LIST OF ABBREVIATIONS

ACD	Acid citrate dextrose
CPDA	Citrate phosphate dextrose
GC-MS	Gas chromatography mass spectrometry
ATP	Adenosine tri phosphate
HIV	Human Immunodeficiency Virus
AIDS	Acquired Immune Deficiency Syndrome
SAG	Saline Adenine Glucose
SAGM	Saline Adenine Glucose Mannitol
NSAIDs	Non-steroidal anti-inflammatory drugs
RBC	Red blood cells
AHD	Alkaline hematin detergent
PBS	Phosphate buffer saline
SEM	Scanning Electron Microscope
TLC	Thin layer chromatography
Hb	Haemoglobin
ROS	Reactive oxygen species
CPD	Citrate phosphate dextrose
TRALI	Transfusion related acute lung injury
2, 3-DPG	2, 3- diphosphoglycerate
NO	Nitric oxide
PGE	Prostaglandin E
HPLC	High performance liquid chromatography

Effect of additives to enhance blood shelf life

Bhagyeshwari chouhan

Delhi Technological University, Delhi, India

ABSTRACT

Blood shelf life is the main problem associated with transfusion medicine because shelf life of stored blood is only about 42-49 days in presence of suitable anticoagulant CPDA and additive solutions which contains glucose, saline, adenine and mannitol which maintains integrity of red blood cells during storage period but during storage of blood various biochemical and physical changes occurred which decreases the shelf life of blood but we know that the in vivo shelf life of erythrocytes is 120 days. In this study for enhancing blood shelf life, Carica papaya leaf extract is used as an additive. It is obtained for dry leaves by soxhlet extraction using different solvents like hexane, acetone, 60%ethanol, 40% ethanol and water, whereas the maceration process is used for fresh leaf extraction by milli-q water. The Papaya leaf has been used in this study as it contains different phyto-chemical like flavonoids, phenolic compound and sterols which exhibit different medicinal values. Some phytoconstituents present in Carica papaya leaf act as antioxidants, antibacterial and antifungal. They also exhibit reactive nitrogen scavenging activity and free radical scavenging activity. It is used in treatment of anemia as a membrane stabilizing agent for stabilizing the membrane of red blood cells. Papaya leaf extract is also used for the treatment of dengue hemorrhagic fever in which platelet count is decreased. So by using different concentration of papaya leaf extract as additive the shelf life of blood is determined.

Keywords: Erythrocytes, red blood cells, Carica papaya leaf, GC-MS analysis, eryptosis, ATP, Phytoconstituents, antimicrobial, anti-inflammatory, antioxidant, AHD, whole blood, phytochemicals, papaya leaf extract, hemoglobin and hemolysis.

Chapter 1

INTRODUCTION

Blood transfusion is the main lifesaving treatment for patients suffering from severe diseases like anaemia, leukaemia and in higher blood loss condition due to any accidental injury. The first transfusion of human was took place in 15th June 1667 by physician Jean Baptiste Denis and the first successful transfusion of blood from human to human was performed in 1818 by British obstetrician James Blundell for the treatment of haemorrhage after deliver a baby. The blood requirement for transfusion is not fulfilled by blood bank because of less number of blood donors and one of the main reason of lacking of blood for transfusion is shelf life of stored blood, we know that the shelf life of blood in in- vivo condition is about 120 days while the shelf life of blood in in- vitro condition is about 42-49 days, after which blood is not so good for transfusion. These transfused aged stored blood in patient evokes many other problems which also be fatal for the patient. The transfusion of old blood into patients causes many health related problem like transfusion related acute lung injury, it also stimulate the immune response and fluid overload which creates problem in patients and some-times this responses causes death of the patient. So to overcome this storage related problems various methods has been developed which improve the blood shelf life in storage condition and maintains the quality of stored blood. Time to time different research is performed to increase shelf life of blood. ACD firstly used as anticoagulant for storing blood but in ACD blood can stored only for 21 days after which the blood pH decreases to acidic so it is dangerous for human creates harmful effects in patients, after use ACD, CPD used as anticoagulant in which the shelf life of blood is about 28 days after using ACD and CPDA now blood is stored in CPDA in which the blood shelf life is 35days. CPDA-1 and CPDA-2 contains citrate as chelating agent which prevent blood clotting, phosphate maintains the pH work as buffer and dextrose maintain red cells viability and adenine required for ATP level and all this anticoagulants are used along with different additive solutions like SAG-M, ADSOL and SAG which contains saline, glucose, adenine and mannitol in different concentration and the function of saline is to maintain tonicity of the cell, for maintaining ATP level adenine required in storage period, glucose provide substrate for glycolysis and mannitol which prevent red blood cells from swelling by adding additives with CPDA the shelf life of RBC increases from 35 to 42 days. Developed countries stored blood in the form of its components and the components of blood separated by apheresis process. By apheresis, components of blood which is required at that time are apheresis from whole blood and remaining part of blood again transfer to the donor. But the shelf life of blood is main problem for developing countries because the lack of resources for storing blood components and awareness about apheresis processes so developing chemical compound from natural sources that will show positive effect on improving shelf life of blood and our study start with papaya leaf extract. Papaya and it's all parts is the rich source of vitamins and antioxidants and it also effective in treatment of dengue fever.

Chapter 2

REVIEW OF LITERATURE

We are using many plants and its products which are obtained from its different parts like leaves, fruits, stem, roots, seeds and flowers as medicine from ancient time and research on the plant and its product is increasing day by day because ultimately the drugs which are available in the market came from plant source directly or indirectly. Our main focus of the research is to enhance shelf life of blood and we are using papaya leaf extract as additive for this purpose. As all knows that papaya and its parts are very useful as a medicinal point of view because papaya fruit, leaves, seeds having secondary metabolites which having different medicinal properties. At the present papaya leaf extract mainly used for treatment of dengue a haemorrhagic fever in which the platelet count is decreased so it causes many problems in a patient because platelets are mainly responsible for blood clotting, so that decreasing platelet count causes internal bleeding as well as it also affects different vital organs like liver and affects the bone marrow which is the site of blood cell production and thus decreasing the production of platelets. The secondary metabolites, mainly present in papaya leaf extract which have medicinal values include alkaloids, terpenoid, cardiac glycosides, phenolic compound, tannins, saponin, anthraquinones, reducing sugar, proteins, amino acids and vitamins C and E. Papaya is used in the treatment of various diseases like cancer, diabetes, dengue, antimicrobial, hypertension, as anti-inflammatory, antifungal, in treatment of HIV-AIDS, malaria, fertility related problems and in the treatment of ulcer and it also used as antioxidant. (Arumugam, N. et al., 2014)

2.1 **Distribution, taxonomy and other properties of papaya plant-** Carica papaya plants belonging to the family Caricaceae and its genus are Carica and we are commonly known as papaya and any other region it is called as paw paw fruit. The main native place of papaya is tropical America and in the 16th century, it was introduced in India. The plant of papaya is mainly weak and unbranched and the clusters of long leaves are found on the top of the plant and the fruits of papaya are found in between leaves and beneath the leaves and the plant of papaya can grow up to the height of 20 meters. (Bhadane, Vishal et al., 2014)

2.2 Carica papaya used as folk medicine from ancient time for treatment of anaemia and blood related disease. Papaya leaf extract contains different phytoconstituents like alkaloid, flavanoids, phenolic compound, saponins, amino acids and vitamins, these constituents shows various medicinal properties and antioxidant activity shown by them are the main activity which prevent free radical formation and these property of phytoconstituents mainly used in the treatment of sickle cell anaemia in which the glutathione a natural antioxidant which are produced by cells endogenously and perform antioxidant activity by removing peroxides by the action of glutathione peroxidase an antioxidant enzyme and it also perform the regulation of action of vitamins such as vitamin C and vitamin E which are nutritive antioxidant present in different plants and show medicinal value. Decrease in the concentration of reduced

form of glutathione in red blood cells then it leads to the increase in concentration of peroxides in the cells which causes red blood cells wall weakening and these leads to the hemolysis of red blood cells which causes deficiency of red blood cells and causing anaemia. Phytoconstituent study shown that the papaya can be used in the treatment of sickle cell disease because of its nutrient property. (Arvind, G. et al., 2013)

2.3 Dengue a hemorrhagic fever caused by virus and transferred in humans by aedes aegypti mosquito. In recent the dengue is the main viral disease which creates a big problem not for specific region but it's an international issue of public health. Symptoms of dengue fever are appears after 5-7 days from the biting of infected mosquito. And the successive infection with different serotypes of dengue viruses causes much life threatening form of disease called dengue hemorrhagic fever (DHF). And the symptoms of dengue fever are rashes on skin, high fever and headache and these three are called dengue triad other than these symptoms include pain in joints and muscles, vomiting, nausea and pain in eye. The primary treatment to be given to patient is acetaminophen and other NSAIDs, antibiotics and corticosteroids are avoided in dengue fever because they cause bleeding in internal organ like in gut. And at present no vaccines are available for dengue fever. At present for the treatment of dengue, mainly research is done on plant like Carica papaya leaves. In-vivo study show that administration of 25ml of papaya leaf extract in water twice a day by patients up to 5 days can increase the platelets counts in patients as well as it also increase other blood cell counts. This medicinal property of papaya leaf extract is mainly due to the presence of different phyto-constituents which exhibit medicinal value. (Ahmad, N. et al., 2011)

2.4 Fruits of papaya mainly exhibit anti-inflammatory and anti-stimulating property and seeds shows post testicular and treatment for infertility, fruit pulps mainly used in treatment of burns, wounds and in skin acne and unripe fruits and seeds have exhibit bacteriostatic action against the pathogens of human enteric part. And last bus not the least the leaves of papaya used in the treatment of asthma and in gastric problems, also in the treatment of fever and amoebic dysentery. The extract of papaya leaves in methanol shows antioxidant and vasodilatory action and by this the extract mainly effective in cardiovascular disease. This study shows the phytochemical analysis of Carica papaya leaf extracts and analysis of secondary metabolites mainly phenolic compounds. Phenolic compounds present in papaya leaf extract exhibit antimicrobial, anti-parasitic, anti-diabetic and anti-cancer and also in the treatment of cardiovascular disease. Gas chromatography-mass spectrometry was used as analytical method for phytochemical analysis of papaya leaf extracts. The extract was prepared by10gm of powdered leaf sample of papaya and extraction was performed in soxhlet extractor and solvent system used for extraction was 70% methanol and volume of solvent was 200ml and acidified with concentrated hydrochloric acid and set pH 2. (Canini, A. et al., 2007)

2.5 Human consumes fruits and vegetables on the daily basis in the form of foods but other than food source they also having medicinal values. And these medicinal

properties of the fruits and vegetables are mainly due to the presence of some chemical compounds called secondary metabolites that exhibit biological action in humans. The whole plant of the papaya shows medicinal value and contains glycosides, flavanoids, alkaloids, phenolic compounds and also contains vitamins and ions. Phytochemical analysis was performed on papaya leaf extract which were obtained from the successive extraction of 200gms of papaya leaf with solvent in increasing polarity and extraction was performed by soxhlet extraction and the final extract obtained after extraction was evaporated by rotary vacuum evaporator and then extract stored at 4°C. GC-MS used for analysis of papaya leaf extract was Perkin-Elmer GC Clarus 500 and data obtained after analysis was compared with NIST library. From these study researchers got about 24 phytochemicals from papaya leaf extract and compounds identified was n-hexadecanoic acid which present in highest percent and acts as anti haemolytic, antioxidant, and as hypocholesterolemic, other phytoconstituents was beta sitosterol, phytol and other compound which we are used as antioxidant, as antiviral, as anticancer drug, antimicrobial, anti-inflammatory, as cell membrane stabilizing agent and many more medicinal properties they have. (Upgade, A. et al. 2013)

2.6 Extraction, isolation and characterization of plant extracts- Natural products which are obtained from medicinal plant either in the form of pure compound or in the form of standardized extracts provide new way of new drug development from that plant. (Cos et al., 2006). According to WHO (world health organization), around 80% or more population of world depends on traditional medicines which are obtained from plant source for the primary treatment of diseases. The primary steps for obtaining biologically active compound from plants involved extraction of metabolites from plant, phytochemical screening, isolation of active compound and characterization than toxicological and clinical evaluation. The extraction to clinical study of new bioactive compound follow by several steps which are-

2.6.1 Extraction- The first step of medicinal plant analysis is extraction because the separation of desired compound totally depends on the extraction process after which it is ready for further process of separation and characterization. The basic steps included in plant extraction such, pre washing of plant part, then drying of plant materials, grinding of dry material to obtain a homogenous mixture for getting increased surface area which helpful in contact with solvent and extraction process. The solvent selection depends on the nature of bioactive compound which want to isolate. Different types of solvent system available for extraction of bioactive compounds from plant source, for isolation of lipophilic compound nonpolar organic solvent is used which includes dichloromethane or mixture of dichloromethane, methanol (1:1) and for hydrophilic compounds, polar solvents are used which include methanol, ethyl acetate or ethanol. In some cases hexane is used as solvent for removing chlorophyll. (Cos et al., 2006). After selection of solvents the selection of extraction process plays an important role, different process are available for plant extraction which includes soxhlet extraction, heating under reflux, sonification and others. In addition to this plant extract also

prepared by process of maceration or percolation for fresh or dried plant material in water or any other organic solvents. (Sasidharan et al., 2011).

Modern techniques of extraction includes solid phase micro-extraction, supercritical fluid extraction, microwave assisted extraction, pressurized liquid extraction, surfactant mediated techniques and solid phase extraction. This processes are more effective than traditional extraction method because this method reduced consumption of solvent, and degradation of sample and extra steps of clean-up and concentration are not required in this process and also improves the extraction efficacy, selectivity and extraction kinetics. (Huie, 2002).

2.6.2 Identification and characterization- Plant extract which are obtained after extraction usually present in the form of combination of different types of bioactive compounds with difference in their polarities. And their identification and characterization is a big challenge. Different types of separation techniques available which include thin layer chromatography, sephedex chromatography, flash chromatography and high pressure liquid chromatography used in purification of compounds. After purification this compound are used for determination of medicinal activity and structure. Other than chromatographic techniques for purification and characterization includes immunoassay which uses monoclonal antibody (MAbs), phytochemical screening assays and fourier-transform infrared spectroscopy (FTIR) can be used for identification and characterization purposes.

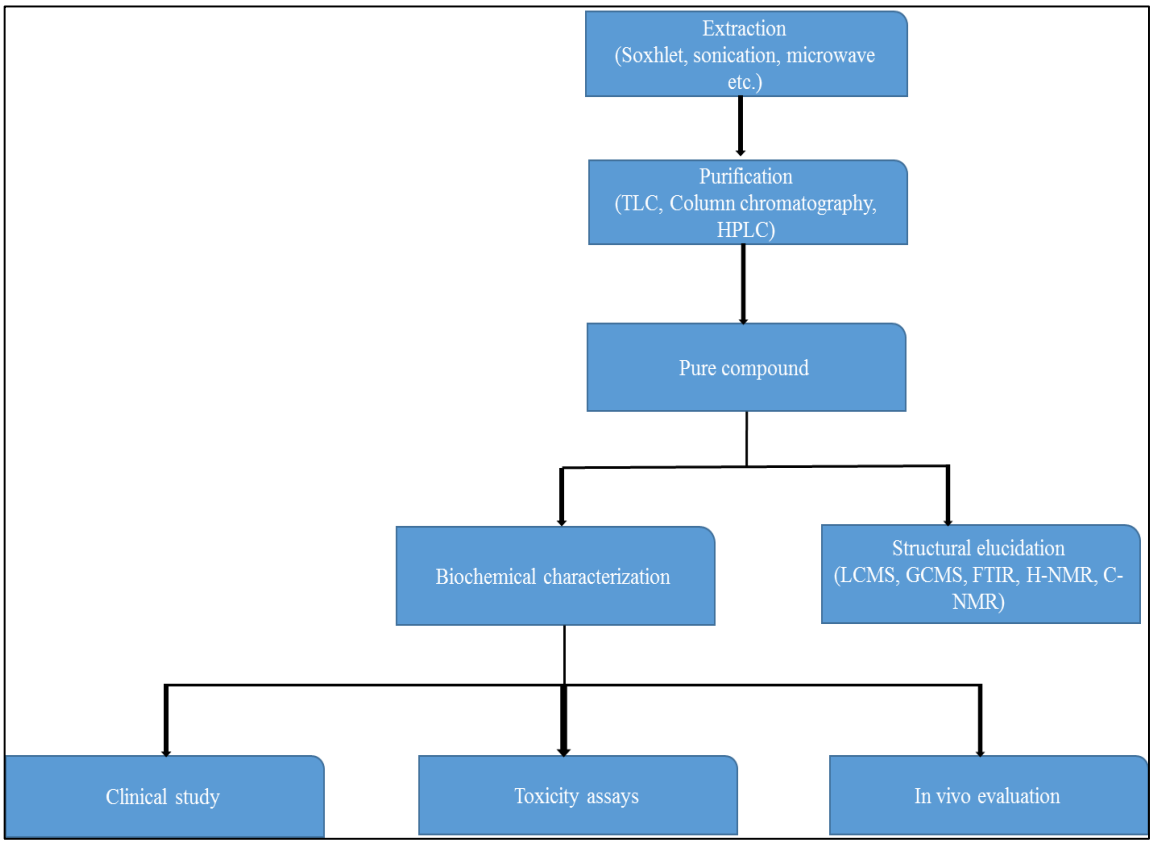

Figure 1. Brief summary of the general approaches in extraction, isolation and characterization of bioactive compound from plant extract. (Sasidharan et al., 2011)

2.7 **Measurement of stored Red blood Cells quality-** The safety of stored RBC is determined by donor's health, needs and patient's condition, cross matching accuracy and storage quality. (Klein HG et al., 2010). The stored Red blood cells are damaged by different processes like accumulation of owns waste product, injury by oxidation and by enzymes, and programmed cell death metabolically. Theses chemical activities causes' storage lesion of RBC which leads to haemolysis, reduce energy and in vivo recovery and loss of membrane, oxygen release altered, ATP and nitric oxide secretion is reduced and shading of toxic substances or products. And these toxic products are lysophospholipid that causes transfusion related acute lung injury, free irons that evoke immune response and causes inflammation and shedding of microvesicles that scavenge nitric oxide and causes inflammation and thrombosis. (Hess J. R. 2014).

Visible changes associated with RBC storage lesions includes the loss of shape from biconcave to Echinocytic spines and microvesicles blabbing. (Antonelou MH et al 2012). Chemical changes includes accumulation of lactate due to consumption of glucose, loss and gain of potassium and calcium respectively, loss of hemoglobin bound nitric oxide and decrease in the concentration of ATP and 2,3-DPG (2,3-diphosphoglycerate). Oxidative and enzymatic injury of lipids, carbohydrates and proteins occurs. And functional changes associated with storage lesion include decreasing capacity of oxygen delivery and decreasing survival rate in circulation and remain intact. (Doctor A et al., 2012).

2.8 Aging of erythrocytes in in-vivo and in-vitro are different thing, in-vivo shelf life of erythrocytes is 120 days while in storage conditions it's only 42-49 days and after that theses erythrocytes are risky for transfusion. Main difference between in-vivo and in-vitro shelf life of erythrocytes are certain biochemical and morphological changes. Recently after stydying with electron microscopy and mass spectrometry various data available which show that biochemical changes during the storage of blood affects the normal morphological condition of erythrocytes. Theses biological changes includechanges in the concentration of cations, reprogramming of energy and metabolism by redox reaction and these biochemical changes causes destruction of enzymatic activity and decrese in high energy containing phosphate molecule and these changes causes oxidative sress within the erythrocytes which includes protein glycation, fragmentation and peroxidation of lipid. And these biochemical chages affect the normal morphology of erythrocytes by causing vesciculation and blebbing of erythrocyte membrane. Transfusion of old stored erythrocytes causes rapid clearance of these erytrhocytes by receipient body within 24hours of administration and these storage lesion interfere in performing normal function of erythrocytes. So it's very necessary to develop such techniques which overcome these problems and maintain the in-vitro shelf life of erythrocytes and these may include adding certain additives, antioxidants changing storage condition to cryopreservation and deoxygenation preservation. (D'Alessandro, A et al., 2015) (Figure 2)

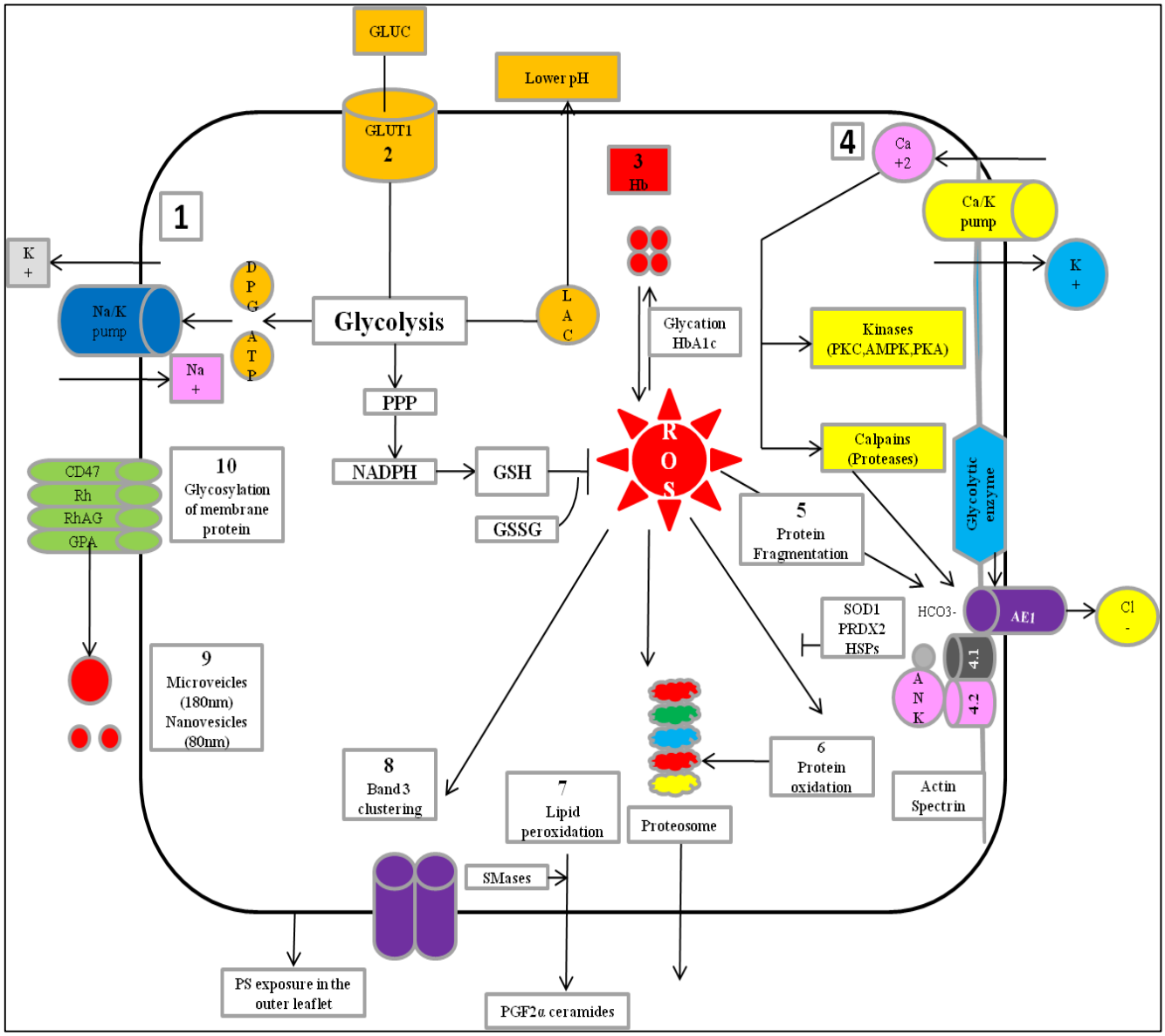

Figure 2. Figure shows an overview of biochemical changes of RBCs in in-invitro condition in blood bank.**1**) Influence of cation homeostasis by depletion of DPG and ATP and low temperature. **2**) Glycolysis consumed glucose and produce ATP and Lactate (LAC) which promote lowering of pH. **3**) Oxidative stress which are mediated by Hb fenton reaction and low temprature promotes the impair GSH and PPP homeostasis. **4**) Alteration of calcium homeostasis (with cAMP and AMP) promote kinases (PKC, AMPK, PKA) or proteolytic enzymes (calpains) targeting Band 3(AE1) and structural protein. **5**) AE1 modulates pH by chloride shift and it influenced indirectly to Hb oxygen affinity and exchanges of gas. Fragmentation of the cytosolic domain of AE1 by ROS,caspeses and calpain displaces structural proteins(ankyrin ANK, Band 4.1 and Band 4.2) and glycolytic enzymes. **6**) Oxidation of protein is partly challenged by antioxidant defences like PRDX2 and SOD1 and heat shock proteins (HSP). Redox modification of proteins (like glycation of Hb, fragmentation and carbonylation) and lipids are still promotes by storage. **7**) Protein degradation is affected by storage via the extruded in supernatant and proteosome and lipid degradation by sphingomylinase dependent accumulation of ceramides. **8**) Membrane accumulation of AE1 clusters, PS exposure in the outer leaflet and formation of lipid raft that could alter the RBCs proimmunogenic potential, promoted by storage. **9**) These all changes affect the membrane deformability, increasing osmotic fragility and promotes vesiculation events in which micro and nanovesicles are shed to eliminate irreversibly altered proteins (traces of glycolytic enzymes). **10**) Exocytic vesicles are enriched with haemoglobin, membrane portions and lipid raft proteins and also exposing some common antigens. (D'Alessandro, A. et al., 2015)

2.9 Programmed cell death of Red Blood Cells or erythrocytes- Production of erythrocyte is a complex and regulated process and in erythropoiesis it starts with a pluripotent stem cells. Erythropoiesis starts with multiple progenitor CFU-GEMM then BFU-E and finally to CFU-E which are first precursor of erythrocyte which are recognized in bone marrow called pronormoblast. And pronormoblast convert in smaller normoblast with increasing haemoglobin content and finally nucleus extruded from normoblast and it converts into reticulocyte first stage of mature erythrocytes then it released into blood stream in the form of mature erythrocytes having biconcave shaped and enucleated and carry oxygen to deliver to the tissues. The matured erythrocytes are unable to mature their own because of lack of nucleus and they don't have capacity to synthesize their proteins. Therefore its life span is higher than other blood cells but if cell environment becomes unfavourable for RBC then they don't repair their own proteins so damaging of RBC proteins is irreversible. In other cells the life and death process is well regulated by cell cycle but it's not possible for red blood cells so they lack the capacity of protein synthesis. (Bosman et al., 2005)

2.9.1 Death of erythrocytes- Destruction of All eukaryotic cells is a regulated process called apoptosis except erythrocytes because of lack of nucleus and other organelles like mitochondria and other. Destruction of mature erythrocytes carried out by their own called self-destruction which includes shrinkage of cells, microvesciculation of plasma membrane, changes in shape, alteration in cytoskeleton which is associated with protein degradation and externalization of phosphatidylserine which causes loss of asymmetry of plasma membrane. And the term for erythrocytes destruction given by some scientist is "eryptosis". (Lang KS et al., 2005).

The life span of RBC is about 120 days and it ended with senescence process in which aged erythrocytes shows molecular changes which are recognized by macrophages which remove aged RBC from peripheral blood reticuloendothelial system. The knowledge of erythrocyte death is very important for blood banking or storage of blood in blood bank by which the age or viability of RBC is improved. (Bratosin et al., 2002).

2.9.2 Induction of premature erythrocyte death- Premature erythrocytes death is triggered by number of factors which are unfavourable for Red blood cells, these factors includes the depletion of energy level in which the antioxidative defence system of red blood cells weakens due to replenishment of glutathione. (Bilmen et al., 2001). And according to this activity cation channels activate which affecting calcium ion flux. (Duronton et al., 2002). Beside this energy depletion also involves activation of protein kinase C and protein Kinase C-dependent phosphorylation of membrane proteins which stimulate eryptosis. (Foller et al., 2008).

2.9.3 Osmotic shock- In hyperosmotic condition erythrocytes released prostaglandins E2 which activates non-selective cation channels (Kaestner and Bernhardt, 2002) (Lang et al., 2005), and thus increasing the level of calcium ions in cytosol and entry of this calcium ions developed the scrambling of cell membrane of RBC. Osmotic cell shrinkage was involved in stimulation of sphingomyelinase which

caused degradation of sphingomyelin with release of ceramide in RBC. (Lang et al., 2004). Ceramide the activate scramblase which lead to breakdown of cell membranes phosphatidylserine asymmetry. (Lang et al., 2004).

2.9.4 **Oxidative stress**- intracellular oxidations due to direct reactions of peroxynitrite with haemoglobin and glutathione lead to lowering of ATP and apoptotic sign such as clustering of Band 3, caspase activation and externalization of phosphatidylserine. (Matarrese et al., 2005).

Chapter 3

OBJECTIVE

1) To perform soxhlet extraction of dry powdered Carica papaya leaves successively with different solvent and extraction of fresh papaya leaf in water by maceration.
2) To study the effect of papaya leaf extract in different solvents on the shelf life of blood.

Chapter 4

METHODOLOGY

4.1 Materials and instruments required for papaya leaf extraction-

Fresh papaya leaf obtained from DTU Campus, mortar pestle, solvents for extraction- Hexane, Acetone, Et60 (Ethanol 60%), Et40 (Ethanol 40%), distilled water, and soxhlet extractor. After extraction all extract were stored in glass bottle at 4°C.

GC-MS instrument description- ionization source EI (electron impact), column used for GC-MS Omega wax (100 mitre), DB-1/RTX-MS (30 mitre), DB-5/RTX5-MS (30 mitre), AB-Innowax (60 mitre) and Rtx-5 (30 mitre).

4.2 Materials and instruments required for study the effect of papaya leaf extract on blood shelf life-

Blood collecting vials, centrifuge, falcon tube, eppendorf, extract in phosphate buffer saline, spectrophotometer (Systronix), scanning electron microscope, inverted microscope, eppendorf, cuvettes, and pH strips and haematology analyzer (Sysmex KX-21).

Chemical requirements- CPDA-1 (citrate phosphate dextrose adenine) as anticoagulant, 2.5% gluteraldehyde, 40, 60 and 70 % of ethanol, amyl alcohol, glass slides for SEM and for haemoglobin determination by alkaline hematin detergent (AHD) method-

Table1.Composition of alkaline hematin detergent (AHD)

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

4.3 Methodology for extraction and phytochemical analysis of C. papaya leaf-

1. Extraction of dried Carica papaya leaves by soxhlet extractor with different solvent successively-

A) For extraction of leaves we collected 400gm fresh leaf sample from DTU campus.
B) After collection of sample, the leaves were washed with tap water 2-3 times with distilled water.
C) After washing, the leaves were dried in the air and in dim sunlight for 10-20 days, and took the weight of sample until we get stable weight. After drying of the sample.
D) After drying plant leaves are powdered in mortar pestle and then powdered leaves stored in airtight container for further process.
E) For soxhlet extraction of sample, we took 50gm of powdered leaves and extracted with 250ml of each solvent in order of increasing their polarity-
N-hexane, acetone, 60% ethanol (Et60), 40% ethanol (Et40) and last with distilled water.

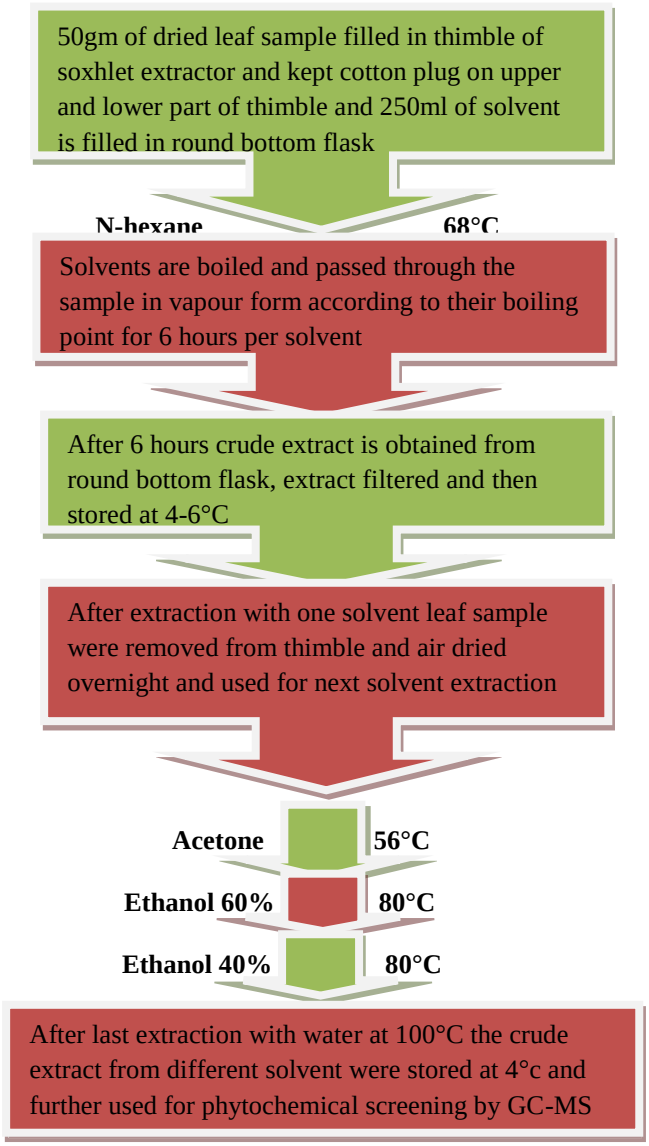

Figure 3. Flow chart of soxhlet extraction procedure.

Figure 4. Soxhlet Extraction of papaya leaf

2. **Extraction of fresh papaya leaf-**
   Fresh leaf extract of papaya leaves were prepared by maceration process in which 50gms of crushed (in mortar pestle) fresh papaya leaves kept in distilled water for 24 hrs. After maceration of 24hrs the extract were filtered by filter paper and stored extract at 4°C to prevent metabolites.

3. **Thin layer chromatography of extract**
   For TLC analysis of extract three different solvent system used for detection of secondary metabolites, for flavonoids chloroform: methanol (9:1), for terpenoid ethyl acetate: benzene (1:1) and for alkaloids hexane: acetone (17:3).

4. **Phytochemical screening of papaya leaf extract**
   A. Following phytochemical screening test were carried out on plant extract for detection of secondary metabolites-
   B. **For saponin (foam test)**- take 1ml of extract and add 10ml of distilled water and shake for 15min, foam formation shows the presence of saponin in extract.
   C. **For phenols and tannins**- ferric chloride test was carried out. Add 2ml of 2% ferric chloride solution in extract, bluish-green or black color formation shows the presence of phenols.
   D. **For flavonoids**- 2ml of 2% sodium hydroxide was added in extract, formation of intense yellow color indicate the presence of flavonoids in extract.
   E. **For glycosides (Salkowski's test)**- 2ml of chloroform were added in extract then 2ml of concentrated Sulphuric acid was added and mix gently, a reddish brown color indicates the presence of glycosides.

5. **Sampling for GC-MS analysis-**
   A. For GC-MS analysis the sample from extracts were prepared by filtering hexane and acetone extract separately with o.2µm syringe filter and stored in eppendorf at 4°C.
   B. For water containing extract sample for GC-MS were prepared by using rotary vacuum evaporator and after drying extract it dissolved in organic solvent like methanol and ethanol and then stored at 4°C.

Figure 5. Gas chromatography-Mass spectrometer

4.4 Methodology for GC-MS:

For GC-MS analysis, we took a 5ml sample of hexane and acetone extract and for extract containing water was vaporized by the rotary vacuum evaporator and after getting the crude dry extract it was dissolved in ethanol and methanol. And sample required for GC-MS analysis was 2µl.

For GC-MS analysis column temperature maintained at 100° and injection temperature was 260°, pressure 95.1 kPa, column flow rate 1.21ml/min, total flow rate 16.3ml/min and the oven temperature programmed for analysis was 100°C for 4 min, 250°C for 5 min and 280°C for 24 min and helium used as carrier gas, and ion source temperature 230°C. (Appendix)

4.5 Methodology for analysis of the effect of C. papaya leaf extract on stored blood sample

1. **Processing of plant extract used in blood**

For using plant extract in blood sample it should be sterilized and it dissolved in PBS for this all extract first evaporated and then dissolved in phosphate buffer saline and after that filtered by 0.22 µm syringe filter.

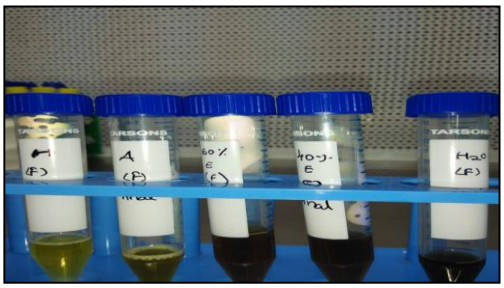

Figure 6. Papaya leaf extract in PBS

2. Collection of blood

A. 40 ml of own fresh blood collected in vials containing CPDA-1 anticoagulant and divide into 19 aliquots for further study.

B. After aliquoting blood add prepared Carica papaya leaf extract (in PBS) which was extracted in hexane, acetone, 60%ethanol, 40% ethanol, water and fresh leaf extract and the concentration of extract is 3%, 6% and 9% with respect to blood. (For 3% extract volume of extract is 0.06ml, for 6% volume is 0.12ml and for 9% volume of extract is 0.18ml for 2ml of blood sample).

3. Methodology of AHD for hemoglobin determination

For hemoglobin determination 20 µl of pellets and supernatant individually mixed with 2 ml of alkaline hematin detergent and measured absorbance at 575±5 nm.

Sample prepared by centrifuging whole blood at 5000 rpm for 5 min and after washing with PBS, pellets were dissolved in PBS.

For standard same procedure is followed with fresh blood, and for 100% lysed blood, whole blood was taken and erythrocytes lysed by Triton X and after centrifugation at 5000 rpm for 15min remove supernatant and then remaining pellets was dissolved in PBS and then add 20µl of pellets and supernatant in 2ml of AHD reagent separately and absorbance was taken at 575nm.

4. Methodology of scanning electron microscopy of blood sample

A. For slide preparation cut glass slide in 1×1 ratio and form a smear of blood by mixing 2.5% gluteraldehyde with blood on slide.

B. After smear formation dry it for 15min then add 40 to 70% ethanol drop wise after each 5min.

C. After that add small amount of amyl alcohol and dry it for 15-30min. Slide was observed in scanning electron microscope.

5. Microscopic evaluation of blood sample

For microscopic evaluation use 5µl of centrifuged pellets dissolved in PBS and form smear on a glass slide and observed in inverted microscope at 40X and picture was taken by camera.

6. pH of blood sample

For pH determination centrifuged blood plasma was taken and measured pH using pH strips of 0.1.

Chapter 5
RESULTS

5.1 Thin layer chromatography of papaya leaf extract-

1. solvent system- chloroform: methanol (9:1) for flavonoids
   Presence of spots in TLC plate shows the presence of flavonoid.

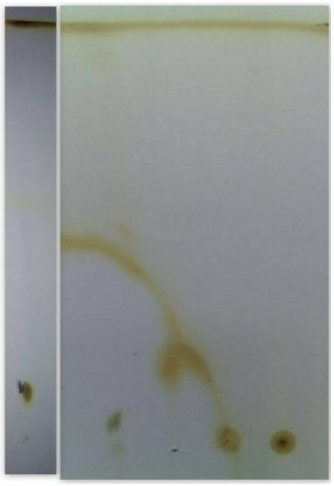

Figure 7. TLC 1 of papaya extract

Table 2. R_f values of TLC 1

S.no	Extract	Rf value
1	Hexane	0.96, 0.14
2	Acetone	0.94, 0.45, 0.06
3	60% ethanol	0.21
4	40% ethanol	0.91
5	Water	0.91

2. Solvent system- ethyl acetate: benzene (17:3) for terpenoid-
   Presence of spots in TLC plate shows the presence of terpenoid.

Figure 8. TLC 2 of papaya leaf extract

Table 3. R_f values of papaya leaf extract by TLC2

S.no	Extract	Rf value
1	Hexane	0.96, 0.98, 0.07
2	Acetone	0.08, 0.15. 0.9, 0.97
3	60% ethanol	No spot found
4	40% ethanol	0.98
5	Water	No spot found

1. Solvent system- hexane: acetone (1:1) for alkaloids-
   Presence of spots in TLC plate shows the presence of flavonoid.

Figure 9. TLC 2 of papaya leaf extract

Table 4. R_f values of papaya leaf extract by TLC 3

S.no	Extract	Rf value
1	Hexane	0.1, 0.17, 0.53, 0.99
2	Acetone	0.08, 0.11, 0.15, 0.38, 0.56
3	60% ethanol	No spot
4	40% ethanol	No spot
5	Water	No spot

5.2 Table 5. Phytochemical screening of papaya leaf extract- Phytochemical analysis shown below represents the secondary metabolites present in different plant extract.

S.no.	Extract	Tannins	Glycosides	Phenols	Saponin	Flavonoids
1	Hexane	-	+	+	-	+
2	Acetone	-	+	+	+	+
3	60% ethanol	+	-	+	+	+
4	40%ethanol	+	-	+	+	+
5	Water	+	-	+	+	-

5.3 GC-MS analysis of papaya leaf extract-

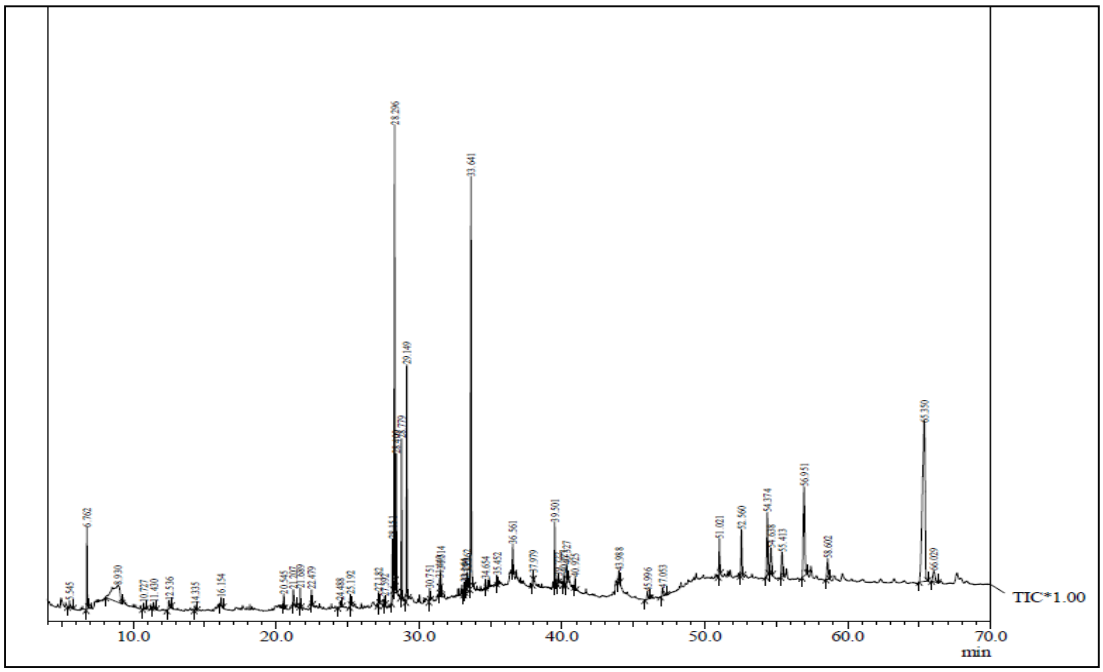

Figure 10. Chromatogram of carica papaya leaf extract in acetone

Table 6. Phytoconstituents identified from the acetone extract of Carica papaya leaves by GC-MS analysis

S. no.	Retention time	Name and Peak area in percentage		Molecular formula/ weight	
1	5.545	Cyclohexanone	0.22	C6H10O	98
2	6.762	2-propanol,1,1,1-trichloro-2-methyl-	1.97	C4H7Cl3O	176
3	8.930	1,2,3-propanetriol	5.76	C3H8O3	92
4	10.727	Benzyl isocyanate	0.19	C8H7NO	133
5	11.430	Cyclopentasiloxane, Decamethyl-	0.16	C10H30O5Si5	370
6	12.536	Cyclopropane, nonyl-	0.36	C12H24	168
7	12.813	Z-11-Tetradecenoic acid	0.59	C14H26O2	226
8	13.703	1-Hexadecanol, 3,7,11,15-Tetramethyl-	0.14	C20H42O	298
9	13.887	2-Butensaeure, 4-(1-methoxycarbonylethyl)-cyclopropyl-, methyl ester, trans	0.70	C12H18O4	226
10	14.262	Neophytadiene	24.72	C20H38	278
11	14.335	Benzene, 1,3-bis(1,1-dimethyl)-	0.12	C14H22	190
12	15.236	Hexadecanoic acid, methyl ester	0.48	C17H34O2	270
13	15.902	1,6-Heptadiene, 2-methyl-6-phenyl-	1.53	C14H18	186
14	16.154	2H-1,4-benzodiazepine-2-one,7-chloro-1,3-dihydro-5-phenyl-1-(trimethylsilyl)-	0.34	C18H19ClN2OSi	342
15	16.489	Hexanoic acid, 2-ethyl-, anhydride	0.29	C16H30O3	270
16	16.551	Isophytol, acetate	0.47	C22H42O2	338

17	16.729	1,11-Hexadecadiyne	0.26	C16H26 218
18	16.913	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	0.94	C19H32O2 292
19	17.318	Unidentified	0.16	
20	18.530	Fumaric acid, 2-dimethylaminoethyl octadecyl ester	0.55	C26H49NO4 439
21	18.986	1-Phenanthrenecarboxylic acid, 7-ethyl-1,2,3,4,4A,4B,5,6,7,9,10,10A-dodecahydro-1,4A,7-trimethyl-, methyl ester	1.08	C21H34O2 318
22	19.136	Dehydroabietic acid	1	C21H30O2 314
23	20.545	1,1,3,3,5,5,7,7,9,9,11,11-Dodecamethyl-hexasiloxane	0.37	C12H38O5Si6 430
24	20.605	Antioxidant 425	0.19	C25H36O2 368
25	20.938	Bis(2-ethylhexyl)phthalate	0.45	C24H38O4 390
26	21.207	Benzene, 1-methyl-4-(methylsulfonyl)-	0.68	C8H10O2S 170
27	21.689	1-Dodecanethiol	0.42	C12H26S 202
28	22.479	Dodecanoic acid	0.46	C12H24O2 200
29	23.062	Tetracontane	0.26	C40H82 562
30	24.488	Cyclododecasiloxane, tetracosamethyl	0.12	C24H72O12Si12 888
31	24.973	Squalene	0.43	C30H50 410
32	25.192	1-Tetradecanol, acrylate	0.31	C17H32O2 268
33	25.952	Hexatriacontane	0.13	C36H74 506
34	27.182	Acetic acid, 3,7,11,15-tetramethyl-hexadecyl ester	0.41	C22H44O2 340
35	27.592	Unidentified	0.17	
36	28.151	2-Hexadecene, 3,7,11,15-tetramethyl-,[R-[R*,R*-(E)]]-	4.79	C20H40 280
37	28.296	2,6,10-Trimethyl,14-ethylene-14-pentadecene	24.48	C20H38 278
38	30.751	Oxirane, hexadecyl-	0.35	C18H36O 268
39	31.440	1,6-Heptadine, 2-methyl-6-phenyl-	0.71	C14H18 186
40	33.164	3,7,11,15-Tetramethylhexadec-2-en-1-ol	14.47	C20H40O 296
41	33.270	Palmitic acid vinyl ester	0.25	C18H34O2 282
42	33.362	9-Octadecenoic acid (Z)-,methyl ester	0.48	C19H36O2 296
43	34.654	3-Nitro-4-(1,3,3-trimethyl-6-AZA-bicyclo[3.2.1]oct-6-yl)-benzoic acid	0.29	C17H22N2O4 318
44	35.452	Phytol,acetate	0.21	C22H42O2 338
45	36.561	3-Cyclopentylpropionic acid,2-dimethylamino ethyl ester	2.39	C12H23NO2 213
46	37.864	Carpaine	14.49	C28H50N2O4 478
47	37.979	9-Octadecenamide	0.55	C18H35NO 281
48	38.315	Isoxazolo(2,3-a)pyridine, 2,7-dicarbaldehyde dioxime-2,4,7-trimethyl-perhydro-(.beta. form)	1.36	C12H21N3O3 255
49	39.547	Benzenepropionic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-,octadecyl ester	0.61	C35H62O3 530

50	39.722	Oxacyclotridecan-2-one	0.15	C12H22O2 198
51	40.127	Cis-10-nonadecenoic acid, methyl ester	0.33	C20H38O2 310
52	40.327	Hexadecanoic acid, 2-hydroxyl-1-(hydroxymethyl)ethyl ester	0.64	C19H38O4 330
53	40.925	1,2-Benzenedicarboxylic acid	0.26	C24H38O4 390
54	43.988	Butyl 9,12,15-octadecatrienoate	0.48	C22H38O2 334
55	47.053	Silikonfett SE30 (Grevels)	0.43	
56	51.021	Gamma-tocopherol	1.31	C28H48O2 416
57	52.560	Vitamin E	1.98	C29H50O2 430
58	54.374	1,54-Dibromotetrapentacontane	2.90	C54H108Br2 914
59	54.638	Ergost-5-en-3-ol, (3.Beta.,24R)-	1.52	C28H48O 400
60	55.413	Stigmasterol	1.36	C29H48O 412
61	56.951	Stigmast-5-en-3-ol, (3.Beta.)-	6.09	C29H50O 414
62	58.602	9,19-Cyclolanost-23-ene-3,25-diol, (3.beta., 23E)-	1.19	C30H50O2 442
63	65.350	1-Alanine, N-(heptafluorobutyryl)-, undec-10-enyl ester	20.34	C18H26F7NO3 437

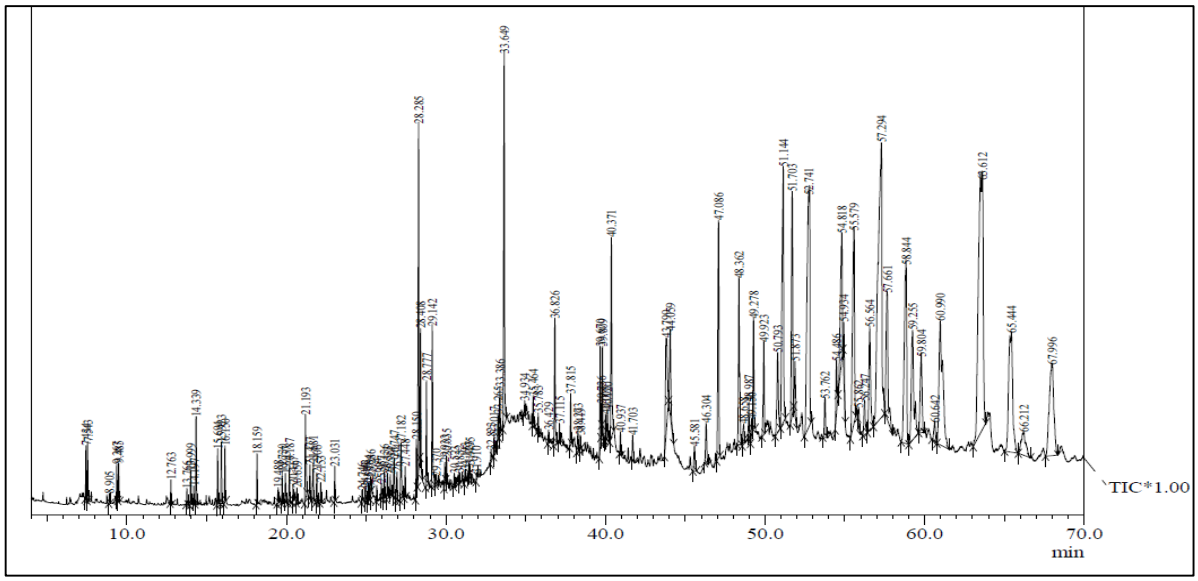

Figure 11. Chromatogram of carica papaya leaf extract in Hexane

Table 7. Phytoconstituent identified from Hexane extract of Carica papaya leaf by GC-MS

S.No.	Retention time	Name of constituent and peak area in percentage		Molecular formula/weight	
1	7.434	Dodecane, 4,6-dimethyl	0.82	C14H30	198
2	8.905	Undecane, 2,8-dimethyl-	0.05	C13H28	184
3	9.367	2-isopropyl-5-methyl-1-heptanol	0.33	C11H24O	172
4	12.763	Dodecane	0.11	C12H26	170
5	14.339	Benzene, 1,3-bis(1,1-dimethylethyl)-	0.46	C14H22	190
6	15.694	1-tridecanol	1.0	C13H28O	200
7	18.159	Tetradecane	0.25	C14H30	198
8	19.488	Hexadecane, 2,6,10,14-tetramethyl-	0.53	C20H42	282
9	19.937	Eicosane	0.37	C20H42	282
10	20.187	Heptadecane, 2,6,10,15-tetramethyl-	0.22	C21H44	296
11	20.659	Heptadecane	0.08	C17H36	240
12	21.193	Phenol, 2,4-bis(1,1-dimethylethyl)-	0.7	C14H22O	206
13	21.477	Hexacosyl heptafluorobutyrate	0.18	C30H53F7O2	578
14	21.681	1-eicosanol	0.3	C20H42O	298
15	21.906	1-dodecanol, 2-hexyl-	0.29	C18H38O	270
16	23.031	Hexadecane	0.18	C16H36	226
17	25.184	1-octanol, 2-butyl-	0.07	C12H26O	186
18	25.286	3-buten-2-one, 4-(4-hydroxy-2,2,6-trimethyl-7-oxabicyclo	0.09	C13H20O3	224
19	25.957	1-dodecanol, 3,7,11-trimethyl-	0.09	C15H32O	228
20	26.136	Tetratriacontyl pentafluoropropionate	0.23	C37H69F5O2	640
21	26.747	Octatriacontyl pentafluoropropionate	0.48	C41H77F5O2	696
22	27.182	14-.beta.-h-pregna	0.50	C21H36	288
23	28.150	2-hexadecene, 3,7,11,15-tetramethyl-, [r-[r*,r*-(e)]]-	0.32	C20H40	280
24	28.285	2,6,10-trimethyl,14-ethylene-14-pentadecne	4.19	C20H38	278
25	29.370	2-methylhexacosane	0.11	C27H56	380
26	29.933	Farnesyl acetone A	0.14	C18H30O	262
27	30.035	Hexadecanoic acid, methyl ester	0.15	C17H34O2	270
28	30.523	Tetrapentacontane	0.08	C54H110	758
29	30.832	1-tridecanol	1.0	C13H28O	200
30	31.423	2-methylhexadecane	0.03	C17H36	240
31	31.495	4,4,6-trimethyl-6-phenyltetrahydro-1,3-oxazine-2-thione	0.08	C13H17NOS	235
32	31.910	Triacontane	0.20	C30H62	422
33	32.823	Oxirane ethanol, 3-butyl-, (2r-cis)-	0.1	C8H16O2	144
34	33.017	5-isopropenyl-3,8-dimethyl-1,2,3,3a,4,5,6,7-octahydroazulene	0.17	C15H24	204
35	33.265	9,12-octadecadienoic acid (z,z)-, methyl ester	0.18	C19H34O2	294
36	33.386	9,12,15-octadecatrienoic acid, methyl ester, (z,z,z)-	0.34	C19H32O2	292
37	33.649	2-hexadecen-1-ol, 3,7,11,15-tetramethyl-, [r-[R*,R*-E]]-	2.96	C20H40O	296
38	34.934	1,54-dibromotetrapentacontane	2.34	C54H10Br2	914
39	35.464	Phytol, acetate	0.17	C22H42O2	338
40	35.783	Tetratriacontyl heptafluorobutyrate	0.62	C38H69F7O2	690

41	36.826	Docosanoic anhydride	0.89	C44H86O3 662
42	37.115	Octanamide, n-(2-hydroxyethyl)-	0.10	C10H21NO2 187
43	37.815	4,8,12,16-tetramethylheptadecan-4-olide	0.29	C21H40O2 324
44	38.273	(2e,6e,10e)-3,7,11,15-tetramethyl-2,6,10,14-hexa	0.16	C22H36O2 332
45	39670	Methyl 5,11,14-eicosatrienoate	0.59	C21H36O2 320
46	39.736	Oxalic acid, decyl 3,5-difluorophenyl ester	0.14	C18H24F2O4 342
47	39.809	Methyl 2-hydroxy-octadeca-9,12,15-trienoate	0.44	C19H32O3 308
48	40.120	15-hydroxypentadecanoic acid	0.24	C15H30O3 258
49	40.371	Hexadecanoic acid, 2-hydroxy-1(hydroxymethyl) ethyl ester	1.76	C19H38O4 330
50	40.937	Bis(2-ethylhexyl) phthalate	0.14	C24H38O4 390
51	44.059	Butyl 9,12,15-octadecatrienoate	0.89	C22H38O2 334
52	45.581	9,12,15-octadecatrienoic acid, phenylmethyl ester, (z,z,z)-	0.29	C25H36O2 368
53	47.086	Squalene	2.46	C30H50 410
54	48.362	Hexatriacontane	5.89	C36H74 506
55	48.658	2-(5-methyl-5-vinyltetrahydrofuran-2-yl)propan-2-yl 2,3,4,5,6-pentafluorobenzoate	0.23	C17H17F5O3 364
56	48.987	Solanesol	0.24	C45H74O 630
57	49.150	Neryl linalool isomer	0.12	C20H34O 290
58	49.278	2h-1-benzopyran-6-ol, 3,4-dihydro-2,8-dimethyl-2-(4,8,12-trimethyltridecyl)-, [2R-[2R*(4R*,8R*)]]-	0.85	C27H46O2 402
59	50.793	Beta.-tocopherol	1.05	C28H48O2 416
60	51.144	Gamma.-tocopherol	3.8	C28H48O2 416
61	51.873	1-heptacosanol	13.19	C27H56O 396
62	52.741	2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydro-2h-chromen-6-yl hexofuranoside	5.91	C35H60O7 592
63	54.818	Ergost-5-en-3-ol, (3.beta.,24r)-	2.22	C28H48O 400
64	54.934	1,30-triacontanediol	0.49	C30H62O2 454
65	55.579	Stigmasta-5,23-dien-3-ol, (3.beta.)-	3.44	C29H48O 412
66	55.862	Octanoic acid, 4-cyano-2,6-diiodophenyl ester	0.16	C15H17I2NO2 497
67	57.294	Stigmast-5-en-3-ol, (3.beta.)-	9.47	C29H50O 414
68	57.661	Fucosterol	2.31	C29H48O 412
69	58.844	9,19-cyclolanost-23-ene-3,25-diol, (3.beta.,23e)-	3.89	C30H50O2 442
70	59.255	9,19-cyclolanost-24-en-3-ol, (3.beta.)-	2.56	C30H50O 426
71	59.804	Longifolenaldehyde	1.29	C15H24O 220
72	60.642	9,19-cyclolanost-23-en-3-ol, 25-methoxy-, acetate, (3.beta.,23e)-	0.28	C33H54O3 498
73	60990	14,16-hentriacontanedione	4.2	C31H60O2 464
74	65.444	L-alanine, n-(heptafluorobutyryl)-, undec-10-enyl ester	5.33	C18H26F7NO3 437
75	67.996	Agatholic acid	3.73	C20H32O3 320

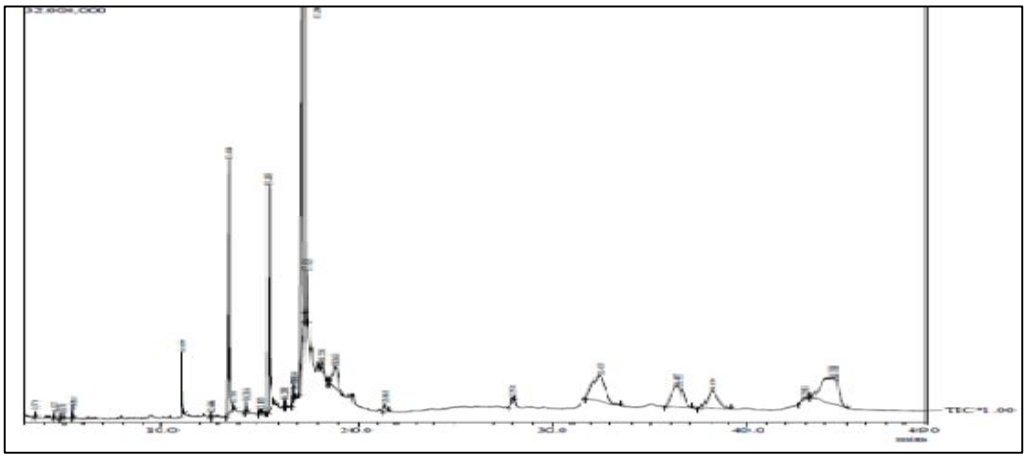

Figure 12. Chromatogram of Carica papaya leaf extract in 60% ethanol

Table 8. Phytochemicals identified in 60% ethanol extract of Carica papaya leaf by GC-MS analysis

S. No.	Retention time	Name of constituent and peak area %	Molecular formula/weight
1	3.571	2-Hydroxy-gamma-butyrolactone 0.18	C4H6O3 102
2	4.527	1,3,5-triazine-2,4,6-triamine 0.12	C3H6N6 126
3	4.876	Cyclopropylmethanol 0.08	C4H8O 72
4	5.476	Benzeneacetonitrile 0.13	C8H7N 117
5	5.537	2,3-dihydro-3,5-dihydroxy-6-methyl-4h-pyran-4-one 0.22	C6H8O4 144
6	11.050	Dodecanoic acid 1.82	C12H24O2 200
7	12.606	1-tetradecanol, acrylate 0.06	C17H32O2 268
8	13.410	Tetradecanoic acid 10.19	C14H28O2 228
9	13.718	Hexadecanoic acid, ethyl ester 0.08	C18H36O2 284
10	14.316	Tetradecanoic acid, trimethylsilyl ester 0.19	C17H36O2Si 300
11	15.105	Hexadecanoic acid, methyl ester 0.05	C17H34O2 270
12	15.485	Pentadecanoic acid 7.92	C15H30O2 242
13	16.288	Hexadecanoic acid, trimethylsilyl ester 0.16	C19H40O2Si 328
14	16.788	9,12-Octadecadienoic acid (z,z)-, methyl ester 0.18	C19H34O2 294
15	16.834	9-octadecenoic acid, methyl ester 0.18	C19H36O2 296
16	17.286	Octadec-9-enoic acid 29.61	C18H34O2 282
17	17.424	Octadecanoic acid 0.79	C18H36O2 284
18	18.228	Tetradecanoic acid, 2-hydroxy-1,3-propanediyl ester 5.27	C31H60O5 512
19	21.461	Cis-9-Hexadecenal 0.63	C16H30O 238
20	27.974	Lanost-24-ene-7,11-dione, 3-(acetyloxy)- 0.44	C32H50O4 498
21	32.435	Cis-1-Chloro-9-octadecene 11.58	C18H35Cl 286
22	36.407	Cholest-24-ene, (5 alpha. 20.xi.)- 8.28	C27H46 370
23	38.139	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester 6.95	C35H68O5 568
24	42.913	Unknown 0.38	
25	44.350	Lauric acid, 2-(hexadecyloxy)-3-(octadecyl oxy)propyl ester 14.53	C49H98O4 750

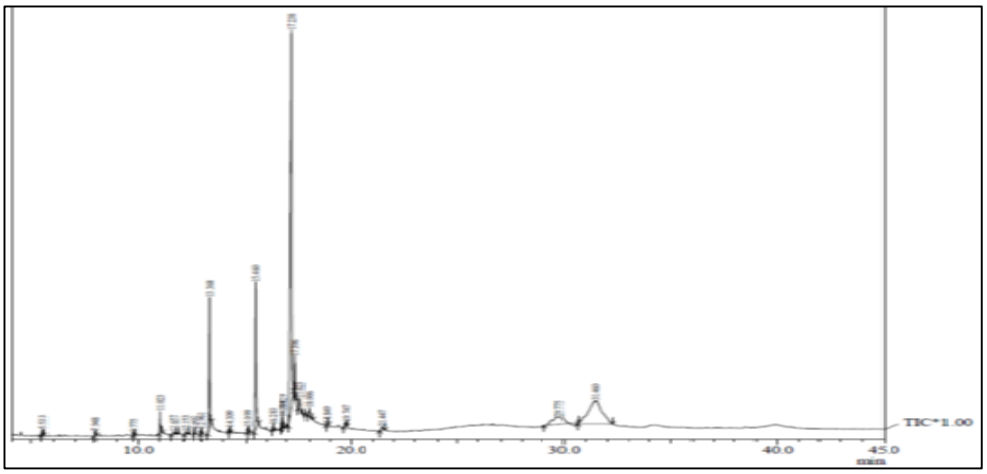

Figure 13. Chromatogram Carica papaya leaf extract in 40% ethanol

Table 9. Phytochemicals identified from 40% ethanolic extract of Carica papaya leaf by GC- MS

S.No.	Retention time	Name of constituent and peak area %		Molecular formula/weight	
1	5.533	2,3-Dihydro-3,5-dihydroxy-6-methyl-4h-pyra	0.29	C6H8O4	144
2	7.948	2-methoxy-4-vinylphenol	0.30	C9H10O2	150
3	9.775	Cis-.beta.-Farnesene	0.12	C15H24	204
4	11.023	Dodecanoic acid	1.35	C12H24O2	200
5	11.657	2-Methyl-4-pyridinamine 1-oxide	0.25	C6H8N2O	124
6	12.153	Alpha.-D-Xylofuranose, 1,2-O-isopropylidene-5-(t-butyldimethylsilyl)-	0.25	C14H28O5Si	304
7	12.602	1-tetradecanol, acrylate	0.08	C17H32O2	268
8	12.961	Tetradecanoic acid, methyl ester	0.20	C15H30O2	242
9	13.368	Tetradecanoic acid	8.12	C14H28O2	228
10	14.309	Tetradecanoic acid, trimethylsilyl ester	0.19	C17H36O2Si	300
11	15.099	Hexadecanoic acid, methyl ester	0.19	C17H34O2	270
12	15.460	Pentadecanoic acid	9.48	C15H30O2	242
13	16.283	Hexadecanoic acid, trimethylsilyl ester	0.19	C19H40O2Si	328
14	16.784	9,12-octadecadienoic acid (z,z)-, methyl ester	0.30	C19H34O2	294
15	16.829	9-octadecenoic acid (z)-, methyl ester	0.50	C19H36O2	296
16	17.238	Heptadecene-(8)-carbonic acid-(1)	44.79	C18H34O2	282
17	17.396	Octadecanoic acid	1.51	C18H36O2	284
18	17.623	(9e,12e)-9,12-octadecadienoyl chloride	1.18	C18H31ClO	298
19	18.006	9,12-octadecadienoic acid	0.76	C18H32O2	280
20	18.849	Hexadecanoic acid	0.10	C16H32O2	256
21	19.747	9-octadecenamide	0.47	C18H35NO	281
22	21.447	4-Cyanobenzoic acid, hexadecyl ester	0.52	C24H37NO2	371
23	29.775	Dodecanoic acid, 1,2,3-propanetriyl ester	4.82	C39H74O6	638
24	31.460	1-octadecanethiol	24.02	C18H38S	286

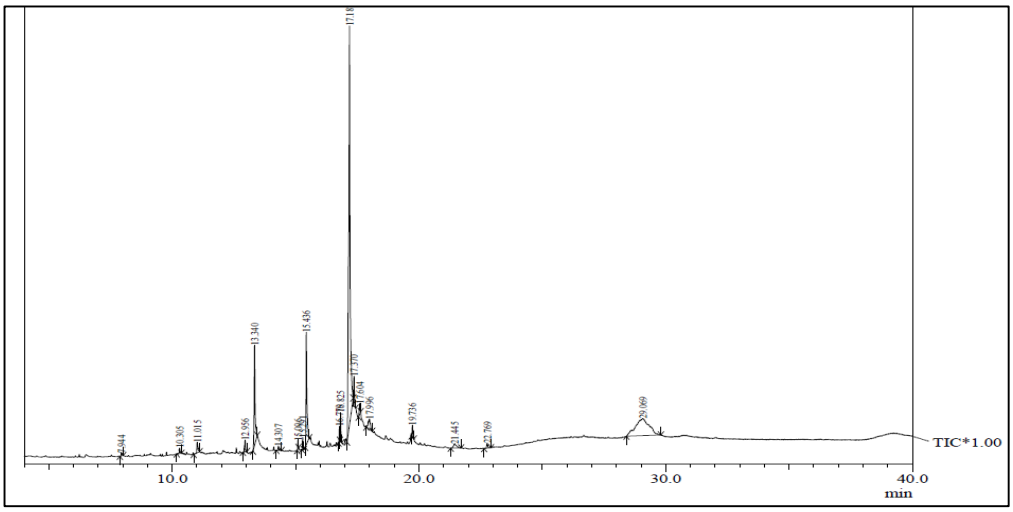

Figure 14. Chromatogram of Carica papaya leaf extract in water

Table 10. Phytochemicals identified from water extract of Carica papaya leaf by GC-MS analysis

S. No.	Retention time	Name of constituent and peak area %		Molecular formula/weight	
1	7.944	2-methoxy-4-vinylphenol	0.30	C9H10O2	150
2	10.305	1,6-cyclodecadiene, 1-methyl-5-methylene-	0.32	C15H24	204
3	11.015	Dodecanoic acid	1.11	C12H24O2	200
4	12.956	Tetradecanoic acid, methyl ester	0.79	C15H30O2	242
5	13.340	Tetradecanoic acid	8.31	C14H28O2	228
6	14.307	Tetradecanoic acid, trimethylsilyl ester	0.35	C17H36O2Si	300
7	15.096	Hexadecanoic acid, methyl ester	0.35	C17H34O2	270
8	15.291	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	0.67	C11H18N2O2	210
9	15.436	Pentadecanoic acid	9.39	C15H30O2	242
10	16.779	9,12-Octadecadienoic acid (z,z)-, methyl ester	0.63	C19H34O2	294
11	16.825	9-octadecenoic acid, methyl ester	1.30	C19H36O2	296
12	17.187	Heptadecene-(8)-carbonic acid-(1)	49.74	C18H34O2	282
13	17.370	Octadecanoic acid	1.44	C18H36O2	284
14	17.604	Octadecanamide	0.85	C18H37NO	283
15	17.996	9,12-Octadecadienoic acid	1.84	C18H32O2	280
16	19.736	9-Octadecenamide	0.82	C18H35NO	281
17	21.445	Z,E-2,13-Octadecadien-1-ol	1.10	C18H34O	266
18	22.769	Bis(2-ethylhexyl) phthalate	0.46	C24H38O4	390
19	29.069	Dodecanoic acid, 1,2,3-propanetriyl ester	20.23	C39H74O6	638

Table 11. Phytoconstituents and their medicinal uses obtained from Carica papaya leaf extract (Hexane)

S.No.	Name	Common name	Nature	Uses
1	2-isopropyl-5-methyl-1-heptanol		Alcoholic	Antimicrobial
2	Heptadecane		Alkane	Antioxidant and anti-inflammatory
3	Phenol, 2,4-bis(1,1-dimethylethyl)-		Phenolic	Antioxidant, antitumor, antimicrobial, antibiotic
4	14-.beta.-h-pregna	14b-pregnane	Glycoside	Parent of progesterone
5	2,6,10-trimethyl,14-ethylene-14-pentadecne	Neophytadiene	Terpenoid	Anti-inflammatory, Antibacterial, antipyretic, analgesic, antioxidant
6	2-hexadecen-1-ol, 3,7,11,15-tetramethyl-, [r-[r*,r*-e]]-	Phytol	Diterpene alcohol	Anti-inflammatory, antioxidant, antimicrobial, cancer preventive
7	Phytol acetate		Diterpene	Antimicrobial, anti-inflammatory, anticancer, diuretic
8	2-hexadecene, 3,7,11,15-tetramethyl-, [r-[r*,r*-(e)]]-			Antimicrobial, anti-inflammatory
9	Hexadecanoic acid, methyl ester	Palmitic acid, methyl ester	Fatty acid ester	Antioxidant, hypocholesterolemic, pesticide
10	9,12-octadecadienoic acid (z,z)-, methyl ester	Linoleic acid,methyl ester	Fatty acid ester	Hepatoprotective, antihistaminic, hypocholesterolemic, antieczemic
11	9,12,15-octadecatrienoic acid, methyl ester, (z,z,z)-	Linolenic acid,methyl ester	Fatty acid ester	Anti-inflammatory, hypocholesterolemic cancer preventive, hepatoprotective, nematicide insectifuge, antihistaminic, antieczemic, antiacne, 5-alpha reductase inhibitor antiandrogenic, antiarthritic, anticoronary
12	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	Glycerol, 2-palmitate	Triglyceride	Hemolytic, pesticide, flavor, antioxidant
13	Squalene		Triterpene hydrocarbon	Antibacterial, antioxidant, antitumor, cancer preventive, immunostimulant, chemo preventive
14	Solanesol		Unsaturated alcohol	Antiulcer, anti-heart failure, treatment of liver injury,adjuvant therapy for cancer, anaemia,muscular

				dystrophy, asthama, diabetes, hypertension
15	2h-1-benzopyran-6-ol, 3,4-dihydro-2,8-dimethyl-2-(4,8,12-trimethyltridecyl)-, [2r-[2r*(4r*,8r*)]]-	Vitamin e	Phenol	Antioxidant, strong immune system, anticancer, heart disease, lung disease, dementia, anaemia
16	Beta-tocopherol		Phenol	antioxidant, anti-inflammatory antimicrobial, oestrogenic and insecticidal
17	Gamma tocopherol		Phenol	Antioxidant, reactive nitrogen scavenging ability, anti-inflammatory
18	1-heptacosanol		Fatty alcohol	Nematicidal , anticancer, antioxidant and antimicrobial
19	1-eicosanol	Arachidyl alcohol	Fatty alcohol	Antimalarial, antifungal, antioxidant
20	2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydro-2h-chromen-6-yl	Delta tocopherol	Phenol	Antioxidant, anticancer, antiinfammatory, alzeimers disease
21	Ergost-5-en-3-ol, (3.beta.,24r)-	Campestrol	Phytosterol	Hypercholesterolemia, benign prostatic hyperplasia
22	Stigmast-5-en-3-ol, (3beta)-	Beta sitosterol	Phytosterol	Cholesterol lowering, immunomodulator
23	Fucosterol		Phytosterol	Antidiabetic, free radical scavenging activity, hepatoprotective, antioxidant

Table 12. Phytoconstituents and their medicinal uses obtained from Carica papaya leaf extract (Acetone)

S.No.	Name	Common name	Compound nature	Uses
1	2-propanol,1,1,1-trichloro-2-methyl-	Chlorobutanol	Alcohol	chemical preservative, sedative hypnotic and weak local anaesthetic, antibacterial and antifungal
2	2-propanol,1,1,1-trichloro-2-methyl-	Glycerol or glycerine	Sugar alcohol or polyol	Food industry and pharmaceuticals
3	Dodecanoic acid	Lauric acid	Saturated fatty acid	Increases HDL and in acne treatment

4	2,6,10-Trimethyl,14-ethylene-14-pentadecene	Neophytadiene		Anti-inflammatory, antibacterial, antipyretic, analgesic, antioxidant
5	3,7,11,15-Tetramethylhexadec-2-en-1-ol	Phytol	Diterpene alcohol	Anti-inflammatory, antioxidant, antimicrobial
6	Phytol acetate		Diterpene alcohol	Antimicrobial, anti-inflammatory, anticancer, diuretic
7	9-Octadecenamide	Oleamide	Amide of fatty acid	Induce sleep in animals
8	Hexadecanoic acid, 2-hydroxyl-1-(hydroxymethyl)ethyl ester	Glycerol, 2-palmitate	triglyceride	Antioxidant
9	1,2-Benzenedicarboxylic acid	Phthalic acid	Carboxylic acid	Manufacturing of medicine, perfume
10	Butyl 9,12,15-octadecatrienoate			Activity unknown
11	Gamma-tocopherol	Vitamin E	Methylated phenols	Antioxidant, reactive nitrogen scavenging ability, Anti-inflammatory
12	Vitamin E	Tocopherol	Methylated phenols	Antioxidant, strong immune system, Anticancer, heart disease, lung disease, dementia, anaemia
13	Ergost-5-en-3-ol, (3.Beta.,24R)-	Campesterol	Phytosterol	In hypercholesterolemia, benign prostatic hyperplasia
14	Stigmasterol		Phytosterols	Anti-inflammatory, tumour promotion inhibitor, anti HIV, anti-hepatotoxic
15	Stigmast-5-en-3-ol, (3.Beta.)-	Beta sitosterol	Phytosterol	Cholesterol lowering, immuno-modulator

16	Squalene		Hydrocarbon, triterpene	Anticancer, antioxidant, immune-stimulant, antibacterial
17	Carpaine		Alkaloids	Cardiovascular, amebicide, antibacterial, antitumor, anti-tubercular

Table 13. Phytoconstituents and their medicinal uses obtained from 60%, 40% ethanol and water extract of papaya leaf

S. No.	Name of constituent	Common name	Nature	Medicinal use
1	2-phenylacetonitrile	Benzyl cyanide	Aromatic nitrile	Precursor of different pharmaceuticals like analgesics, antimalarial, antihistanins etc.
2	2 3-dihydro-3 5-dihydroxy-6-methyl-4h-pyran-4-one	DDMP		Antioxidant, antimicrobial, anti-inflammatory
3	Dodecanoic acid	Lauric acid	Fatty acid	Increases HDL and in acne treatment
4	Tetradecanoic acid	Myristic acid	Fatty acid	In cosmetics
5	Hexadecanoic acid, ethyl ester	Ethyl palmitate	Fatty acid ester	Antioxidant, antiandrogenic, hypocholesterolemic, haemolytic, 5-Alpha reductase inhibitor
6	Hexadecanoic acid, methyl ester	Methyl palmitate	Fatty acid ester	Antioxidant, hypocholesterolemic, antiandogenic
7	Pentadecanoic acid		Fatty acid	Antioxidant
8	9,12-octadecadienoic acid (z,z)-, methyl ester	Linoleic acid, methyl ester	Fatty acid ester	Antimalarial, Hepatoprotective, antihistaminic, hypocholesterolemic, antieczemic
9	9-octadecenoic acid, methyl ester	Oleic acid methyl ester	Fatty acid ester	Antibacterial, antioxidant and antifungal
10	Octadec-9-enoic acid	Oleic acid	Fatty acid	Cancer preventive, Anemiagenic, Antiandrogenic, Dermatitigenic
11	Lanost-24-ene-7,11-dione, 3-(acetyloxy)-	Sterols	Steroid	

12	2-methoxy-4-vinyl phenol		Phenol	Anticancer and anti-inflammatory
13	cis-beta-Farnesene		Sesquiterpens	Antimicrobial
14	Dodecanoic acid	Lauric acid	Fatty acid	Increase serum HDL, acne treatment
15	Tetradecanoic acid, methyl ester	Methyl myristate	Fatty acid ester	Cosmetics
17	9,12-octadecadienoic acid	Linoleic acid	Fatty acid	Antioxidant, anticancer, antidiabetic, antiasthematic,
18	Hexadecanoic acid	Palmitic acid	Fatty acid	Anti-inflammatory
19	9-octadecenamide	Oleamide	Amide	Mood and sleep disorder, antidepressant

5.4 Haemoglobin estimation by AHD (alkaline haematin detergent) method

Table 14. Standard of AHD- Represents the highest and lowest value of haemoglobin in pellets and supernatant by absorbance. In AHD method triton X hemolysed all red blood cells and released free haemoglobin which react with sodium hydroxide and form coloured compound hematin which is measured in spectrophotometer.

S. No.	Sample	Absorbance of pellets	Absorbance of plasma
1	Fresh blood 0^{th} day	0.285	0.006
2	100% lysed blood by triton X	0.125	0.657

Table.15 Absorbance of different blood sample by AHD method (Pellets=P, supernatant=SN).

Extract %	Day 0		Day 14		Day 21		Day 28		Day 35		Day 42	
	P	SN	P	SN	P	SN	P	SN	P	SN	P	SN
Control	0.285	0.006	0.253	0.014	0.244	0.027	0.209	0.031	0.158	0.017	0.105	0.049
3% Hex	-	-	0.277	0.012	0.267	0.023	0.232	0.026	0.165	0.017	0.119	0.03
6% Hex	-	-	0.271	0.017	0.262	0.027	0.229	0.03	0.178	0.014	0.103	0.037
9% Hex	-	-	0.256	0.018	0.271	0.025	0.244	0.028	0.146	0.021	0.118	0.035
3% Ace	-	-	0.259	0.023	0.268	0.034	0.234	0.041	0.157	0.016	0.126	0.048
6% Ace	-	-	0.248	0.026	0.263	0.035	0.226	0.045	0.163	0.015	0.118	0.053
9% Ace	-	-	0.255	0.019	0.271	0.027	0.241	0.032	0.15	0.014	0.129	0.045
3% Et60	-	-	0.264	0.017	0.272	0.024	0.251	0.029	0.168	0.016	0.159	0.036
6% Et60	-	-	0.249	0.022	0.27	0.025	0.246	0.035	0.172	0.015	0.144	0.048
9% Et60	-	-	0.259	0.026	0.269	0.034	0.238	0.041	0.152	0.013	0.116	0.053
3% Et40	-	-	0.245	0.021	0.25	0.027	0.232	0.034	0.153	0.014	0.115	0.058
6% Et40	-	-	0.256	0.015	0.247	0.022	0.223	0.039	0.152	0.01	0.106	0.047
9% Et40	-	-	0.263	0.029	0.236	0.036	0.211	0.045	0.159	0.019	0.112	0.048
3% water	-	-	0.268	0.011	0.276	0.02	0.256	0.025	0.153	0.015	0.141	0.028
6% water	-	-	0.259	0.017	0.262	0.028	0.241	0.033	0.159	0.015	0.139	0.041
9%water	-	-	0.261	0.014	0.256	0.023	0.234	0.038	0.16	0.019	0.126	0.046
3% fresh leaf	-	-	0.253	0.012	0.28	0.018	0.264	0.023	0.157	0.016	0.136	0.029
6% fresh leaf	-	-	0.264	0.015	0.264	0.019	0.24	0.021	0.167	0.017	0.127	0.035
9% fresh leaf	-	-	0.249	0.016	0.26	0.02	0.231	0.028	0.158	0.021	0.118	0.037

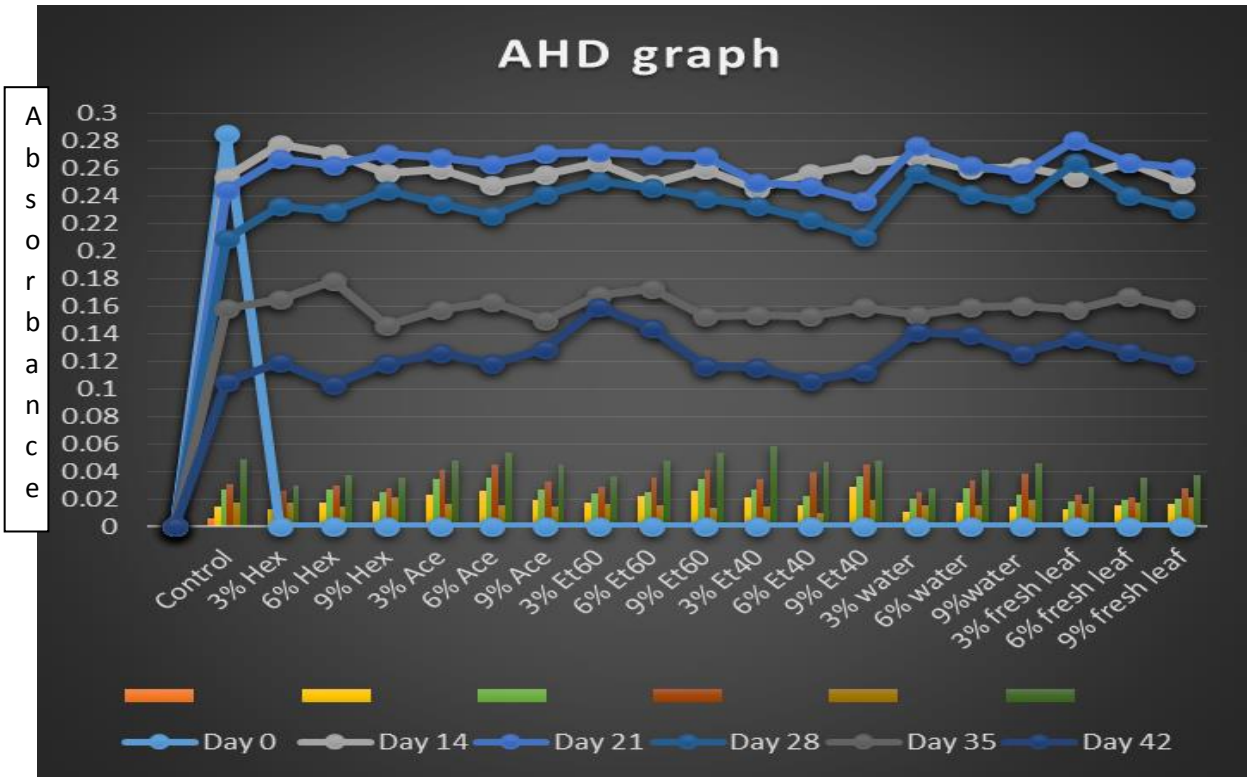

Figure 15. AHD absorbance of blood sample- day by day during storage of blood the absorbance of supernatant is increasing and absorbance of pellets decreasing because the haemolysis of RBC increases day by day during storage period

5.5 Table 16. pH of blood sample up to 7th week-

S.no.	Sample name	0th day	14th day	21st day	28th day	35th day	42nd day
1	**Control**	7.4	7.2	7.0	6.9	6.8	6.7
2	**3% Hex**	-	7.3	7.2	7.0	7.0	6.7
3	**6% Hex**	-	7.3	7.2	7.0	7.0	6.8
4	**9% Hex**	-	7.4	7.2	7.0	7.0	6.9
5	**3% Ace**	-	7.4	7.2	7.0	7.0	6.9
6	**6% Ace**	-	7.3	7.1	7.0	7.0	6.7
7	**9% Ace**	-	7.3	7.2	7.0	7.0	6.9
8	**3% Et60**	-	7.4	7.3	7.1	7.0	6.9
9	**6% Et60**	-	7.4	7.3	7.0	7.0	6.9
10	**9% Et60**	-	7.3	7.2	7.0	7.0	6.7
11	**3% Et40**	-	7.3	7.2	7.0	7.0	6.9
12	**6% Et40**	-	7.2	7.0	7.0	7.0	6.7
13	**9% Et40**	-	7.2	7.0	7.0	7.0	6.8
14	**3% Water**	-	7.4	7.2	7.0	7.0	6.9
15	**6% Water**	-	7.4	7.2	7.0	7.0	6.9
16	**9% Water**	-	7.4	7.2	7.0	7.0	6.8
17	**3% Fresh leaf**	-	7.4	7.2	7.0	7.0	6.9
18	**6% Fresh leaf**	-	7.4	7.2	7.0	7.0	6.9
19	**9% Fresh leaf**	-	7.4	7.2	7.0	7.0	6.8

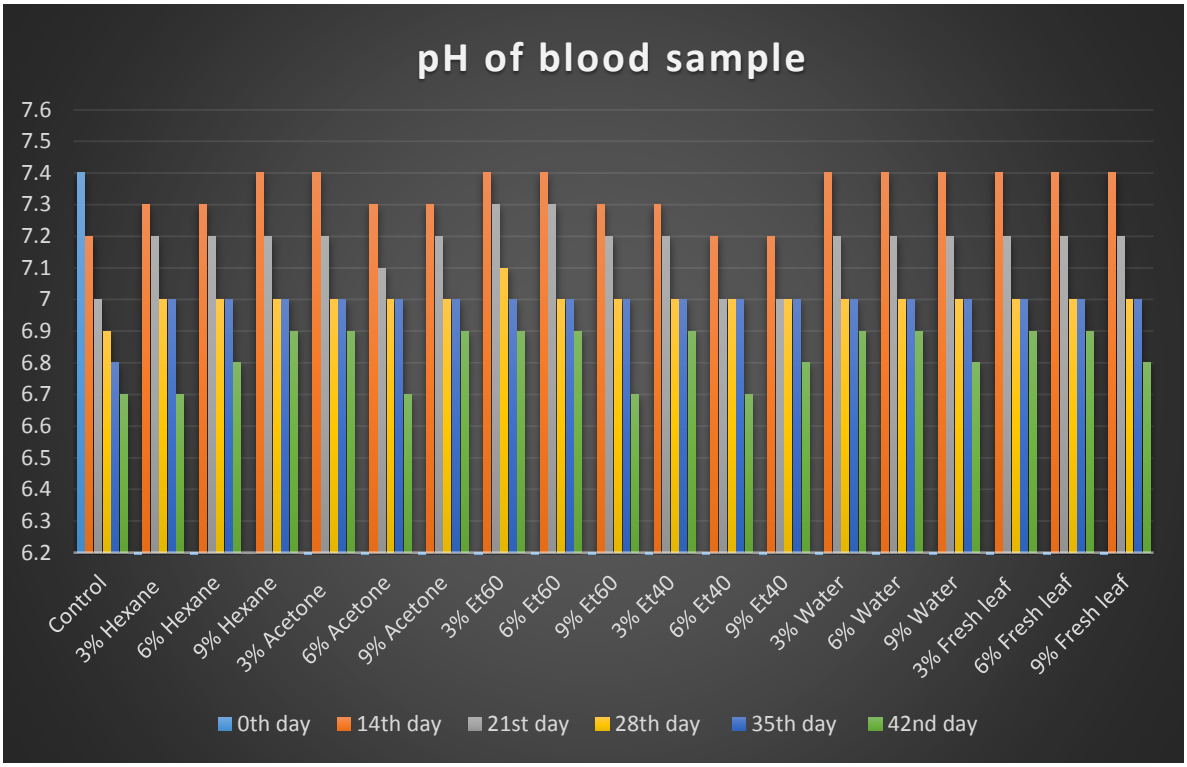

Figure 16. pH of different extract containing blood sample during storage period- Show changes in pH from day 0 to day 42, pH changes shows that the acidity of stored blood is increased day by day but in presence of extract the effect is slow. pH is acidic due to accumulation of lactic acid by glycolysis.

5.6 Erythrocyte morphology by inverted microscope (40X) - morphology changes from discoid or biconcave shape to Echinocytes and then spherocytes but changes in shape is different in all extract. Morphological studies explained about the cell death and shape of the red blood cells.

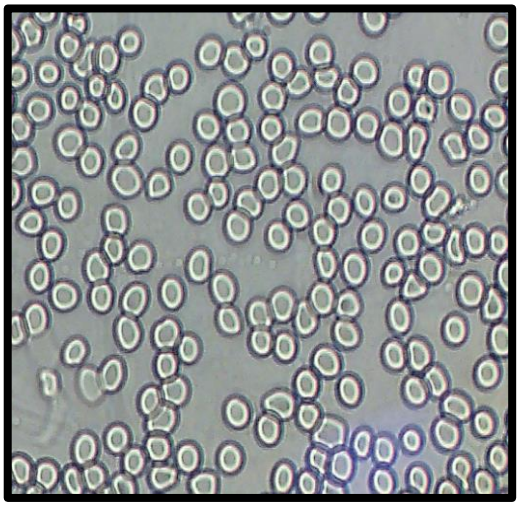

Figure 17. Morphology of fresh blood sample (40X)

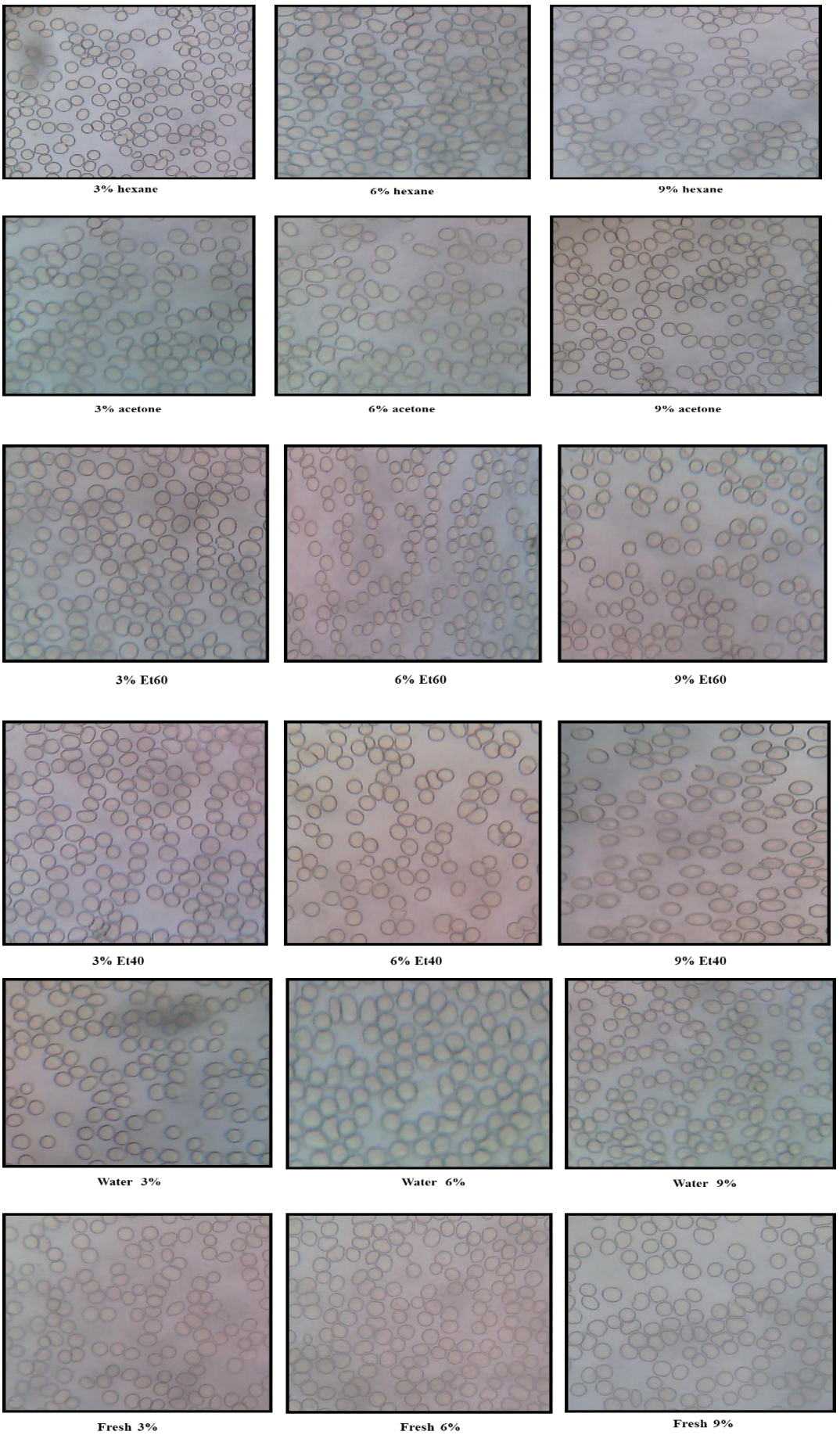

Figure 18. Morphology of erythrocytes after 2 weeks of storage- biconcave shaped

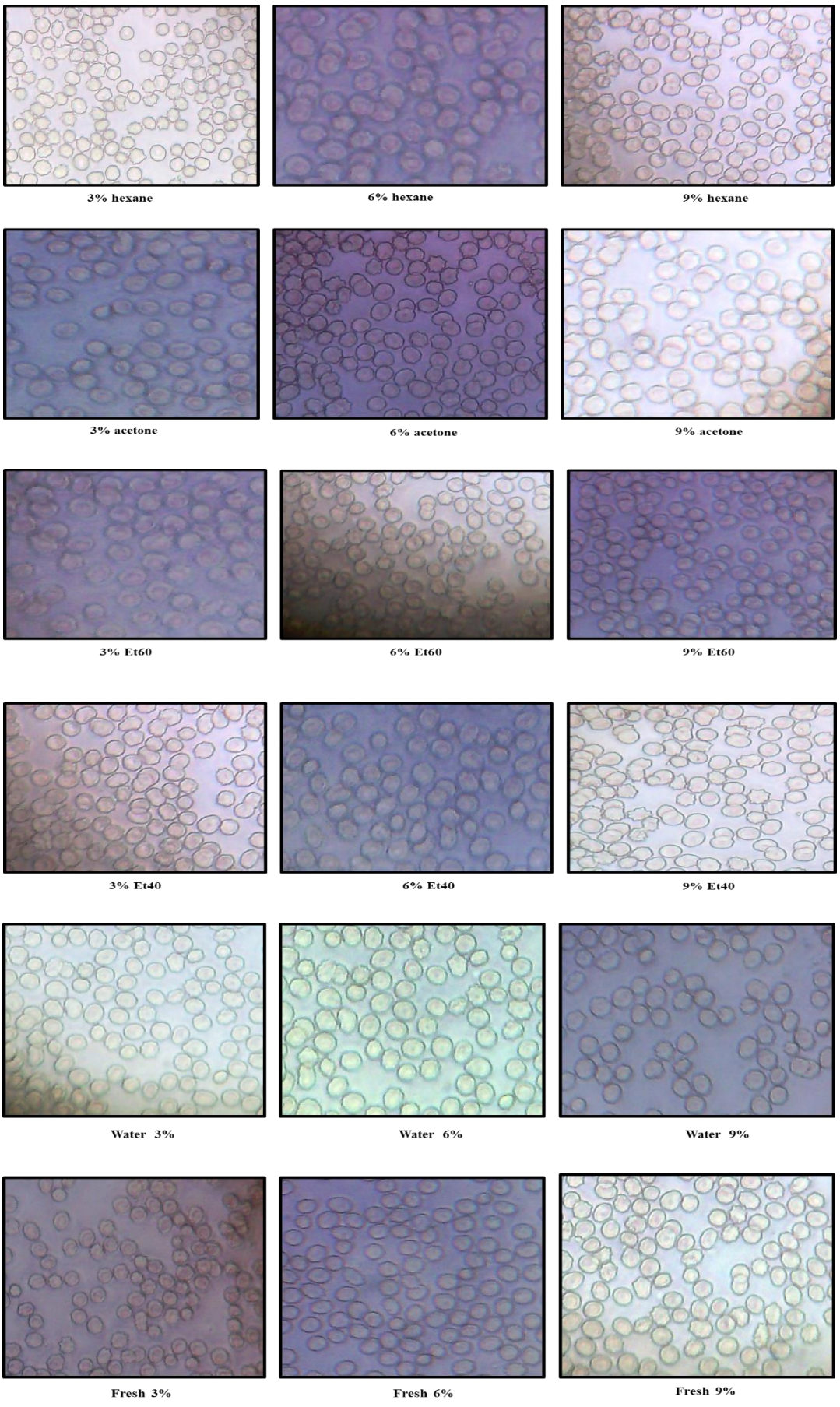

Figure 19. Morphology of a erythrocyte after 4weeks of storage- biconcave and some echinocytes

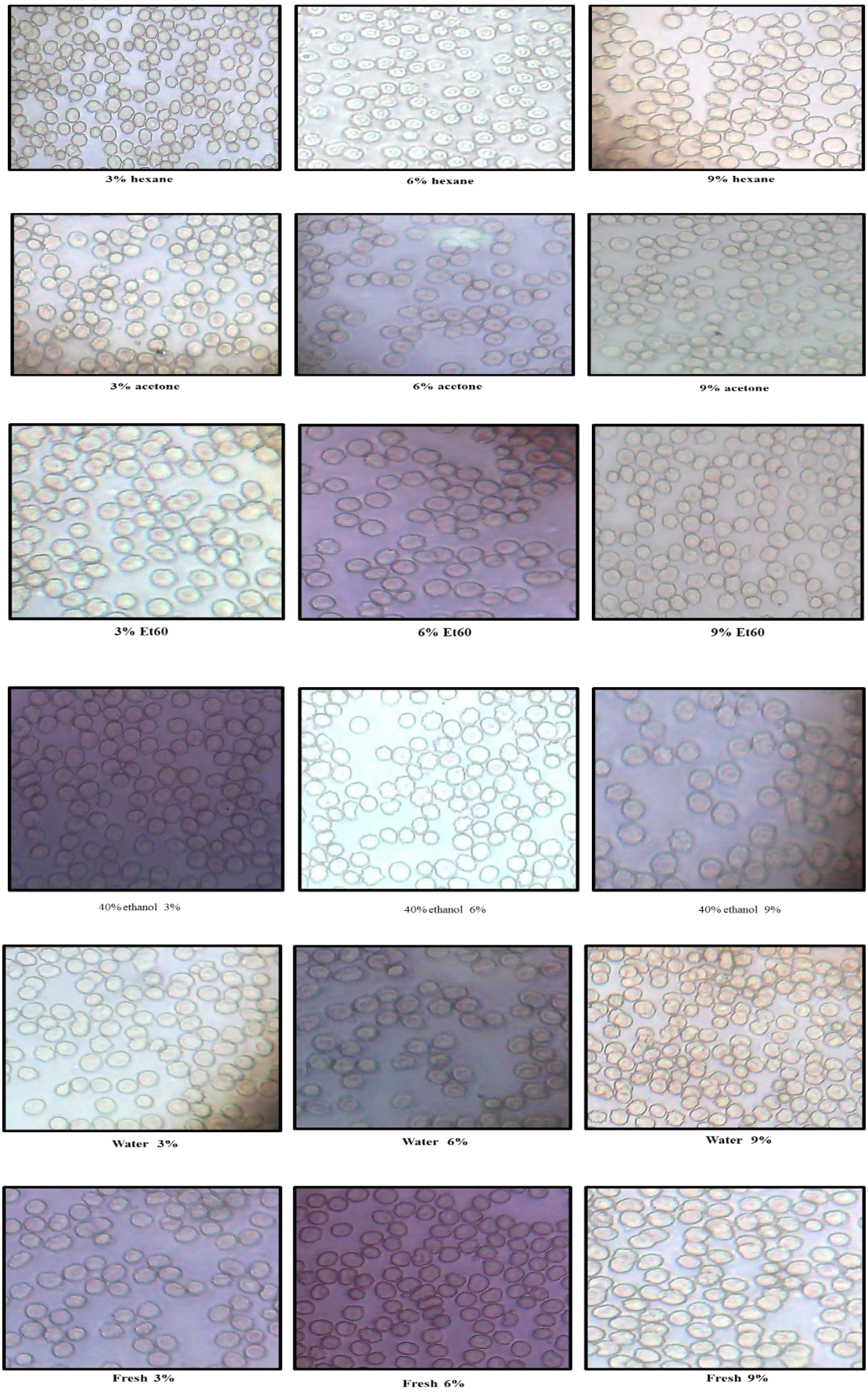

Figure 20. Morphology of erythrocytes after 5 weeks of storage- biconcave and echinocytes.

5.7 Red blood cells morphological changes during storage period by scanning electron microscope (SEM)-

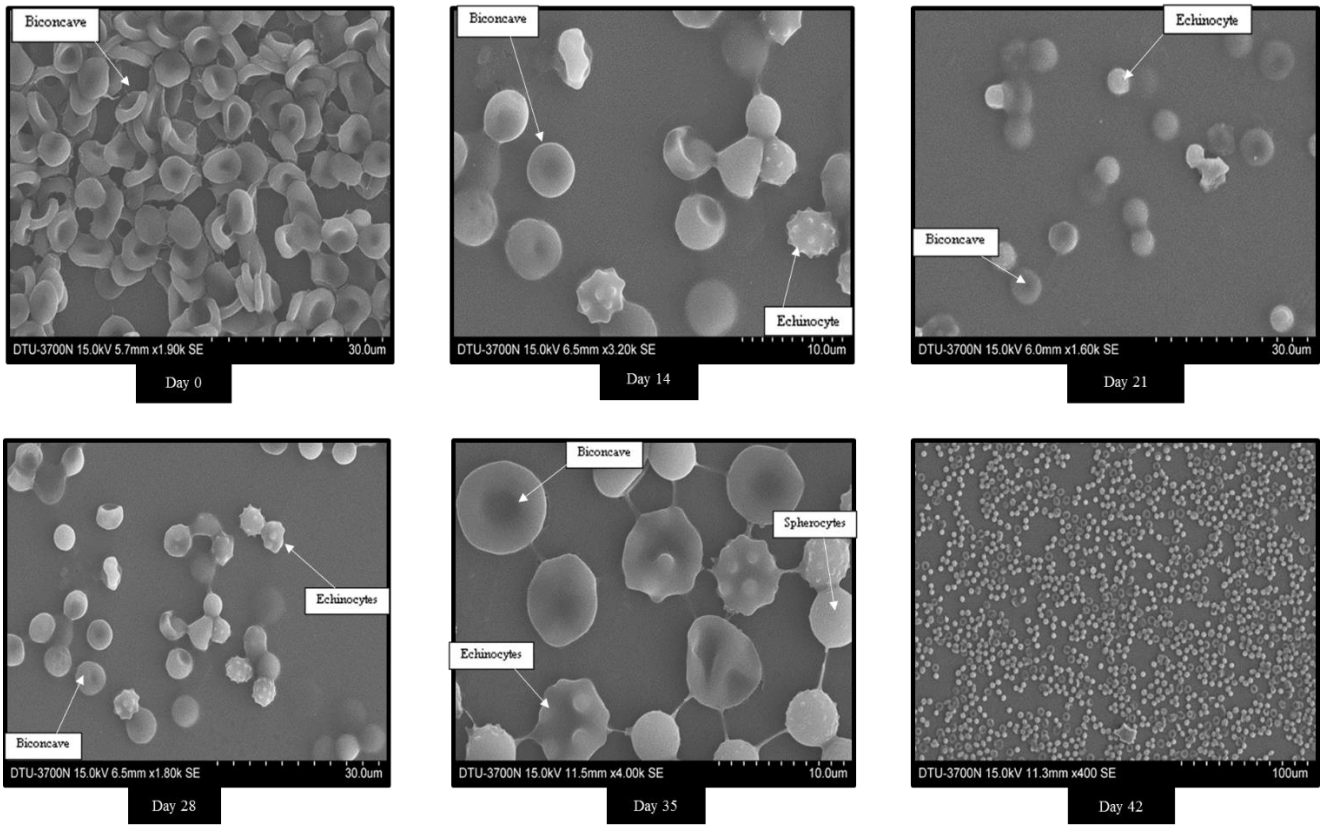

Figure 21. Study of RBC morphological changes from 1st week to 5th week during storage by scanning electron microscopy. During storage period the shape of red blood cells changed from discoid or biconcave to Echinocytes and then spherocytes or microvesicles. The reason for change in the shape of red blood cell is externalization of phosphatidylserine of plasma membrane due to which spike like projection formed on outer surface.

5.8 Table 17. Red Blood Cell count by haematology analyzer (Sysmex KX-21N) - Cell count of blood sample containing different concentration of extract by haematology analyzer represents the effect of different concentration of plant extracts on blood shelf life. Highest blood count shown by 3% concentration of Et60 (60% ethanol) extract of papaya leaf which shows about 91.55 % of viability with respect to fresh blood cell viability which is 100 % and it is effective for storage of blood. Below table explains the comparison of cell count with control and the effect of different extracts on blood shelf life.

S. No.	Extract %	Cell count $\times 10^{12}$ cells/ml	% viability of erythrocytes	Haemoglobin gm./dl	Remark
1	Fresh sample	3.79	100 %	10.6	Fresh sample
2	3% Hexane	2.82	74.40 %	7.9	Negative effect
3	6% Hexane	2.89	76.25 %	7.8	Negative effect
4	9% Hexane	3.04	80.21 %	8.3	Positive effect
5	3% Acetone	3.14	82.84 %	8.5	Positive effect
6	6% Acetone	2.94	77.57 %	8.0	No effect
7	9% Acetone	3.06	80.73 %	8.2	Positive effect
8	3% Et60	3.47	91.55 %	9.8	Best positive effect
9	6% Et60	3.07	81.00 %	8.4	Positive effect
10	9% Et60	2.93	77.30 %	8.0	Negative effect
11	3% Et40	3.04	80.21 %	8.3	Positive effect
12	6% Et40	2.87	75.72 %	7.9	Negative effect
13	9% Et40	2.92	77.04 %	8.0	Negative effect
14	3% Water	3.25	85.75 %	8.9	Positive effect
15	6% Water	3.15	83.11 %	8.5	Positive effect
16	9% Water	3.07	81.00 %	8.4	Positive effect
17	3% Fresh leaf	3.27	86.27 %	9.0	Positive effect
18	6% Fresh leaf	3.13	82.58 %	8.6	Positive effect
19	9% Fresh leaf	2.98	78.62 %	8.1	Positive effect
20	Control	2.94	77.57 %	8.1	Control

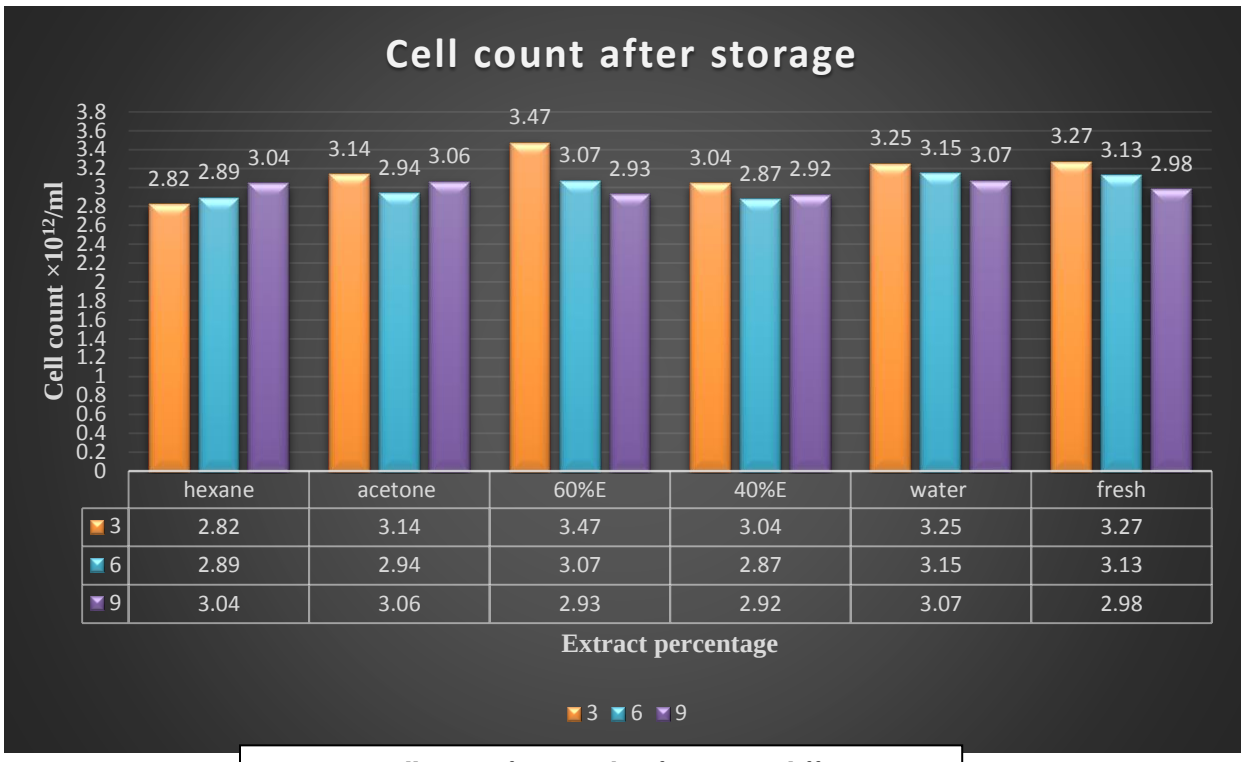

Figure 22. Cell count after 5 weeks of storage in different

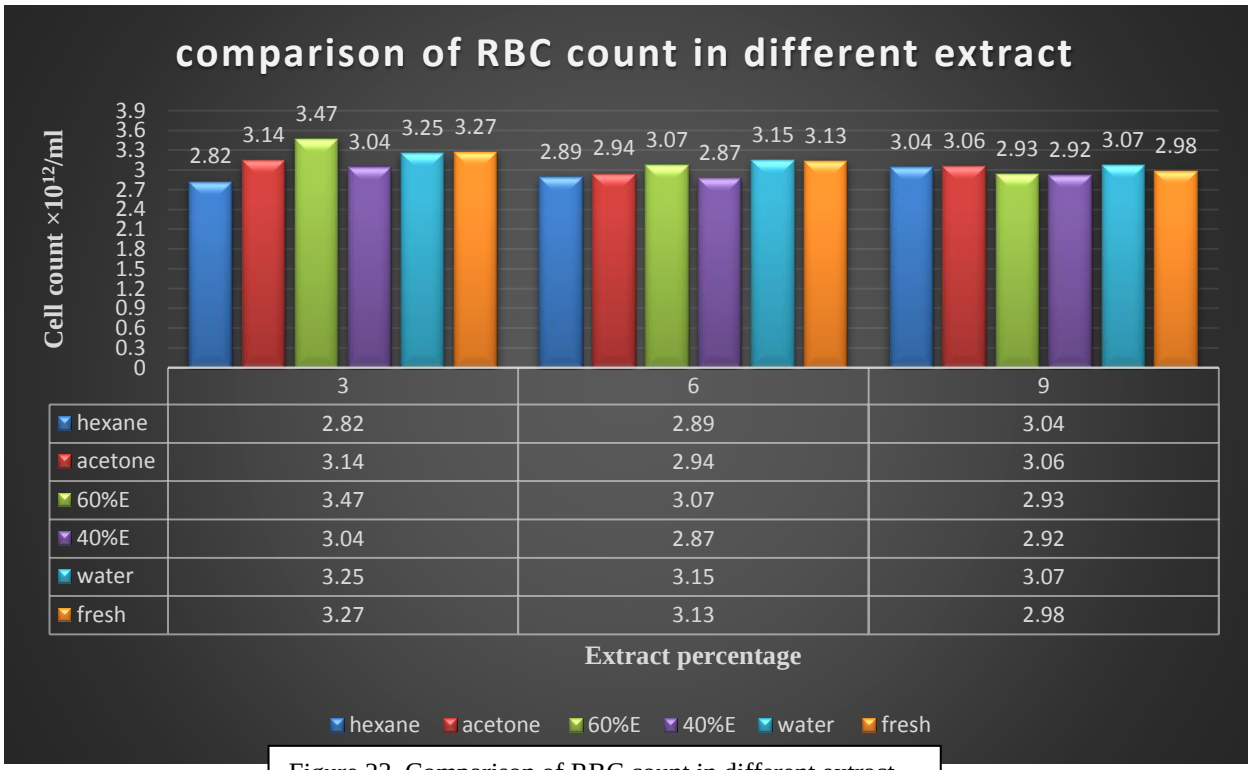

Figure 23. Comparison of RBC count in different extract

Highest blood count shown by 3% concentration of Et60 (60% ethanol) extract of papaya leaf which shows about 91.55 % of viability with respect to fresh blood cell viability which is 100 % and it is effective for storage of blood. Below table explains the comparison of cell count with control and the effect of different extracts on blood shelf life.

DISCUSSION

In our work about 75 phytochemicals was identified by GC-MS analysis from hexane extract, 63 from acetone extract, 25 from 60% ethanol, 24 from 40%ethanol and 19 from water extract. Study shown that the Carica papaya leaf extract in different solvent are effective on stored blood and it helps in maintaining cellular viability of red blood cells during storage of blood because extract of papaya in different solvent contains phytochemicals like vitamin E and tocopherol and also plant sterols and phenolic compounds which are effective in storage condition which exhibit antioxidant effect, nitrogen scavenging effect, free radical scavenging activity, antimicrobial activity, antifungal activity and it also effective in treatment of anaemia and some chemicals work in membrane stabilization. Other activity includes immunomodulation, anti-inflammatory and as anticancer, and in treatment of HIV. Antioxidant and free radical scavenging activity are effective for blood cells during storage because the main reason of red blood cell blood death due to the production of reactive oxygen species inside the RBC and oxidation reaction due to production of lactate in glycolysis process, so we can conclude from this that it may show effect in storage of blood. And from this study we concluded that the concentration of different solvents are effective as additives on shelf life of blood because from experimental analysis shows that the hexane 3%, acetone 3 and 9%, 60% ethanol 3 and 6%, 40% ethanol 3% and water and fresh extract 3,6,9% are effective on blood shelf and in future it may be used as additives in storage of blood in blood bank and this work will open the new door of study the effect of plant extract on blood shelf life. Other studies shown that the fresh papaya leaf extract contains vitamin C, vitamin A and other phyto-constituents. So this study open the new door in the field of transfusion medicine because all additives used in blood storage are chemicals but the plant extract are naturally obtained from plants and easily available in nature. The research on the papaya leaf extract is very helpful for patients which are suffering from lack of blood in blood banks or old blood during any trauma, diseased or accidental condition. From this study we can concluded that if we used this extract so we can alter the shelf life of stored blood in storage condition.

CONCLUSION

From the above result it is concluded that 3% of the ethanol 60% extract of Carica papaya leaf extract shows positive effect on the shelf life of stored blood and this explained by red blood cell counts and hemoglobin content, so in future 3% of the 60% ethanolic extract will be use as additive for enhancing stored blood shelf life which is very helpful in the field of transfusion medicine, and also reduce the loss of stored blood which is discarded after 42 days of storage.

REFERENCES

1. Adias, T.C.; Moore-igwe, B.; Jeremiah, Z. A.; (2012). Storage Related Haematological and Biochemical Changes of CPDA-1 Whole Blood in a Resource Limited Setting. Blood disorders and transfusion. 3(3), 3–6.
2. Ahmad, N.; Fazal, H.; Ayaz, M.; Abbasi, B. H.; Mohammad, I.; Fazal, L. (2011). Dengue fever treatment with Carica papaya leaves extracts. Asian Pacific Journal of Tropical Biomedicine, 1(4), 330–333.
3. Aravind, G.; Bhowmik, D.; Duraivel, S.; Harish, G. (2013). Traditional and Medicinal Uses of Carica papaya. Journal of Medicinal Plants Studies, 1(1), 7–15.
4. Arumugam, N.; Boobalan, T.; Rajeswari, P. R.; Duraimurugan, M. D.; (2014). Antimicrobial activity and phytochemical screening of Cynodon dactylon and Carica papaya, 5(5), 21–31.
5. Aubron, C.; Nichol, A.; Cooper, D. J.; Bellomo, R. (2013). Age of red blood cells and transfusion in critically ill patients. Annals of Intensive Care, 3(1), 2.
6. BashiAhmed Y. Dallal; Saleh Bashar M. (2009). Effect of blood storage on certain hematological parameters. Tikrit Medical Journal. 15(1): 171-180.
7. Bhadane, Vishal; Belemker, Sateesh; Mali, Bhupesh. (2014).The nature's potential multipurpose gift-Papaya (Carica papaya lin.): A complete overview. Asian journal of pharmaceutical research and development. Vol. 2(1) Jan- Feb, 75-82.
8. Blakney G. B.; Dinwoodie a. J. (1975). A spectrophotometric scanning technique for the rapid determination of plasma hemoglobin. Clin.Bioehem.8, 96-102.
9. Blood, S. (2005). Manual on the management, maintenance and use of blood cold chain. World Health organization.
10. Burger, P.; Korsten, H.; Verhoeven, A. J.; De Korte, D.;Van Bruggen, R. (2012). Collection and storage of red blood cells with anticoagulant and additive solution with a physiologic pH. Transfusion. 52(6), 1245–1252.
11. Canini, A.; Alesiani, D.; D'Arcangelo, G.; Tagliatesta, P. (2007). Gas chromatography-mass spectrometry analysis of phenolic compounds from Carica papaya L. leaf. Journal of Food Composition and Analysis, 20(7), 584–590.
12. Chakravarthy V.Kalyan; Chandra D. Naveen; Prasanna B. Santhoshi; Rao T. Jaya Mastan; Rao D. Ranga, (2012) Haemoglobin estimation by non-cyanide method. Journal of Clinical and Diagnostic Research. Vol-6(6): 955-958.
13. Cluitmans, J. C.; Hardeman, M. R.; Dinkla, S.; Brock, R.; Bosman, G. J. C. G. M. (2012). Red blood cell deformability during storage: towards functional proteomics and metabolomics in the Blood Bank. Blood Transfusion .Trasfusione Del Sangue, 10 Suppl 2, s12–8.
14. 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.
15. D'Alessandro, A.; Kriebardis, A. G.; Rinalducci, S.; Antonelou, M. H.; Hansen, K. C.; Papassideri, I. S.; Zolla, L. (2014). An update on red blood cell storage lesions, as gleaned through biochemistry and omics technologies. Transfusion. 55(January), 205–219.

16. D'almeida, M. S.; Jagger, J.; Duggan, M.; White, M.; Ellis, C.; Chin-Yee, I. H. (2000). A comparison of biochemical and functional alterations of rat and human erythrocytes stored in CPDA-1 for 29 days: Implications for animal models of transfusion. Transfusion Medicine, 10(4), 291–303.
17. Dinkla Sip; PeppelmanMalou; RaadtJori Van Der; AtsmaFemke; Novotny Vera M. J.; Kraaij Marian G.J.;Van, Joosten Irma; BosmanGiel J.C.G.M. (2014) Phosphatidylserine exposure on stored red blood cells as a parameter for donor-dependent variation in product quality. Blood Transfuse 12. 204-9.
18. Fairbanks Virgil F.; Ziesmer Steven C.; O'Brien Peter C. (1992).Methods for Measuring Plasma Hemoglobin in Micro molar Concentration Compared. Clin. Chem.38/1, 132-140.
19. Hess J. R. (2014). Measures of stored red blood cell quality. International society of blood transfusion, 107(1) 1-9.
20. Hess, J. R. (2010). Conventional blood banking and blood component storage regulation: opportunities for improvement. Blood Transfusion, 8(Suppl 3), s9–s15.
21. Hess, J. R.; Sparrow, R. L.;Van Der Meer, P. F.; Acker, J. P.; Cardigan, R.A.; Devine, D. V. (2009). Red blood cell hemolysis during blood bank storage: Using national quality management data to answer basic scientific questions. Transfusion, 49(12), 2599–2603.
22. Hillyer, C. D.; Shaz, B. H.; Zimring, J. C. (2009). Transfusion Medicine and Hemostasis: Clinical and Laboratory Aspects.
23. Hillyer, C. D. (2007). Blood Banking and transfusional medicine: Clinical and laboratory aspects.
24. Hod, E.; Zhang, N.; Sokol, S.; Wojczyk, B. S.; Francis, R. O.; Ansaldi, D.; Spitalnik, S. L. (2010). Transfusion of red blood cells after prolonged storage produces harmful effects that are mediated by iron and inflammation. Blood, 115(21), 4284–4292.
25. Ikeyi Adachukwu P.; Ogbonna Ann O.; Eze Faith U. (2013) Phytochemical Analysis of Paw-Paw (Carica Papaya) Leaves. Volume 2, no. 3. 2250-3137
26. Imaga, N. A. (2013). Phytomedicines and nutraceuticals: Alternative therapeutics for sickle cell anemia. The Scientific World Journal.
27. Imaga, N. A.; Gbenle, G. O.; Okochi, V. I.; Adenekan, S.; Duro-emmanuel, T.; Oyeniyi, B.; Ekeh, F. C. (2010). Phytochemical and antioxidant nutrient constituents of Carica papaya and Parquetina nigrescens extracts. Scientific Research and Assays, 5(16), 2201–2205.
28. Krishnaneni, S.; Venkatalakshmi, P. (2014). Antimicrobial, larvicidal and acricidal activities of the ethanolic extract of Andrographis paniculata and Carica papaya leaves. World journal of pharmaceutical research. Vol. 3, issue 4, 660-669.
29. Lelubre, C.; Vincent, J. (2013). Relationship between red cell storage duration and outcomes in adults receiving red cell transfusions : a systematic review. Critical Care, 17(2), R66.
30. Liumbruno, G.; Bennardello, F.; Lattanzio, A.; Piccoli, P. (2009). Recommendations for the transfusion of red blood cells. 49–64.
31. Makroo RN; RainaVimarsh; Bhatia Aakanksha; Gupta Richa; Majid Abdul; Thakur Uday Kumar; Rosamma NL. (2011). Evaluation of the red blood cell hemolysis in

packed red cells during processing and storage. Asian Journal of transfusion Science. Vol. 5 issue 1 page 15-17.

32. Mansy, E.; Tanahy, E.; Waseif, E. (2015). Chemical Composition, Antioxidant and Antimicrobial Properties of the Essential Oils and Extracts of Some Aromatic Plants. 344–352.
33. Manuscript, A. (2010). NIH Public Access, 35(5), 382–388.
34. Mukherjee, P. K.; Venkatesh, P.; Ponnusankar, S. (2010). Ethnopharmacology and integrative medicine - Let the history tell the future. Journal of Ayurveda and Integrative Medicine, 1(2), 100–109.
35. Ng, L. Y.; Ang, Y. K.; Khoo, H. E.; Yim, H. S. (2012). Influence of Different Extraction Parameters on Antioxidant Properties of Carica Papaya Peel and Seed.Pdf. Research Journal of Phytochemistry.
36. Parikh S.; Parikh B.; Shah C.; Bhansali P.; Patel J.; Joshi D. (2010). Haemoglobinometry by a Novel Alkaline Haematin Detergent-575 Method. Gujarat medical journal. Vol. 65 No. 1.
37. Ranasinghe Priyanga; Ranasinghe Pathmasiri; Gunatilake Saman B. (2012). In vitro erythrocyte membrane stabilization properties of Carica papaya L. leaf extracts. Pharmacognosy research, 4(4) 196-202.
38. Roa-de, L. F.; Lobato-garcía, C. E.; Ble-castillo, J. L. (2014). Phytochemical screening and hypoglycemic activity of Carica papaya leaf in streptozotocin-induced diabetic rats. 24, 341–347.
39. Romasi, E. F.; Karina, J.; Jan, A.; Parhusip, N. (2011). Antibacterial avtivity of papaya leaf extracts against pathogenic bacteria. Makara Technology, 15(2), 173–177.
40. Sohmer PR; Moore GL; Beutler E; Peck CC. (1982). In vivo viability of red blood cells stored in CPDA-2. Transfusion. Nov-Dec 22(6), 479-84.
41. Sparrow, R. L. (2012). Time to revisit red blood cell additive solutions and storage conditions: a role for "omics" analyses. Blood Transfusion. 10(Suppl 2), s7–s11.
42. Upgade Akhilesh; Bhaskar Anusha. (2013). 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. Vol. 6, suppl.4, 101-106.
43. Vittori, D.; Vota, D.; Nesse, A. (2005). Erythrocyte : Programmed Cell Death.
44. Wallas, C. H. (1979). Sodium and potassium changes in blood bank stored human erythrocytes. Transfusion, 19(2), 210–5.
45. Weinstein Robert. (2012). Red blood cell transfusion: A clinical practice guidelines from the AABB. American society of hematology. 157: 49-58.
46. Yao-Xiong Huang. (2011). Human red blood cell aging: correlative changes in surface charge and cell properties. J. Cell. Mol. Med. Vol.15, No 12. pp. 2634-2642
47. Yazdanbakhsh, K.; Bao, W.; Zhong, H. (2011). Immunoregulatory Effects of Stored Red Blood Cells. Hematology. (1), 466–469.
48. Yushua'u, M.; Onuorah, F. C.; Murtala, Y. (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.

49. Zander Rolf; Lang Werner; Wolf H. Uwe. (1984). Alkaline Hematin D-575, a new tool for the determination of hemoglobin as an alternative to the cyanhaemoglobin method. ClinicaChimicaActa. 136(1984) 83-93.
50. Zimrin, A. B.; Hess, J. R. (2009). Current issues relating to the transfusion of stored red blood cells.93–103.
51. Zimring, J. C. (2013). Fresh versus old blood: are there differences and do they matter? Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program, 2013, 651–5.

APPENDIX

Table- System requirement for plant extract analysis by GC-MS-

Instrumentation and system requirement for GC-MS analysis of hexane extract	
===== Analytical Line 1 =====	
[AOC-20i+s]	
# of Rinses with Pre solvent	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 Temperature	60.0 °C
Injection Temperature	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]		
Ion Source Temperature	230.00 °C	
Interface Temperature	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	

Table- System requirement for GC-MS analysis of plant extract

Instrumentation and system requirements for GC-MS analysis of Acetone extract		
[GC-2010]		
Column Oven Temperature	100.0 °C	
Injection Temperature	260.00 °C	
Injection Mode	Split	
Flow Control Mode	Linear Velocity	
Pressure	95.1 kPa	
Total Flow	16.3 mL/min	
Column Flow	1.21 mL/min	
Linear Velocity	40.9 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)
-	100.0	4.00
10.00	250.0	5.00
15.00	280.0	24.00
< 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 Plus]		
Ion Source Temperature	230.00 °C	
Interface Temperature	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	49.98min
ACQ Mode	Scan
Event Time	0.50sec
Scan Speed	1250
Start m/z	40.00
End m/z	600.00
Sample Inlet Unit	GC
[MS Program]	
Use MS Program	OFF