Investigating the Effects of *Chenopodium album* and *Caralluma tuberculata* on High Aggressive Acute Myeloid Leukemia

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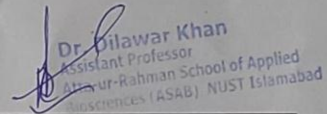

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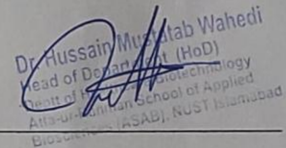

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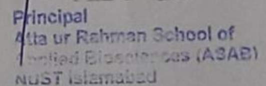

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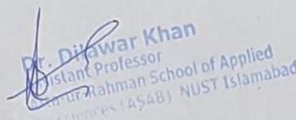

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“And ˹surely˺ your Lord will give so much to you that you will be pleased.”

Quran 93: 5

DEDICATION

"This work is dedicated to our beloved parents and teachers. Their continuous love and support have been our motivation to strive and move forward.

To our honored Supervisor for his immense guidance, support, and inspiration.

To our friends and siblings who motivated and cheered us during these years,

And to all patients who have faced the challenges of cancer with courage and hope."

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LIST OF ACRONYMS

A

ABL	Abelson murine leukemia virus homology gene
ALL	Acute lymphatic leukemia
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia

B

BCR	B cell receptor
BCR/*ABL*	Breakpoint cluster region/Abelson murine leukemia virus
bp	Base pairs
BCR	B cell receptor

C

CDC25A	Cyclin-Dependent kinases by Cell division cycle 25A
cDNA	Complementary DNA
CMP	Common Myeloid Progenitors

D

DEK-NUP214	DEK proto-oncogene- Nucleoporin 214kDa
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

dNTPs	Deoxynucleoside triphosphate

F

FBS	Fetal Bovine serum
FLT3	Fms like tyrosine kinase 3 (CD 135)
FLT3-ITD	Fms like tyrosine kinase 3/internal tandem duplications
FBS	Fetal Bovine serum

H

HSCs	Hematopoietic stem cells
HSCT	Hematopoietic stem cell transplantation
HSPCs	Hematopoietic stem and progenitor cells
HSCs	Hematopoietic stem cells

I

IL	Interleukin

J

JAK	Janus kinase

K

KIT	KIT Proto-Oncogene, Receptor Tyrosine Kinase

L

LSC	Leukemic stem cell
LT	Long term
LT-HSC	Long-term hematopoietic stem cells

M

MAPK	Mitogen activated protein kinase
mTOR	Mammallian target of rapamycin
MTT	Tetrazolium salt

N

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NUP214	Nucleoporin 214

P

PBS	PBS Phosphate-buffered saline
PCR	PCR Polymerase chain reaction
PI3K	Phosphatidylinositol-3-kinase
PKC	Phospho kinase C
PML	Promyelocytic leukemia protein
PML/RARα	Promyelocytic leukemia/retinoic acid receptor α

Q

qPCR	quantitative polymerase chain reaction

R

RBC	Red blood cells
RA	Retinoic acid
RAR	Retinoic acid receptor
RAS	Rat sarcoma Rnase Ribonuclease
rIFN-α	Recombinant interferon-alpha
rpm	Revolution per minute
RPMI	Roswell Park Memorial Institute

S

SOCS	Suppressors of Cytokine Signaling
STAT	Signal transducers and activators of transcription

T

t (6;9)	Translocation of chromosome 6 and 9
TET2	Tet methylcytosine dioxygenase 2
TYK	Tyrosine Kinase

Units

M Molar	M Molar
mM	Millimolar

µl	Microliter
µM	Micromolar
µg	Microgram

+ Positive

- Negative

% Percent

°C Degree Celsius

ABSTRACT

Background: Acute myeloid leukemia (AML) t (6;9), which is also called High Aggressive AML is a subtype of AML. This form of AML makes up only 1% of all cases. High Aggressive AML has an early onset, aggressive disease progression, poor prognosis, low complete remission rate along with low survival rates in patients. Non-specific chemotherapy followed by Bone marrow transplantation is the only treatment currently available for High Aggressive AML; however, this subtype of AML has been seen to be resistant to chemotherapy. *C. album* and *C. tuberculata* are plant species found commonly in Asia and other regions of the world and have been used in traditional medicine for hundreds of years. Recently, research has shown that extracts of these plants show anticancer effects against solid tumor cancers, but more investigation needs to be done to determine their anticancer potential against liquid tumors.

Aim: Our aim was to evaluate the anti-proliferative role of *C. album* and *C. tuberculata* in High Aggressive AML cell line FKH1 having t (6:9), DEK-NUP214 fusion oncogene and ETV6-ABL1 mutation. We also aimed to evaluate the anti-proliferative role of *C. album* and *C. tuberculata* on the U937 cell line, which is a commonly used AML cell line having no DEK-NUP214 or *ABL* mutation.

Methodology: Extracts of C. *album* and *C. tuberculata* were separately assessed for potential antiproliferative effects in FKH1 and U937 cells using MTT assays. In FKH1, the activities of each extract were also compared with those of imatinib. DNA fragmentation assay and gene expression analysis of c-*Myc*, *Eya3, p21* and *p53* was conducted to assess the mechanism by which the two extracts interfered with proliferation.

Results: *C. album* and *C. tuberculata* both decreased the proliferation of FKH1 and U937 cells in a dose dependent manner. In FKH1 cells, the recorded IC-50 values of *C. album* and *C. tuberculata* were found to be approximately 150ug/ml and 7.5ug/ml respectively with high significance of growth inhibition at these concentrations ($p<0.0001$). Upon comparing the effects of imatinib and *C. album* in FKH1 cells, comparable antiproliferative activity was observed near IC-50 values of both. Likewise, similar results were recorded in FKH1 cells when comparing *C. tuberculata* with imatinib. However, when comparing the effective concentrations of *C. album* and *C. tuberculata*, it is evident that *C. tuberculata* shows much stronger inhibition of proliferation.

DNA fragmentation assay performed in FKH1 cells showed no apoptosis caused by either of the extracts. Gene expression analysis showed that both extracts downregulated the expression of the *c-Myc* and *Eya3* genes, with *C. tuberculata* showing a stronger downregulation effect ($p<0.001$ for both c-*Myc* and *eya3*). Gene expression of *p53* and *p21* was upregulated in FKH1 cells upon treatment with the plant extracts. *C. tuberculata* showed a higher gene upregulation effect in both *p53* and *p21*.

Conclusion: Through this study, we found that both *C. album* and *C. tuberculata* extracts interfered with the proliferative potential in U937 and FKH1 with *C. tuberculata* showing much more potent activity. In FKH1 cells, this interference was in a manner that was comparable to that induced by a standard inhibitor of growth in *ABL* positive cell lines i.e. imatinib. Furthermore, in terms of the underlying mechanisms of the observed anti-proliferative effects, our results show that *C. album* and *C. tuberculata* exert these through cell cycle arrest. In order to corroborate these results, translational studies, cell cycle analysis and more sensitive experiments such as Taq-man PCR need to be conducted. A detailed GCMS analysis of *C. album* and *C. tuberculata* plant extract and further studies with focus on the molecular aspects are needed.

Keywords

AML, FKH1 cells, U937 cells, DEK-NUP214, MTT assay, c-Myc, eya3, p21, p53, Chenopodium album, Caralluma tuberculata

CHAPTER 1

INTRODUCTION

Leukemia is a form of cancer that starts in the bone marrow and enhances the production of white blood cells abnormally. It is divided into two main categories based on which type of blood cells it affects: myelogenous leukemia, which involves the development of abnormal myeloid cells, and lymphocytic leukemia, which affects lymphoid cells (Szczepański et al., 2003).

This disease can also be classified as acute or chronic, depending on how quickly it progresses and how aggressive it is. Acute leukemia progresses rapidly and is more aggressive, while chronic leukemia progresses more slowly, and patients may not experience symptoms for a longer period (Szczepański et al., 2003).

Hematopoiesis is the natural process by which hematopoietic stem cells and immature blast cells in the body undergo multiplication and differentiation to produce various types of blood cells. This vital process mainly takes place within the bone marrow (Cumano & Godin, 2007).

Leukemia cells behave somewhat like stem cells because they either originate from hematopoietic stem cells with specific mutations or from more specialized progenitor cells that acquire the ability to renew themselves due to abnormalities. This abnormal growth and development of leukocytes disrupt normal blood cell production and can lead to various symptoms and complications (Passegué et al., 2003).

Similar to the normal process of hematopoiesis, leukemia follows a hierarchical organization, with leukemic stem cells, that are a subset of leukemic cells (LSCs), playing a pivotal role in initiating and sustaining the disease. These abnormal cells give rise to more differentiated malignant cells. They infiltrate the bone marrow, enter the bloodstream, and interfere with normal hematopoiesis by displacing healthy blood cells. Additionally, they can infiltrate organs like the spleen, liver, and lymph nodes, leading to compromised immune function and contributing to symptoms such as susceptibility to infections, anemia, and bleeding disorders. Consequently, the genetic mutations, disruption of the immune system, and changes in the bone marrow microenvironment drive the progression of leukemia (Riether et al., 2015).

According to WHO statistics Leukemia has been ranked 13th in incidence rate, with 487,294 new cases in 2022 remains a significant concern in public health initiatives worldwide. It also ranks 10th in mortality rate with 305,405 of all cancer related deaths worldwide reflecting the ongoing challenges in combating this disease despite advancements in treatment and care (WHO, 2022).

In Pakistan, leukemia holds the 6th rank among all cancers, with 7,722 (4.2%) of new cases and 5,345 (4.5%) of all cancer related deaths, emphasizing its significant burden on the country's healthcare system and population health according to WHO statistics 2022 (WHO, 2022).

1.1. Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a cancer that originates from progenitor stem cells that are involved in red blood cell production, platelets production, and in the production of certain types of white blood cells excluding B and T cells. Like other forms of cancer, it occurs due to genetic alterations that trigger neoplastic clonal proliferation (Pelcovits & Niroula, 2020).

Acute myeloid leukemia (AML) is a heterogenous disease marked by genetic changes leading to reduced production of normal blood cells. These changes hinder the maturation of cells and promote the excessive growth or buildup of immature cells known as blasts (Pelcovits & Niroula, 2020).

The buildup of immature cells typically initiates in the bone marrow but often swiftly progresses into the bloodstream, and occasionally disseminates to other organs including lymph nodes, spleen, liver, testes, and the central nervous system (Prada-Arismendy et al., 2017a). In regular conditions, the bone marrow usually contains fewer than 5% blasts. However, leukemia leads to a substantial increase in blast count. Individuals diagnosed with acute leukemias typically have blast counts exceeding 20% (Marshall, 2014). Because of its genetic roots, AML frequently exhibits certain cytogenetic abnormalities, including but not limited to translocation mutations like t (8;21), t (15;17) and other cytogenetic abnormalities involving inversion 16, trisomy 8, deletions involving parts or the entirety of chromosomes 5 or 7 are also frequently exhibited in AML (Prada-Arismendy et al., 2017b).

In over 90% of AML patients, next-generation sequencing has revealed numerous recurring somatic mutations. Among these mutations, frequently observed genes (occurring in more than 5% of cases) include receptor tyrosine kinases like FLT3 and other genes such as NPM1, TET2, CEBPA, and WT1 (Kantarjian et al., 2021).

A fusion gene generated by the chromosomal translocation t (6;9)(p23;q34.1) is DEK-NUP214, which accounts for approximately 1% of genetic aberrations observed in AML. It frequently accompanies FLT3-ITD mutations in 70% of cases and KRAS mutations in 20% of cases. DEK-NUP214 is classified as an adverse-risk genetic anomaly, indicating a dismal prognosis for affected patients (Wilcher et al., 2023a).

A specific intron on chromosomes 6 and 9 harbors the breakpoints involved in the translocation, resulting in the transcription of a chimeric mRNA. This mRNA encodes a novel protein weighing 165-kd, which holds significance in the process of leukemogenesis (Ko et al., 2006).

DEK-NUP214 has the capability to transform CD34+ human bone marrow cells, contributing to the development of an especially aggressive form of acute myeloid leukemia (AML) (Wilcher et al., 2023a).

Currently, there is no specific treatment available for high-risk AML because of the limited understanding of the leukemogenic potential of the DEK/NUP214 fusion protein. Additionally, patients with this fusion protein typically exhibit poor responses to chemotherapy. Consequently, there's a lack of definitive treatment options for this particular subtype of AML that directly addresses the underlying issue (Ishiyama et al., 2012).

1.2. Anti-Proliferative Potential of *C. album* and *C. tuberculata* against AML

Studies in epidemiology have indicated that medicinal plants may play a significant role in reducing the genetic damage and cancer-causing potential of chemotherapeutic drugs.

C. album, commonly known as bathua, is an underutilized wild herb with diverse pharmacological properties. This herb shows a variety of effects that are antiviral, antifungal, and anti-inflammatory in nature. *C. album* is also shown to possess antiallergic, antiseptic, and immunomodulatory properties. Despite these attributes, its potential as an anticancer agent has been largely overlooked. It contains phytochemicals such as flavonoids, isoflavonoids, and polyphenols, which are known for their potential in promoting human health and reducing the risk of cancer. The presence of these bioactive compounds has prompted its consideration for further investigation in cancer research (Khoobchandani et al., 2009).

Caralluma, including *C. tuberculata* (Chunga), species possess magnitudes of beneficial properties that are pharmacologically significant. These plants exhibit anti-inflammatory properties, pain-relieving effects

and are shown to have antidiabetic, liver-protective and gastric mucosa-protecting effects. They are consumed for their roles as antioxidant, and appetite-suppressing supplements as well (Sana et al., 2022).

Different constituents or extracts from plants have varying effects on different types of cancer. Some may inhibit the cell cycle, while others may induce apoptosis, and some may target pathways involved in cell proliferation. Researchers are investigating factors such as the ease of modifying drugs, their effectiveness, targeted delivery to cancer cells, and potential side effects using various cancer cell lines (Engel et al., 2016).

In this research we aimed to evaluate the antiproliferative potential of two plants *C. album* and *C. tuberculata* against aggressive form of AML

1.3. Hypothesis

In leukemia treatment, two primary challenges are cancer relapse and the development of adaptive resistance to current therapies. The interaction among bone marrow microenvironment stromal cells, leukemia stem cells (LSCs), and soluble mediators is crucial for the survival of blasts and their resistance to chemotherapy (Rashidi & Uy, 2015).

Current research is delving into novel targeted strategies for addressing leukemia, with one avenue being the exploration of plant extracts. These extracts have shown promising anti-cancer activity both in laboratory settings and in vivo, while also exhibiting favorable safety profiles and ease of adjustment. In this study we examined the anti-proliferative effects of extracts from two medicinal plants, *C. album* and *C. tuberculata* against AML.

1.4. Research objectives:

The objectives of our research were to:

- To explore the anti-proliferative action of *C. album* and *C. tuberculata* in AML cell lines (FKH1 and U937).
- To compare the antiproliferative effects of *C. album* and *C. tuberculata* with that of imatinib in FKH1 cell line.
- To perform the mechanistic studies of the action of *C. album* and *C. tuberculata* against FKH1 cell line.

CHAPTER 2

LITERATURE REVIEW

2.1. Hematopoiesis

The formation of all blood cells is termed hematopoiesis. In humans, it is a complex process and begins in the yolk sac. This process is then taken up by the liver, followed by bone marrow and thymus. There are two sites of definitive hematopoiesis in adults i.e. bone marrow and thymus. Definitive hematopoiesis occurs later in development, and involves the production of HSC, which can produce every type of blood cells including myelocytes and lymphocytes (Jagannathan-Bogdan et al., 2013). Hematopoiesis thus occurs within the specialized microenvironment of the bone marrow. Hematopoietic stem cells reside within the bone marrow in a dormant state until they receive signals triggering their activation and differentiation.

This resident environment involves niches, or specified areas, which include specific ECM components, specialized cell populations, and molecules that help in cell differentiation, specialization, renewal, and migration. The dynamics within the bone marrow microenvironment are thus finely regulated by an interplay of molecular signals, transcriptional factors, and cellular interactions which allow for both self-renewal, as well as the transformation of hematopoietic stem cells (HSCs), which possess multipotency, into various mature blood cells of all lineages (Yin et al., 2006; Pinho et al., 2019).

2.1.1. Hematopoietic Stem Cells: Types and Differentiation

Hematopoietic stem cells are divided into two types based on their capacity for self-renewal i.e. long-term hematopoietic stem cells, which retain this capacity throughout an organism's life span, and short-term hematopoietic stem cells, which can maintain self-renewal up to eight weeks. (Morrison & Weissman, 1994). Hematopoietic stem cells generally follow a hierarchical system where each degree of differentiation is irreversible (Figure 1). Within this hierarchy, the order of formation begins with LT-HSC's. These then give rise to ST-HSC's, which form multipotent progenitors (Morrison et al., 1997). These cells retain the ability to give rise to multiple blood cell lineages. As these progenitors develop, they become increasingly restricted in their differentiation potential. Ultimately, they producing lineage-

committed progenitors known as oligopotent or unipotent progenitors (Lee et al., 2020). In mice, the common lymphoid progenitor and common myeloid progenitor have been identified as two sets of the previously mentioned oligopotent progenitors. These then give rise to cells of the lymphoid and erythromyeloid lineages respectively (Akashi et al., 1999; Kondo et al., 1997; Mebius et al., 2001). The processes that give rise to each respective lineage are known as lymphopoiesis and myelopoiesis. Through lymphopoiesis, three types of cells are formed, these are T, B and natural killer cells. During myelopoiesis, CMPs differentiate into myelomonocytic progenitors (GMPs) and megakaryotic/erythroid progenitors (MEPs) (AKASHI et al., 1999). Thus, during myelopoiesis, all blood cells excluding the previously mentioned lymphoid cells are formed. GMPs from monoblasts, which give rise to monocytes, and granulocytes (neutrophils, basophils, and eosinophils). MEPs form erythroblasts which give rise to erythrocytes, and megakaryocytes which form platelets (Lee & Hong, 2020; Passegué et al., 2003).

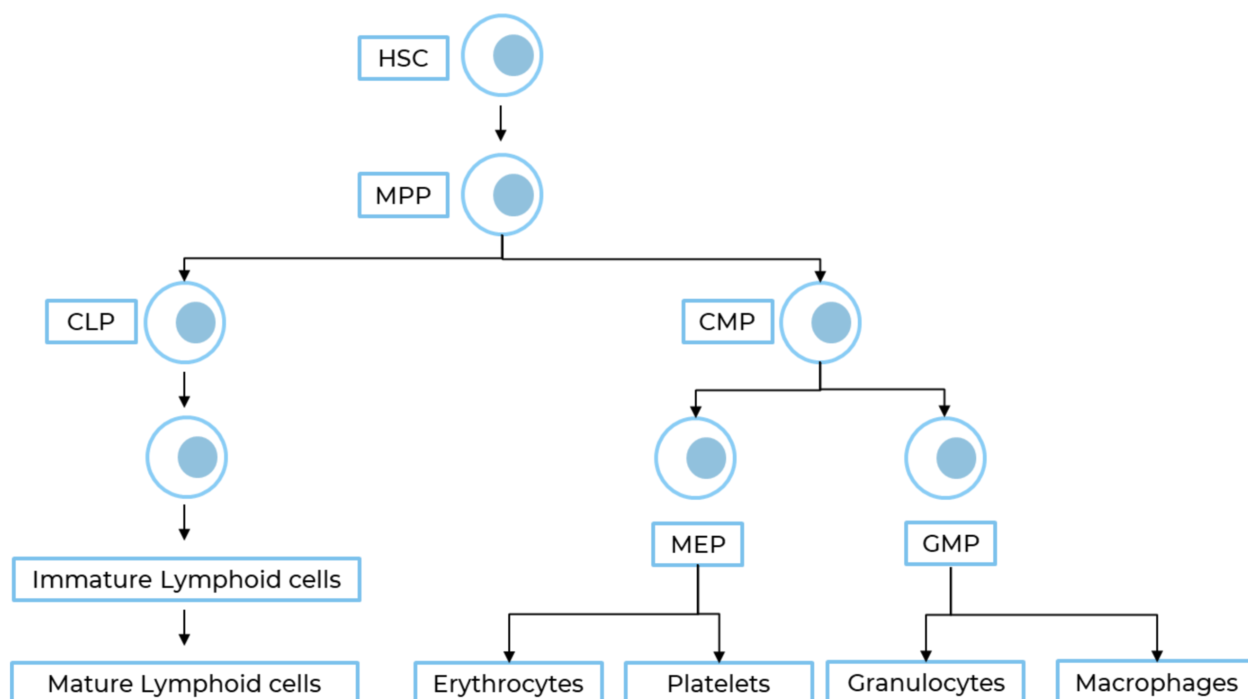

Figure 1: Flow Chart of Normal Hematopoiesis

2.2. Leukemia

The highly regulated process of hematopoiesis, when disrupted, causes several abnormalities including leukemogenesis. Leukemia is a cancer of the blood and bone marrow. In leukemia, there is uncontrolled and abnormal production of blood cells. These blood cells do not function normally and suppress the

production of normal cells. Symptoms are nonspecific and can include fever, fatigue, shortness of breath, bone pain, weight loss, swollen lymph nodes, bruising, or bleeding.

In order to develop, the multistep process of oncogenesis requires increased proliferation, mutations that block differentiation and the involvement of proto-oncogenes with a role in apoptosis (Figure 2) (Passegué et al., 2003).

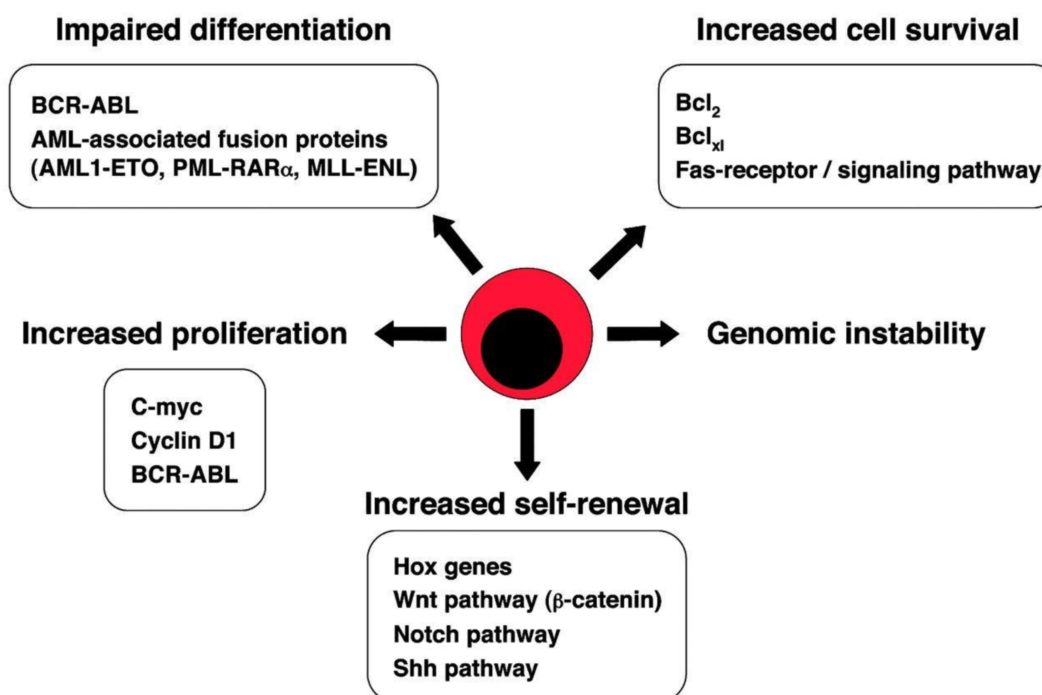

Figure 2: Deregulated Pathways Leading to Leukemia (Passegué et al., 2003)

2.2.1. Risk Factors

Numerous genetic and environmental risk factors are involved in the development of leukemia. Benzene exposure, ionizing radiations, viral infections, several genetic syndromes, a history of any hematological malignancy, Previous exposure to chemotherapy, especially inhibitors of topoisomerase II and alkylating agents increase the risk of leukemia (Chennamadhavuni A et al., 2023).

2.2.2. Types of Leukemia

There are two types of leukemia based on disease progression, acute leukemia and chronic leukemia and based on cell of origin, it is classified into myelocytic or lymphocytic. In acute leukemia, percentage of

blasts in the peripheral blood smear or in bone marrow is greater than 20%, causing more rapid onset of symptoms. In chronic leukemia, blasts percentage is less than 20%, leading to persistent and long-term onset of symptoms. The four major subtypes of leukemia include (Chennamadhavuni A et al., 2023):

Acute lymphoblastic leukemia (ALL): There is blastic transformation of B & T cells in ALL. It is the most common form of liquid cancer in pediatric population, with up to 80% of cases in children and only 20% in adults (Chennamadhavuni A et al., 2023).

Acute myelogenous leukemia (AML): There are greater than 20% myeloid blasts in AML and it is the most common type of acute leukemia in adults. It also occurs in children. It is the most aggressive cancer with highly varying prognosis (Chennamadhavuni A et al., 2023).

Chronic lymphocytic leukemia (CLL): There is proliferation of monoclonal lymphoid cells. It is the most common chronic leukemia in adults. Symptoms CLL may not appear for many years (Chennamadhavuni A et al., 2023).

Chronic myelogenous leukemia (CML): It results from cytogenic aberration in which there is reciprocal translocation and fusion of chromosome 22 and chromosome 9 leading to formation of oncogene called BCR-ABL. The truncated chromosome 22 formed due to this mutation is known as the Philadelphia chromosome (Ph). It is more common in the elderly but onset can be at any age (Chennamadhavuni A et al., 2023).

2.3. Acute Myeloid Leukemia

Acute myeloid leukemia is a heterogenous disorder resulting from abnormal proliferation of myeloid progenitor cells in the bone marrow and peripheral blood. It is the most commonly occurring acute form of leukemia in adults, being responsible for 80% of the cases (Lagunas-Rangel et al., 2017). The incidence of this cancer increases with age and 1.3 cases per 100,000 population are in patients under the age of 65. Additionally, 12.2 cases occur per 100,000 population in patients above the age of 65 (De Kouchkovsky & Abdul-Hay, 2016).

2.3.1. Pathophysiology

Chromosomal translocations and molecular genetic mutations lead to abnormal cellular proliferation in myeloid stem cells.

Non-random chromosomal abnormalities (e.g., deletions, translocations) account for approximately half of all adults diagnosed with primary AML. Cytogenetic abnormalities include inv(16) (p13q22) [CBFB/MYH11], [t(15;17) (q22,q21)], t (8:21) etc. These cytogenetic abnormalities are linked with periods of remission that last longer as well as longer and survival rates. While other cytogenetic aberrations including t (6;9)(p23;q34) [DEK/NUP214], t (1;22)(p13;q13) [RBM15/MKL1], inv(3)(q21q26) [RPN1/EVI1] are rarely linked with a positive response to therapy and tend to show shorter rates of survival (Saultz & Garzon, 2016).

Additionally, 97% of cases include genetic mutations. Class I mutations are categorized as such due to their role in the activation of specific cell signaling pathways. These are pathways that tend to mediate cellular proliferation and survival. Common class I mutations are FLT3-ITD, FLT-3-TKD, and tyrosine kinase domain mutations, TKD), c-KIT, TP53, JAK2 and K/NRAS. Class II mutations impair normal hematopoietic differentiation and apoptosis. It includes PML-RARA, RUNX1-RUNX1T1, NPM1 (Lagunas-Rangel et al., 2017).

2.3.2. Classification of Acute Myeloid Leukemia

The current WHO classification defines six subtypes of AML (Hwang, 2020). These include:

AML with recurrent genetic abnormalities:

This subtype refers to a subtype of AML which includes balanced translocations e.g. t (8;21), inv(16), t(15;17). The type with inversions or translocations often has distinct clinical features. Additionally, there are several other recurrent genetic abnormalities in AML, such as mutations involving genes like FLT3, NPM1, CEBPA, and others (Hwang, 2020).

AML with myelodysplasia-related changes (MRC):

This refers to AML that develops after a history of Myelodysplastic/myeloproliferative neoplasms. Certain cytogenetic abnormalities are also associated with AML-MRC. There are several strict diagnostic criteria for this subtype, including those relating to blast count, percentage of dysplasia, number of cell lineages displaying dysplasia, a history of several dysplastic syndromes, and exclusion of genetic abnormalities associated specific to AML with recurrent genetic abnormalities. Patients with this subtype of AML generally do not have a promising prognosis and are less likely to undergo complete remission (Hwang, 2020).

Therapy-related myeloid neoplasms (t-MN):

This subtype involves malignancies that develop due to previous exposure to cytotoxic chemotherapy or radiation therapy (Given for a different disorder). Certain chemotherapeutic drugs such as inhibitors of topoisomerase II and alkylating agents increase the risk of t-MN. Certain inherited risk factors have also been implicated in the pathogenesis. This AML subtype also has a poor prognosis (Hwang, 2020).

AML, not otherwise specified (NOS):

AML-NOS is a diagnosis of exclusion. It is used when a clear subtype of AML cannot be identified despite comprehensive testing. This category includes AML subtypes that do not fit into the other defined categories and are classified based on their morphology and/or cytochemistry, and other parameters such as immunophenotype (Hwang, 2020).

Myeloid sarcoma:

Myeloid sarcoma is an extramedullary tumor consisting of underdeveloped cells having myeloid origin. It may present de novo or concurrently with acute myeloid leukemia (AML) (Hwang, 2020).

Myeloid proliferations related to Down syndrome (DS):

This subtype of AML is specific to patients with Down syndrome. Two types of DS associated myeloid proliferations are included in this sub-category: transient abnormal myelopoiesis (TAM) and myeloid leukemia. TAM in children with DS typically resolves spontaneously but can progress to AMKL in some cases. The majority of myeloid leukemia in DS patients is acute megakaryoblastic leukemia. The underlying genetic abnormalities in Down syndrome contribute to this increased risk of myeloid proliferation (Hwang, 2020).

2.4. Aberrant Signaling Pathways in Leukemia

2.4.1. Ras/Raf pathway

The mitogen-activated protein kinase (MAPK) cascade regulates different cellular functions such as cellular survival, cellular proliferation, cellular differentiation, angiogenesis, and migration (Figure 3). Ras proteins are signaling proteins activated by binding of ligand to receptor tyrosine kinase at the cell surface. Activation of Ras leads to activation of downstream components Raf, MEK (mitogen-activated protein kinase kinase), extracellular signal-regulated kinase (ERK) and PI3K/AKT (Knight & Irving, 2014). RAS

proto-oncogenes encode HRAS, NRAS and KRAS, that are involved in homeostatic mechanisms of normal cells. Ras proteins are monomeric membrane associated GTPases. Ras activation is dependent on some factors. These include guanine nucleotide exchange factors (GEFs). One of the GEFs that catalyze the activation of Ras is son of sevenless (SOS). SOS primarily functions by removing GDP and instead allowing GTP to occupy the now empty nucleotide binding site. In order to de-activate Ras proteins, this process is reversed as GTPase activating proteins (GAPs) catalyze the hydrolysis of GTP and the restore the formation of RAS–GDP (Knight & Irving, 2014). Activating KRAS and NRAS mutations are common in AML while HRAS mutations are rare (Huang et al., 2016). Abnormal activation of RAS signal transduction happens through two routes. This can be due to the activation of this pathway via mutated proteins and/or the overexpression of signaling molecules such as FLT3, KIT, and Ras proteins which leads to phosphorylation and activation of downstream effectors thereby promoting cell survival.

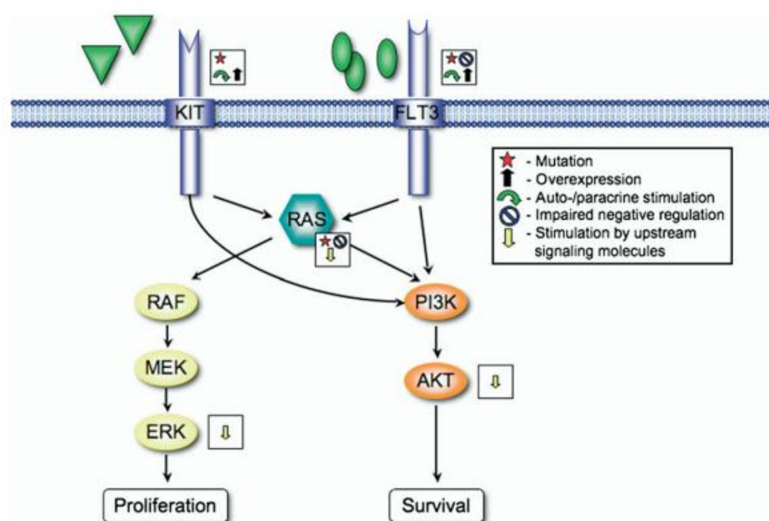

Figure 3: Ras/Raf Pathway (Scholl et al. 2008)

2.4.2. Phosphoinositide 3-Kinase (PI3K)-Akt-Mammalian Target of Rapamycin (mTOR) Pathway

PI3K/Akt mTORC1 pathways are important in the regulation of hematopoiesis and occupy the place of important mediators in this process under both normal as well as malignant conditions (Nepstad et al., 2020). The pathway plays an important function in proliferation, survival, differentiation, cell cycle progression and apoptosis. Abnormal activation of this pathway is present in 50–80% of human AML and

is associated with decreased overall survival (Annageldiyev et al., 2020). The pathway is activated by many extracellular signals. Some examples of these are receptor tyrosine kinases (RTK), lymphocyte receptors, and various integrins etc.

Akt has the potential to function as a proto-oncogene and it exists in three different structurally active forms. Akt is present in cytosol, however an activation mediated by PI3K requires translocation of Akt to the membrane. PDK1 phosphorylates Akt at Thr308 followed by phosphorylation of Akt at Ser473 by mTOR complex 2 (mTORC2). This activation relocates Akt to the cytosol or the nucleus.

The Ser/Thr protein kinase mTOR belongs to PI3K-related kinase (PIKK) family. The mTORC1 consists of five units. The mTORC2 is composed of mTOR, mLST8, Deptor, the rapamycin insensitive companion of mTOR (Rictor), Protor and the mammalian stress-activated protein kinase-interacting protein 1 (mSIN1) (Nepstad et al., 2020). The main downstream substrates of mTORC1 include proteins with various essential cellular functions. E.g. the eukaryotic initiation factor-4E (eIF4E) -binding protein 1 (4EBP1) & the ribosomal protein S6 kinase (S6K) which play integral roles in cell growth and in the regulation of translation respectively (Figure 4) (Nepstad et al., 2020).

This pathway shows overactivation in AML cells which leads to aberrant changes in key cellular pathways. Dysregulation of this signaling pathway is caused by the oncogene amplification or the oncogene activating more mutations.

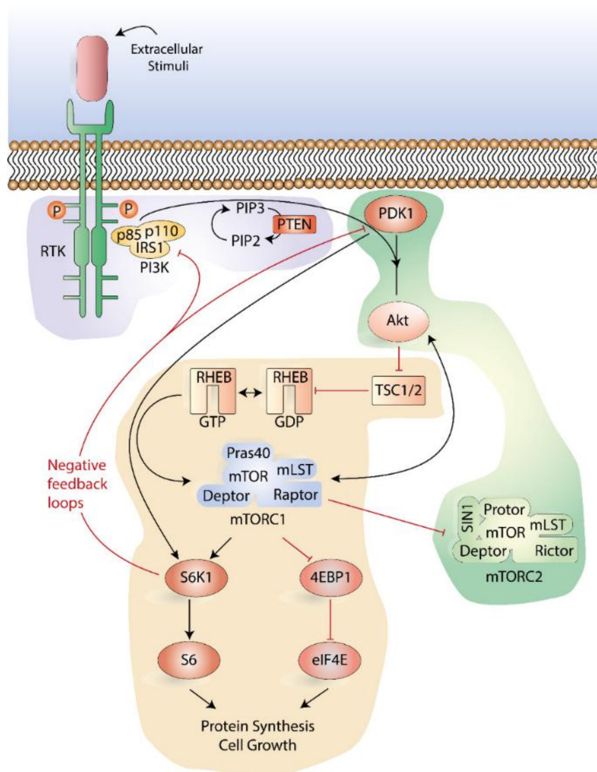

Figure 4: PI3K-Akt-mTOR Pathway (Nepstad et al., 2020)

2.4.3. FLT3 ITD Signaling

In early blood cells flt3 is a tyrosine kinase receptor and plays a role in their normal growth and immune system development. Its corresponding activating molecule is produced by bone marrow stromal cells and other cell types and when combined with other growth factors, this molecule works together with FLT3 to enhance the multiplication of SC, DC and NK cells and progenitor cells (Gilliland & Griffin, 2002).

FLT3 is commonly found in numerous types of human and mouse cell lines belonging to both the myeloid and B-lymphoid lineages. Within healthy bone marrow, its presence seems to be limited to early progenitor cells, particularly those marked by the presence of CD34 and exhibiting elevated levels of CD117 (also known as c-KIT). (Rasko et al., 1995; Rosnet et al., 1996)

When FLT3 ligand (FL), binds to FLT3 receptor it triggers its dimerization and activates the intrinsic tyrosine kinase activity. This activation brings about FLT3 phosphorylation, setting off various signaling pathways. Signal transduction molecules are then recruited to the activated FLT3 and bind to the receptor.

Upon activation FLT3 plays important roles in promoting the proliferation, survival, and differentiation of HSPCs. (Kazi & Rönnstrand, 2019).

One of the most common mutations in AML includes FLT3 and is present in approximately 25-35% of cases. Insertion mutations within the juxta membrane region are the most commonly found FLT3 mutations in AML and are known as internal tandem duplication (ITD) mutations (Figure 5) which are prevalent in approximately 25% of cases. According to a study in 1996 it was found that ITD mutations involve the in-frame duplication of a segment of the juxta membrane region, and they can also extend beyond this area. About 33% of ITD mutations occur at the tyrosine kinase domain's beginning region. Size of these duplications varies widely, ranging from 3 to 1,236 base pairs. Studies have also shown that FLT3-ITD mutations can serve as a significant prognostic factor for the risk of relapse in AML patients is the presence of demonstrating its clinical relevance (Kazi & Rönnstrand, 2019).

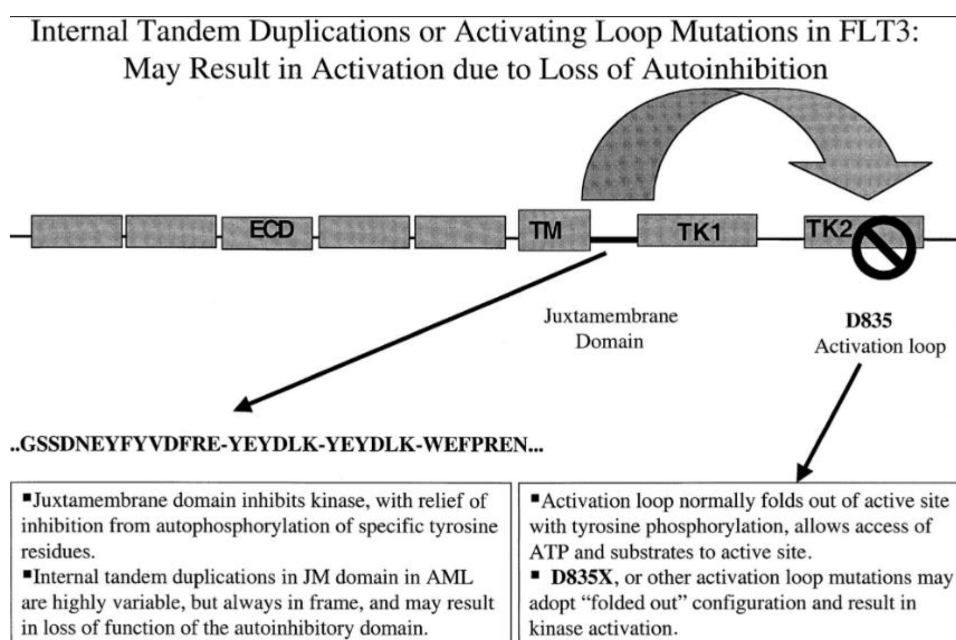

Figure 5: Two types of activating mutations in FLT3 are associated with AML. (D. Gary Gilliland, 2002)

FLT3-ITD expression contributes to heightened proliferation rates and enhanced resistance to cell death (Yuan et al., 2014).

2.4.4. JAK/STAT pathway

Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway is an intracellular signaling pathway which is involved in multiple biological functions, including cell growth, apoptosis, differentiation, and immune system regulation. The JAK-STAT pathway initiates with external signals such as cytokines, hormones, and growth (Xin et al., 2020).

The process starts with cytokines binding to their specific receptors, causing receptor dimerization, and coupling with JAK kinases. This event triggers the phosphorylation of tyrosine residues on the receptor's catalytic domain, creating a binding site for STAT proteins with SH2 domains. Upon recruitment to these sites, STATs undergo phosphorylation and become activated, allowing them to dimerize. These dimerized STATs then translocate from the cytoplasm to the nucleus, where they regulate gene expression responsive to cytokine signaling by binding to specific DNA segments as shown in Figure 6.

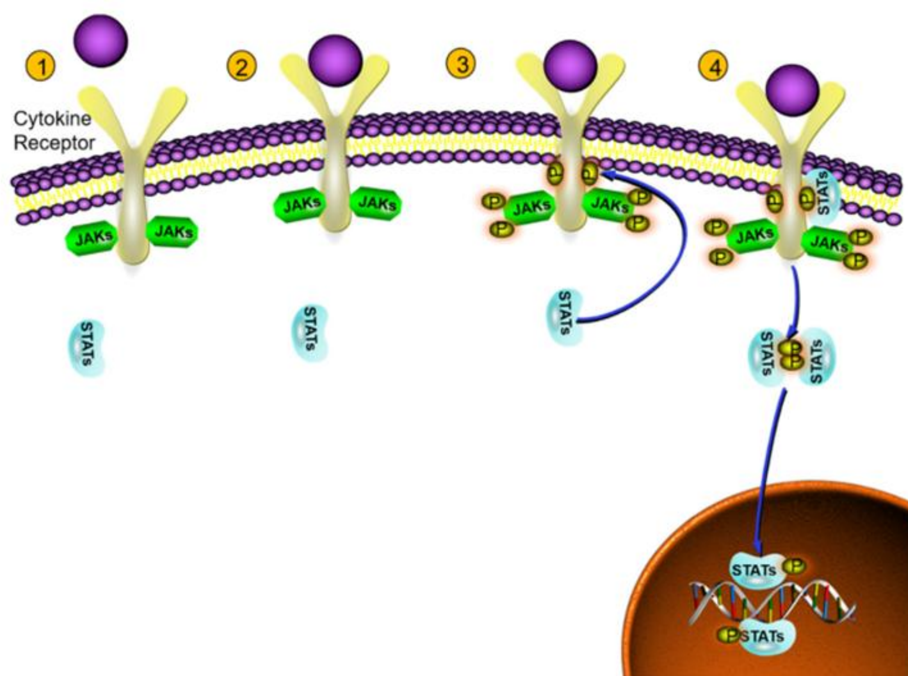

Figure 6: JAK/STAT signalling pathway (Xin, 2020)

JAK1, JAK2, JAK3, and TYK2 form the family of JAK kinases. Within mammals, the STAT protein comprises of STAT1-4, STAT5a, STAT5b, and STAT6, all characterized by a conserved structure (Fasouli & Katsantoni, 2021). Activation of this pathway leads towards STATs phosphorylation. Phosphorylation of

STATs plays a crucial role in regulating hematopoiesis, as well as maintaining HSC proliferation, survival, and cellular renewal. Disruption of this pathway is seen to initiate different types of malignancies (Fasouli & Katsantoni, 2021).

The development of acute myeloid leukemia (AML) is recognized as a complex, multi-step process involving various causes. Translocations and inversions, which often result in fusion oncogenes utilizing the JAK-STAT pathway, have been involved in this process. Initial mutations activating receptor tyrosine kinases promote the proliferation of HSC. Subsequent transcription factor mutations that affect cellular differentiation and apoptosis are involved in the transformation of these cells into leukemic cells. (Fasouli, JAK-STAT in Early Hematopoiesis and Leukemia, 2021) Hyperactive JAK-STAT signaling is involved in the development of myeloid malignancies. About 66% of AML patients carry mutations that upregulate JAK-STAT pathway (Venugopal et al., 2020).

The JAK2 V617F mutation is commonly observed as a somatic mutation in most cases of myeloproliferative neoplasms (MPNs), resulting in the increase in JAK2 activity and which leads to activation of STAT5 (Goyal et al., 2020). According to a study the constitutive activation of STAT1, STAT3, and STAT5 in cells derived from acute leukemias was observed. STAT1 is a promoter of leukemia development, while STAT5 contributes towards myeloid and lymphoid leukemia development. Mutant forms of STAT5A, characterized by constitutive activation and the formation of stable tetramers at elevated levels, have been associated with the onset of multilineage leukemias. In human leukemias, an excess of STAT5 tetramers compared to dimers has been detected (Fasouli & Katsantoni, 2021).

2.4.5. Current Treatments

Different treatment options are available for the patient of AML. These include chemotherapy, radiation therapy, immunotherapy, HPSC transplant and targeted therapy (De Kouchkovsky & Abdul-Hay, 2016).

- Chemotherapy includes drugs to stop the growth of cancer cells either by blocking their division or killing them. It is given in different cycles, consisting of induction therapy and consolidation therapy which work towards achieving complete remission and prevention of relapse respectively.
- Radiation therapy involves the use of high energy radiations such as X-rays to kill cancer cells.

- Immunotherapy aims to boost the immune system and help it to recognize cancer cells. Gemtuzumab-ozogamicin (GO) is an anti-CD33 antibody-drug conjugate (ADC) approved by FDA for AML.
- HPSC transplantation uses healthy blood-forming cells from matched healthy donor. After chemotherapy, these cells are infused into patients that help to restore the patient's blood cells which were destroyed during chemotherapy.

2.5. DEK-NUP214 Acute Myeloid Leukemia

DEK-NUP214 is an oncogene present in the High Aggressive AML cases (Figure 7). The chromosomal translocation between DEK gene on chromosome 6 and NUP214 gene on chromosome 9 leads to formation of fusion protein DEK-NUP214 (165-kDa) which leads to development of leukemia. DEK gene is present on chromosome 6p23. DEK gene plays an important role in differentiation, particularly myelopoiesis, hematopoietic stem and progenitor cell (HSPC) proliferation (Wilcher et al., 2023b). NUP214 is present on chromosome 9q34 is a component of the nuclear pore complex (NPC) and is involved in protein and mRNA nuclear export (Wilcher et al., 2023b).

It is a rare subtype present in 1-2% of AML patients. It is a aggressive subtype of AML with a shorter overall survival rate and poor prognosis.

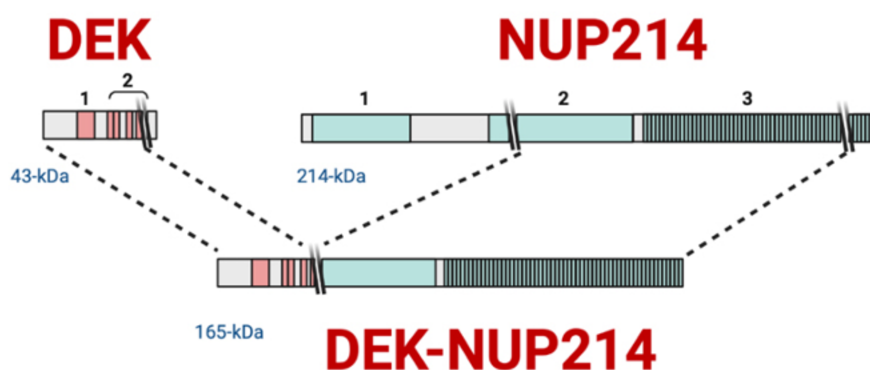

Figure 7: Formation of DEK-NUP214 fusion protein (Rattanathammethee 2023)

2.5.1. DEK

The DEK proto-oncogene, located on chromosome 6, constitutes one of the two of the parts of the fusion oncogene in the AML sub-type under study. While it forms a fusion oncogene in high aggressive acute myeloid leukemia, the DEK gene has been shown to be individually over expressed in several cancer types. (Carro et al., 2006) DEK overexpression has generally been linked to proliferation and elongation of cellular lifespan (Riveiro-Falkenbach & Soengas, 2010). High expression of the DEK protein has also shown to interfere with cellular differentiation in certain cell types, whereas it is expressed in lower amounts in cells undergoing differentiation (Wise-Draper et al., 2009). In addition to these pro-cancerous roles, DEK has also been implicated to have an anti-apoptotic role, with lower DEK levels corresponding to cellular apoptosis through different mechanisms including *p53* stabilization. (Wise-Draper et al., 2006)

The DEK protein, on account of its unique structural organization, is a chromatin associated protein. DEK has two DNA-binding domains. The first domain, located in the central part of the protein, is the scaffold attachment factor, which is a conserved motif shared by a number of other chromatin binding proteins. The second domain, as seen in figure 8, occurs in the C-terminus of the protein. (B hm, 2005) Thus it plays a significant role in various cellular processes, including epigenetic and transcriptional regulation.

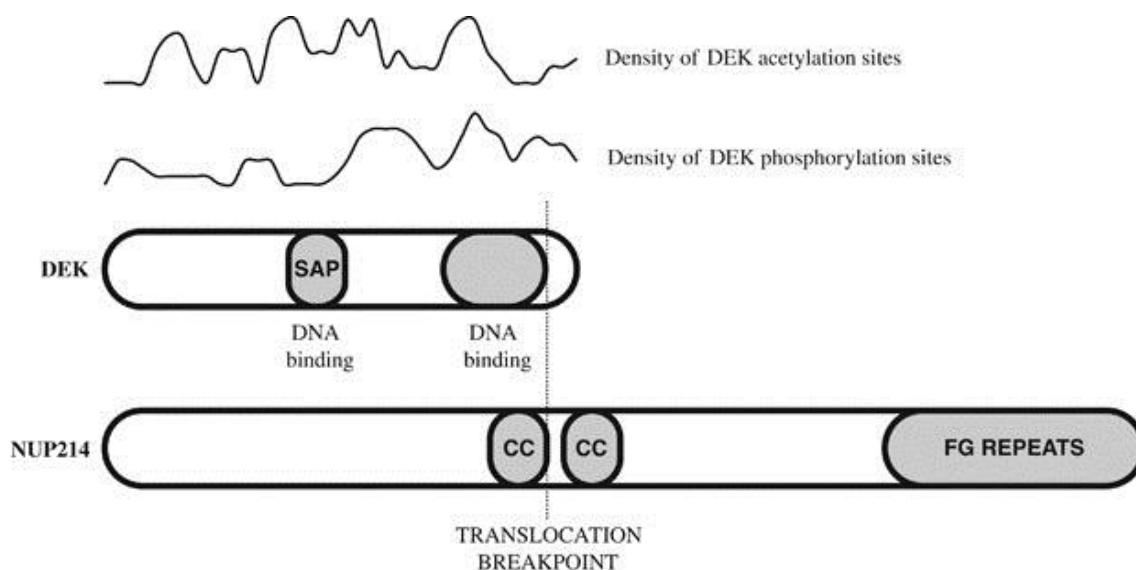

Figure 8: Structure of DEK and NUP214 Genes (Sandén and Gullberg 2015)

2.5.2. NUP-214 / CAN

NUP-214 is the second gene that forms part of the fusion oncogene in the AML subtype under study. NUP-214 or CAN, is a protein involved in the transport of mRNA from the nucleus to the cell cytoplasm. Mutations or translocations in this gene are thereby particularly detrimental owing to the crucial role of this protein's involvement in transport of transcribed genetic material towards the cytoplasm (thereby allowing RNA to be translated and functional proteins to be added to the cellular proteome at any given period of time). For the purpose of exporting mRNA, a nuclear pore complex is assembled. NUP-214 forms one part/subunit of this larger protein complex, alongside the products of many other nucleoporin genes (Mendes & Fahrenkrog, 2019).

As a whole, the structure of the NUP-214 protein may be divided into three parts: a central domain, flanked by N and C terminal regions. The central domain consists of two coiled motifs, that bring about interactions with other nucleoporins within the NPC. NUP-214 is referred to as an "FG" protein. This is due to the structural repetition of lengths of phenylalanine-glycine motifs. The presence of these repeated motifs allows for dynamic interactions with other nuclear transport proteins. This property, as well as inherent structural properties of these repeats, confer selective permeability to the process of nuclear transport (Patel et al., 2007). NUP-214 is also involved in the nuclear export of proteins. This is due to the fact that it is one of the two nucleoporins that interact with CRM1 or exportin 1 (Fornerod et al., 1997).

2.6. Ethnopharmacology and Medicinal Plant Usage

Ethnopharmacology is a scientific discipline that involves the study of biologically active agents with a history of traditional and indigenous use, for medicinal purposes (Süntar, 2020). It involves the interdisciplinary exploration of such agents by combining knowledge from fields including botany, chemistry, and pharmacology. It aims to understand the utilization, bioactivity, and effect of traditional remedies. This approach generally includes field observations, phytochemical analysis, pharmacological research utilizing crude extracts, and identification of plant materials (Figure 9). The rich metabolites produced by plants offer an array of compounds with potential medicinal effects. Thus, ethnopharmacological studies have been a driver of drug discovery in the past, as is further evident by several modern-day medicines that have been derived from plant constituents. (Pirintsos et al., 2022).

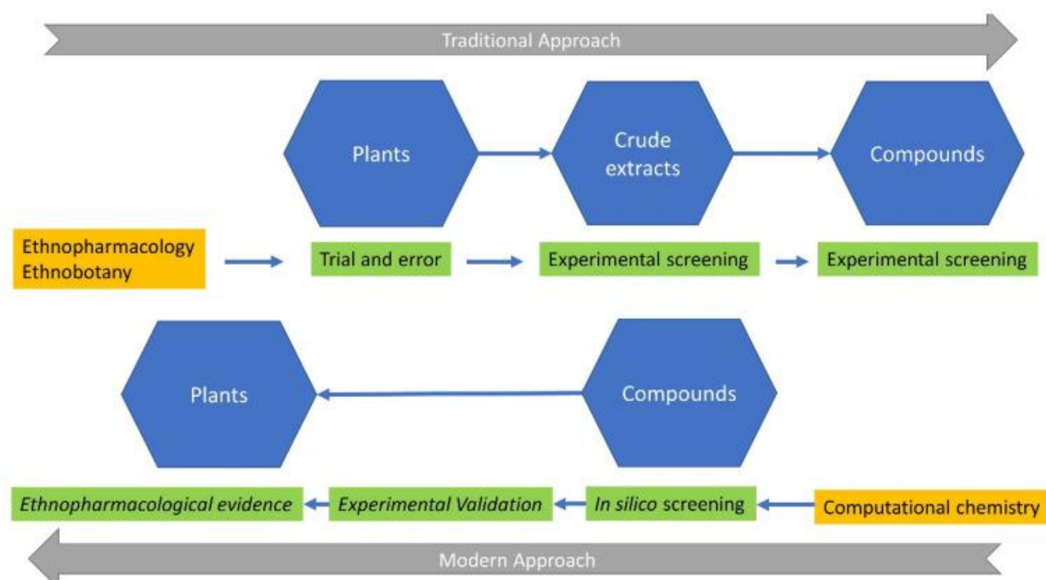

Figure 9: Approaches Used in Ethnopharmacology (Pirintsos et al., 2022)

For example, Artemisinin and Taxol are drug compounds isolated using an ethnopharmacological approach. Artemisinin, previously known as qinghaosu, is a potent antimalarial drug. It was discovered through the ethnopharmacological study of the traditional Chinese herb Artemisia annua. Having been used for around 2000 years by Chinese herbalists, in 1972, the active ingredient was isolated. The traditional use of this herb in Chinese medicine led to the isolation and development of artemisinin (Meshnick, 2002).

Taxol, a widely used anticancer drug, was also discovered through ethnopharmacological investigation of the Pacific yew tree (Taxus brevifolia). It was in fact, isolated as a result of a study aiming to find anti-cancer compounds from plants (Meshnick, 2002). Such compounds highlight the value of traditional medicinal knowledge in modern drug development.

2.6.1. Chenopodium

Introduction:

Chenopodium, is a genus of plants in the Chenopodiaceae family, includes around 200 species of herbaceous plants. The species in this genus contain a number of compounds to which their bioactive properties can be attributed (Fig 10 and 11). These compounds include phenols, saponins, terpenes, alkaloids, glycosides, and amides (Kokanova-Nedialkova et al., 2009). (Compounds from several species

including *Chenopodium bonus-henricus L.*, and *C. album L.*, have also been investigated for their anti-cancerous potential.

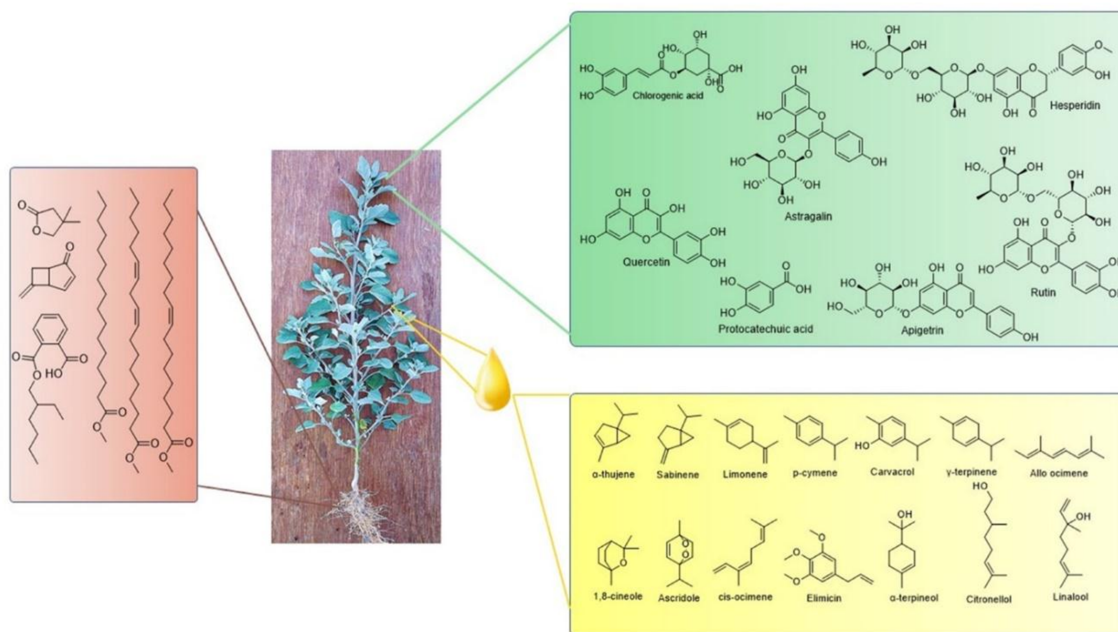

Figure 10: Photoactive compounds in different parts of *C. album* (Singh et al., 2023)

Anti-cancerous role:

Recently, a study isolated six compounds including two new glycosides and four saponins from the roots of the plant *Chenopodium bonus-henricus L.*, which showed moderate anti-cancerous activity in five leukemic cell lines including HL-60, SKW-3, Jurkat E6-1, BV-173 and K-562 (Kokanova-Nedialkova et al., 2019).

C. album leaf extract has been extensively explored for its efficacy against various types of cancers. It demonstrated anti-tumor effects in Swiss albino mice bearing Ehrlich ascites carcinoma cells through initiation of pro-apoptotic signaling, tumor shrinkage and an increased live span (Rana et al., 2020). Several solvent extracts of *C. album* leaves, have displayed anti-cancerous activity in estrogen dependant breast cancer cells (MCF-7) (Khoobchandani et al., 2009). In human non-small cell lung cancer cells (A549), petroleum ether extracts of *C. album* exhibited anti-proliferative effects via induction of apoptosis through cell cycle arrest at the G1 phase (Zhao et al., 2016).

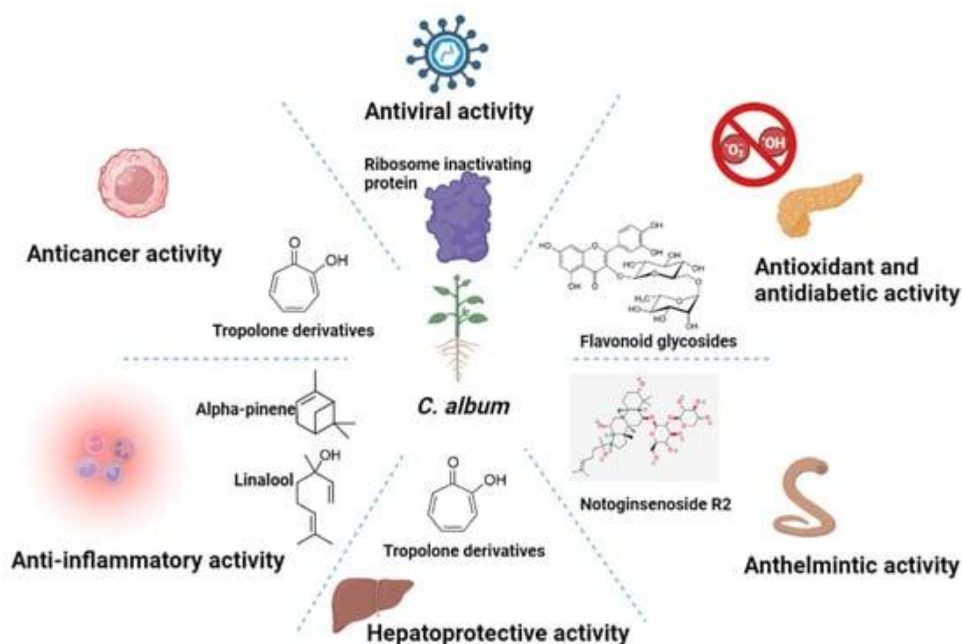

Figure 11: Biological activities of photoactive compounds in *C. album* (Singh et al., 2023)

2.6.2. Caralluma

Introduction:

Caralluma is a genus of plants belonging to the family Apocynaceae, also known as the milkweed family. *Caralluma* species, cultivated in Asia and the Mediterranean region, are recognized for their significant phytochemical composition, which includes numerous components showing strong therapeutic potential i.e. glycosides, saponins, triterpenes, and various flavonoids (Noreen, 2017). These components have been investigated for their therapeutic potential against various pathologies, malignancies and metabolic disorders including cancer, rheumatic pain, obesity, hyperglycemia, hypercholestrolemia, hypertesnion wound healing, gastric problems and infectious diseases (Adnan et al., 2014).

In Pakistan, particularly Punjab, *Caralluma* is known as “Chunga” or “Chung” and has historically served as a famine vegetable in the country’s semi-arid areas (Noreen, 2017).

Anti-Cancerous Role:

The *Caralluma* species are of particular interest to anti-cancer drug development because of a class of compounds known as pregnane glycosides. These have been shown to have anti-tumor activity in recent

years (Si et al., 2022). In a certain study, the impact of several *C. tuberculata* extracts, with varying degrees of refinement, were tested against three types of human cancers. The preparations included unrefined extracts, fractions, and fully isolated compounds. Two novel compounds (a pregane glycoside and an androstane glycoside) were isolated from *C. tuberculata* in this study, and subsequently showed cytotoxic activity via caspase dependent apoptosis in human breast cancer cells and human colonic cancer cells (Waheed et al., 2011).

In our research extracts from both plants, previously unused for high aggressive acute myeloid leukemia cells, have been tested in FKH1 cells. Their prominent anti-proliferative and cytotoxic roles in other forms of human cancers, as well as their well-established phytochemical profiles containing numerous potentially therapeutic compounds make them ideal candidates for testing against aggressive AML.

CHAPTER 3

MATERIALS AND METHODS

3.1. Cell Lines in Study

In this study, two cell lines were used, these included FKH1 and U937.

- The FKH1 cell line model is typically used in research to study Acute Myeloid Leukemia. This cell line is indicated by a specific chromosomal mutation of t (6;9) (p23; q34). The translocation mutation between Chromosome 6 and 9 leads to the formation of DEK/NUP214 fusion gene, also known as the DEK/CAN fusion gene (Zhou and Yang 2014).
- U937 is a cell line model obtained from a patient with histiocytic lymphoma, exhibiting monocytic morphology (Sundström and Nilsson 1976). This cell line is commonly used in research involving AML studies. It does not contain a t (6;9) chromosomal mutation and hence lacks a DEK/NUP214 (DEK/CAN) fusion gene.

Cell Line Used	Description
FKH1	Acute Myelocytic Leukemia Cell line
U937	Human Monocytic Leukemia Cell line

Table 1: Cell Lines Selected for Study

3.2. Culturing of Cells

To avoid contamination and keep cell lines sterile, the cells were cultured in cell culture flasks and were supplemented with pre-warmed Roswell Park Memorial Institute medium (RPMI-1640 medium) made by Gibco life technologies. 10% Fetal Bovine serum (FBS) manufactured by Gibco life technologies, was added into the RPMI medium for U937 cell line (Chanput et al., 2015), while for the FKH1 cell line, 20% FBS was added. For the prevention of bacterial contamination, 1% concentrated Pen-strep solution (by Gibco life technologies) was used which consisted of Penicillin G and streptomycin, to prevent fungal

growth, an antimycotic agent Amphotericin B of concentration 0.25 – 0.5 ug/ml was added to the cell culture medium as well.

The optimum growth of these cell lines requires 95% air, 5% CO2, temperature of 37°C, and a water jacketed incubator that maintained the humidity, temperature, and the atmosphere at optimum conditions.

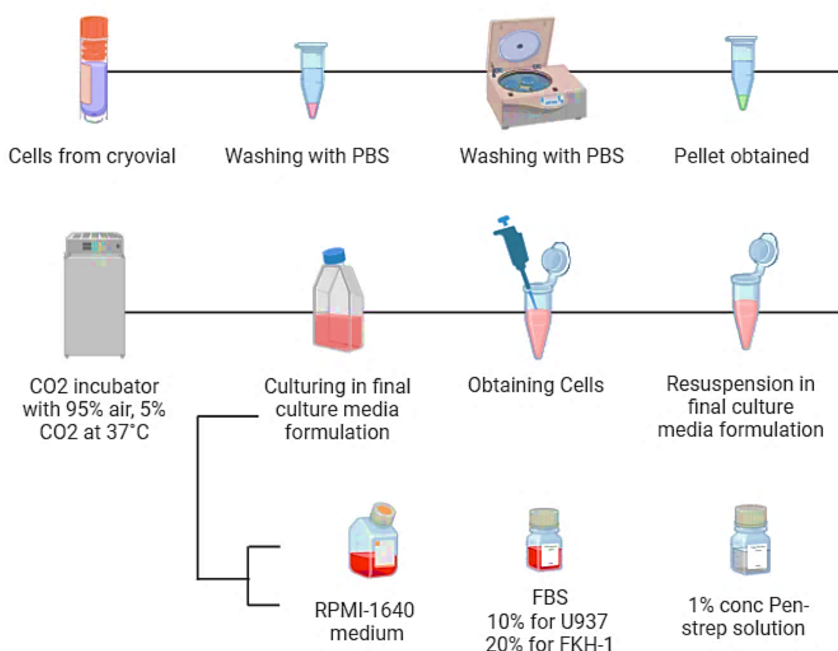

Figure 12: Thawing and Culturing of Cells

3.3. Freezing and Thawing

3.3.1. Freezing cells

Cryopreservation is done to preserve the cells by cooling them to around -80°C temperature (Jang et al., 2017). For this procedure, two solutions were prepared which are denoted as freezing solution I and freezing solution II. Freezing solution I contained 70% (v/v) RPMI and 30% (v/v) FBS while freezing solution II was composed of 80% (v/v) RPMI and 20% (v/v) DMSO. The cells were pipetted into 2 ml Eppendorf tubes and centrifuged for 5 minutes at 1500 rpm to obtain a cell pellet. The supernatant was removed, and PBS was added to the Eppendorf tube without disturbing the pellet. The Eppendorf tubes were centrifuged again, and the supernatant was once more discarded. Through this washing procedure unwanted components present in the sample were removed (Budi et al., 2024). The cell pellet was then

dissolved in 2 ml of freezing solution I and all the content of the Eppendorf was shifted into a properly labelled cryovial. 1 ml of Freezing solution II was added to the cryovial drop by drop as DMSO holds cytotoxic properties and without mixing freezing solution II with the contents of the cryovial, the cryovial was placed in Mr. Frosty kept at -80°C temperature. Mr. Frosty is a freezing container made by ThermoFisher which is filled with 100% Isopropanol alcohol and reduces the temperature of the cryovials by 1°C per minute. The cooling rate of Mr. Frosty provides an ideal condition for cell freezing and after 24 hours, the cryovials were taken out of Mr. Frosty and placed directly in the -80°C freezer.

3.3.2. Thawing Cells

To thaw the cryopreserved cells, the cryovials were first placed in the incubator at 37°C. The cells were then washed once through PBS washing to rid the cells of DMSO that was present in *freezing solution II.* After washing, the fresh culture media was mixed in the cell pellet. The cells were plated in cell culture flasks which were then placed in the CO2 incubator at 37°C and the flasks were oriented in a tilted manner to boost cell growth by minimizing available volume.

3.4. Trypan Blue Exclusion Assay

Trypan blue exclusion assay is a cytotoxic assay is performed to calculate the number of viable cells in a cell population. This assay utilizes a trypan blue dye that stains the dead cells in a blue tint while the live cells retain their original transparent appearance (Strober 2001). This assay was performed before conducting MTT assay to estimate the number of viable cells present in the cell culture flask so that a calculated and required number of cells would be introduced and utilized for the subsequent experimentation.

To execute this assay, firstly 1-3 ml of the cell culture was taken from the cell culture flask and put into 1-3 Eppendorf tubes (1 ml each). The tubes were centrifuged for 5 minutes at 1500 RPM to obtain a cell pellet. The supernatant was removed, and all the pellets of different Eppendorf tubes were concentrated into a final volume of 1 ml fresh RMPI media with 10% FBS and 20% FBS for U937 and FKH1 respectively. For U937, a 4:1 dilution ratio (40 µl U937, 10 µl Trypan Blue dye) was used and for FKH1, a 1:1 dilution ratio (10 µl FKH1, 10 µl Trypan Blue dye) was used. 10 µl of the dilution was loaded into the counting chambers of the hemocytometer and cell counting was done across all four grids of the chamber.

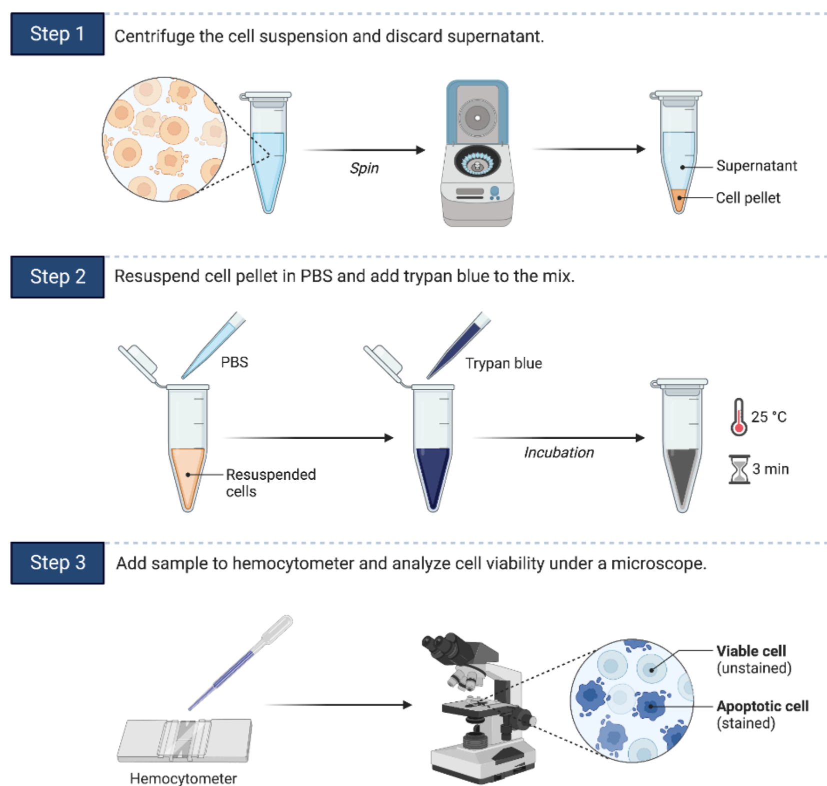

Figure 13: Procedure for Trypan Blue Exclusion Assay

The formula for cell counting through a hemocytometer is as follows:

$$\textit{Viable cell \%} = \frac{\textbf{Number of cells in 4 grids (Grid1+ 2+3 +4)}}{4} \; x \textit{ Dilution factor} \times 10^4 \textit{ cells/ml}$$

3.5. Drug Dilutions

Both the plant extracts *C. album* and *C. tuberculata* were added in DMSO to make a stock solution of 10mg/ml. Different drug concentrations were prepared from the stock solution in order to perform various experiments. Each drug from stocks was further diluted in 90% RPMI and 10% FBS media (80% RPMI and 20% FBS in the case of FKH1). The dilution factor in each growing step of serial dilution remains constant. The protocol of serial dilution from the stock is as follows:

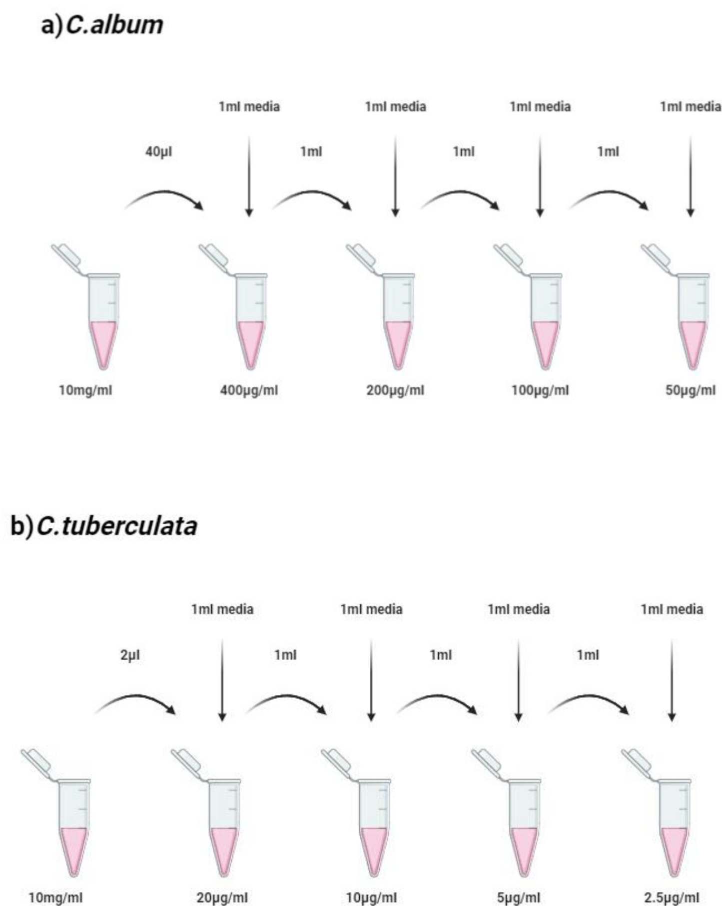

Figure 14: Serial Dilution Concentration for a) *C. album* and b) *C. tuberculata.*

3.6. Cell Viability Assay/MTT assay

MTT assay is performed to calculate cell viability and is essentially a colorimetric assay in nature. In this assay the main reagent is MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) which is a mono-tetrazolium salt. MTT reagent is lipophilic in nature and has a positive charge due to which it can enter the plasma and mitochondrial membranes of viable cells. Inside mitochondria, MTT is reduced to Formazan through the action of NADPH-dependent cellular oxidoreductase enzymes at different stages from the glycolytic pathway to mitochondrial electron transport chain in live cells.

Cells were picked from the culture and added in the 96-well plate with a seeding density of 10,000 cells per 100 µl in each well. After 24 hours, 50 µl of 0.01% DMSO was added into each of the triplicate wells of negative control containing 50 µl of media with 10,000 cells seeded. In triplicate wells of experimental group, 50µl of plant extracts with varying concentrations *of C. album* (200µg/ml, 150 µg/ml, 100µg/ml, 50µg/ml, 25µg/ml and 12.5µg/ml) and *C. tuberculata* (10µg/ml, 7.5ug/ml, 5µg/ml, 2.5µg/ml and 1.25ug/ml) were added to the 50 µl media containing 10,000 cells. A triplicate containing only 100 µl of media was also added to the 96 well-plate to serve as a control to negate any impact of drug solvents or the media. The media used in this triplicate was the same as the one used to make drug dilutions (RPMI media with 10% FBS for U937 and 20% FBS for FKH1). The control group, the treatment groups and the media group all were done in triplicates to remove standard errors. After the plating step, the 96 well-plate was incubated at 5% CO2 and 37°C for 48 hours in the case of U937 and 72 hours in the case of FKH1. After incubation, 15µl of the MTT reagent (5mg/ml) was dissolved in PBS (Made by Bio-west) and was then added into each of the well of the 96 well-plate containing control group, treatment groups and media group. This step was performed to determine the cell viability of the treatment groups after being treated with the plant extracts and compare the viability level with the untreated negative control group. After the addition of MTT reagent, the plate was again incubated at 5% CO2 and 37°C for 3-4 hours to allow the conversion of MTT dye into Formazan crystals. After the second incubation, 115 µl of the media was removed carefully from each well without disturbing the purple crystals that formed and settled at the base of the well and 100 µl of DMSO was added to each well, the crystals were then dissolved in the DMSO through pipetting up-and-down. The plate was again incubated at 5% CO2 and 37°C for 10-15 minutes to properly allow the crystals to dissolve into the DMSO. Using a spectrophotometric plate reader, absorbance value was determined at 550 nm for each of the well and the raw data given by the Magellan plate-reader software was analyzed statistically to obtain results.

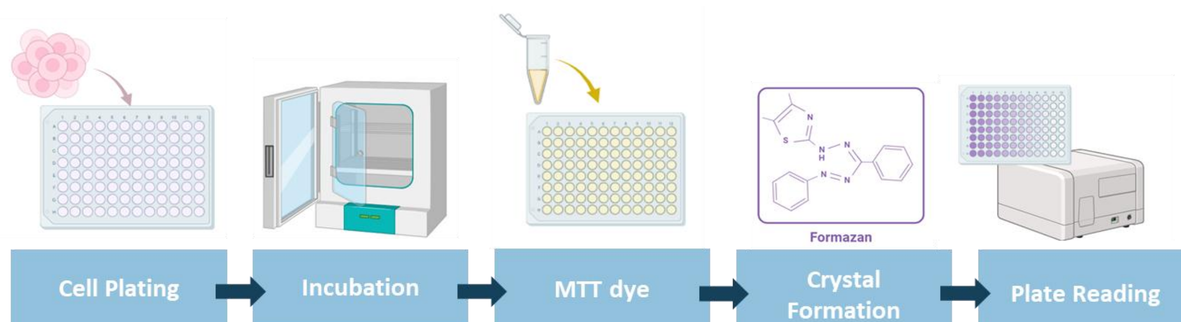

Figure 15: Procedure of MTT Assay

Single target MTTs were also performed that were similar in procedure with the addition of a treatment group containing varying concentrations of Imatinib (0.0625 µM, 0.125 µM, 0.25 µM) which is an anti-cancer drug that specifically targets *ABL* mutations and is typically used to treat Chronic Myeloid Leukemia.

3.7. DNA Fragmentation Assay

DNA Fragmentation Assay is used to measure the anti-proliferative role of a drug on cellular proliferation through confirming whether the drug is causing apoptosis or not. When the cells are treated with a compound that causes cell death through apoptosis, the DNA fragmentation analysis shows DNA smear bands on agarose gel through gel electrophoresis.

For this assay, 1 million FKH1 cells were plated per well. 200µg/ml of *C. album*, 20ug/ml of *C. tuberculata*, and 2-3 µl of DMSO as negative control were added to individual wells. Subsequently, the cells were grown for 72 hours at 37°C with 5% CO2. Following cell treatment, the cells were placed into an Eppendorf tube that were labeled appropriately and they were short-spined for five minutes at 1500 rpm to produce a solid pellet. This pellet was subsequently utilized for obtaining DNA using a DNA isolation kit from Thermo Scientific. After removing the excess supernatant, the cell pellet in each of the three tubes (control, *C. album*, *C. tuberculata*) was mixed with 200 µl PBS, 200 µl lysate solution, and 20 µl Proteinase K. The tubes were agitated to ensure that all the pellets were equally dispersed in the fluid. The tubes were incubated for ten minutes at 56°C and were vortexed periodically to create a homogenous mixture. In each Eppendorf tube 20µl RNase A was added, vortexed, and incubated at room temperature for 10 minutes. 400 µl of 50% ethanol was added to each tube and they were vortexed. The resultant lysate solution was then put onto a DNA extraction column placed in a collecting tube, and it was centrifuged for 1 minute at 6000xg. The DNA specimen stayed within the extraction column while the contents of the collecting tube were discarded. The collecting tube was disposed of and replaced with a new 2 ml collecting tube, 500 µl of ethanol-diluted wash buffer mixture-I was added to the DNA extraction column and the tubes were centrifuged for 1 minute at 8000xg. The flow-through was again disposed of and a new collecting tube was attached to the extraction column. After adding 500µl of ethanol-diluted wash buffer mixture-II to the column, it was subjected to centrifugation once more for 3 minutes at 12000xg. The extraction column was placed into a 1.5 ml Eppendorf tube and the extraction tube along with the flow-through mixture was discarded. 100 µl of elution buffer was added to the extraction column which eluted

the Genomic DNA into the Eppendorf tube. For the extraction of a larger quantity of DNA from the extraction column, the resulting mixture underwent centrifugation two more times at 8000xg for 1 minute after a 2-minute incubation at room temperature. The required genomic DNA was obtained within the Eppendorf tube, which was then kept at -20°C for future use.

3.7.1.DNA Quantification

The concentration and purity of genomic DNA were assessed utilizing Nanodrop 2000 from Thermo Scientific USA. The sample's purity was determined based on the A260/A280 ratio, and the samples having a ratio of 1.8 and a sufficient DNA concentration were used for apoptosis analysis.

3.7.2. Apoptosis Analysis

For the preparation of a 1.5% agarose gel, 0.75g of agarose was dissolved in 50 ml of 1X TAE buffer and heated in a microwave for 1-2 minutes until completely dissolved, ensuring not to overheat and alter the gel percentage. Following this, 4µl of ethidium bromide was added to the solution and allowed to cool for a few minutes. The gel solution was carefully poured into a casting tray to prevent the formation of bubbles and left to solidify for 20 minutes. Once solidified, the gel was placed in a gel tank, and 3µl of DNA ladder was loaded into the first well. Subsequently, 4µl of the DNA sample and 2µl of loading dye were added to each subsequent well. Gel electrophoresis was performed at 90V and 500A for 40 minutes. Gel bands were visualized and analyzed using the ChemiDoc Imaging System.

3.8. Mechanistic Study

3.8.1. FKH1 treatment with *C. album* and *C. tuberculata* extracts

Our research included both U937 and FKH1 AML cell lines, with a core focus on the High Aggressive AML cell line FKH1. Hence for gene expression analysis, only FKH1 was selected. From FKH1 cell culture, cells were poured in a 6-well plate with a seeding density of 1 million cells per well. FKH1 cells were supplemented with RPMI media containing 20% FBS and 1% pen strep. In the 6-well plate, a negative control group was formed that contained 1 million cells exposed to 0.01% DMSO. The rest of the wells were treated with different concentrations of *C. album* (200µg/ml) and *C. tuberculata* (20µg/ml) and after the addition of drugs, the 6-well plate for FKH1 was incubated at 37°C and 5% CO2 concentration for a duration of 72 hours. The purpose of this treatment was to conduct gene expression analysis through

quantitative PCR (qPCR) and so after this treatment, RNA extraction was done followed by cDNA synthesis that was then used in the qPCR technique.

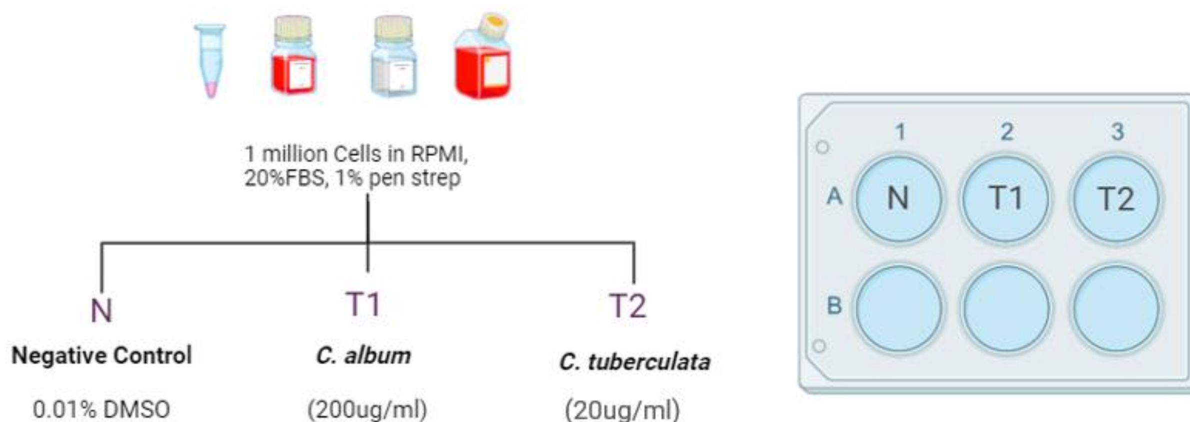

Figure 16: FKH1 Cells Treatment with 0.01% DMSO, 200 ug/ml *C. album* and 20ug/ml *C. tuberculata* for Mechanistic Studies.

3.8.2. RNA Extraction

After treating FKH1 cells with *C. album* and *C. tuberculata* and Imatinib, 1-2 million cells from each of the wells as well as the control were taken into 2 ml Eppendorf and centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded without disturbing the pellet and 500 µl of chilled PBS was added to the Eppendorf tubes. The tubes were again centrifuged at 1200 rpm for 5 minutes. The supernatant was again discarded, and the obtained pellet was dissolved in 1 ml of TRIZOL (life technologies), each Eppendorf was incubated on ice for 5 mins. After incubation, 200 µl of chilled chloroform was added to each Eppendorf and they were vortexed for 15 seconds and again were incubated on ice for 15 minutes. After this step, Eppendorfs were centrifuged at 12000xg and 4°C for 20 minutes. The centrifugation step led to the formation of three distinct phases in the Eppendorf: Aqueous phase (containing RNA), Interphase (containing DNA) and Organic phase (containing proteins).

350 µl volume was picked from the aqueous phase of the Eppendorf and was shifted to new Eppendorf. This phase contained RNA along with some impurities and to remove those impurities, 500 µl of chilled isopropanol was added, the tube was vortexed and incubated on ice for 15 minutes. The Eppendorf were then centrifuged at 12000xg and 4°C for 20 minutes. The supernatant was discarded and a pellet was

obtained that was then rinsed with 75% chilled ethanol and centrifuged at 7500xg and 4°C for 5 minutes. After removing most of the ethanol with a pipette, the remaining ethanol was air dried by keeping the lid of the Eppendorf open and placing it on ice for 20 minutes. The pellet was resuspended in 20 µl of Nuclease Free (NF) water and was put in -80 °C freezer for future use of cDNA synthesis.

3.8.3. RNA Quality and Quantity Assessment

Using Thermo scientific, USA Nanodrop 2000, the quality of the RNA was measured, and the 260/280 ratio was examined which is used to validate the purity of RNA sample.

3.8.4. Synthesis of Complementary DNA (cDNA)

In this technique, the DNA formed is termed Complementary DNA (cDNA) because it is synthesized from an RNA template. The RNA is first converted to DNA through RNA-dependent DNA polymerases and then the DNA copies are amplified through polymerase chain reaction (PCR). This process is regarded as Reverse transcription Polymerase Chain Reaction. For the synthesis of cDNA, 1,000 nanograms of RNA template is used. Subsequently, this procedure requires the addition of 1µl of 10µM random primers to the RNA template (adjusting the total volume to 12µl), 2µl of dNTPs, 1µl of Revert aid, 1µl of ribolock, and 4µl of reaction buffer. This entire reaction mixture is made in a PCR tube and is subjected to a Thermocycler that is set on the experimental parameters as outlined by the kit manufacturer (Thermoscientific).

3.8.5. Gel Electrophoresis for the Confirmation of cDNA Synthesis:

To confirm that cDNA was produced through cDNA amplification procedure, Gel Electrophoresis was performed. In this step, the PCR product, which is the obtained cDNA, was loaded onto agarose gel submerged in a tank containing TAE buffer and was allowed to run across the gel under the influence of electric field. cDNA moves across the gel due to its negative charge and is run parallel to a DNA ladder of known length (kbps), through this we can confirm the presence and quality of the obtained cDNA.

To execute this procedure, 2% agarose gel was required. Firstly, a mixture of 50 ml of 1X TAE Buffer was prepared that contained 1 g agarose, 1 ml of 50x TAE buffer, and 49 ml distilled water. This mixture was microwaved for about 60 seconds and was stirred until it turned clear, and the agarose was fully dissolved in the buffer solution. The solution was allowed to cool down for 10 minutes and 4 µl of ethidium bromide

was added into the solution carefully as it is a hazardous reagent. The glass beaker was then shaken gently to evenly mix the added ethidium bromide. Subsequently, the mixture was poured into a casting tray (with gel combs placed at equal intervals) slowly to avoid the formation of any bubbles. The gel was allowed to cool down at room temperature. After the formation of the gel, it was transferred to a gel tank containing 200 ml of 1X TAE Buffer and 6 µl of PCR product mixed with 2 µl of loading dye was added to each well. 1 kb of Thermo Scientific DNA ladder was used as a marker and was added to a separate well. The gel was run for about 40 minutes at 90 volts and 500 amperes.

3.8.6. Real-time PCR (qPCR) for Gene Expression Analysis:

Real-time PCR technique was used to examine the impact of *C. album* and *C. tuberculata* on the gene expression of the FKH1 cells. To determine the anti-proliferative role of these extracts on FKH1 cells, the genes of interest selected were *c-Myc, Eya3, p53* and *p21*. The forward and reverse primers for these genes of interest are shown in table 2.

Gene/ Primer	Primer Sequence (5'- 3')	GC Content (%)	Annealing Temperature (°C)
Beta Actin Forward	AGCGAGCATCCCCCAAAGTT	55	66.9
Beta Actin Reverse	GGGCACGAAGGCTCATCATT	55	65
C-Myc Forward	CAGCGACTCTGAGGAGGAAC	60	59.8
C-Myc Reverse	TCGGTTGTTGCTGATCTGTC	50	58.2
Eya3 Forward	GGGCAAGAGGAAAGCTGA	55.6	59.9
Eya3 Reverse	GCCACTGAAACCATCTGTTG	50	60.4
p53 Forward	CTTTGAGGTGCGTGTTTGTGC	52.3	65.57
p53 Reverse	GGTTTCTTGGCTGGGGA	58.8	61.69

p21 Forward	TGAGCCGCGACTGTGATG	61.1	64.92
p21 Reverse	CTAGACGCTGGCTCCTCAGTA	57.1	65.35

Table 2: Characteristics of gene specific primers selected for PCR.

The basic principle of this assay was to analyze the genes of interest, which were *p21, p53, Eya3* and *c-Myc* by using specific primers to amplify the product at 10 mM. The control group used for normalization was a housekeeping gene *BETA-ACTIN*. The whole procedure was performed on ice inside a fume hood. Specific PCR strips were created for each primer and the reaction mixture contained 5 µl cDNA, 1 µl forward primer (10 mM), 1 µl reverse primer (10 mM), 12.5 µl of 2X SYBR Green master mix (Solar Bio) and 5.5 µl Nuclease Free water to make a final volume of 25 µl in a qPCR tube. The qPCR tube was short-spined to settle down all the contents at the bottom of the tube and then the tube was placed inside the qPCR machine.

The reaction proceeded through three distinct steps. Initially, the reaction mixture was heated to 50°C for 2 minutes, followed by a subsequent heating to 95°C for 10 minutes. Then, 40 cycles of PCR were initiated. Each PCR cycle involved three incubation phases: Phase I at 95°C for 15 seconds, Phase II at 60°C for 45 seconds, and Phase III at 72°C for 30 seconds. Additionally, a dissociation stage was included to analyze the melt curve, comprising three stages: Stage I at 95°C for 30 seconds, Stage II at 60°C for 30 seconds, and Stage III at 95°C for 30 seconds. Data collection occurred at 72°C. For data analysis, the SDS software for the ABI 7300 system was utilized. The relative quantification of gene expression employed the 2-C technique.

3.9. Statistical Analysis:

The Standard Error of the Mean (SEM) was calculated using both Microsoft Excel and GraphPad Prism 10.2.2 software. Significance values were determined through One Way ANOVA analysis (GraphPad Prism 10.2.2), with P-values below 0.05 being considered noteworthy. For data presentation, GraphPad Prism 10.2.2 and Apache OpenOffice software were utilized.

CHAPTER 4

RESULTS

4.1. Results from Culturing of Acute Myeloid Leukemia Cell lines

The U937 and FKH1 cell lines were successfully revived, cultured, and maintained at the conditions described previously with maximum cell viability.

Figure 17: U937 Cell Culture

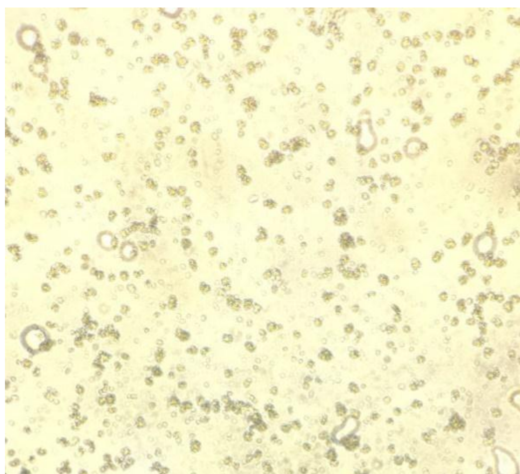

Figure 18: FKH1 Cell Culture

4.2. Effect of *C. album* and *C. tuberculata* on Acute Myeloid Leukemia Cell Viability

4.2.1. Effect of *C. album* on the proliferation potential of FKH1 cells

In this study, the anti-proliferative potential of *C. album* was assessed against FKH1 cells. Cells were treated with different concentrations of *C. album* (12.5µg/ml, 25µg/ml, 50µg/ml and 100µg/ml) and an MTT assay was performed after 96 hours to analyze its effects on the proliferation potential of FKH1 cell line and results were collected. The *C. album* plant extract was found to interfere with the proliferation potential of FKH1 cell lines in a dose dependent manner.

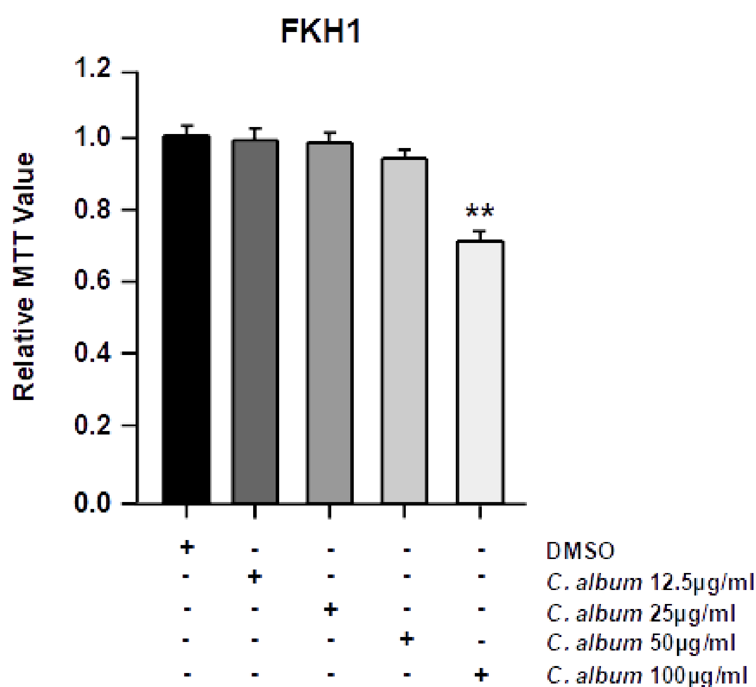

Figure 19: FKH1 Cell Viability Assay for *C. album*. The cells were grown in liquid media (79% RPMI + 20% FBS + 1% Pen-Strep + Amphotericin B) and the specified concentrations of *C. album*. Following 96 hours, cell growth was determined using the MTT test. One-way ANOVA was used to evaluate for statistically significant results ($p < 0.05$). The horizontal lines above each bar show ±SEM.

Increasing the dose concentration of *C. album* extract (50µg/ml, 100µg/ml, 150µg/ml and 200µg/ml) for FKH1 revealed its 1C50 value to be 150µg/ml.

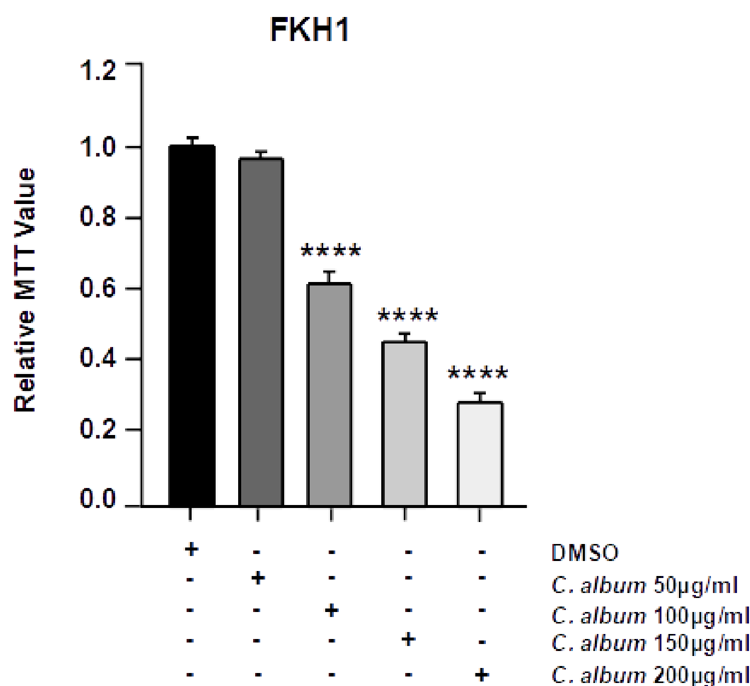

Figure 20: Cell Viability Assay to determine *C. album* IC50 value for DEK-NUP214 positive FKH1 cells. The cells were grown in liquid media (79% RPMI + 20% FBS + 1% Pen-Strep + Amphotericin B) and *C. album* extract concentrations were increased to determine the IC50 value for FKH1 cells. Following 96 hours, cell growth was determined using the MTT test. One-way ANOVA was used to evaluate for statistically significant results ($p < 0.05$). The horizontal lines above each bar show ±SEM.

4.2.2. Comparison between the anti-proliferative effect of *C. album* and Imatinib on FKH1 cells

It has been shown that *ABL* is active in DEK/CAN-positive FKH1 cells (Chiriches et al., 2022). This is significant as Imatinib is used as a frontline therapy for CML and acts as a selective inhibitor of tyrosine kinase that binds to the ATP-binding site of BCR-*ABL* gene (Chandrasekhar et al., 2019). This makes FKH1 a suitable model for analyzing the effects of both Imatinib and *C. album* extract on the anti-proliferative potential of DEK-CAN and *ABL* positive leukemic cells.

FKH1 cells were targeted with different concentrations of Imatinib (0.0625µM, 0.125µM and 0.25µM) and *C. album* extracts (50µg/ml, 100µg/ml, 200µg/ml). Proliferation was assessed through MTT assay after 96 hours to compare the antiproliferative activity of both treatments (Figure, Left: Imatinib and Right: *C. album* extract). No significant difference between the anti-proliferative ability of the two treatments was seen. Both the treatments show almost equal effect on the cell proliferation ability of

FKH1 cells in a dose-dependent manner, though the *C. album* extract required a relatively higher dose concentration compared to Imatinib. Hence both, *C. album* and Imatinib might be an effective therapeutic option in the future for both DEK-CAN and *ABL* positive leukemia through more research and validations.

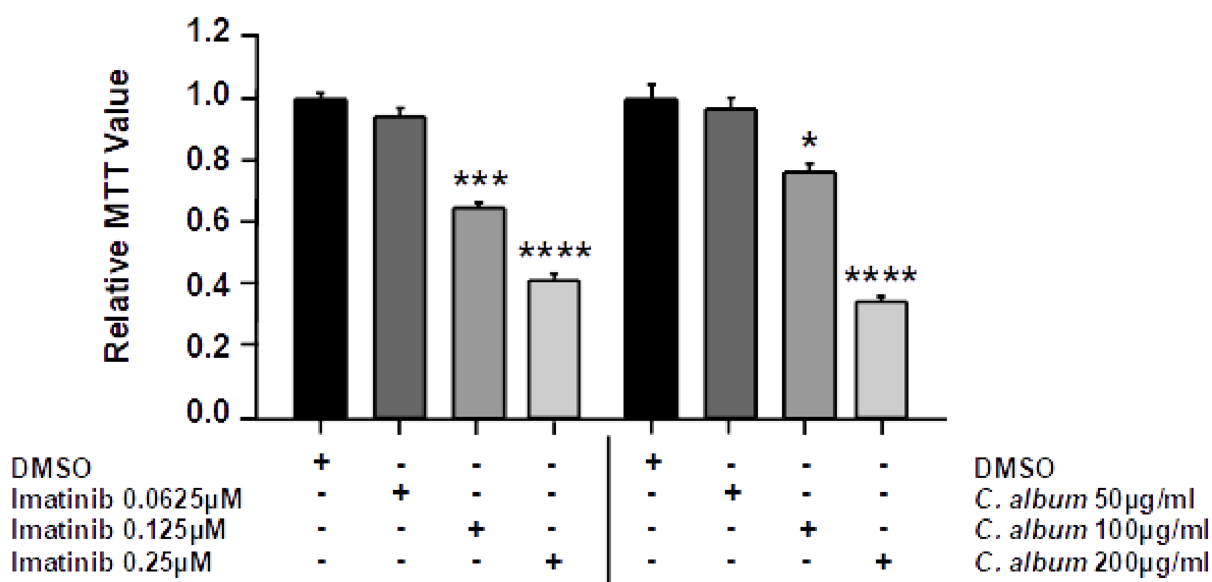

Figure 21: Comparison between Anti-proliferative potential of Imatinib (Left) and *C. album* extract (Right) in FKH1 cells. The anti-proliferative effects of each of the given treatments on DEK-NUP214 and *ABL* positive FKH1 cells were evaluated using MTT assay. One-way ANOVA was used to evaluate for statistically significant results ($p < 0.05$). The horizontal lines above each bar show ±SEM

4.2.3 Effect of *C. tuberculata* on the proliferation potential of FKH1 cells

Similar to *C. album*, the anti-proliferative potential of *C. tuberculata* was assessed against FKH1 cells. Cells were treated with different concentrations of *C. tuberculata* (1.25µg/ml, 2.5µg/ml, 5µg/ml and 10µg/ml) and an MTT assay was performed after 96 hours to analyze its effects on the proliferation potential of cell lines and results were collected. *The C. tuberculata* extract was found to interfere with the proliferation potential of FKH1 cells in a dose dependent manner.

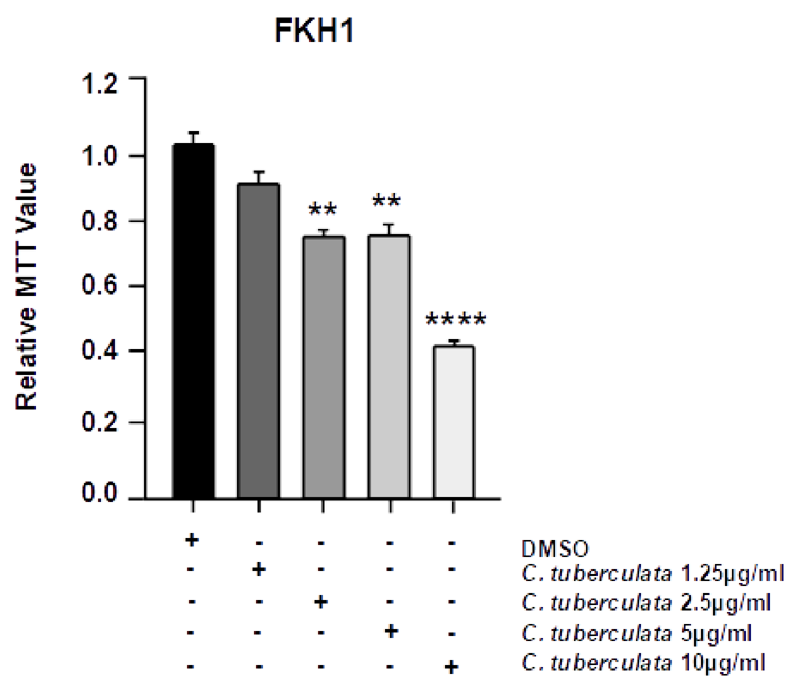

Figure 22: FKH1 Cell Viability Assay for *C. tuberculata*. The cells were grown in liquid media (79% RPMI + 20% FBS + 1% Pen-Strep + Amphotericin B) and the specified concentrations of *C. tuberculata*. Following 96 hours, cell growth was determined using the MTT test. One-way ANOVA was used to evaluate for statistically significant results ($p < 0.05$). The horizontal lines above each bar show ±SEM.

Increasing dose concentration of *C. tuberculata* extract (2.5µg/ml, 5µg/ml, 7.5µg/ml and 10µg/ml) for FKH1 revealed its 1C50 value to be approximately 7.5µg/ml.)

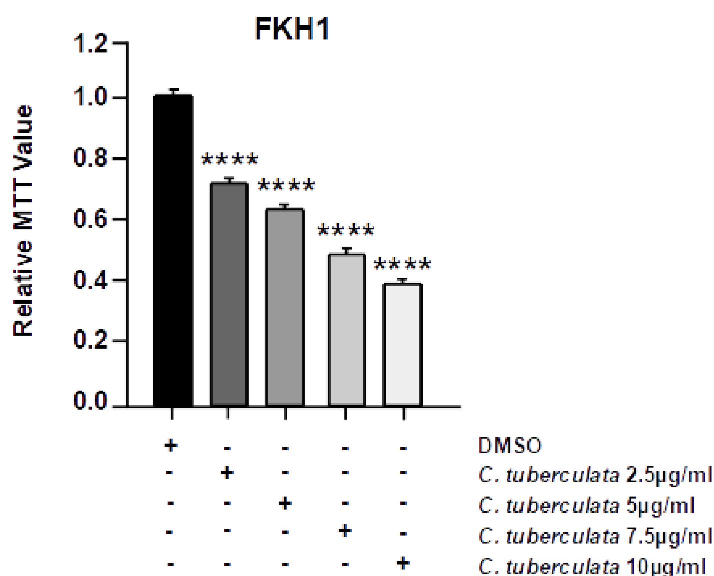

Figure 23: Cell Viability Assay to determine *C. tuberculata* IC50 value for DEK-NUP214 positive FKH1 cells. The cells were grown in liquid media (79% RPMI + 20% FBS + 1% Pen-Strep + Amphotericin B) and *C. tuberculata* extract concentrations were increased to determine the IC50 value for FKH1 cells. Following 96 hours, cell growth was determined using the MTT test. One-way ANOVA was used to evaluate for statistically significant results ($p < 0.05$). The horizontal lines above each bar show ±SEM

4.2.4. Comparison between the anti-proliferative effect of *C. tuberculata* and Imatinib on FKH1 cells

It has been shown that *ABL* is active in DEK/CAN-positive FKH1 cells (Chiriches et al., 2022). This is significant as Imatinib is used as a frontline therapy for CML and acts as a selective inhibitor of tyrosine kinase that binds to the ATP-binding site of BCR-*ABL* gene (Chandrasekhar et al., 2019). This makes FKH1 a suitable model for analyzing the effects of both Imatinib and *C. tuberculata* extract on the anti-proliferative potential of DEK-CAN and *ABL* positive leukemic cells.

FKH1 cells were targeted with different concentration of Imatinib (0.0625µM, 0.125µM, 0.25µM) and *C. tuberculata* extracts (7.5µg/ml, 10µg/ml, 12.5µg/ml). Proliferation was assessed through MTT assay after 96 hours to compare the anti-proliferative activity of both treatments (Figure Left: Imatinib and Right: *C. tuberculata* extract). No significant difference between the anti-proliferative ability of the two treatments was seen. Both the treatments show almost equal effect on the cell proliferation ability of FKH1 cells in a dose dependent manner. Hence both, *C. tuberculata* and Imatinib might be an effective therapeutic option

in the future for both DEK-CAN and *ABL* positive leukemia through more research and validations.

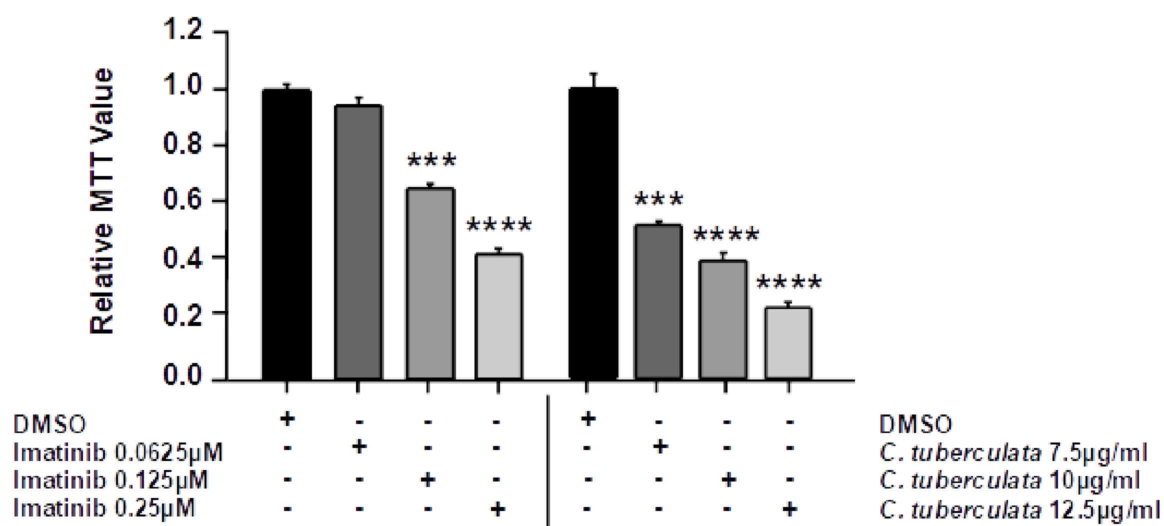

Figure 24: Comparison between Anti-proliferative potential of Imatinib (Left) and *C. tuberculata* extract (Right) in FKH1 cells. The anti-proliferative effects of each of the given treatments on DEK-NUP214 and *ABL* positive FKH1 cells were evaluated using MTT assay. One-way ANOVA was used to evaluate for statistically significant results ($p < 0.05$). The horizontal lines above each bar show ±SEM

4.2.5. Effect of *C. album* on the proliferation potential of U937 Cells

In this study, the anti-proliferative potential of *C. album* was assessed against U937 cells. Cells were treated with different concentrations of *C. album* (12.5µg/ml, 25µg/ml, 50µg/ml and 100µg/ml) and an MTT assay was performed after 48-72 hours to analyze its effects on the proliferation potential of cell line and results were collected.

The *C. album* plant extract was found to interfere with the proliferation potential of U937 cell line in a dose dependent manner.

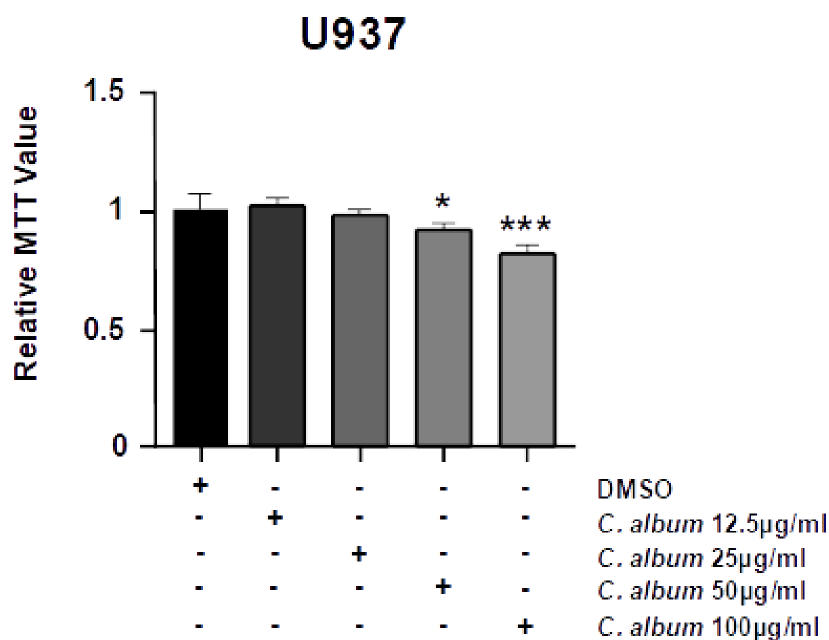

Figure 25: U937 Cell Viability Assay for *C. album*. The cells were grown in liquid media (89% RPMI+ 10% FBS + 1% Pen-Strep + Amphotericin B) and increasing concentration of *C. album*. Following 72 hours, cell growth was determined using the MTT test. One-way ANOVA was used to evaluate for statistically significant results ($p < 0.05$). The horizontal lines above each bar show ±SEM.

4.2.6. Effects of *C. tuberculata* extracts on U937 Cells

The anti-proliferative potential of *C. tuberculata* was assessed against U937 cells. Cells were treated with different concentrations of *C. tuberculata* (1.25µg/ml, 2.5µg/ml, 5µg/ml and 10µg/ml) and an MTT assay was performed after 48-72 hours to analyze its effects on the proliferation potential of cell lines. MTT assay results were collected after 72 hours.

The *C. tuberculata* extract was found to interfere with the proliferation potential of U937 cell lines in a dose dependent manner.

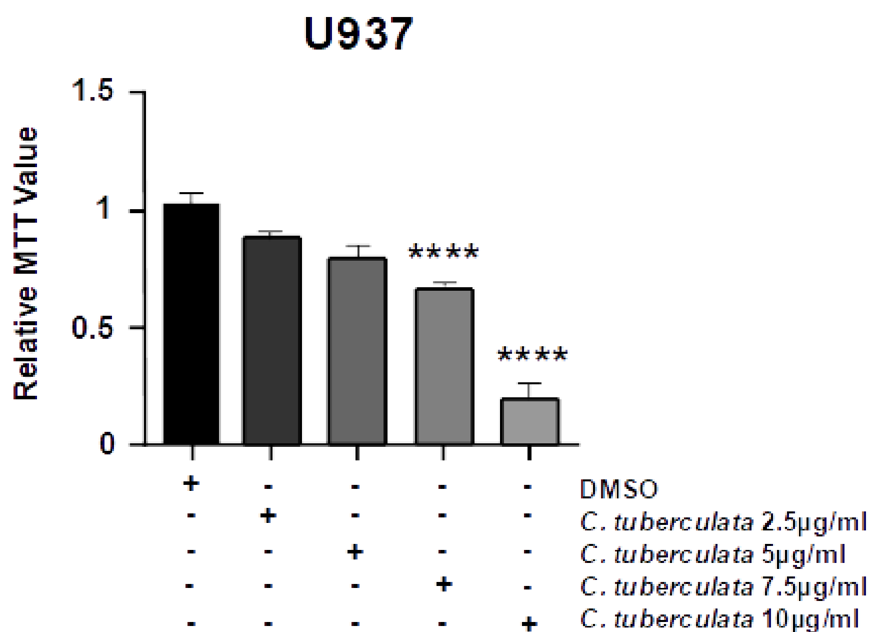

Figure 26: U937 Cell Viability Assay for *C. tuberculata*. The cells were grown in liquid media (89% RPMI+ 10% FBS + 1% Pen-Strep + Amphotericin B) and increasing concentration of *C. tuberculata*. Following 72 hours, cell growth was determined using the MTT test. One-way ANOVA was used to evaluate for statistically significant results ($p < 0.05$). The horizontal lines above each bar show ±SEM

4.2.7. Comparison between the anti-proliferative effect of *C. album* and *C. tuberculata* on U937 cells

U937 cells were targeted with different concentrations of *C. album* extracts (50µg/ml, 100µg/ml, 200µg/ml) and *C. tuberculata* extracts (7.5µg/ml, 10µg/ml, 12.5µg/ml). Proliferation was assessed through MTT assay after 72 hours to compare the anti-proliferative activity of both treatments.

C. album and *C. tuberculata* both interfered with the proliferation potential of U937. However, *C. tuberculata* significantly reduced the proliferation potential of U937 cells at comparatively lower concentrations in a dose dependent manner.

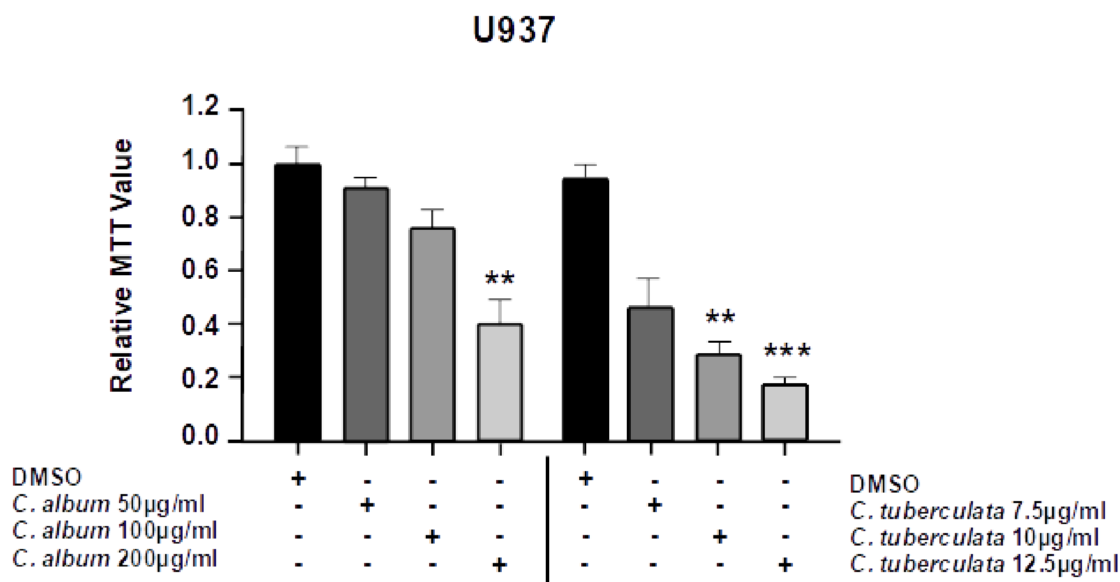

Figure 27: Comparison between Anti-proliferative potential of *C. album* (Left) and *C. tuberculata* extract (Right) in U937. The two drugs were compared for their anti- proliferative effect on U937 cells through MTT assay. One-way ANOVA was used to evaluate statistically significant results ($p < 0.05$). The horizontal lines above each bar show the mean ± SEM.

4.3. DNA Fragmentation Analysis

To determine the exact mechanism through which *C. album* and *C. tuberculata* were imparting their anti-proliferative effect on FKH1 cells, DNA fragmentation Analysis was performed.

FKH1 cells were treated with 200 ug/ml of *C. album* and 20 ug/ml of *C. tuberculata* in separate treatment groups for 72 hours and DNA extraction was performed followed by Gel Electrophoresis. No DNA fragmentation was observed in the *C. album* and *C. tuberculata* treated FKH1 groups as there were no DNA band smears. The results of this experiment are not shown.

4.4. Effect of *C. album* and *C. tuberculata* on Oncogenes Upregulated in DEK-NUP214 positive FKH1 Cells

c-Myc and *Eya3* genes are upregulated in patients having DEK-NUP214 leukemogenesis. *C-Myc* is a proto-oncogene involved in cellular proliferation and cell cycle hijacking. *Eya3* is involved in the stabilization of *c-Myc* by binding to a PP2A enzyme which dephosphorylates and hence stabilizes *c-Myc*.

Gene expression analysis of *c-Myc* and *Eya3* genes was done through qPCR. For this step, FKH1 cells were treated with 200 ug/ml of *C. album* and 20ug/ml of *C. tuberculata*.

4.4.1. Effect of *C. Album* and *C. tuberculata* on *c-Myc* gene expression in DEK-NUP214 positive FKH1 Cells

For the gene expression analysis of *c-Myc* in FKH1 cells, qPCR was done, and the results showed that both *C. album* and *C. tuberculata* downregulated the expression of this gene as compared to its expression in untreated FKH1 control group. *C. tuberculata* showed a stronger downregulation effect on the *c-Myc* gene expression in FKH1 cells at 20 ug/ml as compared to *C. tuberculata* extract's downregulation effect at 200 ug/ml.

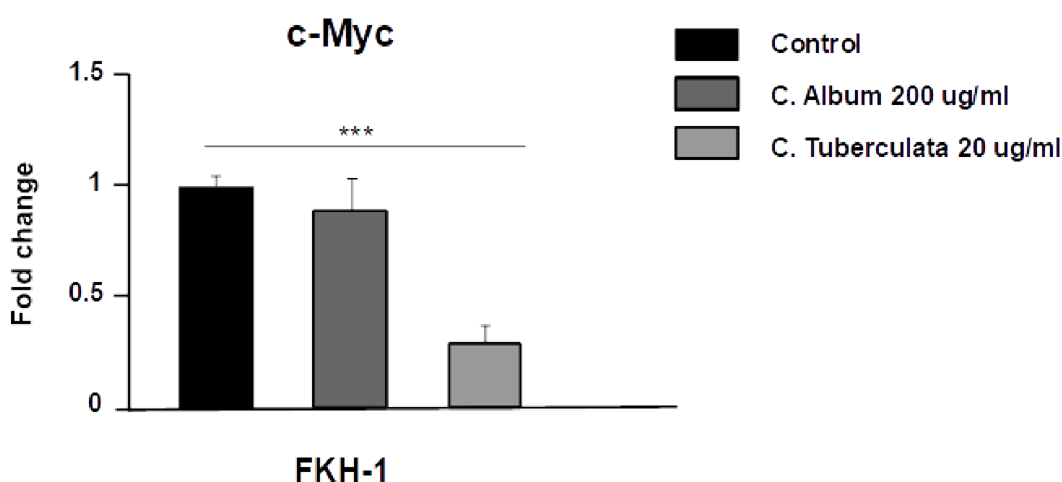

Figure 28: Effect of *C. album* and *C. tuberculata* on *c-Myc* expression in DEK-NUP214 positive FKH1 cells. Cells were cultured in liquid medium (79% RPMI + 20% FBS + 1% Pen-Strep + Amphotericin B) in the presence of 0.01% DMSO and indicated concentrations of *C. album* and *C. tuberculata*. Expression analysis was performed via qPCR on cDNA samples from cells treated for 72 hours. Statistical significance was tested using One-Way ANOVA ($p < 0.05$). Horizontal lines above each bar indicate ±SEM.

4.4.2. Effect of *C. album* and *C. tuberculata* on *Eya3* gene expression in DEK-NUP214 positive FKH1 Cells

For the gene expression analysis of *Eya3* in FKH1 cells, qPCR was done, and the results showed that both *C. album* and *C. tuberculata* downregulated the expression of this gene as compared to its expression in

untreated FKH1 control group. *C. tuberculata* showed a more potent down regulation effect on the gene expression of *Eya3* in FKH1 cells compared to the downregulation effect of *C. album*.

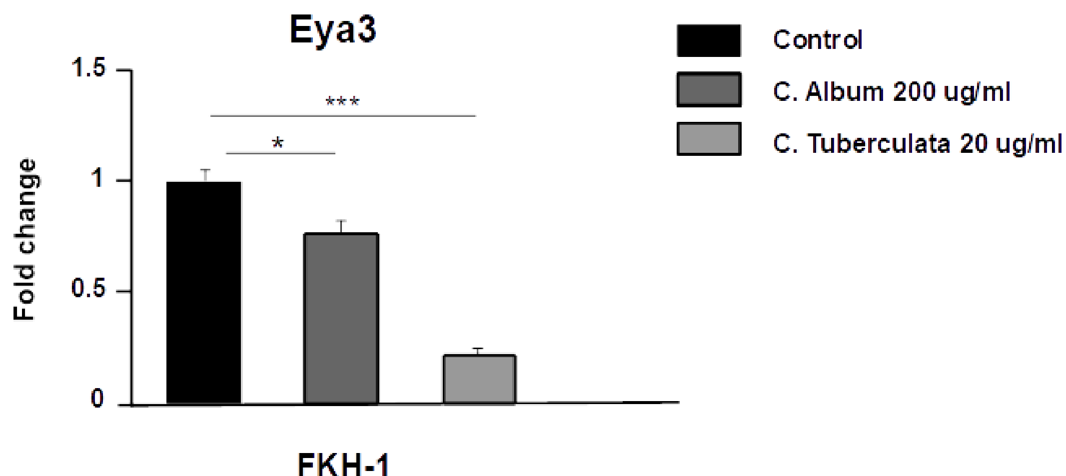

Figure 29: Effect of *C. album* and *C. tuberculata* on *Eya3* expression in DEK-NUP214 positive FKH1 cells. Cells were cultured in liquid medium (79% RPMI + 20% FBS + 1% Pen-Strep + Amphotericin B) in the presence of 0.01% DMSO and indicated concentrations of *C. album* and *C. tuberculata*. Expression analysis was performed via qPCR on cDNA samples from cells treated for 72 hours. Statistical significance was tested using One-Way ANOVA ($p < 0.05$). Horizontal lines above each bar indicate ±SEM.

4.5. Effect of *C. album* and *C. tuberculata* on Cell Cycle regulators in DEK-NUP214 positive FKH1 Cells

p53 is a tumor suppressor gene involved in cell cycle arrest through *p21* mediation pathways and it is also involved in cell apoptosis. *p53* is dysregulated in many cancers that results in uncontrolled cellular proliferation. *p53* is also responsible for *c-Myc* downregulation. *p21* is a cell cycle regulator that is involved in cell cycle arrest. It is the downstream target of *p53*. Both genes are cell cycle regulators.

Gene expression analysis of *p21* and *p53* was done through qPCR. For this step, FKH1 cells were treated with 200 ug/ml of *C. album* and 20ug/ml of *C. tuberculata*.

4.5.1. Effect of *C. album* and *C. tuberculata* on *p53* gene expression in DEK-NUP214 positive FKH1 Cells

For the gene expression analysis of *p53* in FKH1 cells, qPCR was done, and the results showed that both *C. album* and *C. tuberculata* upregulated the expression of this gene as compared to its expression in untreated FKH1 control group. *C. tuberculata* and *C. album* had a comparable upregulation effect on *p53* gene expression in FKH1 cells.

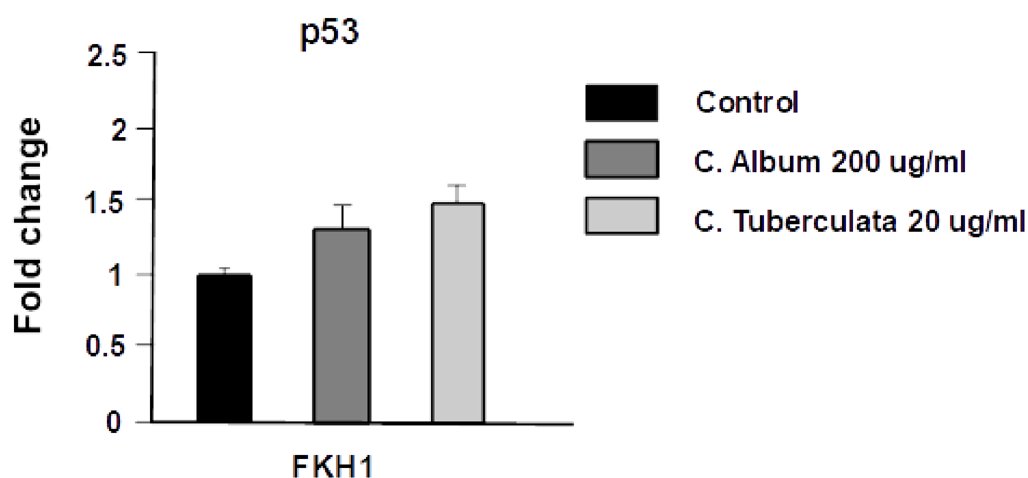

Figure 30: Effect of *C. album* and *C. tuberculata* on *p53* gene expression in DEK-NUP214 positive FKH1 cells. Cells were cultured in liquid medium (79% RPMI + 20% FBS + 1% Pen-Strep + Amphotericin B) in the presence of 0.01% DMSO and indicated concentrations of *C. album* and *C. tuberculata*. Expression analysis was performed via qPCR on cDNA samples from cells treated for 72 hours. Statistical significance was tested using One-Way ANOVA ($p < 0.05$). Horizontal lines above each bar indicate ±SEM.

4.5.2. Effect of *C. album* and *C. tuberculata* on *p21* gene expression in DEK-NUP214 positive FKH1 Cells

For the gene expression analysis of *p21* in FKH1 cells, qPCR was done, and the results showed that both *C. album* and *C. tuberculata* upregulated the expression of this gene as compared to its expression in untreated FKH1 control group. *C. tuberculata*, however, showed a strong upregulation effect on *p21* gene expression compared to *C. album* at a lower concentration.

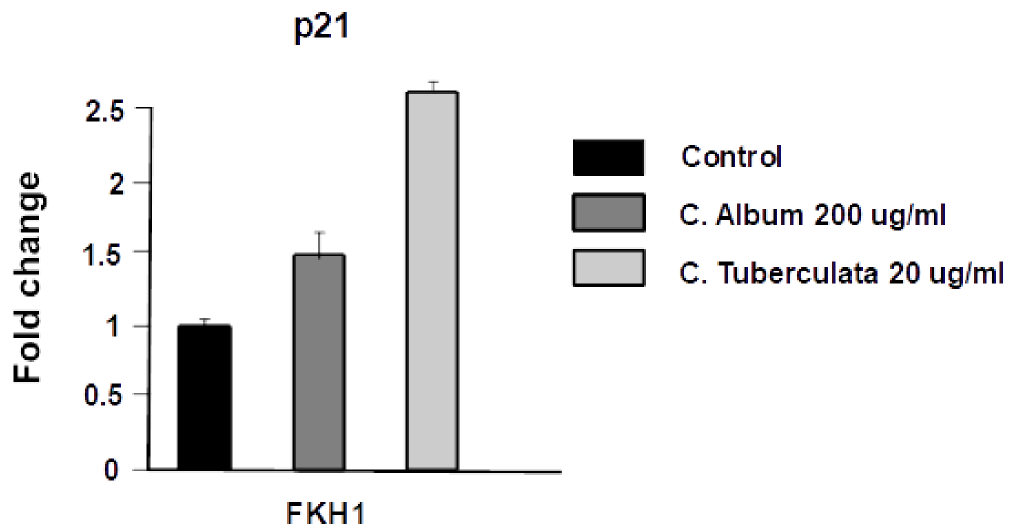

Figure 31: Effect of *C. album* and *C. tuberculata* on *p21* gene expression in DEK-NUP214 positive FKH1 cells. Cells were cultured in liquid medium (79% RPMI + 20% FBS + 1% Pen-Strep + Amphotericin B) in the presence of 0.01% DMSO and indicated concentrations of *C. album* and *C. tuberculata*. Expression analysis was performed via qPCR on cDNA samples from cells treated for 72 hours. Statistical significance was tested using One-Way ANOVA ($p < 0.05$). Horizontal lines above each bar indicate ±SEM.

CHAPTER 5

DISCUSSION

The aim of the present project was to explore the potentially therapeutic effects of *C. album* and *C. tuberculata* against AML cell lines, studying the potential efficacy of these extracts against cell proliferation. For DEK-NUP214 and *ABL* positive AML, we also aimed at comparing the activity of the plant extracts to that of imatinib and determining if the extracts influenced cell cycle regulation or apoptosis.

DEK-NUP214 or DEK-CAN is an AML subtype belonging to the category of AML with recurrent genetic abnormalities. Thus, sub-classification tends to have an overall poor prognosis, lower chance of complete remission, poor overall survival, and greater resistance to chemotherapy. In 2 studies, the overall 5-year survival rates have been reported to be 9 and 28 percent. As compared to other types of AML, this subtype also has a younger median age (Slovak et al., 2006).

Recently, it has also been shown that the incidence of remission, and other prognostic parameters, are highly comparable among patients of high aggressive, DEK-NUP214 positive AML with, or without an accompanying FLT-3 ITD mutation. This signifies the inherent role of the associated translocation in poor patient outcomes. The need for the development of targeted therapies in leukemias is thus coinciding with the identification of relevant targets since cancer cell survival is seemingly independent of certain damaging mutations. Thus, it is imperative to discover therapeutic options against high aggressive AML, that target the pro-oncogenic mechanisms of the cancer cells in an effective way. (Tarlock et al., 2014). Currently, the two treatments for high aggressive AML involve chemotherapy and Hematopoietic stem cell transplant. Chemotherapy has not shown to be effective in the case of t (6;9) AML HSCT, if given in the first remission cycle, has been shown to improve survival (Kayser et al., 2020).

As discussed above, the anti-proliferative effects of *C. album* and *C. tuberculata* have been previously explored in a number of pathologies, including cancer. However, to the best of our knowledge, the efficacy of these compounds against high aggressive AML has not been tested before. FKH1 is a t (6;9) positive cell line, and the only available cell model to study diseases with this translocation. For the purpose of our study, it was used as a model for high aggressive AML.

To understand if the *C. album* and *C. tuberculata* were effective at inhibiting cell proliferation in high aggressive AML, we performed MTT assays at differing concentrations of the two extracts each. Through the results of our MTT assays, it was shown, for the first time, that both *C. album* and *C. tuberculata* have dose dependent a negative effect on the proliferation of FKH1 cells after 72 hours of incubation time. Among the two observed extracts, *C. tuberculata* shows a much stronger effect, with a markedly lower IC50 value. The observed value, as per the MTT, at which *C. album* inhibits cells growth by half in FKH1 cells been observed to be approximately 150ug/ml, whereas the IC-50 of *C. tuberculata* is approximately 7.5ug/ml. Thus, against FKH1, *C. tuberculata* showed much higher efficacy than *C. album*.

Since the therapeutic potential of both *C. album* and *C. tuberculata* extracts have also been explored and validated against other cell lines, it is noteworthy to compare the IC-50 we observed with other pre-established IC-50 values in literature. When compared to the results present against other cancers, the IC-50 that we obtained for C. album is a relatively high value (This statement holds true for most, however not all priorly conducted experiments). This difference could be a consequence of several different factors including changes in the method of extraction or inherent phytochemical changes in the composition of starting plant material. This may also signify that C. album extracts contain compounds with a markedly higher or lower affinity for other targets found in certain human cancers. This parameter requires further validation. In the case of *C. tuberculata*, the previously cited study showed certain interesting findings. While crude extract of *C. tuberculata* showed an IC-50 of nearly 200ug/ml in MTT assays performed on MCF-7, MDA-MB-468, Caco-2, certain compounds isolated from crude extracts showed an IC-50 that resembled that obtained in FKH1 cells.

Ethnopharmacological studies against cancer often begin by testing the potential of crude plant extracts against a particular malignancy. In case of promising results, refining of crude extracts and isolation of compounds that are the active phytochemicals contributing to antiproliferative effects is often the next step (Pirintsos et al., 2022). Our research undertook step one i.e. researching the efficacy of crude extracts with promising anti-cancerous activity, against high aggressive AML. However, in the case of *C. tuberculata*, potent compounds responsible for the anti-cancerous role have been isolated and determined in other studies.

The antiproliferative potential of both the extracts was also tested against that of imatinib. FKH1 cells have been established to harbor several other mutations apart from the primary DEK-NUP214 driver translocation. Among others, the ETV6-ABL1 mutation is especially noteworthy due to its role in the

survival of DEK/NUP-214 positive AML cells. This role is evident through findings that have detected the strong sensitivity of this cell line to inhibition by imatinib. FKH1 cells have been shown to be inhibited by imatinib and dasatinib in a manner similar to K562 cells, which harbor the BCR-*ABL* translocation (Chiriches et al., 2022). Thus, we decided to investigate whether the activity of *C. album* and *C. tuberculata* is comparable to that of imatinib. Our results indicated that both extracts were capable of producing comparable inhibition at their IC-50 values. This is a promising result, as *C. album* and *C. tuberculata* are both edible plants, thus they could provide a safe and effective source for the derivation of anti-cancer drugs in the future.

As a part of our study, *C. album* and *C. tuberculata* extracts were also tested against an *ABL* and DEK-NUP214 negative AML cell line. For this purpose, the U937 cell line isolated from the histiocytic lymphoma of a 37-year-old male patient, and used as a model of pro-monocytic leukemia, was selected. (Sundström & Nilsson, 1976). Thus, in line with our first aim, we conducted MTT assays, to test the antiproliferative effects of *C. album* and *C. tuberculata* in U937 cells. The results indicated that both extracts possessed antiproliferative effects in a dose dependent manner. As was the case with FKH1, comparison studies showed that *C. tuberculata* was reducing the proliferative potential of U937 Cells more significantly than *C. album*.

The second major part of our project undertook mechanistic studies to elucidate the pathways through which *C. album* and *C. tuberculata* bought about their anti-proliferative effects. For this purpose, we chose to compare the expression levels of four genes between treated and untreated cells. Cells were thus treated with 20ug/ml of *C. tuberculata*, and 150ug/ml of *C. album* for a period of 72 hours before expression levels were checked through real time PCR. The four selected genes were: *c-Myc*, *Eya3*, *p53* and *p21*.

c-Myc and *Eya3* are overexpressed in multiple cancers on account of their pro-oncogenic activities and have thus been proposed as therapeutic targets in cancer (Blevins et al., 2015). The over expression of the transcription factor protein Myc has also been shown to be sufficient for the induction of certain types of cancers (Adams et al., n.d.; Stock et al., 2000). Thus, we decided to investigate whether *c-Myc* downregulation was one of the mechanisms through which FKH1 proliferation was being blocked. We observed that both *C. album* and *C. tuberculata* were found to downregulate expression levels of the *c-Myc* gene. However, *C. tuberculata* was found to do so in a highly significant manner, with a P value less than 0.001 showing that the antiproliferative mechanism of *C. tuberculata* likely operates by causing the reduction of *c-Myc* transcription.

In a study of 62 patients with t (6;9) AML, a unique pattern of gene expression was discovered which showed among other genes, increased levels of *Eya3*. *Eya3* has been shown to interact with a subunit of PP2A. PP2A or protein phosphatase 2A is a trimeric phosphatase enzyme. *Eya3* interacts with a subunit of this enzyme and causes it to dephosphorylate *c-Myc* at a specific position. This dephosphorylation of *c-Myc* at PT58 causes it to stabilize, thus allowing it to carry out its various pro-cancerous roles (Sandahl et al., 2014). In our research, we found both the extracts to decrease expression levels of *Eya3* in a significant manner, however, with *C. tuberculata* doing so more significantly. (*C. album* = $p<0.05$, *C. tuberculata* $p<0.001$). This implies that the method through which *C. album* and *C. tuberculata* block proliferation is also influenced by reducing *Eya3* aided oncogenic activity of *c-Myc*. However, as seen above, *C. tuberculata* extracts have been implied to directly decrease *c-Myc* transcription as well, however, further studies are important for corroboration with our results.

The other two genes selected as a part of our mechanistic studies were *p53* and *p21*. *P53* and *p21* are well documented tumor suppressor genes. The *p53* protein is a transcription factor that responds to various forms of cellular stress, including DNA damage, oncogene activation, and hypoxia. It has been referred to as the "guardian of the genome" due to its critical function in maintaining genomic integrity. *p53* achieves this by inducing cell cycle arrest, apoptosis, or senescence in response to DNA damage. This prevents the propagation of genetically altered cells that could give rise to cancer. The cell cycle arrest function of *p53* occurs in a *p21* dependent manner. *p21* is a cyclin-dependent kinase inhibitor that is a prominent transcriptional target of *p53*. *p21* achieves cell cycle arrest by inhibiting the activity of cyclin-dependent kinases (CDKs), which are essential for cell cycle progression. Both these genes are downregulated in cancer (Engeland, 2022).

These genes were selected in order to further corroborate the findings of DNA apoptosis assay, which showed no DNA fragmentation, suggesting that the extracts were showing cell cycle arrest dependent inhibition of proliferation. Both genes were seen to be upregulated after treatment with *C. album* and *C. tuberculata*, with *C. tuberculata* treated cells showing more significant upregulation of both genes. Additionally, *c-Myc* has been shown to be repressed in a *p53* dependent manner, with ectopic expression of *c-Myc* interfering with the ability of *p53* to drive the cell towards cell cycle arrest i.e. *p53* dependent cell cycle arrest has also been shown to depend on the repression of *c-Myc* (Ho et al., 2005). Thus, the elevated *p53* levels, and reduced *c-Myc* levels, further indicate that the anti-proliferative mechanism of our extracts, particularly *C. tuberculata*, can be ascribed to cell cycle arrest via *p21*.

CONCLUSION

C. album and *C. tuberculata* induced antiproliferative effects in AML cell lines of FKH1 and U937. *C. tuberculata* had a more potent antiproliferative affect in FKH1 cell line by showing a lower IC-50 value of approximately 7.5µg/ml as compared to c album with IC-50 value of approximately 150µg/ml. In FKH1 cell lines, both *C. album* and *C. tuberculata* show similar cytotoxicity in comparison to imatinib which is a known inhibitor of cell growth in the *ABL* positive FKH1 cell line. DNA fragmentation analysis of FKH1 cells after treatment with *C. album* and *C. tuberculata* revealed no apoptosis, while real time expression analysis showed low levels of proto-onco genes *c-Myc* and *Eya3* and elevated levels of tumor suppressor genes *p53* and *p21*. The results overall indicated the potential antiproliferative mechanism of *C. album* and *C. tuberculata* to be cell cycle arrest. Thus, it can be stated that both of these extracts are potential therapeutic options in both DEK-NUP214 and *ABL* positive, as well as negative leukemias.

FUTURE PROSPECTS

Our study confirms the antiproliferative role of *C. album* and *C. tuberculata* on FKH1 and U937 and the roles of *c-Myc*, *Eya 3*, *p53* and *p21* in the induction of antiproliferation through cell cycle arrest which can be further confirmed through cell cycle specific analysis. Moreover, to confirm our mechanistic studies at translational level western blotting is required. Additional in silico investigations are advised to study the plant active ingredients with other proteins and the reproduction of results in mouse models. Further research is required to be done to confirm our findings on patient derived (primary) DEK-NUP214 positive cells.

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