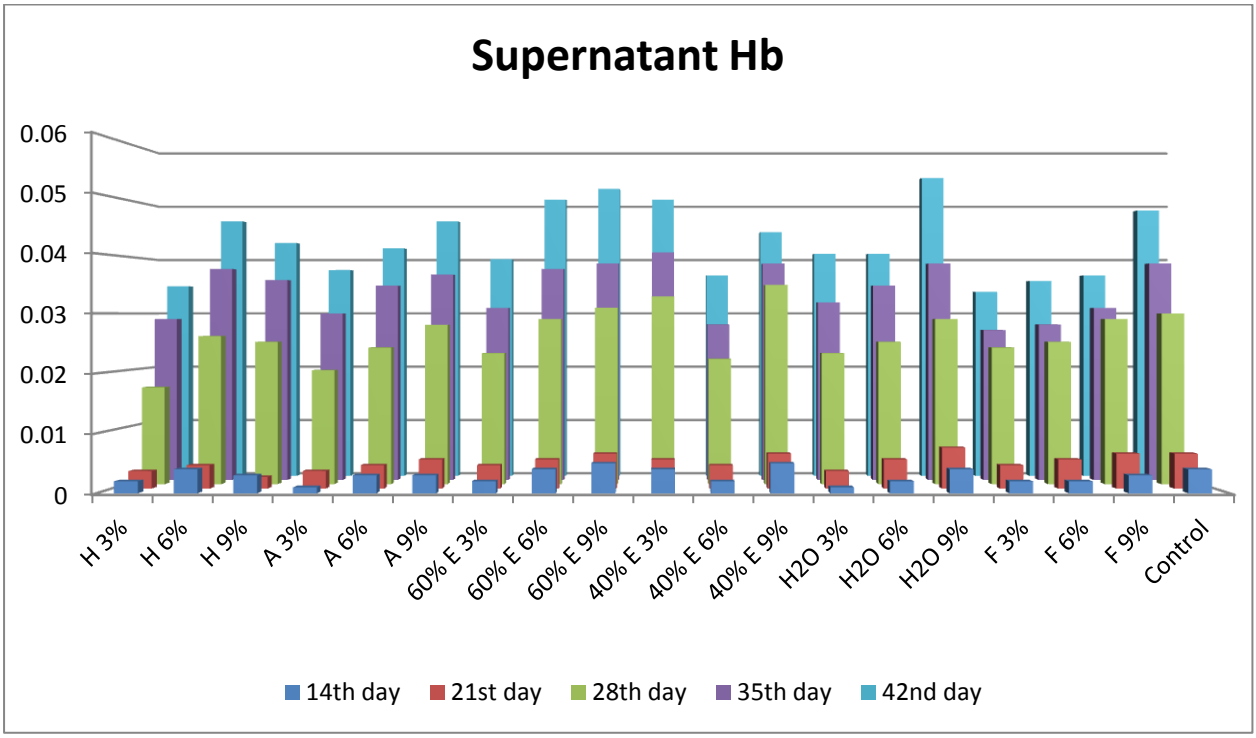


In pellet the absorbance of 3%, 6% & 9% fresh (F) leaves extracts increase while in supernatant the absorbance of each fresh extracts decrease as compared to control. This shows the haemolysis of RBCs in extracts is less than control during storage period.

Figure 15: comparative analysis of hemoglobin of each extract with control.



Highest haemoglobin content of pellet is shown by fresh leaves extract and the least is shown by 9% of H_2O extract. The haemoglobin content of hexane, acetone, fresh leaves, 60% E 3%, 40% E 6% and H_2O 3% & 6% extract is more with respect to control.



The absorbance of 9% of H_2O is highest which shows the haemoglobin release in supernatant is more and the least is shown by fresh leaves extract. The haemoglobin content in supernatant of hexane, acetone, fresh leaves, 60% E 3%, 40% E 6% and H_2O 3% & 6% extract is less with respect to control means haemolysis of RBCs in these extracts is less than control.

Figure 16: comparative analysis of haemoglobin in each extract during storage period.

6.7 pH

Table 12: change in pH during storage period

Samples	Blood with ACD	Blood with CPDA and Extracts	Blood with CPDA (CONTROL)
Fresh blood	7.2	7.4	7.4
After 7 days	7.0	7.2	7.2
After 14 days	7.0	7.2	7.1
After 23days	6.9	7.0	6.9
After 35 days	6.8	6.9	6.9
After 42 days	6.8	6.9	6.8

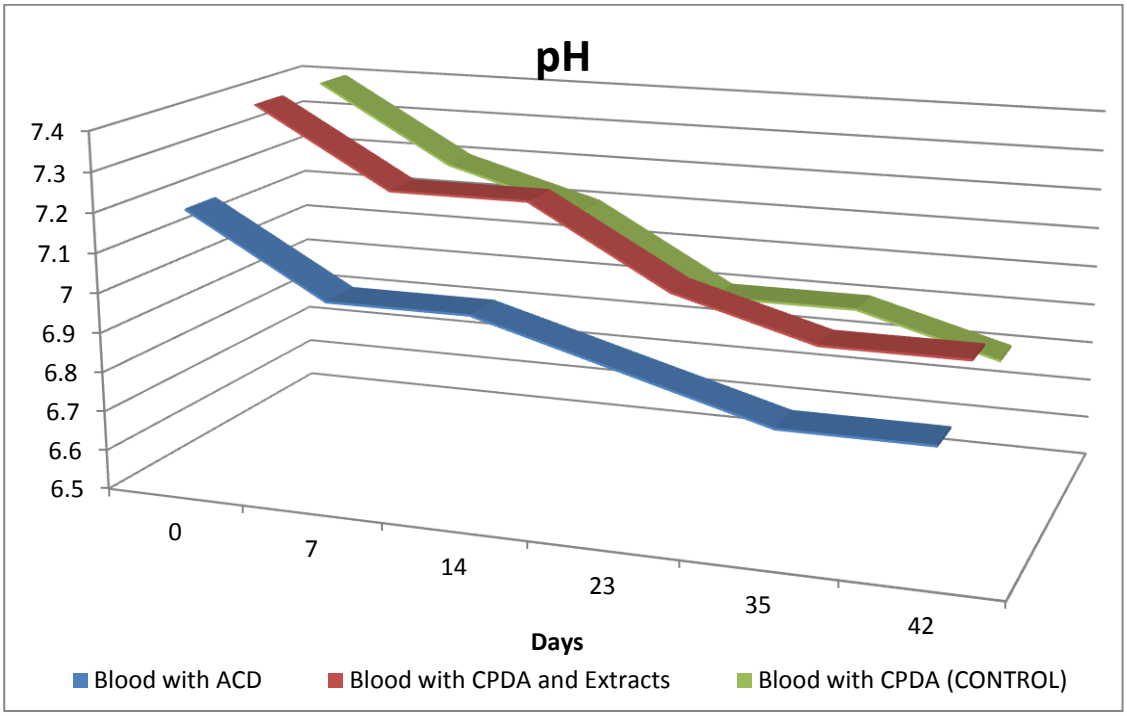


Figure 17: Comparative analysis of pH of blood with CPDA + extracts with control and blood with ACD. [Changes in pH from day 0 to day 42, pH changes show that the acidity of stored blood is increased day by day but in presence of CPDA decrease in pH is less than blood with ACD.]

6.8 Cell count

Table 13: Relative viability of RBCs of each extract

S. No.	Samples	RBCs Cell count ($x10^6$ cells/µl)	Relative viability (%)
1.	Fresh blood (0 day)	4.32	100
	Extracts (after 40 days)		
2.	H 3%	3.57	82.6
3.	H 6%	3.37	78.0
4.	H 9%	3.50	81.0
5.	A 3%	3.67	84. 9
6.	A 6%	3.50	81.0
7.	A 9%	3.43	79.4
8.	60% E 3%	3.60	83.3
9.	60% E 6%	3.35	77.5
10.	60% E 9%	3.28	75. 9
11.	40% E 3%	3.35	77.5
12.	40% E 6%	3.38	78.2
13.	40% E 9%	3.34	77.3
14.	H_2O 3%	3.65	84.5
15.	H_2O 6%	3.50	81.0
16.	H_2O 9%	2.24	51.8
17.	F 3%	3.64	84.3
18.	F 6%	3.55	82.2
19.	F 9%	3.45	79. 9
20.	Control	3.37	78.0

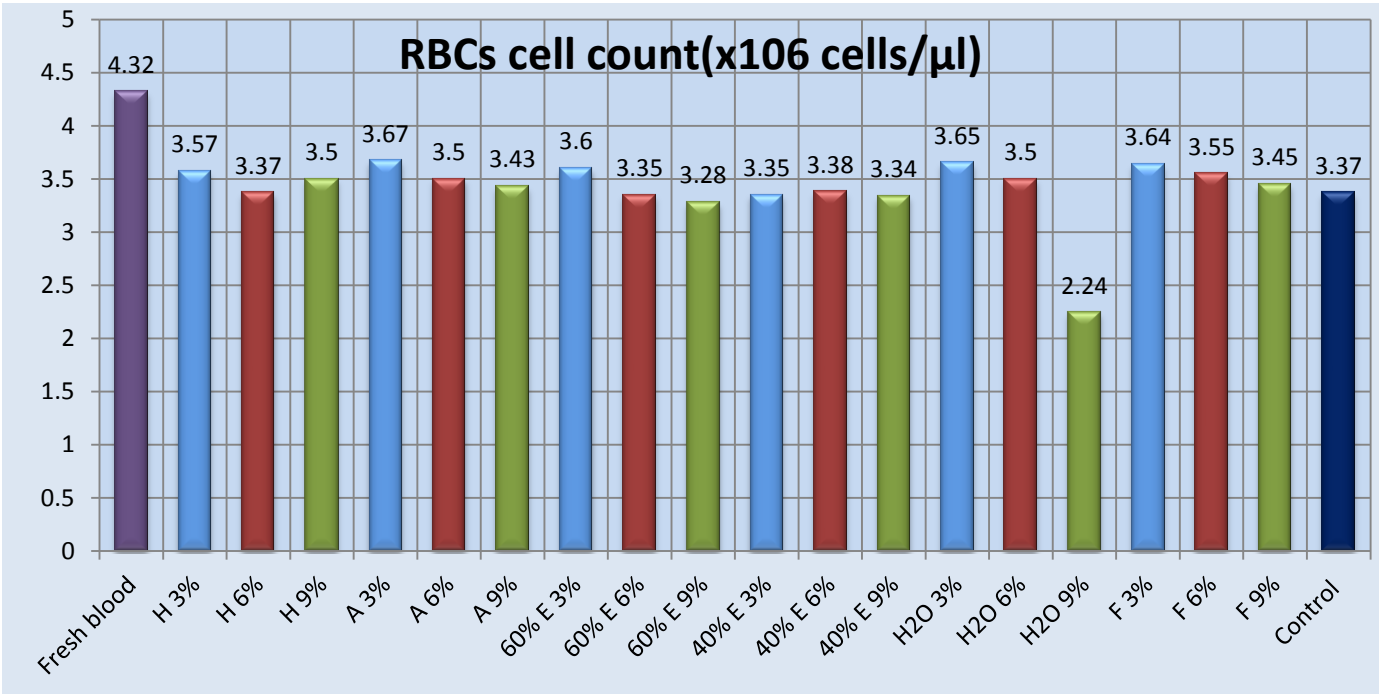


Figure 18: comparative study of RBCs cell count of each extract with fresh blood and control

[The RBCs cell count of hexane, acetone, fresh leaves, 60% E 3%, 40% E 6% and H_2O 3% & 6% extract is more with respect to control means these extracts increases the cell viability of blood during storage and the least cell count of 9% of H_2O extract shows the less viability of cells.]

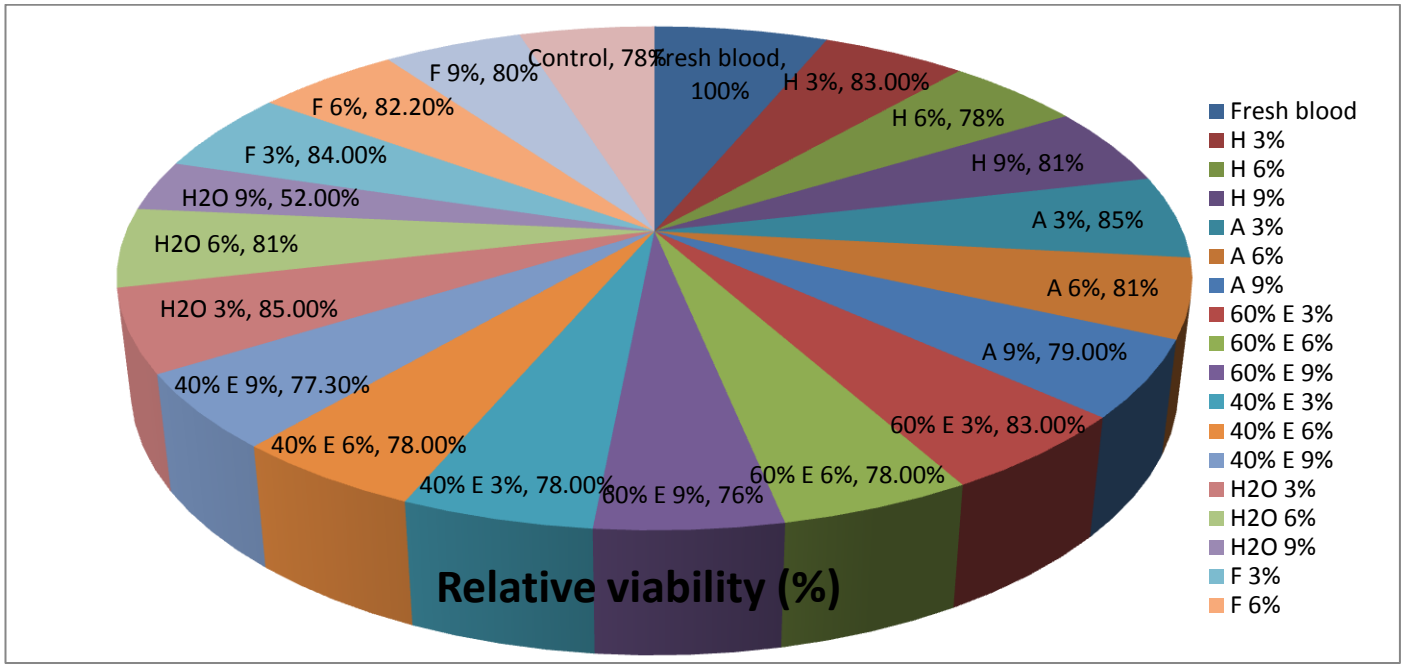


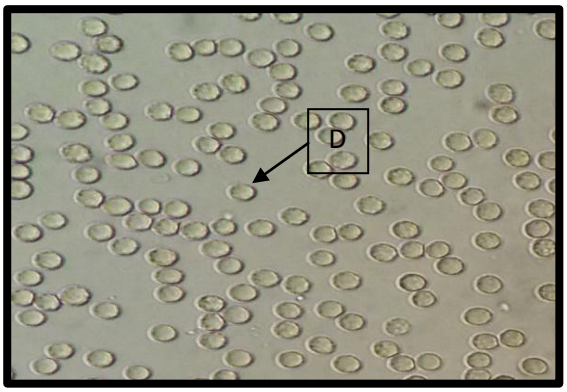
Figure 19: relative viability of RBCs of each extract after 40 days

[Relative viability of hexane, acetone, fresh leaves, 60% E 3% and H_2O 3% & 6% extract with respect to fresh blood cell viability (100%) is more than control means these extracts increases the cell viability of blood during storage and the least viability is shown by 9% of H_2O extract. The comparison of cell count with control has shown the effect of different extracts on blood shelf life.]

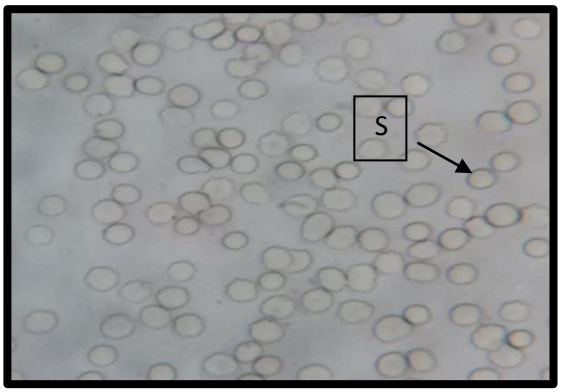
6.9 **Morphology**

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

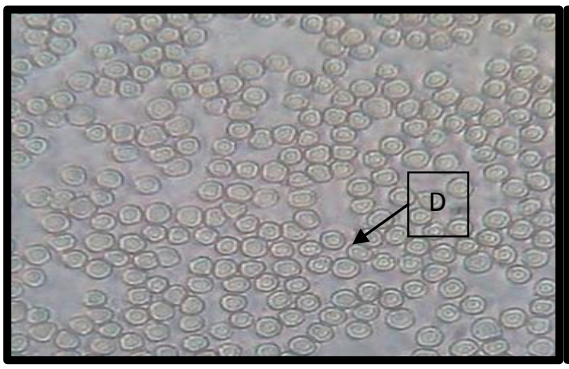
Hexane



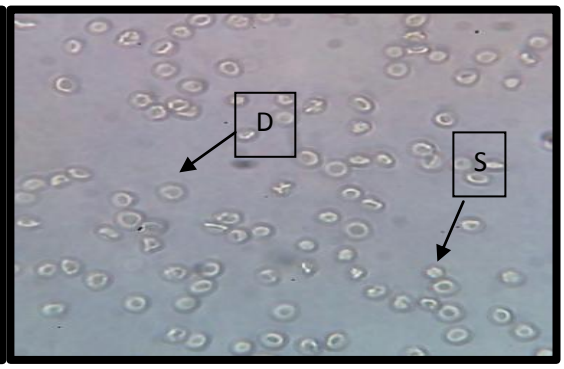
H 3% (Day 14)
Discocytes – biconcave disc (D)



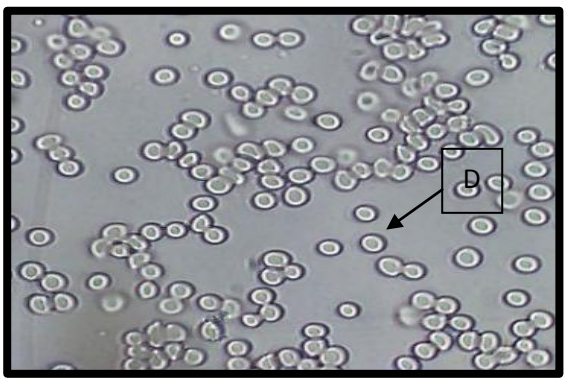
H 3% (Day 28)
Spherocytes (S)



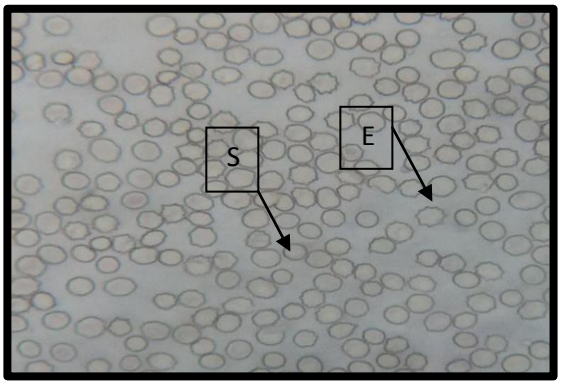
H 6% (Day 14)
Discocytes – biconcave disc (D)



H 6% (Day 28)
Discocytes – biconcave disc (D) and few spherocytes cells (S)

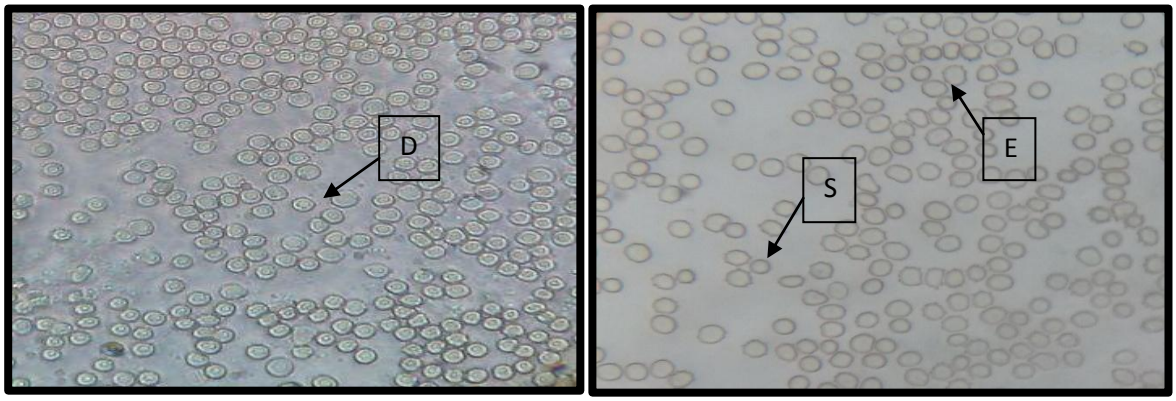


H 9% (Day 14)
Discocytes – biconcave disc (D)



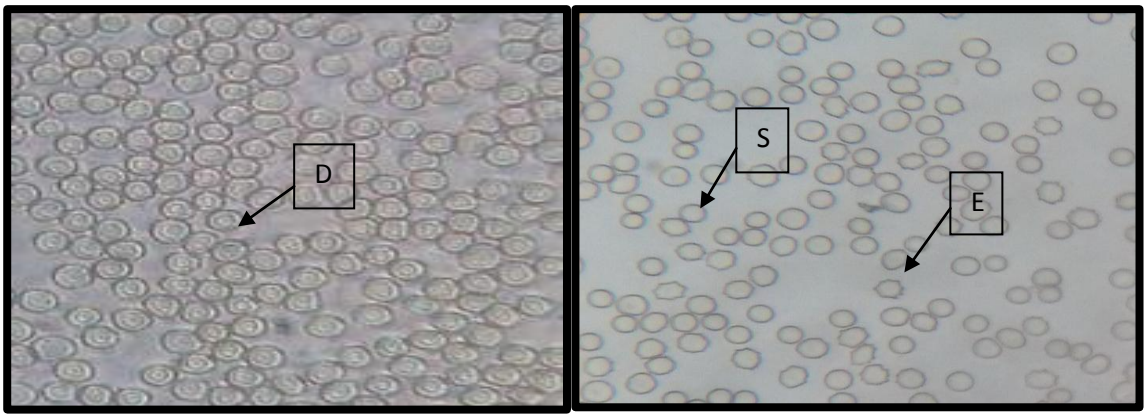
H 9% (Day 28)
Spherocytes (S) & few echinocytes-
Speculated burr cells (E)

Acetone



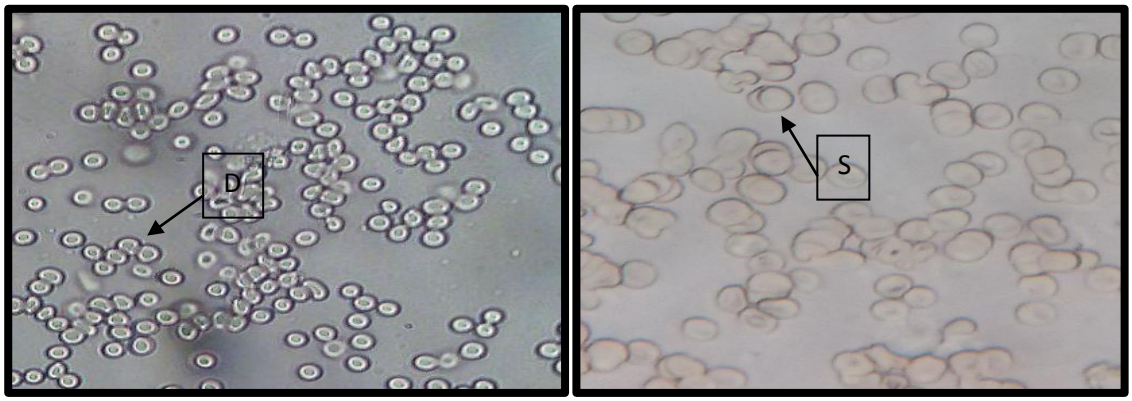
A 3% (Day 14)
Discocytes – biconcave disc (D)

A 3% (Day 28)
Spherocytes (S) & few echinocytes-
Speculated burr cells (E)



A 6% (Day 14)
Discocytes – biconcave disc (D)

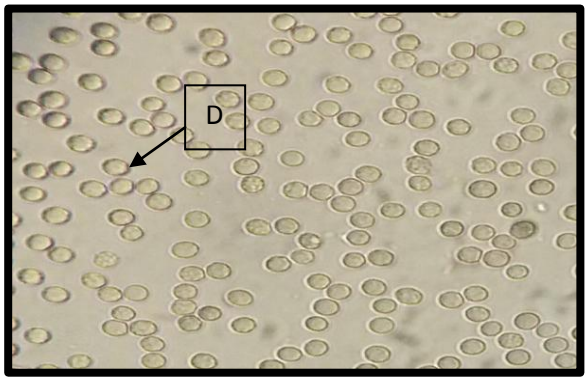
A 6% (Day 28)
Spherocytes (S) &echinocytes-
speculated burr cells (E)



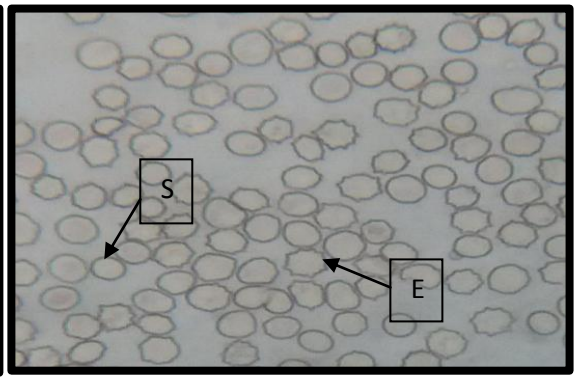
A 9% (Day 14)
Discocytes – biconcave disc (D)

A 9% (Day 28)
Spherocytes (S)

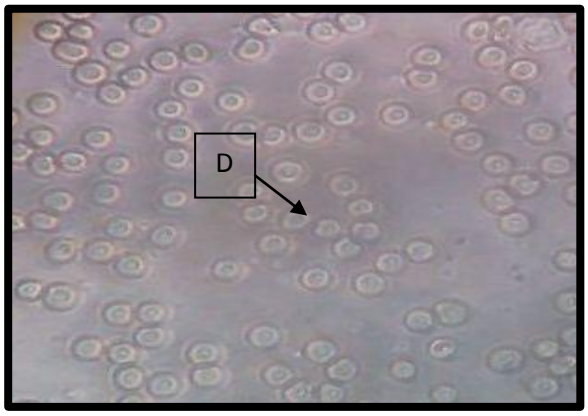
60% Ethanol



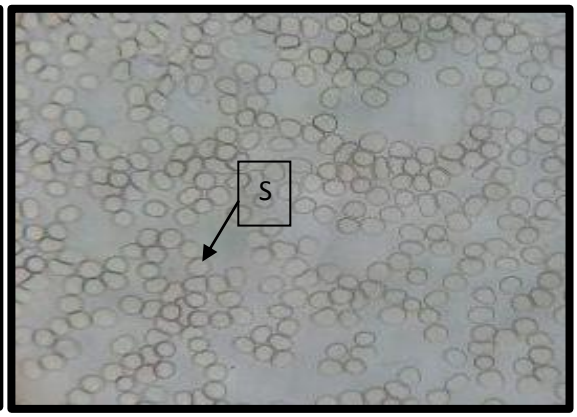
60% E, 3% (Day 14)
Discocytes – biconcave disc (D)



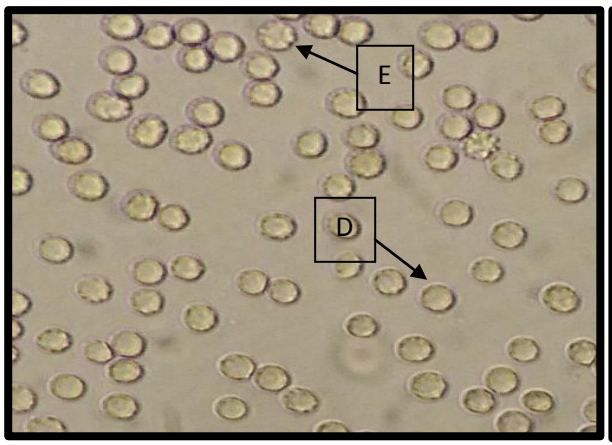
60% E, 3% (Day 28)
Spherocytes (S) & echinocytes-speculated burr cells (E)



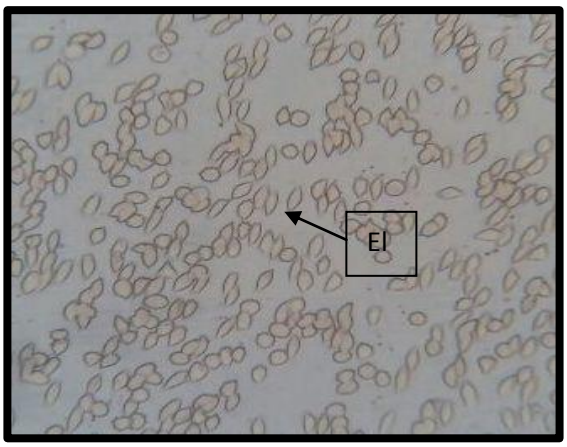
60% E, 6% (Day 14)
Discocytes – biconcave disc (D)



60% E, 6% (Day 28)
Spherocytes (S)

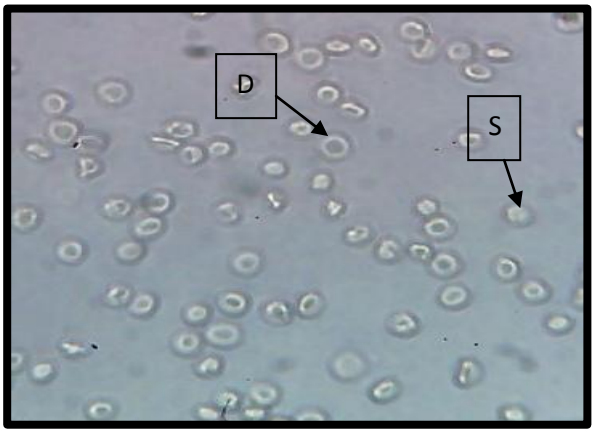


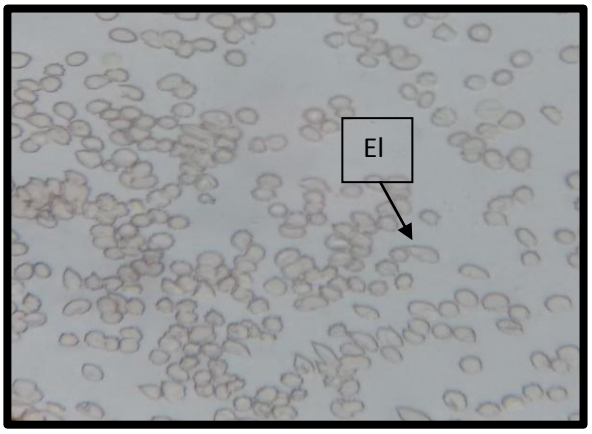
60% E, 9% (Day 14)
Discocytes – biconcave disc (D), echinocytes (E)



60% E, 9% (Day 28)
Eliptocytes or dumbbell shaped (El)

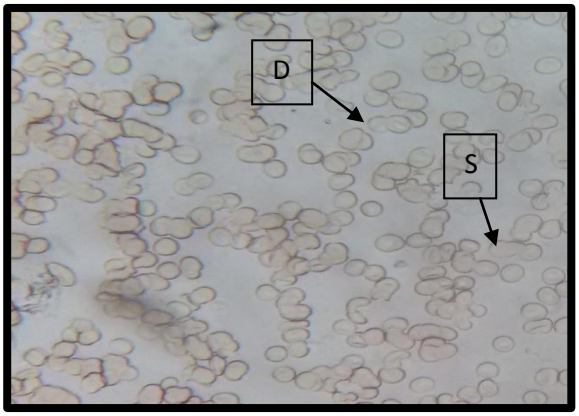
40% Ethanol

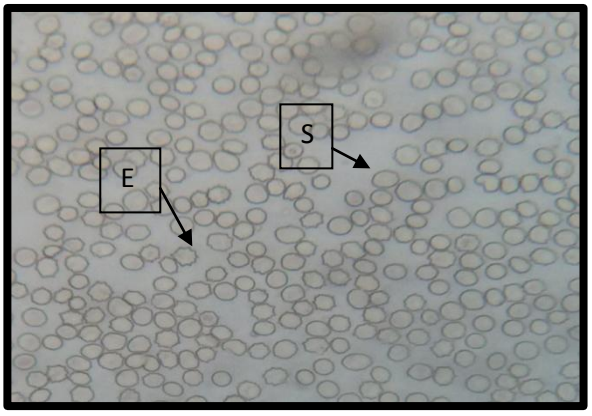




40% E, 3% (Day 14)
Discocytes – biconcave disc (D) & spherocytes (S)

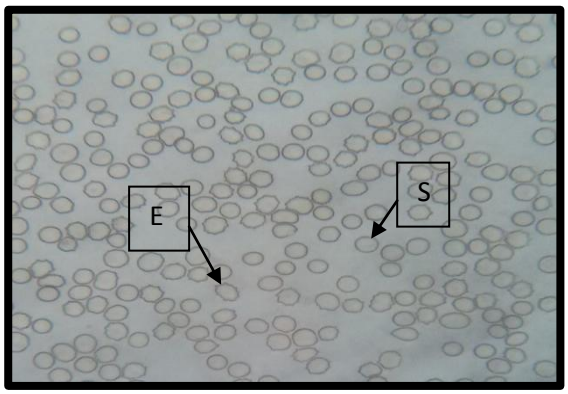
40% E, 3% (Day 28)
Eliptocytes or dumbbell shaped (El)

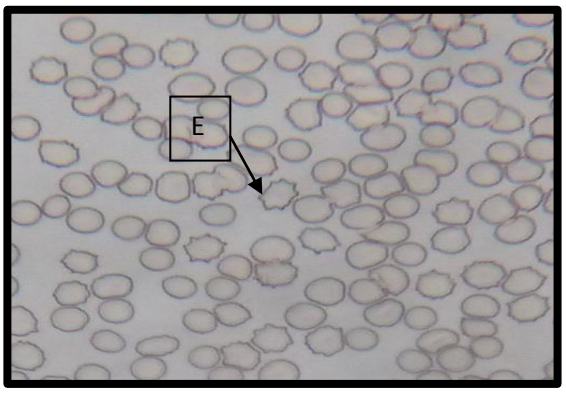




40% E, 6% (Day 14)
Discocytes – biconcave disc (D) & spherocytes (S)

40% E, 6% (Day 28)
Spherocytes (S) &very less echinocytes-
Speculated burr cells (E)

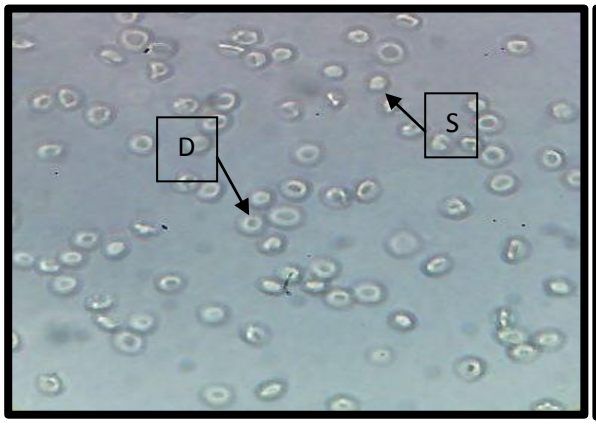




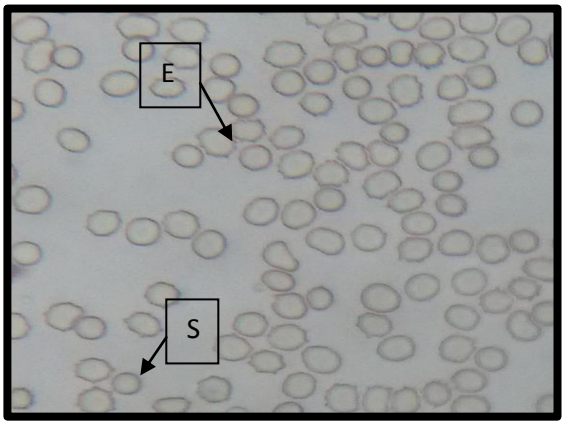
40% E, 9% (Day 14)
Spherocytes (S) & few echinocytes-
speculated burr cells (E)

40% E, 9% (Day 28)
Echinocytes – speculated burr cells (E)

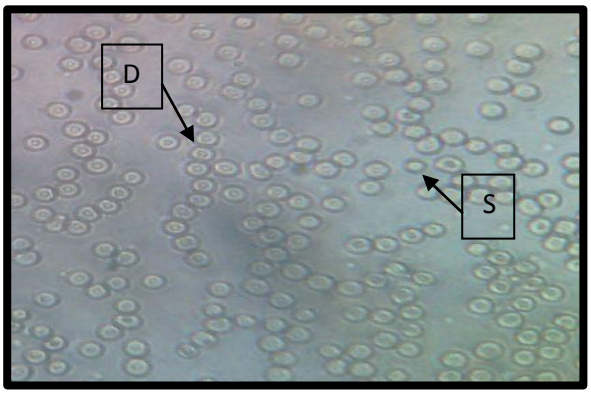
Water



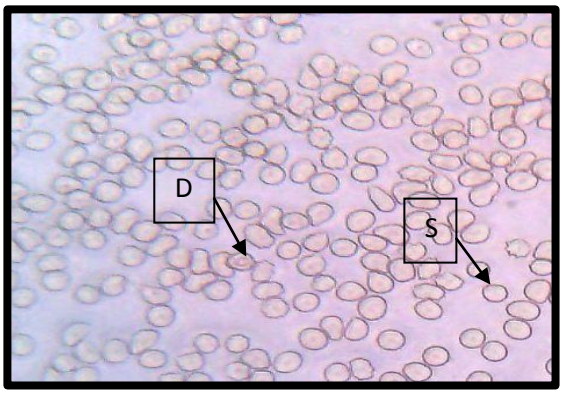
H_2O, 3% (Day 14)
Discocytes – biconcave disc (D) & spherocytes (S)



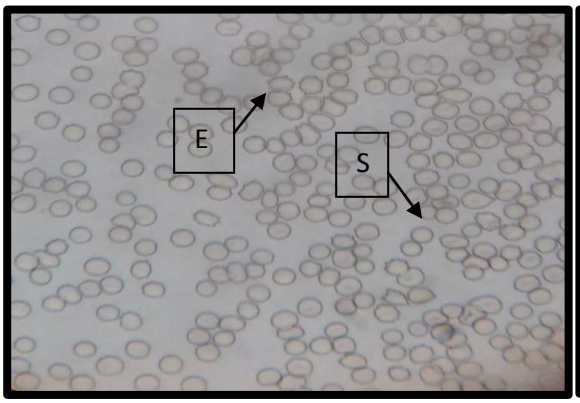
H_2O, 3% (Day 28)
Spherocytes (S) & Echinocytes – Speculated Burr cells (E)



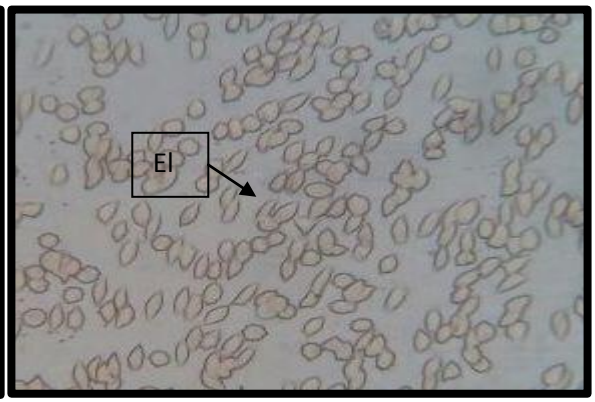
H_2O, 6% (Day 14)
Discocytes – biconcave disc (D) & spherocytes (S)



H_2O, 6% (Day 28)
Spherocytes (S) & Echinocytes – speculated Burr cells (E)

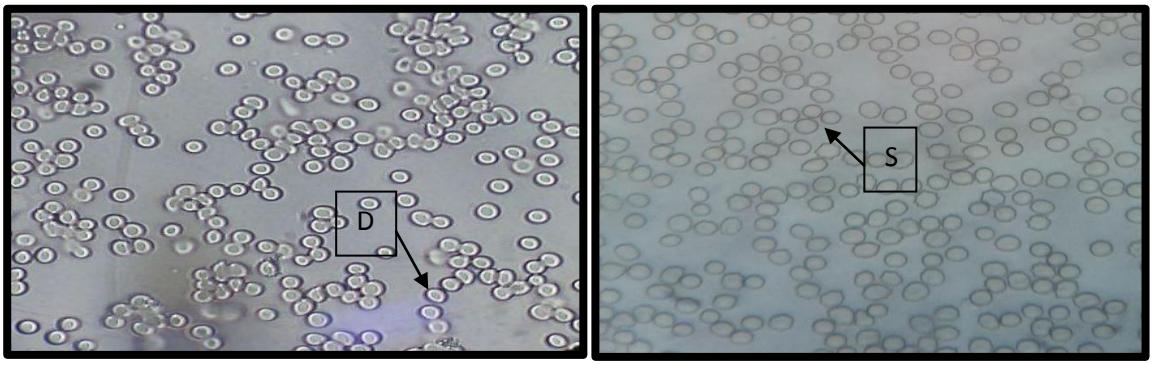


H_2O, 9% (Day 14)
Spherocytes (S) & Echinocytes – Speculated burr cells (E)



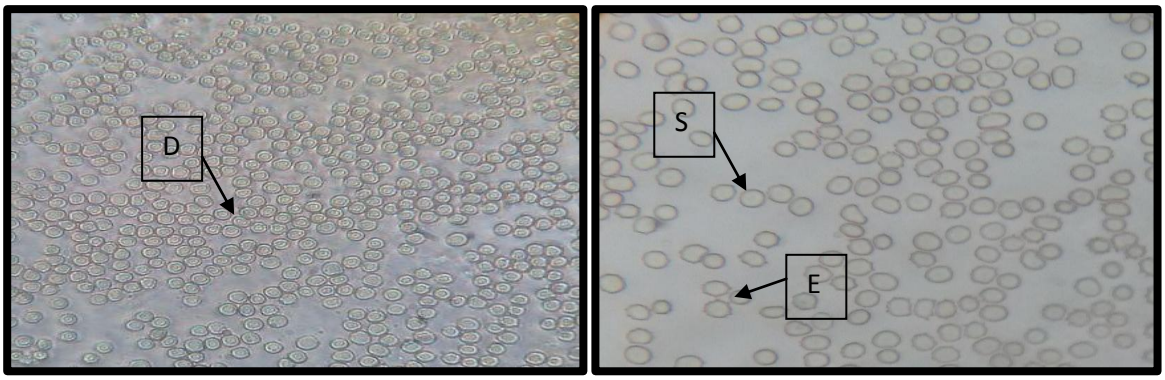
H_2O, 9% (Day 28)
Elliptocytes or dumbbell shaped (El)

Fresh leaves extract



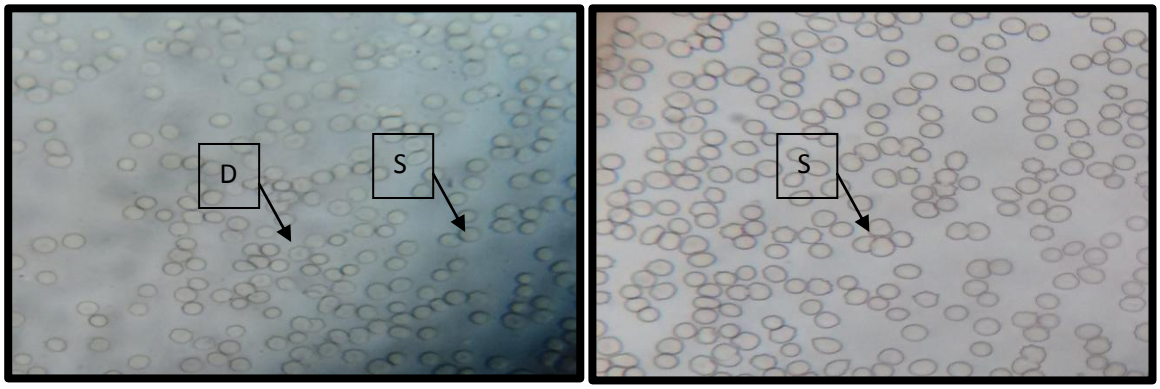
F 3% (Day 14)
Discocytes – biconcave disc (D)

F 3% (Day 28)
Spherocytes (S)



F 6% (Day 14)
Discocytes – biconcave disc (D)

F 6% (Day 28)
Spherocytes (S) & Echinocytes – Speculated burr cells (E)



F 9% (Day 14)
Discocytes – biconcave disc (D) & spherocytes (S)

F 9% (Day 28)
Spherocytes (S)

Figure 20: Morphology of RBCs over the period of time

[Morphology changes from discoid or biconcave shape to spherocytes and then elliptocytes/acanthocytes but changes in shape are different in all extract. Morphological studies explained about the cell death.]

b) SEM

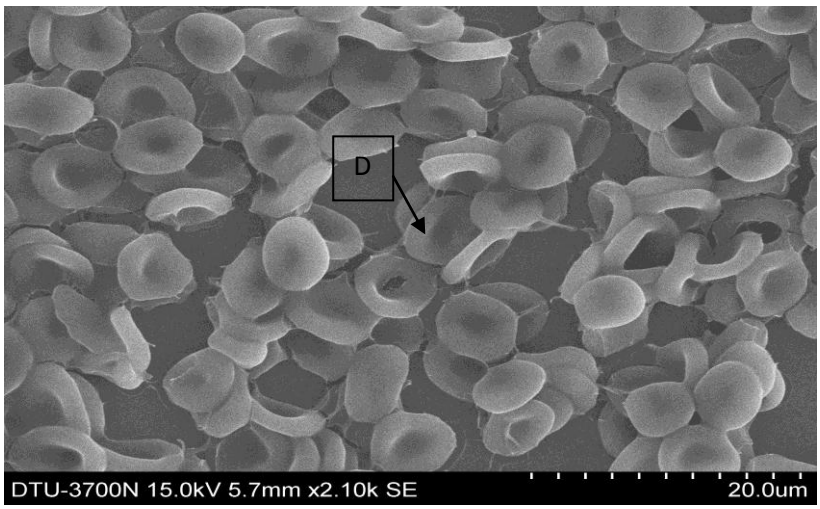
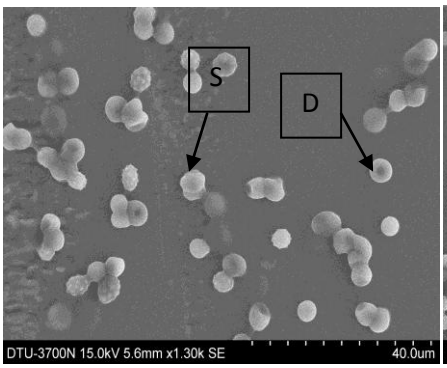


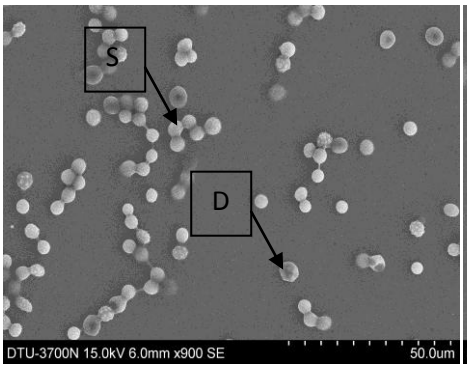
Figure 21: SEM image of Fresh Blood Sample

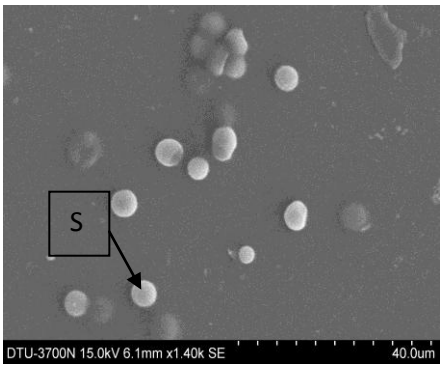
Discocytes – biconcave disc (D)

After 42 days

Hexane



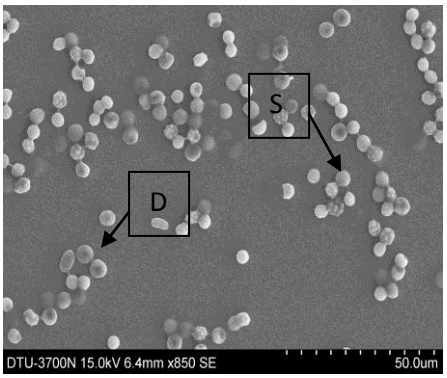


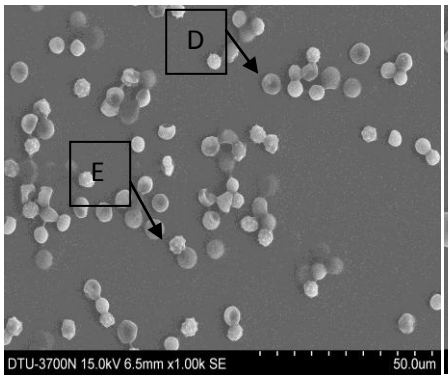


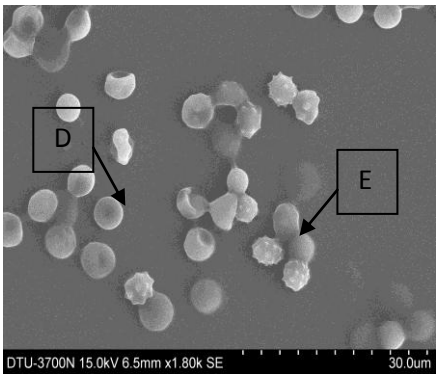
H 3% H 6% H 9%

Discocytes – biconcave disc (D) & spherocytes (S)

Acetone



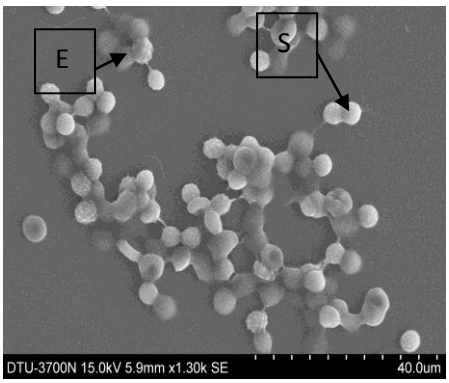


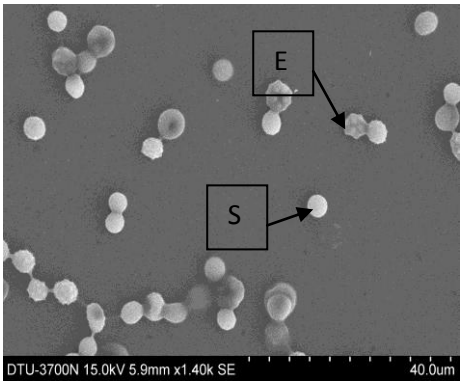


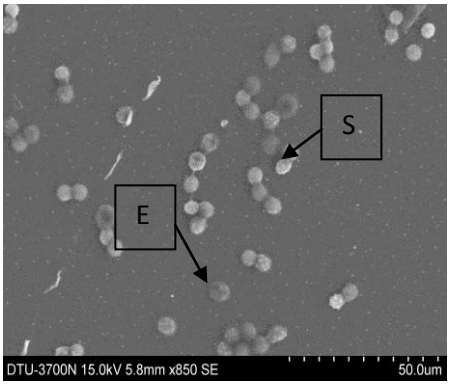
A 3% A 6% A 9%

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

60% Ethanol



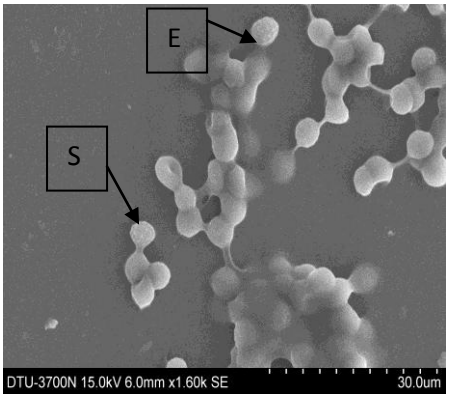


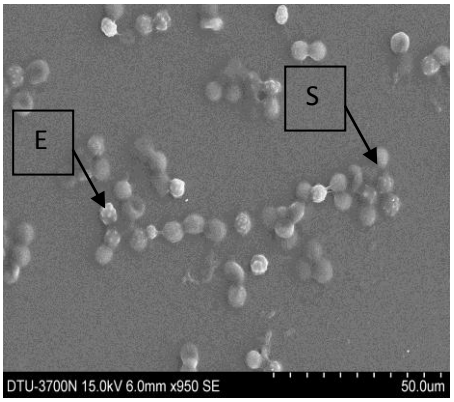


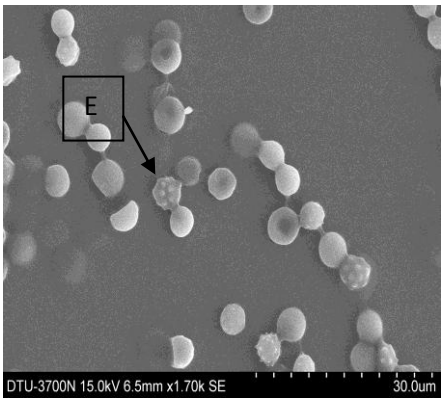
60% E, 3% 60% E, 6% 60% E, 9%

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

40% Ethanol



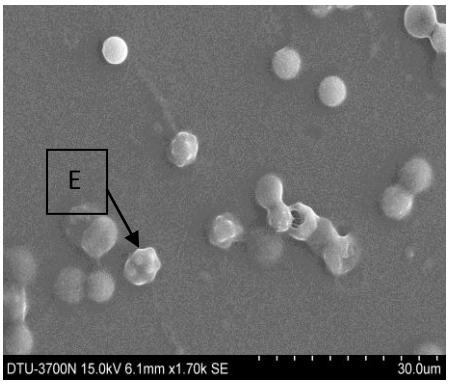


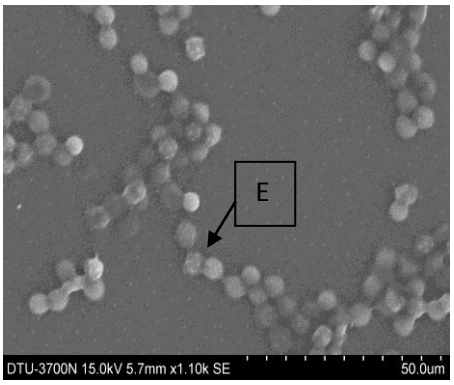


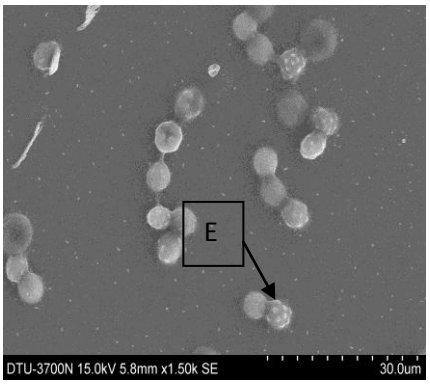
40% E, 3% 40% E, 6% 40% E, 9%

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

Water



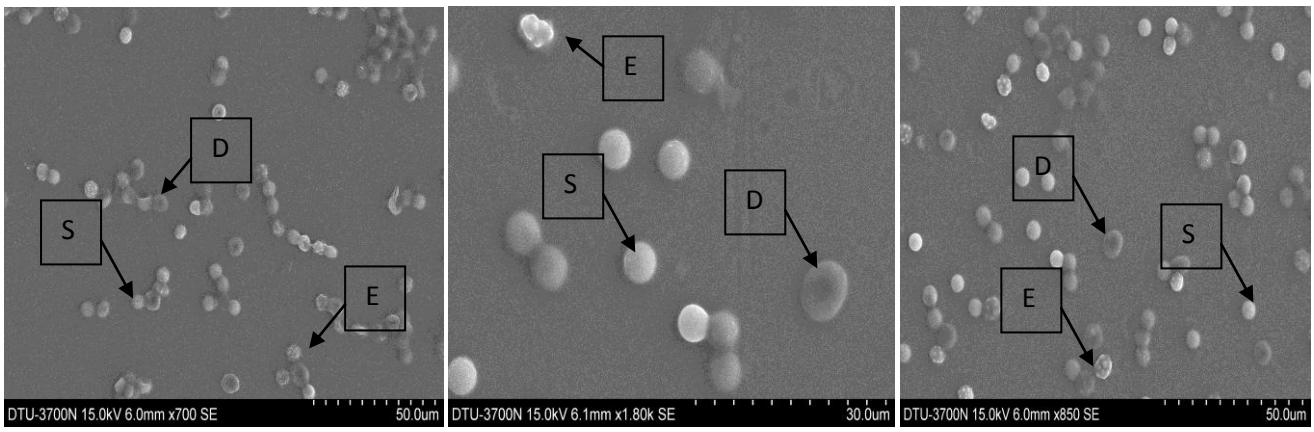




H_2O, 3% H_2O, 6% H_2O, 9%

Echinocytes – speculated burr cells (E)

Fresh leaves



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

Figure 22: SEM images of RBCs.

[Morphology changes from discoid or biconcave shape to spherocytes and then elliptocytes/acanthocytes but changes in shape are different in all extract. Morphological studies explained about the cell death.]

CONCLUSION

Carica papaya leaves were evaluated for phytochemical analysis by using different organic solvents to determine the type of phytoconstituents present in the extracts. Phytochemical analysis of the leaf extracts confirmed the presence of alkaloids, glycosides, saponins, tannins, flavonoids, phenolic etc. TLC profiling of all 5 extracts showed presence of a number of phytochemicals. Different R_f values of the compounds in different solvent systems provided information about their polarity. This information helped in solvent system selection for further separation of pure compounds from plant extracts. From GC-MS results we concluded that Papaya leaf extracts mainly 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 from this study we concluded that different concentrations of extracts are effective as additives for improving the quality of blood while storage and in future, these papaya leaves extracts can be used as additives for increasing the shelf life of stored blood.

DISCUSSION AND FUTURE PERSPECTIVE

The experiments performed in this project showed a significant improve in the cellular viability, pH, morphology and haemoglobin content of blood stored in CPDA and papaya leaves extracts compared to blood stored in CPDA only. Hexane, acetone and fresh leaves extracts show maximum activity as compared to other extracts. The results of Phytochemical analysis of the extracts confirmed the presence of glycosides, saponins, tannins, flavonoids, phenolic etc. These metabolites have been reported to possess medicinal properties like antimicrobial, antioxidant, anti-inflammatory, analgesic, anti-atherogenic, antithrombotic, anticoagulant, neuroprotective, antiviral, immune-modulatory, cell membrane-stabilizing and antiproliferative activities. These metabolites help in overcoming the decrease in haemoglobin content and damage due to acidic ph and hence increases the viability of cells while storage. They also improve the morphology of stored blood cells by stabilizing the membrane of cells. This shows the effect of papaya leaves extracts during storage of blood. Since bioactive compounds present in papaya leaves consist of multi-components but their separation and purification still creates problems. Isolation of these compounds will help to detect their biological activities. We plan to isolate each of these metabolites and study individual effect on blood in storage condition to improve its shelf life. Further we also plan to observe other parameters like glucose consumption, ATP deterioration, membrane protein degradation and electrolyte analysis during storage.

REFERENCES

1. 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).
2. Alsolami, R. (n.d.). Estimation of Hemoglobin Concentration.
3. 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
4. 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
5. 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.
6. 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
7. Building, T. (2010). Electron microscopy procedures manual, (July), 1–25.
8. 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
9. 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

10. 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
11. 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
12. Corporation, P. (n.d.). NAD / NADH-Glo ™ Assay NAD / NADH-Glo ™ Assay.
13. 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
14. 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.
15. 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
16. 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
17. Facts, Q. (2005). ATP Determination Kit (A22066) Quick Facts. *Microscope*, 1–3.
18. gaat10i1p14 (1).pdf. (n.d.).
19. 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
20. Gujjeti, R. P., & Mamidala, E. (2013). Phytochemical Analysis and TLC Profile of Madhuca indica Inner Bark Plant Extract, *4*(10), 1507–1510.
21. Hb estimation from dried blood spot by cyanmethaemoglobin method. (n.d.).
22. Hess, J. R. (2009a). Red cell storage: When is better not good enough? *Blood Transfusion*, *7*(3), 172–173. http://doi.org/10.2450/2009.0110-09

23. Hess, J. R. (2009b). 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
24. Hess, J. R., Kagen, L. R., Van Der Meer, P. F., Simon, T., Cardigan, R., 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
25. 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
26. In vivo. (n.d.), (12).
27. 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.
28. Julianti, T., De Mieri, M., Zimmermann, S., Ebrahimi, S. N., Kaiser, 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
29. 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.
30. 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.
31. 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
32. 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
33. 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

34. 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
35. 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
36. 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
37. 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
38. 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.
39. 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
40. 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
41. 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
42. Riss, T. L., Niles, A. L., & Minor, L. (2004). Cell Viability Assays Assay Guidance Manual. *Assay Guidance Manual*, 1–23.

43. Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K. M., & Latha, L. Y. (2011). 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.
44. 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
45. 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.
46. 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
47. 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.
48. Subenthiran, S., Choon, T. C., Cheong, K. C., Thayan, R., Teck, M. B., Muniandy, 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
49. 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.
50. Technical, P., & Sheet, I. (2012). Photometric Detection of Nicotinamide Adenine Dinucleotides, (July).
51. 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
52. Volume, B. (2010). Escherichia coli, klebsiella pneumoniae and proteus vulgaris isolates, *3*(1), 195–198.
53. Wolf, H. U., Diagnostics, M., Zander, R., & Lang, W. (2010). Development of the AHD 575 method, 1–21.

54. 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
55. 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.
56. 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

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