CULTURE AND CHARACTERIZATION OF STEM CELLS ISOLATED FROM BOVINE UMBILICAL CORD BLOOD AND STUDY THE EFFECT OF *Clematis buchananiana DC EXTRACT*

M.Sc. Thesis

(2022)

Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

For partial fulfillment of the requirement of the

M. Sc. degree in Biotechnology

Ganesh Adhikari

Roll No.: BT 506/074

T.U. Regd. No. 5-1-48-110-2009

CULTURE AND CHARACTERIZATION OF STEM CELLS ISOLATED FROM BOVINE UMBILICAL CORD BLOOD AND STUDY THE EFFECT OF *Clematis buchananiana DC EXTRACT*

M.Sc. Thesis

(2022)

Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University

Kirtipur, Kathmandu, Nepal

By

Ganesh Adhikari

Supervisor

Prof. Dr. Gaurishankar Manandar

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

Date: 2079/09/

Recommendation

This is to certify that the research work entitled "**CULTURE AND CHARACTERIZATION OF STEM CELLS ISOLATED FROM BOVINE UMBILICAL CORD BLOOD AND STUDY THE EFFECT OF *Clematis buchananiana DC EXTRACT*"** has been carried out by **Mr. Ganesh Adhikari** under my supervision.

This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. The result presented here is his/her original findings. I/we, hereby, recommend this thesis for final evaluation.

……………………………….

Supervisor

Prof. Dr. Gaurishankar Manandhar

Central Department of Biotechnology

Tribhuvan University,

Kirtipur, Kathmandu, Nepal

Central Department of Biotechnology

Tribhuvan, University.

Kirtipur, Kathmandu. Nepal

Date: 2079/09/

Certificate of Evaluation

This is to certify that this entitled "**CULTURE AND CHARACTERIZATION OF STEM CELLS ISOLATED FROM BOVINE UMBILICAL CORD BLOOD AND STUDY THE EFFECT OF *Clematis buchananiana DC EXTRACT*"** presented to evaluation committed by **Mr. Ganesh Adhikari** is found satisfactory for the partial fulfillment of master of Science and Technology.

……………………………………… ……………………………

Prof. Dr. Krishna Das Manandhar

Head of Department

Central Department of Biotechnology

Kirtipur, Kathmandu, Nepal

External Examiner

……………………… …………………………………….

Internal Examiner

Supervisor

Prof. Dr. Gaurishankar Manandhar

Central Department of Biotechnology

Kirtipur, Kathmandu, Nepal

Dedicated

To

MY FAMILY

AND

COUNTRY

ACKNOWLEDGEMENT

I would like to express my sincere and deepest gratitude to all the people who had directly and indirectly helped me during and till the journey of this Dissertation.

Foremost, I am highly indebted and grateful to my supervisor Prof. Dr. Gaurishankar Manandhar, whose expertise, understanding and generous guidance made this project possible and Prof. Dr. Krishna Das Manandhar (Head, Central Department of Biotechnology), who believed in me and motivated me by providing all the lab facilities. Words cannot be enough to thank Dr. Umesh Mandal sir, Senior veterinarian of Nepal who dedicated his life to save animals, for providing me samples and supporting me. I am very grateful to Mr. Anil Singh Basnet research officer of National Ayurvedic Research center who helped me with the related reagents and accessories for the MTT assay.

It was a pleasure to get seniors like Mr. Mahesh Lamsal and Ms. Roji Raut who guided me in each and every step, Ms. Srijan Shrestha for providing me PBS, nuclease free water and continuous guidance, Ms. Chetana Khanal for helping me in MTT assay, Ms. Apashara Parajuli, Mr. Machhendra Thapa, Mr. Ramanuj Rauniyar, Ms. Tinmaya Rai, Ms. Jayaeshori Sharma, Ms. Sabina Bhandari, Ms. Elisha Regmi, Mr. Shrawan Kumar Chaudhary, Ms. Bandana Thakur, Ms. Samikshya Kandel and all my seniors for helping me all the time I needed and guided me through. I feel myself very lucky to have so many caring and loving seniors who make me feel this department is like a family.

I would also like to thank Mr. Suman Gauttam, Mr. Suresh Joshi, Mr. Sailesh Adhikari, Ms. Puja Bhatt, Mr. Ashwin Bahadur Chand, Mr. Uttam Belbase and all my friends from 9Th batch and junior Mr. Binod Khadka, Ms. Smita Shrestha, Ms. Binita khadka for their support.

I am very thankful to Mr. Sudip Timilsina for Introducing me to Biotechnology and all other friends who were there for me.

I am also Thankful to Mr. Madan Rokaya, Mr. Gunanidhi Dhakal, Ms. Elian Pardhan, Ms. Binu Magar, Ms. Shova Magar, Mr. Rajendra Maharjan and all the office staff of CDBT. And Mr. Lalit Gurung security in charge of the building.

I would like to express my heartfelt gratitude to all my teachers since my childhood who make me who I am today.

Finally, and most Importantly, none of this could have been possible without the love, patience and trust of my Family. I would like to dedicate this works and express my heartfelt gratitude to my family for their constant encouragement, Prayers, support and trust.

ACRONYM AND ABBREVIATIONS

ADC:	Adherent cells
DMEM:	Dulbecco's modified Eagle's Medium
EDTA:	Ethylene di amine tetra acetic acid
HSC:	hematopoietic stem cell
MSC:	Mesenchymal stem cell
NAC:	Non- adherent cells
NK cells:	Natural killer cells
PBMC:	Peripheral blood mononuclear cells
PBS:	phosphate buffer saline
RBC:	red blood cells
WBC:	White blood cells
RPM:	revolution per minute
TU:	Tribhuvan University
UCB:	Umbilical cord blood
GVHD:	Graft versus host disease
ESC:	Embryonic stem cells
MAPK:	Mitogen-activated protein kinase
mg :	milligram
ml :	milliliter
gm:	gram
°C:	degree centigrade
µl:	microliter
µm:	micrometer
MTT:	3-(,5-dimethylthiazol-2yl-2,5-diphenyltrazoliumbromide0

ELISA:	Enzyme-linked immunosorbent assay
DMSO:	Dimethyl sulfoxide
S.D:	standard deviation
C.V.:	coefficient of variance
Conc:	concentration
-OH:	hydroxide
HPLC:	high performance liquid chromatography

TABLE OF CONTENTS

Recommendation ii

Certificate of Evaluation iii

ACKNOWLEDGEMENT v

ACRONYM AND ABBREVIATIONS vi

TABLE OF CONTENTS viii

LIST OF FIGURES xii

LIST OF TABLES xiii

ABSTRACT xiii

CHAPTER 1 1

INTRODUCTION 1

1.1 BACKGROUND 1

1.1.1 Mesenchymal stem cells 1

1.1.2 Hematopoietic stem cells 2

1.1.3 The role of medicinal plants in cell proliferation 2

1.1.4 in vitro cell culture 4

1.1.5 Bioactive Phytochemicals 5

1.2 Project Rationale 5

1.3 Research Hypothesis 6

1.4 RESEARCH OBJECTIVES 6

1.4.1 General objective 6

1.4.2 Specific Objectives 6

1.5 Research plan 7

CHAPTER 2 8

LITERATURE REVIEW 8

2.1 Stem cells 8
2.2 Umbilical cord blood as the source of stem cells 9
2.3 Hematopoietic stem cells 10
2.4 in vitro expansion of umbilical cord blood stem cells 12
2.5 The role of bioactive phytochemicals in cell culture 12
2.6 Phytochemical Extraction 13
2.7 PHYTOCHEMICAL SCREENING 14
2.8 Clematis buchananiana DC. as ethnomedicine 15
2.9 MTT assay 15
2.10 Bovine umbilical cord blood 16
2.11 Wright staining 16
2.12 Gram staining 17
2.13 Trypan Blue staining 17
2.14 Hemocytometer 17
CHAPTER 3 19
METHODOLOGY 19
3.1 Collection of plant sample 19
3.2 Identification of plant species 19
3.3 Extraction of phytochemicals from plant 19
3.4 Qualitative analysis of phytochemicals 20
3.5 Detection of carbohydrates 20
3.6 Detections for alkaloids 21
3.7 Detection of proteins and amino acids 21
3.8 Detection of phenolic compounds and tannins 21
3.9 Detection of flavonoids 21
3.10 Preparation of complete DMEM media 21

3.11 Collection of umbilical cord blood 22

3.12 Isolation of peripheral blood mononuclear cells and cell culture 22

3.13 Isolation of hematopoietic stem cells and passaging of cells 23

3.14 Trypan blue staining and cell counting by hemocytometer 23

3.15 Wright staining 23

3.16 Gram staining 24

3.17 Cell Proliferation Assay 24

3.18 Data and graphical analysis: 25

CHAPTER 4 26

RESULTS 26

4.1 Identification of plant sample 26

4.2 Phytochemical extraction of Clematis buchaniana. 27

4.3 Qualitative phytochemical analysis of plant extract 28

4.4 Isolation and culture of Peripheral blood mononuclear cells (PBMC) from bovine umbilical cord blood. 28

4.5 Isolation of stem cells from PBMC of bovine umbilical cord blood 29

4.6 The isolated cells have higher viability rate and have the ability to divide and differentiate 30

4.7 Cell cultures contain healthy and viable cells without contamination 33

4.8 The cell culture contains hematopoietic stem cells 34

4.9 MTT assay and cellular viability 35

CHAPTER 5 39

DISCUSSION 39

5.1 Verification for *Clematis buchaniana* DC 39

5.2 Phytochemical extraction of *Clematis buchaniana* DC 39

5.3 Qualitative phytochemical analysis of extract 40

5.4 Isolation and culture of peripheral blood mononuclear cells (PBMC) from bovine umbilical cord blood 41

5.6 Isolations of stem cells from PBMC cells 41

5.7 Presence of hematopoietic stem cells 41

5.8 The phytochemical extract of *Clematis buchaniana* helps in cell proliferation 42

SUMMARY 45

Conclusions 46

REFERENCES 47

APPENICES 60

LIST OF FIGURES

Figure 1: Different sources of stem cells from where they can be isolated and the stemness of cells on the basis of age and origin 9
Figure 2: Umbilical cord blood contains hematopoietic and mesenchymal stem cells 10
Figure 3: Different possible progenitor and specialized cells developed from the hematopoietic stem cells 11
Figure 4: The role phytochemicals in the proliferation and differentiation of stem cells 13
Figure 5: Hemocytometer gridlines, a cell counting technique 18
Figure 6: The Herbarium verification by National herbarium and botanical laboratory 26
Figure 7: Phytochemical extraction and phytochemical screening of Clematis buchaniana. 27
Figure 8: Isolation and culture of peripheral blood mononuclear cells (PBMC) from bovine umbilical cord blood (UCB). 29
Figure 9: Isolation of stem cells from peripheral blood mononuclear cells (PBMC) 30
Figure 10: 3rd passaging of cells observed in inverted microscope 30
Figure 11: 3rd passaging of cells observed in inverted microscope. 31
Figure 12: 4th passaging of cells observed in inverted microscope 31
Figure 13: 5th passaging of cells observed in inverted microscope 31
Figure 14: 6th passaging of cells observed in inverted microscope 32
Figure 15: 7th passaging of cells observed in inverted microscope 32
Figure 16: 8th passaging of cells observed in inverted microscope 32
Figure 17: 9th passaging of cells observed in inverted microscope 33
Figure 18: Microscopical observation of cells from 4th passage to see if there is any contamination 33
Figure 19: Microscopical observation of cells to check the possible contamination and cell viability 34
Figure 20: Microscopic observation of cells from 8th passage showing differentiation to monocytes, lymphocytes, granulocytes and erythrocytes 35
Figure 21: Cell culture in a 96 well plate culture flask before and after the treatment of MTT reagents 36
Figure 22: Microscopic observation 96 well plate cultured cells treated with 36
Figure 23: Cell viability by MTT assay. The columns represent mean values calculated from three repeats and bars represent standard error. 37
Figure 24: Bar diagram presentation of One way ANOVA test with comparision of different treatment on cell. 38

LIST OF TABLES

Table 1: The qualitative phytochemical analysis of *Clematis Buchaniana.* 28

Table 2: One way-ANOVA analysis to compare the effect of different treatment on cell (plant extract, ethanol, amfol plus, and control). 37

ABSTRACT

Stem cells are undifferentiated cells that can perpetually divide and differentiate into different types of cells. These cells possess high potential for regenerative therapy. Umbilical cord blood (UCB) is residual blood contained in the umbilical cord discarded after the birth of the baby. It is rich in hematopoietic stem cells and different types of other mesenchymal stem cells. Sample was collected from bovine umbilical cord and cultured in DMEM low glucose medium. Two different types of cells were isolated, among them the floating cells were subcultured for up to 9 passages. Till the 9th passage very active growth of cells was observed. These dividing cells on wright staining microscopy show different types of cell population morphologically similar to blood cells i.e., lymphocytes, erythrocytes, granulocytes, monocytes. Presence of these cells till 8th, 9th passage indicate there is the source of these blood cells i.e., hematopoietic stem cells. These cells were treated with the phytochemical extracted from the ethnomedicinal plant *Clematis buchananiana.* On treatment with phytochemicals, cell proliferation was observed.

Keywords: Umbilical cord blood, stem cell, *Clematis buchananiana,* Alkaloids, Phenolics, wright stain, MTT assay, Phytochemicals, PBMC, Regenerative Therapy, Proliferation

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Stem cells are the undifferentiated cells of multicellular organisms that have the ability to replicate indefinitely to similar kinds of identical daughter cells and potency to differentiate into specialized progenitor cells giving rise to different kinds of cells (Weiss & Troyer, 2006). The children are born with umbilical cord and placenta attached which are discarded normally. Umbilical cord blood is a very good source of stem cells. Umbilical cord and cord blood along with placenta which are normally discarded, possess a great significance. Umbilical cord blood is considered as a good source of the non-embryonic stem cells containing multipotent stem cells. These stem cells are naïve cells so very less chances of graft versus host immunological rejection (Goldstein et al., 2007). It can be the future for regenerative therapy treating many kinds of abnormalities, disorder diseases etc. It is like the backup for the future that a child is born with. Regenerative therapy is a safe and effective way of naturally repairing the body, restoring function and improving mobility by creating functional tissues. The use of stem cells in regenerative therapy has opened a hope for new dimensions which possess a huge possibility. Umbilical cord blood possesses two major types of stem cell that carry a wide range of applications and hope for the regenerative therapy i.e., hematopoietic stem cells and Mesenchymal stem cells (Hutton et al., 2009; Javed et al., 2008)

1.1.1 Mesenchymal stem cells

Mesenchymal stem cells are fibroblast like multipotent stem cells. That can differentiate into almost all three kinds of germ layer i.e., ectoderm (epithelial cells, neuron cells glial cells etc.), endoderm (muscle cells, gut epithelial cells, lung cells, endothelial cells, hepatocytes etc.), mesoderm (connective stromal cells, chondrocyte osteoblast, adipocytes etc., (Chang et al., 2006; Divya et al., 2012). Mesenchymal stem cells can be isolated from different source such as, bone marrow, deciduous teeth adipose tissue, hair follicles peripheral blood, periodontal ligament, trabecular bone, scalp subcutaneous

tissue, skeletal muscle, corneal stroma, liver, lung, spleen, amniotic fluid, placenta, umbilical cord, umbilical cord blood (Berebichez-Fridman & Montero-Olvera, 2018). The UCB is the non-invasive source for collecting mesenchymal stem cells that contain the naive cells which are nonimmunogenic to the donor hence low chance of graft versus host rejection after transplantation (Goldstein et al., 2007; Rocha et al., 2004).

1.1.2 Hematopoietic stem cells

Hematopoietic stem cells are the multipotent stem cells that are the continuous source of blood and immune cells throughout life. HSC have the ability to differentiate into all type of mature blood cells through progenitor cells including myeloid lineage (granulocytes, monocytes, leukocytes, erythrocytes, and megakaryocytes) and lymphoid lineage (T-cells, B-cells, lymphocytes). HSC are first developed in the embryonic yolk sac and the aorta-gonad-mesonephros region from where it migrates to the placenta, cord blood, fetal liver, spleen and bone marrow. After the child birth hematopoietic stem cells are found in the bone marrow which regulate the blood cells and immune cells throughout the life. The cord blood derived HSC could be an alternative source of HSC to treat any blood related disorder and disease by using regenerative therapy. The HSC from UCB contains immature cells with very low GVHD (Pang et al., 2021; Saudemont & Madrigal, 2017).

The UCB contains about 0.5-1% HSC of total mononuclear cells isolated, which is comparatively higher than in the bone marrow but still is not sufficient for the therapeutic use. So, to use the HSC for therapeutic purposes, *in vitro* expansion of the cells is necessary (Watt et al., 2022).

1.1.3 The role of medicinal plants in cell proliferation

Many medicinal herbs have been used traditionally to treat many diseases, wounds and injury. Studies show that these medicinal plants can be utilized to control cell proliferation. The extract obtained from medicinal herbs contain bioactive phytochemicals that promote cell proliferation (Olatunbosun et al., 2012; Osibote et al., 2011; Saud et al., 2019).

Studies have shown the plant derived phytochemicals regulate the cell signaling, metabolic mechanism and transcriptional mechanism which plays a major role in cell proliferation, lineages and differentiation. The bioactive phytochemicals such as phenols, alkaloids, sterols, aldehydes, sterols regulate the different signal pathways and control the fate of stem cells (Zhang et al., 2020a). The medicinal plants and the plant derived phytochemicals have shown the significant roles to enhance the adult stem cell proliferation and inhibit the proliferation of cancerous cells (Kornicka et al., 2017). Several studies have shown that these phytochemicals are effective stimulants for both *in vivo* and *in vitro* cell proliferation (Kornicka et al., 2017).

According to World Health Organization, the majority of world population still depends on the ethnomedicine for primary health care. Around 80%of gloabal population still rely on the plants, plant derived medicines (Tugume & Nyakoojo, 2019) and other traditional methods like yoga, acupuncture and other therapies. In Nepal and India from the ancient periods of time, people used to follow the ayurvedic practices described in Ayurveda, but with the modernization and the development of allopathy the practices of ayurvedic medicine and knowledge have decreased and slowly forgotten. But they are still used as a cultural practice and tradition, treatment process and the use of medicinal plants are still in use as ethnomedicine. There are around 2000 different plant species that have been used by various ethnic groups of Nepal among the 6500 species of flowering plants and ferns recorded in Nepal (Ambu et al., 2020; Kunwar et al., 2022). Among the various medicinal plants that have been used as ethnomedicine from the ancient periods *Clematis* species shrubs from the family of Ranunculaceae are also commonly used popular ethnomedicine. These plants are distributed in the temperate region of northern hemisphere and are composed of vigorous, woody, climbing vines. They have been used in Chinese traditional medicines from the ancient times and considered as the sources of various pharmaceutically important compounds. There are around 355 species of clematis among which around 70 species are used as ethnomedicine. The *Clematis* species are mainly used externally to cure sores, wound, ulcers and injuries broken bones and orally for syphilis, gout, rheumatism, bone disorder, chronic skin conditions etc. (Chawla et al., 2012; Hao et al., 2015). Among the several species of clematis, the *Clematis buchananiana* DC. found in the altitudinal range of 900-3900m is used in several ways for the treatment

of different disease and injuries. These are found climbing around the small trees and rock (Ghimire et al., 2020).

1.1.4 in vitro cell culture

in vitro cell culture is the process in which the cells are grown in an artificial growth media under an artificial and controlled environment. This technique is used for studying behavior of animal cells in a specific controlled condition. The cell culture lab is the basic requirement of biotechnology. Three different types of cell culture are practiced. 1) differentiated cells that have lost the capacity to further differentiate, 2) precursor cells which are undifferentiated but committed to differentiation into a particular cell lineage, 3) stem cell culture (Verma et al., 2020). Studying stem cells helps to understand the development of the whole organism and how our body recovers dead cells, causes birth defects, abnormal cell division and differentiation and cancer cells (Ogliari et al., 2014). Because of the regenerative capacity and plasticity, stem cells offer great potential as regenerative medicine for cell-based therapies to treat disease and different disorders. The embryonic stem cell can become any tissue and cell of the body and the placenta (Asch et al., 1995; Seydoux & Braun, 2006). Embryonic stem cells supply new cells to an embryo that develops into a baby. The embryonic stem cells are pluripotent which can differentiate and develop into many types of tissue cells of the body. But despite its high potency in stem cell therapy embryonic stem cells have few disadvantages. It involves destroying the embryo to acquire cells which gives rise to ethical issues. Embryonic stem cells possess more risk of rejection and development of teratomas. The use of adult stem cells has less chances of rejection and does not harm the donor. Umbilical cord blood is collected from useless, discarded tissue without harming the mother or baby. So, it does not raise ethical issues and the research has shown there are less chances of rejection by the body. Umbilical cord blood is rich in hematopoietic stem cells and mesenchymal stem cells that can be used to treat different types of blood disease such as leukemia, cancer and is comparatively safer and appears to have lower immune system incompatibility. Mesenchymal stem cells can be used to treat the terminal stage of liver disease, bone injuries and skin transplant etc. Cord blood isolation is a non-invasive technique and can be easily cryopreserved. Hence, in the developed countries cord banking is available and

peoples can cryopreserve cord cells for future use (Covas et al., 2003; Hill et al., 2019; Yu et al., 2018; L. Zhou et al., 2020).

1.1.5 Bioactive Phytochemicals

The phytochemicals are the chemical compounds produced by plants. Bioactive phytochemicals have biological activity due to which they could be used to treat injuries and diseases. These naturally occurring phytochemicals are divided into two types on the basis of their function and production. The primary metabolites are the necessary nutrients for plant life such as carbohydrates, amino acids, proteins, lipids, purines, and pyrimidines of nucleic acids. The secondary metabolites are the byproduct of the primary metabolic pathways such as alkaloids, phenolics and terpenoids, flavonoids etc. (Rabizadeh et al., 2022; Xiao & Bai, 2019). The secondary metabolites have various properties based on its uses and applications e.g., dyes, medicines, insecticides, herbicides, perfumes etc. Secondary metabolites are usually produced by the plants under special conditions i.e., during the time of injury, stress, invasion or reproductive stages etc. (Mendoza et al., 2018). These phytochemicals thus produced are accumulated in different parts of the plants, such as in the roots, stems, leaves, flowers, fruits and seeds. They possess antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, modulations of hormones. These secondary metabolites were found to be not just protecting plants but also protecting humans and other animals against diseases (Saxena et al., 2013).

1.2 Project Rationale

Clematis buchananiana is selected for this study because of their ethnomedicinal values and the folk tales about the effect of its use. The plant is called Chautajod in the community from where the sample was collected due to its ability to join the wound.

Amfol plus is a common supplement recommended by the clinician in case of anemia. These amfol plus contain folic acid which is an important mineral for red blood cell formations. So, Amfol plus was used to observe its effect in cell proliferation.

Cord blood is an easily available source of embryonic stem cells which can be collected non-invasively posing no harm to the mother or newborn child. Cord blood possesses hematopoietic stem cell and mesenchymal stem cells which can differentiate into wide range of progenitor cells so can be good source of different cell lines and tissues which can be used for different purposes i.e., regenerative therapy, antibody production vaccine development, *in vitro* culture of different kinds of viruses and parasites. In the present work, we have investigated the putative cell proliferative property of *Clematis buchananiana* extract on hematopoietic stem cells obtained by umbilical cord blood culture.

1.3 Research Hypothesis

Ethanolic extract of *Clematis buchananiana* enhances proliferation of hematopoietic stem cells isolated from bovine umbilical cord blood.

1.4 RESEARCH OBJECTIVES

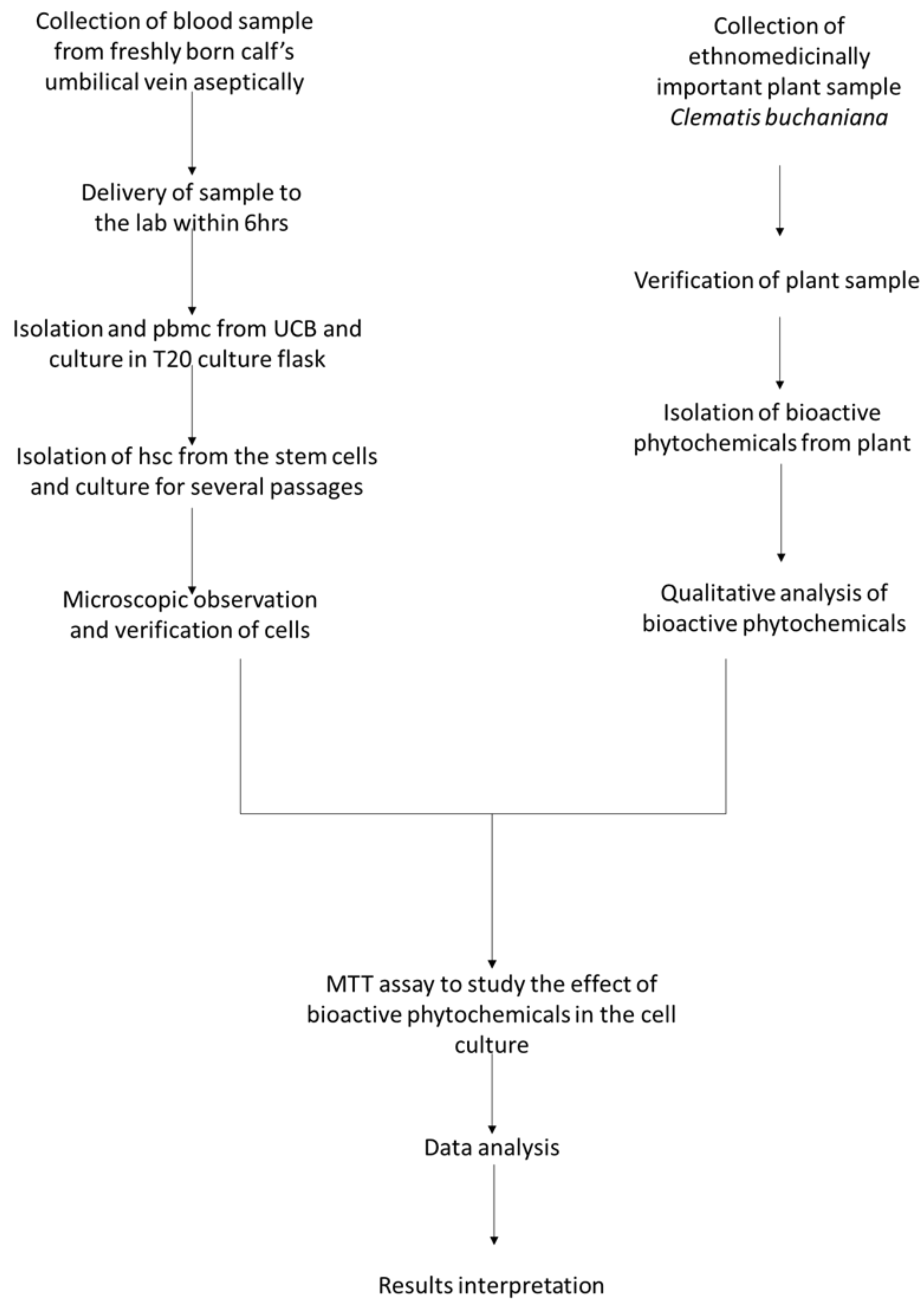
1.4.1 General objective

- To isolate and culture hematopoietic stem cells from bovine umbilical cord blood.
- To study the role of bioactive phytochemical present in *Clematis buchananiana* DC in cell proliferation and/or differentiation.

1.4.2 Specific Objectives

- To verify the selected ethnomedicinal plant
- To extract the bioactive phytochemical from the selected plants
- To isolate and culture peripheral blood mononuclear cells from bovine umbilical cord blood
- To isolate and expand stem cells from PBMC
- To isolate and expand hematopoietic stem cells
- Study the effect of bioactive phytochemical present in *Clematis buchananiana* DC in cell proliferation of isolated stem cells in different lineages

1.5 Research plan



CHAPTER 2

LITERATURE REVIEW

2.1 Stem cells

To understand the disease, aging, body healing mechanism, effect of drug, immune system, vaccine development, mutation and genetic disorders the study of cells is the basic requirement. In *in vivo* conditions there may be many known and unknown factors which are influencing cell biology directly or indirectly. To know and understand individual cells, *in vitro* cell culture is the most important technique available (Segeritz & Vallier, 2017).

There are cells which have the ability to divide indefinitely into identical daughter cells and differentiate different types of progenitor cells. These progenitor cells give rise to mature cells. Based on their ability to differentiate into different types of cells, stem cells are divided into five different types. i.e., 1) Totipotent stem cells, these cells can differentiate into any kind of cells of an organism. These cells are the original source of all cells of the organism for example, zygote. 2) Pluripotent stem cells, these cells also can differentiate into almost all kinds of cells e.g embryonic stem cells. 3) Multipotent stem cells, these cells can differentiate into many types of cells but closely related only e.g., Hematopoietic stem cells can differentiate into only blood related cells but all kinds of it. 4) Oligopotent stem cells, these cells can differentiate into few types of cells only for example myeloid stem cells can white blood cells only, 5) Unipotent stem cells, these cells can only differentiate into specific type of cells e.g., erythropoietic cells (Zakrzewski et al., 2019).

These cells originate from different sources in a different phase of life and some last for only a specific period of time. The source of stem cells is differentiated into four types i.e., Embryonic, fetal, perinatal and adult stem cells. Among the various sources of stem cells only perinatal is the source from which stem cells can be isolated without harming the donor organism. Perinatal sources are available just for a few times after the delivery of

the baby (Ekblad-Nordberg et al., 2020). Although embryonic and fetus derived stem cells have better potency and low risk of GVHD, there are many hurdles for the isolation and use of embryonic and fetus derived stem cells. The process of isolation of stem cells from embryo or fetus may involve harming the life and have ethical issues. The isolation of adult stem cells involves the painful process and the potency of isolated stem cells is limited and weak stemness. The perinatal sources such as Amniotic fluid, placenta umbilical cord and cord blood are readily available sources of stem cells which are generally discarded after the birth of child. These cells possess naïve cells with more potency than adult stem cells and can be isolated without harming the child. Among prenatal stem cells umbilical cord derived stem cells are most commonly used and studied. Umbilical cord is an elastic connecting organ of the fetus to placenta. These umbilical cord derived stem cells show many promising possibilities in regenerative therapy (Si et al., 2015).

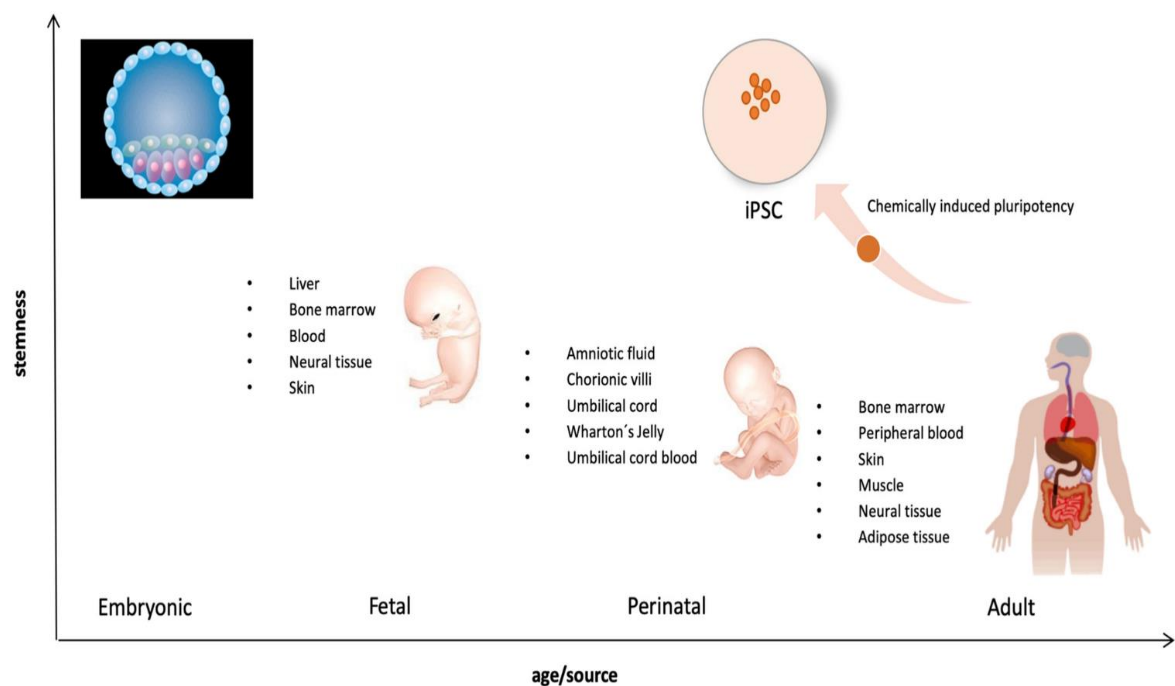


Figure 1: Different sources of stem cells from where they can be isolated and the stemness of cells on the basis of age and origin (Ekblad-Nordberg et al., 2020)

2.2 Umbilical cord blood as the source of stem cells

The cure of HIV infection by the use of umbilical cord blood has shown the whole world about the potential of stem cells in regenerative therapy (Marley et al., 2022). It has been almost six decades since the first detection of stem cells. In 1963, two Canadian scientists

first detected the unspecialized cells that can differentiate into specialized cells. In 1907 A.D. American embryologist Ross Granville Harrison first developed the techniques of cell culture in *vitro* (Mak, 2018). Small pieces of living frog embryonic tissue were isolated and cultured in the *in vitro* condition outside of the body. This has been a milestone in the study of cell biology (Jedrzejczak-Silicka & Jedrzejczak-Silicka, 2017). The isolation of cells and study in *in vitro* condition has made it possible to learn the nature of cells and cell biology in the controlled condition. This enables us to know the characteristics of cells and its potency under direct observation. Two types of stem cells, hematopoietic stem cells and mesenchymal cells are found in umbilical cord blood.

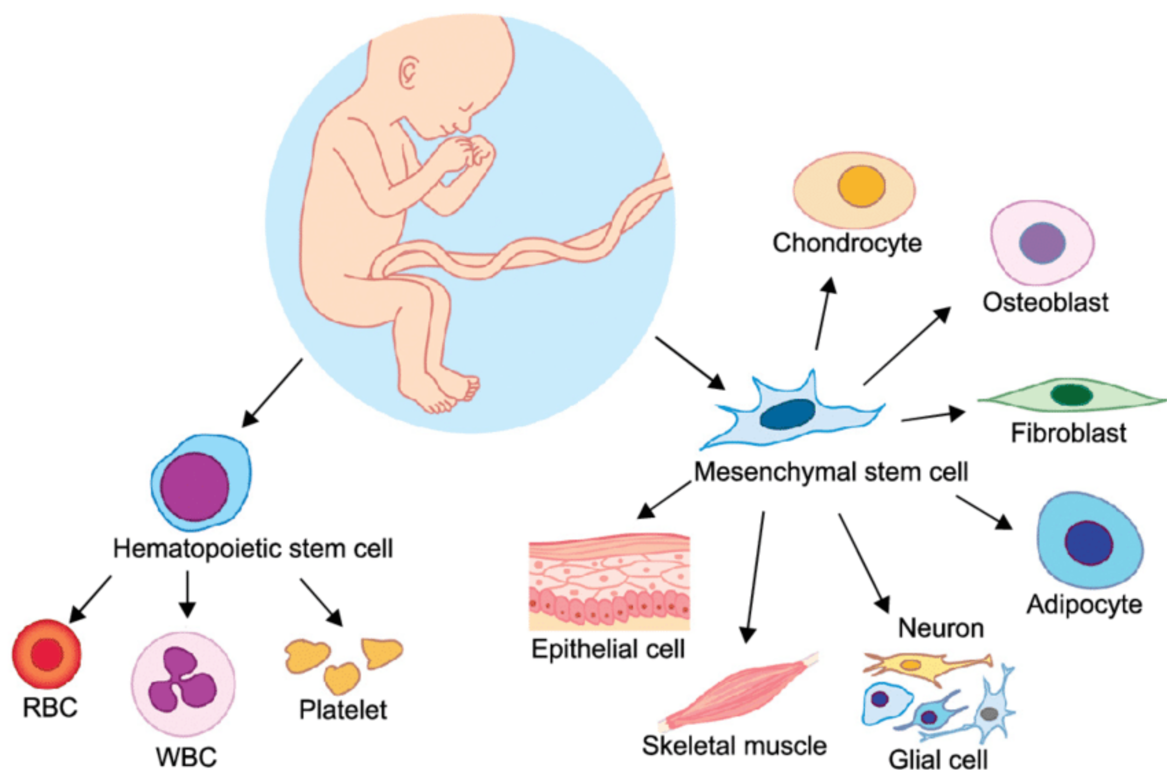


Figure 2: Umbilical cord blood contains hematopoietic and mesenchymal stem cells (Lee, 2010)

2.3 Hematopoietic stem cells

After the discovery of stem cells which have the ability to divide and form colonies in the umbilical cord blood culture by Knudtzon in 1974, cord blood became a potential candidate for the source of hematopoietic stem cells (Knudtzon, 1974). The use of hematopoietic stem cells to cure human disease has already been more than 60 years. The first successfully transplanted cells were the hematopoietic stem cells by bone

marrow cells transplantation from the identical twins of a patient suffering from acute leukemia in 1959 "E Donnall Thomas (1920–2012). The first cord blood transplant was performed after 30 years of successful bone marrow transplant in 1989 by Gluckman and colleagues. Some features of cord blood like easy availability and the presence of more primitive and naïve cell than in bone marrow which increases the tolerance for human leukocytes, antigen mismatches and decreases the incidence of graft-versus-host disease, make it more popular and established source for the hematopoietic stem cells for the transplantation (Metheny et al., 2013). In the last 33 years after the first transplantation of umbilical cord blood stem cells, there has been a huge progress in stem cell therapy. The use of umbilical cord blood for regenerative have been so popular that more than 450 cord banks have been established worldwide (Global Cord Blood Industry Database, 2021) in which more than 5,800,000 cord blood units have been stored in private and public cord bank and more than 35,000 UCB transplantation has been performed worldwide (Murata et al., 2022). The isolation of cord blood can be done once in life and it possesses a huge possibility. Cord banking is getting popular for future use. These hematopoietic stem cells have the ability to develop into all types of matured blood cell types through the organized hierarchy i.e., myeloid lineages such as granulocytes, monocytes, leukocytes, erythrocytes and megakaryocytes, and lymphoid lineage cells such as T and B lymphocytes (Kondo, 2010).

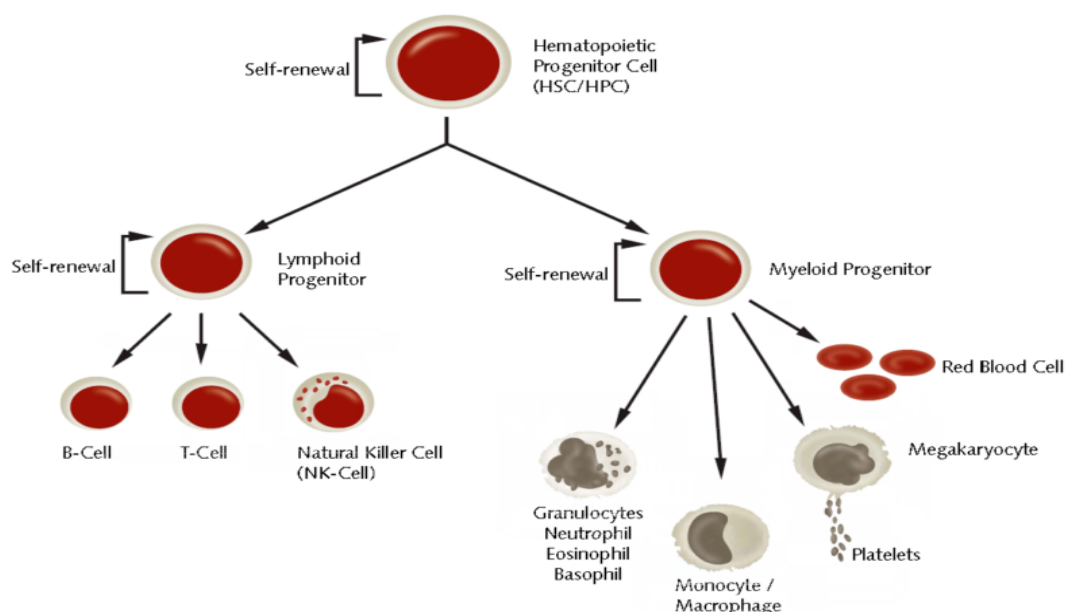


Figure 3: Different possible progenitor and specialized cells developed from the hematopoietic stem cells (From https://www.americordblood.com/)

This potential of umbilical cord blood to develop into all kinds of blood and immune cells has attracted the interest of researchers. Several clinical studies on cancers, lymphoma, leukemia, autoimmune disease immunodeficiency, cardiac failure, neural disorder, metabolic and genetic disorder etc., are in progress to explore the hematopoietic stem cells therapies potential and minimize the possible risk of the rejection, teratoma formation and autoimmune disease (Chivu-Economescu & Rubach, 2017). Cord blood has already proven treatment for many diseases related to various types of cancers, inherited metabolic disorders, blood disorders and immune deficiencies. Among the different types of stem cells, hematopoietic stem cell transplantation is the most applied therapy to treat and cure different diseases and disorders. Both allogeneic and autologous hematopoietic stem cell transplantation has been performed (Talib & Shepard, 2020).

2.4 in vitro expansion of umbilical cord blood stem cells

The number of stem cells present in umbilical cord blood is low as compared to bone marrow. *ex vivo* cell culture of the umbilical cord blood has been proposed to increase the cell number available for the transplant (McNiece et al., 2000). The *in vitro* cultures of the cells have been useful for studying cell biology, effects of the different drugs and other chemicals to the cells and cytotoxicity, productions of vaccines and proteins etc. (Arango et al., 2013). The study of behavioral and differential characteristics in *in vitro* culture condition are very effective and important for the cell differentiation such as the hematopoietic stem cells are non-adherent cells floating in the culture media while the mesenchymal stem cells adhered in the plastic surface of the culture plate (Mantri & Mohapatra, 2014).

2.5 The role of bioactive phytochemicals in cell culture

Stem cells are the cells which can divide indefinitely through the self-renewal and can differentiate into mature daughter cells, this phenomenon is regulated by various intrinsic and extrinsic mechanisms and factors. Metabolism plays a very important role in maintaining self-renewal and differentiation. The studies have shown that fatty acid metabolism has an important role in the self-renewal of hematopoietic stem cells by controlling over the fate of stem cells (Ito & Suda, 2014). Self-renewal and the

differentiation into different lineages are affected by the multiple signal pathways and signal transduction. The different bioactive phytochemicals are found to stimulate self-renewal and differentiation of stem cells by activating or inhibiting the signal pathways (MAPK, canonical Wnt/B-catenin, AKT, etc.,) and key transcription factors (e.g., OCT4, NANOG, Runx2, etc.). In regenerative therapy the controlling of proliferation and differentiation of stem cells have been a major challenge. Study shows Phytochemicals such as flavonoids, sterols, phenols and alkaloids derived from the foods and herbs have shown clear results on modulation and self-renewal function of stem cells. The cooperative application of bioactive phytochemicals with stem cells shows promising effects and possess huge possibilities (S. Zhang et al., 2020b).

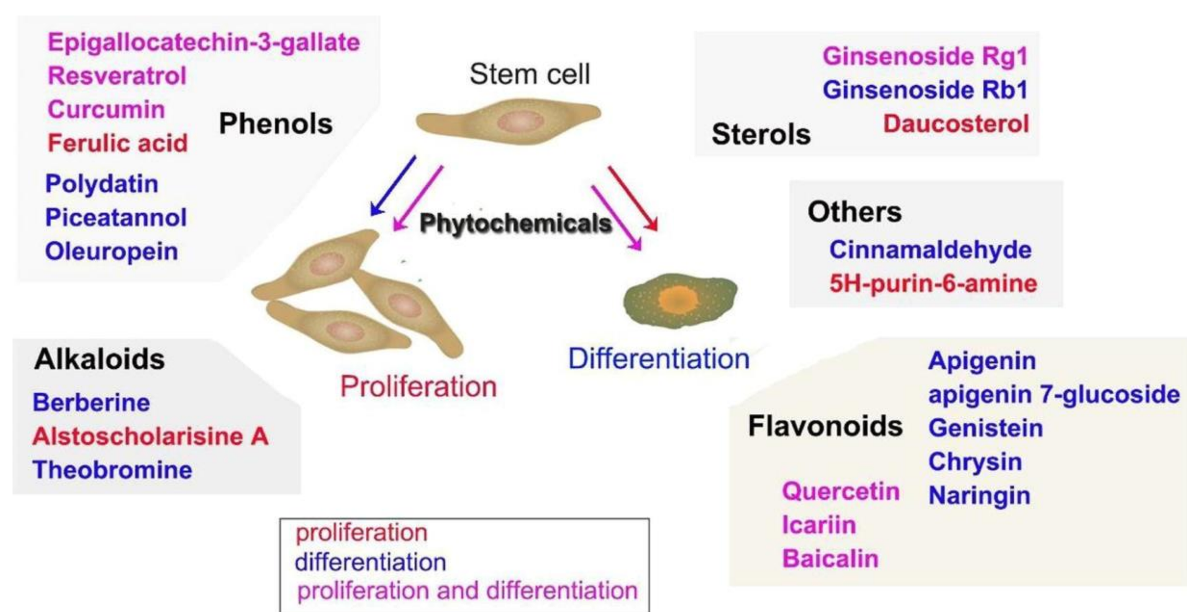


Figure 4: The role of phytochemicals in the proliferation and differentiation of stem cells (From S. Zhang et al., 2020b).

2.6 Phytochemical Extraction

Phytochemicals are the chemical compounds that naturally occur and secreted by the living plant tissues. These bioactive phytochemicals have the ability to interact with other components of living tissue in various effective ways as antioxidants, cell stimulants, enzymes, nutrients etc. Based on the chemical structure and characteristics these phytochemicals are classified into six major groups i.e., lipids, carbohydrates, phenolics, alkaloids, terpenoids and other nitrogen-containing compounds (Huang et al., 2016).

More than 1000 phytochemicals have been known and different phytochemicals have different characteristics and functions. Single plant and plant parts contain many different types of phytochemicals in different proportions. To isolate the desired phytochemicals of interest the phytochemical extraction is carried out. Medicinal plants contain many important phytochemicals along with other building compounds. To isolate the specific and effective phytochemicals in sufficient amounts specific extraction methods should be followed (Thakur et al., 2020). Different kinds of solvents are used for the extraction of phytochemicals from the plant based on the polarity of the desired phytochemicals. The solvents are selected on the basis of polarity of the desired compounds. A solvent of similar polarity will dissolve the solute properly. The study has shown that ethanolic extracts of plants extracted higher concentrations of phenolics compounds than by acetone, water or methanol (Koffi et al., 2010). To achieve a desired compound form phytochemical extraction, the proper selection of the solvent and extraction technique plays an important role. For the extraction there are several methods such as maceration, infusion, decoction, percolation, Soxhlet extraction, sonication, digestion and microwave assisted extraction etc., the choice of appropriate extraction methods depends on the nature of plant materials, solvent used, temperature and mainly the desired compounds and its purpose (Abubakar & Haque, 2020a).

2.7 PHYTOCHEMICAL SCREENING

Pre-phytochemical screening is a very important step to study the significance of bioactive phytochemicals. Before continuing the study, the analysis of phytochemicals helps us to examine if the extraction was performed well or not and the desired phytoconstituent is present or not in the extraction. The phytochemical screening is considered as the preliminary test or a qualitative analysis of the presence of primary and secondary metabolites such as carbohydrates, lipids, alkaloids, flavonoids, phenols and tannins and proteins (Pant et al., 2017). The phytochemicals like phenols and alkaloids exhibit antioxidant properties which protects the cellular constituent from oxidative stress and improve cell survival. The studies have shown that these antioxidants not only improve cell proliferation but also help to maintain the potency and differentiation of stem cells (Shaban et al., 2017).

2.8 *Clematis buchananiana* DC. as ethnomedicine

Clematis buchananiana are called by different names in different places based on the locality and community and the names are normally given based on its applicability. The knowledge of ethnomedicine is passed from generation to generation verbally. So, it's hard to find the written literature since when the plant was started to be used as the medicine. Although there are many related plants like this mentioned in Ayurveda, the knowledge of Ayurveda is written in Sanskrit and not available online. The detailed study of the mode of action and its efficacy to treat the disease and disorder and injury is not well known but still it has been practiced for centuries. The literature has shown that not just in Nepal in many parts of the world *Clematis* spp. have been used as ethnomedicines. Traditionally, it has been used to treat sinusitis, a paste of the roots is used to treat inflammation, toothache, juice of root is used in the treatment of peptic ulcers. It has also found to be administered orally and topically to treat sexually transmitted infections, bone disorders, chronic skin diseases, rheumatoid arthritis, chronic skin disease in different parts of the world (Adhikari et al., 2019; Bhat et al., 2013; Saha & Chattopadhyay, 2011; Subba et al., 2018). The *in vitro* study in the Sikkim University has shown the C*lematis buchnaaniana* has shown the inhibition of hemolysis and protein denaturation (Subba et al., 2018). The phytochemical screening of methanolic extract of *Clematis buchananiana* have shown the presence of anti-inflammatory metabolites such as flavonoids and phenolics and the results have also shown the evidence for the anti-inflammatory property (Subba et al., 2018).

2.9 MTT assay

To measure the cell viability, proliferation and cytotoxicity MTT assay was performed. MTT is abbreviated from 3-(,5-dimethylthiazol-2yl-2,5-diphenyltrazoliumbromide a tetrazolium salt. It is a yellow water-soluble dye that is cleaved by the mitochondrial succinate dehydrogenase produced by the metabolic activity of the cell, into violet color formazan. This is also used as a quantitative cytotoxicity assay. If there is no color change cells are dead or absent (Ghasemi et al., 2021). It is a colorimetric assay discovered by Tim Mosmann in 1983. This tetrazolium salts consist of a positively charged quaternary

tetrazole ring core containing four nitrogen atoms surrounded by three aromatic rings including two phenyl moieties and one thiazolyl ring which can pass through the cell membrane as well as the mitochondrial inner membrane of the viable cells. The reduction of MTT by metabolic activity of cells results in the color change by the disruption of the core tetrazole ring and the formation of a violet-blue water insoluble molecule formazan. The change in color is measured by reading optical density by ELISA reader and the change in color is compared with the controls to determine the percentage of viable cells (Mosmann, 1983)

2.10 Bovine umbilical cord blood

There are many obstacles and issues to using human umbilical cord blood although these are discarded after the birth of a child, mainly due to ethical issues and parent's consent. So, as an alternative bovine umbilical cord blood was chosen as an alternative for human umbilical cord blood for the present study. The umbilical cord blood samples from the calf could be taken as a model for the study. Several studies have shown that the bovine umbilical cord blood has similar properties and expresses similar surface markers as human umbilical cord stem cells. Therefore, bovine umbilical cord blood can be taken as the model for the study of animal cell culture which is applicable for both veterinary and human cell culture and regenerative therapy, vaccine and hormone production etc (Kusindarta & Wihadmadyatami, 2021).

2.11 Wright staining

Wright Stain is a commonly used stain in hematology laboratory for the staining of peripheral blood smears. Wright's stain is named after James Homer Wright who first made this stain by modifying Romanowsky stain in 1902. This stain is used as a differential stain to distinguish between blood cells. Wright stain is a mixture of basic dye methylene blue and acidic dye eosin. These induce multiple colors when applied to the cells. The basic dye methylene blue helps to stain the acidic components e.g., nucleus with nucleic acid as a result it becomes blue to purple shades. And the acidic dye eosin helps to stain the basic component of cells e.g., hemoglobin, eosinophilic granules and cytoplasm and form orange to pink color. The neutral component of cells could take both dyes resulting

in variable colors. It does not require any special fixative agent as it is methanol-based stain (Giri, 2019).

2.12 Gram staining

Gram staining is the most commonly used technique in Microbiology laboratory. It was first used in 1884 by Hans Christian Gram while searching for the method to visualize cocci present in pneumonia. This stain is the fundamental to phenotypic characterization of bacterial cells but even the cells of fungi and yeast can be stained by it. The staining procedure differentiates organisms according to the cell wall structure. Gram-positive cells have a thick peptidoglycan layer and stain blue to purple while Gram-negative cells have a thin peptidoglycan layer and stain red to pink (*Gram Stain Protocols*, 2019).

2.13 Trypan Blue staining

Trypan blue staining is the dye exclusion test used to determine the number of viable and number of viable cells in a cell suspension. This staining process is based on the principle that the live cells have intact cell membranes that exclude the penetration of dye whereas the dead cells don't have intact cell membranes and the cell absorbs dye. Trypan blue is diazo dye that, being negatively charged, doesn't interact with the cells and penetrate membranes, thus it couldn't take dye, so unstained cells were considered as viable cells. In other hand, the cells with damaged membranes are stained in a distinctive blue color readily observed under microscope (Tran et al., 2011).

2.14 Hemocytometer

Cells can’t be counted directly from the flask, even on slides counting all cells is not possible. There is no order of magnitude of the volume of cells in normal glass slides. Hence to calculate the density of a cell in a suspension hemocytometer is designed. Hemocytometer is a thick glass slide that contains the square chamber carved in it that has a specific depth (Liu, 2017). Fig.5 shows the structure and cell counting technique of Hemocytometer.

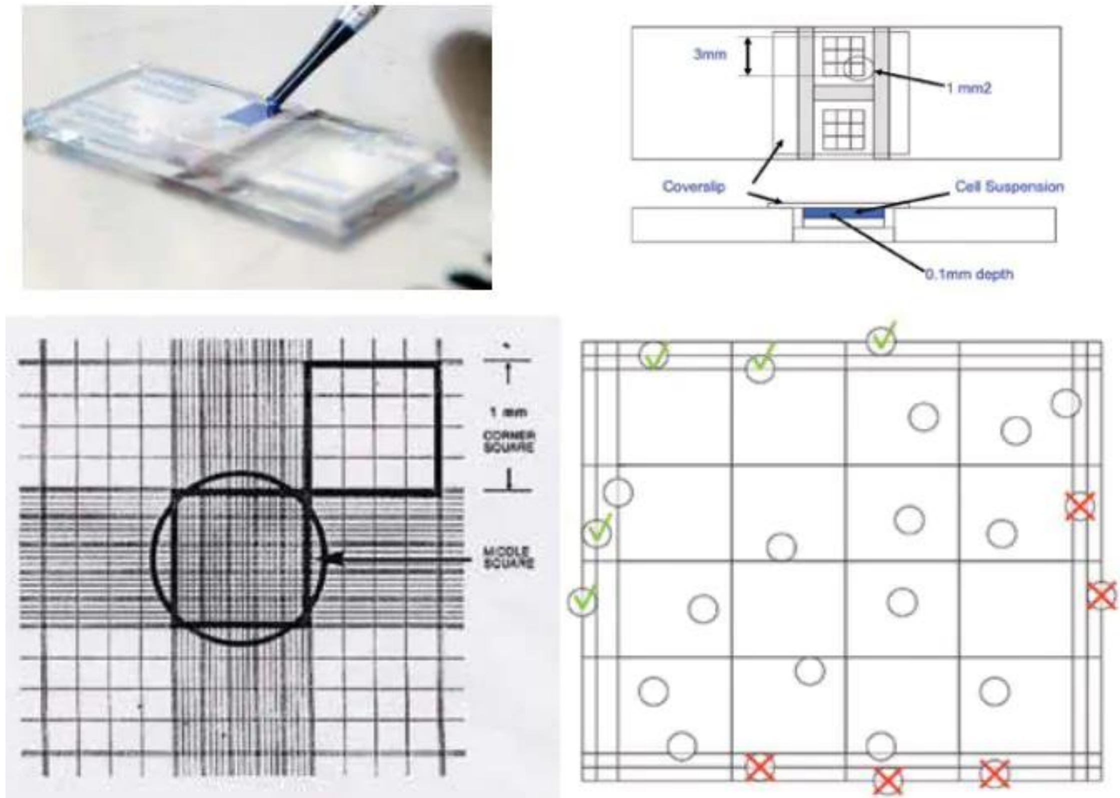


Figure 5: Hemocytometer gridlines, a cell counting technique (After sigmaaldrich.com).

CHAPTER 3

METHODOLOGY

All the laboratory works were performed in the Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu, Nepal. The plant sample was collected from the Western region of Nepal (https://goo.gl/maps/3neu8fhuh74qXbo67) from Kaski district and the umbilical cord blood was collected from the agriculture farm of (https://goo.gl/maps/ati84YoEubePBFUy50) Gatthaghar, Bhaktapur, Nepal.

3.1 Collection of plant sample

The plant sample was selected on the basis of its ethnomedicinal value and the practices in the local community in the villages located at the 1300 m altitude western region of Nepal Gandaki province. The aerial part of the plant sample including leaf, stems and flowers were collected in a clean plastic bag and delivered to laboratory central department of Biotechnology, Kirtipur, Kathmandu, Nepal.

3.2 Identification of plant species

To identify the Plant sample species, the plant (stem, leaves, flower) herbarium was prepared on a white paper. The plant was kept pressed in layers of newspaper for 15 days. The papers were changed periodically to avoid the humidity. The dried plant sample without losing its color was sent to the National herbarium and botanical laboratories, Godawori, Lalitpur, Nepal.

3.3 Extraction of phytochemicals from plant

The leaves of the plant sample were collected and air dried at room temperature for a week. Powder was made from the completely dried leaf by using a grinder. 10 g of powder was added in a thimble prepared by filter paper in the lab (Whatman grade 1 filter paper) and kept in the extraction chamber of Soxhlet apparatus. 250 ml of 70% ethanol was added in the round bottom flask of Soxhlet. The apparatus was fitted as required and

started heating at 60^0 C by using a heating mantle. In and out flow of cold water was maintained in a Soxhlet condenser. For the condensation of the ethanol evaporated by heating in the heating mantle. Each drop of the condensed ethanol falls on the powder which mixes with the phytochemical of the plant and separates it. The extracted solution falls back to the round bottom flask. This process was repeated for 10 hours. After 10 hours of continuous running of the Soxhlet apparatus the collected solution in the round bottom flask was transferred to the round bottom flask of rotavapor. The round bottom flask of the rotavapor was fixed to the rotavapor and the rotavapor was run at 60°C and 50 rpm till the ethanol was completely evaporated (Abubakar & Haque, 2020b; Redfern et al., 2014a, 2014b). The dried extract was removed from the round bottom flask of Rotavapor and stored in a 15 ml falcon tube in a dark condition.

3.4 Qualitative analysis of phytochemicals

The qualitative tests for preliminary phytochemical screening were performed to detect the presence of bioactive phytochemicals (Pandey & Tripathi, 2014; Shaikh &Patil, 2020). Preliminary phytochemicals are effective for extraction and to analyze the possible utility of the plant extract. This test was performed to detect the presence of phytochemical groups like Alkaloids, carbohydrates and glycosides, proteins and amino acids, phenolic compounds and tannins and flavonoids.

3.5 Detection of carbohydrates

100 mg extract solid was dissolved in 5 ml distilled water and filtered. Filtrates were used to test for the presence of carbohydrates and glycosides. To detect the presence of carbohydrates and glycosides Benedict’s test was performed. Two different solutions i.e., solution A was prepared by mixing 17.3 gm sodium citrate and 10 gm sodium carbonate and 80 ml water and dissolved by boiling to make clear solution and solution B was prepared by dissolving 1.73 gm of copper sulphate in 10 ml distilled water, these two solutions were mixed to prepare the Benedict’s reagent. 2 ml Filtrates were treated with benedict’s reagents and heated gently. Orange red precipitate indicates the presence of Carbohydrates.

3.6 Detections for alkaloids

Wagner's test was performed to detect the presence of Alkaloids. Wagner's reagents were prepared by mixing 1.27 gm of iodine with 2 gm of potassium iodide and dissolved in distilled water to make the final volume 100 ml. 100 mg of extracts were dissolved individually in dilute hydrochloric acid and filtered. 2ml Filtrates were treated with Wagner's reagents. Formation of brown/reddish precipitate indicates the presence of alkaloids.

3.7 Detection of proteins and amino acids

Ninhydrin test was performed to detect the presence of proteins and amino acids. 2 drops of Ninhydrin solution (10 mg ninhydrin + 200 ml acetone) were added in the 2 ml of filtrate. A formation of purple color indicates the presence of proteins and amino acids.

3.8 Detection of phenolic compounds and tannins

Ferric chloride test was performed to detect the presence of phenolic compounds and tannins. Extract aqueous solution was treated with 3-4 drops of ferric chloride solution. Formation of greenish/blueish to black color indicates the presence of phenols.

3.9 Detection of flavonoids

Alkaline reagent Test was performed to detect the presence of flavonoids. 1 ml aqueous solution was treated with 2 ml of 2% NaOH solution A formation of intense yellow color which again disappeared on adding few drops of diluted HCl indicates the presence of flavonoids.

3.10 Preparation of complete DMEM media

The complete DMEM media was prepared by adding 0.4 gm incomplete DMEM {Dulbecco's Modified Eagle's Medium (DMEM) -low glucose with 1000 mg/L glucose and L-glutamine, without Sodium bicarbonate} powder media of sigma analytical in 1 L reagent bottle containing 400 ml distilled water and was shaken gently for a while till it

mixed properly. Then 1.85 gm of sodium bicarbonate was added on it and filtered through 0.2 micron pore size syringe filter and was stored at 4°C. 10% FBS (5 ml) and 1% antibiotics (500 mg streptomycin sulfate) was mixed in the 45 ml of incomplete DMEM media (Clouthier, 2012.).

3.11 Collection of umbilical cord blood

The umbilical cord blood was drained from the umbilical cord blood vein just after the delivery of the calf by the senior veterinary expert Dr. Umesh Mandel. Doctor was called by the farmer due to an abnormality in delivery. The mother cow was a Red Jersey breed giving 3rd baby and was having a delivery complication. The calf was delivered successfully alive without harming the mother cow by the doctor, a 20 ml blood sample was collected by 10 ml syringe from the vein of umbilical cord and then collected in the EDTA vial placed in a zip-logged bag aseptically. The collected blood sample was delivered to the lab within 6 hours in an ice bag maintaining a cold temperature for processing. There were no signs of leakage, coagulation or hemolysis observed after delivery to the laboratory.

3.12 Isolation of peripheral blood mononuclear cells and cell culture

Whole Blood isolated from the cord was mixed with PBS 1:1 ratio for dilution. Divided into two falcon tubes of 15 ml containing 5 ml Ficoll gradient in each carefully, without mixing two separate layers. Then centrifuged at 1850 rpm for 45 min. Mononuclear cell layer was separated and taken in another sterile clean falcon tube. 5 ml PBS (“Biotech corporations” manufactured for RNA extraction) was added, mixed and centrifuged at 1300 RPM for 5 min (washing). Supernatant was discarded and pellet was mixed with 5 ml complete media and again centrifuged at 1300 rpm for 5 min. Supernatant was discarded. The pellet was resuspended with 1 ml complete DMEM media. Then the suspension was transferred to a T25 flask containing 9 ml DMEM-low glucose complete media (10% FBS and 1% antibiotics). And then incubated at 37^0 C and 5% CO_2 (Mantri et al., 2014).

3.13 Isolation of hematopoietic stem cells and passaging of cells

After 2 days T25 flask was examined in the inverted microscope. The cell growth was observed. Adherent cells (ADC) and non-adherent cells (NAC) were differentiated by pouring the media in a falcon tube. The adherent cell contained in the flask was fed by adding 10 ml complete DMEM media. While the floating was centrifuged at 1300 RPM for 5 min. The media was removed and the pellet was resuspended with 1 ml of DMEM media, then it was transferred to the new T25 flask containing 9 ml complete DMEM media. Both T25 flasks were incubated at 37^0C and 5% CO_2. In every 24 hours the culture flask of the adherent cell containing flask was changed for feeding and after observing more than 90% cell density the passaging was done. The process was repeated for several passages.

3.14 Trypan blue staining and cell counting by hemocytometer

100 µl of cell suspension was mixed with 100 µl of 0.4% trypan blue by using micropipette incubated for 3-5 minutes. 100 µl mixed suspension was placed in the edge of hemocytometer glass slides underneath the coverslip on it. The cell suspension was drawn out due to the capillary action. The hemocytometer was placed in the stage of the microscope and focused on the cells of the grid lines in the hemocytometer with 10X objectives. The stained and unstained cells were counted separately in the hemocytometer within the square. The hemocytometer contains 9 squares out of which 4 corner squares contain 16 squares in it. Only the cells within those 4 corner squares were counted. Total number of viable cells per ml of was calculated by, multiplying the total number of viable cells by 2 (the dilution factor) multiplied by 10,000. The cell viability percentage was calculated by dividing the total viable cells by the total sum of both viable and dead cells (total cell count) multiplied by 100.

3.15 Wright staining

A thin film of cell suspension was made in a clean grease free microscopic slides and allowed to air dry. The slides were placed facing upwards for faster drying. The smear was flooded with Wright's stain and left for 3 minutes. The distilled water was added to the surface of the slides for dilution and left for 5 minutes. After 5 minutes the slides were

washed with a distilled water till the slides have thinner film of pinkish red color. The slides were then placed facing upwards on top of tissue paper till the slides dried completely. Then the dried slide was observed in the microscope (*Giri., 2019*.).

3.16 Gram staining

A thin smear was made in the clean grease free slides, the smear was exposed to small flame for a very short time for heat fix and left for air dry. After air dry the cells were flooded with crystal violet staining reagents and left for one minute. The slide was washed in a gentle stream of tap water for 2-3 seconds. Then the slide was flooded with mordant Gram's Iodine and left for one minute. After one minute the slide was washed with a gentle indirect stream of water for 2-3 seconds. Then the slide was flooded by decolorizer and left for 15 seconds again then washed gently by tap water then the slide was flooded with counterstain (safranin) and left for 1 minute. Then again, the slide was washed with a gentle and indirect stream of tap water until no color was observed in the effluent. Then the slide was left for drying, placed facing upward over a blotting paper. After drying, a slide was observed in the microscope. Oil immersion was used to observe under 100X magnification (*Gram Stain Protocols*, 2019).

3.17 Cell Proliferation Assay

MTT (Thiazolyl Blue Tetrazolium Bromide) assay was performed in this study to detect the condition of cell proliferation. MTT solution and MTT solvent were prepared fresh just before starting the assay. MTT solution was prepared by mixing 25 mg of MTT salt with 5 ml PBS (5 mg/ml) solution and vortexed to mix. It was filtered by using a syringe filter. DMSO was taken as MTT solvent. The cell suspension culture media from the culture flask were harvested in 15 ml falcon tubes and centrifuged at 500 rpm for 5 min. The supernatant was discarded and the pellet was resuspended with 5 ml complete media. In a 96 well plate culture flask the 100 µl cell suspension (containing 1216*10^3 cells) was seeded in 39 wells (13x3) in 3 wells 100 µl distilled water were placed as a negative control and in 3 well complete cell free media was placed and left for 24 hrs incubation. On 2nd day, 4 different concentrations (62.5, 125, 250, 500 µg/ml) of plant extract, or Amfol plus (iron chakki) or 70% ethanol (vehicle control) was were prepared in distilled water and

added to 3 well as a triplicate of each concentration in a plate seeded and left for overnight incubation. On the third day 20 µl of MTT solvent was added in each well and incubated for 3.5 hrs at 37^{0}C, after incubation 150 µl MTT solvent was added into each well. After that the culture flask was wrapped with foil and shaken for 15 minutes. And then absorbance was read at OD=551 nm. by ELISA reader (https://www.abcam.com/kits/mtt-assay-protocol)

3.18 Data and graphical analysis:

The data of MTT assay were analyzed by using MS Excel (Microsoft Inc) and GraphPad Prism 9.5.0

CHAPTER 4

RESULTS

4.1 Identification of plant sample

The plant sample selected was identified by comparing herbarium of the plant with the other plant herbarium and other related information in the National Herbarium and botanical Laboratories of Nepal, Godawori. The plant was found to be the family of Ranunculaceae named *Clematis buchaniana* DC. These shrubs are found in warm broad-leaved to cool mixed forests growing epiphytically on the small trees and bushes and rock at the elevation of 460-3650 m.

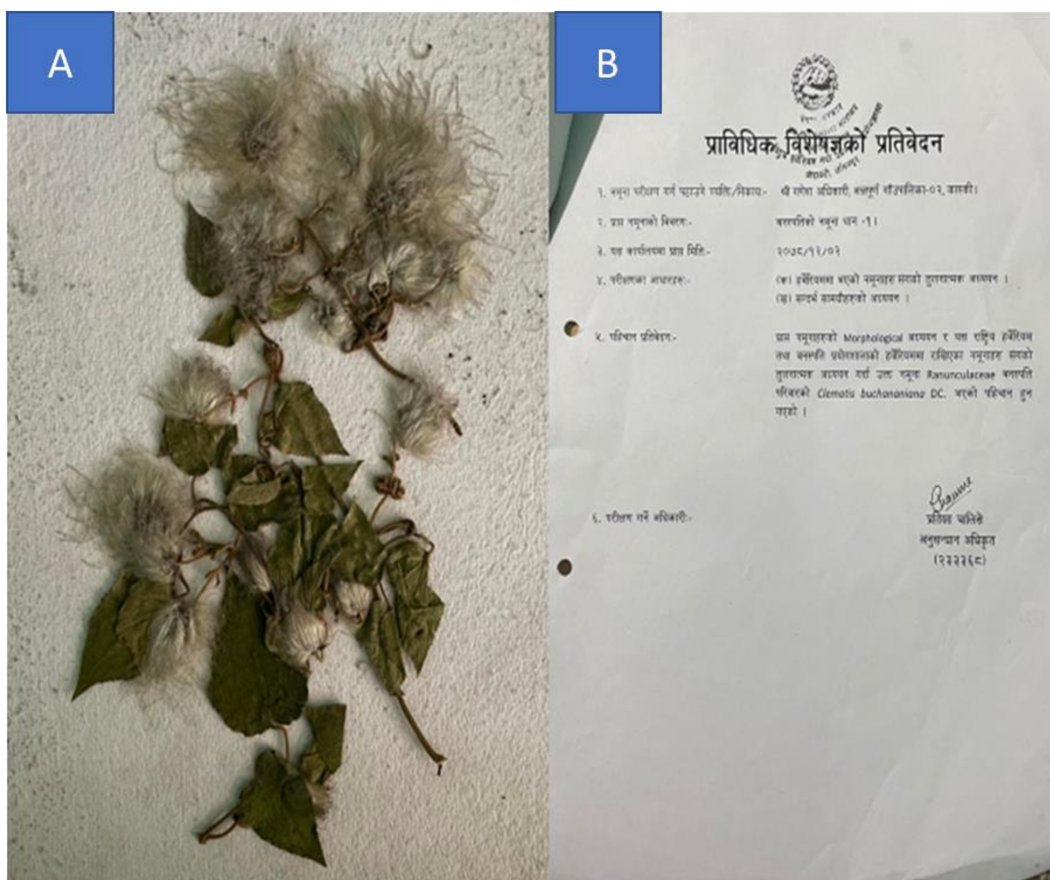


Figure 6: The Herbarium verification by National herbarium and botanical laboratory A) Herbarium sample sent for verification, B) Verification certificate given by National Herbarium and botanical Laboratory, Godawori, Lalitpur

4.2 Phytochemical extraction of Clematis buchaniana.

Figure 7: Phytochemical extraction and phytochemical screening of *Clematis buchaniana.* A) Fresh *Clematis buchaniana,* B) Soxhlet extraction of *Clematis buchaniana,* C) Rotavapor evaporation of solvent ethanol D) Phytochemical analysis results

The phytochemical extraction of *Clematis buchaniana* was carried out successfully by using soxhlet apparatus and the solvent was evaporated by using rotavapour. 0.2 g/ml extract was obtained in a greasy state from the extraction of 10 g of dried powdered plant leaves with 300 ml of 70%. In total the ethanolic extraction exhibited 20% yield i.e. 2 gram extract was obtained.

4.3 Qualitative phytochemical analysis of plant extract

Qualitative phytochemical screening of 70% ethanolic extract of *Clematis buchaniana* showed the presence of both primary metabolites and secondary metabolites. The primary metabolites like carbohydrate and glycosides, protein and amino acids were found in extract while the secondary metabolites like alkaloids, phenolic compounds and tannins. Flavonoid was undetectable.

Table 1: The qualitative phytochemical analysis of *Clematis Buchaniana.*

S.N.	Chemical constituent	Tests	Results
1	Carbohydrates and glycosides	Benedict's test	+
2	Proteins and amino acid	Ninhydrin test	+
3	Alkaloids	Wagners Test	+
4	Phenolic compounds and tannins	5% FeCl3 test	+
5	Flavonoids	Alkaline reagents test	-

4.4 Isolation and culture of Peripheral blood mononuclear cells (PBMC) from bovine umbilical cord blood.

The PBMC cells were isolated successfully from the whole blood of the bovine umbilical cord by using the density gradient centrifugation method. The isolated cells were cultured in a complete DMEM medium at 37°C and 5% CO_2. Fig

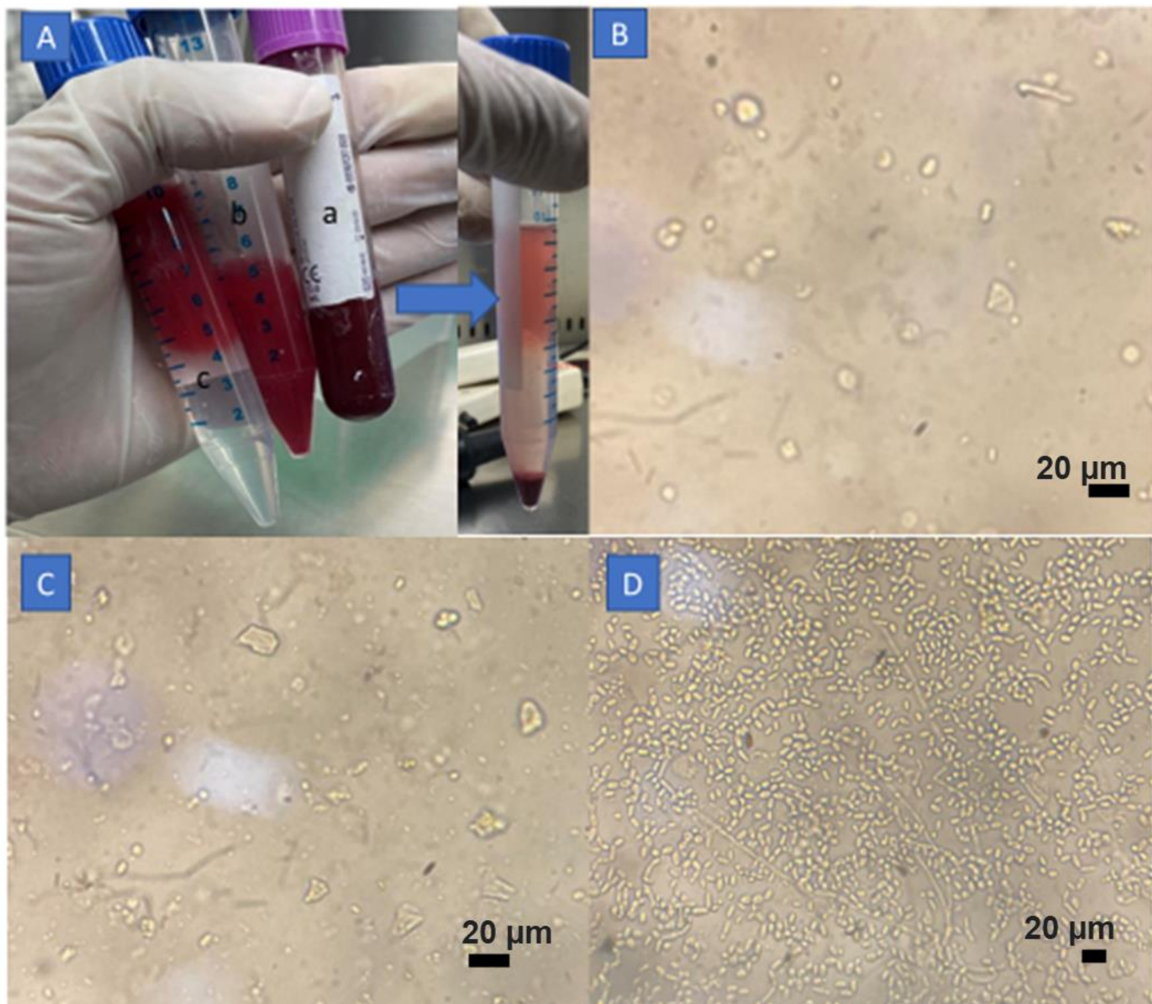


Figure 8: Isolation and culture of peripheral blood mononuclear cells (PBMC) from bovine umbilical cord blood (UCB). A) Blood sample from the vein umbilical cord of freshly born calf (a) Diluted blood sample with pbs in 1:1 ratio (b), and tube containing two separate layers of diluted blood sample and ficoll (c). B) Seeding of PBMC isolated from UCB in T-20 culture flask containing culture medium. C) Growth of cells observed under inverted microscope after 24 hrs of incubation. D) Cell growth observed after 48 hrs. of incubation.

4.5 Isolation of stem cells from PBMC of bovine umbilical cord blood

After 2 days of incubation the confluent growth of the cell culture was observed. The culture flask contained two types of cells on it i.e., floating and adherent cells. On sub-culturing of these two types of cells the regenerative capacity was intact. And the cells were found to be capable of dividing continuously. The floating cells were subcultured for up-to 9 passages and still had the ability to divide.

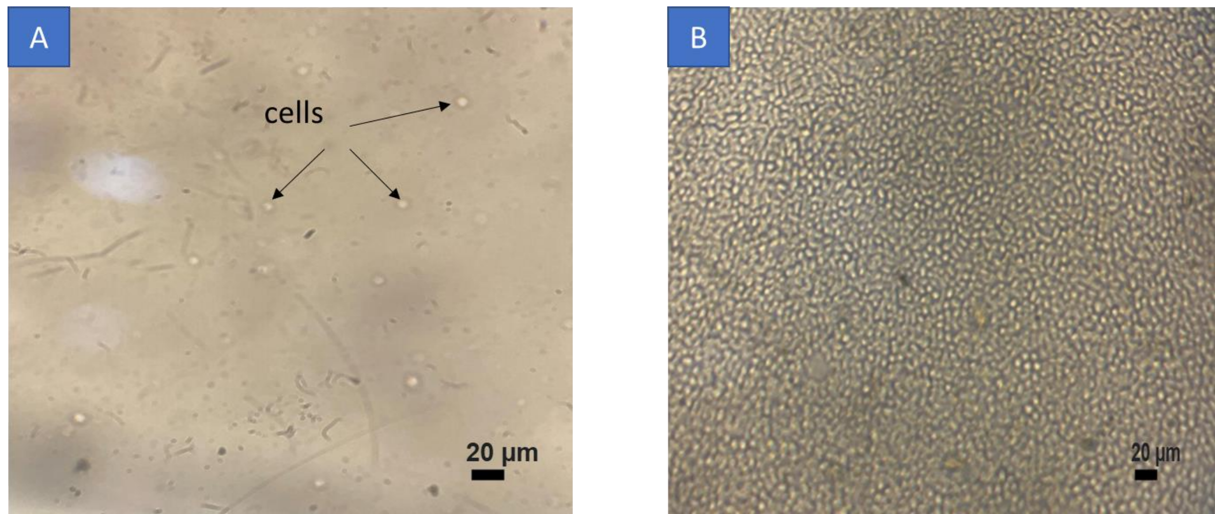


Figure 9: Isolation of stem cells from peripheral blood mononuclear cells (PBMC) i.e., passaging of cells observed in inverted microscope, A) seeding of cells isolated from PBMC culture, B) confluent cell growth observed after 2 days of incubation

4.6 The isolated cells have higher viability rate and have the ability to divide and differentiate

Isolated cells from PBMC show very active growth. On further subculturing the cells upto 9th passage there is no any visible loss of growth rather, it still has active cell division. The fig 11- fig 17 shows the cells seeding in each passage in new culture flask and their growth till 9th passage. The cells were found to be confluently grown within 48 hours so, each passaging was done after 48 hours of incubation of cells in 5% CO_2 at 37°C.

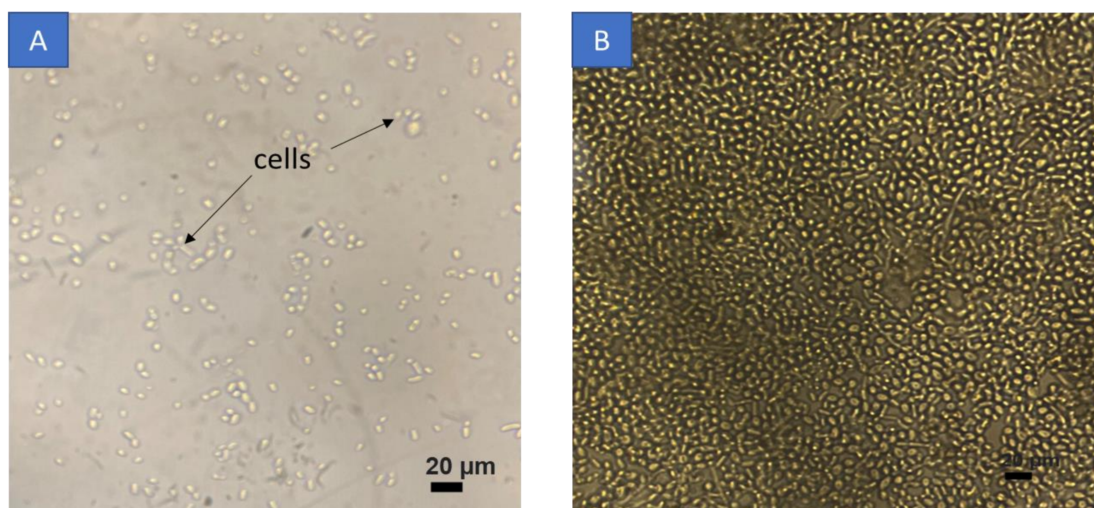


Figure 10: 2nd passaging of cells observed in inverted microscope, A) Seeding cells from 1st passage, B) Confluent cell growth observed after 2 days of incubation

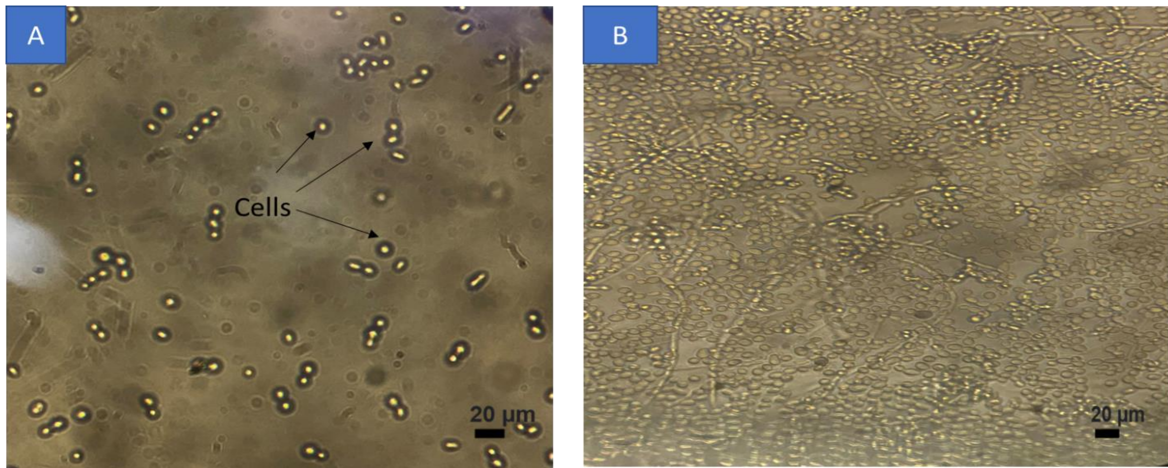


Figure 11: 3rd passaging of cells observed in inverted microscope. A) Seeding of cells isolated from 2nd passage, B) Growth observed after 2 days of incubation

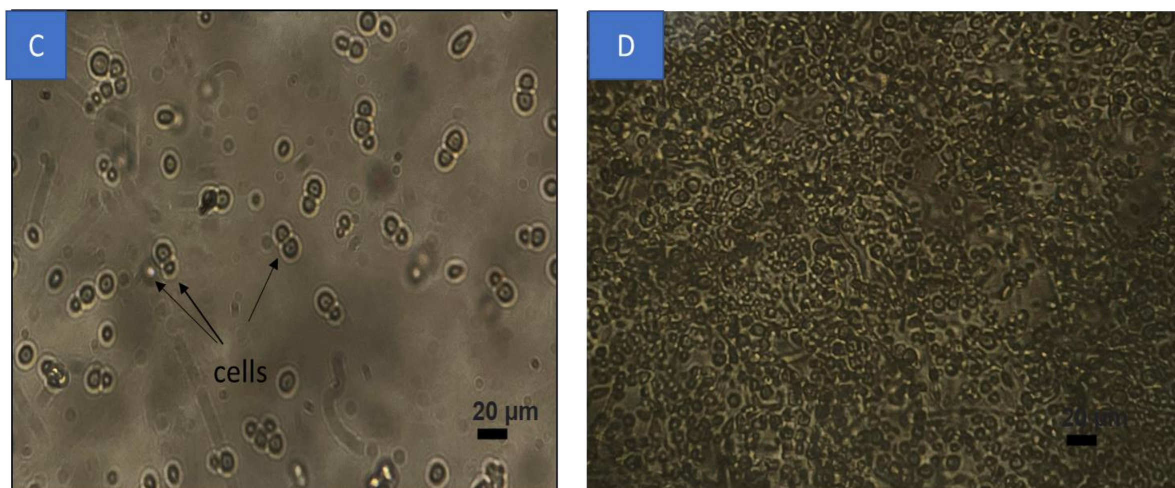


Figure 12: 4th passaging of cells observed in inverted microscope, A) Seeding of cells isolated from 3rd passage, B) Growth observed after 2 days of incubation

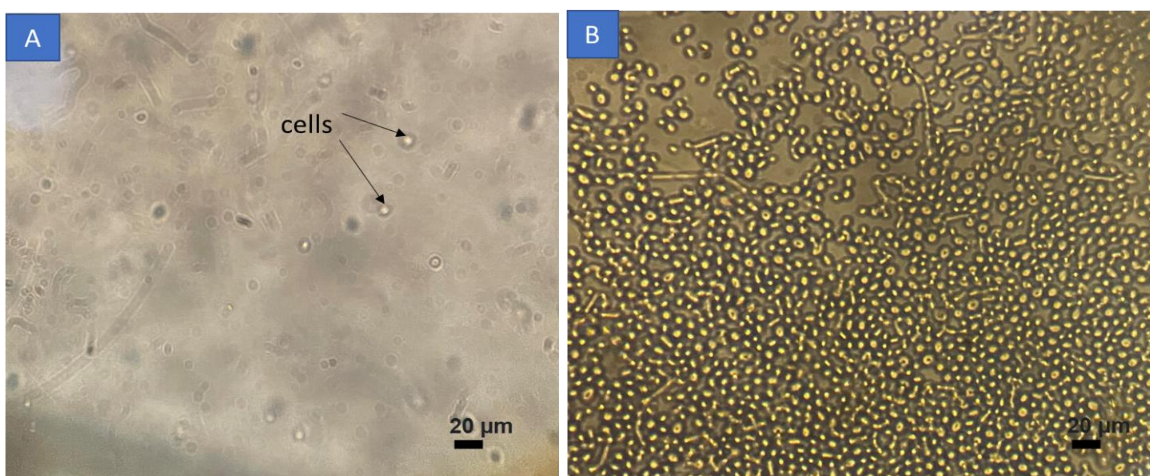


Figure 13: 5th passaging of cells observed in inverted microscope. A) Seeding of cells isolated from 4th passage, B) Growth observed after 2 days of incubation

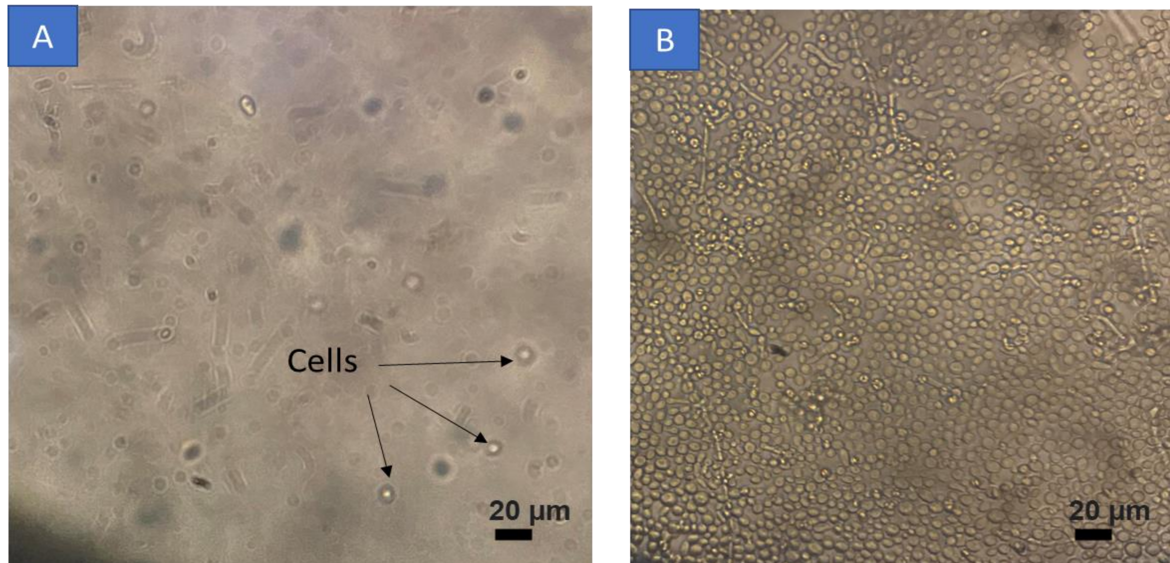


Figure 14: 6th passaging of cells observed in inverted microscope. A) Seeding of cells isolated from 5th passage, B) Growth observed after 2 days of incubation

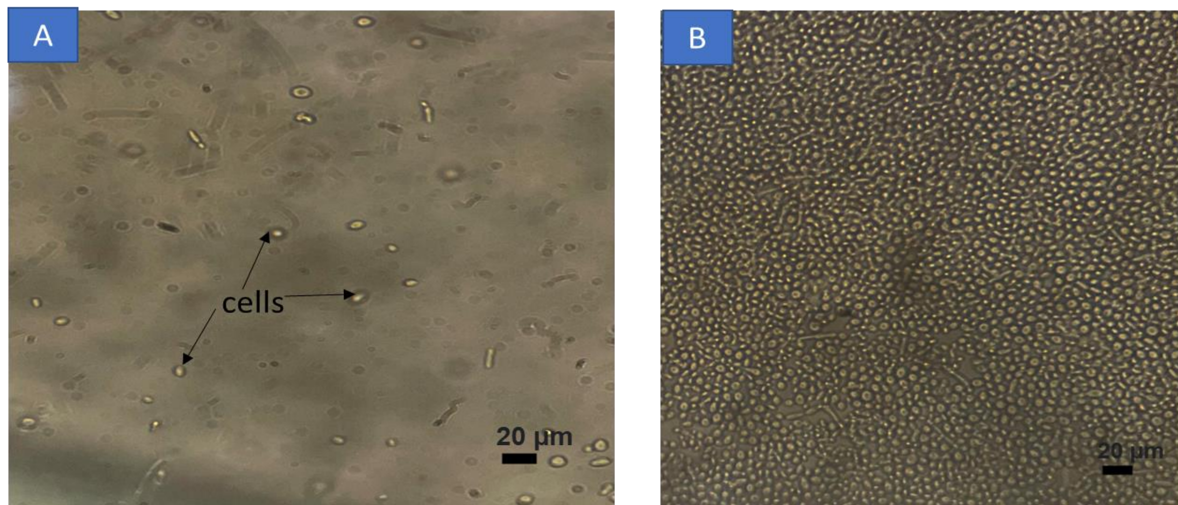


Figure 15: 7th passaging of cells observed in inverted microscope. A) Seeding of cells isolated from 6th passage, B) Growth observed after 2 days of incubation

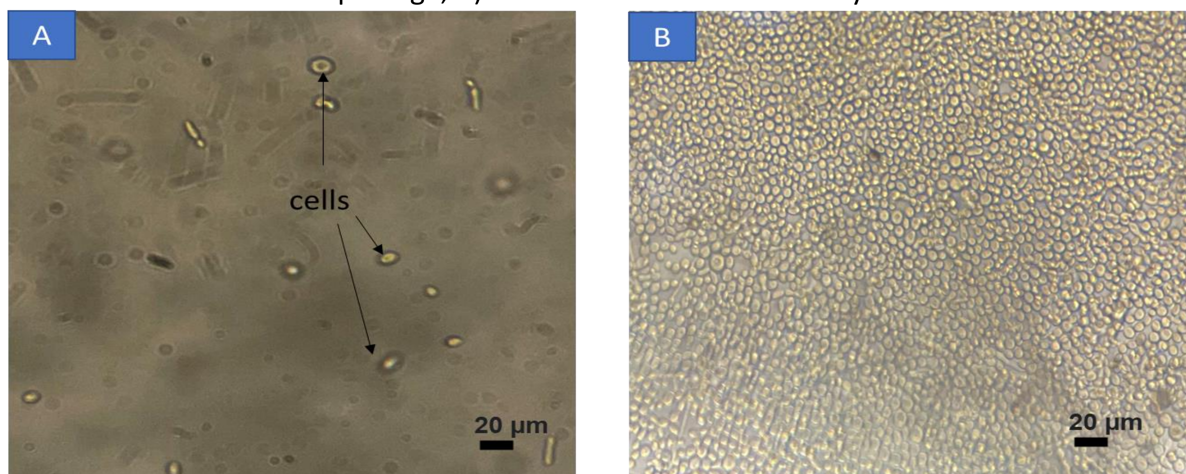


Figure 16: 8th passaging of cells observed in inverted microscope. A) Seeding of cells isolated from 7th passage. B) Growth observed after 2 days of incubation

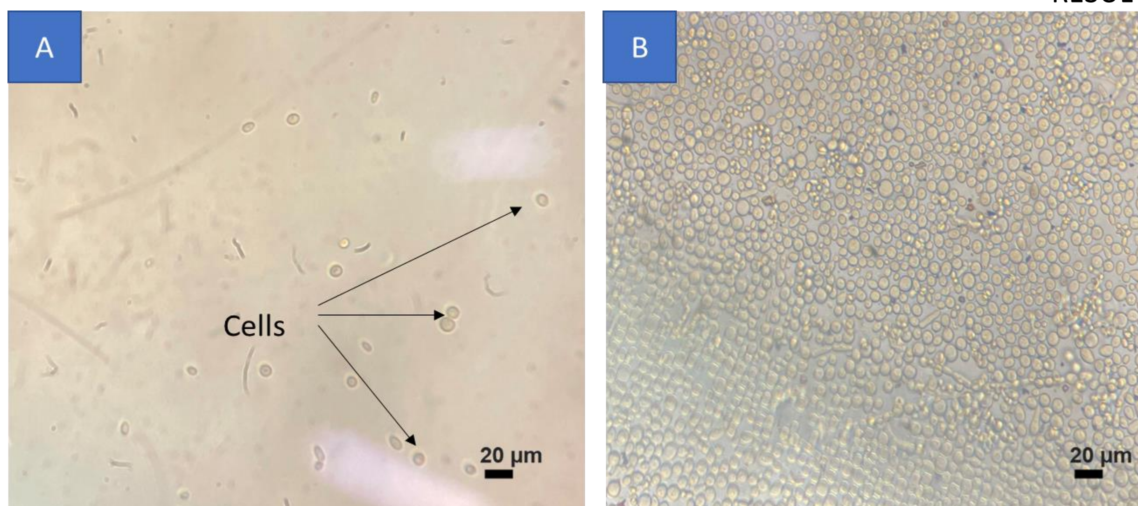


Figure 17: 9th passaging of cells observed in inverted microscope. A) Seeding of cells isolated from 8th passage, B) Growth observed after 2 days of incubation

4.7 Cell cultures contain healthy and viable cells without contamination

The hemocytometer reading of the cells from cell culture shows the cell viability is higher than 95%. In 4th passaging it was 97.32%, in 6th passaging it was 95% and in 8th passaging it was 98%. Which indicates the cell's viability on passaging was not affected with passaging. The isolated cells were healthy and had high potency. The microscopic observation of the cells with different staining like Gram staining, Giemsa staining and direct microscopic observation shows the structure of the cells and the cell culture were free from bacterial fungal and yeast contamination.

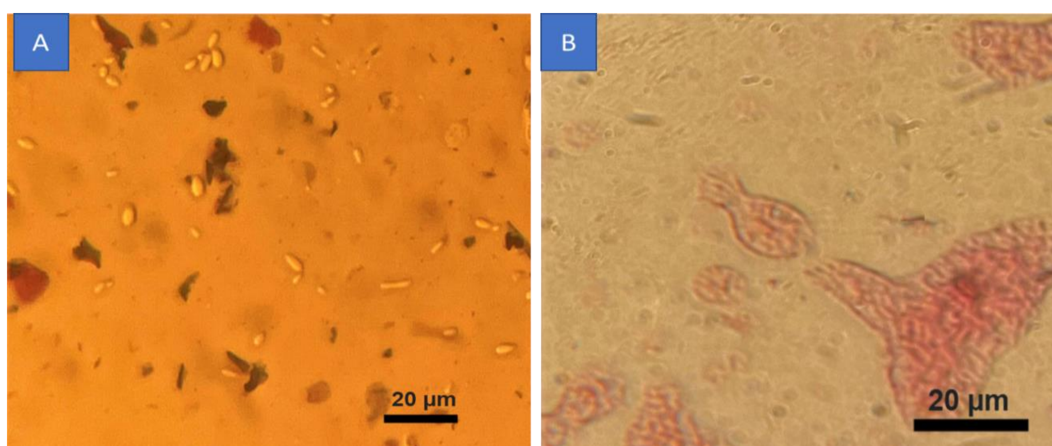


Figure 18: Microscopical observation of cells from 4th passage to see if there is any contamination. A) Cells observed in compound microscope at 100X magnification after Geimsa staining, B) Cells observed in compound microscope at 100X magnification after Gram's staining

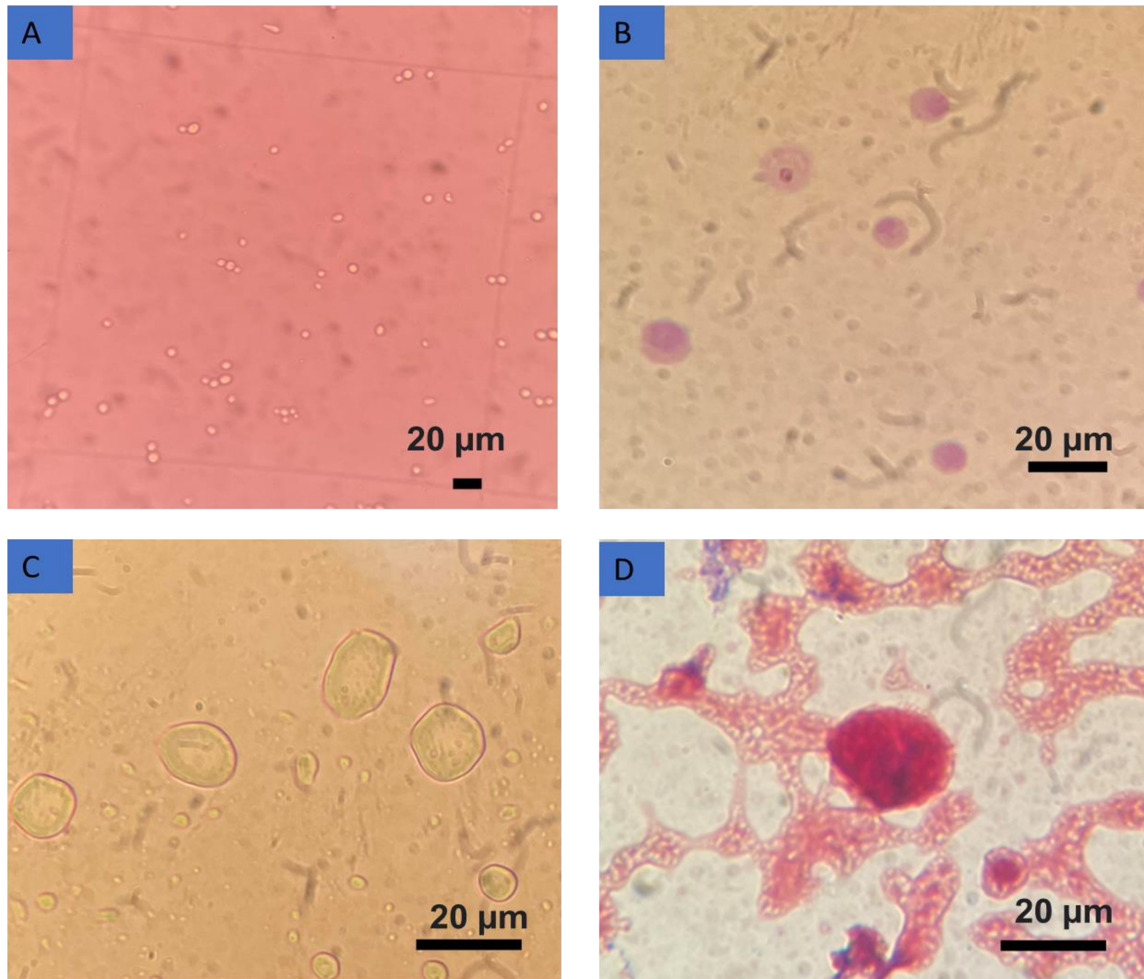


Figure 19: Microscopical observation of cells to check the possible contamination and cell viability, A) viable cells observed in hemocytometer by using trypan blue stain. B) Cell morphology observation by using Geimsa stain, C) Direct microscopic observation for cell morphology, without staining. D) Microscopical observation of cells to detect the possible fungal and yeast contaminations

4.8 The cell culture contains hematopoietic stem cells

By Wright staining, different kinds of cell structure were observed. Many cells are small spherical cells without nucleus, cells with round nucleus, cells with bilobed nucleus and kidney like nucleus as shown in fig.

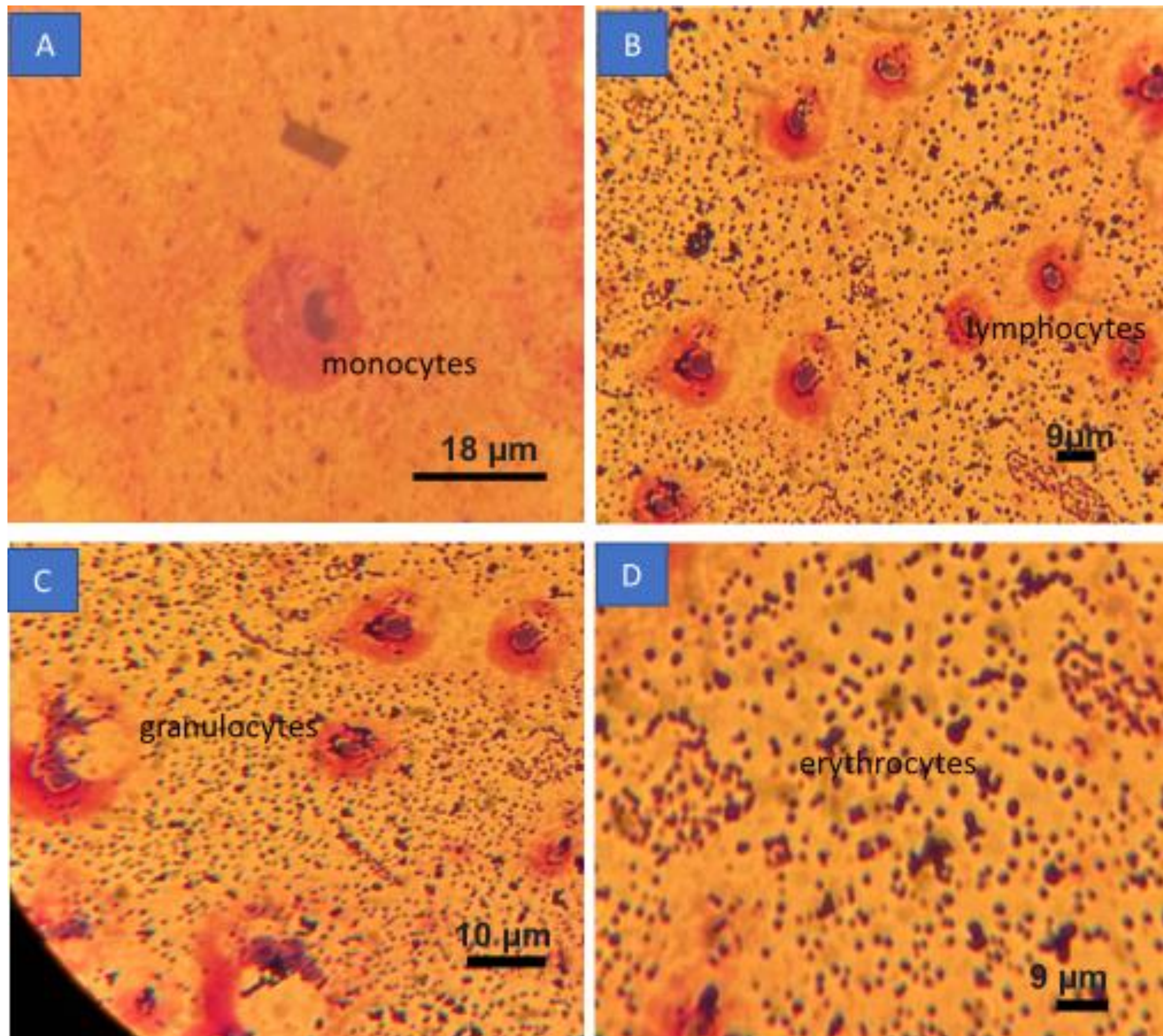


Figure 20: Microscopic observation of cells from 8th passage showing differentiation to monocytes, lymphocytes, granulocytes and erythrocytes.

4.9 MTT assay and cellular viability

The color change in the treated well of the culture flask indicates the presence of viable cells and cell metabolism. No color change in the blank well which just contains distilled water and color change in the well which contain control indicates the test is reliable.

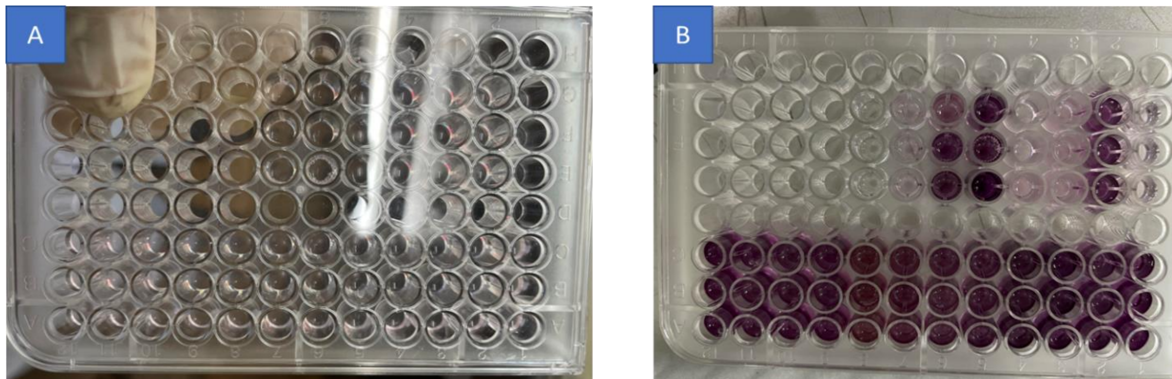


Figure 21: Cell culture in a 96 well plate culture flask before and after the treatment of MTT reagents. A) 96 well plate culture flask seeded with cells. B) The effect of MTT reagents in cell cultured well.

The microscopic observation of different well of culture flasks treated with cells and MTT reagents show higher cellular density on the strong color well and lower cellular density in light colored well. The data reading of these cells by ELISA reader shows similar results.

Cells treated with phytochemical extract have higher cell viability then the untreated control cells. The MTT assay was performed with plant extract. As negative control, the cultures were treated with Amfol plus (iron chakki) and as vehicle control, the cultures were treated with different concentrations of ethanol. The data obtained from the MTT assay on ELISA reader showed plant extract treated cells showed higher rate of cell viability in comparisons to the negative, and vehicle controls.

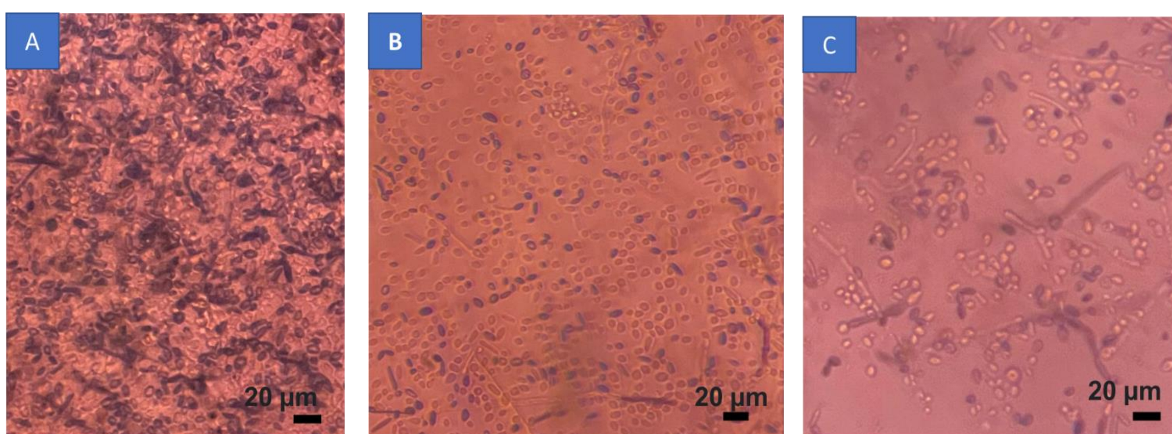


Figure 22: Microscopic observation 96 well plate cultured cells treated with A) phytochemical extract, B) Amfol plus (iron chakki), C) 70% ethanol respectively before taking reading in an ELISA reader after MTT assay.

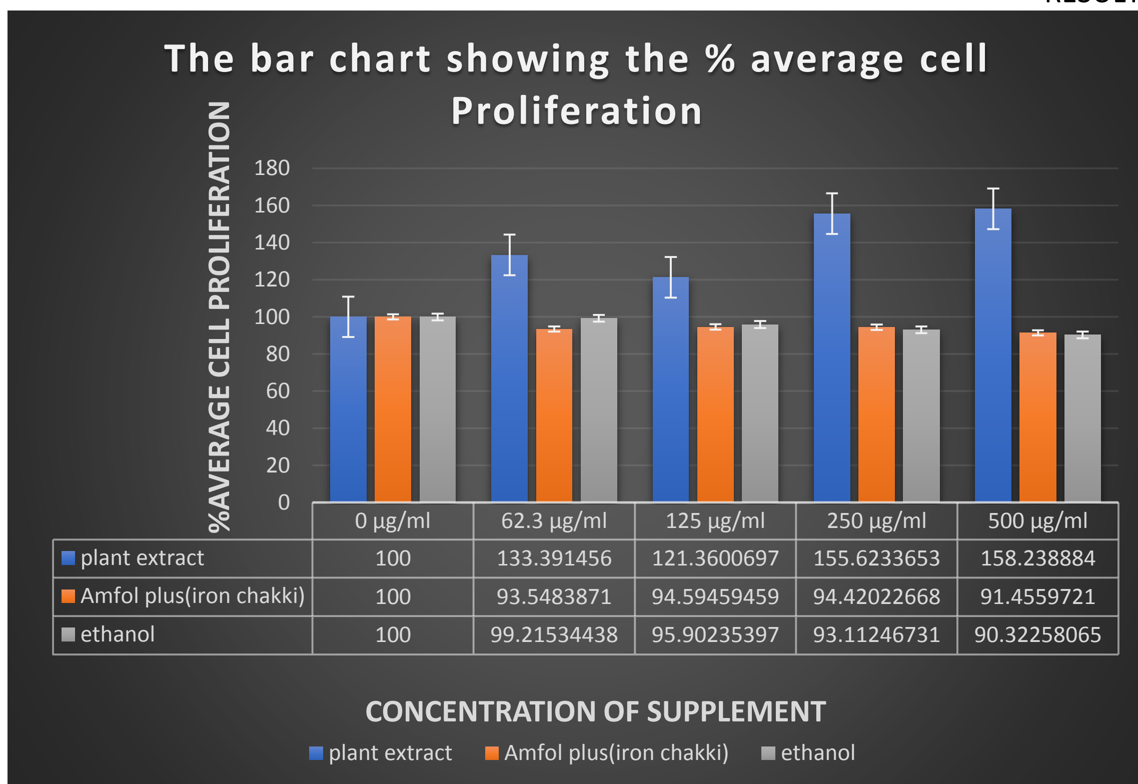


Figure 23: Cell viability by MTT assay. The columns represent mean values calculated from three repeats and bars represent standard error. The values of treatment, amfol and vehicle controls have been shown as the comparative values considering the blank control as 100%.

Table 2: One way-ANOVA analysis to compare the effect of different treatments on cells (plant extract, ethanol, amfol plus, and control).

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value	
Plant extract vs. Amfol plus	48.65	29.49 to 67.80	Yes	****	<0.0001	A-B
Plant extract vs. Ethanol	47.52	28.36 to 66.67	Yes	****	<0.0001	A-C
Plant extract vs. Control	42.15	23.00 to 61.31	Yes	***	0.0001	A-D
Amfol plus vs. Ethanol	-1.133	-20.29 to 18.02	No	ns	0.9980	B-C
Amfol plus vs. Control	-6.495	-25.65 to 12.66	No	ns	0.7486	B-D

{note: ***=P value(0.0001), ****=P value(<0.0001)}

On a one way-ANOVA test to compare the effect of cells with different treatments, the cells treated with plant extracted show significant growth in comparison to the cells treated with Amfol plus and ethanol then the control.

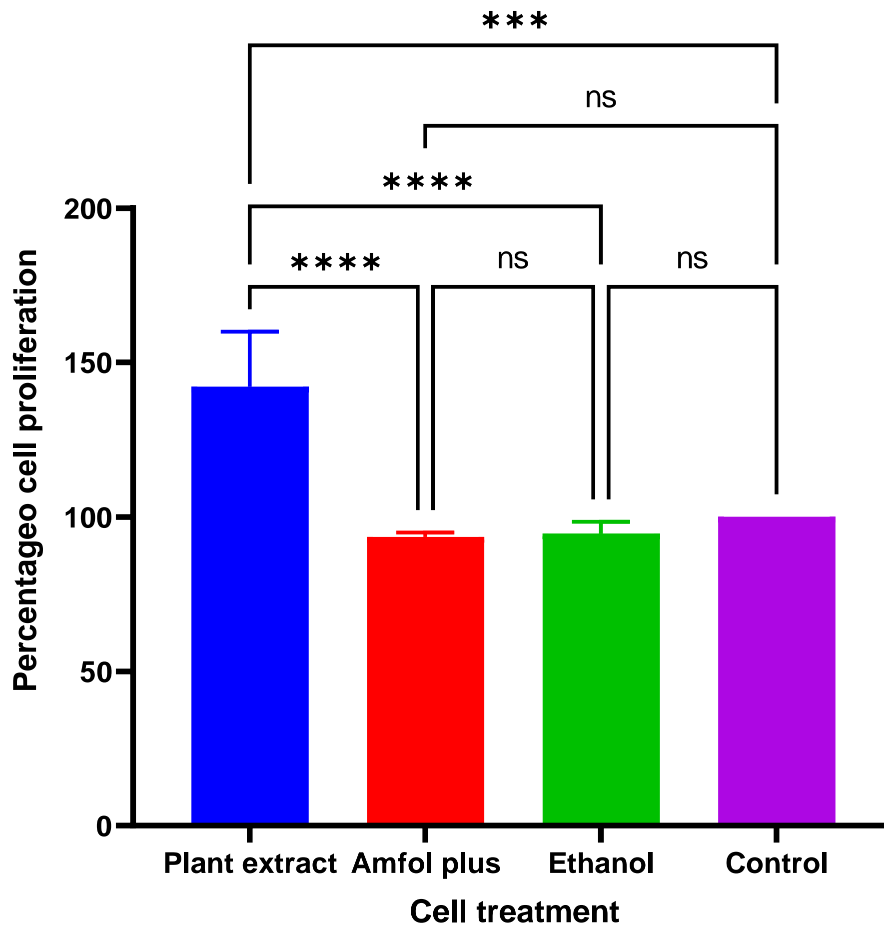


Figure 24: Bar diagram *presentation* of One way ANOVA *test with* comparison of different treatments on cells (i.e., plant extract, amfol plus, ethanol and untreated cells). *The columns represent mean values of treatment and bars represent standard deviation.*

{note: ***=P value(0.0001), ****=P value(<0.0001)}

CHAPTER 5

DISCUSSION

The recent success in the development of corona vaccines and treating HIV has shown that today's medical science has advanced a lot. But with that the pandemic of SARS-COV2 has brought new challenges ahead. There are many diseases which are still not fully understood. The indigenous therapies and ethnopharmacological uses could be of great significance. The use of stem cell therapy in combination with the phytochemicals from medicinal plants could be a new hope for the upcoming and existing medical challenges.

5.1 Verification for *Clematis buchaniana* DC

Every plant has a local name in every locality. Since the plant sample was selected on the basis of local use and its significance as ethnomedicine its scientific name had to be verified by National Herbarium and Botanical Laboratory. To verify this plant sample a Herbarium was prepared and sent to the National herbarium and botanical laboratory. Based on the morphological comparison of plant herbarium sample with the standard herberium in their depository and studying related literature, the selected plant sample was verified to be *Clematis buchaniana* DC.

5.2 Phytochemical extraction of *Clematis buchaniana* DC

The whole plant physiology is very complex. It contains lots of primary and secondary metabolites along with other structural components which may have different characteristics. Sometimes one component may mask the effect of another and the desired component may be present in very small quantities. To isolate the desired chemical compound the phytochemical extraction is a very important process (Mosić et al., 2020). For effective extraction the good choice of extraction solvent and its concentration is also very critical. The selection of extraction solvent not only plays an important role in extraction but also to the purpose of extract. Based on these features 70% ethanol for extraction holds many advantages over other solvents. 70% ethanol has a higher polarity than the absolute ethanol, as it contains water in it. The solvents with

higher polarity have a more ability to extract compounds with wider polarity, the extraction of phenolic as well as non-phenolic polar compounds such as carbohydrates and proteins could be extracted effectively. The phenolic compounds play a very important role in cell proliferation. The phenolic compound has a tendency to donate hydrogen atoms or electrons from its hydroxyl group to free radicals, because phenolics are plant produced metabolites which have one or more -OH groups in its aromatic form. These aromatic hydroxyl groups in their structure make them effective antioxidant compounds. The extraction of more phenolics compounds make the extract more antioxidant (Hikmawanti et al., 2021). Studies have shown, total antioxidant activity of phenolic compounds extracted by using ethanol solvent is more than that of methanol and acetone as solvent (Do et al., 2014). This may be because of the presence of phenolic compounds which exhibit more antioxidant properties. The use of Soxhlet apparatus involving a continuous heating process has advantages as it is a continuous extraction method that takes less time and solvent for extraction with high extraction efficiency. The high temperature and long extraction time might cause the thermal degradation so, although the boiling temperature of ethanol is 78^0C and could be heated below 78^0C but to prevent the thermal degradation of phytochemicals it was heated only up to 60^0C (Kusumawati et al., 2019; Q. W. Zhang et al., 2018). The use of rotavapor after soxhlet extraction make the extraction fast due to the continuous heating and rotation followed by the vacuum pressure

5.3 Qualitative phytochemical analysis of extract

This preliminary qualitative phytochemical analysis shows there is presence of primary and secondary metabolites. In the test, the absence of flavonoid was observed and presence of other phytochemicals compounds like phenols and tannins, proteins and amino acids, carbohydrates and glycosides, and alkaloids shows that the extraction process was successful. The presence of phenolics compounds shows this plant as a possible candidate for stimulating cell proliferation. The cell proliferation effect may be because of the phenolic compound present in it. There are more than 8000 phenolic compounds identified till now which naturally occur. The cell proliferation may be because of any of these compounds so further study of the extract by the separation of

components by HPLC and other separation methods could be done (Tungmunnithum et al., 2018).

5.4 Isolation and culture of peripheral blood mononuclear cells (PBMC) from bovine umbilical cord blood

The cell growth observed in the T20 culture flask shows the isolation of peripheral blood mononuclear cells from the blood of the newly born calf's umbilical cord vein was successfully done without any contamination. DESCRIBE WHAT CELLS YOU OBSERVED. The isolation process was effective and the culture media was effective and contamination free. DMEM low glucose with 10% FBS was used as culture media for their better performance to culture stem cells (Ayatollahi et al., 2012). The 0.2 microns syringe filter of the media was effective to prevent contamination.

5.6 Isolations of stem cells from PBMC cells

There are very less stem cells in a umbilical cord blood; around 1% (Watt et al., 2022), and rest is red blood cells (erythrocytes) and white blood cells i.e., Granulocytes (neutrophils, eosinophils, and basophils), monocytes, and lymphocytes (T and B cells, Dean, 2005). Both mature red blood cells and white blood cells do not divide. RBC don't have Nucleus and the ability to respond to the growth factors while WBC do possess their nucleus which is terminally differentiated so even they can no longer undergo mitosis (Watt et al., 2022). (https://www.frsd.k12.nj.us/cms/lib01/NJ01001104/Centricity/Domain/639/what_types_of_cells_do_not_undergo_mitosis.pdf). Due to which these cells do not divide on subculture (passaging). The cell growth in subsequent subculture indicates the presence of stem cells.

5.7 Presence of hematopoietic stem cells

The microscopic observation of cells stained with Wright stain to identify blood cells. On Wright staining, different kinds of cell structure were observed. Many cells are small spherical cells without nucleus, cells with round nucleus, cells with bilobed nucleus and kidney like nucleus. Based on the morphological structure and the nucleus the cells were

found to be similar with the blood. The study clearly showed the nucleated and enucleated cells whose morphology resembles the erythrocytes, granulocytes, monocytes and lymphocytes. The presence of these mature cells till 7-8 passaging indicates the presence of hematopoietic stem cells in the culture (Zhou et al., 2020a).

5.8 The phytochemical extract of *Clematis buchaniana* helps in cell proliferation

MTT assay was performed to detect the cell proliferation of cells on different kinds of treatment. Cells from 8th passage was taken for MTT assay. With MTT assay we found that the plant extract exerts 133.39%, 121.36%, 155.623%, 158.23% cell proliferations at the concentration of 62.3 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml respectively. This data in comparison to the positive control clearly indicates the proliferation of cells. Amfol plus (iron chakki) is a supplement capsule of vitamin B12 and folic acid which is commonly used during pregnancy and anemia. Treatment with Amol plus showed 100%, 93.54%, 94.59%, 94.42%, 91.45% . Hence cell growth is relatively lower than the blank control (untreated cells). DMEM complete media do not possess some essential supplements (folate, vitamin B12 and vitamin A) to support the erythropoiesis. So, erythropoiesis might be suppressed. The imbalance of erythroid proliferation and differentiation shows the increase in erythroblast proliferation and which couldn't produce enucleated RBCs or produce unhealthy RBC (Gupta et al., 2018; P. Zhou et al., 2020b). The use of Amfol plus supplement might treat this condition and decrease the erythropoiesis or the supplement might mask cell proliferation ability of the hematopoietic stem cells.

70 % ethanol was used for the extraction of phytochemicals. So, 70% ethanol was also used to treat the cells to observe its effect in cell proliferation (vehicle control). The data obtained from the cells treated with 70% ethanol at different concentration i.e., 0 µg/ml, 62.3 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml was found to be 100%, 99.21%, 95.90%, 93.11%, 90.32258% cell growth decreasing respectively. The cell growth was found to be decreasing with increasing concentration. This is in accordance with the characteristics of ethanol. i.e., ethanol causes inhibitory effect in cell growth in cell culture (Le et al., 2012).

In comparison to the untreated cells, the cells treated with phytochemical extract from plant *Clematis buchaniana* resulted in significantly higher cell growth with P value 0.0286 (P<0.05). The cell proliferation was also significantly higher when treated with *Clematis buchaniana* extract when compared with Amfol plus (iron chakki) treatment (P value 0.0286). The cells are treated with *Clematis buchaniana* DC. Shows significantly higher cell proliferation than the cells treated with 70% ethanol (P value 0.0286). No significant difference was observed between the use of Amfol plus and ethanol P value 0.8857 (P>0.05). The overall data analysis clearly indicates the higher cell proliferation was observed when treated with *Clematis buchaniana* DC.extract than the cell treated with Amfol plus or 70 % ethanol and untreated cells.

Amfol plus is a supplement commonly referred by the clinician during pregnancy to promote maternal health and the child's health and treat anemia. These Amfol plus contains folic acid and iron (III) Hydroxide Polymaltose complexes. Folic acids were found slightly inhibiting cell proliferation when treated to myoblast (Rednic et al., 2022). Similar results were observed when treated with isolated stem cells. Since these supplements were commonly prescribed by the doctors during menstruation and pregnancy. Proper study should be done to know about its effect on long term use.

The phytochemicals have been found to inhibit some enzymes which are associated with the development of many human diseases and disorders. Such as, inhibition of angiotensin-converting enzyme (ACE) which is associated with hypertension (Rahman et al., 2022). They also exhibit the inhibition of carbohydrate hydrolyzing enzymes responsible for type 2 diabetes (Rahman et al., 2022). The inhibition of cholinesterase is helpful to treat Alzheimer’s disease. The phenolic compound extracted from plants has been found to exhibit protective activities against oxidative stress and anti-inflammatory properties which are helpful to treat skin related diseases, rheumatoid arthritis, and inflammatory disease, anti-cancer, anti-aging, antibacterial activities (Rahman et al., 2022). Some alkaloids extracted from plant have shown to express Alkaloids express the antiplatelet, antioxidant, antinociceptive, anticancer and antibacterial effects and inhibit the cell growth by regulating various mechanism pathway of cell division (Khan et al., 2022). The main objective of stem cell therapy is to treat and heal the disease and disorders, regenerate the dysfunctional and damaged cells. Main challenge in stem cell

therapy was found to be to control cell growth. The Phyto alkaloids and phenolic compounds in proper concentration play a very important role in stem cell therapy. The combination of stem cells and plant extracts could be the solution for stem cell therapy challenges (Chan et al., 2018). The proliferative phenomenon of cells treated with the extract of *Clematis buchaniana* has shown this plant does help in cell proliferation. The further study must be carried to understand the mechanism of this proliferation and the types of cell proliferated by the treatment. The effect of plant phytochemicals in stem cell culture may help in stem cell proliferation or differentiation to tissue cells. Phytochemicals like alkaloids, phenolics, tannins, flavonoids, etc contain many types of chemical compounds in them with different characteristics. The isolation of such specific compounds could be performed by using various techniques i.e., column chromatography, HPLC.

SUMMARY

Stem cells are undifferentiated cells that can perpetually divide and differentiate into different types of cells. These cells possess high potential for regenerative therapy. Umbilical cord blood (UCB) is residual blood contained in the umbilical cord discarded after the birth of the baby. It is rich in hematopoietic stem cells and different types other mesenchymal stem cells. Sample was collected from vein of bovine umbilical cord. PBMC was isolated from the cord blood and cultured in DMEM low glucose medium. Two different types of cells were isolated, among them the floating cells were subcultured for up to 9 passages. Gram's staining, Giemsa staining, trypan blue staining and wright staining were performed to observe the cell and check contamination. Till the 9th passage very active growth of cells were observed. These dividing cells on wright staining microscopy shows different types of cell population morphologically similar to blood cells i.e., lymphocytes, erythrocytes, granulocytes, monocytes. Presence of these cells till 8th,9th passage indicate there is the source of these blood cells i.e., hematopoietic stem cells.

The ethnomedicine *Clematis buchananiana* was collected and verified from the National Herbarium and plant laboratory. Phytochemicals from *Clematis buchananiana* were extracted by heating method Using Soxhlet apparatus with a 70% ethanol solvent. The preliminary phytochemical qualitative analysis of these phytochemicals express the presence of both Primary and secondary metabolites like carbohydrates, proteins, alkaloids and phenolic compounds. The isolated cells from 9th passages were treated with the phytochemical extracted from the ethnomedicine *Clematis buchananiana* along with Amfol plus and ethanol. On treatment with phytochemicals, cell proliferation was observed while cell inhibition was observed by amfol plus and ethanol. This proliferation might be because of a phenolic compound present in it which is screened during preliminary qualitative analysis.

The data obtained from MTT assay were analyzed by using MS Excel and GraphPad software.

Conclusions

The present research has demonstrated that the umbilical cord blood of calves could be isolated and cultured in the laboratory and the MTT assay can be used to detect the cell proliferation. The umbilical cord blood collected from the umbilical vein of the newly born calf was aseptically isolated from and safely delivered to the laboratory. From that blood the PBMC isolation could be done successfully and cultured in *in vitro* medium for up to 9 passages. In control cultures, peripheral and lymphocytic differentiation was observed in late passages. Umbilical blood stem cells incubated with phytochemical extract showed a higher rate of stem cell proliferation. Thus it was concluded that the extract of ethnomedicinal plant *Clematis buchaniana* DC contains putative principle (possibly phenolics) which helps in mesenchymal stem cell proliferation.

REFERENCES

Abubakar, A. R., & Haque, M. (2020a). Preparation of Medicinal Plants: Basic Extraction and Fractionation Procedures for Experimental Purposes. *Journal of Pharmacy & Bioallied Sciences*, *12*(1), 1. https://doi.org/10.4103/JPBS.JPBS_175_19

Abubakar, A. R., & Haque, M. (2020b). Preparation of Medicinal Plants: Basic Extraction and Fractionation Procedures for Experimental Purposes. *Journal of Pharmacy & Bioallied Sciences*, *12*(1), 1. https://doi.org/10.4103/JPBS.JPBS_175_19

Adhikari, M., Thapa, R., Kunwar, R. M., Devkota, H. P., & Poudel, P. (2019). Ethnomedicinal Uses of Plant Resources in the Machhapuchchhre Rural Municipality of Kaski District, Nepal. *Medicines*, *6*(2), 69. https://doi.org/10.3390/MEDICINES6020069

Ambu, G., Chaudhary, R. P., Mariotti, M., & Cornara, L. (2020). Traditional Uses of Medicinal Plants by Ethnic People in the Kavrepalanchok District, Central Nepal. *Plants 2020, Vol. 9, Page 759*, *9*(6), 759. https://doi.org/10.3390/PLANTS9060759

Arango, M.-T., Quintero-Ronderos, P., Castiblanco, J., & Montoya-Ortíz, G. (2013). *Cell culture and cell analysis*. https://www.ncbi.nlm.nih.gov/books/NBK459464/

Asch, R., Simerly, C., Ord, T., Ord, V. A., & Schatten, G. (1995). Fertilization and development: The stages at which human fertilization arrests: microtubule and chromosome configurations in inseminated oocytes which failed to complete fertilization and development in humans. *Human Reproduction*, *10*(7), 1897–1906. https://doi.org/10.1093/oxfordjournals.humrep.a136204

Ayatollahi, M., Salmani, M. K., Geramizadeh, B., Tabei, S. Z., Soleimani, M., & Sanati, M. H. (2012). Conditions to improve expansion of human mesenchymal stem cells based on rat samples. *World Journal of Stem Cells*, *4*(1), 1. https://doi.org/10.4252/WJSC.V4.I1.1

Berebichez-Fridman, R., & Montero-Olvera, P. R. (2018). Sources and Clinical Applications of Mesenchymal Stem Cells: State-of-the-art review. *Sultan Qaboos University Medical Journal*, *18*(3), e264. https://doi.org/10.18295/SQUMJ.2018.18.03.002

Bhat, J. A., Kumar, M., & Bussmann, R. W. (2013). Ecological status and traditional knowledge of medicinal plants in Kedarnath Wildlife Sanctuary of Garhwal Himalaya, India. *Journal of Ethnobiology and Ethnomedicine*, *9*(1), 1. https://doi.org/10.1186/1746-4269-9-1

Chan, M. M., Chen, R., & Fong, D. (2018). Targeting cancer stem cells with dietary phytochemical - Repositioned drug combinations. *Cancer Letters*, *433*, 53. https://doi.org/10.1016/J.CANLET.2018.06.034

Chang, Y. J., Tseng, C. P., Hsu, L. F., Hsieh, T. B., & Hwang, S. M. (2006). Characterization of two populations of mesenchymal progenitor cells in umbilical cord blood. *Cell Biology International*, *30*(6), 495–499. https://doi.org/10.1016/J.CELLBI.2005.12.009

Chawla, R., Kumar, S., & Sharma, A. (2012). The genus Clematis (Ranunculaceae): Chemical and pharmacological perspectives. *Journal of Ethnopharmacology*, *143*(1), 116–150. https://doi.org/10.1016/J.JEP.2012.06.014

Chivu-Economescu, M., & Rubach, M. (2017). Hematopoietic Stem Cells Therapies. *Current Stem Cell Research & Therapy*, *12*(2), 124–133. https://doi.org/10.2174/1574888X10666151026114241

Clouthier, S. (n.d.). *Complete Media Preparation*.

Counting cells using a hemocytometer | Abcam. (n.d.). Retrieved December 14, 2022, from https://www.abcam.com/protocols/counting-cells-using-a-haemocytometer

Covas, D. T., Siufi, J. L. C., Silva, A. R. L., & Orellana, M. D. (2003). Isolation and culture of umbilical vein mesenchymal stem cells. *Brazilian Journal of Medical and Biological Research*, *36*(9), 1179–1183. https://doi.org/10.1590/S0100-879X2003000900006

Dean, L. (2005). *Blood and the cells it contains*. https://www.ncbi.nlm.nih.gov/books/NBK2263/

Divya, M. S., Roshin, G. E., Divya, T. S., Rasheed, V. A. bdul, Santhoshkumar, T. R., Elizabeth, K. E., James, J., & Pillai, R. M. (2012). Umbilical cord blood-derived mesenchymal stem cells consist of a unique population of progenitors co-expressing mesenchymal stem cell and neuronal markers capable of instantaneous neuronal differentiation. *Stem Cell Research & Therapy*, *3*(6), 57. https://doi.org/10.1186/SCRT148

Do, Q. D., Angkawijaya, A. E., Tran-Nguyen, P. L., Huynh, L. H., Soetaredjo, F. E., Ismadji, S., & Ju, Y. H. (2014). Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of Limnophila aromatica. *Journal of Food and Drug Analysis*, *22*(3), 296. https://doi.org/10.1016/J.JFDA.2013.11.001

E Donnall Thomas (1920–2012). (2012). *Bone Marrow Transplantation 2012 48:1*, *48*(1), 1–1. https://doi.org/10.1038/bmt.2012.254

Ekblad-Nordberg, Å., Walther-Jallow, L., Westgren, M., & Götherström, C. (2020). Prenatal stem cell therapy for inherited diseases: Past, present, and future treatment strategies. *STEM CELLS Translational Medicine*, *9*(2), 148–157. https://doi.org/10.1002/SCTM.19-0107

Ghasemi, M., Turnbull, T., Sebastian, S., & Kempson, I. (2021). The mtt assay: Utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis. *International Journal of Molecular Sciences*, *22*(23). https://doi.org/10.3390/IJMS222312827/S1

Ghimire, B., Park, B. K., Son, D. C., & Oh, S. H. (2020). Achene Morphology and Anatomy of Clematis L. (Ranunculaceae) in Korea and Its Taxonomic Implications. *Plants*, *9*(10), 1–27. https://doi.org/10.3390/PLANTS9101279

Global Cord Blood Industry Database, 2021. (n.d.). Retrieved December 13, 2022, from https://bioinformant.com/product/global-cord-blood-industry-database/

Goldstein, G., Toren, A., & Nagler, A. (2007). Transplantation and Other Uses of Human Umbilical Cord Blood and Stem Cells. *Current Pharmaceutical Design*, *13*(13), 1363–1373. https://doi.org/10.2174/138161207780618759

Gram Stain Protocols. (2019). www.asmscience.org

Gupta, R., Musallam, K. M., Taher, A. T., & Rivella, S. (2018). Ineffective Erythropoiesis, Anemia and Iron Overload. *Hematology/Oncology Clinics of North America*, *32*(2), 213. https://doi.org/10.1016/J.HOC.2017.11.009

Hao, D. C., Gu, X. J., & Xiao, P. G. (2015). Medicinal Plants: Chemistry, Biology and Omics. *Medicinal Plants: Chemistry, Biology and Omics*, 1–681. https://doi.org/10.1016/C2014-0-01090-8

Hemocytometer protocol • Hemocytometer. (n.d.). Retrieved December 14, 2022, from https://www.hemocytometer.org/hemocytometer-protocol/

Hill, A. B. T., Bressan, F. F., Murphy, B. D., & Garcia, J. M. (2019). Applications of mesenchymal stem cell technology in bovine species. In *Stem Cell Research and Therapy* (Vol. 10, Issue 1, p. 44). BioMed Central Ltd. https://doi.org/10.1186/s13287-019-1145-9

Huang, Y., Xiao, D., Burton-Freeman, B. M., & Edirisinghe, I. (2016). Chemical Changes of Bioactive Phytochemicals during Thermal Processing. *Reference Module in Food Science*. https://doi.org/10.1016/B978-0-08-100596-5.03055-9

Hutton, J. F., Gargett, T., Sadlon, T. J., Bresatz, S., Brown, C. Y., Zola, H., Shannon, M. F., D’Andrea, R. J., & Barry, S. C. (2009). Development of CD4+CD25+FoxP3+ regulatory T cells from cord blood hematopoietic progenitor cells. *Journal of Leukocyte Biology*, *85*(3), 445–451. https://doi.org/10.1189/JLB.1008620

IOP Conference Series: Earth and Environmental Science The Effect of Ethanol Concentrations as The Extraction Solvent on Antioxidant Activity of Katuk (Sauropus

androgynus (L.) Merr.) Leaves Extracts. (n.d.). https://doi.org/10.1088/1755-1315/755/1/012060

Ito, K., & Suda, T. (2014). Metabolic requirements for the maintenance of self-renewing stem cells. *Nature Reviews Molecular Cell Biology 2014 15:4*, *15*(4), 243–256. https://doi.org/10.1038/nrm3772

Jawad Javed, M., Mead, L. E., Prater, D., Bessler, W. K., Foster, D., Case, J., Scott Goebel, W., Yoder, M. C., Haneline, L. S., & Ingram, D. A. (2008). *Endothelial Colony Forming Cells and Mesenchymal Stem Cells are Enriched at Different Gestational Ages in Human Umbilical Cord Blood*.

Jedrzejczak-Silicka, M., & Jedrzejczak-Silicka, M. (2017). History of Cell Culture. *New Insights into Cell Culture Technology*. https://doi.org/10.5772/66905

Khan, H., Alam, W., Alsharif, K. F., Aschner, M., Pervez, S., & Saso, L. (2022). Alkaloids and Colon Cancer: Molecular Mechanisms and Therapeutic Implications for Cell Cycle Arrest. *Molecules 2022, Vol. 27, Page 920*, *27*(3), 920. https://doi.org/10.3390/MOLECULES27030920

Knudtzon, S. (1974). In Vitro Growth of Granulocytic Colonies From Circulating Cells in Human Cord Blood. *Blood*, *43*(3), 357–361. https://doi.org/10.1182/BLOOD.V43.3.357.357

Koffi, Dodehe, T., & Soro S. (2010). Effect of solvent type on extraction of polyphenols from twenty three Ivorian plants. *Journal of Animal & Plant Sciences*, *5*, 550–558. http://www.biosciences.elewa.org/JAPS;ISSN2071-7024

Kondo, M. (2010). Lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. *Immunological Reviews*, *238*(1), 37. https://doi.org/10.1111/J.1600-065X.2010.00963.X

Kornicka, K., Kocherova, I., & Marycz, K. (2017). The effects of chosen plant extracts and compounds on mesenchymal stem cells—a bridge between molecular nutrition and

regenerative medicine- concise review. *Phytotherapy Research*, *31*(7), 947–958. https://doi.org/10.1002/PTR.5812

Kunwar, R. M., Baral, B., Luintel, S., Uprety, Y., Poudel, R. C., Adhikari, B., Adhikari, Y. P., Subedi, S. C., Subedi, C. K., Poudel, P., Paudel, H. R., Paudel, B., Kunwar, L. M., Upadhayaya, K. S., Bhattarai, S., Pyakurel, D., Kutal, D. H., Pandey, P., Bhandari, A., … Bussmann, R. W. (2022). Ethnomedicinal landscape: distribution of used medicinal plant species in Nepal. *Journal of Ethnobiology and Ethnomedicine*, *18*(1), 1–11. https://doi.org/10.1186/S13002-022-00531-X/FIGURES/4

Kusindarta, D. L., & Wihadmadyatami, H. (2021). Conditioned medium derived from bovine umbilical mesenchymal stem cells as an alternative source of cell-free therapy. *Veterinary World*, *14*(10), 2588. https://doi.org/10.14202/VETWORLD.2021.2588-2595

Kusumawati, N., Bahar, A., & Sianita Maria, M. (n.d.). *Effect of Extraction Time on Averrhoa bilimbi Leaf Ethanolic Extracts Using Soxhlet Apparatus You may also like Impact of Curing and Extraction Time on Yield and Quality of Base Gelatin from Goat Skin*. https://doi.org/10.1088/1757-899X/543/1/012018

Le, T. do, Do, T. A. T., Yu, R., & Yoo, H. (2012). Ethanol Elicits Inhibitory Effect on the Growth and Proliferation of Tongue Carcinoma Cells by Inducing Cell Cycle Arrest. *The Korean Journal of Physiology & Pharmacology : Official Journal of the Korean Physiological Society and the Korean Society of Pharmacology*, *16*(3), 153. https://doi.org/10.4196/KJPP.2012.16.3.153

Lee, Y. H. (2010). Implication of Cord Blood for Cell-Based Therapy in Refractory Childhood Diseases. *International Journal of Stem Cells*, *3*(1), 22. https://doi.org/10.15283/IJSC.2010.3.1.22

Mak, T. W. (2018). Ernest Armstrong McCulloch. 21 April 1926—20 January 2011. *Biographical Memoirs of Fellows of the Royal Society*, *64*, 317–339. https://doi.org/10.1098/RSBM.2017.0019

Mantri, S., & Mohapatra, P. C. (2014). In-vitro Behavior of Human Umbilical Cord Blood Stem Cells Towards Serum Based Minimal Cytokine Growth Conditions. *Indian Journal of Clinical Biochemistry*, *29*(3), 279. https://doi.org/10.1007/S12291-013-0346-8

Mantri, S., Praksh, •, & Mohapatra, C. (n.d.). *In-vitro Behavior of Human Umbilical Cord Blood Stem Cells Towards Serum Based Minimal Cytokine Growth Conditions*. https://doi.org/10.1007/s12291-013-0346-8

Marley, G., Tan, R. K. J., & Tang, W. (2022). Stem cell research finds possible HIV cure with cord blood transplant. *The Innovation*, *3*(3), 100238. https://doi.org/10.1016/J.XINN.2022.100238

McNiece, I., Kubegov, D., Kerzic, P., Shpall, E. J., & Gross, S. (2000). Increased expansion and differentiation of cord blood products using a two-step expansion culture. *Experimental Hematology*, *28*(10), 1181–1186. https://doi.org/10.1016/S0301-472X(00)00520-8

Mendoza, N., Silva, E. M. E., Mendoza, N., & Silva, E. M. E. (2018). Introduction to Phytochemicals: Secondary Metabolites from Plants with Active Principles for Pharmacological Importance. *Phytochemicals - Source of Antioxidants and Role in Disease Prevention*. https://doi.org/10.5772/INTECHOPEN.78226

Metheny, L., Caimi, P., & de Lima, M. (2013). Cord Blood Transplantation: Can We Make it Better? *Frontiers in Oncology*, *3*. https://doi.org/10.3389/FONC.2013.00238

Mosić, M., Dramićanin, A., Ristivojević, P., & Milojković-Opsenica, D. (2020). Extraction as a Critical Step in Phytochemical Analysis. *Journal of AOAC INTERNATIONAL*, *103*(2), 365–372. https://doi.org/10.5740/JAOACINT.19-0251

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*(1–2), 55–63. https://doi.org/10.1016/0022-1759(83)90303-4

MTT assay protocol | Abcam. (n.d.). Retrieved December 15, 2022, from https://www.abcam.com/kits/mtt-assay-protocol#reagent-preparation

Murata, M., Kawabe, K., Hatta, T., Maeda, S., & Fujita, M. (2022). Current status of umbilical cord blood storage and provision to private biobanks by institutions handling childbirth in Japan. *BMC Medical Ethics*, *23*(1), 1–7. https://doi.org/10.1186/S12910-022-00830-8/TABLES/2

New Research: Placental MSCs are Effective in the Expansion of Cord Blood HSCs | Americord®. (n.d.). Retrieved December 13, 2022, from https://www.americordblood.com/new-research-placental-mscs-effective-in-the-expansion-of-cord-blood-hscs/

Ogliari, K. S., Marinowic, D., Brum, D. E., & Loth, F. (2014). Stem cells in dermatology. In *Anais Brasileiros de Dermatologia* (Vol. 89, Issue 2, pp. 286–291). Sociedade Brasileira de Dermatologia. https://doi.org/10.1590/abd1806-4841.20142530

Olatunbosun, L. O., Caxton-Martin, A., Jimoh, O. R., Biliaminu, S. A., & Ghazal, O. K. (2012). Proliferative Effect of Aqueous Extracts ofParquetina Nigrescens on HaemopoieticMultipotent Stem Cells in Irradiated Guinea Pigs. *Journal of Applied Pharmaceutical Science*, *:*(Issue), 108–111. https://doi.org/10.7324/JAPS.2012.2608

Osibote, E., Noah, N., Sadik, O., McGee, D., & Ogunlesi, M. (2011). Electrochemical sensors, MTT and immunofluorescence assays for monitoring the proliferation effects of cissus populnea extracts on Sertoli cells. *Reproductive Biology and Endocrinology*, *9*(1), 1–14. https://doi.org/10.1186/1477-7827-9-65/TABLES/1

Pandey, A., & Tripathi, S. (2014). Concept of standardization, extraction and pre phytochemical screening strategies for herbal drug. ~ *115* ~ *Journal of Pharmacognosy and Phytochemistry*, *2*(5), 115–119.

Pang, M., Han, S., Ren, M., Guan, D., Kuang, T., Liu, S., Liu, Y., Chen, J., Wang, F., Shi, C., & Ye, Z. (2021). Isolation and Ex Vivo Expansion of Human Hematopoietic Stem Cells

Derived from Umbilical Cord Blood 2 Stem Cell Discovery. *Stem Cell Discovery*, *11*, 1–13. https://doi.org/10.4236/scd.2021.111001

Pant, D. R., Pant, N. D., Saru, D. B., Yadav, U. N., & Khanal, D. P. (2017). Phytochemical screening and study of antioxidant, antimicrobial, antidiabetic, anti-inflammatory and analgesic activities of extracts from stem wood of Pterocarpus marsupium Roxburgh. *Journal of Intercultural Ethnopharmacology*, *6*(2), 170. https://doi.org/10.5455/JICE.20170403094055

Qualitative tests for preliminary phytochemical screening: An overview Junaid R Shaikh and MK Patil. (2020). https://doi.org/10.22271/chemi.2020.v8.i2i.8834

Rabizadeh, F., Mirian, M. S., Doosti, R., Kiani-Anbouhi, R., & Eftekhari, E. (2022). Phytochemical Classification of Medicinal Plants Used in the Treatment of Kidney Disease Based on Traditional Persian Medicine. *Evidence-Based Complementary and Alternative Medicine : ECAM*, *2022*. https://doi.org/10.1155/2022/8022599

Rahman, M. M., Rahaman, M. S., Islam, M. R., Rahman, F., Mithi, F. M., Alqahtani, T., Almikhlafi, M. A., Alghamdi, S. Q., Alruwaili, A. S., Hossain, M. S., Ahmed, M., Das, R., Emran, T. bin, & Uddin, M. S. (2022). Role of Phenolic Compounds in Human Disease: Current Knowledge and Future Prospects. *Molecules*, *27*(1), 233. https://doi.org/10.3390/MOLECULES27010233

Redfern, J., Kinninmonth, M., Burdass, D., & Verran, J. (2014a). Using Soxhlet Ethanol Extraction to Produce and Test Plant Material (Essential Oils) for Their Antimicrobial Properties. *Journal of Microbiology & Biology Education*, *15*(1), 45. https://doi.org/10.1128/JMBE.V15I1.656

Redfern, J., Kinninmonth, M., Burdass, D., & Verran, J. (2014b). Using Soxhlet Ethanol Extraction to Produce and Test Plant Material (Essential Oils) for Their Antimicrobial Properties. *Journal of Microbiology & Biology Education*, *15*(1), 45. https://doi.org/10.1128/JMBE.V15I1.656

Rocha, V., Labopin, M., Sanz, G., Arcese, W., Schwerdtfeger, R., Bosi, A., Jacobsen, N., Ruutu, T., de Lima, M., Finke, J., Frassoni, F., Gluckman, E., Hôpital Saint-Louis, F. V., saint Antoine, H., & Vergata, T. (2004). *Transplants of Umbilical-Cord Blood or Bone Marrow from Unrelated Donors in Adults with Acute Leukemia* (Vol. 22). www.nejm.org

Saha, J., & Chattopadhyay, S. (2011). A survey of ethnomedicinal plants of Darjeeling hills for their antimicrobial and antioxidant activities. *Indian Journal of Natural Products and Resources*, *2*(4), 479–492.

Saud, B., Malla, R., & Shrestha, K. (2019). A Review on the Effect of Plant Extract on Mesenchymal Stem Cell Proliferation and Differentiation. *Stem Cells International*, *2019*. https://doi.org/10.1155/2019/7513404

Saudemont, A., & Madrigal, J. A. (2017). Immunotherapy after hematopoietic stem cell transplantation using umbilical cord blood-derived products. *Cancer Immunology, Immunotherapy*, *66*(2), 215–221. https://doi.org/10.1007/S00262-016-1852-3

Saxena, M., Saxena, J., Nema, R., Singh, D., & Gupta, A. (2013). Phytochemistry of Medicinal Plants. *Journal of Pharmacognosy and Phytochemistry*, *1*(6). www.phytojournal.comwww.phytojournal.com

Segeritz, C. P., & Vallier, L. (2017). Cell Culture: Growing Cells as Model Systems In Vitro. *Basic Science Methods for Clinical Researchers*, 151. https://doi.org/10.1016/B978-0-12-803077-6.00009-6

Seydoux, G., & Braun, R. E. (2006). Pathway to Totipotency: Lessons from Germ Cells. In *Cell* (Vol. 127, Issue 5, pp. 891–904). Elsevier. https://doi.org/10.1016/j.cell.2006.11.016

Shaban, S., El-Husseny, M. W. A., Abushouk, A. I., Salem, A. M. A., Mamdouh, M., & Abdel-Daim, M. M. (2017). Effects of Antioxidant Supplements on the Survival and Differentiation of Stem Cells. *Oxidative Medicine and Cellular Longevity*, *2017*. https://doi.org/10.1155/2017/5032102

Si, J.-W., Wang, X.-D., & Shen, S. G. (2015). Perinatal stem cells: A promising cell resource for tissue engineering of craniofacial bone. *World Journal of Stem Cells*, *7*(1), 149. https://doi.org/10.4252/WJSC.V7.I1.149

Subba, B., Thapa, S., & Sapkota Armila Rajbhandari Assoc Gopi Chandra Kaphle, K. (2018). ANALYSIS OF PHYTOCONSTITUENTS AND BIOLOGICAL ACTIVITIES ON THE SELECTED MEDICINAL PLANTS OF DOLAKHA AND SINDHUALCHOWK DISTRICT OF NEPAL. *JIST*, *2*(2), 2467–9240.

Talib, S., & Shepard, K. A. (2020). Unleashing the cure: Overcoming persistent obstacles in the translation and expanded use of hematopoietic stem cell-based therapies. *STEM CELLS Translational Medicine*, *9*(4), 420–426. https://doi.org/10.1002/SCTM.19-0375

Thakur, M., Singh, K., & Khedkar, R. (2020). Phytochemicals: Extraction process, safety assessment, toxicological evaluations, and regulatory issues. *Functional and Preservative Properties of Phytochemicals*, 341–361. https://doi.org/10.1016/B978-0-12-818593-3.00011-7

Tran, S. L., Puhar, A., Ngo-Camus, M., & Ramarao, N. (2011). Trypan Blue Dye Enters Viable Cells Incubated with the Pore-Forming Toxin HlyII of Bacillus cereus. *PLoS ONE*, *6*(9). https://doi.org/10.1371/JOURNAL.PONE.0022876

Tungmunnithum, D., Thongboonyou, A., Pholboon, A., & Yangsabai, A. (2018). Flavonoids and Other Phenolic Compounds from Medicinal Plants for Pharmaceutical and Medical Aspects: An Overview. *Medicines*, *5*(3), 93. https://doi.org/10.3390/MEDICINES5030093

Verma, A., Verma, M., & Singh, A. (2020). Animal tissue culture principles and applications. *Animal Biotechnology*, 269. https://doi.org/10.1016/B978-0-12-811710-1.00012-4

Watt, A. P., Kirkland, M., Nekkanti, L., Pham, Y., McDonald, C., Malhotra, A., Moeneclaey, G., Miller, S. L., & Jenkin, G. (2022). Effect of expansion of human umbilical cord blood CD34 + cells on neurotrophic and angiogenic factor expression and function. *Cell and*

Tissue Research, *388*(1), 117–132. https://doi.org/10.1007/S00441-022-03592-2/FIGURES/5

Weiss, M. L., & Troyer, D. L. (2006). Stem cells in the umbilical cord. *Stem Cell Reviews*, *2*(2), 155–162. https://doi.org/10.1007/s12015-006-0022-y

Wright Stain Principle, Procedure, Result. (n.d.). Retrieved December 14, 2022, from https://microbiologynote.com/wright-stain-principle-procedure-result/

Xiao, J., & Bai, W. (2019). *Bioactive phytochemicals*. https://doi.org/10.1080/10408398.2019.1601848

Yu, Y. bin, Song, Y., Chen, Y., Zhang, F., & Qi, F. Z. (2018). Differentiation of umbilical cord mesenchymal stem cells into hepatocytes in comparison with bone marrow mesenchymal stem cells. *Molecular Medicine Reports*, *18*(2), 2009–2016. https://doi.org/10.3892/mmr.2018.9181

Zakrzewski, W., Dobrzyński, M., Szymonowicz, M., & Rybak, Z. (2019). Stem cells: Past, present, and future. *Stem Cell Research and Therapy*, *10*(1), 1–22. https://doi.org/10.1186/S13287-019-1165-5/FIGURES/8

Zhang, Q. W., Lin, L. G., & Ye, W. C. (2018). Techniques for extraction and isolation of natural products: a comprehensive review. *Chinese Medicine*, *13*(1), 20. https://doi.org/10.1186/S13020-018-0177-X

Zhang, S., Lam, K. K. H., Wan, J. H., Yip, C. W., Liu, H. K. H., Lau, Q. M. N., Man, A. H. Y., Cheung, C. H., Wong, L. H., Chen, H. B., Shi, J., Leung, G. P. H., Lee, C. K. F., Shi, Y. G., Tang, S. C. W., & Zhang, K. Y. B. (2020a). Dietary phytochemical approaches to stem cell regulation. *Journal of Functional Foods*, *66*, 103822. https://doi.org/10.1016/J.JFF.2020.103822

Zhang, S., Lam, K. K. H., Wan, J. H., Yip, C. W., Liu, H. K. H., Lau, Q. M. N., Man, A. H. Y., Cheung, C. H., Wong, L. H., Chen, H. B., Shi, J., Leung, G. P. H., Lee, C. K. F., Shi, Y. G., Tang, S. C. W., & Zhang, K. Y. B. (2020b). Dietary phytochemical approaches to stem

cell regulation. *Journal of Functional Foods*, *66*, 103822. https://doi.org/10.1016/J.JFF.2020.103822

Zhou, L., Mcdonald, C., Yawno, T., Jenkin, G., Miller, S., & Malhotra, A. (n.d.). *Umbilical Cord Blood and Cord Tissue-Derived Cell Therapies for Neonatal Morbidities: Current Status and Future Challenges*. https://doi.org/10.1093/stcltm/szab024

Zhou, P., Ouchari, M., Xue, Y., & Yin, Q. (2020a). In Vitro Generation of Red Blood Cells from Stem Cell and Targeted Therapy. *Cell Transplantation*, *29*. https://doi.org/10.1177/0963689720946658

Zhou, P., Ouchari, M., Xue, Y., & Yin, Q. (2020b). In Vitro Generation of Red Blood Cells from Stem Cell and Targeted Therapy. *Cell Transplantation*, *29*. https://doi.org/10.1177/0963689720946658

APPENICES

1. Data obtained from MTT assay analyzed by using MS Excel

	plant extract				ironchakki				ethanol				Blank	cell+mtt	cell only
conc(microgram)	62.3 µg	125 µg	250 µg	500 µg	62.3 µg	125 µg	250 µg	500 µg	62.3 µg	125 µg	250 µg	500 µg			
Rep1	0.629	0.477	0.54	0.629	0.408	0.385	0.349	0.375	0.379	0.389	0.35	0.38	0.05	0.405	0.215
Rep2	0.448	0.465	0.629	0.573	0.315	0.333	0.399	0.355	0.352	0.336	0.34	0.34	0.05	0.372	0.212
Rep3	0.453	0.45	0.616	0.613	0.35	0.367	0.335	0.319	0.407	0.375	0.37	0.32	0.05	0.37	0.19
average	0.51	0.464	0.595	0.605	0.358	0.362	0.361	0.35	0.3793	0.367	0.36	0.35	0.05	0.3823	0.2057
s.d	0.1031	0.014	0.048	0.029	0.047	0.026	0.034	0.028	0.0275	0.027	0.02	0.03	0	0.0197	0.0137
c.v	20.213	2.915	8.079	4.768	13.13	7.301	9.32	8.116	7.25	7.49	4.6	8.15	1.92	5.1409	6.6371
average cell viabilit	133.39	121.4	155.6	158.2	93.55	94.59	94.42	91.46	99.215	95.9	93.1	90.3	13.6	100	53.793

2. Cell viability percentage data generated from MS Excel analyzed Graph Pad to find P-value

Group A	Group B	Group C	Group D
plant extract	Amfol plus	etanol	pc
133.391456	93.54839	99.21534	100
121.3600697	94.59459	95.90235	100
155.6233653	94.42023	93.11247	100
158.238884	91.45597	90.32258	100

i. *Clematis buchananiana vs* Positive control

Table Analyzed	Data 1
Column D	Pc
vs.	vs.
Column A	plant extract
Mann Whitney test	
P value	0.0286
Exact or approximate P value?	Exact
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,D	26 , 10
Mann-Whitney U	0
Difference between medians	
Median of column A	144.5, n=4
Median of column D	100.0, n=4
Difference: Actual	-44.51
Difference: Hodges-Lehmann	-44.51

ii. Ethanol vs plant extract

Column C	etanol
vs.	vs.
Column A	plant extract
Mann Whitney test	
P value	0.0286
Exact or approximate P value?	Exact
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,C	26 , 10
Mann-Whitney U	0
Difference between medians	
Median of column A	144.5, n=4
Median of column C	94.51, n=4
Difference: Actual	-50.00
Difference: Hodges-Lehmann	-49.74

iii. Amfol plus vs positive control

Table Analyzed	Data 1
Column D	pc
vs.	vs.
Column B	amfol plus
Mann Whitney test	
P value	0.0286
Exact or approximate P value?	Exact
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column B,D	10 , 26
Mann-Whitney U	0
Difference between medians	
Median of column B	93.98, n=4
Median of column D	100.0, n=4
Difference: Actual	6.016
Difference: Hodges-Lehmann	6.016

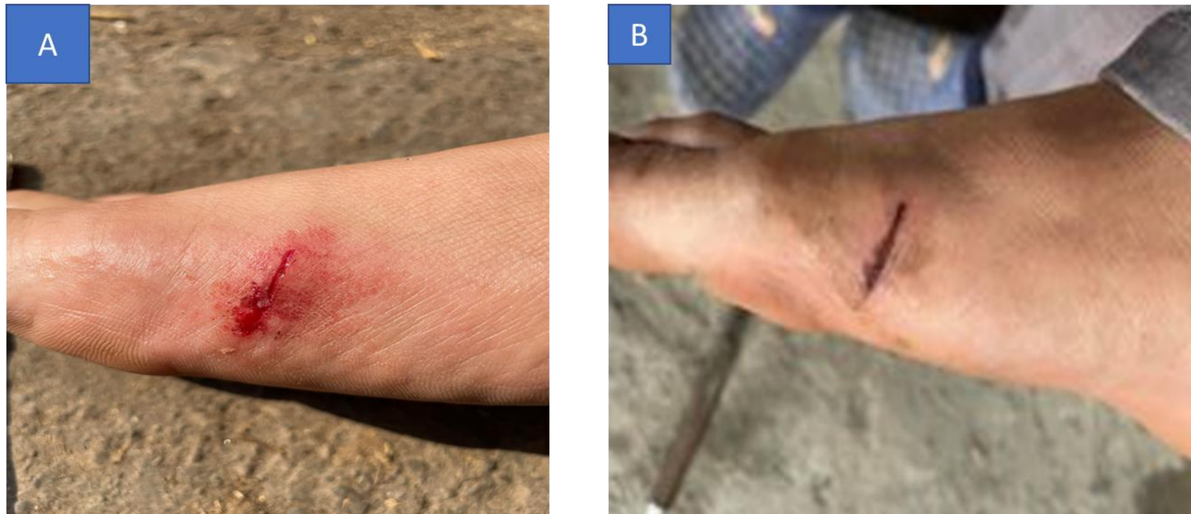
iv. Ethanol vs Pocitive control

Table Analyzed	Data 1
Column D	pc
vs.	vs.
Column C	etanol
Mann Whitney test	
P value	0.0286
Exact or approximate P value?	Exact
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column C,D	10 , 26
Mann-Whitney U	0
Difference between medians	
Median of column C	94.51, n=4
Median of column D	100.0, n=4
Difference: Actual	5.493
Difference: Hodges-Lehmann	5.493

v. Plant extract vs Amfol plus

Table Analyzed	Data 1
Column B	amfol plus
vs.	vs.
Column A	plant extract
Mann Whitney test	
P value	0.0286
Exact or approximate P value?	Exact
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	26 , 10
Mann-Whitney U	0
Difference between medians	
Median of column A	144.5, n=4
Median of column B	93.98, n=4
Difference: Actual	-50.52
Difference: Hodges-Lehmann	-51.48

3. The results of using crude extract of *Clematis buchananiana* after 24 hrs in wound



3 How scales in microscopic image were labeled? Why the magnification is not uniform?

The microscopic image was labeled on the basis of size compared to the size of hemocytometer chamber and cell size. The magnification of microscopic images were not uniform because they were magnified for better visualization of cell structure. The scales were placed in fig to understand and compare the cell size and magnification.

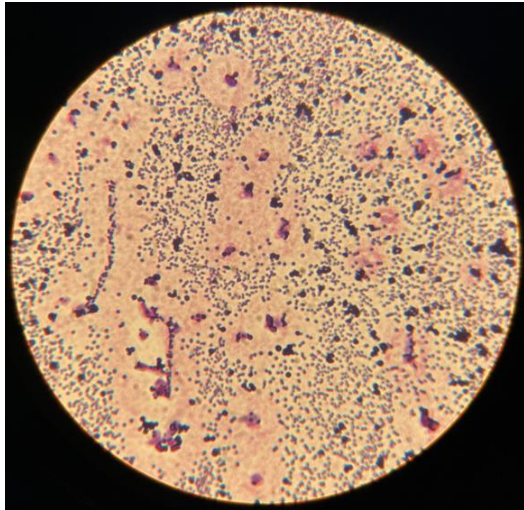
4 What concentration of treatments were used as described in Table 2??

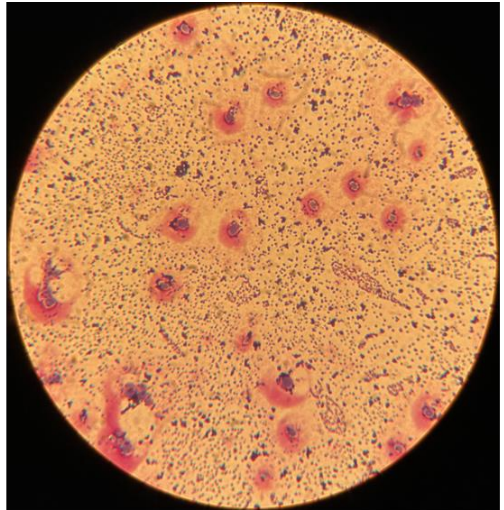
The overall comparison of different concentrations replicates was performed by using Graph Pad Prism tool for One-way Anova test.

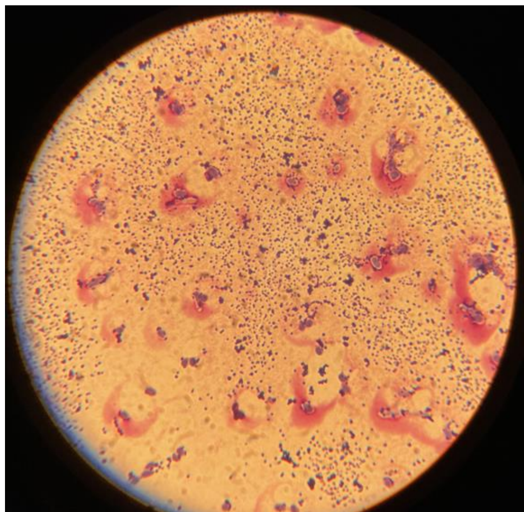
5 Why only 9 passages of cells were cultured??

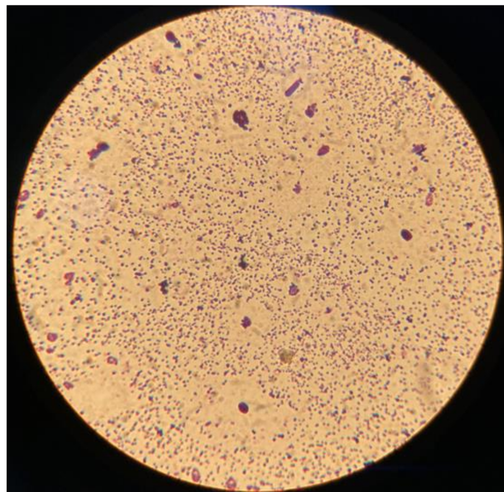
The cell passaging was performed to observe the potency of cells. till 9th passage there was no loss of cells ability to divide which was evident it has a potential to divide for several passages which meet our objectives and we had a limited time and resources which limits us for further study.

6 Microscopic photos of cells









iThenticate

thesis

BY GANESH ADHIKARI

Quotes Excluded

Bibliography Excluded

13%

SIMILAR

12 CULTURE AND CHARACTERIZATION OF STEM CELLS ISOLATED FROM BOVINE UMBILICAL CORD BLOOD AND STUDY THE EFFECT OF *Clematis buchananiana DC EXTRACT*

M.Sc. Thesis

(2022)

Match Overview

#	Source	Similarity
1	**Internet** 333 words crawled on 12-Oct-2022 repository.untar.ac.id	2%
2	**Internet** 68 words crawled on 30-Jan-2023 www.researchgate.net	<1%
3	**Internet** 61 words crawled on 13-Oct-2022 www.coursehero.com	<1%
4	**Internet** 55 words crawled on 08-Dec-2021 www.mdpi.com	<1%
5	**Internet** 50 words crawled on 06-Jan-2023 helda.helsinki.fi	<1%
6	**Internet** 36 words crawled on 20-Nov-2018 ulir.ul.ie	<1%

PAGE: 1 OF 72

Text-Only Report