An experimental approach to unearth the immunomodulatory aspects of *Withania somnifera* towards altered physiology and Hedgehog signalling in the bone marrow of leukemic mouse

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PROFORMA 1

"Statement of Originality"

I, **Sayantan Ghosh** registered on **September 24th, 2019**, **Registration number: SLSBT1110619; Ref.No. D-7/SC/423/2020** do hereby declare that this thesis entitled "**AN EXPERIMENTAL APPROACH TO UNEARTH THE IMMUNOMODULATORY ASPECTS OF *WITHANIA* *SOMNIFERA* TOWARDS ALTERED PHYSIOLOGY AND HEDGEHOG SIGNALLING IN THE BONE MARROW OF LEUKEMIC MOUSE**" contains literature survey and original research work done by the undersigned candidate as part of Doctoral studies.

All the information in this thesis have been obtained and presented in accordance with existing academic rules and ethical conduct. I declare that, as required by these rules and conduct, I have given fully cited and have referred all materials and results that are not original to this work.

Signature of Candidate

Date

Certified by Supervisor

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Certificate from supervisor

This is to certify that the thesis entitled "**An experimental approach to unearth the immunomodulatory aspects of *Withania somnifera* towards altered physiology and Hedgehog signalling in the bone marrow of leukemic mouse"** submitted by **Mr. Sayantan Ghosh**, who registered on **24.09.2019** for the award of **Ph.D** (**Life Science and biotechnology**) degree of **Jadavpur University** is absolutely based upon his own work under my supervision and that neither his thesis nor any part of the thesis has been submitted for any degree/diploma or any other academic award anywhere before.

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Declaration of originality and compliance of academic ethics

I hereby declare that the thesis contains a literature survey and original research work by the undersigned candidate as part of the Doctor of Philosophy (Ph.D.) course.

All information in this research work have been obtained and presented as per the academic and ethical rule.

I also declare that as per the rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

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Titles of the thesis: **An experimental approach to unearth the immunomodulatory aspects of *Withania somnifera* towards altered physiology and Hedgehog signalling in the bone marrow of leukemic mouse"**

Signature with date

I dedicate this thesis to my respected and beloved

Maa and Baba

Mon coeur

Dadu

&

The mice who sacrificed their life for science

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Contents

Introduction & aims and objectives

List of tables

List of figures

Chapter 03

Ashwagandha and Withanolide D improved physiological and hematological scenario in the bone marrow of NOC induced leukemic murine model

Chapter 04

Ashwagandha and Withanolide D modulated components of deregulated Hedgehog signalling pathway in NOC induced leukemic bone marrow cells

List of Abbreviations

5'UTR- 5' untranslated region

ALL-Acute lympholytic leukemia

AML- Acute myeloid leukemia

AMPK-AMP-activated protein kinase

AGO- Argonaute,

ATG12- Autophagy-related protein

β-TrCP - Beta-transducin repeat-containing E3 ubiquitin

BMI-1-B-lymphoma Moloney murine leukaemia virus insertion region-1

BCL2- B-cell lymphoma 2

CD 90- Cluster of differentiation 90

CD 31- Cluster of differentiation 31

CML-Chronic myeloid leukemia

CTD- C-terminal domain

CYLD- Cylindromatosis

DC – Differential count.

DHH - Desert Hedgehog

DGCR8- DiGeorge syndrome critical region gene 8

EC_{50}- Effective concentration 50

ENU- N-ethyl-N-nitrosourea

FBS- Foetal bovine serum

FGF-fibroblast growth factor

GAS1-growth arrest-specific 1

GSK-3β- Glycogen synthase kinase 3β

GLI – Glioma associated oncogene

HH – Hedgehog pathway

HHIP – Hedgehog interacting protein

HSPC - Hematopoietic stem and progenitor cell

HPLC- High performance liquid chromatography

IHH – Indian Hedgehog

KEAP1- Kelch-like ECH-associated protein 1

LD_{50}- Lethal dose 50

MEKK1-Mitogen-activated protein kinase kinase 1

MFI- Mean fluorescence intensity

mRNA- messenger RNA

miRNA – microRNA

MTT- 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

N- CAD- Neural Cadherin

HhN - N-terminal Hh signalling domain

NOC- N-Nitoso compounds

NRF2- Nuclear factor erythroid 2-related factor 2

N-MYC- Neuroblastoma-Derived V-Myc Avian Myelocytomatosis

P62/SQSTM1- Sequestosome 1

PECAM-1- Platelet and endothelial cell adhesion molecule -1

PTCH – Patched

PUMA- p53 upregulated modulator of apoptosis

PBS- Phosphate buffer saline

RPMI-1640 –Roswell Park memorial institute 1640

RBC – Red blood cells/corpuscles

RNAi- RNA interference

RISC -RNA- induced silencing complex

RLC- *RISC-loading complex*

SHH – Sonic Hedgehog

SMO – Smoothend

SUFU – Suppressor of fused

TC – Total count

TRAF2-TNF receptor-associated factor 2

TERT- Telomerase reverse transcriptase

Wnt – Wingless

WBC –White blood cells/corpuscles

WD – Withanolide D

ABSTRACT

N-nitroso compounds (NOC) is a potent DNA alkylating agent having serious carcinogenic effects on biological system. Environmental exposure to NOC can impart genomic insults to the bone marrow cells leading to the malignant transformation of hematopoietic stem/ progenitor population as well as to the haematopoiesis supportive microenvironmental components resulting in the development of leukaemia. In this study, we would like to unearth the detrimental consequences of NOC-induced leukaemia and the ameliorating effects of Ashwagandha and its isolated counterpart, Withanolide D, on the dreadful disease condition.

Four groups of animals were taken for experimental purposes viz; group I= Ethyle nitroso urea (ENU) mediated leukemic mice (L); group II=control mice (C); group III = leukemic mice treated Ashwagandha (L+A); and group IV =leukemic mice treated with extracted Withanolide D, (L+WD). Group III and IV received Ashwagandha and isolated Withanolide D respectively via oral route, and the other two groups received an equal volume of distilled water. Various physiological, haematological, cytological, immunofluorescence, and flow cytometric studies were taken into consideration before and after the administration of Ashwagandha and Withanolide D. Various doses of Ashwagandha and Withanolide D were administered to evaluate the LD_{50} and EC_{50} values. Estimated LD_{50} and EC_{50} values of Ashwagandha were 1175 mg/kg bodyweight and 600mg/kg bodyweight, respectively, whereas the LD_{50} and EC_{50} values of Withanolide D were 75 mg/kg bodyweight and 37.5 mg/kg bodyweight, respectively.

A significant decline in locomotor activity and bodyweight was noted due to the leukemic insult caused by ENU. Haematological profiling depicted an increase in WBC count, reticulocyte count, abnormal blasts, and other parameters indicative of leukemic pathophysiology. The reflection of the disease condition was further noted in the cytological analysis. Excessive cellular proliferation, the presence of abnormal cells, and significantly fewer dead cells were key indicators. Upon further investigation, various cellular signalling pathways and key cytological processes were found to be deregulated. Components of the hedgehog signalling pathway, viz; SHH, PTCH, SMO, and GLI1, were over-expressed, while expressional decline was observed in case of HHIP, SUFU, and GLI3. Overexpression of GSK3β and β-TrCP was found to be associated with the activation of the NRF2/KEAP1 axis. Simultaneously, expressional increase of key cytoskeletal proteins, viz; β actin, β tubulin, N-

cadherin, and vimentin, was also noted. Over expression of RNAi machinery components viz; DICER, DROSHA, AGO1, and AGO2, was found to be associated with the malignant transformation of bone marrow during leukaemia. LIN28, a key component modulating many cellular functions was also found to be up-regulated in diseased condition, which can be correlated with the over-expression of N-MYC oncogene. Molecular components associated with autophagy viz; P62 and ATG12, were also found to be down-regulated which might have aided in the accumulation of defective proteins as well as diminished the DNA repair mechanism. Elevated levels of BCL2, TERT, and decreased expression of PUMA, Caspase 3, CD11b, and CD95 can be correlated with the malignancy associated anti-apoptotic phenomenon. Thus, a wide range of molecular expressional alterations was found to be associated with the ENU mediated leukemic transformation of the bone marrow.

With the administration of Ashwagandha and the isolated Withanolide-D, body weight and locomotory activity were found to be shifted towards normalcy in the treated. The confirmation of the positive effects of Ashwagandha and Withanolide D was also noted in the haematological as well as in case of cytological profiling. Expressions of Hedgehog signalling components were found to be shifted towards their normal values. The NRF2/KEAP1 signalling axis was also found to be modulated, which imparted a co-modulatory effect on the components of the hedgehog signalling cascade, viz; SHH, SUFU, GLI1, etc., with a shift towards normal condition. Expressions of cytoskeletal proteins in the phytochemical treated groups were also found to be shifted towards normal values which can be correlated with the disruption of spindle structure, microtubular pathways, and protein-protein interactions in the treated leukemic cells resulting in checking of abnormal proliferation and migration. Simultaneously, expressions of RNAi components as well as that of tumorogenic N-MYC and BMI-1 were also found to be declined significantly with the effect of Ashwagandha and Withanolide-D. Expressional restoration of autophagic machineries viz; P62 and ATG12 were also noted. Malignancy related anti-apoptotic phenomenon also got reversed by the effects of the phytochemicals as shown by the expressional alterations of TERT, BCL2, Caspase 3, CD11b, CD95, and PUMA.

Introduction & aims and objectives

INTRODUCTION

Haematopoiesis is a meticulously regulated process in which hematopoietic stem cells (HSCs) differentiate into various specialized blood cells such as red blood cells (erythrocytes), white blood cells (leukocytes), and platelets (thrombocytes). This process begins with the proliferation and differentiation of HSCs, which are multipotent cells capable of self-renewal and the generation of a variety of progenitor cells with distinct developmental destinations (Mikkola et al., 2006). Under the influence of numerous growth factors, cytokines, and signalling molecules, these progenitors mature into particular blood cell lineages.

The regulation of haematopoiesis is finely tuned to ensure a proper balance between different blood cell types. For instance, erythropoietin stimulates the production of red blood cells, while granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) influence the formation of white blood cells. This harmonious interplay ensures that the body maintains sufficient levels of each blood cell type for optimal functioning (Winkler et al., 2012; Chatterjee et al., 2015).

Leukemia, a cancer that develops from the bone marrow and blood-forming organs, is a striking example of the consequences that can occur when hematopoiesis fails. Genetic mutations or chromosomal abnormalities disturb the normal process of blood cell development and proliferation in leukemia. As a result, immature and dysfunctional blood cells known as leukemic blasts rapidly accumulate in the bone marrow and bloodstream, crowding out healthy cells and impeding the formation of functional blood cells (Maillard et al., 2008).

Leukemia is classified as acute or chronic based on the rate of disease progression and based on the type of blood cell involved i.e., myeloid or lymphoid lineages. Acute leukemias are characterized by the fast proliferation of immature cells that do not mature into functioning blood cells, whereas chronic leukemia is characterized by the overproduction of mature but defective cells. The existence of these defective cells causes a wide range of complications, including anaemia from decreased red blood cell synthesis, susceptibility to infections from

impaired white blood cell activity, and bleeding tendencies from low platelet levels (Chattopadhyay et al.2018).

Understanding hematopoiesis and its sophisticated regulatory processes have had a substantial impact on the development of leukemia treatments. Chemotherapy, targeted treatments, and stem cell transplantation have emerged as critical strategies for combating leukemia by either removing leukemic cells or restoring normal blood cell production balance. In this study, we explored the relationship between disease etiology, haematological disruption, and modulation in important signalling components during a NOC-induced leukemic mouse model and effects of Ashwagandha on the same after administration. The study's findings are expected to contribute significantly to the development of effective therapeutic modalities for the dreadful disease.

AIMS AND OBJECTIVES OF THE STUDY

The present study broadly aims in delineating the intricate mechanism of Hedgehog signalling pathway in the bone marrow and microenvironment in experimentally induced leukemia following therapeutic modulation of aforementioned signalling pathway along with cell cycle inhibitors, apoptotic scenario, autophagy mechanism, cyto-architectural modulation, RNAi machinery and hematopoietic niche structure by Ashwagandha (*Withania somnifera)* and/or its bioactive compound, Withanolide D.

With the view of above, the work proposal employs the following objectives:

1. Establishment and characterization of Swiss albino mouse model of leukaemia by ENU administration and determination of $LD_{50,}$ and EC_{50} value of *Withania somnifera* in control and experimental mice.
2. Study of Hedgehog signalling pathway following therapeutic modulation by *Withania somnifera.*

3. Unearthing the intricate microenvironmental status within bone marrow with special reference to niche structure in leukaemia.
4. Assessment of cyto- architectural arrangement and apoptotic mechanism in the bone marrow cells following therapeutic intervention.
5. Expressional study of RNAi machinery and autophagy mechanisms in the diseased and treated bone marrow cells.

Chapter 01

Literature review

CHAPTER SUMMARY

This chapter of the thesis will provide in-depth insights into the history of cancer with special focus on leukemia and its pathophysiology, demographics, and therapeutic regimens. The overarching purpose of this chapter is to convey the existing information on N-Nitroso compounds and their devastating effect on hematological physiology, as well as the pathophysiological conversion of the same in leukemia in the context of the Hedgehog signalling system. The chapter also mentioned prospective characteristics of Ashwagandha that could potentially be explored in order to uncover the therapeutic techniques during the dreadful disease in discussion.

1. INTRODUCTION

1.1. The history of Cancer and leukaemia

Life is all about balance. It is a finely tuned equilibrium between creation and destruction. When this very balance is off to any side it commences a cascade of events that lead to either excessive proliferation or destruction and both are detrimental to thriving life. This very essence of life is also applied to the cells and tissues of our body too. In Cancer, cells evade the fine balance of creation and destruction and as a result it proliferates abnormally.

In medicine, the earliest written description of diseases and cancer is found in the Edwin Smith Papyrus, which was written approximately 3000 BC and documented a case of breast cancer ("The Edwin Smith Surgical Papyrus, 1930). 1500 years later, the Ebers Papyrus depicts the first reference to a soft-tissue tumour, a fatty tumour, and includes references to possible cancers of the stomach, skin, uterus, and rectum as well (Hajdu, 2004). The Egyptians not only detected the disease, but they also attempted to treat tumours and cancers with cautery, knives, and salts. The introduction of arsenic paste by the Egyptian physicians remained in use as ‘‘Egyptian ointment’’ until the 19th century. Hippocrates (460–375 BC) and his disciples believed that cancer was initiated by natural causes. They rationalised that cancer may be induced by excess or deprivation of mucus, blood, bile, and other body secretions, particularly at old age (Elgood, 1939). Hippocrates found similarities between cancerous growths and a moving crab, which led to the term karkinos." Aulus Celsus (25

BC–AD50) was an influential Roman physician who made Latin the language of medicine. He continued the Hippocratic tradition by comparing cancer to a crab that adheres to surrounding structures with his claws and translating the Greek word into Latin, cancer (Sarton, 1931).

In later years, great advancements in the detection, description, and treatment of cancer and other diseases were documented. Pliny the Roman (AD 23–79) compiled remedies for cancer. In his book, Materia Medica, he recommended various herbal and other remedies before or after attempted surgery in the advanced stage of cancer (Churchill, 1857). Aretaeus (AD 81–138) first noted down the comprehensive description of symptoms, signs, and treatment of uterine cancer. In his notes, he depicted that there were two distinct forms of cancer: one was foul-smelling and ulcerated, whereas the other was firm to the touch and non-ulcerated. Both forms of tumours were associated with swelling and pain in the groin. According to his notes, both lesions are chronic and deadly, but the ulcerated one was worse with no chance of cure (Die Geschichte Unserer Pflanzennahrung Von Den Urzeiten Bis Zur Gegenwart," 1928, Churchill; 1857). After Aretaeus, the whole known world at that time came under the influence of Claudius Galen (130–200). Though he contributed to the understanding of various diseases, including cancer, he also named swellings and fleshy tumours with the appearance of raw meat as sarcoma, but his dogmatic theories delayed advances in understanding the nature of cancer and its treatment for several centuries (Sarton, 1931; Thomas, 1954).

Baghdad and Constantinople became the centers of knowledge after the decline of the Roman Empire. Oribasius of Baghdad (325–403) noted that cancers were mostly painless and were not as red as inflammatory lesions. Aetius (527–565), physician to Emperor Justinian in Constantinople, introduced the treatment of breast cancer by amputation of the whole breast. He applied cautery for the treatment of non-ulcerated lesions of the cervix, labia, and anus, but he did not prescribe any treatment for ulcerated cancers (Ricci, 1950). Paulus of Aegina (625–690) was aware of hepatic and gastrointestinal cancers. He was well versed in the surgical excision of cancers but was advised not to because they are incurable. Rhazes of Baghdad (860–932), who practiced surgery and introduced new operative techniques and instruments, advised surgeons that no surgery should be attempted if the obstruction was caused by cancer unless the cancer could be excised completely ("Arabian Medicine. Edward G. Browne, Thomas Adams's," 1921). Avenzoar (1070–1162), a Jewish physician who

practiced in Cordova, Spain, described the signs and symptoms of cancer of the esophagus. He first introduced esophageal and gastric cannules for relief of strictures caused by cancer and also for injection of nourishment into the patient (Hajdu, 2005).

Theodoric (1205–1296), a bishop and physician, advised that cancers can be excised widely with healthy tissue around them because of the unknown anatomic extent of tumours (Hajdu, 2005; Berdoe, 1893). Lanfranc (1252–1315) was the first to differentiate benign tumours of the breast from cancer. Henri de Mondeville (1260–1320) rejected Galen's nearly 1000-year-old theories and classified cancers into simple and compound forms on the basis of the history of prior lesions. He also introduced the classification of cancers with consideration of anatomic site, size, and superficial or deep location of the tumours (Hajdu, 2005; Hajdu, 1981; Sarton, 1929). He also conceptualized the effects of external carcinogens on the development of cancer. Another French surgeon, Guy de Chauliac (1300–1388), delineated hot and cold lesions. The hot abscessic growths were pustules, abscesses, and gangrenes, whereas the cold ones were identified as cancers ("HENRI DE MONDEVILLE.," 1895). He administered wide excisions for the treatment of operable cancers. John Arderne (1307–1399) recommended mucus, blood, and constriction as cardinal signs of cancer and advised only local excision for cancers. (Arderne 1910).

The dawn of the Renaissance in Europe opened up new avenues in the arts and sciences, including medicine. Antonio Benivieni (1443–1502) published the first case report of cancer. Paracelsus (1493–1541) and Gabriele Fallopius (1523–1562) pioneered chemistry and chemotherapy in various cancers. Ambroise Pare (1510–1590) also implemented chemotherapy to ameliorate cancer and described cancers as far more common and dangerous in women than in men. Jean Fernel (1497–1558) and Felix Platter (1526–1614) described the differentiation methods of carcinomas and sarcomas. Marco Aurelio Severino (1580-1656), Peter Lowe (1552-1612), Zacutus Lusitani (1575-1642), and Nicholas Tulp (1593-1674) contributed to the understanding of various cancers and their contagious nature. The findings of Jean Riolan (1577–1657), Johannes Scultetus (1595–1645), Guilielmus Hildanus (1560–1644), Fredericus Ruysch (1638–1731), Charles Le Clerc (1644–1727), and Deshaies Gendron (1663–1750) greatly influenced the genesis, detection, and differentiation of various cancers along with treatments (Sarton, 1931; Hajdu, 2005; Hajdu, 2010).

During the 4000 years of noted history, physicians and surgeons who cared for cancer patients were the pioneers in oncology. They all contributed greatly to the understanding of the development, detection, diagnosis, and treatment of cancer.

Until 1800, detection and treatment of cancers were limited to various soft tissue carcinomas and sarcomas. In 1811, Peter Cullen, a surgeon, reported a case of splenitis acutus" (acute hyperplasia of the spleen). He examined a 35-year-old male patient who presented with pronounced abdominal pain and symptomatic fever, along with the milky appearance of blood serum. Cullen believed that due to the rapid absorption of fat, a natural emulsion had formed in the patient's blood. However, he did not have any plausible explanation for the milky serum and its relation to the acute hyperplasia of the spleen (Cullen 1811). With our current knowledge, Cullen most likely described a case of a patient suffering from chronic leukemia for the first time.

Alfred Armand Louis Marie Velpeau reported a detailed case report in 1827 with symptoms of pronounced swelling of the abdomen, fever, weakness, and urinary stones. Contrary to Peter Cullen's findings, the first accurate description of a leukemia patient at post-mortem dissection was provided by Velpeau. He described the symptoms of leukemia as being related to the increase in white blood cells (milky serum). Velpeau speculated on an association between this particular disease and the circulatory system (Velpeau, 1827; Dunn, 2005).

A minor contribution to the understanding of leukaemia at that time was provided by Duplay in 1833. He found a large amount of pus in the arterial and venous systems, along with slight hypertrophy in the liver and intestinal walls (Duplay, 1834).

In 1839, Alfred Francois Donné, a pioneer in microscopy for the study of disease, examined the blood of a patient with splenomegaly. He reported that half of the corpuscles in the blood appeared like "mucous globules". According to him, the accumulation of mucous or purulent globules (leucocytes) in the blood had been mistaken for pus by the physicians. He stated that due to the differentiation arrest of the white blood cells, excess globules were formed. Donné was the first physician to perform an accurate microscopic examination, description, and illustration of the altered composition of the blood observed in acute-phase leukemia patients (Donne, 1844).

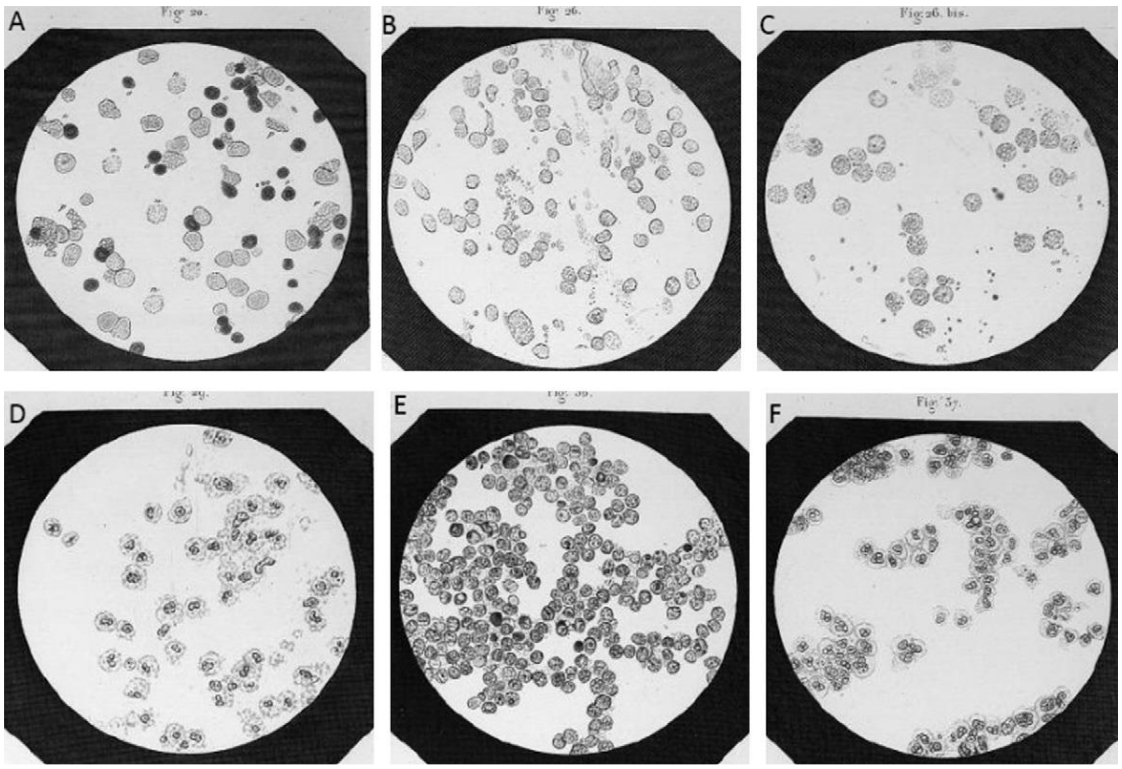
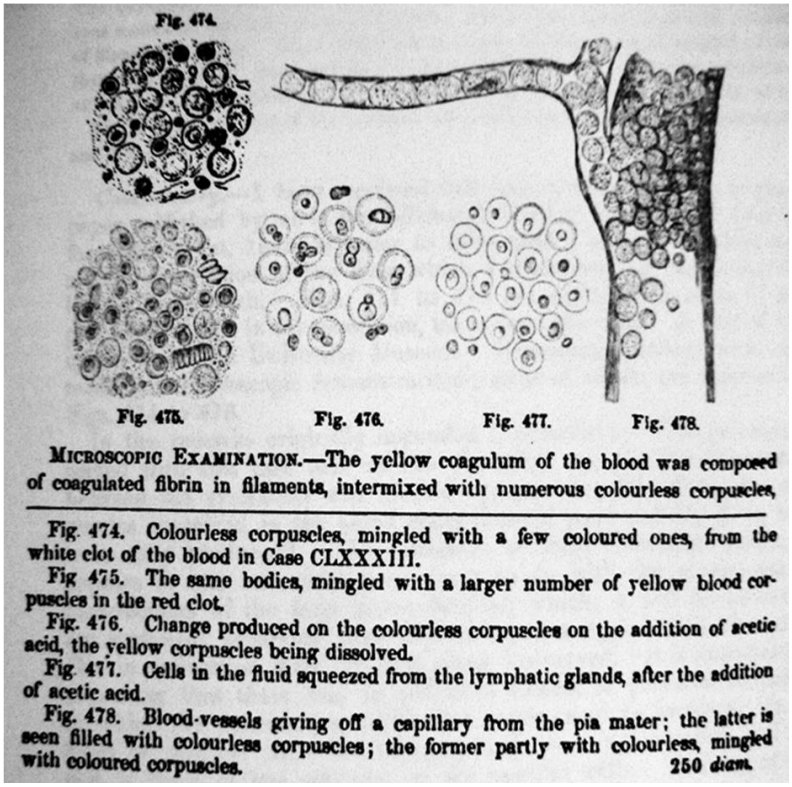


Figure. 1. The globules that Donné presented in the supplementary Atlas published in 1845. These figures demonstrate the similarity between the globules of pus and mucous globules. (A) Presentation of white globules (white blood cells) in the blood, extracted from a leukemic patient. (B and C) Mucous globules (purulent globules). (D) Mucous globules after acetic acid treatment to visualize the nuclei. (E) The globules of pus. (F) Globules of pus after acetic acid treatment. (Kampen, 2012)

In 1845, John Hughes Bennett and David Craigie individually reported that the serum of the blood was composed of coagulated fibrin filaments mixed with numerous colourless corpuscles (white blood cells) (Bennett 1845, Craigie 1845).

The most decisive moment in the history of leukemia came when Rudolf Ludwig Karl Virchow published his case report in 847 (Virchow,1856). He described the altered appearance of the blood components as a reversed balance between normal levels of erythrocytes and leukocytes. This reversal in balance may suppress the formation of red blood cells in diseased patients. In Virchow's opinion, the white blood cells did not display a purulent origin, and he also termed this disease Leukämie' (Virchow, 1856). At the same time, in London, Henry William Fuller and his colleague Cantab published a case report describing enlargement of the spleen and liver, dilation of all the blood vessels of the body,

and an altered condition of the blood in a male patient (Fuller & Cantab, 1845). After the first report in 1846, Fuller reported another case in 1850, which is of particular historical interest. He depicted a 9-year-old girl with frequent haemorrhage, spongy gums, and acute splenomegaly, and her haematological analysis showed a large number of colourless, granular, spheroidal globules. This may be the first recorded case of childhood leukemia.



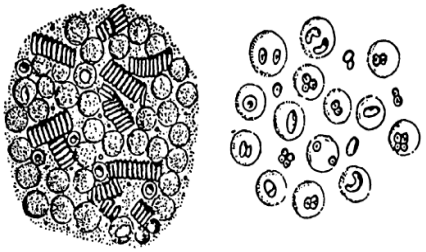


Figure 2. A. Bennett's colourless corpuscles in the blood of a patient suffering from leukemia. Fig. 478 demonstrates the accumulation of the colourless corpuscles in the circulatory system. These figures were included in the manuscript where Bennett described leucocythemia as a primary systemic disease of the blood in 1845(Kampen, 2012). B. The microscopic appearance of blood as seen by J. H. Bennett, Edinburgh (Piller, 2001).

In France, six consecutive meetings were held in Paris during 1855–1857 by some of the leading physicians at that time. This lasting debate gradually accepted leukemia as a distinct disease, and in the following years, published case reports increased in number. Clinical and pathological descriptions of the disease became more detailed, but the aetiology remained elusive (Friedreich, 1857).

A vital discovery came in 1868 when Ernst Neumann reported changes in the bone marrow of leukemia patients and established the link between the bone marrow and the source of blood. He devoted his whole life to understanding and studying the origin of blood pigments. His study led to two fundamental discoveries: that the marrow is the source of blood formation and that it is a continuous process. At the same time, his findings were supported by the studies conducted by Giulio Bizzozero. Neumann continued his studies and reported leukemia as a disease of the marrow (Neumann, 1872).

The next significant step in the understanding of leukemia was noted when Paul Ehrlich developed a triacid stain that provided a clear definition of the nucleus, cytoplasm, and other details of the blood cells in a thin dried film for the first time. (Ehrlich, 1877). His findings classified leukemia into two varieties: the lymphoid group (lymphocytes) of non-granular cells and the myeloid group (granulocytes) of cells from the bone marrow. He also depicted the primitive cell; according to him, it was a cell in a semi-transformed state, an ancestor cell from which other cells were developed into distinct cellular lineages. An ancestral cell in the hemopoietic system had been described for the first time, probably the earliest concept of the stem cell.

In 1900, the Swiss hematologist Naegeli made an important discovery that supported Ehrlich's view that the various cell lineages were distinct (Naegeli, 1900). He described a new cell in the myeloid cell line, which he named the myeloblast, and he showed this to be an ancestor.

Table I. Some known cases from the available literature on cases of uncommon or peculiar alteration of the blood before the identification of leukaemia as a disease (Piller, 2001).

Date	Physician/Surgeon	Patient	Sex	Age	Place	Reference
1811	P. Cullen	Thomas Halke	Male	35	Skegness	Cullen (1811)
1827	A. Velpeau	Mr. Vernis	Male	63	Paris	Velpeau (1827)
1829	M. Collineau		Male	29	Paris	Collineau et al (1829)
1833	M. A. Duplay	Josephine Guevis	female	27	Paris	Duplay (1834)

1839	Barth	Housewife	Female	44	Paris	Dreyfus (1957)
1839	Rayner		Male?		Paris	Donne (1844)
1841	D. Craigie	Peter Campbell	Male	30	Edinburgh	Craigie (1845)
1845	Rudolf Ludwig Karl Virchow	Marie Straide	Female	50	Berlin	Virchow (1845)
1846	Fuller and Cantab	Thomas Windsor	Male	22	Manchester	Fuller and Cantab (1856)

Since 1800 advancement in science and technology greatly expanded our understanding of the origin, etiology and pathophysiology of leukemia.

1.2. DEMOGRAPHICS OF LEUKEMIA: WORLD AND HOME

Cancer ranks as leading cause of death and a major barrier to the life expectancy in almost every country in the world. According to a report by World Health Organization (WHO) in 2019 there were estimated 309 thousand deaths and 437 thousand new cases of cancers were recorded due to leukemia worldwide (Sung et al., 2021).

Table II. New Cases and Deaths in 2020.

Incidence	**NO. OF NEW CASES (% OF ALL SITES)**	**NO. OF NEW DEATHS (% OF ALL SITES)**
Leukemia	474,519	311,594

Table III. Incidence and Mortality Rates (Age-Standardized Rate, Cumulative Risk) for 24 World Areas and Sex for leukemia in 2020.

Cancer site	INCIDENCE					
	MALES			FEMALES		
	Cases	Age standardized Rate (world)	Cumulative Risk, ages 0-74 years%	Cases	Age standardized Rate (world)	Cumulative Risk, ages 0-74 years, %
Leukemia	269,503	6.3	0.59	205,016	4.5	0.41
	MORTALITY					
	MALES			FEMALES		
	Cases	Age standardized Rate (world)	Cumulative Risk, ages 0-74 years, %	Cases	Age standardized Rate (world	Cumulative Risk, ages 0-74 years, %
	177,818	4.0	0.38	133,776	2.7	0.26

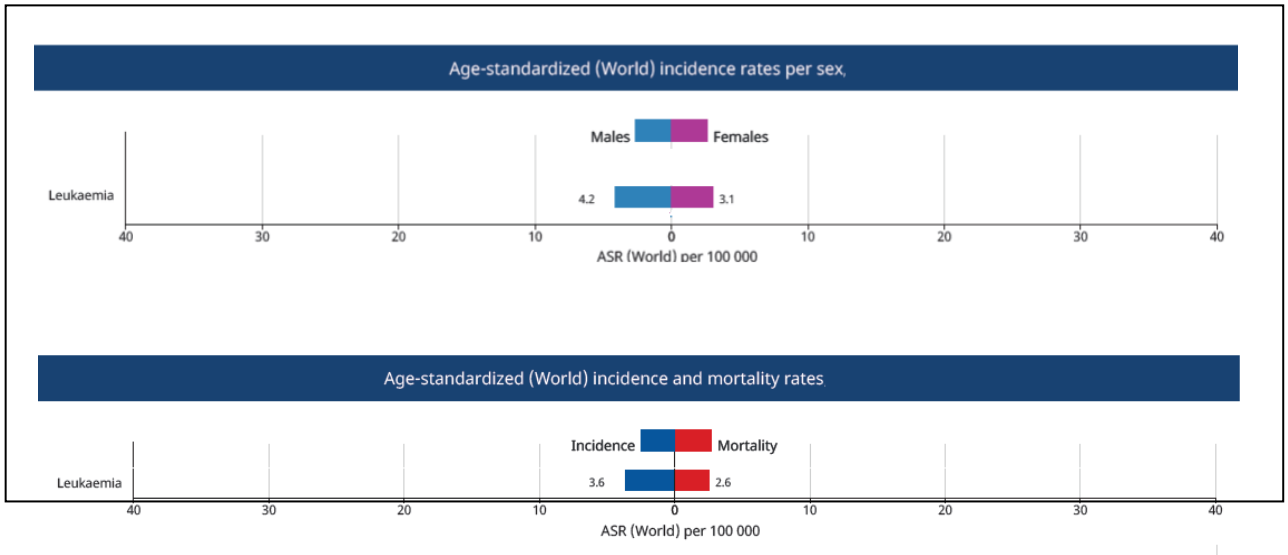


Figure 3. Age standardized incidence of leukaemia and mortality rate per sex worldwide (Globocan, 2020)

Table IV. Indian scenario of leukemia in 2019 (source; India, Globocan, 2020)

Cancer	New cases				Deaths				5-year prevalence (all ages)	
	Number	Rank	(%)	Cum.risk	Number	Rank	(%)	Cum.risk	Number	Prop. (Per 100 000)
Leukaemia	48,419	7	3.7	0.31	35,392	7	4.2	0.24	127,493	9.24

India is developing at a fast speed. To match this speed of development and to provide food to almost 1.4 billion people demand of crops has increased, at the same time use of artificial fertilizers, chemical pesticides, genetically modified food etc. has also increased. All these cumulatively increases and keep on increasing rate of incidence of various types of cancers including leukemia.

1.3. LEUKEMIA: ETIOLOGY AND CLASSIFICATION

Leukemia is classified into four major groups. Etiology of the disease is still shrouded under the veil of mystery, but numerous studies revealed many aspects of the disease.

1.3.1. Acute Myeloid Leukemia (AML)

Around the world, highly developed regions have a greater incidence of AML. Age-standardised incidence is 2.7 and 2.0 per 100,000 for males and females, respectively, in the United Kingdom, 2.7 and 2.2 per 100,000 in Austria, and 2.8 and 2.0 per 100,000 in Australia. Miranda-Filho et al. (2018)

White people are more likely than other ethnic and racial groupings to develop AML and die from it. According to studies, the age-adjusted incidence in white males is 5.4 per 100,000 in SEER (Surveillance, Epidemiology, and End Results), compared to 4.5 per 100,000 in blacks and 4.1 per 100,000 in Hispanics (Noone et al. 2017). The death rate (3.8, 2.7, and 2.3 per 100,000 for whites, blacks, and Hispanics, respectively) shows a similar racial pattern. These racial and ethnic patterns are consistent across all age and sex categories, according to SEER data (Zhao et al. 2018).

The precise genetic mutation that caused AML to grow is still mostly unknown. The most important initiating causes of the disease are smoking, chemotherapy, radiation therapy, and other environmental exposures. According to diagnoses made by patients who underwent chemotherapy for their first primary malignancy, the incidence rate of AML was 4.7 times higher (Morton et al. 2013). In an early case-control study of AML in breast cancer patients, Curtis et al. (1992) showed relative risks of 2.4 for receiving radiation treatment alone, 10.0 for alkylating drugs, and 17.4 for combined radiation-alkylating agent regimens. Smoking in adulthood is a significant risk factor for AML.

Numerous meta-analyses describe increased AML risk in smokers. According to Ugai et al. (2018), present and former smokers have an elevated risk of AML of 40% and 25%, respectively, compared to non-smokers. According to recent studies, occupational exposure to benzene in the United States is most strongly linked to environmental-induced leukemia. It is one of the most commonly used compounds in the rubber, oil refining, shoe manufacturing, and gasoline industries (Carlos-Wallace et al. 2016; Raaschou-Nielsen et al. 2018).

1.3.2. Acute Lymphocytic Leukemia (ALL)

While the prevalence of other leukemia normally rises with age, the incidence of ALL is clearly bimodal, with paediatric populations experiencing higher incidence rates than older age groups. As with other leukemia, all exhibit a male predominance. The incidence is generally steady (about 1 per 100,000) throughout adulthood, increasing to 1.9 per 100,000 in elderly persons aged 80–84 (Noone et al. 2017). According to a study (Miranda-Filho et al. 2018), the incidence of ALL is highest in nations in South and Central America, including Ecuador (2.8 and 3.3 per 100,000 for males and females, respectively), Costa Rica (2.4 and 2.3 per 100,000), and Colombia (2.3 and 2.1 per 100,000).

As the most prevalent chromosomal translocation in adult ALL (present in 25% of patients), the Philadelphia chromosome, or t (9; 22), differs from paediatric ALL in that it has a much more aggressive clinical course (Gleissner et al. 2002; Pui et al. 2004; Thomas and Heiblig 2016). Numerous risk factors, such as chemotherapy (for primary malignancies, for example), ionising radiation (from therapy or exposure to atomic bombs), and chemical

poisons like benzene, are the same for both adult and paediatric ALL. We still don't know the precise risk pathways associated with these exposures.

1.3.3. Chronic Myeloid Leukemia (CML)

A recent study depicted that CML made up about 15% of leukemia diagnoses in the US in 2018 (Siegel et al. 2018). Although the most common age of diagnosis is between 65 and 74 years of age (comprising 21% of CML diagnoses), the incidence rate continuously rises with age and peaks at 10.3 cases per 100,000 among people in the 80-to-84-year age group.

Due to the reciprocal translocation t (9; 22) (q32; q11) caused by the shortening of chromosome 22, the Philadelphia chromosome is cytogenetically linked to the pathogenesis of CML. Ionising radiation exposure is the only other known risk factor for CML besides advancing age. 1960 (Heyssel et al.).

1.3.4. Chronic Lymphocytic Leukemia (CLL)

The incidence of CLL is highest in France and Canada, two nations with relatively high HDIs, where the annual rate of new cases per 100,000 males is above 4. Particularly in Malaysia (0.1 per 100,000), Japan (0.1 per 100,000), and the Philippines (0.2 per 100,000), incidence is remarkably low in Asian nations. CLL has more sex-specific abnormalities than other leukemias. Globally, the prevalence of CLL in men is over two times higher than in women (Miranda-Filho et al. 2018).

People over 65 had a 67% rate of CLL diagnoses, which indicates that the risk of the disease is substantially age-dependent. The incidence for people aged 65 and older is 26.4 per 100,000, and for people aged 85 and older, it is 35.8 per 100,000 (Noone et al. 2017).

The best and most reliable indicator of hereditary genetic pathways in CLL aetiology is a family history of hematologic malignancy. According to extensive research by the Swedish Cancer Registry, relatives of CLL patients have 7.5 to 8.5 times increased risk of incidence than that of controls (Goldin et al., 2004b, 2009). There are currently no known germline alterations that serve as a chance antecedent to CLL (Goldin and Caporaso 2007). Monoclonal B-cell lymphocytosis (MBL), which is more common in high-risk CLL families than the general population (Rawstron et al. 2002; Goldin et al. 2013), may be an early

genetic component indicative of hereditary propensity. The fact that CLL is uncommon among Asians, both domestically and internationally, supports the idea that there is a significant hereditary component to illness risk.

Agent Orange, a dioxin-containing herbicide used in Vietnam, is linked to CLL, despite the fact that data on chemical exposures are often conflicting (Institute of Medicine 2009). CLL is typically regarded as being non-radiogenic, in contrast to other leukemias. Major challenges in observational studies that look at radiation (and environmental exposures) as a risk factor for CLL include a lack of diagnostic specificity, a decades-long latency period for CLL and SLL, historical underreporting bias, a low case-fatality rate, and difficulty obtaining an adequate sample size (Richardson et al., 2005; Bispo et al., 2019).

1.4. THERAPEUTIC STRATEGIES AGAINST LEUKEMIA: PAST TO PRESENT.

Therapeutic intervention started immediately after the detection of the disease. Historically, treatment of cancer started in Egypt approximately 3000 years BC. The aetiology of the disease remained elusive for more than 4800 years, and because of that, the implementation of a proper therapeutic regimen remained ineffective most of the time. In 1930, a physician based in Zurich, Dr. Gloor, met an American businessman with anaemia, fever, and an elevated level of white blood cells, a classical representation of acute leukemia. He was treated with arsenic, mesothorium, radiation, and blood transfusions, which resulted in complete remission of the disease. The curative effect of treatment might be due to the blood transfusion, which acted as the first stem cell transplantation, rather than the effects of anti-leukemic agents alone (Beutler, 2001; Lo-Coco et al., 2013).

Table V. Development of anti-leukemia treatments (adapted from Paul S. Gaynon, Toska J. Zomorodian, and Donald Pinkel. History of leukemia: Historical perspectives. In childhood Leukemias: Third edition, by Ching-hon Pui, Ed. Cambridge university press 978–0–521-19661-1**).**

Year	Development
1865	Heinrich Lissauer administered potassium arsenite to a woman with chronic myelogenous leukemia
1895	Radiation therapy was administered with transient benefit

1930	Walther Gloor cured the first leukemic patient with arsenic trioxide, irradiation, mesothorium and transfusion
1943	Isolation of folic acid
1948	Nitrogen mustard for Hodgkin disease; Antifols: aminopterin then amethopterin (methotrexate) for acute lymphoblastic leukemia
1951	Adrenocorticotropic hormone then prednisone for acute lymphoblastic leukemia
1953	Mercaptopurine, methotrexate licensed by the FDA
1955	Prednisone licensed by FDA
1958	Dexamethasone licensed by FDA
1958	Cyclophosphamide licensed by FDA
1963	Vincristine licensed by FDA
1969	Cytarabine licensed by FDA
1978	Native L-asparaginase licensed by FDA
1979	Daunorubicin licensed by FDA
1983	Etoposide licensed by FDA
1987	Mitoxantrone licensed by FDA
1994	Pegylated L-asparaginase licensed by FDA
1995	All-trans-retinoic acid approved for acute promyelocytic leukemia
2000	Arsenic trioxide licensed for acute promyelocytic leukemia by FDA
2001	Imatinib licensed for chronic myelogenous leukemia by FDA*

Pre-clinical studies in 1950 revealed that association of bone marrow engraftment after sub-lethal irradiation led to the disappearance of leukemia in mice. This finding sparked further studies in humans, and in 1957, the first intravenous fusion of bone marrow in humans was noted by Dr. Donald Thomas. Afterwards, tremendous progress was recorded regarding successful bone marrow transplantations in acute leukemia patients with relapsed or refractory disease and in complete remission (Pui, 1995).

At the beginning of the 1960s, the gracious efforts of Don Pinkel and colleagues marked progressive and impressive improvements in the therapeutic regimen. They systematically altered and improved their chemotherapy regimens, resulting in a terrific improvement for a disease that had remained fatal until then. The discovery and application of the combination

of daunorubicin and cytarabine in 1973 were documented to be effective in acute myeloid leuksemia (Pui & Evans, 2006).

Table VI. Evolution of transfusion services and stem cell transplantation (Paolo Piccaluga, 2021)

Date	Development
1873	Blood transfusion first applied to leukemic patients (Callender)
1901	First description of human blood groups (Landsteiner)
1937	First hospital blood bank
1954	Introduction of platelet transfusion
1957	First successful syngeneic bone marrow transplantation
1968	First successful sibling donor bone marrow transplant (immunodeficiency)
1972	First successful matched sibling donor marrow transplantation (aplastic anemia)
1974	Anthony Nolan Bone Marrow Donor Registry (UK)
1977	Evidence of survivals >1 for 18/110 patients with advanced leukemia transplanted from matched donors
1979	Report of Success >50% for matched sibling donor marrow transplantation for acute myeloid leukemia in first remission
1986	National Marrow Donor Registry Program (USA)
1983	First successful haploidentical T-cell depleted bone marrow transplant
1989	First successful transplant using umbilical cord blood
1997	First reduced-intensity bone marrow transplantation
2002	First generation CAR-T cells
2017	The FDA approves CD19-directed CAR T cells for the treatment of relapsed, refractory acute lymphoblastic leukemia in children and young adults.

In the following decades, success of anti-leukemic treatments not only led to discoveries of new drugs but also in the improvements in supportive cares strategies.

1.5. N-NITROSO COMPOUNDS (NOC)

N-nitroso compounds (NOCs) are a class of synthetic compounds containing the N-nitroso functional group (R-NO). These compounds have received considerable attention due to their potential role in carcinogenesis. N-nitroso compounds are found in a variety of environmental sources, including food, water, air, and workplaces. They have been extensively studied for their carcinogenic potential and are known to play an important role in the initiation, promotion, and progression of various cancers (Lijinsky, 1999).

N-nitroso compounds are of particular interest because they have the ability to cause DNA damage and induce genetic mutations, converting healthy cells into cancer cells These compounds can act as direct alkylating agents, altering DNA structure and inhibiting the normal course of cellular processes. In addition, they can induce the generation of reactive oxygen species (ROS), cause oxidative stress, and further contribute to DNA damage and cellular dysfunction (Mirvish, 1975).

The association between N-nitroso compounds and cancer is supported by epidemiological studies, which show that the use of these compounds increases the risk of several types of cancer associated with N-nitroso compounds including cancer of the stomach, esophagus, colon, bladder, pancreas and lungs and others (Cross & Sinha., 2004).

Understanding the origin, availability, and mechanism of action of N-nitroso compounds is critical for identifying potential prevention strategies and therapeutic targets. Efforts to reduce the use of these compounds have revealed the need for dietary changes and the implementation of stringent occupational safety measures.

1.5.1. Origin and sources of NOCs

N-nitroso compounds (NOCs) can arise from both internal and external sources, contributing to their presence in nature. To evaluate potential exposure and associated health risks, it is crucial to understand these origins. In this discussion, we explore the natural sources of N-nitroso compounds:

1. **Endogenous Sources**:

   a. Endogenous sources can contribute to the formation of N-nitroso compounds in the body. One such source is nitric oxide (NO), which acts as a signalling molecule and

can undergo oxidation and nitrosation reactions. Nitrite, a common component in biological systems, reacts with endogenously produced NO, resulting in the generation of N-nitroso compounds.

b. Another source is nitrous acid (HONO), formed through the atmospheric oxidation of nitric oxide. In addition to this, enzymatic and non-enzymatic processes within the body can also generate HONO. When HONO reacts with amines, it produces N-nitroso compounds (Turesky, 2007; Ma et al., 2018).

2. Exogenous Sources:

a. Dietary Sources: Certain foods, like vegetables (such as spinach and lettuce) and cured or processed meats, contain naturally occurring nitrate and nitrite. When these are converted in the gastrointestinal tract under specific conditions, N-nitroso compounds can form (Cross et al., 2010).

b. Environmental Sources: N-nitroso compounds may also originate from environmental sources. For example, water sources can become contaminated with nitrate and nitrite pollutants from agricultural runoff, potentially exposing individuals to N-nitroso compounds (Essien et al., 2020).

c. Occupational source: Certain industries, like rubber and latex production, can expose workers to N-nitroso compounds. This exposure can happen through inhalation, skin contact, or ingestion of contaminated materials (Tricker, 1997).

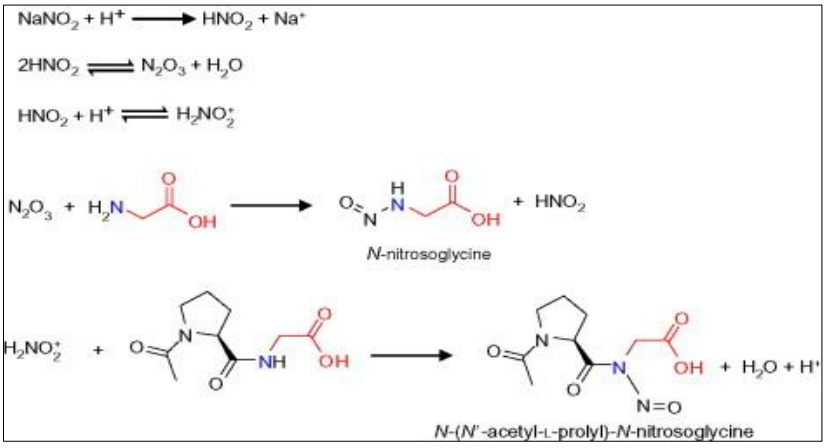


Figure 4: Chemical pathway of formation of Nitroso compounds

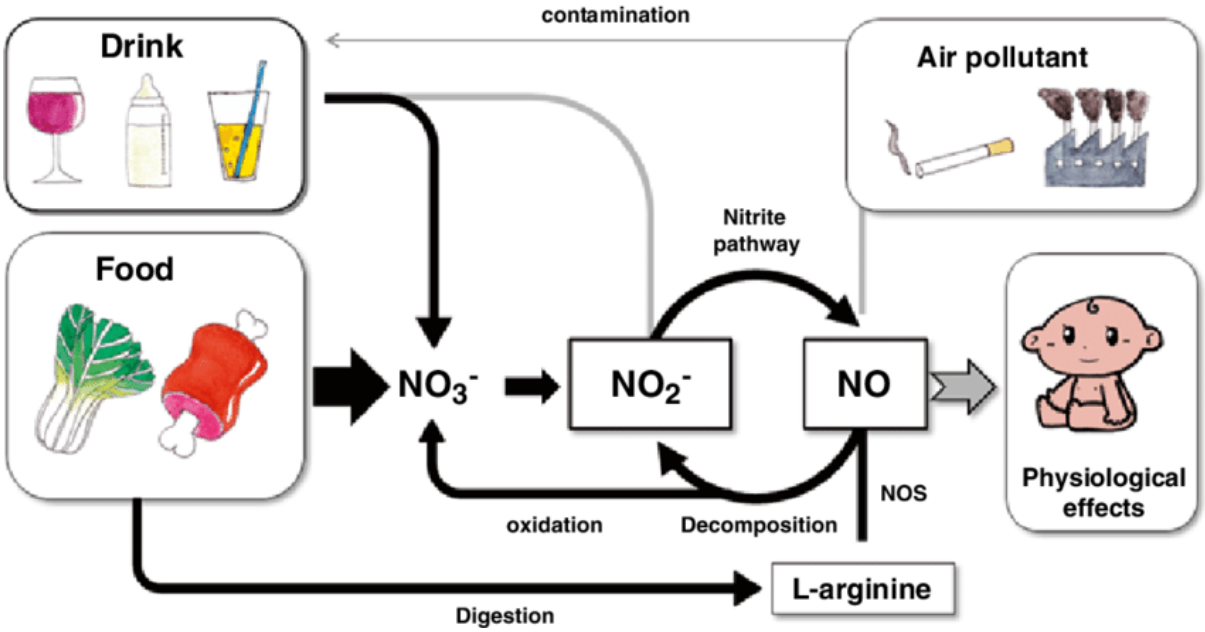


Figure 5: Graphical representation of endogenous and exogenous pathway of Nitroso compound formation

1.5.2. First Evidence of N-Nitroso Compounds in Nature and Animal Systems:

The mid-twentieth century marked the initial documentation of N-nitroso compounds in living organisms. Investigations early on centered on examining the relationship between N-nitroso compounds and carcinogenesis. Here are some notable early observations:

1. Magee and Barnes (1956): By means of semen studies, those investigators showed the carcinogenic capabilities of N-nitroso compounds. Findings showed that N-nitroso compounds may lead to tumor development in various animal populations, including those of rodent and mouse origin. During this investigation, substantial proof emerged to support a connection between N-nitroso compounds and cancer development.

2. Nitrosamines in tobacco smoke: Nitrosamines, a tiny class of N-nitroso compounds, were uncovered in tobacco fumes. Numerous studies shed light on the pivotal part these chemicals play in bringing about lung and other cancers linked to smoking. The discovery of N-nitroso compounds in tobacco products shed light on their carcinogenic nature (Schenk & De boer, 1980).

3. Dietary nitrosamines: Analytical studies on dietary intake revealed the presence of N-nitroso compounds, specifically nitrosamines, in various types of food. During various

food processes like preparation, cooking, and fermentation, chemical reactions occur between nitrites and amines/amides, resulting in the formation of harmful N-nitrosochemicals (Mackor et al., 1967).

The foundational discoveries provided a springboard for probing N-nitrosochemistry's function in carcinogenesis in greater detail. Expanding on earlier findings, subsequent studies have illuminated the mechanisms of action, exposure, and associated health consequences of these drugs.

1.5.3. Effects of NOCs on ROS generation

The mechanism by which NOCs cause cellular oxidative stress by producing reactive oxidative stress (ROS) is a critical process. Through different routes, NOCs generate damaging compounds (ROS), jeopardizing cells' normal operation and DNA integrity. Here, we explore the mechanisms by which N-nitroso compounds induce ROS formation and their subsequent impact on cellular oxidative stress:

1.5.3.1. Direct ROS Generation:

- In enzymatic and non-enzymatic reactions, some N-nitroso compounds give rise to ROS. When an electron gets transferred from the N-nitroso group to molecular oxygen (O2), superoxide anion radicals (O2•-) and other ROS may form.
- Direct generation of ROS by NOCs may trigger oxidative stress, leading to an unstable balance between ROS production and antioxidant thereby causing a defense within cells (Radaković et al., 2018).

1.5.3.2. Indirect ROS Generation:

- Indirectly N-nitroso compounds can also promote ROS formation which may interfere with cellular processes and trigger oxidative stress.
- Mitochondrial Dysfunction: Some NOCs can disrupt mitochondrial function, leading to electron leakage from the electron transport chain (ETC) and superoxide radicals (O2•-) formation subsequently within the mitochondria. This damages the cellular antioxidant defense system and aids to increase in ROS levels.
- Enzyme Activation: Certain enzymes, such as NADPH oxidases can be activated by NOCs, , which are responsible for production of ROS. The activation of these

enzymes by NOCs can result in the formation and production of superoxide radicals and hydrogen peroxide (H2O2), resulting in oxidative stress.

- DNA Damage: DNA damage occurs if N-nitroso compounds directly interact with DNA. DNA lesions induced by NOCs can trigger cellular responses that produce ROS as a byproduct, causing severe oxidative stress (Denysenko et al., 2019).

3. Impact on Cellular Oxidative Stress:

ROS produced by N-nitroso compounds can have several destructive effects on cells and tissues:

a. DNA Damage: ROS causing DNA damage, can result in base alterations, breaks, and cross-links. Disruption in cellular processes can result in genomic instability and mutations (Dornas & Lagente, 2019).

b. Lipid Peroxidation:The lipids oxidised by ROS deteriorate the cell membrane. This process generates lipid peroxides and other reactive substances that rapidly increase oxidative stress and cellular harm.

c. Protein Oxidation: ROS can oxidize proteins, causing alterations in protein structure and function. Protein oxidation can damage enzymatic activities, disrupt signalling pathways, and lead to cellular dysfunction.

d. Cellular Dysfunction: Chronic exposure to ROS produced by N-nitroso compounds can hamper the cell's natural defenses, leading to cellular malfunctioning and eventually cell death (Mu & Liu, 2017).

In a nutshell, N-nitroso compounds accelerate the creation of ROS via direct and indirect pathways. The increase in ROS triggers oxidative stress, culminating in DNA harm, lipid peroxidation, protein oxidation, and cellular malfunction. These effects add to N-nitroso compounds' carcinogenic characteristics and link to many malignancies, including leukemia.

1.5.4. Cellular mechanisms affected and altered by NOC

N-nitroso compounds (NOCs) causing DNA damage, mutations, and changed cell signalling, can lead to cancer development through multiple pathways. All these factors initiate and accelerate the process of cancer cell growth and multiplication. The

potential mechanisms by which N-nitroso compounds can induce cancer are stated here:

1.5.4.1. DNA Damage and Mutagenesis:

- Nitroso compounds directly engage with DNA, causing genetic alterations. NOCs can alter DNA structure by forming alkyl groups with DNA bases. Such disruptions can distort the fundamental structure of DNA, impairing its regular operations.

- The influence of pollutants on DNA replication causes deleterious effects, leading to mutations that disrupt the integrity of the genetic material. Persistent DNA links with potential carcinogens contribute to chromosomal instability and tumour development.

- Additionally, N-nitroso compounds create ROS, a group that can lead to DNA oxidative damage, including base pair modification and DNA strands' breakage. DNA damage occurring through exposure to ROS facilitates the development of mutations and the collection of genetic irregularities (Mu & Liu, 2017).

1.5.3.2. Altered Cell Signalling:

- Cellular signalling pathway modification related to cell proliferation, survival, and programmed cell death promote tumour growth.

- Activation of oncogenic signalling pathways: Activation of signalling pathways such as the RAS-MAPK and PI3K-AKT routes by NOCs leads to proliferation of cell and their survival. NOCs mediated cellular pathways can show unrestrained growth and resistance to signals causing cell death, according to Ledda et al. (2016).

- Inactivation of tumor suppressor genes: The activity of genes, such as p53 get disrupted by N-nitroso compounds, which majorly protect against cancer, by interfering with their normal function through DNA damage or epigenetic modifications. The loss or inaction of tumour suppressor genes hampers their cellular regulatory functions, fostering uncontrolled cell growth and DNA damage, which contribute to cancer emergence.

- Altered gene expression: NOCs create an explicit gene expression change such as altering epigenetic marks. Epigenetic changes exhibits irregular gene expression, oncogene activation, and silencing tumour suppressor genes.

1.5.4.3. Genomic Instability:

- Cancer's defining trait, genomic instability, can arise from exposure to N-nitroso compounds. Generally, genetic mutations occur within cells, including chromosomal anomalies and DNA alterations.
- DNA synthesis may also be hampered by NOCs by intervening with DNA repair and replication process. Genomic alterations cause accumulation of genetic anomalies which promote tumor progression (Metayer et al., 2013).

1.5.4.4. Epigenetic Alterations:

- Gene expression patterns undergo alteration of N-nitrosomes, wherein no DNA modification is needed.
- The actions of NOCs have a profound impact on gene expression by altering these epigenetic markers. Epigenetic modification hinders the function of tumour suppressor genes leading to oncogenes activation, fostering tumorigenesis.

The various ways in which N-nitroso compounds facilitate the growth of cancer are summarized. DNA damage, mutations, and genome instability result in the buildup of genetic anomalies. Beyond affecting cell-to-cell communication and gene expression, NOCs create an environment conducive to unrestrained cell growth, endurance, and evasion of programmed cell death triggers. Involved mechanisms interact intricately, leading to the progression of cancer formation.

1.6. HEDGEHOG SIGNALLING AND ITS PIVOTAL ROLE IN THE CANCER AND LEUKEMIA

Christiane Nusslein-Volhard and Eric F. Weischaus first identified the hedgehog (HH) gene in 1980 through a screen for embryonic fatal mutations of Drosophila melanogaster that disturbed the larval body plan (Nüsslein-Volhard & Wieschaus, 1980). It is named appropriately for the short, "spiked" phenotype of the mutant Drosophila larvae's cuticle. HH signalling is evolutionarily conserved from flies to humans and has been identified as a key regulator of several fundamental processes in vertebrate embryonic development, including cell fate, patterning, proliferation, survival, and differentiation (Varjosalo & Taipale, 2008)

(Ingham & McMahon, 2001). Controlled Hh signalling activity is essential for maintaining homeostasis, promoting tissue regeneration, and healing wounds in adults (Beachy et al., 2004) (Petrova & Joyner, 2014). The carcinogenesis, development, metastasis, and resistance therapy of numerous malignancies, including basal cell carcinoma (BCC), medulloblastoma (MB), and many other solid and haematological malignancies, have been linked to activation of ectopic Hh pathway (Briscoe & Thérond, 2013). The Hh pathway has been a popular target for cancer therapy as a result of its broad involvement in malignancies. The discovery of clinical-grade Hh pathway modulators as well as the fundamental comprehension of the Hh pathway have received enormous amounts of attention over the past two decades.

The transmembrane Patched 1 (PTCH1) receptor acts as where canonical activation initiates when an HH ligand binds to it (Figure 1) (Taipale et al., 2002). PTCH1 naturally suppresses SMO activity, however this inhibition ceases when a ligand interacts with PTCH1. The cytoplasmic sequestration of GLI transcription factors by suppressor of fused (SUFU) is eventually eliminated as an outcome of activated SMO conveying the HH activating signals there. This enables GLI to translocate into the nucleus and bind transcriptional targets to control cellular gene expression (Dunaeva et al., 2003). Mammals have three GLI isoforms that function as the HH pathway's terminal effectors: (1) GLI1, which is regarded to be a reliable biomarker of pathway activation, (2) GLI12, which can either promote or repress gene expression, and (3) In most of the contexts GLI3 functions as a transcriptional repressor. (Ruiz i Altaba, 1997).

1.6.1. Components of hedgehog signalling pathway

Hh protein/gene The Hh gene, a crucial regulator of embryonic development, is predominantly conserved from fruit flies to humans (Varjosalo & Taipale, 2008). The Sonic Hedgehog (SHh), Indian Hedgehog (IHh), and Desert Hedgehog (DHh) genes have been found in vertebrates, unlike *Drosophila melanogaster* , having one Hh gene (Echelard et al., 1993) (Krauss et al., 1993) (Marigo & Tabin, 1996). Any of these three genes' products can bind to the Ptch1 receptor and activate the Hh signalling pathway (Marigo & Tabin, 1996), although they do so in different organ systems (Echelard et al., 1993, Marigo & Tabin,1996). Hh proteins can function at greater or lesser distances, throughout various developmental stages, and in various tissues as mitogens, morphogens, and differentiation factors (Cross & Bury, 2004). SHh is the most explored Hh ligand. It is highly vigorous and contributes to the development of several organs during embryogenesis. During development, SHh is expressed

in the lungs, teeth, intestines, central nervous system, and hair follicles (Goodrich et al., 1996, Bellusci et al., 1997). Both autocrine (binds to the same cell from which it is secreted) and paracrine (binds to neighboring cells or produces changes in cells at further distances) forms of SHh signalling are possible. IHh is a negative regulator of chondrocyte differentiation (Vortkamp et al., 1996), involves in the formation of the gastrointestinal tract (van den Brink et al., 2004) and mammary glands (Lewis & Veltmaat, 2004), and is involved in the production of endochondral bone. The D. melanogaster Hh ligand's closest homologue among all known Hh proteins is DHh (Robbins et al., 2012). Its expression is primarily confined to the gonads, significantly in the Sertoli cells of the testis that is necessary for the development of germ cells. Before the active ligand is produced, released from the cell, and the Hh signalling pathway is activated, all Hh proteins go through a maturation process. A 45 kDa long polypeptide that has had its N-terminal signal sequence deleted is then autocatalytically cleaved between glycine and cysteine to produce an N-terminal fragment. A 19 kDa N-terminal Hh signalling domain (HhN), linked to a number of recognised signalling functions, is produced when the C-terminal domain (autoprocessing domain) of the Hh polypeptide stimulates the binding of cholesterol to glycine at the C-terminus of the N-terminal segment (Mann & Beachy, 2004). The acyltransferase Skinny Hedgehog (Ski) then adds a palmitic acid moiety to the cysteine on the N-terminus of Hh, creating the dually modified Hh signalling domain (HhNp), or active Hh protein (BuGLIno & Resh, 2008).

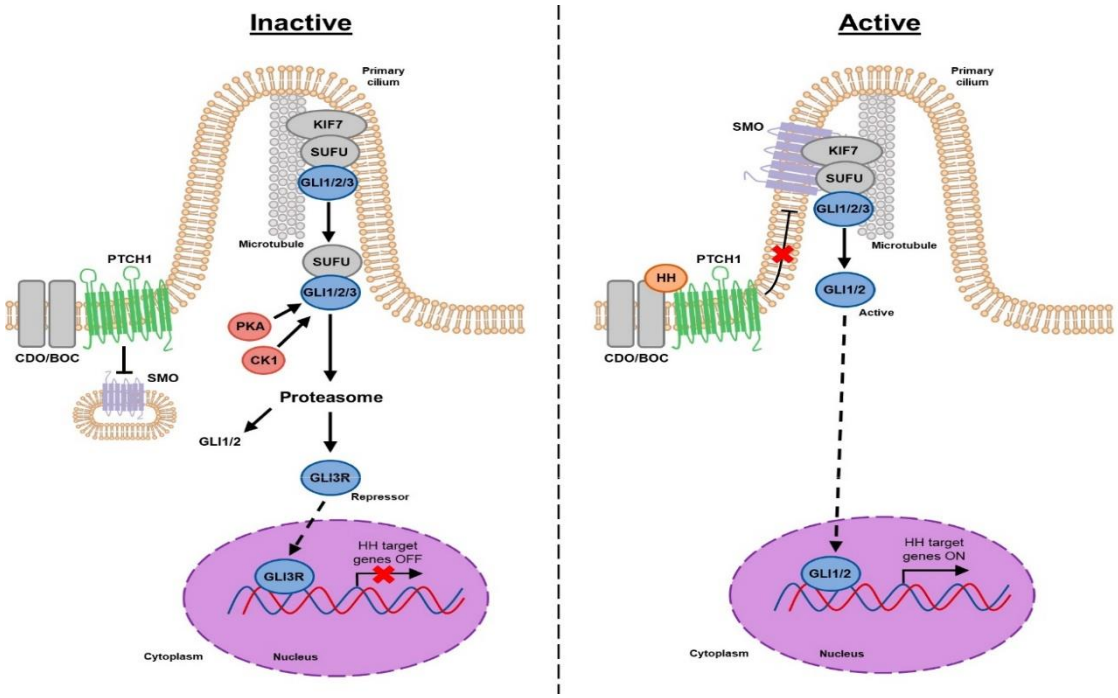


Figure 6 : Active and inactive state of Hedgehog signalling pathway.

1.6.1.1. PTCH protein and gene

PTCH is the receptor of the Hh protein. PTCH1 and PTCH2 are the homologs that have been discovered in vertebrates (Burns et al., 2002). Since SHh, IHh, and DHh ligands bind to them with equal affinity, both of these proteins may restrict the activity of the SMO protein (Gailani et al., 1996). The human Ptch1 gene has 23 exons, and it encodes a glycoprotein of 1447 amino acids which is found on chromosome 9q22.3. The Ptch2 gene, which codes for a 1203-amino-acid protein, is found on 1p34.1. Because PTCH1 and PTCH2 express differently during epidermal development, their functions are distinct. Ptch2 is found in skin and testicular epithelial cells, whereas PTCH1 is mostly expressed in mesenchymal cells that make SHh proteins (Larsson et al., 1998) (Smyth, 1999). PTCH inhibits the pathway's activity when Hh is not present. However, Hh removes Ptch's inhibition when it's ligand binds to it, activating signal transmission. As a result, SMO blockage is no longer present, and GLI transcription factors are modulated. Additionally, PTCH entraps Hh ligand and limits the free ligand's signalling range (Johnson et al., 2000). Hh protein is more widely distributed in cells without PTCH and it drives the desired gene expression over a wider area. Although the exact method by which PTCH controls SMO is still unknown, it has been demonstrated that Hh binding leads PTCH to internalize from the cell surface and encourages the buildup of SMO there (Johnson et al., 2000).

Hedgehog-interacting protein (HHIP) is a negative regulator of Hh signalling, whereas Cdo and Boc bind vertebrate Hh proteins and positively control Hh signalling. It is still unclear whether they cooperate or compete with Ptch1 to bind to Hh (Tenzen et al., 2006). The membrane protein growth arrest-specific 1 (GAS1), a well-known Hh signalling negative regulator, has been demonstrated to modify Hh signalling positively in some instances (Jeong & McMahon, 2005). The amplitude and scope of the Hh signalling pathway are most likely closely monitored by these negative and positive receptors (Martinelli & Fan, 2007).

1.6.1.2. SMO protein/gene

SMO protein is a co-receptor in the Hh signalling pathway, it is an integral part of the receptor complex that sends signals downstream. The seven-pass transmembrane protein shares structural homology with G-protein-coupled receptors with an essential extracellular cysteine-rich domain (CRD) for its function (Rana et al., 2013). SMO is regarded as a

positive Hh signalling pathway regulator since it stimulates the activation of downstream Hh signalling pathway components and is constitutively active in the absence of inhibitory PTCH1. It is still unclear how PTCH1 prevents SMO from functioning. The proposed mechanism based on SMO and PTCH1 physically interact producing a membrane-associated receptor complex hasn't been validated *in vivo*. PTCH1 may work by altering the concentration or distribution of a small molecule that influences SMO (Taipale et al., 2002). One of the key functions in the suppression of SMO is played by ciliary localization of PTCH1, which is made possible by the ciliary localization sequence (CLS) within its carboxyl-terminal tail. PTCH1 transfer into the PC is likewise enabled by CLS (Bijlsma et al., 2006). It is yet to be fully understood how PTCH1 represses SMO and how Hh ligand overrides this impact, despite these new insights into the factors that control SMO activity, (Corcoran & Scott, 2006). SMO is phosphorylated by PKA and CK1 and its endocytosis and degradation are prevented after the binding of the Hh ligand and the destruction of the PTCH1 protein. SMO sends a signal to the cytoplasm, with the GLI protein as the ultimate target, in a phosphorylation cascade (Kim et al., 2015) (Figure 6).

1.6.1.3. GLI protein/gene

Zinc finger proteins make up the GLI family of proteins, it was nomenclated after the GLIoblastomas from which they were first discovered. The three members of the GLI gene family in vertebrates include GLI1, GLI2, and GLI3. The GLI1 protein activates transcription (Kinzler et al., 1987). GLI1 is likewise positively receptive to Hh signalling and is expressed in response to Hh ligands (Regl et al., 2002) (Regl et al., 2003). With 18 amino acids, GLI1's activator domain likely forms a negatively charged helix similar to viral protein 16. In Hh signalling, GLI2 largely acts as a transcriptional activator whereas GLI3 mostly acts as a repressor (Persson et al., 2002). By physically attaching to the promoters of the target genes, GLI proteins control how those genes are expressed. The 5'-GAACCACCCA-3' sequence in target gene promoters is recognized by the GLI1 and GLI3 proteins, and GLI2 recognizes a nearly identical 5'-GAACCACCCA-3' motive (Pan et al., 2006). GLI1L is phosphorylated by PKA, GSK3, and CK1 in the absence of Hh ligand, and it is then recognized by -transducing-repeat-containing protein (β-TrCP). As a result, GLI1L is proteolytically broken down into the C-terminally short repressor form, GLIR (Humke et al., 2010). GLIR moves into the nucleus, where it interacts with Hh target gene promoters to inhibit the production of those

genes (Figure 1A). The synthesis of GLI1A and the release of GLI from SUFU are caused by the binding of the Hh ligand. The transcription of Hh target genes is subsequently activated because of GLI1A's nuclear translocation and binding to target gene promoters (Figure 1B). The transcription of target genes in the Hh signalling pathway is induced by the activation of classical Hh signalling, which inhibits the proteolytic degradation of GLI proteins and increases their cytoplasmic and nuclear levels. Studies have revealed that additional signalling mechanisms also control the production of GLI transcription factors and their activation. For instance, in esophageal cancer mTOR/ribosomal protein S6 kinase beta-1 (S6K1) signalling pathway phosphorylates GLI1, releasing it from the SUFU protein complex and translocating into the nucleus (Wang et al., 2012). Additionally, it has been noted that by the transforming growth factor beta (TGF-β), epidermal growth factor receptor (EGFR), mitogen-activated protein kinases (MAPK), and fibroblast growth factor (FGF) signalling pathways, the expression of GLI transcription factors can be induced (Mimeault et al., 2015).

1.6.1.4. Suppressed fusion protein (SUFU)

Suppressed fusion protein or SUFU works as a mediator between the transcription factors Smo and GLI and is an essential negative regulator of the Hh pathway. It interacts with GLI proteins directly (Stone et al., 1999). Active SMO causes the recruitment of SUFU-GLI to cilia in a stimulated Hh signalling pathway, which is followed by a quick dissociation of the complex and the beginning of target gene transcription (Tukachinsky et al., 2010). By inhibiting their translocation into the nucleus, SUFU inhibits GLI proteins (Méthot & Basler, 2000). It ensures precise Hh signalling by playing a crucial part in the stabilization and processing of GLI. SUFU can bind to GLI-binding regions in DNA molecules and stop gene transcription when it localises to the nucleus (Chen et al., 2009).

1.6.1.5. Other key proteins

KIF7 The kinesin protein, a part of Hh signalling that can function as both a positive and a negative regulator (Liem et al., 2009). KIF7 localises at the tip of the PC in response to Hh activation, and it has the ability to regulate cilium shape and arrange a specific compartment required for Hh signalling (He et al., 2014). Dispatched (DISP) protein controls the secretion

of mature, functionally active Hh proteins, which permits their discharge from the cells (Shirras, 2000).

1.6.2. CANONICAL SIGNALLING IN HEDGEHOG PATHWAY

One of the essential components of the canonical pathway in mammals are the hedgehog glycoproteins, Sonic, Desert, and Indian hedgehog (SHH, DHH, IHH), the 12-transmembrane protein Patched1 (PTCH1), the 7-transmembrane protein Smoothened (SMO), the suppressor of fused (SUFU), and the GLI zinc-finger transcription factor serve as the pathway's terminal effectors. The family of GLI transcription factors comprise three distinct proteins, GLI1 – generally a transcriptional activator, GLI2 - can be either an activator or a repressor, and GLI3 - a transcriptional repressor (Ruiz i Altaba, 1997). GLI1 expression serves as a readout of pathway activation and is highly dependent on active HH signalling. SHH is the most widely expressed and powerful of the three HH glycoproteins (Varjosalo & Taipale, 2008).

SHH goes through a number of processing steps before being secreted, including the removal of its signalling sequence, cleavage catalysed by its own C-terminal protease domain (Lee et al., 1994), addition of cholesterol to the C-terminal domain to facilitate association with the plasma membrane (Fan et al., 1995), and finally the addition of a palmitate at the N-terminal domain to create the fully active SHH signalling molecule (Pepinsky et al., 1998, BuGLIno & Resh, 2008). Recent research states that there are at least two possible mechanisms for mediating repression: (1) by alteration of SMO localisation, and (2) by catalytic inhibition of SMO (Taipale et al., 2002, Denef et al., 2000). The primary cilium, a single cell surface projection that serves as a key signalling hub in mammalian cells, is a need for SMO-dependent GLI1 activation. As PTCH1 localises at the base of the primary cilium, it prevents SMO from building up and inhibits its activation (Denef et al., 2000, Goetz & Anderson, 2010, Rohatgi et al., 2007). When SHH binds to PTCH1, PTCH1 is delocalized from the primary cilium, which causes it to degrade and cause SMO to relocate into the cilium in response. Furthermore, PTCH1 has sequence similarities to members of the prokaryotic resistance-nodulation-division (RND) family of transporters, such as the bacterial proton-driven multidrug resistance exporter AcrB, which raises the possibility that PTCH1 functions as an SMO ligand transporter. (Bijlsma et al., 2006). It's significant that PTCH1 has a sterol-sensing domain that is shared by regulating enzymes for sterol biosynthesis, perhaps giving it the ability to bind sterols (Incardona, 2005).

SMO is eventually unrepressed when SHH binds to the PTCH1 receptor, releasing GLI1 transcription factors from cytoplasm. However, it is still doubtful how the activation signal travels from the ciliary SMO to the cytoplasmic GLI1 effectors (Kogerman et al., 1999). As was before mentioned, SMO localization to the primary cilia is necessary for the SHH signal to reach GLI1 effectors. SMO is phosphorylated and activated, causing it to bind with β-arrestin and move to the primary cilium, as a result of its relationship with CK1 and GPCR kinase 2 (GRK2) (Chen et al., 2011, Kovacs et al., 2008). There is further evidence linking the lateral migration of SMO in mammalian cells to the cyclic AMP (cAMP)-protein kinase (PKA) pathway (Milenkovic et al., 2009).

A protein complex containing kinesin protein (KIF7) and SUFU coupled to GLI1 transcription factors is dynamically trafficked to the active SMO in the primary cilia after SMO builds up in the primary cilia (Humke et al., 2010, Liem et al., 2009, (Cheung et al., 2009). However, there is no proof that SMO works with SUFU directly to release GLI1. These proteins function downstream of SMO to encourage the dissociation of SUFU from the GLI proteins. They co-associate and localise at the primary cilia in a mutually dependent way. Through direct binding to GLI1 effectors via an N-terminal interaction site to sequester GLI1 in the cytoplasm, SUFU is crucial for reducing GLI1 activity in unstimulated cells (Dorn et al., 2012, Han et al., 2015). After being attracted to the main cilia by activated SMO, endogenous SUFU-GLI1 complexes quickly dissolve, allowing GLI1 proteins to initiate gene transcription (Tukachinsky et al., 2010). Through phosphorylation, inhibitors like SUFU, Ren, protein kinase A (PKA), glycogen synthase kinase 3 (GSK3), and activators like Dyrk1, Ras, and Akt control the activation of GLI1 at various degrees (Ferretti et al., 2005).

1.6.3. NONCANONICAL SHH SIGNALLING

Noncanonical SHH signalling is the phrase used to describe cellular and tissue reactions to HH isoforms that are not mediated by GLI1 isoform transcriptional activity. The three types of noncanonical HH signalling are as follows:

Type I acts through PTCH1 via functions distinct from its inhibition of SMO.

Type II is mediated by GLI1-independent activities of SMO, such as activating small GTPases.

Type III refers to all other mechanisms of upstream PTCH1-SMO signalling independent GLI1 transcription factor activation (Jenkins, 2009).

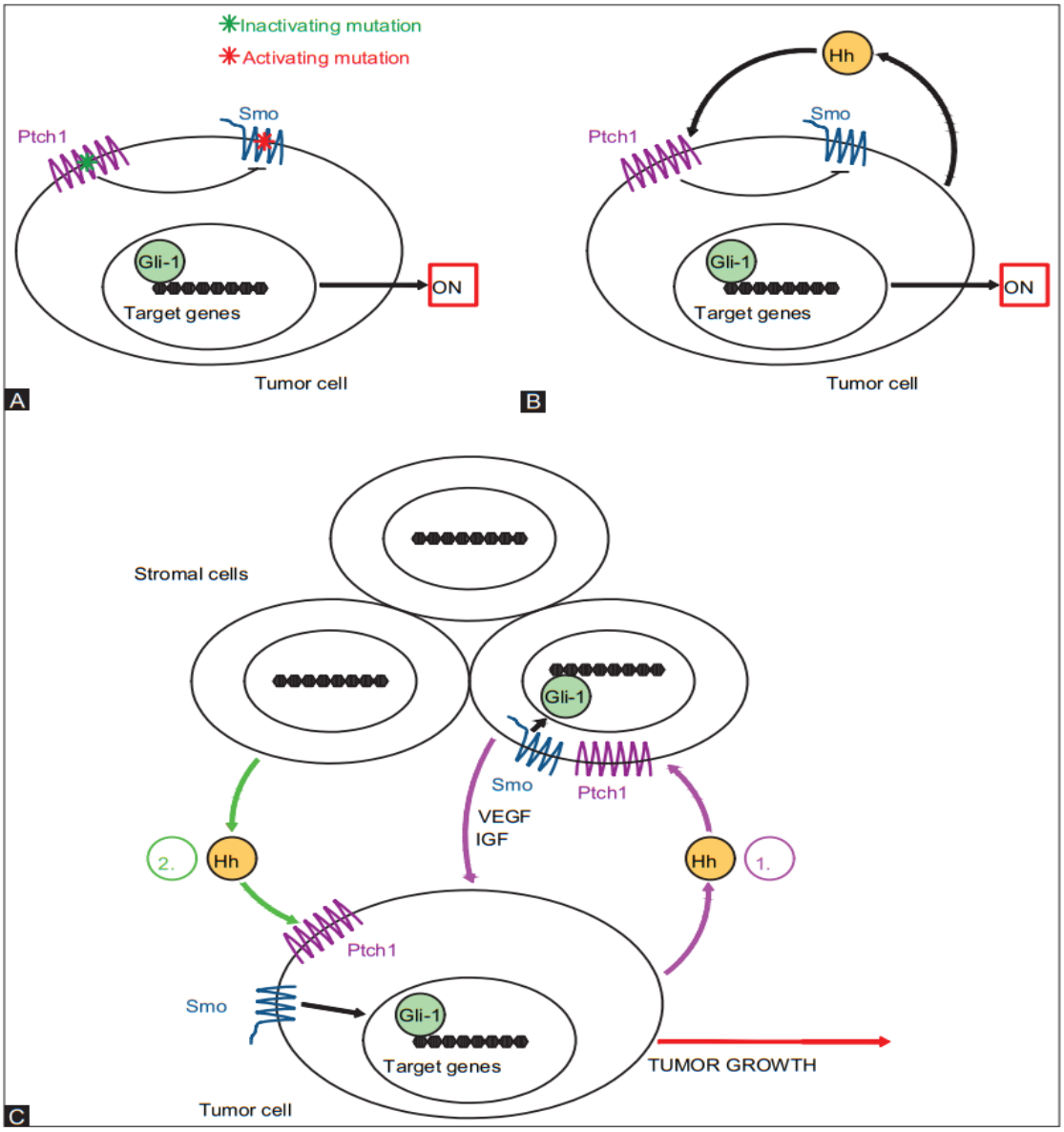


Figure 7. Different non canonical SHH signalling

1.6.3.1. Type I—PTCH1 Functions Distinct from SMO Inhibition

Canonical SHH signalling inhibits apoptosis by activating the pro-survival kinase Akt, the Type I noncanonical pathway, and upregulating pro-survival genes (including Bcl-2) via GLI1-dependent transcription. Recent studies have shown that PTCH1 can stimulate apoptosis without relying on traditional SHH signalling (Chinchilla et al., 2010, (Ferretti et al., 2005). Because SMO overexpression was insufficient to prevent cell death, it is possible

that ectopic PTCH1 synthesis in vitro caused apoptosis without the help of SMO (Ferretti et al., 2005). Due to its inhibition of SMO, PTCH1 is usually referred to as a tumour suppressor. Chinchilla et al. (2010)'s findings suggest, however, that PTCH1's capacity to inhibit cancer growth goes beyond its relationship with SMO. However, the findings of Chinchilla et al. (2010) suggest that PTCH1's capacity to inhibit tumour growth goes beyond its relationship with cancer. It's interesting to note that PTCH1 has been suggested to act as a dependency receptor because its activity at the time it is produced completely depends on the presence of the SHH ligand. Similarities between PTCH1 and other dependency receptors are many. Additionally, it was discovered that cleaving the PTCH1 C-terminal domain (CTD) causes cell death and controls PTCH1 localization and degradation (Lu et al., 2006). For the fidelity of PTCH1-mediated SMO inhibition, the CTD does not appear to be required, albeit (Nieuwenhuis et al., 2007). In addition to SMO, SHH also activates the ERK pathway, most likely via altering how the PTCH1 CTD interacts with SH3-containing proteins like Grb2 (Chang et al., 2010). Additionally, SMO-independent noncanonical signalling can affect cell cycle control and proliferation in specific cell types. In particular, cerebellar granule precursor cells may cause medulloblastoma through GLI1-mediated activation of N-MYC and Cyclin D1 (Wechsler-Reya & Scott, 1999, Hatten & Roussel, 2011). The cyclin protein family functions as regulatory subunits for cyclin-dependent kinases, which control the transcriptional landscape of a cell to directly control the advancement of the cell cycle (Marak et al., 2020). Activated Cyclin B1-Cdk1, which is crucial for mitotic progression, can be controlled by noncanonical SHH signalling. This signalling is specifically engaged in getting through the G2/M checkpoint. The wide intracellular loop between the sixth and seventh transmembrane domains of PTCH1 allows it to engage with phosphorylated Cyclin B1, preventing it from translocating into the nucleus and so decreasing proliferation. The discovery of Cyclin B1 sequestration by PTCH1 proves that this protein has tumor-suppressive properties separate from downstream SHH signalling elements. As anticipated, the addition of SHH reinstates canonical signalling, prevents the interaction of PTCH1 with Cyclin B1, and allows for nuclear translocation and mitotic completion (Barnes, 2001). The variety of PTCH1 tumor-suppressive activities support the idea that PTCH1 mutations are a potent indicator of breast cancer recurrence and warrant additional investigation.

1.6.3.2. Type II—SMO-Mediated Functions Independent of GLI1

A functional G protein-coupled receptor (GPCR) called SMO exhibits selectivity for heterotrimeric Gi proteins (Riobo et al., 2006). Small GTPases serve important functions in cytoskeletal reorganisation and are known to be activated by GPCRs (Hall & Nobes, 2000). These monomeric G proteins, or tiny GTPases, function as molecular switches to swiftly control cellular activities (Cherfils & Zeghouf, 2013). Rapid stimulation of Rac1 and RhoA small GTPases by SMO has been found to be a cause of fibroblast migration in the setting of SMO-dependent noncanonical SHH signalling (Polizio et al., 2011). Purmorphamine, a powerful SMO agonist, and SHH ligand both stimulate NIH3T3 fibroblast migration in this study, however SMO-knockout mouse embryonic fibroblasts (MEFs) are unaffected. Ho Wei et al. (2018) discovered that Gi proteins and RhoA were induced by SHH and purmorphamine-mediated SMO activation without the need for primary cilia. After stimulating endogenous SMO with SHH or purmorphamine, KIF3A-deficient fibroblasts, which are unable to generate primary cilia and thus activate the canonical SHH pathway, preserved their capacity to activate RHOA. (Ho Wei et al., 2018).

1.6.3.3. Type III—Mechanisms Independent of Upstream PTCH1-SMO Signalling

All other GLI1 activation mechanisms that take place independently of the noncanonical PTCH1 and SMO signalling activities mentioned above are referred to as type III noncanonical hedgehog signalling. The post-translational modification of GLI1 proteins and the interaction of GLI1 genes with other oncogenic pathways leads to GLI1 activation. The most often researched GLI1 PTM is phosphorylation, which is a significant post-Phosphorylation modification of GLI1 proteins, primarily by protein kinase A (PKA). In the absence of Sonic hedgehog ligand, PKA-mediated phosphorylation of GLI1 serine residues is the essential inhibitory signal that restricts the conversion of GLI1 proteins into transcriptional activators. Both the synthesis of GLI1 repressor proteins and the stabilisation of active GLI1 proteins following CUL3/SPOP-mediated proteosomal degradation depend critically on casein kinase 1 (CK1) (Shi et al., 2014). It is also known that the dual-specificity tyrosine kinase family (DYRK) of proteins controls GLI1 proteins via PTM. In part via keeping GLI1 in the nucleus, DYRK1 directly phosphorylates GLI1, increasing its transcriptional activity (Schneider et al., 2015; Ehe et al., 2017). Additional kinases that have been shown to phosphorylate GLI1 includes - ULK3, S6K1, atypical PKA (Maloverjan et al.,

2010; Wang et al., 2012), AMP-activated protein kinase (AMPK) (Mirza et al., 2019; Atwood et al., 2013), Mitogen-activated protein kinase kinase 1 (MEKK1), and HCK (Di Magno et al., 2016; Zhang et al., 2017, Li et al., 2015).

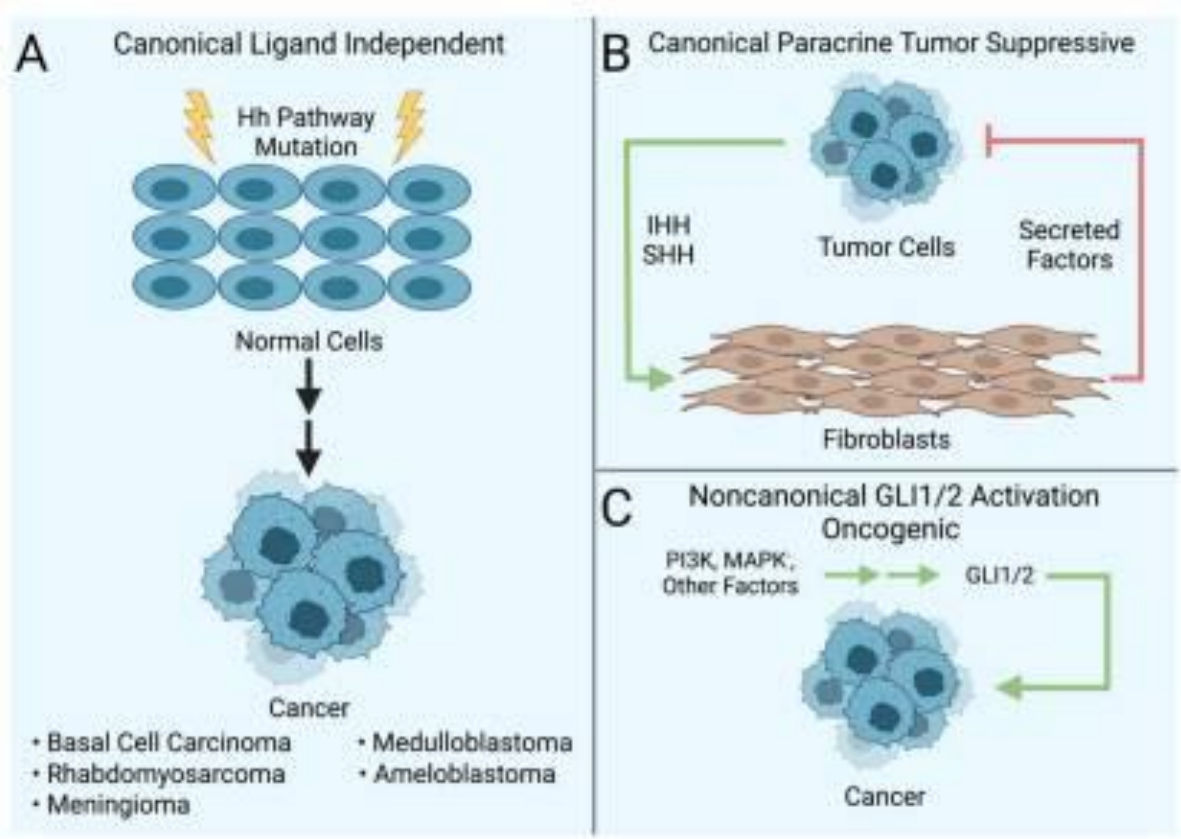


Figure 8: Alternative activation pathway of hedgehog signalling

1.7. ASHWAGANDHA STORY

Withania somnifera (L.) Dunal (Solanaceae), also known as Ashwagandha in Sanskrit, has been used extensively as an herbal remedy since it was first developed around 6000 B.C. W. somnifera is a woody shrub that is evergreen and belongs to the Solanaceae family. It has been given a number of names that have different meanings, including "Indian ginseng" and "Indian winter cherry" in English, "Punir" or "Asgandh" in Hindi, and "Asgand" in Urdu (Ahmad & Dar, 2017). The species name has been given as "somnifera," which is Latin for "sleep-inducer," because of its extraordinary anti-stress properties; "Ashwagandha" (from the words "ashwa" and "gandha," which mean smell of horse) because the roots have a distinctive "wet horse" smell; and "Indian ginseng" because of its familiarity with the

pharmacological effects and traditional uses of Korean ginseng tea (Dar et al., 2015). Ashwagandha is known as the "Sattvic Kapha Rasayana" in Ayurveda, which is widely used as an astringent, anti-carbuncle, anti-stress, tonic, narcotic, diuretic, and for the treatment of worms, piles, goitre, leucoderma, nervous breakdown, sleeplessness, and constipation (Agarwal et al., 1999; Machiah & Gowda, 2006). In Kitab-al-Hashaish, a book authored by Dioscorides in 78 A.D., Withania is referred to as "Asgand" and is mentioned in relation to the Unani medical system. W. somnifera is listed as an official medication in the Indian Pharmacopoeia of 1985 (Renuka S Solunke et al., 2022). The plant's roots have been heavily utilised in both the Unani and Ayurvedic medical systems. Alkaloids, volatile oils, starch, amino acids, glycosides, reducing sugars, and steroids are all said to be present in roots to varying degrees (0.13–0.31%) (Paul et al., 2021). Flowers have been used as astringents and diuretics in addition to the root, fruits are used to treat skin ulcers, tumours, and carbuncles, and seeds are good for promoting testicular expansion and boosting sperm count (Singh et al., 2011).

Withania somnifera is the most widely distributed species in the genus . Natural it occurs in the Mediterranean through tropical Africa, South Africa, Afghanistan, Baluchistan, Pakistan, Sri Lanka, China, Nepal, and the Middle East and Arabia. It is grown in gardens in the warmer regions of Europe, and South Australia and New South Wales have accepted it as a weed (Mirjalili et al., 2009; Renuka S Solunke et al., 2022).

1.7.1. ANTICANCER ACTIVITIES OF WITHANIA SOMNIFERA

1.7.1.1. Lung Cancer

Lung cancer being the most common malignancy and the primary cause of cancer-related deaths worldwide. Studies have shown that oral administration of WS extract can prevent urethane-induced lung adenoma in albino mice and suppress lung carcinogenesis in Swiss albino mice. WS root extracts restore immune cells, suppress cell proliferation, and display antioxidant activity, protecting mice from reactive oxygen species-induced damage (Singh et al., 1986). Withaferin-A or WA (a steroidal lactone purified from WS) inhibits the binding of U937 monocytic cells with A549 cells stimulated by TNF-α. In NSCLC A549 and H1299

cells, WA has been shown to be highly effectual in suppressing TGF-β1 and TNF-α-induced Epithelial-Mesenchymal Transition (EMT) (Senthilnathan et al., 2006).

a

b

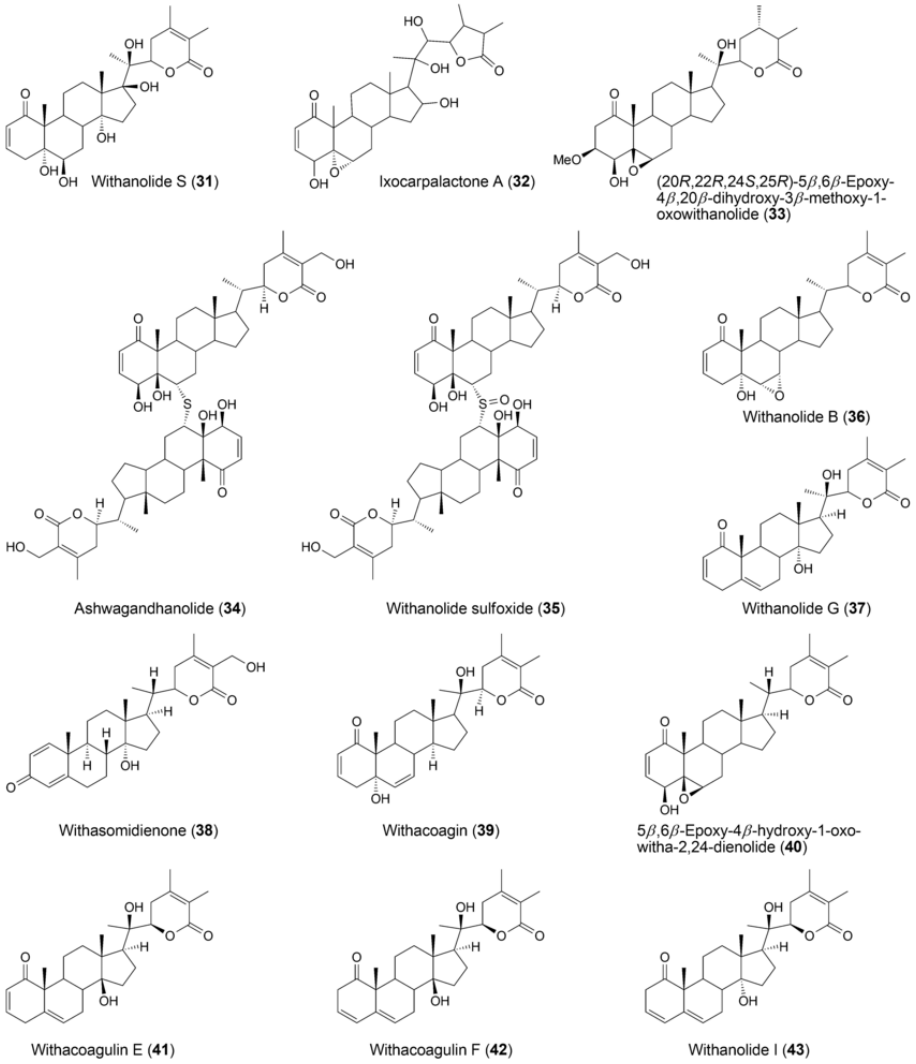


Figure 9: a. Ashwagandha (Withania somnifera) plant with fruits. **b**. Major bioactive compounds identified from Ashwagandha**.**

Apoptosis, cell cycle arrest, and inhibited proliferation are induced by WS, suppressing the activation of the PI3K/AKT pathway (Oh & Kwon, 2009). In silico drug screening suggests that WS is a potential candidate for anticancer therapy, potentially overcoming cisplatin resistance by targeting Cancer Stem Cells (CSCs). Further testing for WS's efficacy should be

conducted alone or in combination with other chemotherapeutic drugs for lung cancer (Senthilnathan et al., 2006).

1.7.1.2. Breast Cancer

Breast cancer is an usual malignancy among women, with aggressive and metastatic breast tumors. Studies have shown that Withaferin A (WA) has potential therapeutic potential against breast cancer by suppressing tumor cells' proliferative and invasive characteristics. (Stan et al., 2008) Early studies on cell lines indicated that WA induced mitotic arrest in MCF7 and MDA-MB-231 cells, and female nude mice injected with MDA-MB-231 cells displayed significant tumor retardation when treated with 5 weekly intraperitoneal injections of 4mg WA/kg body weight (Stan et al., 2008). WA has also been involved in reactive oxygen species (ROS)-induced apoptosis and inhibited oxidative phosphorylation in breast cancer cells (Hahm & Singh, 2013).

The Ser56 residue of vimentin has been phosphorylated by WA, providing anti-invasive and anti-metastatic properties in both *in vitro* and *in vivo* settings (Mulabagal et al., 2009). Withania root extracts have also been reported to inhibit Epithelial-Mesenchymal Transition (EMT) in breast cancer cell lines and in xenograft mice models injected with MDA-MB-231 cells. Withaferin A inhibited the growth of Estrogen receptor (ER) positive MCF7 and T47D cells, exhibiting anti-estrogen properties (Lee et al., 2012; Szarc vel Szic et al., 2017).

1.7.1.3. Brain cancer and Glioblastoma

Alcoholic leaf extracts of WS have been shown to induce differentiation, apoptosis, and growth arrest in Glioma cells. The combined use of WA and Withanone showed anti-invasive and anti-angiogenic activity by down-regulating hnRNP-K, VEGF, and MMP-3. Ashwagandha water extracts (ASH-WEX) have been found to have anti-tumor effects, including differentiation, apoptosis induction, morphological changes, cell cycle arrest, and downregulation of EMT markers (Shah et al., 2009). In vitro studies have shown reduced intracranial tumor volumes and downregulation of tumor-promoting proteins, further strengthening the anti-glioma efficacy of ASH-WEX. Ashwagandha extract also effectively mitigated LPS-induced nitric oxide and ROS production, upregulating the NRF-2/HO1 pathway, and downregulating filopodia formation in BV-2 cells (Grogan et al., 2010).

Studies by Grogan et al. suggest that WA derived from *Vassobia breviflora* activates both extrinsic and intrinsic apoptosis pathways, decreasing protein expression of AKT/mTOR pathway components and cell surface receptors (Kataria et al., 2013). The study also reported a dose-dependent decrease in cellular proliferation and resensitization of temozolomide-resistant Glioblastoma multiforme cells when treated with WA, suggesting potential use in combination with standard chemotherapeutic agents like TMZ (Grogan et al., 2014).

1.7.1.4. Prostate Cancer

Prostate cancer is the second most common malignancy in men worldwide, accounting for 3.8% of all deaths in 2018. WS root extracts have been reported to induce multiple anticancer effects, including apoptosis response-4 (PAR-4) dependent apoptosis in prostate cancer cells, regressions of PC-3 xenografts in nude mice, and a dose-dependent decrease in cell viability and Wee1 accumulation. WA also mediates vimentin disruption, induced ROS generation, and reduced c-FLIP levels. A 3-azido derivative of WA (3-azidoWA) inhibited invasion by extracellular Par-4-dependent inhibition of MMP-2 in HeLa and PC-3 cells. In vitro studies showed that WA inhibited prostate tumor development, decreased Akt levels, and restored Par4 protein function, inhibiting Akt-induced EMT (Moselhy et al., 2017). Oral administration of WA inhibited EMT and lung metastasis in treated mice, suggesting the medicinal potential of WS in prostate cancer. Recent research suggests that WS root extract may inhibit lipogenesis in 22Rv1 cells by downregulating c-MYC and p-AKT levels, suggesting the role of fatty acid metabolism in cancer cells and a new mechanism of imparting antitumor activity in prostate cancer (Kim et al., 2020).

WA has also been shown to suppress JAK2 and STAT-3 phosphorylation, inhibit NF-kB, activate NOTCH-2 and NOTCH-4, restrain the self-renewal of cancer stem cells, and upregulate Death Receptor five mediated by Elk1. Epigenetic investigations have revealed that WA could lead to changes in the methylation status of genes involved in Triple-negative Breast Cancer (TNBC) and suppress TNBC-specific characteristics, leading to less aggressive luminal breast cancer with improved therapeutic response and sensitivity. Overall, these studies suggest that WS active constituents work via multiple molecular modes to suppress tumor cells' proliferative and invasive characteristics, offering immense therapeutic potential against breast cancer (Nagesh et al., 2018).

1.7.1.5. Colon cancer

Prostate cancer is the second most common malignancy in men worldwide, recording 3.8% of all deaths in 2018. WS root extracts have been reported to induce multiple anticancer effects, including apoptosis response-4 (Par-4) dependent apoptosis in prostate cancer cells, regressions of PC-3 xenografts in nude mice, and a dose-dependent decrease in cell viability and Wee1 accumulation. WA also mediates vimentin disruption, induced ROS generation, and reduced c-FLIP levels (Koduru et al., 2010). A 3-azido derivative of WA (3-azidoWA) inhibited invasion by extracellular PAR-4-dependent inhibition of MMP-2 in HeLa and PC-3 cells (Das et al., 2014). In vitro studies showed that WA inhibited prostate tumor development, decreased Akt levels, and restored Par4 protein function, inhibiting Akt-induced EMT. Oral administration of WA inhibited EMT and lung metastasis in treated mice, suggesting the medicinal potential of WS in prostate cancer. Recent research suggests that WS root extract may inhibit lipogenesis in 22Rv1 cells by downregulating c-Myc and p-Akt levels, suggesting the role of fatty acid metabolism in cancer cells and a new mechanism of imparting antitumor activity in prostate cancer (Choi & Kim, 2015).

1.7.1.6. Other Cancers

WS has been reported as a potent antitumor agent in various cancer types, including melanoma, skin papilloma genesis, and osteosarcoma. It induces DNA fragmentation, ROS generation, and mitochondrial mediated apoptosis in melanoma cells, and can prevent skin papilloma genesis in mice (LV & WANG, 2015). Treatment with WS root extract leads to weight gain and skin lesions reduction in Swiss albino mice. Cell cycle arrest in osteosarcoma, gastric, and oral cancer cells is also induced by it. Detailed studies on its molecular mechanisms have been limited however (Chang et al., 2017).

In oral cancer, researchers found oxidative stress-mediated selective killing of oral cancer cells, depolarization of mitochondrial membrane potential, and DNA damage (Li et al., 2015). In pancreatic cancer cells, combined administration of oxaliplatin with WA led to increased intracellular ROS levels, apoptosis induction, and inactivation of Akt phosphorylation (Munagala et al., 2011). Further studies on mice demonstrate the strongest anti-tumor activity of WS (Mehta et al., 2020).

Chapter 02

General Remarks

General Remarks

2.1. Materials and Reagents required for experimental studies

We have procured ENU (N-ehtyl N-nitrosourea) and 3− (4, 5-Dimethytthiazol-2-yr) −2, 5-diphenyltetrazolium bromide (MTT) from Sigma-Aldrich (USA). Paraformaldehyde Sigma glutaraldehyde, Xylene, Ethyl alcohol and methyl alcohol were used as fixatives and cleaning reagents and obtained from Giemsa stain Merck, USA). Various histological and cytological stains e.g. Haematoxylin and Eosin stain, Janus Green B, Trypan blue, Brilliant Cresyl Blue stain were obtained also from Merck (USA). Drabkins solutions were procured form Stanbio Reagent, India for the estimation of Hemoglobin by colorimetric method. Ashwagandha from Himalaya Drugs Co. India used for preparation of Ashwagandha solution. All solutions were passed through a 0.22 µm fitter (GVMP 01230, Millipore) and stored at 4C until use.

Low melting agarose (1%) normal agarose. EDTA (ethylene di amine tetra acetate), and NaOH were procured form SRL, India. Tris-HCI was obtained from Himedia , India were used for comet assay. RPMI-1640 (Sigma, USA), FBS (Gibco, Thermo Fisher Scientific, USA) and Penicillin and Streptomycin (HiMedia, India) were procured and used for various cell culture works.

Cell culture dishes (35mm and100mm) Cell culture plates (6 well,12 well and 24 well plates), Transwell membrane plate or invasive chamber with an 8-µm pore with polycarbonate membrane were obtained from Corning, Inc., USA. 15mL and 50mL falcon tubes and 0.5mL, 1.5mL and 2mL were obtained from Tarsons Products Pvt.Ltd, India. Glass slides and cover slips were procured from Blue star, India. Sterile syringe with 24 gauge needle and 23 gauge needle from Dispovan, India were used for administration of ENU and flush out bone marrow form long bones.

2.2. Primary and secondary antibodies used for experimental studies

Activity of deferent components of hedgehog pathway was assessed by using primary antibody e.g. Anti-HHIP, Anti-SHH, Anti-PTCH and Anti-SMO, Anti-SUFU, Anti-GLI 3, Anti-GLI1 , for apoptotic pathway anti- PUMA, anti-BCL2 and anti-caspase 3 were used. Anti-NRF2 and Anti-KEAP1 were used for evaluation of NRF2-Keap1 pathway, anti-Cyclin

D1 anti-NCAD, anti-VIMENTIN, anti-BETA ACTIN, anti-BETA TUBULIN, anti-CYLD, anti-NAK and anti-CD31 were used to assess cytoskeletal and cell cycle processes. Anti-ATG12 , anti-DICER, anti-DROSHA, anti- AGO1 and anti-AGO2 antibody were used to fathom miRNA processing pathway. To evaluate autophagy and cell cycle activity Anti-TRAF2 and Anti- P62, anti-BMI-1, anti-LIN28A, anti-N-MYC were used. All these aforementioned primary antibodies were procured from Cell signalling technologies, USA). To assess various activities in Hedgehog pathway, NRF2-KEAP1 pathway, cytoskeletal architecture modulation anti-GSK3β and anti-βTrCP from Santa Cruze Biotechnology, USA were obtained. In all the flow cytometric assays Alexa488 from Invitrogen USA were used as secondary antibody. On the other hand, for immunocytochemical analysis DAPI shield from Sigma-Aldrich, USA was used for nuclear staining.

2.3. Animal maintenance for experimental purpose

Inbred Swiss albino mice (***Mus musculus***) were maintained at the animal house of Calcutta School of Tropical Medicine abiding by the regulations of Institutional Animal Ethical Committee (IAEC). Animals were kept in sterile condition, with proper temperature (22 ± 2 ºC), humidity (50±5%) and 12 hours light and dark cycle. Maximally six animals were kept in each cage and provided with proper diet and water ***ad libitum*** throughout the experimental period.

2.4. Equipment and Instrument in use

For hematological and cytological estimation improved Neubeur chamber from Rohem, India was used. Cell cultures were maintained in Heracell VIOS 160i CO2 incubator (Thermo scientific, USA) and cultures were optically evaluated with an inverted microscope (Olympus, Japan). Hematological, cytological and histological slides were periodically evaluated by light microscope (Ch 20i Olympus, Japan). For immunofluorescence evaluation slides were examined under Axio Scope.A1, Zeiss, Germany, fluorescence microscope and for confocal microscopy LSM700 confocal microscope (Zeiss, Germany) was used.

BD FACS Calibur (Becton-Dickinson, USA) using CellQuest Pro software (v9.1 Becton-Dickinson, USA) were used for Flow cytometric data acquisition and analysis. Statistical

evaluation was conducted with Graph pad prism (version 8.4). Comet Score software (version 2.0.0.8, USA) was used for the assessment of Comet assay.

Chapter 03

Ashwagandha and Withanolide D improved physiological and hematological scenario in the bone marrow of NOC induced leukemic murine model

CHAPTER SUMMARY

The current chapter gives a brief discussion on the development and assessment of the leukemic condition, as well as the estimation of LD_{50} and EC_{50} values of Ashwagandha and Withanolide D. Effects of Ashwagandha and Withanolide D on N-Ethyl N-Nitrosourea (ENU) induced mouse model was assessed through physiological assessment, peripheral blood hemogram, bone marrow cytology, and other methods. Our results successfully established a mouse model of the disease that closely imitates human bone marrow leukemia. The findings of the study helped in the investigation of disease mechanisms at the deeper molecular level.

3.1. INTRODUCTION

N-nitroso compounds and Nitrosamines are broad acting carcinogens. Nitrosamine-mediated injury and mutagenesis is heavily influenced by frequency of exposure, chemical nature of the compound, and dose. Chemical reactions between nitrites and secondary amines or proteins helped in the formation of Nitrosamines (R1N(-R2)-N=O). Sodium nitrite, a nitroso compound, is added to fish and meat to prevent *Clostridium botulinum* induced toxin production. It also helps to preserve colour and flavour of meat. Acidification, heating, or oxidation of nitrite leads to nitrous acid formation. The resulting nitrosonium cation (N=O+) reacts with dimethylamine to generate nitrosamines. Due to the high protein content of bacon, ground beef and cured meats contain abundant amounts of amines, and they also have high levels of nitrates and nitrites (Lijinsky 1999). Other important sources of nitrosamine exposure include cheese products, fish byproducts, non-fat dry milk, tobacco, and water (Hotchkiss 1989). Apart from dietary sources pesticides, fumes from rubber factories, industrial waste materials are also major sources of nitroso compounds (Swann et al. 1968; Kearney 1980; Pegg 1980). Moreover, nitrosamines are easily generated under high temperatures associated with frying, flame broiling or various industrial manufacturing processes or with strong acid conditions, as exist in the stomach (Fournier 1990; Robbiano et al. 1996; Kyrtopoulos et al. 1997).

Destabilization and increased breakage of DNA were caused due to toxic and mutagenic effects exerted by alkylating N-7 of guanine by Nitrosamines (Otteneder et al. 1999; Chuang

et al. 2006; Yadav et al. 2007; Espey et al. 2002; Lim 2002; de la Monte et al. 2008). Activated nitrosamines aid in the generation of hydrogen peroxide (H_2O_2) and reactive oxygen species such as superoxide (O2−). Generation of ROS thereby increases DNA damage, oxidative stress, protein adduct formation and lipid peroxidation (Alaba et al., 2017). Oxidative stress and DNA damage activate pro-inflammatory cytokines, promote insulin resistance, alterations in various signalling pathways, stoppage in apoptotic and autophagy machinery etc.; all of which play crucial role in the pathogenesis of leukaemia (Shaik et al., 2020).

Hematopoiesis is a finely orchestrated process and any alterations in this endroit lead to leukemic pathophysiology with an abundance of immature cellular population reflected in bone marrow as well as in peripheral circulation (Law et al,.2001; 2003; 2008; 2011). In the battle against this dreadful disease many strategies, therapeutic regimen, were formulated and implemented which bears various side effects and other complications so we turned towards mother nature in search of more effective, natural alternatives.

Withania somnifera or Ashwagandha is one of the most ancient and widely used herbs in traditional Indian Ayurvedic medicine mainly found in Indian subcontinent along with many parts of south Asia (Henrich et al., 2015; Issa et al., 2017). In the Ayurvedic system this plant is used in various diseases and disorders. It is extensively studied for its biologically active constituents and contains several pharmacologically important steroidal lactones i.e., Withanolides, Glycowithanolides, Withaferins etc. Various studies on Withanolides portrayed their effect on inhibition of metastasis, angiogenesis, cell cycle arrest, cytotoxicity, apoptosis, cytoskeletal destabilization etc.(Ichikawa et al., 2006; Lacombe et al., 2020; Wijeratne et al., 2018).

In this present chapter we tried to unveil the establishment of NOC induced leukaemia in Swiss albino mice and dosimetry of Ashwagandha and Withanolide D on the same.

3.2. MATERIALS AND METHODS

3.2.1. Disease induction

Inbred Swiss albino mice (***Mus musculus***) were maintained at the animal house of Calcutta School of Tropical Medicine abiding by the regulations of Institutional Animal Ethical Committee (IAEC). Animals were kept in sterile condition, with proper temperature (22 ± 2 ^{o}C), humidity and 12 hours light and dark cycle. Maximally six animals were kept in each cage and provided with proper diet and water ***ad libitum*** throughout the experimental period.

Four groups of animals were taken into consideration for experimental purposes. Group-II (denoted as L): Litter pups of 10-14 days weighing 4-5 gms were challenged with intra-peritoneal injection of ENU, a potent carcinogen, at a dose of 80 mg/kg body weight (Druckery et al. 1967) for developing leukemic (N=30) condition in 6-8 months as confirmed by peripheral blood hemogram. Group-I (denoted as C): Control group of mice(N=15) received equal volume of saline in similar condition. Group III (denoted as L+A): After the development of the disease animals were administered aqueous extract of Ashwagandha via oral route (N=30) and in group IV(denoted as L+WD) : animals were administered with Withanolide D solution via oral route.

3.2.2. Isolation of Withanolide D

20 grams of Ashwagandha powder was dissolved in 100ml of distilled water over night at 4°C, followed by filtration under sterile condition. Filtrate was treated as 100%. This filtrate was filtered again through a 0.22-micron filter membrane and used for High Performance Liquid chromatography. Withanolide D (WD) was separated and collected from Ashwagandha whole extract.

3.2.3. Estimation of LD_{50} and EC_{50} administration of Ashwagandha and Withanolide D.

To determine the **LD_{50}** value of Ashwagandha and isolated Withanolide D was administered via oral route in the leukemic mice. In leukemic mice 1000,1100,1200,1300 mg /kg body weight of Ashwagandha were administered. No mortality was found up to 1100 mg/kg body weight, but animals were susceptible to death after administration at doses of 1200mg /kg body weight and above. Further fractions of doses of 1125, 1150, 1175 mg/kg bodyweight of

Ashwagandha were administered and 50 % animals were susceptible to death at 1175 mg/kg bodyweight. On the other hand, Withanolide D was administered at a dose of 50, 60, 70, 75 and 80 mg/kg of bodyweight. It was found that at the dosimetry of 75 mg/kg of bodyweight 50 percent of all the experimental animals died.

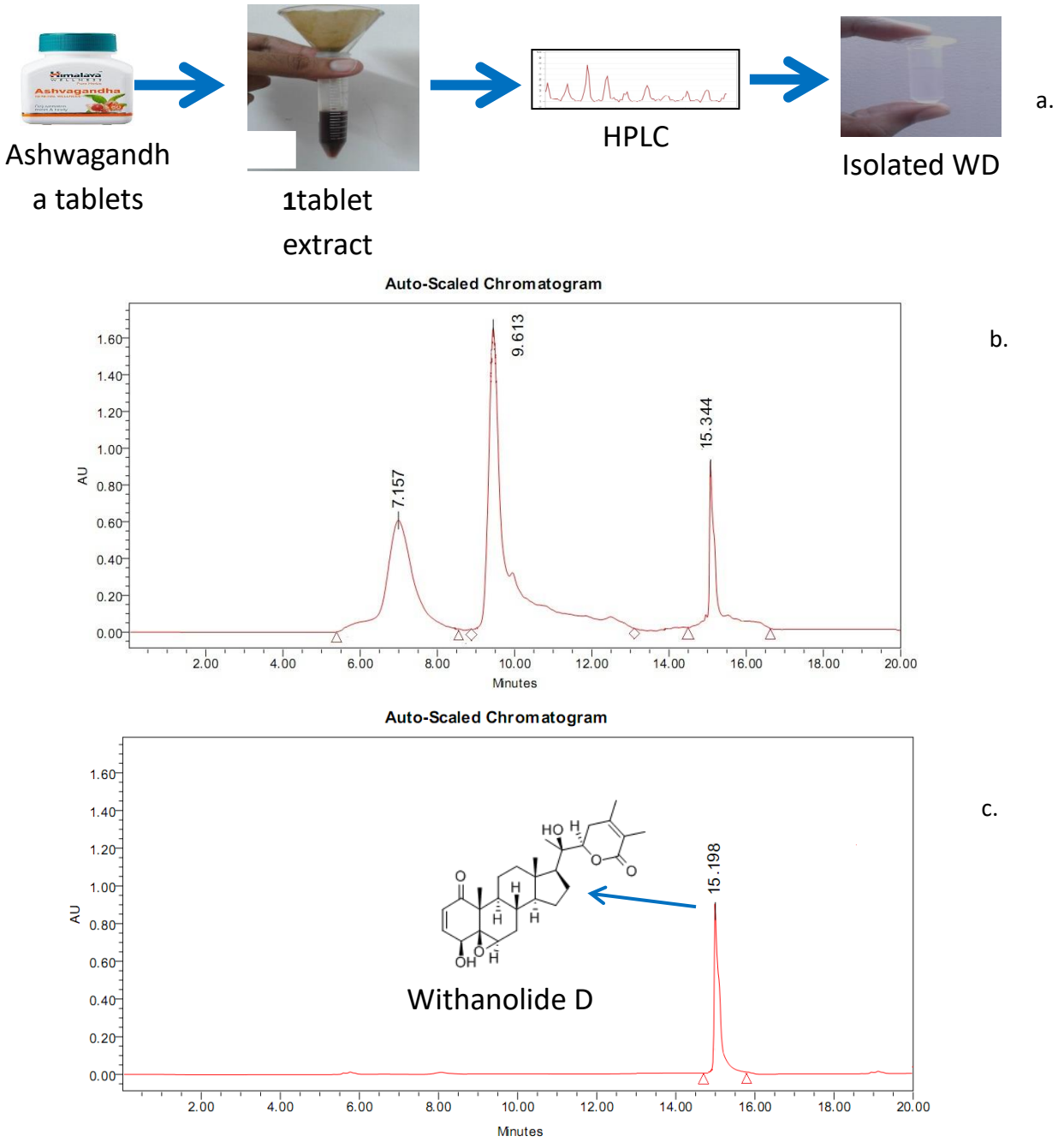


Figure 1. a. Isolation of Withanolide D from Ashwagandha. b. HPLC graph of Ashwagandha extract c. HPLC graph of Withanolide D standard.

To denote **EC_{50}** value half of **LD_{50}** value was taken as per previous studies. Ashwagandha was administered at a dose of 500, 550, 600, 700, and 800 mg/kg of bodyweight. Similarly, Withanolide D was administered at a dose of 27.5, 37.5 and 47.5mg/kg of bodyweight. After the dosing, 50 % of experimental animals showed change in their physiological, haematological, and cytological parameters at a dose of 600mg/kg bodyweight of Ashwagandha and 37.5 mg/kg bodyweight for Withanolide D.
So, Ashwagandha and Withanolide D were administered via oral route for 30days at a dose of 600mg/kg bodyweight and 37.5mg/kg of bodyweight respectively.

3.2.4. Assessment of physiological status

Bodyweight measurement: Change in bodyweight of an animal clearly reflects any internal alterations taken place inside the system. Animals of all four groups were regularly weighed for the entire experimental period.

Locomotor Activity: The open field activity monitoring system comprehensively assesses locomotor and behavioral activity levels of mice of all four experimental groups. Animals of all four groups were regularly placed in a 4' X 4' open field box and their movements were recorded for 3 minutes. Each animal was evaluated 3 times at regular intervals.

3.2.5. Hemogram profiling of peripheral blood

After the development of disease (4-6 months) and post Ashwagandha and Withanolide D administration approximately 200 µl of peripheral blood was collected by tail vein puncture method for assessing various hematological parameters. Differential count (DC) of WBC was evaluated by Leishman-stained blood smears and Total count (TC) of WBC, RBC by standard laboratory procedure utilizing improved Neubauer chamber (Rohem, India). The percentage of reticulocytes was determined by using Brilliant Cresyl Blue staining method. Hemoglobin percentage was to delineate by colorimetric method using Drabkins solutions (Stanbio Reagent, India).

3.2.6. Dye exclusion method

Animals of all four groups (e.g. control, leukemic, leukemic administered with Ashwagandha and leukemic administered with Withanolide D) were euthanized by cervical dislocation and their long bones e.g. femur, tibia were aseptically collected. Marrow from the aforementioned bones were flushed out using sterile syringe with 24-gauge needle (Dispovan, India) filled with RPMI-1640 (Sigma, USA Single cells were prepared from the extracted marrows portions. Single cell suspension was divided into two parts, one part was used for other cytological analysis and other for the dye exclusion method. Single cells were incubated with 0.04% Trypan blue (Sigma, USA) stain at 1:1 ratio for 10 minutes at room temperature. Post incubation cells were charged into hemocytometer (Rohem, India) for evaluation.

3.2.7. Statistical evaluation

Intergroup comparison was evaluated by student's t test and for multiple comparisons one way analysis of variance (ANOVA) was used. All data are presented as mean ± standard deviation. Each experiment was performed three times for statistical significance. Significance was defined as $P < 0.05$.

3.3. RESULTS

3.3.1. Establishment of ENU induced leukemic mouse

N-Ethyl N-nitrosourea (ENU) is a chemical compound known for its mutagenic properties. ENU is known to induce DNA damage through the formation of alkylating agents. Alkylating agents generated by ENU can directly interact with DNA, leading to the formation of DNA adducts and subsequent mutations. (Lijinsky, 1999.; Ledda et al. 2017;).Post ENU induction Swiss albino mice were under strict observation and their physiological and hematological parameters were tested at a regular interval of every 15 days. 60 to 65 days post administration of ENU animals started to show the initial signs of leukemic conditions, e.g., increase in blast burden, presence of abnormal cells in peripheral blood, reduced locomotor activity etc. After 90 to 120 days most of the animals showed specific signs of leukemic pathophysiology, described as increased blast burden in peripheral blood, abnormal levels of lymphocytes and neutrophils, reticulocytes etc. Associated symptoms of leukemic conditions

such as ruffled hairy coat, weight loss, reduced movement, loss of appetite, stupor etc. also indicated the onset of the disease in the ENU administered mice group.

ENU-induced DNA damage can impair specific repair pathways, such as nucleotide excision repair and base excision repair involved in repairing ENU-induced DNA damage in bone marrow cells. (Metayer et al. 2017). ENU-induced mutations lead to altered proliferation, differentiation, and survival of hematopoietic cells, as well as hematopoietic stem cells and progenitor cells affecting the overall balance of hematopoiesis which ultimately leads to leukemic conditions in experimental animals.

3.3.2. Dosimetry and estimation of LD_{50} and EC_{50} values

Solution of Ashwagandha and Withanolide D were administered in experimental leukemic animal groups in order to estimate the LD_{50} value. LD_{50} refers to a dose at which 50% of all test subjects die. Previous studies have estimated LD_{50} value of Ashwagandha and Withanolide D in normal Swiss albino mice but LD_{50} value of Ashwagandha and Withanolide D in leukemic mice were unavailable. Ashwagandha was administered at a dose of 1000 mg /kg ,1100 mg /kg ,1200 mg /kg and1300 mg /kg bodyweight via oral route. 100% mortality was observed at 1200 mg /kg and1300 mg /kg. At the dose of 1150 mg/bodyweight 33.34% mortality, 16.67% was observed at 1125 mg/bodyweight and no mortality was noted at 1125 mg/bodyweight 1100 mg/bodyweight and 1000 mg/bodyweight. At 1175 mg/bodyweight 50% of all test animals were succumbed to death (Table I).

Table I: LD_{50} value of Ashwagandha

Ashwagandha	Dead	survived	Total	Rate	% mortality	% survival
1300	30	0	30	30/0	100	0
1200	30	0	30	30/0	100	0
1175	15	15	30	15/30	50	50
1150	10	20	30	10/30	33.34	66.66
1125	5	25	30	5/30	16.67	83.33
1100	0	30	30	0/30	0	100
1000	0	30	30	0/30	0	100

Similarly, Withanolide D was administered at a dosimetry of 50mg/kg, 60 mg/kg, 70mg/kg, and 80mg/kg of bodyweight via oral route. 100% mortality rate was noted at 80mg/kg of bodyweight dose and at 70 mg/kg of bodyweight 33.33% mortality rate was observed. A fraction between 70 and 80mg/kg bodyweight, i.e., 75mg/kg bodyweight was administered and 50% of all the test animals died (Table II).

Table II: LD_{50} value of Withanolide D

Withanolide D mg/kg	Dead	survived	Total	Rate	% mortality	% survival
80	30	0	30	30/0	100	0
75	15	15	30	15/30	50	50
70	10	20	30	10/30	33.34	66.66
60	5	25	30	5/30	16.67	83.33
50	0	30	30	0/30	0	100

After the LD_{50} values determination EC_{50} values were also evaluated for Ashwagandha as well as for Withanolide D. Experimental animals were divided into five groups and all of them were administered with Ashwagandha solution via oral route. Five different doses were evaluated for the estimation of EC_{50} value, i.e., 500 mg/kg 550 mg/kg 600 mg/kg 700 mg/kg and 800 mg/kg bodyweight. At 600 mg/kg bodyweight dose changes in hematological, cytological and physiological parameters were observed (Table III).

Table III: EC_{50} value of Ashwagandha

Ashwagandha mg/kg	Total	Activity observed	Rate	%
500	30	5	5/30	16.67
550	30	10	10/30	33.33
600	30	15	15/30	50
700	30	15	15/30	50
800	30	30	30/0	100

Similarly effective concentration or EC_{50} value was evaluated. Experimental animals were divided into 3 groups, and they received an aqueous solution of Withanolide D via oral route. Three different doses of Withanolide D i.e., 27.5mg/kg, 37.5mg/kg and 47.5mg/kg of bodyweight were administered. At a dose of 37.5mg/kg of bodyweight Withanolide D showed maximum effect in 50% of the experimental animals (Table IV).

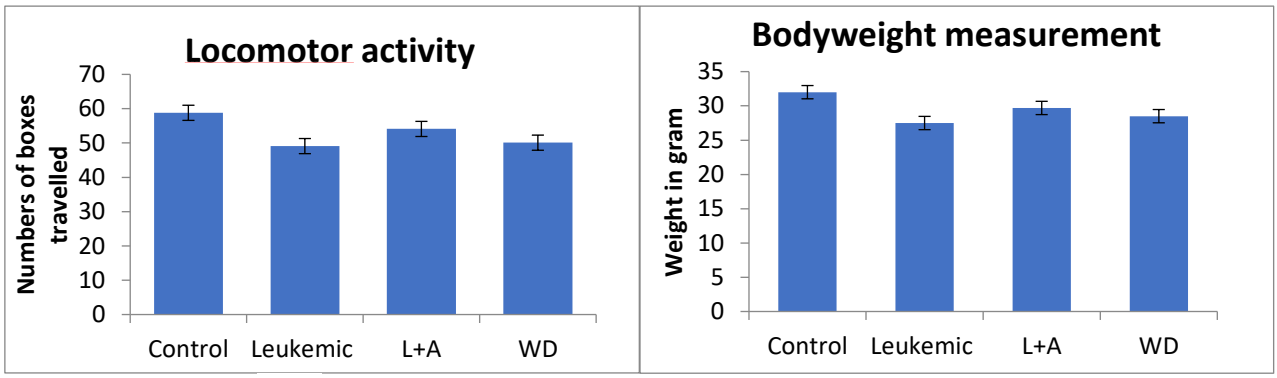
Table IV: EC_{50} value of Withanolide D

Withanolide D mg/kg	Total	Activity observed	Rate	%
27.5	30	5	5/30	16.67
37.5	30	3	15/30	50
47.5	30	30	30/0	100

Summarily, at a dose of 1175mg/kg body weight, Ashwagandha and at a dose of 75mg/kg of bodyweight, Withanolide D showed severe toxicity in leukemic mice and 50 percent of animals died. The estimated Effective concentration of Ashwagandha was 600mg/kg bodyweight and Withanolide D was 37.5mg/kg of bodyweight.

3.3.3. Ashwagandha and Withanolide D improves physiological activity

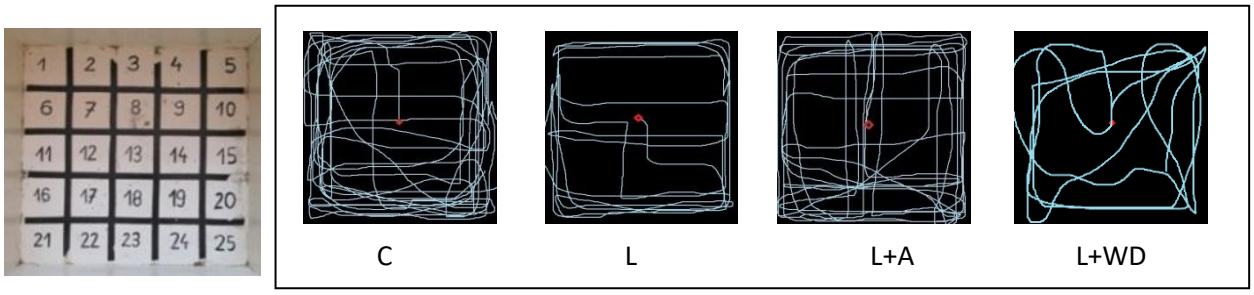
Bodyweight and locomotor activity are two very basic but important physiological parameters that can give a basic idea of the health conditions of the experimental animals. Measurement of bodyweight was also evaluated for its value in toxicity testing for drugs and progression of disease. On the other hand, open field activity monitoring system comprehensively assess locomotor and behavioral activity levels of mice. It is a useful tool for assessing locomotive impairment in animal models to see the progression of disease and efficacy of therapeutic drugs that may improve locomotion and/or muscle function. (Tatem *et al.* 2014). As we know leukaemia results in severe weight loss, fatigue, restricted movements and many other physiological issues. Signs of disease progression and effects of Ashwagandha and Withanolide D administration against the dreadful disease were evaluated at a regular interval, bodyweight and locomotor activity of animals among all four groups were recorded. Progression of the disease clearly left it's mark on the bodyweight of leukemic mice and as a result bodyweight in leukemic group has markedly decreased to an average of 27±2.2gms in comparison to control group with an average of 33.5±3.5gms. Post Ashwagandha and Withanolide D administration bodyweight of leukaemic mice were increased to an average of 29.2±1.9gms and 28.5± 1.2 gms respectively (Figure.1a, b & c.).



a. b.

Figure 2. **a.** Graphical representations of locomotor activity and **b.** bodyweight measurement in all four experimental groups.

On the other hand, locomotor activities of the leukemic mice were limited to an average of 48.3± 3.62 boxes in comparison to control group with an average of 58.5± 2.74 numbers of boxes. After Ashwagandha and Withanolide D administration locomotor activity has increased to 54.2± 2.9 boxes and 50.1± 1.4 boxes respectively on average (Figure.1d & e). Locomotor activity and body weight measurements clearly portrayed positive changes imparted by Ashwagandha and Withanolide D administration.



a b

Figure 3. **a**. Representative image of box plot **b.** Representative image of locomotor activities in all four experimental animals' groups.

3.3.4. Ashwagandha and Withanolide D administration improved hematological parameters:

Hematological assessment showcased a deeper insight into the disease development and progression as well as reversal of the scenario caused by Ashwagandha and Withanolide D administration. Leukaemia as a hematopoietic disorder definitely left its mark on the hematological parameters. To ascertain the clinical status of the disease hematological analysis was performed and it revealed sharp increase ($P<0.0001$) of total white blood cell

(WBC-TC) count in leukemic mice (35.3 ± 2.57 × 10^3 cells/mm^3) as compared to control (6.3 ± 1.28 cells/mm^3) but in Ashwagandha treated group.

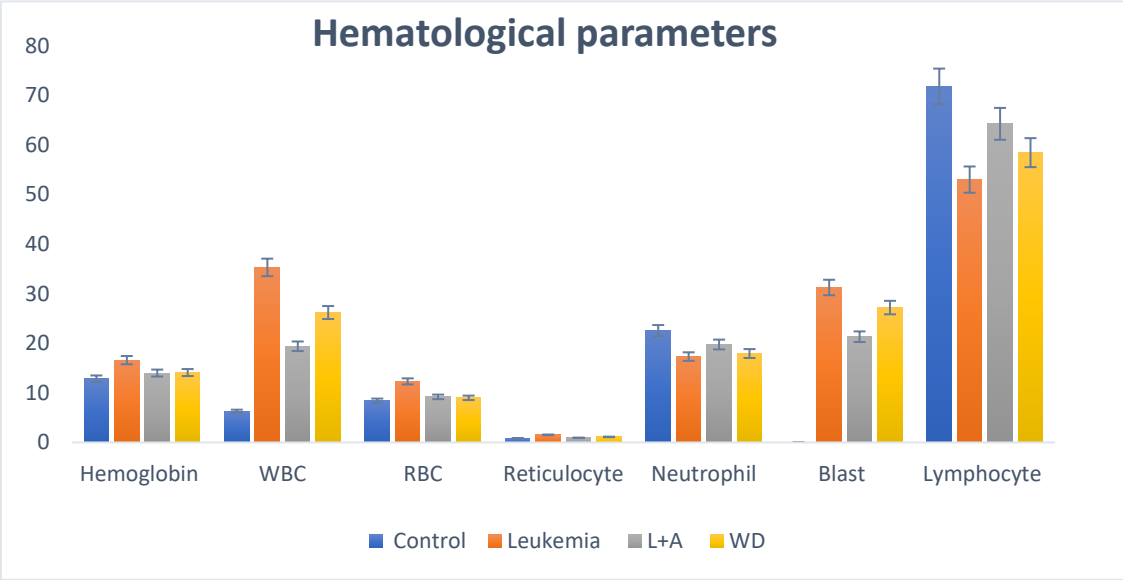


Figure 4. Graph depicting various hematological parameters in all four experimental groups.

WBC-TC values started to decrease towards normal values (19.4 ± 0.68 cells/mm^3) (Table XI). Withanolide D treated group also showed significant decrease in WBC-TC values (26.2± 0.72 cells/mm^3). Red blood cell (RBC) count as well as hemoglobin content also increased.

Significantly (L= 12.31 ± 0.23 x 10^6 cells/mm^3 and 16.6± 1.97gm/dl) in leukaemic condition as compared to control (C = 8.43 ± 0.32x 10^6 cells/mm^3 and 12.87 ± 0.26 gm/dl), a clear sign of progression of the disease. Post Ashwagandha and Withanolide D administration directed a significant decrease in red blood cell count along with hemoglobin content (L+A= 9.19± 0.86 x 10^6 cells/mm^3 and 14.0 ± 0.47 gm/dl) and (L+WD = 9.0± 0. 95 cells/mm^3 and 14.1± 0.32 gm/dl). Ashwagandha along with Withanolide D not only ameliorated aforementioned blood parameters but also imparted positive effects on reticulocyte count and blast percentage. Increased reticulocyte percentage ($P < 0.01$) was observed in leukemic mice (L=1.53 ± 0.09%) in comparison to control (C=0.81 ± 0.15%) and decreased significantly post Ashwagandha and Withanolide D administration (L+A = 0.94 ± 0.07%) and (L+WD =1.12± 0.08). Moreover, a sharp rise in blast percentage (L=31.25 ± 6.65%) in leukemic condition started to decrease in both the treated group (L+A = 21.33± 4.38%) and (L+WD =

27.21±1. 41%) clearly depicted positive effect of Ashwagandha and Withanolide D hematological aspects (Figure.5).

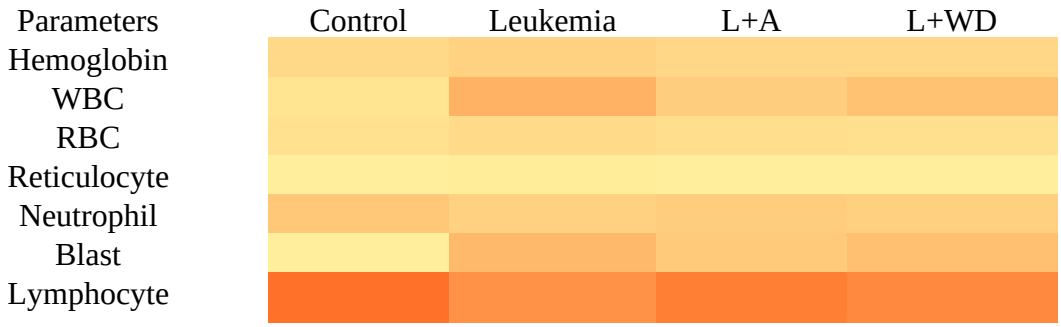


Figure 5. Heat map depicting various hematological parameters in four experimental groups.

Table V. Hematological parameters in the four experimental groups (C=control , L=leukemic, L+A=leukemic treated with Ashwagandha, Leukemic+WD =Leukemic treated with Withanolide D)

Parameters	Control (X±SD)	Leukemic (X± SD)	L+A (X±SD)	Leukemic+WD (X±SD)
Hemoglobin (g/ dL)	12.87 ± 0.26	16.6± 1.97	14.0 ± 0.47	14.1± 0.32
(WBC) (X 10^3/µl)	6.3 ± 1.28	35.3 ± 2.57	19.4 ± 0.68	26.2± 0.72
(RBC) (X 10^6/ µl)	8.43 ± 0.32	12.31 ± 0.23	9.19± 0.86	9.0± 0. 95
Reticulocyte (%)	0.81 ± 0.15	1.53 ± 0.09	0.94 ± 0.07	1.12± 0.08
Neutrophil (%)	22.55 ± 2.21	17.32 ± 2.21	19.75 ± 2.75	17.93± 2. 54
Blast (%)	-	31.25 ± 6.65	21.33± 4.38	27.21±1. 41
Lymphocyte (%)	71.8 ± 2.16	53 ± 2.54	64.25 ± 7.63	58.44±5. 74

3.3.5. Assessment of cellular death by dye exclusion method

Post Ashwagandha and Withanolide D administration bone marrow cells were assessed for the cellular death ratio. In Control(C) group (Group I) percentage of live cell was 91± 0.85% and dead cell was 9±1.04 %. On the other hand, leukemic (L) group (Group II) showed increased (P = 0.01) survivability with a percentage of 97.78±1.12% and decreased mortality, 2.22±0.95%. Post Ashwagandha and WD administration the percentages of live cells have decreased to 92.87±1.12% and 94.15±1.33% respectively and percentage of dead cells increased to 7.13± 0.71% and 5.85±0.63% in group III (L+A)and in group IV(L+WD) respectively. Cytochemical and immunocytochemical methods revealed augmented cellular death in the treated group compared to the leukemic group (Figure 6 a-d)

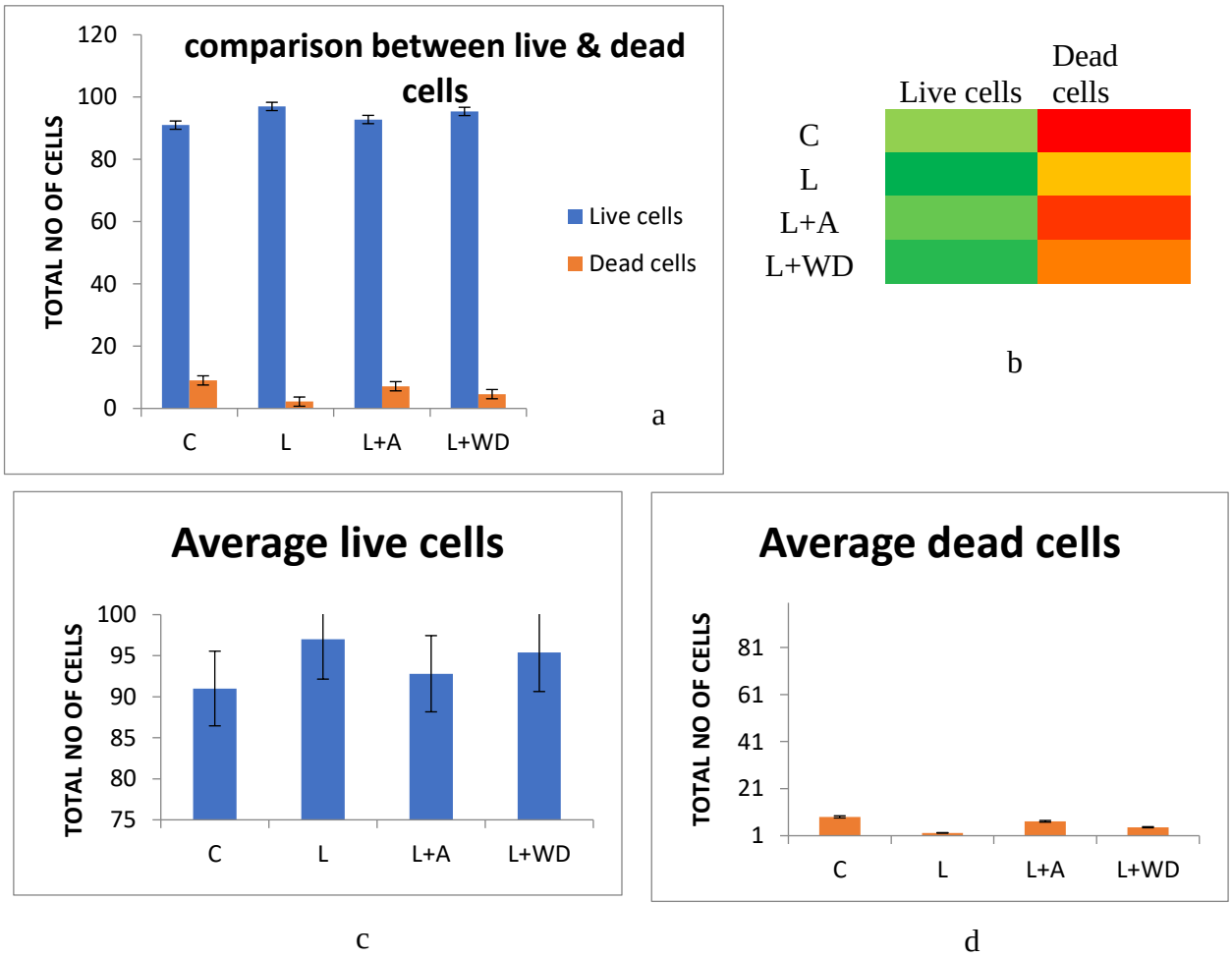


Figure 6. a. Bar graph depicting comparison between living and dead cells in control, leukemic and leukemic treated with Withanolide D. **b.** Heat map depicting live and dead cells among all three experimental groups. **c.** Graph describing dead cell numbers in all three groups. **d.** Graph describing live cell numbers in all three groups.

3.4. DISCUSSION

A healthy cellular/ tissue environment in an organism is maintained by a fine balance between cell proliferation and death, and any imbalance causes abnormality and ultimately to a diseased condition. In our NOC-induced leukemic model, abnormal cellular proliferation and decreased cellular death hindered the balance. As NOCs are mostly accumulated from dietary sources through endogenous sources, we chose intraperitoneal administration rather than other routes. Oral administration may lead to buccal, esophageal or gastrointestinal cancer, which was not desired for our experimental purpose. Both the subclasses of NOCs, nitrosamines and nitrosamides, form highly reactive disazonium ions and can induce alkylating DNA damage. Diazonium ion species alkylated DNA bases at the N7and O6 positions of guanine and the O4 position of thymine. GC-AT transition mutations were caused by O6-alkylguanine adducts, which have been identified as the primary cause of premutagenic lesions. O4-alkylthymine led to TA-GC transitions (Chuang 2006; Espey 2002). Cumulatively, the DNA damage led to abnormal protein production and trafficking, activation of oncogenes, and deregulation of the finely orchestrated hematopoietic machinery that ultimately helped in the progression of the NOC-induced leukaemia.

The catastrophic effects of ENU caused NOC-induced leukaemia in the experimental animals. Results of leukemic progression in the animals led to a decline in physiological parameters, less movement, weight loss, etc. In the haematological assessment, a decline in haemoglobin level, a steep increase in the blast population, total WBC counts, and reticulocyte counts clearly indicated deregulation in the hematopoietic machinery. Deregulation in the hematopoietic machinery led to the overproduction of abnormal blast cells and reticulocytes, which ultimately ended up in the peripheral blood and eventually helped in the progression of the disease.

Locomotor activity is generally used to assess the effect of any drug, medication, and disease condition in animals. In our experimental design, Ashwagandha and its active compound Withanolide D imparted their positive effect, and as a result, body weight and locomotor activity in treated animals started to increase. The positive effects of Ashwagandha and Withanolide D were revealed on hematological assessment of the treated animals. The decline in blast population, total WBC count, and reticulocyte counts, along with other hematological parameters, started shifting towards their control values reflecting the positive effects clearly. Cytological analysis also supported the fact that abnormal cell numbers

decreased and increasing dead cell numbers. Ashwagandha and Withanolide D must have removed the repression of the suppressed apoptotic and/or autophagy machinery, which ultimately caused the destruction and removal of the abnormal cells, as reflected in the cytological evaluation.

As Ashwagandha and Withanolide D showed promising results, we can conclude that both Ashwagandha and Withanolide D may provide an inexpensive, safer, and more easily available alternative for the therapeutic management of leukemia.

Chapter 04

Ashwagandha and Withanolide D modulated components of deregulated Hedgehog signalling pathway in NOC induced leukemic bone marrow cells

CHAPTER SUMMARY

This chapter discussed the immunomodulatory properties of Ashwagandha (*Withania somnifera*) and Withanolide D in the context of NOC-induced leukemic pathogenesis. Components of the aforementioned signalling pathway, SHH, PTCH, SUFU, and GLI1, showed a stronger positive shift in expression than their leukemic counterparts after administering Ashwagandha and Withanolide D. As a result, aberrant cell proliferation and disease development were significantly reduced in the treated bone marrow cells. We hope that our findings will be helpful in providing improved leukemia therapy techniques.

4.1. INTRODUCTION

Bone marrow functions as the principal repository of hematopoietic stem and progenitor cell (HSPC) population for the production of diverse lineages of blood cell, a complex mechanism referred as haematopoiesis (Mikkola et al., 2006). The vigorous process is choreographed by the complex interplay of stem-progenitor cells with highly specialized niche or microenvironment comprised of mesenchymal derivatives such as osteoblasts, multilaminar cells, stromal fibroblasts etc. (Weiss et al., 1984, Dorshkind K. 1990, Winkler et al., 2012). Anomalies in the normal process of blood cell formation may lead to various malignant and non-malignant pathophysiological conditions such as leukaemia, aplastic anaemia, myelodysplasia etc. (Law et al., 2007, Chatterjee et al., 2008, Chatterjee et al., 2015). One of the most common hematopoietic disorders is Leukaemia, develops due to malignant transformation of hematopoietic machinery due to various environmental, xenobiotic stress, chemical pollutants related insults.

However, the identity of the molecular mechanisms and signals which govern hematopoietic cells fate mostly remains illusory. Because of that, identification of mediators of HSPC function is of prime importance in haematopoiesis. The roles of developmentally important signalling pathways, such as Notch, Wingless (Wnt), and Hedgehog (HH) in HSPC homeostasis, proliferation and functions have been extensively studied (Maillard et al., 2008, Stier et al., 2002, Cobas et al., 2004, Reya et al., 2003, Chattoadhyay et al.2018).

HH is a secreted protein family with three member Sonic hedgehog (SHH), Indian hedgehog (IHH,) dessert hedgehog (DHH). In the absence of HH, the Patched (PTCH) receptor acts as

a negative regulator of signalling as it inhibits the action of Smoothened (SMO) (Hammerschmidt et al., 1997). HH protein binds and inhibits PTCH function, induces SMO for onward signal transduction. This signalling cascade activates and localizes the GLI family of transcription factors in nucleus. Although HH is a key regulator of body segment polarity and cell fate decision (Nusslein-Volhard and Wieschaus, 1980), its role in hematopoietic cell differentiation and homeostasis remains elusive. Several reports have portrayed that HH signalling is crucial for hematopoietic progenitor differentiation (Zeng et al. 2018, Cortes et al. 2019).

Furthermore, a recent study indicated that bone marrow cells require SMO-mediated signals for their homeostasis using a HH deficient *in vivo* model (Zhao et al., 2009). Upon deletion of PTCH1 a defect in the common lymphoid progenitor (CLP) population was observed by Uhmann (Uhmann et al., 2007). Moreover, HH signalling has been depicted to be decisive for the proliferation and differentiation of hematopoietic progenitors in the thymus (El Andaloussi et al., 2006; Crompton et al., 2007). Finally, a recent report also showed SMO as a drug target for BCR-ABL+ human leukaemic stem cells and tried to establish the Hedgehog pathway as an important one regarding malignant haematopoiesis and the maintenance of leukaemia. (Dierks et al., 2007, 2008).

Advancement in scientific knowledge gives us newer, more accurate arsenal of molecules and drugs to fight the battle against cancer. The chemotherapeutic agents in cancer are characterized by an escalated level of inherent toxicity and the development of drug resistance. Thus, there is an undeniable need for new interference strategies with an enhanced therapeutic outcome. Natural products showcase an opulent source of biologically active compounds which interact simultaneously with different targets involved in cell differentiation, migration, growth, and apoptosis.

Ashwagandha, (*Withania somnifera*) also known as "Indian winter cherry" or 'Indian ginseng" is a medicinal plant that has been utilized in Ayurvedic system of treatment in India as well as many other parts of South Asia for ages. Ashwagandha is known for a wide range of biological activities including neuroprotective (Jain et al. 2001) immunomodulating (Nosalova et al.,2013), anti-inflammatory (Khanna et al.,2007), and anticancer activities (Rai et al. 2016). Isolated active compounds of Ashwagandha have been studied on leukaemic cell lines as an anti-leukaemic drug but Ashwagandha root extract as potent modulator of

hedgehog pathway in *in-vivo* leukemic model is yet to be investigated on which we have emphasized in the study.

4.2.MATERIALS AND METHODS

4.2.1. Animal maintenance and disease development

Inbred Swiss albino mice (***Mus musculus***) were maintained at the animal house of Calcutta School of Tropical Medicine abiding by the regulations of Institutional Animal Ethical Committee (IAEC). Animals were kept in sterile condition, with proper temperature (22 ± 2 ºC), humidity and 12 hours light and dark cycle. Maximally six animals were kept in each cage and provided with proper diet and water ***ad libitum*** throughout the experimental period.

Four groups of animals were taken into consideration for experimental purposes. Group-II (denoted as L): Litter pups of 10-14 days weighing 4-5 gms were challenged with intra-peritoneal injection of ENU, a potent carcinogen, at a dose of 80 mg/kg body weight (Druckery et al. 1967) for developing leukemic (N=30) condition in 6-8 months as confirmed by peripheral blood hemogram. Group-I (denoted as C): Control group of mice(N=15) received equal volume of saline in similar condition. Group III (denoted as L+A): After the development of the disease animals were administered aqueous extract of Ashwagandha via oral route (N=30) and in group IV(denoted as L+WD) : animals were administered with Withanolide D solution via oral route.

4.2.2. Administration of Ashwagandha and Withanolide D

Animals of group IV/L+A were administered with Ashwagandha at a dose of 600mg/kg orally for 30 days. Similarly, animals of Group III/ L+WD were administered with Withanolide D Solution at a dose of 37.5mg/kg bodyweight via oral gavage for 30 days. Equal volume of distilled water administered via oral route in the animals of group I and group II.

4.2.3. Bone marrow isolation and single cell preparation

Animals of all four groups (e.g., control, leukemic, leukemic administered with Ashwagandha and leukaemic administered with Withanolide D) were euthanized by cervical dislocation and their long bones e.g. femur, tibia were aseptically collected. Marrow from the

aforementioned bones were flushed out using sterile syringe with 24-gauge needle (Dispovan, India) filled with RPMI-1640 (Sigma, USA). Large portions of the marrows were used for bone marrow smear studies. Remaining part of the marrows was triturated into single cells by repeat pipetting in cold media. Finally, the cell suspensions were passed through a 100µm strainer to get a debris free pure single cell suspension.

4.2.5. Bone marrow smears study

Bone marrow smears were prepared from the intact marrow by conventional method and stained with Giemsa (Merck, USA) for observation under light microscope (Ch 20i Olympus, Japan).

4.2.6. Flowcytometric analysis

Bone marrow single cell suspensions from control, leukemic Ashwagandha and Withanolide D treated groups were fixed in 1.5% paraformaldehyde (PFA) for 15 minutes in dark at 37º C. Cells were then washed twice with phosphate buffered saline (1XPBS) and divided into two parts. One part of the non-permeabilised cells was directly suspended in FACS fluid to detect the surface proteins (Becton-Dickinson, USA). Another part was treated with 90% chilled methanol for membrane permeabilizations for 20 minutes, then washed thoroughly with PBS and suspended in FACS fluid for the detection of intracellular signalling molecules (Becton- Dickinson, USA). From the suspension of non-permeabilized cells, 2 x 10^6 cells/ ml were collected in respective sorting tubes and incubated for 60 minutes with 2 µl primary antibody (Anti-HHIP, Anti-SHH, Anti-PTCH and Anti-SMO, Cell signalling, USA). After incubation tubes were centrifuged; supernatant was discarded, and cells were suspended in FACS fluid. 2 µl of secondary antibody (Alexa488, Invitrogen USA) was added in respective tubes and excess stain was washed off by centrifugation after incubation period.

Permeabilized cells, 2 x 10^6 cells/ ml were collected in respective sorting tubes and incubated for 60 minutes with 2µl primary antibody (Anti-SUFU, Anti-GLI 3, Anti-GLI 1 (Cell signalling, USA). After incubation tubes were centrifuged; supernatant was discarded, and cells were resuspended in FACS fluid. 2µl of secondary antibody (Alexa488, Invitrogen, USA) was added in respective tube and excess stain was washed off by centrifugation after incubation period.

4.2.7. Immunocytochemistry

Immunofluorescence (IF) analysis was done to assess expression pattern of HHIP, SHH, PTCH, SMO, SUFU, GLI3, and GLI1 in bone marrow cells from control, leukemic Ashwagandha and Withanolide D treated samples with primary antibody (1: 200 dilution, Cell signalling, USA) and secondary antibody (1: 600 dilutions, Alexa488, Invitrogen, USA). Nuclei were stained using DAPI shield (Sigma-Aldrich, USA). Slides were examined under fluorescence microscope (Axio Scope.A1, Zeiss, Germany).

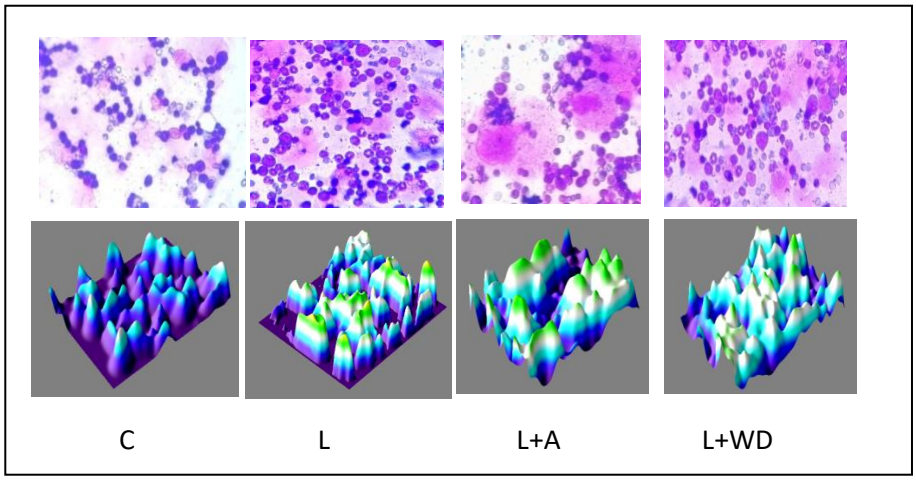
4.2.8. Statistical analysis:

All data are presented as mean ± standard deviation. Student t test was performed for intergroup comparison and one way analysis of variance (ANOVA) was used for multiple comparisons. Probability of < 0.05 was considered to be statistically significant.

4.3. RESULTS

4.3.1. Ashwagandha and Withanolide D administration decreased abnormal cellular growth and number

Bone marrow cytological analysis portrayed vivid image of catastrophe caused by NOC induced leukaemia. Microscopic analysis of bone marrow cells from control, leukaemic Ashwagandha and Withanolide D treated animals revealed interesting insights. Control bone marrow cells showed a heterogenous population of cells devoid of any abnormal cells or apoptotic bodies, a sign of healthy hematopoietic milieu. On the other hand, leukaemic bone marrow smears showed increased cellularity and abundant abnormal cells clearly indicating towards deregulated hematopoietic machinery. In contrast to the leukaemic condition bone marrow cells from Ashwagandha treated animals showed significant decline in abnormal cell number and presence of many apoptotic cells. Bone marrow cells from Withanolide D treated cells showed decline in abnormal cells count and also portrayed apoptotic cells but less than Ashwagandha treated cells. Withanolide D and Ashwagandha administration-imposed suppression on the proliferation mechanism of the abnormal cell in the bone marrow. This suppression resulted in a decline in abnormal cellular number as well as halt in the progression of the disease (Figure.1f-j, Figure.2).



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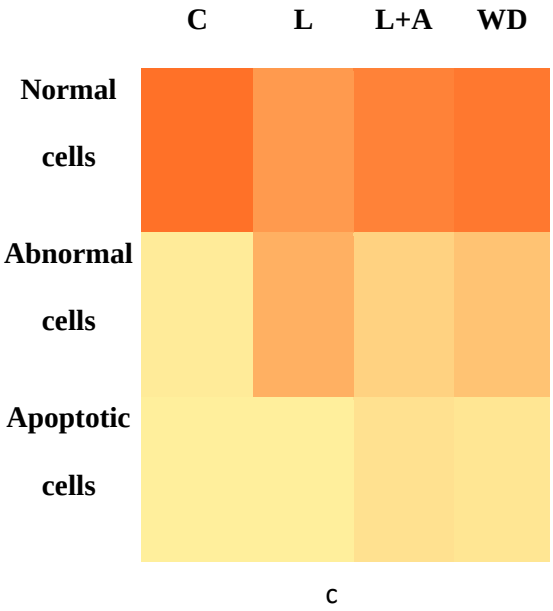

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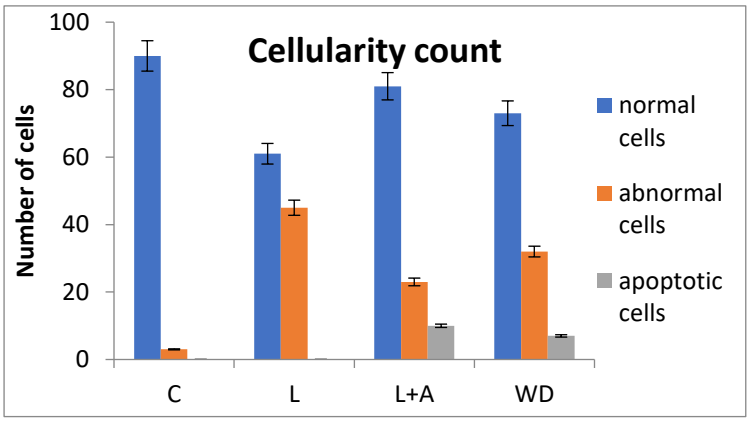


b

Figure 1a. Upper row: Leishman stained bone marrow cells of Control, Leukemic, Leukemic treated with Ashwagandha and Leukemic treated with Withanolide D. Bottom row: 3D interactive plots for the same **b**. Bar graph depicting comparison between living, abnormal and dead cells in control, leukemic and leukemic with Ashwagandha and leukemic with Withanolide D. **c.** Heat map depicting live, abnormal and dead cells among all four experimental groups.

4.3.2. Ashwagandha and Withanolide D modulated Hedgehog signalling components in leukemic bone marrow

Hedgehog pathway being indispensable part of developmental signalling is often reported to be impaired during leukemia and this scenario undoubtedly coincides with our findings. In our study Sonic hedgehog (SHH) expression was increased in leukemic condition (MFI 62.55±2.11) when compared to control (MFI 36.07±3.2). Expression of HHIP in the control (MFI 72.03± 1.92) and in leukemic (MFI 23.77±2.01) showed significant changes. Significant change in the expression of PATCH noted between control (MFI 42.51±2.41) and leukemic (MFI 87.18±2.33); clearly indicating the activation of the pathway. Upon further investigation elevated levels of SMO (MFI 78.69±3.1), and decreased SUFU (MFI 39.56 ±2.6) expression in the leukemic group was noted compared to control SMO (MFI 109.57±2.43) and SUFU (54.75±3.01) indicating removal of repression over the SMO and because of that SMO is now communicating with SUFU. As a result, SUFU is degraded, and downstream signalling is activated. GLI-3 expression has decreased in the leukemic group (MFI 55.22± 2.01) than control (MFI 80.44±1.93). Simultaneously, expression of GLI-1 has increased (MFI 103.62±2.13) in leukemic group in comparison to control GLI-1(MFI 63.96±2.21). Thus, the activation of Hedgehog signalling in NOC induced leukemia has taken place.

Post Ashwagandha and Withanolide D administration SHH expression started to shift towards their normal values (L+A=MFI 47.62±2.01 and L+WD =56.34±2.11). This expressional shift in SHH modulated the expression of other signalling components. Increase in HHIP was found in the Ashwagandha treated (MFI 54.83±2.14) group as well as in Withanolide D treated group (MFI 43.27±1.87). Expressional shift towards normal values were noted of PTCH. PTCH expression in Ashwagandha (MFI 61.32±1.93) treated group was better than Withanolide D (MFI 74.59±4.33). Expressional decline in PTCH led to decrease in the SMO expression in both the treated groups (L+A MFI 88.28±1.98 and L+WD MFI 96.76 ±2.61). As a result of this decline in SMO expression communication between SMO and SUFU hindered and resulted in expressional increase in Ashwagandha (67.87±2.09) as well as in Withanolide D (MFI 53.77±2.10) treated groups. Expression of GLI3 is also started to increase in both the treated groups (L+A MFI 71.16±2.63 & L+WD MFI 61.91±2.62). Elevated levels of SUFU and GLI3 imposed a repression over GLI 1 resulted in a noted decrease in GLI1 expression (L+A MFI 81.11±2.44 & L+WD MFI 94.21±1.87) in Ashwagandha treated group and in Withanolide D (MFI) treated groups.

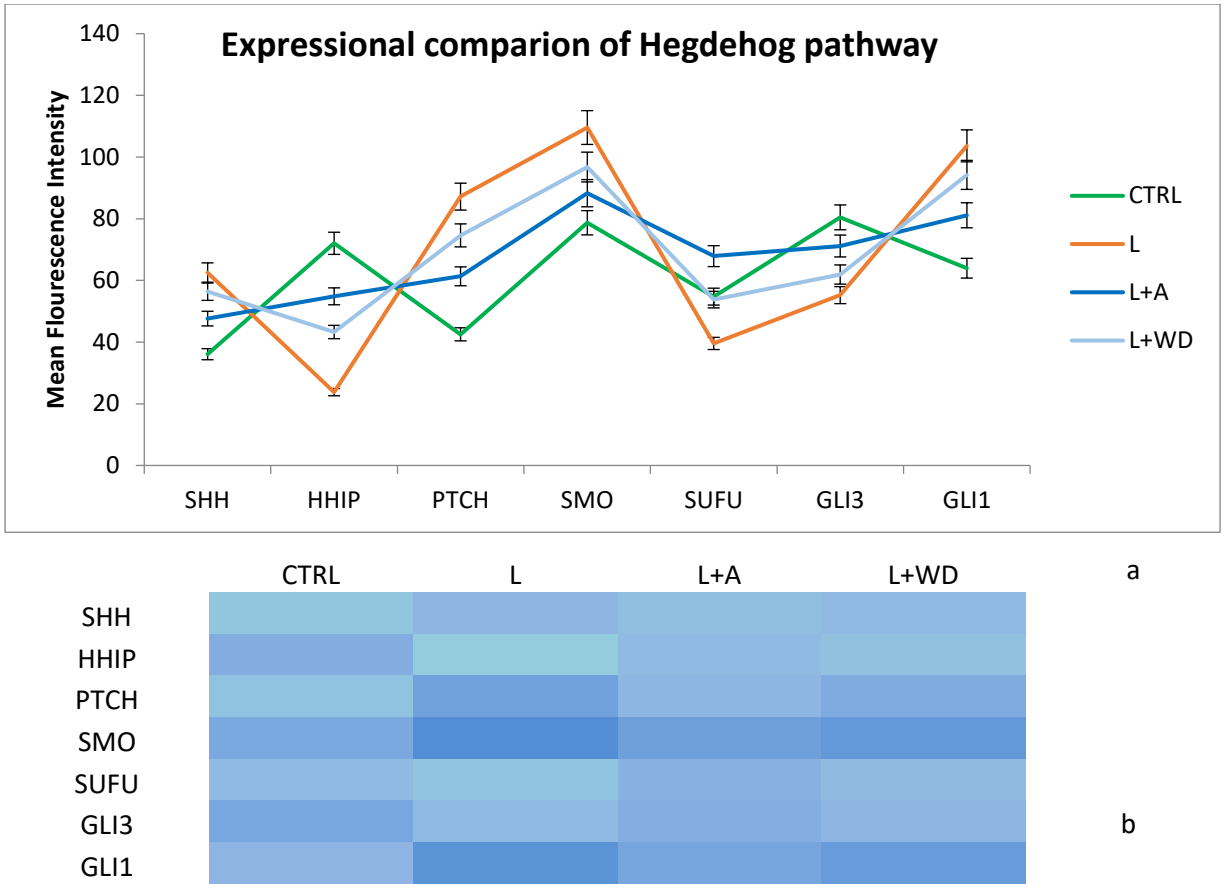


Figure 2 a. Graph depicting expressional pattern of SHH, HHIP, PTCH, SMO, SUFU, GLI3 & GLI1 in all four experimental groups **b**. Heat map depicting expressional intensity of the different hedgehog signalling components across all four experimental groups.

After Ashwagandha treatment the expression was still on the higher side when analysed in the treated group. both in the Ashwagandha treated group and Withanolide D treated group SUFU expressions increased to the control value. GSK3β and GLI-3 has showed some interesting results. Expression of GSK3β has significantly decreased in leukemic group (MFI 4.54± 3.77) than control (MFI 69.15±2.52) and increased in the treated group but further decreased in the treated (MFI 16.17±3.11) group (Figure 3).

4.4. DISCUSSION

The present study delineated the mechanistic scenario behind the disruption of normal haematopoiesis during ENU induced murine model of leukemia and the notable alteration towards normalcy post administration of Ashwagandha. Abnormalities in erythropoiesis

leukopoiesis and thrombopoiesis were evident by the presence of structurally abnormal neutrophils, enlarged platelets, reticulocytes, blasts etc. The occurrence of the blood anomalies was the direct consequence of the hematopoietic defect in leukemia and decline in abnormalities indicated a shift towards normalcy.

Cytological observations by bone marrow smear studies depicted alteration in the bone marrow cellular architecture and morphology among all the four groups. In comparison to control, leukemic marrow smear showed more abnormal cells along with very small number of apoptotic cells.

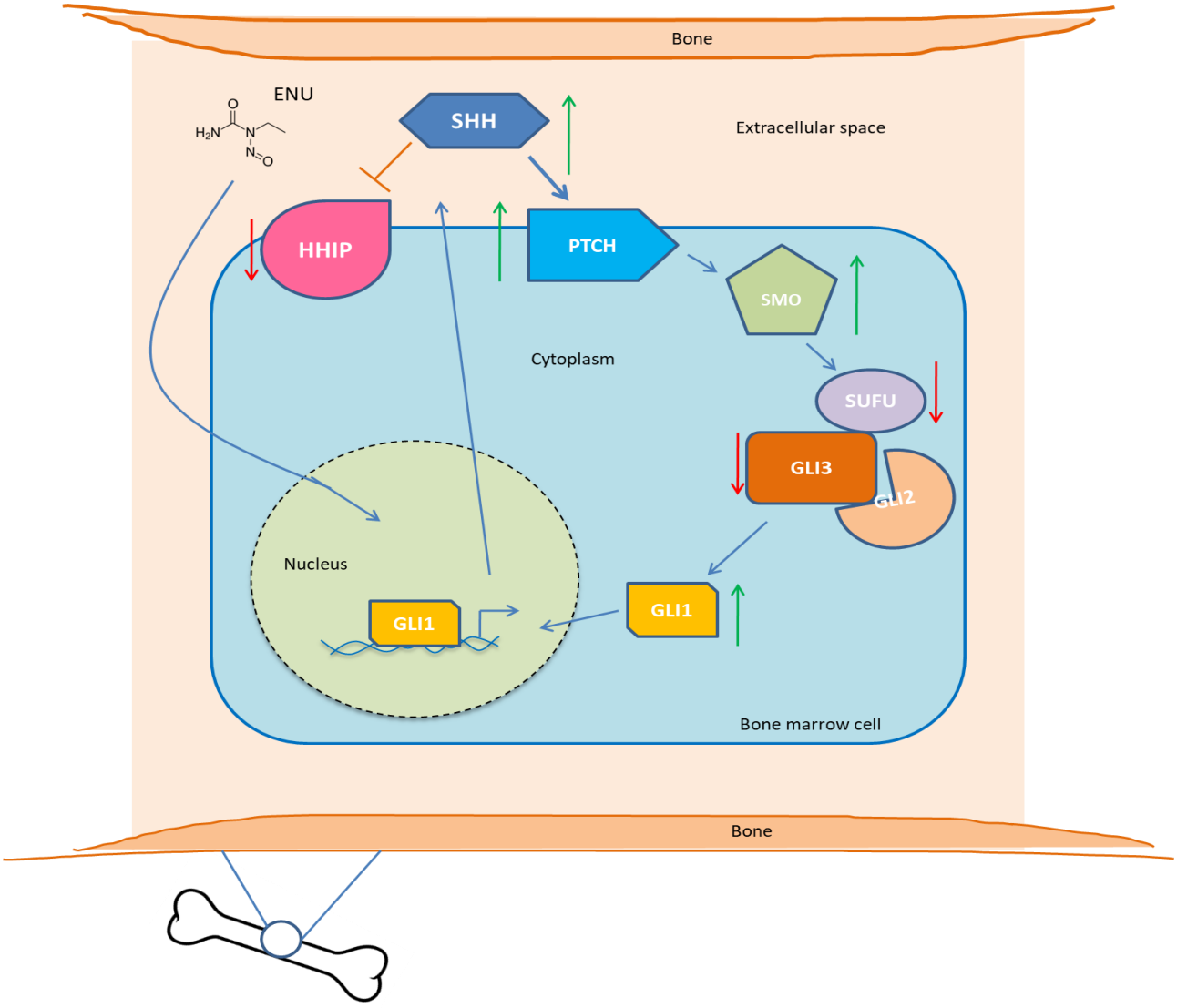


Figure 3. Graphical representation of deregulation of hedgehog pathway in the bone marrow cells in caused by ENU

In contrast to that Ashwagandha treated marrow cells showed low number of abnormal cells with high number of apoptotic bodies. Similarly, in Withanolide D treated group increased apoptotic cell numbers, decrease in abnormal cells and rise in normal cell numbers were noted. Ashwagandha as well as Withanolide D may have rearranged the cellular mechanism inside the marrow resulting in a positive shift in the cellular proliferation, morphology and architecture.

Proliferation in bone marrow compartment during leukemia was found to be associated with the expressional alterations of hedgehog signalling. Sonic hedgehog (SHH), patched (PTCH)

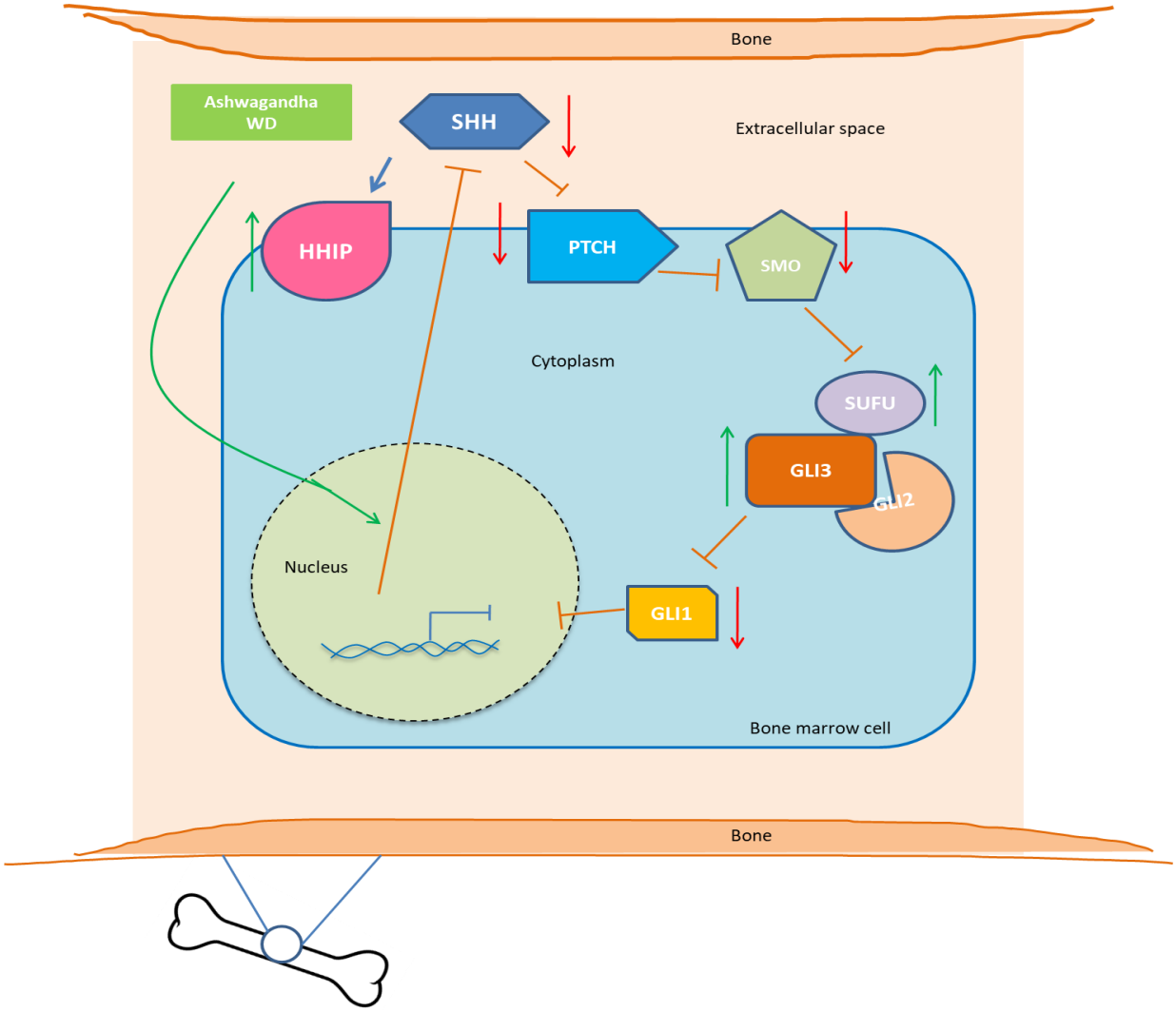


Figure 4. Graphical representation of Ashwagandha and Withanolide D mediated modulation of Hedgehog signalling in the leukemic bone marrow cells.

smoothened (SMO), suppressor of fused (SUFU) and Glioma-Associated Oncogene (GLI) seemed to have played crucial roles behind the deregulation of Hedgehog signalling during leukemia. According to recent studies (Terao et al. 2019, Zeng et al, 2018) the expressional disruption of the major suppressor of the pathway decline in pro apoptotic markers also helps in the hyperproliferative nature of leukemia. It is the complex interplay between the active and inactive state of the pathway and the positive and negative feedback loops that maintain the careful balance of HH signalling in normal tissue.

HHIP is an endogenous Hh ligand inhibitor, with a binding affinity comparable to PTCH1, has the capacity of preventing pathway activation. In our study HHIP expression in leukemic condition was decreased and as a result it was unable to bind to the excess levels of SHH molecules. Unbound excess SHH then bound to the PTCH and activated the signalling cascade. After SHH and PTCH binding repression over SMO was removed. As a result, SMO internalized and communicated with the SuFu GLI3 GLI2 complex. Interaction between SMO and SuFu GLI3 GLI2 resulted in the degradation of Sufu and GLI 3 protein. SuFu and GLI3 act as repressor of the GLI1 from translocating into the nucleus and activating the pathway Decrease in the GLI3 expression points out towards the degradation of the molecule and because of that repression has removed and pathway is now on. Increased expression of GLI1 in the leukemic group clearly indicated that no repressor molecule is obstructing the pathway anymore and thus showing activity of the pathway. Activated Hedgehog signalling pathway led to the augmented cellular proliferation rate, decreased cellular death which ultimately aided in the progression of the disease.

In the treated groups, expression of SHH decreased as well as HHIP was increased. Elevated HHIP bound the excess SHH and very less SHH molecules were able to bound to the PTCH. Simultaneous increase in HHIP and decrease in SHH expression created a bidirectional pressure over the Hedgehog signalling cascade. In the absence of SHH, PTCH mounted repression over SMO and as a result interaction between SMO and SuFu GLI3 GLI2 was hindered. Loss in interaction helped in the expressional elevation of SUFU and GLI3. Elevated levels of SuFu and GLI3 mounted repression over GL1. Thus, GLI1 was unable to translocate into the nucleus and activate the pathway. This alteration in the expression in the Ashwagandha and Withanolide D treated groups showed shifting towards normalcy or slowing down the activity of the pathway.

In the presence of Ashwagandha and Withanolide D, components of the Hedgehog pathway has started to shift towards the normal expression level. This positive shift in the expression of the signalling pathway components also reflected in the cytological profile, haematological parameters and as well as in the physiological parameters.

Ashwagandha and Withanolide D mediated positive immune modulation of the key components of the hedgehog pathway has reversed the devastating pathology of NOC induced leukaemia to great extent. Moreover, further studies will reveal elaborate view of Ashwagandha and Withanolide D as potent hedgehog modulator in leukaemia with a promise to open new therapeutic avenues in the battle against the disease.

Chapter 05

Ashwagandha and Withanolide D ameliorated deregulated apoptotic mechanism and modulated aberrantly activated NRF2/KEAP1/SHH signalling axis in experimental leukemic bone marrow

CHAPTER SUMMARY

In this chapter, we assessed the immunomodulatory qualities of Withanolide D and Ashwagandha on the Apoptosis mechanism modulated by NRF2/KEAP1/Hedgehog signalling axis in NOC-induced leukaemia. In the Withanolide D and Ashwagandha treated bone marrow cells, cytological, immunological, haematological, microscopic, and flow cytometric analysis demonstrated an expressional shift towards normalcy in the unregulated NRF2/KEAP1/Hedgehog signalling axis, as well as activation of repressed apoptotic pathway due to leukemic stress. Taken together, the findings suggested that Ashwagandha and Withanolide D could serve as an efficient regulator of the NRF2/KEAP1/Hedgehog axis in leukemia, with a hope to open up a novel therapeutic direction.

5.1. INTRODUCTION

Leukemia is the most common hematopoietic disorder that involves malignant transformation of hematopoietic stem or progenitor or other cells under the support of altered microenvironment (Basak et al., 2010; das et al., 2010) Pathophysiological changes of hematopoietic accouterment during leukaemia results into the exuberance of abnormal immature population in bone marrow as well as in peripheral circulation at the cost of proper functional cells (Law et al., 2001; Law et al., 2003; Chatterjee et al., 2009; Chatterjee et al., 2010) . According to Globecon 2018, World Health Organization (WHO) report 4, 37,033 new leukaemia cases was reported worldwide and in India 42,055 new cases of leukaemia had been reported which was 3.6% of total cancer occurrence (Globecon 2018) Increasing population burden along with scarcity in basic and natural resources accompanied by various forms of pollutants, use of pesticides and other harmful chemicals in our foods augmenting the risks of N-nitroso compounds (NOC) induced leukaemia. In this battle against NOC induced leukaemia a better, affordable and abundant source of arsenal is the need of the hour.

NRF2 (nuclear factor erythroid 2-related factor 2) is ubiquitously expressed transcription factor belongs to a cap “n” collar family of transcription factor. Generally NRF2 regulates the expression of proteins which are involved in drug metabolism, oxidative stress related response and cytoprotection (Bryan et al., 2013; Jaramillo & Zhang, 2013; Menegon et al.,

2016). Apart from this NRF2 also plays a crucial role in cellular proliferation, differentiation, growth, autophagy, and apoptosis. On the other hand, KEAP1 (Kelch-like ECH-associated protein 1 is a homodimer protein with three functional domains and binds to NRF2. Upon binding with KEAP1, NRF2 undergoes proteosomal degradation. A change in conformation in KEAP1 molecule mediated via its cysteine residues takes place when introduced to chemical insult or oxidative stress. This conformational change results in dissociation of NRF2 from KEAP1 molecule. Subsequently ubiquitination and degradation of NRF2 halts resulting in accumulation of the same in the cytosol. This newly synthesized NRF2 heterodimers then translocate to nucleus and binds to antioxidant responsive elements (ARE) and activate the cytoprotective genes (Leung et al., 2020; Ma et al., 2020; Panieri & Saso, 2019; Robledinos-Antón et al., 2019).

Initial studies described cytoprotective and chemopreventive function of the NRF2/KEAP1 pathway in carcinogenesis. Contrary to the initial findings, aberrant activation of the mentioned pathways has been noted frequently in tumor progression, metastasis, survival, and resistance to therapies. Recent studies have depicted NRF2 bind to the promoter of sonic hedgehog and subsequently activates the pathway (Abdul-Aziz et al., 2015; Katsuragi et al., 2016; Martin-Hurtado et al., 2020). Aberrantly activated hedgehog pathway leads to leukaemogenesis.

Since ages Ashwagandha, (*Withania somnifera*) also known as “Indian winter cherry” or ‘Indian ginseng” is extensively applied in Ayurvedic and Unani system of treatment. Ashwagandha and its biologically active compounds have portrayed wide range of biological activities including cytostatic, anti-neoplastic, anti-inflammatory (Rai et al. 2016), anti-depressant, neuroprotective (Jain et al. 2001), and immunomodulating (Hanna D et al. 2007; Archana R.1998; Abbas et al.1993). Myriad implementation of Ashwagandha and its biologically active compounds on various cancerous milieus without any serious side effects entice us to explore its potential in NOC induced leukemic pathophysiology.

Involvement of NRF2/KEAP1 and Hedgehog pathway has been studied separately in various haematological malignancies but the modulation of NRF2/KEAP1/SHH axis has not been studied previously in the presence of Ashwagandha and Withanolide D in NOC induced leukemia. In addition, we have also emphasized on the assessment of micro-environmental status and apoptotic machinery in the presence of Ashwagandha and Withanolide D.

5.2. MATERIALS AND METHODS

5.2.1. Animal maintenance and disease development

Four groups of inbred Swiss albino mice (*Mus Musculus*) were taken into consideration (Group I: control denoted as C, Group II: leukemic denoted as L, Group III: leukemic treated with Ashwagandha, denoted as L+A and Group IV: leukemic treated with Withanolide D, denoted as L+WD). Pups of both sexes aged between 10-15 days were administered via intra peritoneal route with ENU (N-ethyl-N-nitrosourea) at a dose of 80mg/kg body weight for the development of leukaemia. An equal amount of 1XPBS was administered in the pups of control groups. 4-6 months after ENU administration blood hemogram was performed to assess the disease status. Post leukemic development group III was administered with Ashwagandha extract via oral route. Animals of group IV were orally administered with Withanolide D.

During the experimental period all the animals were on a normal diet and water *ad libitum* with 12 hour light and dark cycle. All the animals were maintained according to the Institutional Animal Ethical Committee (IAEC) guidelines and protocols.

5.2.2Ashwagandha and Withanolide D administration

Animals of Group III/L+A a received Ashwagandha extract at a dose of 600mg/kg bodyweight and Group IV/L+WD was administered with Withanolide D at a dose of 35mg/kg bodyweight via oral route for 30 days. An equal amount of distilled water was administered by oral route in the animals of group I/C and group II/L.

5.2.3. Single cell preparation from bone marrow

Animals of all four groups (e.g., control, leukemic, leukemic administered with Ashwagandha and leukemic administered with Withanolide D) were sacrificed by cervical dislocation and their long bones e.g., femur, tibia were aseptically collected. Marrow from the aforementioned bones were flushed out using sterile syringe with 24 gauge needle (Dispovan, India) filled with RPMI-1640 (Sigma, USA). Marrow fragments were triturated into single cells by repeat pipetting in cold media. Finally, the cell suspensions were passed through a 100µm strainer to get a debris free pure single cell suspension.

5.2.4. Short term cell culture of bone marrow cells

The single cell suspensions were from each group seeded in RPMI-1640 media (Sigma, USA) complemented with 30%FBS (Gibco, Thermo Fisher Scientific, USA) and Penicillin and Streptomycin (HiMedia, India) (1:1) at a concentration of $1x10^5$ cells/ml. Cells were seeded in 12 well plates (Corning, USA) and incubated at 37ºC humidified incubator with 5% CO_2. The media was drained off at every 48-hour interval and fresh FBS supplemented media was added for the maintenance of the culture. The culture was maintained for 96 hours and observed at a regular interval using inverted microscope (Olympus, Japan).

5.2.5. Assessment of apoptotic pathway in bone marrow cells

Bone marrow cells from all four groups (i.e., control, leukemic, Ashwagandha treated and Withanolide D treated) were fixed with 1.5% PFA. Post fixation cells were washed with 1X PBS and then permeabilized with 90% chilled methanol and incubated at 4 °C for 15 min to 20 min. Finally cells were washed with 1X PBS and resuspended in FACS fluid and were divided into different sorting tubes with $2x10^6$ cells. 2µl of anti- PUMA, anti-BCL2 and anti-caspase 3 antibody (Cell signalling technology, USA) were added per 2 X 10^6 Cells of each tube and incubated at 37ºC for 30minutes. Post incubation cells were washed to get rid of unbound excess antibodies and then secondary antibody, Alexa Fluor 488 (Invitrogen, USA) was added in each tube followed by 30minutes of incubation in dark at 37ºC. Finally, cells were washed by centrifugation and resuspended in FACS fluid for acquisition and analysis BD FACS Calibur (Becton-Dickinson, USA) using CellQuest Pro software (v9.1 Becton-Dickinson, USA).

5.2.6. Measurement of NRF2-KEAP1 levels in marrow cells

Bone marrow cells from control, leukemic, Ashwagandha treated and treated with Withanolide D were subjected to NRF2 and KEAP-1 immunostaining to determine their levels. 2µl of Anti-NRF2 and Anti-KEAP1 (Cell signalling technologies, USA), anti-GSK3β, anti-βTrCP (Santa Cruze Biotechnology, USA) antibodies were added in each tube containing PFA fixed and permeabilized 2 X 10^6 cells. Post addition of antibodies cells were incubated at 37 ºC for 30 minutes. After that cells were added with 2µl of Alexa Fluor 488 (Invitrogen, USA) tagged secondary antibody followed by incubation at 37ºC. Finally, cells

were washed and resuspended in FACS fluid for acquisition and analyzed with BD FACS Calibur (Becton-Dickinson, USA) and Cell Quest Pro software (v9.1 Becton-Dickinson, USA).

5.2.7. Immunocytochemistry

Expression pattern of KEAP1, NRF2, PUMA, BCL2, TERT, GSK3β and βTrCP were accessed by Immunofluorescence (IF) analysis from four different groups viz. control, leukemic Ashwagandha and Withanolide D treated bone marrow cells samples with primary antibody (1: 200 dilution, Cell Signalling, USA) and secondary antibody tagged with Alexa 488 (1: 600 dilution, Invitrogen, USA). Nuclei were stained using DAPI shield (Sigma-Aldrich, USA). Slides were examined under fluorescence microscope (Axio Scope.A1, Zeiss, Germany).

5.2.8. Statistical analysis

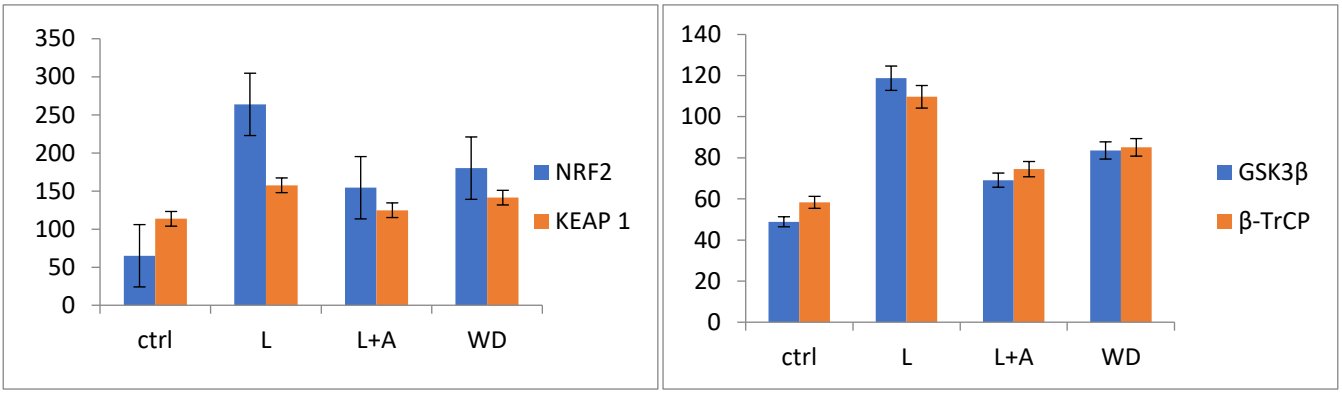
All data are presented as mean ± standard deviation. Student t test was performed for intergroup comparison and one way analysis of variance (ANOVA) was used for multiple comparisons. Probability of < 0.05 was considered to be statistically significant.

5.3. RESULTS

5.3.1. Ashwagandha and Withanolide D reversed activation of NRF2/KEAP1 axis due to leukaemic insult

In leukemia, there was a significant increase in the expression of NRF2 (MFI 263.79±2.33) compared to the control group (MFI 64.97±1.75), while there was a simultaneous decrease in the expression of KEAP1 in leukemic cells (MFI 113.52±3.21) compared to control cells (MFI 157.52±1.56). This alteration in the expression of KEAP1 and NRF2 in leukemic cells resulted in the activation of the signalling axis. Our study also revealed elevated levels of GSK-3β (MFI 118.74±2.43) and β-TrCP (MFI 109.7±1.67) in the leukemic group compared to the control groups (MFI 48.47±0.98 and 58.33±0.99). Increased levels of GSK-3β, β-TrCP, and NRF2 indicated the activation of NRF2 by the glycogen synthase kinase 3 beta

(GSK-3β) and the E3 ligase adapter β-TrCP. This activation of NRF2 suggested an alternative activation of the NRF2/KEAP1 pathway (Figure 1a-g).



a b

Figure 1. a. Graph depicting NRF2/KEAP1 expression pattern**. b.** Graph depicting expressional pattern of GSK-3β/ β-TrCP.

Following the administration of Ashwagandha and Withanolide D, the expression levels of NRF2 (MFI 154.31 ± 2.71 and 180.02±2.47) and KEAP1 (MFI 124.77±1.67 and 141.3±3.45) began to shift towards their baseline values. This shift implies the increasing repression of NRF2 by KEAP1. Simultaneously, a decline in the expression levels of GSK-3β (MFI 75.3±1.88 and 98.6±1.61) and β-TrCP (MFI 71.98±2.07 and 89.63±3.01) indicated the effective influence of Ashwagandha and Withanolide D on these molecules. Ultimately, by modulating the NRF2 expression, Ashwagandha and Withanolide D hindered the activation of the NRF2/KEAP1 axis.

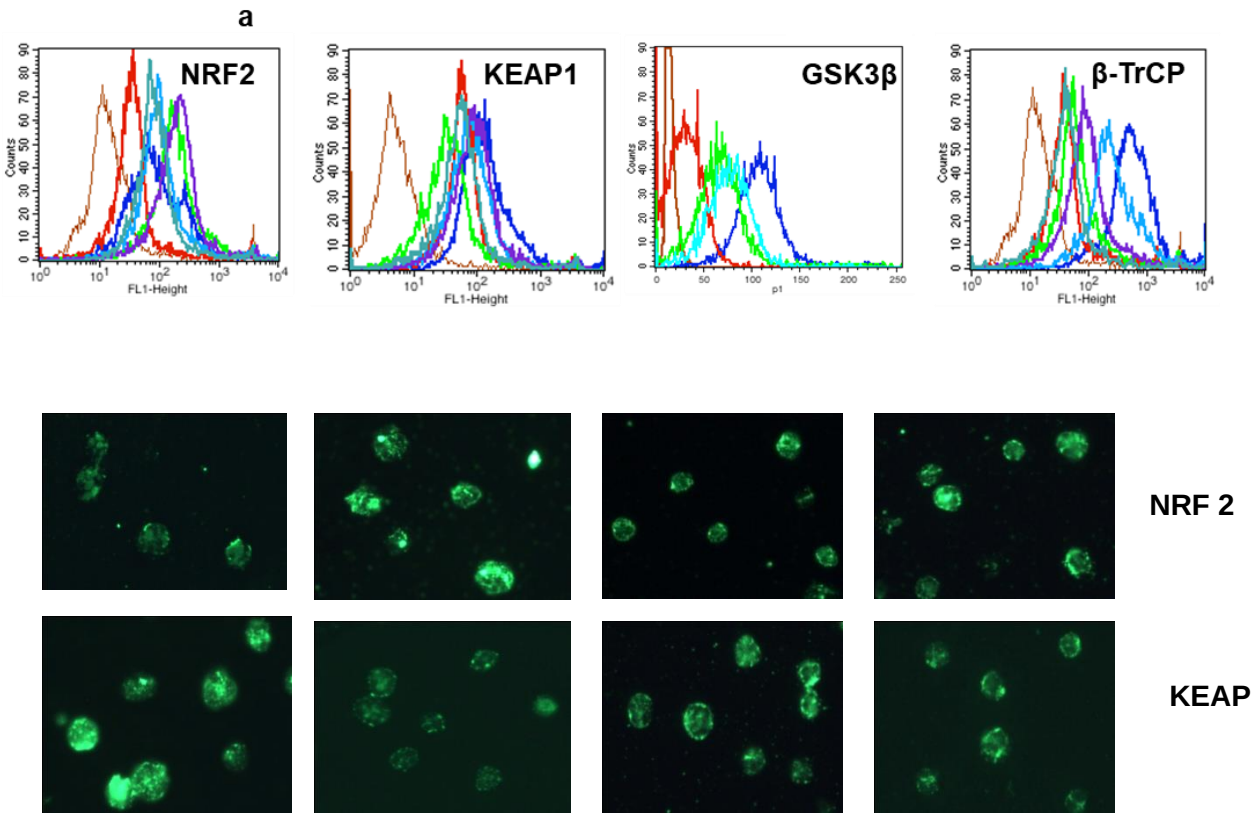


Figure 2. **a.** Histogram plot of NRF2 , KEAP1 , GSK-3β and β-TrCP expression in all four groups **b.** .Immunofluorescence image of NRF2 (upper row) and KEAP1 (lower row) Immunofluorescence image of GSK-3β (upper row) and β-TrCP (lower row)

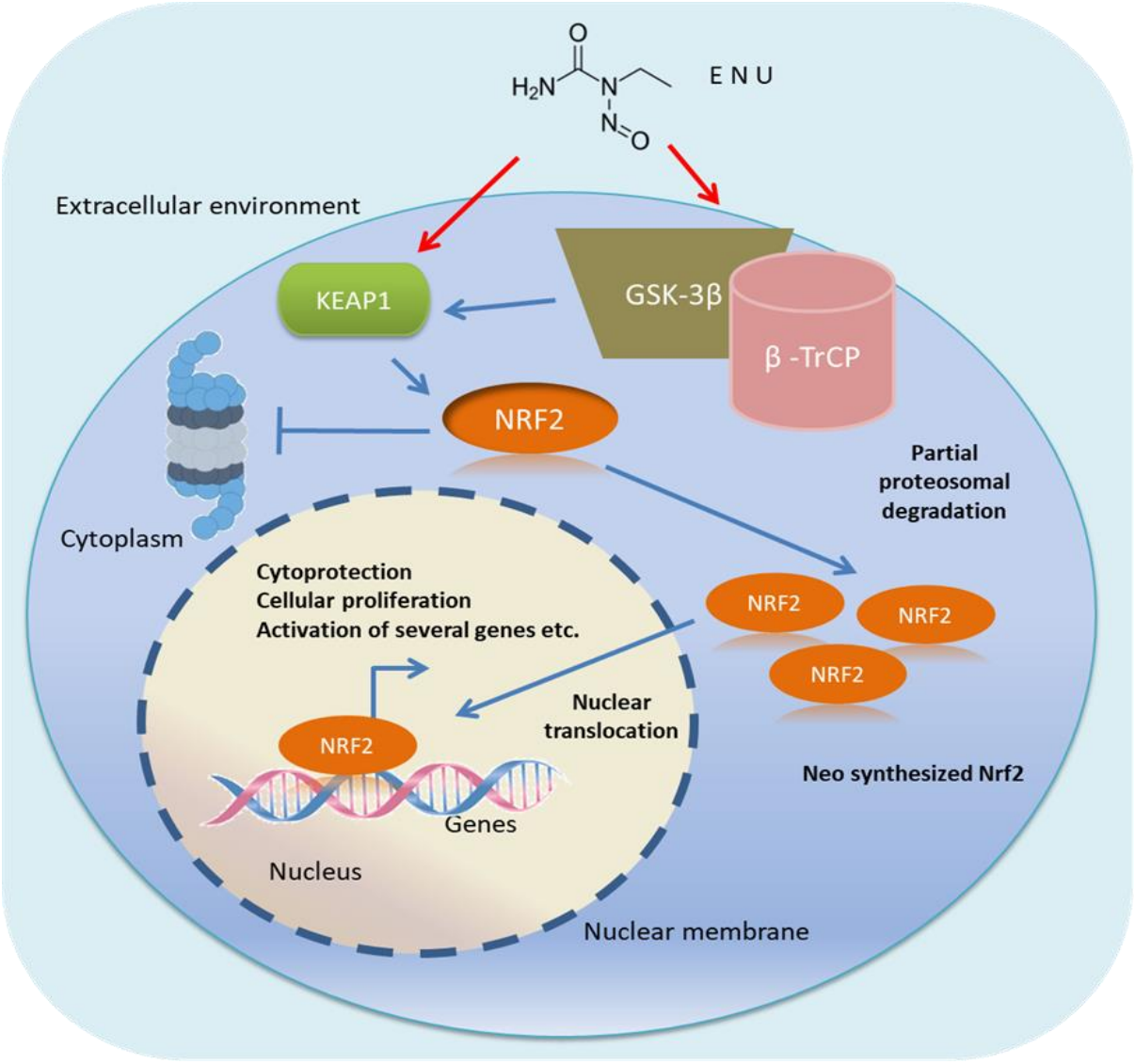


Figure 3. Diagrammatic representation of NRF2 activation

GSK-3β/ β-TrCP complex proteolytically degrades NRF2 and created neo synthesized NRF2. This newly synthesized NRF2 translocated in the nucleus and binds to ARE region. Binding to ARE region helped in cellular proliferation, cytoprotection, activation of many other pathway components.

5.3.2. Ashwagandha and Withanolide D modulated leukemic microenvironmental status.

In our experimental setup, after an incubation period of 24 hours, the cells in each well, which were assigned to different culture groups (C, L, L+A & L+WD), were captured through photography. This process was then repeated at 48 hours and 96 hours after the initial

seeding. A total of 20 random images per well were taken and subjected to analysis using ImageJ. This entire procedure was replicated three times for all the groups. Through our study of cellular culture and its digital analysis, we have observed an increase in the rate of cellular proliferation in all four groups after seeding. Specifically, in the C group, the rate of cell number increase was found to be 17%±2.05 as compared to the leukemic group's 36%±3.11. Furthermore, after 48 hours, the rate of proliferation in the C group increased by 1.5 times, while the L group experienced 2.5 times increase. At the 72-hour mark, the L group exhibited a 2.1 times growth in cellular numbers compared to the C group's 1.2 times increase. This excessive rate of proliferation in the L group serves as a clear indication of the pathophysiology induced by NOC.

In the groups that received treatment, the rate of cell division began to decline in comparison to the condition of leukemic cells. At the onset, after a period of 24 hours, the groups treated with Ashwagandha demonstrated a growth increase of 28%±1.56, while the group treated with Withanolide D showed a growth increase of 30%±3.03. After a duration of 48 hours, the rate of cell division in the L+A group was 1.7 times greater, and in the L+WD group it was 1.89 times greater. Further assessment after 72 hours revealed that the rate of cell division in the L+A group was 1.58 times greater, and in the L+WD group it was 1.78 times greater. The reduction in the rate of cellular proliferation was indicative of the reversal of the damage caused by NOC-induced leukemia.

The L group exhibits a significantly greater magnitude compared to the C group. Within the treated group, the proliferation rate of seeded cells began to decline. The cells in the Ashwagandha treated groups displayed a reduced propensity for proliferation as compared to the Withanolide D treated groups. The alteration in the proliferation rate and the evident change in the microenvironmental state resulted from the insult induced by ENU (Figure 2a - c).

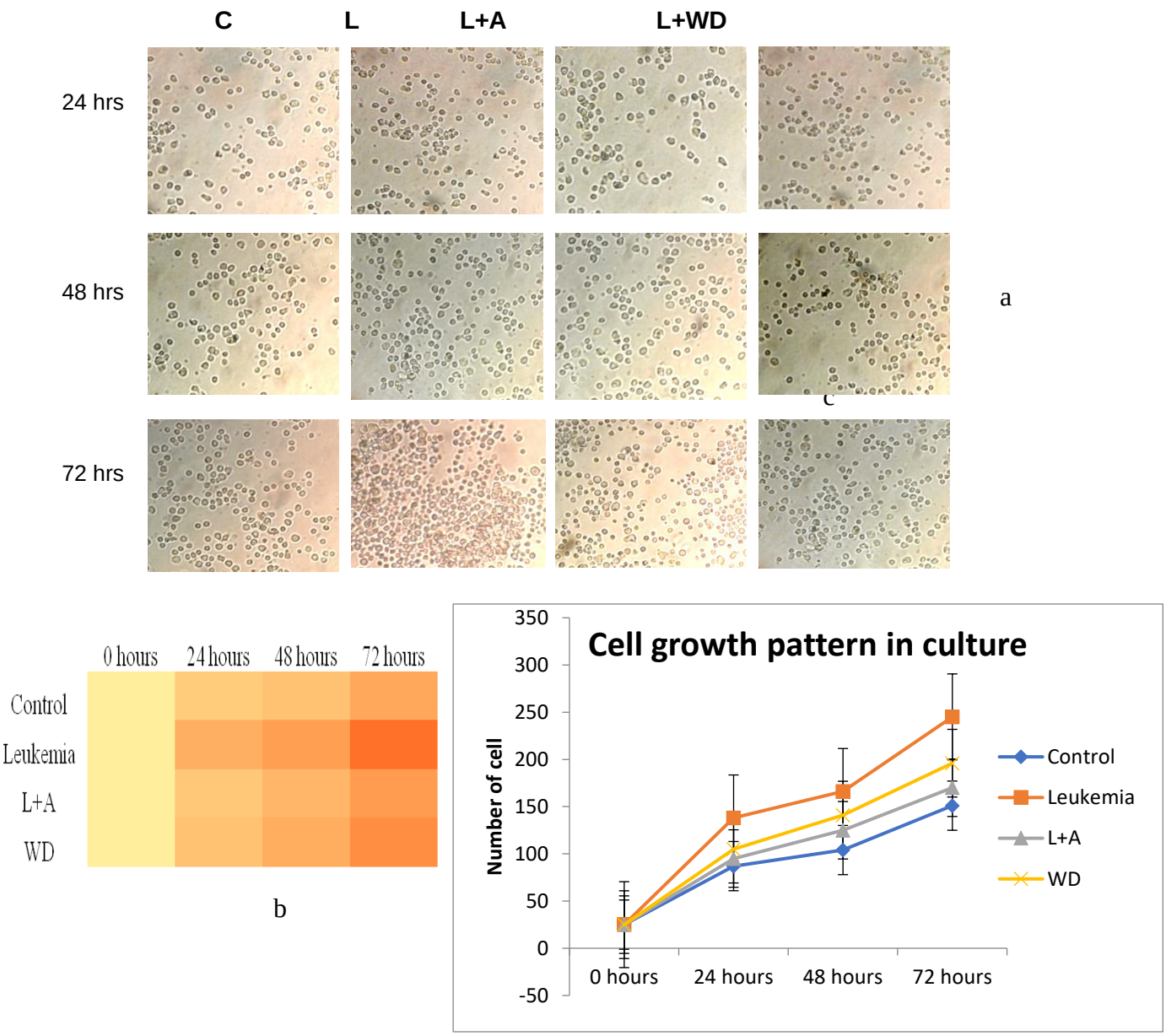


Figure 4. **a**. Bone marrow cell in culture**. b.** Graph depicting cellular growth pattern of all four groups for 24hours, 48 hours and 72 hours. **c**. Heat map depicting cellular growth pattern of all four groups.

5.3.3. Ashwagandha and Withanlolide D impeded the NRF2-mediated activation of NRF2/SHH axis in leukemic cells.

Proteolytically degraded and activated NRF2 translocates into the nucleus of the cell, where it binds in the ARE region and exerts control over the activation of more than 200 genes, including Hedgehog. In the preceding chapter, we observed the activation of the Hedgehog signalling pathway as a result of leukemic insult induced by ENU. In line with this observation, NRF2 was found to induce the expression of SHH protein, which ultimately triggered the signalling cascade. The heightened levels of NRF2 and SHH directly indicated the activation of the Hedgehog pathway.

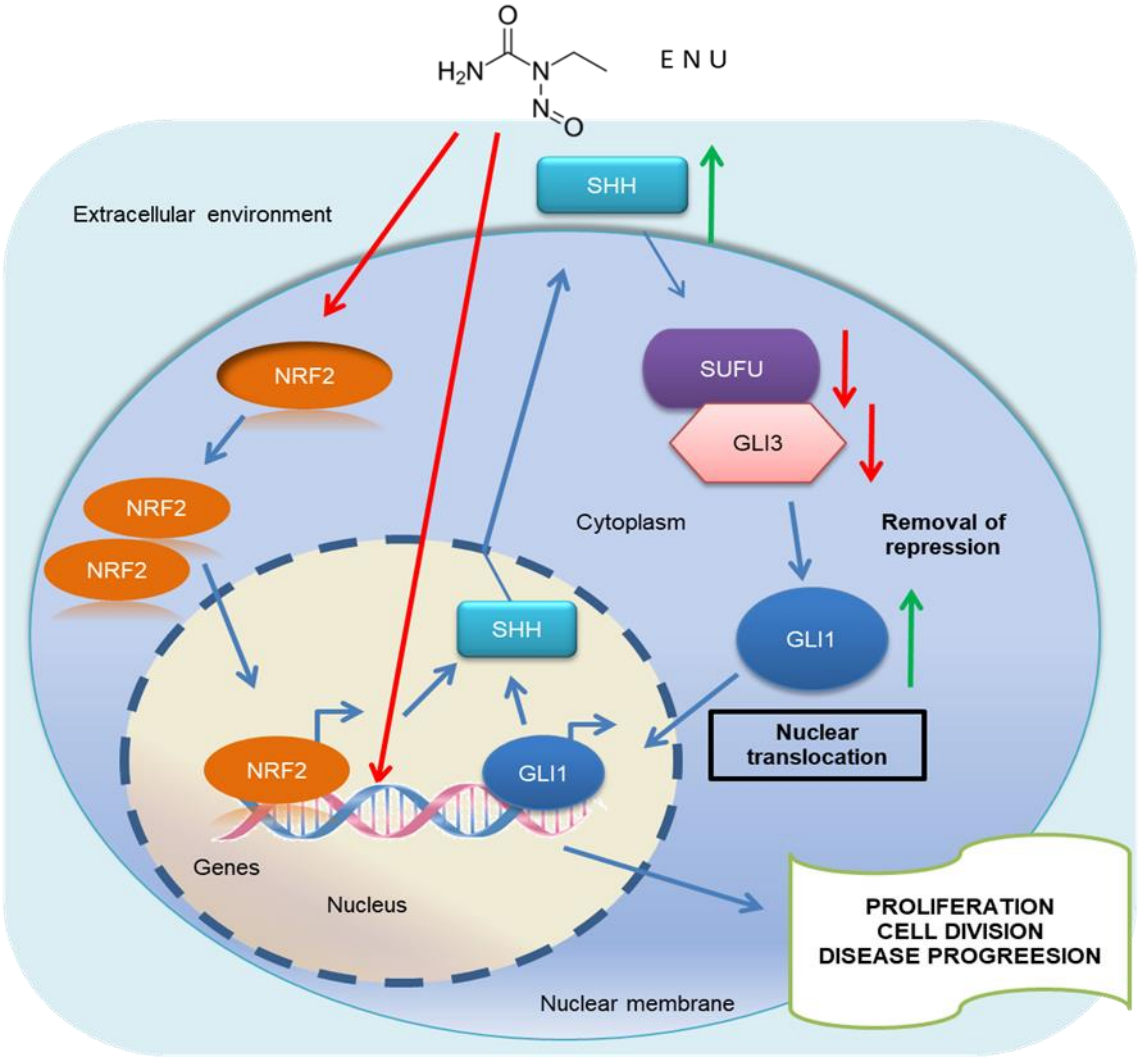


Figure 5. Diagrammatic representation of NRF2 mediated HH activation.

NRF2 translocated in the nucleus and bound to ARE region. Upon binding to ARE region SHH gene has been activated. Upregulated SHH expression then initiated the

downstream signalling cascade via SUFU, GLI3 and GLI1. GLI1 then translocated in the nucleus and helped in cell proliferation, cell division, disease progression etc.

Following the administration of Ashwagandha and Withanolide D, the expressional pattern of Hedgehog pathway components began to shift towards their normal values, along with the expression of NRF2 and KEAP1. As a consequence of this favourable shift, a decrease in cellular proliferation rate and an improvement in disease condition were observed (Figure 4).

5.3.4. Ashwagandha and Withanolide D increased pro apoptotic activity in Leukaemic bone marrow cells

The delicate balance between pro-apoptotic and anti-apoptotic mechanisms determines the fate of cells. Pro-apoptotic function has been strongly linked to Caspase 3 and PUMA expression levels. In our examination, the expression level of PUMA declined significantly in the leukemic group (MFI 138.09±1.32) compared to the control group (MFI 297.16±2.01). Similarly, Caspase 3 expression sharply declined in the leukemic bone marrow (MFI 90.74±3.93) compared to the control group (MFI 123.23±3.11). Another key component-BCL2, demonstrated a significant increase in expression level in the leukemic group (MFI 323.66±1.36) compared to the control group (MFI 184.95±2.42). The simultaneous decrease in the expression level of PUMA and Caspase 3, along with the increase in BCL2 expression, shows a clear indication of dysregulation in apoptotic mechanisms and the survival of abnormal cells.

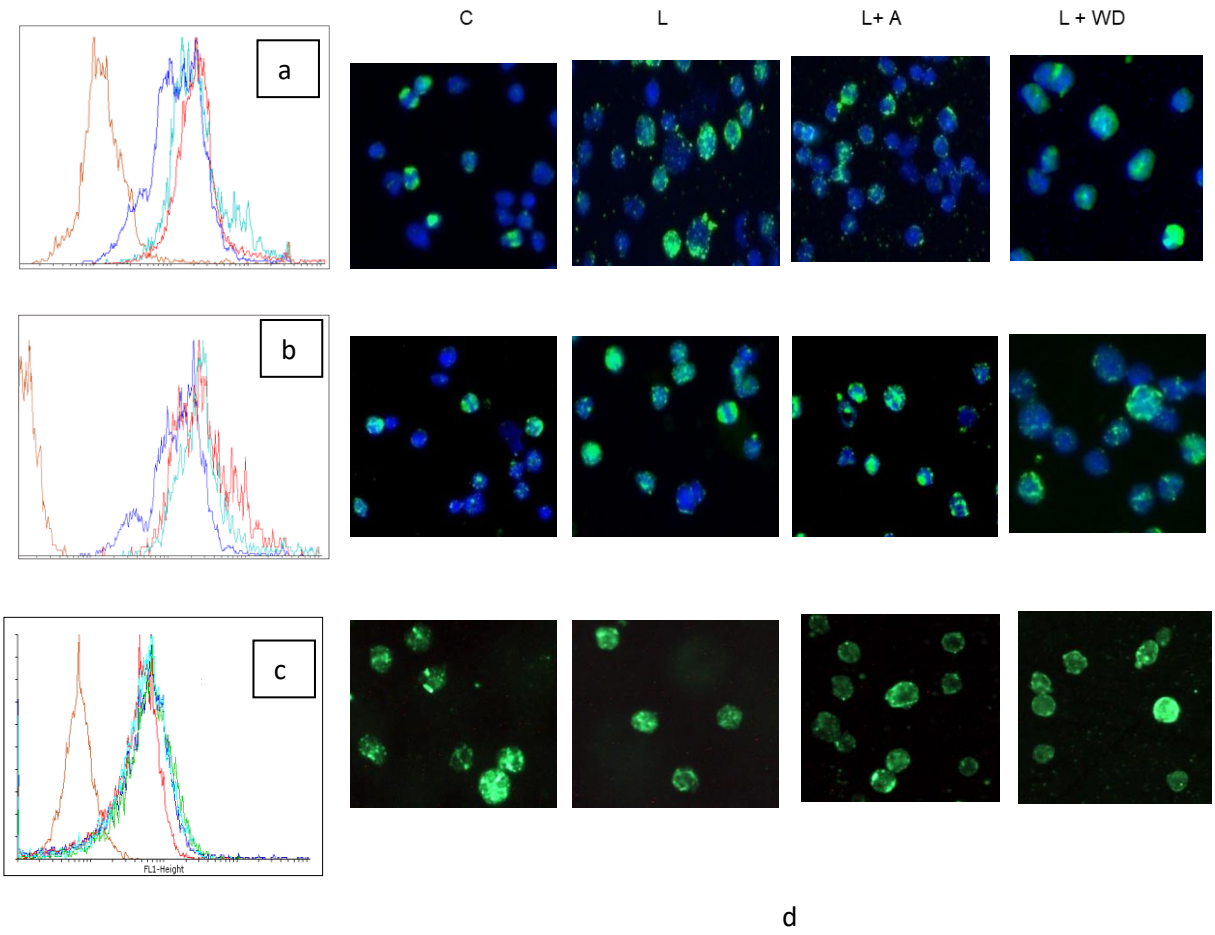
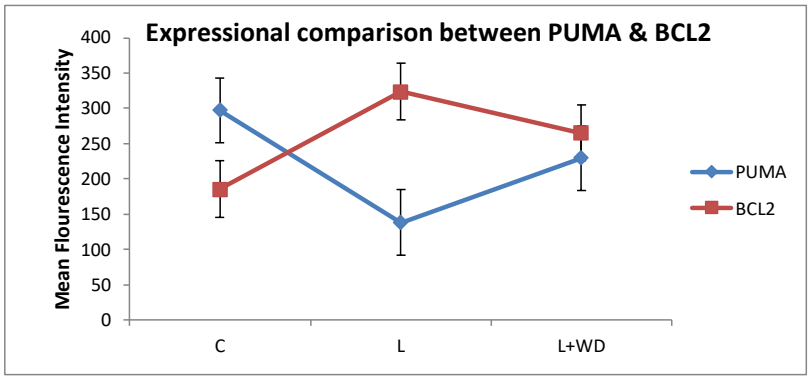


Figure 6. **a**. Histogram plot depicting expression of BCl2 in all four groups. **b.** Histogram plot depicting expression of CASPASE3 in all four groups. **c**. Histogram plot depicting expression of PUMA. **d**. Immunofluorescence image of BCL2 (upper row), PUMA (middle row) and Caspase 3 (lower row).

On the contrary, when the leukemic mice underwent treatment with Ashwagandha and Withanolide D, the levels of PUMA expression in L+A and in L+WD (MFI 229.66±2.11) as well as Caspase 3 in L+A (MFI 111.23±2.49) and in L+WD (MFI 102.27±1.97) experienced a notable increase. Moreover, BCL2 in L+A (MFI 226.38±2.55) and in L+WD (MFI 265.33±1.98) showed a significant decline ($P \leq 0.05$) compared to their leukemic counterparts. The administration of Ashwagandha and Withanolide D effectively mitigated the repression on the apoptotic machinery, causing the rate of apoptosis to escalate This heightened apoptosis rate not only impeded the proliferation of abnormal cells but also aided

the bone marrow microenvironment in overcoming the catastrophic effects induced by leukemia (Figure 1h-i, figure5)



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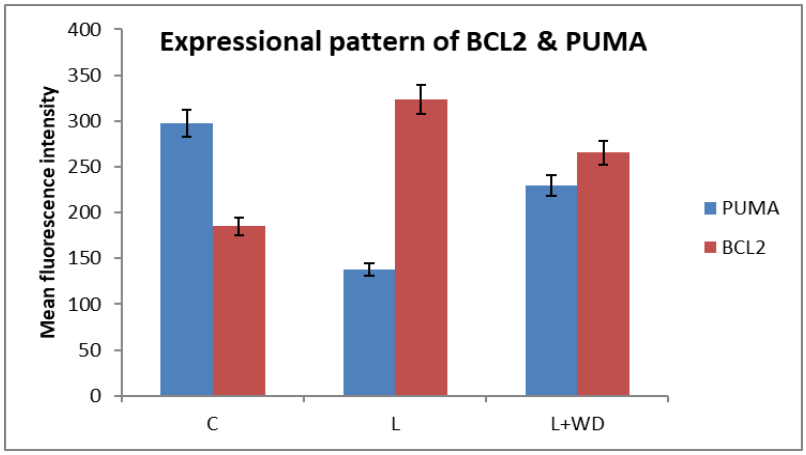


b

Figure 7. **a.** Comparison between PUMA and BCL2 expression in all four experimental groups. **b**. Graph depicting comparative expressional pattern of BCL2 and CASPASE3 in all four groups.

5.3.5. The alteration in TERT expression following the administration of Ashwagandha and Withanolide D.

In our immunocytochemical investigations, it was observed that the level of TERT expression in the leukemic state was higher compared to the control. Other studies have also reported an increased level of TERT in most tumor cells, which aligns with our findings. Elevated levels of TERT are often linked to the proliferative ability of non-reproductive cells, and in the case of leukemia, this heightened proliferative phenomenon contributed to the progression of abnormal leukemic cells by inhibiting their anti-apoptotic function. After the administration of Ashwagandha and Withanolide D, the expression of TERT moved towards

a more normal level, thereby activating the apoptosis mechanism. This decrease in telomerase activity has been directly correlated with an increased susceptibility to apoptosis, as depicted in Figure 1. This modification in the apoptotic mechanism resulted in an amplified occurrence of apoptosis in the treated bone marrow cells. The heightened apoptosis effectively aided in reducing the burden of abnormal cells in the bone marrow microenvironment affected by leukemia.

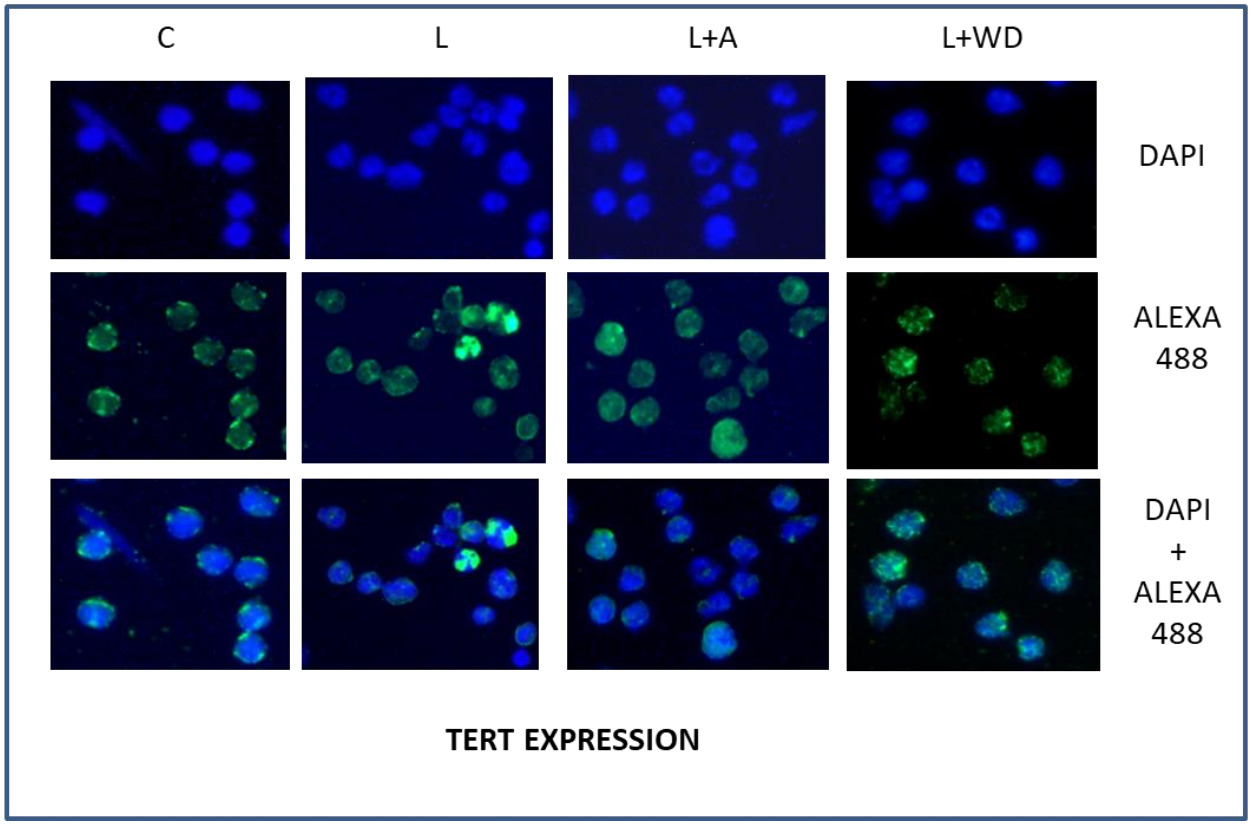


Figure 8. TERT expression in bone marrow cells. Upper row depicts DAPI stained bone marrow cells of Control, Leukaemic, Leukemic with Ashwagandha and Leukemic administered with Withanolide D. Middle row depicts Alexa 488 stained bone marrow cells of Control, Leukemic, Leukemic with Ashwagandha and Leukemic administered with Withanolide D. Bottom row depicts composite image of DAPI + Alexa 488 stained bone marrow cells of Control, Leukemic, Leukemic with Ashwagandha and Leukemic administered with Withanolide D.

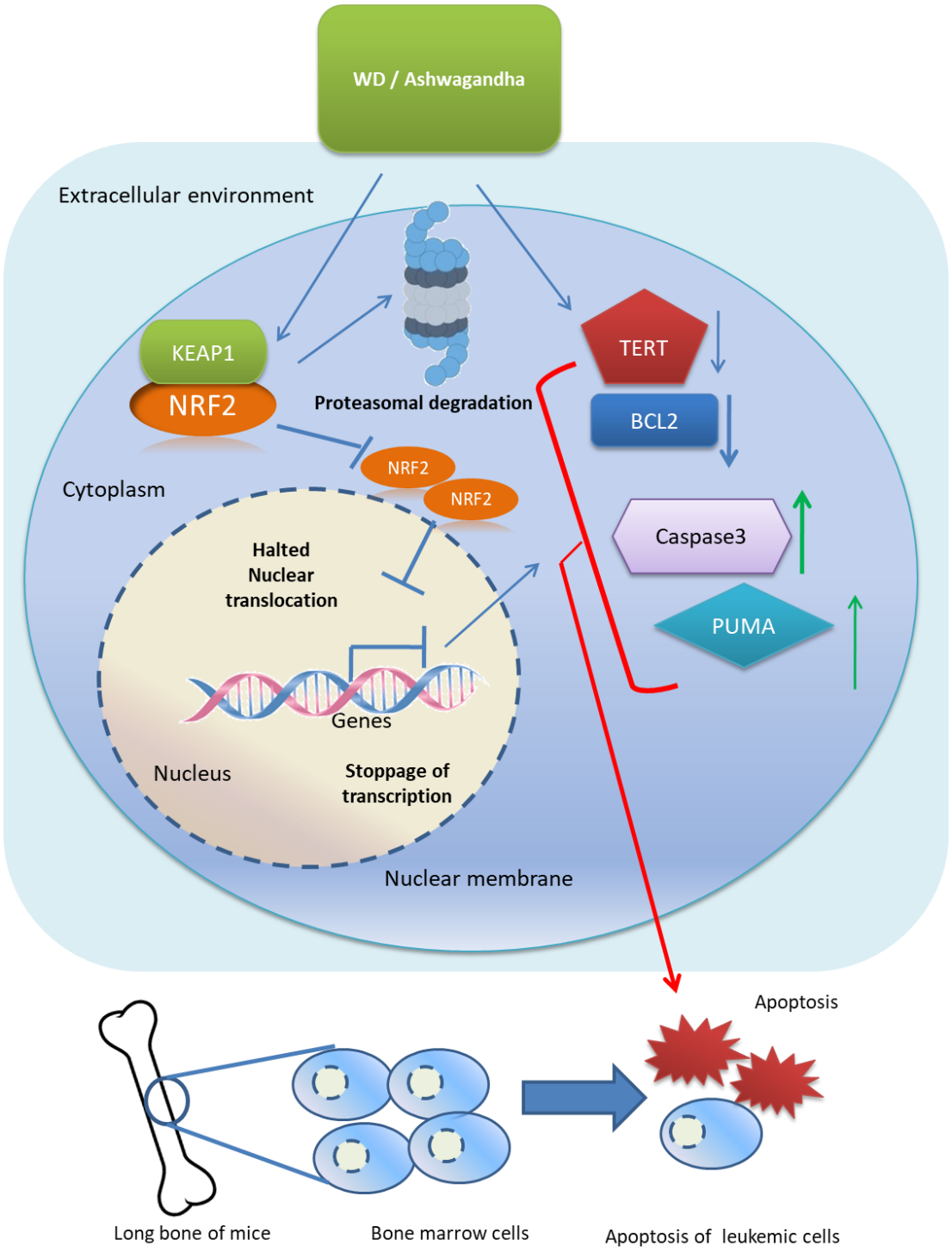


Figure 9. Ashwagandha/Withanolide D stops nuclear translocation of NRF2. This halt in the translocation resulted in the expressional increase in Caspase 3 , PUMA and decrease in BCL 2, TERT. Increased Caspase 3 expression led to apoptosis of the treated cells.

5.4. DISCUSSION

A delicate equilibrium between cellular proliferation and cellular death helps to maintain a healthy environment in an organism. Any interruption to this fine balance can result in abnormalities and ultimately cause the development of disease. Within the cancerous milieu, numerous signalling molecules and pathways experience deregulation.

The present study assesses the NRF2/KEAP1 signaling axis in leukemia. Exogenous sources such as pharmaceuticals, environmental agents, and industrial chemicals generate reactive oxygen species (ROS). The conformation of the KEAP1 protein changes in the presence of oxidative stress. Consequently, NRF2 is released from its low affinity binding site and the transfer of ubiquitin is interrrupted. As a result, KEAP1 molecules become saturated with NRF2, preventing its destruction. Simultaneously, newly synthesized and free NRF2 accumulates in the cytosol. Subsequently, NRF2 translocates to the nucleus where it binds to the antioxidant response element (ARE), hence activating the transcription of various cell defense genes.

The ARE region bears a multitude of genes and transcription factors. The activation of the ARE region results in the upregulation of the SHH gene and simultaneous expression of the corresponding protein. The elevated levels of SHH subsequently initiated a downstream signalling cascade within the hedgehog signaling pathway. This activation of the Hedgehog signalling pathway ultimately caused cellular proliferation and the suppression of the apoptotic pathway, thereby facilitating the disease progression. Additionally, NRF2 plays a crucial role in the differentiation of early-stage hematopoietic cellular lineages. Abnormal differentiation of hematopoietic lineages, disturbances in iron metabolism, changes in the microenvironment, and inhibition of the apoptotic mechanism were observed as a result of the aberrant activation of the NRF2/KEAP1 pathway, induced by NOC-induced stress.

A reversal in the xenobiotic stress-mediated insult commenced in the presence of Ashwagandha and Withanolide D, resulting in the decrease in dissociation of NRF2 from KEAP1. Consequently, due to the simultaneous degradation of free and ubiquitinated NRF2, the translocation of NRF2 into the nucleus and activation of the pathway were impeded. Further, the downregulation of the NRF2/KEAP1 axis caused a decline in SHH expression and the inactivation of the Hedgehog pathway. Consequently, there was a reduction in abnormal hematopoietic lineages, alterations in iron metabolism, and the reactivation of previously halted cellular processes. In the hemoglobin concentration, reticulocyte counts,

and the overall white blood cell scenario, these changes were reflected. Hence, it can be concluded that all the parameters that were aberrantly expressed in the leukemic condition began to shift towards normal condition.

In our model of leukemia, the equilibrium between cellular proliferation and death was severely impeded by aberrant cellular proliferation and reduced cellular death, also known as apoptosis. Apoptosis, a highly intricate mechanism that encloses various crucial components of both the extrinsic and intrinsic pathways. In our experiment, we have focused on four representative key components - TERT, BCL2, Caspase 3, and PUMA, associated with specific anti-apoptotic and pro-apoptotic functions. In the leukemia model, increased levels of TERT disrupted the apoptotic machinery and facilitated the survival of the abnormal cells. The increase in abnormal cells subsequently resulted in the disease progression. Notably, the heightened activity of the anti-apoptotic and proliferative mechanisms contributed to the progression of leukemia, along with pro-apoptotic system. PUMA and Caspase 3 are highly potent activators of apoptosis. The balance of the apoptotic system was disrupted by the augmented levels of TERT and BCL2, coupled with the decreased expression of PUMA and Caspase 3. As a consequence, the abnormal cells proliferated exponentially.

Ashwagandha and Withanolide D exhibited interactions with key components, namely TERT, BCL2, Caspase 3, and PUMA, when introduced into the leukemic system. In the abnormal hematopoietic cells, the downregulation of TERT and BCL2 expression suggested an enhanced susceptibility to apoptosis. The upregulation of PUMA and Caspase 3 expression initiated the apoptotic process concurrently. The dual impact on the apoptotic machinery eventually resulted in cellular death. This enhanced mortality rate and lowered proliferative activity of leukemic cells, significantly impeded disease progression. (Figure 4)

In summary, it can be stated that the hematopoietic niche in the leukemic state underwent changes upon exposure to Ashwagandha and Withanolide D. The introduction of Ashwagandha and Withanolide D led to a significant decrease in the nuclear translocation of NRF2, resulting in an increase in the expression of pro-apoptotic proteins and a decline in the expression of anti-apoptotic proteins. The reversal of the disease condition was notable due to the inhibition of the NRF2/KEAP1 signaling axis and the induction of apoptosis. Ultimately, it can be concluded that Ashwagandha and Withanolide D present a promising potential for the exploration of novel therapeutic approaches in the fight against leukemia.

Chapter 06

Ashwagandha and Withanolide D mediated cytoskeletal arrangement and modulated cyto-architecture in bone marrow cells of leukemic mouse.

CHAPTER SUMMARY

In this chapter, we investigated the modulatory effects of Ashwagandha and Withanolide D on aberrant cyto-architectural proteins (β-actin, β-tubulin, N-cadherin, and Vimentin) as well as on other deregulated cellular proliferation, migration, and adhesion functions in NOC-induced leukaemic bone marrow cells. Cytological, immunological, microscopic, and flow cytometric analysis demonstrated that the aberrant expression of cytoskeletal proteins returned to normal level in the treated groups of animals. Simultaneously, the augmented rate of cellular proliferation and migration of leukaemic cells were severely decreased. Ashwagandha and Withanolide D exhibited a positive impact on the regulation of the leukaemic cyto-architectural modulation and contributed to the curative process of the disease, with the potential to offer up new therapeutic approaches in the treatment of leukaemia.

6.1. INTRODUCTION

Skeletons form the structure or frame on which a body stands. It also defines the shape of the body of an organism. A cytoskeleton does the same function to a cell as a skeleton does for an organism or an animal: it defines the shape and also provides a structure on which a cell retains its shape. The functions of the cytoskeleton are not limited to the shape or architectural stability of a cell. It also helps in intracellular traffic, cell-to-cell communication, cell-to-matrix interaction, cellular migration, division, etc. Various cancerous endroits depict alteration in the basic functions of cytoskeletal architecture and arrangement due to genetic, epigenetic, and translational modification (Garitano-Trojaola et al., 2021; Jekely, 2014; Rivero & Cvrková, 2007; Trendowski, 2014).

One key cytoskeletal protein, Actin is a globular multi-functional protein that stays in two forms: either in a free monomer form, also known as globular actin (G-actin), or as part of a microfilament polymer known as filamentous actin (F-actin). Actin acts as an ATPase and also executes more interactions than any known protein, which grants it the ability to enact an astounding variety of functions important for cellular functions, including cytokynesis and

chemotaxis. (Borys et al., 2020; Bunnell et al., 2011; Izdebska et al., 2018, 2020; Khan, 2014).

Another key cytoarchitechtural protein is Vimentin (VIM), a type III intermediate filament ubiquitously present in an array of cell types, including smooth muscle cells, fibroblasts, and leukocytes, It is a major cytoskeletal component of mesenchymal cells and plays a pivotal role in the maintenance of cellular shape. Vimentin aids in the anchoring and supporting of organelles, a type of stabilising cytoskeletal interaction. It is often used as a marker for cells undergoing EMT processes during metastatic progression or physiological processes. (Madsen et al., 2019; Strouhalova et al., 2020; Wu et al., 2018)

Tubulin, a key component in the cytoskeletal scenario, has different isotypes expressed by specific genes that are regulated spatially and temporally. The α and β monomers of tubulin are present as isotypes. They differ in their amino acid sequence as they are encoded by different genes. α/β heterodimers polymerize into microtubules, which are extremely important for cellular growth and division (Binarová & Tuszynski, 2019; Faizan et al., n.d.; Liaw et al., 2008; Parker et al., 2014). Along with microtubules, microfilaments are crucial for fruitful cellular proliferation.

Tubulin, plays a crucial role in chronic myeloid leukaemia (CML). A study depicts that expression of tubulin isoforms from the tubulin gene is elevated and up-regulated in the chronic phase of leukaemia compared to the blast phase of leukaemia (Faizan et al., 19). Vimentin (VIM) is a type III intermediate filament that maintains cellular integrity and is involved in cellular adhesion, motility, and migration. In acute myeloid leukaemia (AML), VIM upregulation is associated with poor clinical outcomes (Wu et al., 2018). N-Cadherin belongs to a family of classic cadherins that mediate calcium-dependent intercellular adhesion. A study portrayed N-cadherin expression as playing a pivotal role in maintaining the quiescent state of LSCs in niches (Zhi et al., 2016).

Since microtubules are pivotal for mitosis, cell trafficking, and, in some circumstances, cell movement, inhibiting the dynamic instability of these polymers can be absolutely devastating for rapidly proliferating cells, henceforth making them ideal for helpful in tumorigenic growths. (Trendowski, 2014)

Ashwagandha and Withanolide D, a steroidal lactone abundantly present in Ashwagandha (*Withania somnifera),* has been studied for its pro-apoptotic, anti-neoplastic, and cytostatic effects but has not been studied as a NOC induced leukaemic cytoarchitectural modulator. In the current study, we tried to unearth the effects of Ashwagandha and Withanolide D on cytoskeletal architecture in leukaemic bone marrow cells.

6.2. MATERIALS AND METHODS

6.2.1. Maintenance and development of leukaemic animals

Four groups of inbred Swiss albino mice (*Mus Musculus*) were taken into consideration (Group I: control denoted as C, Group II: leukemic denoted as L, Group III: leukemic treated with Ashwagandha, denoted as L+A and Group IV: leukemic treated with Withanolide D, denoted as L+WD). Pups of both sexes aged between 10-15 days were administered via intra peritoneal route with ENU (N-ethyl-N-nitrosourea) at a dose of 80mg/kg body weight for the development of leukaemia. An equal amount of 1XPBS was administered in the pups of control groups. 4-6 months after ENU administration blood hemogram was performed to assess the disease status. Post leukemic development group III was administered with Ashwagandha extract via oral route. Animals of group IV were orally administered with Withanolide D.

During the experimental period all the animals were on a normal diet and water *ad libitum* with 12-hour light and dark cycle. All the animals were maintained according to the Institutional Animal Ethical Committee (IAEC) guidelines and protocols.

6.2.2. Ashwagandha and Withanolide D administration

Animals of Group III/L+A a received Ashwagandha extract at a dose of 600mg/kg bodyweight and Group IV/L+WD was administered with Withanolide D at a dose of 37.5mg/kg bodyweight via oral route for 30 days. An equal amount of distilled water was administered by oral route in the animals of group I/C and group II/L

6.2.3. Confocal microscopy

Bone marrow cells from all three groups were fixed and permeabilized. Fixed cells were treated with anti-CD90, NCAD, and PHALLOIDIN (Cell Signalling Technology, USA) primary antibodies and incubated for 45 minutes at 37ºC. Post-incubation, excess primary

antibody was rinsed off and followed by an Alexa 488 (Invitrogen, USA) secondary antibody. Finally, cells were placed on a slide and covered with DAPI. Slides were analysed with a Zeiss LSM700 confocal microscope (Zeiss, Germany).

6.2.4. Scanning electron microscopy

A small portion of the intact marrow tissues containing the total niche were removed aseptically from the femur and tibia without damaging the inner cell mass. Bone marrow portions were kept in 2.8% glutaraldehyde (Merck, USA) overnight for fixation. Post fixation tissue portions were repeatedly passed through a 30%, 50%, 70%, and 100% gradient of alcohol (Merck, USA), and finally critical point drying was done. Then the samples will be coated with gold (Au) in a gold coater. Finally, the gold-coated samples were placed on metal studs and analysed by a Zeiss scanning electron microscope (Zeiss, Germany).

6.2.5. Cellular migration assay

For the migration assay, the invasive chamber (Corning, Inc.) with an 8-µm pore polycarbonate membrane was pretreated for 2 h in serum-free RPMI 1640 (Sigma, USA) medium at 37 °C and 5% CO2. After the removal of the medium, 1.5 x 10^4 cells were added to the upper chamber and incubated overnight at 37 °C and 5% CO2. Cell samples were collected, and cells on the lower surface of the chamber were fixed with 4% paraformaldehyde for 30 min. After washing three times with PBS, the samples were stained with crystal violet (0.5 mM) dye for 30 min and observed and photographed under the microscope. (Huang et al. 2019)

6.2.6. Explants culture

Bone marrows were flushed out from the long bones of the animals in all three experimental groups. Small portions of the isolated marrow were cut into small pieces and cultured in triplicate. Bone marrow fragments were placed in a 10 mm^2 culture dish (Corning, USA) containing RPMI-1640 (Sigma, USA) complemented with 30% FBS (Gibco, Thermo Fisher Scientific, USA) and penicillin and streptomycin (HiMedia, India). The cultures were incubated at 37 °C in an incubator supplemented with 5% CO2. The cultures were maintained

for 96 hours and observed at regular intervals using an inverted microscope (Olympus, Japan).

6.2.7. LAI and NAI

Isolated bone marrow cells from four experimental groups (C, L, L+A and L+WD) were charged into four different hemocytometers (Rohem, India), and their numbers were counted. After that, the hemocytometer and the cells were submerged in 1X PBS. Nonadherent cells were washed off. Again, hemocytometers were placed under the microscope (Ch20i, Olympus, Japan), and the remaining numbers of cells were counted. Pre-wash and post-wash cell numbers indicated the level of adherence in the bone marrow cells of the three experimental groups. Adherent and non-adherent cell numbers of all three groups were evaluated.

6.2.8. Assessment of cellular proliferation

Expression of Cyclin D1 was assessed in the four experimental groups (C, L, L+A, and L+WD). Briefly, 1.5% PFA-fixed bone marrow cells were permeabilized with 90% chilled methanol, followed by a PBS wash and resuspension in FACS fluid. Tubes were added with an anti-Cyclin D1 antibody (Cell Signalling Technology, USA) and incubated for 30 minutes at 37ºC. Fluorochrome-labelled secondary antibodies (Alexa Fluor 488, Invitrogen, USA) were added after incubation with the primary antibody. Finally, cells were analysed with BD FACS Calibur (Becton-Dickinson, USA) and Cell Quest Pro software.

Bone marrow cells from the four experimental groups (C, L, L+A, and L+WD) were cultured in RPMI 1640 media (Sigma, USA) complemented with 30% FBS (Gibco, Thermo Fisher Scientific, USA) and penicillin and streptomycin (HiMedia, India). The media was removed, and cells were washed with 1X PBS. MTT (Sigma, USA) solution was added with a final concentration of 0.5 mg/mL, followed by incubation for 4 hours at 37°C. Post-incubation cells were checked under the microscope for intracellular purple formazan crystals. MTT solution was carefully removed, and MTT solvent or solubilizing solution was added with incubation for 30 minutes at 37°C. Finally, the absorbance was measured at 570 nanometres.

6.2.9. Flow Cytometric evaluation of cytoskeletal proteins

Flow cytometric studies were taken into consideration and described in detail in previous chapters. Briefly, 1.5% PFA-fixed bone marrow cells were permeabilized with 90% chilled methanol, followed by a PBS wash and resuspension in FACS fluid. Each tube was added with the respective antibodies (anti-N-CAD, anti-VIMENTIN, anti-β ACTIN, anti-β TUBULIN, anti-CYLD, anti-NAK, and anti-CD31 from Cell Signalling Technology, USA) and incubated at 37ºC for 30 minutes. Thereafter, each tube was added with fluorochrome-labelled secondary antibodies (Alexa Fluor 488, Invitrogen, USA) at similar conditions. Finally, cells were analysed using BD FACS Calibur (Becton-Dickinson, USA) and Cell Quest Pro software.

6.2.10. Immunofluorescence

Expression patterns of CYCLIN D1, N-CAD, VIMENTIN, β-ACTIN, β-TUBULIN, CYLD, NAK, and CD31 were accessed by immunofluorescence (IF) analysis from four different groups, viz. control, leukaemic, and Withanolide D (C, L, L+A, L+WD) treated bone marrow cell samples with primary antibody (1:200 dilution) and secondary antibody (1:600 dilution). Nuclei were stained using DAPI shield (Sigma-Aldrich, USA). Slides were examined under a fluorescence microscope (Axio Scope A1, Zeiss, Germany).

6.2.11. Statistical assessment

Experimental data from all three groups (C, L, L+A, and L+WD) are presented as the mean ± standard deviation. A student t test and one-way analysis of variance (ANOVA) were used for intergroup comparison. A probability of ≤ 0.05 was considered statistically significant. GraphPad Prism (version 8.4) was used for statistical calculations.

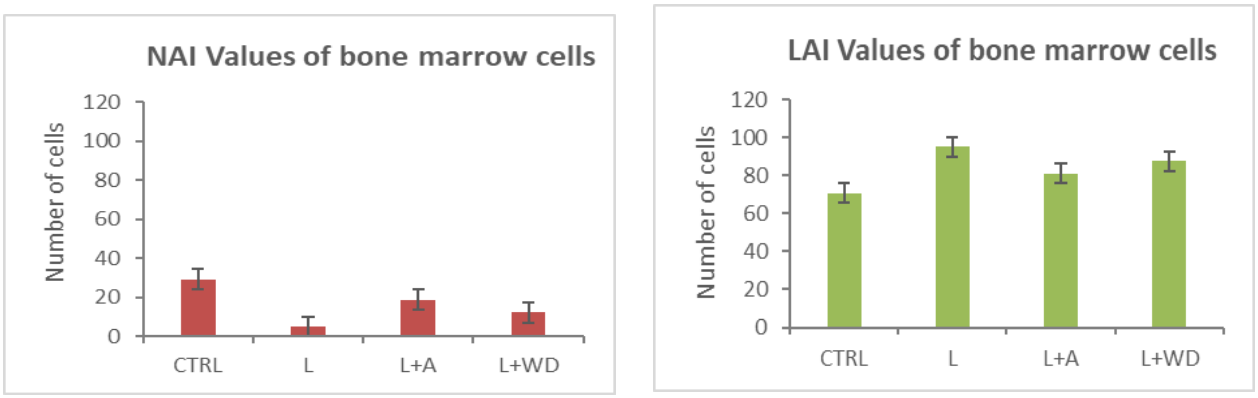
6.3. RESULTS

6.3.1. Ashwagandha and Withanolide D impaired cellular adhesion in leukaemic cells

LAI and NAI values serve as indicators of alterations in the adhesive characteristics of specific cell groups under specific conditions. Our investigation revealed an increase in LAI

values for leukemic bone marrow cells; however. In the leukemic group, the LAI value (95.7±0.38) displayed a significant increase compared to the control group (70.81±0.87). The enhanced LAI values clearly signify an augmentation in adhesion properties. Further, the non-adherent values for both groups were examined, supporting the observation that detached cells were less prevalent in the leukemic groups (4.93±0.56) than in the control groups (29.19±1.34).

In the groups administered with Ashwagandha and Withanolide D, the LAI and NAI values were (L+A)81.2±1.32, (L+WD) 87.7±1.95, and (L+A) 18.8±1.44, (L+WD)12.3±1.76, respectively. This change in LAI and NAI values unequivocally demonstrated a reduction in both the quantity of adherent cells and the quantity of non-adherent or detached cells in the treated group, in comparison to the Leukemic group.



a b

Figure 1. a. NAI or Non-Adhesive Index of cells depicted in graph. **b**. Bar graph showing LAI or Leukocyte Adhesion index value in the three experimental groups.

CD31, known as PECAM, is a cell adhesion molecule that is significantly expressed on leukocytes and platelets (Chen et al., 2010). The expression of CD31 was predominantly higher (90.43±3.11) in leukemic bone marrow cells compared to the control group (32.91±2.35). Similarly, the expression of another important adhesion molecule called N cadherin was also considerably elevated in the leukemic group (82.58±2.10) compared to the control group (48.39±1.78).

Interestingly, in the groups treated with Ashwagandha and Withanolide D the expression of both CD31 and N-CAD significantly decreased, with CD31 expression measuring (L+A MFI

58.3±1.34 & L+WD MFI 76.39±3.52) and N cadherin expression measuring (L+A MFI 61.45±3.18, L+WD MFI 72.13±2.54). The decrease in expressional values of CD31 and N-CAD clearly indicated a reduction in the adhesion properties of the treated cells. These findings were further established with fluorescence imaging and confocal imaging, as shown in figure 1a to b.

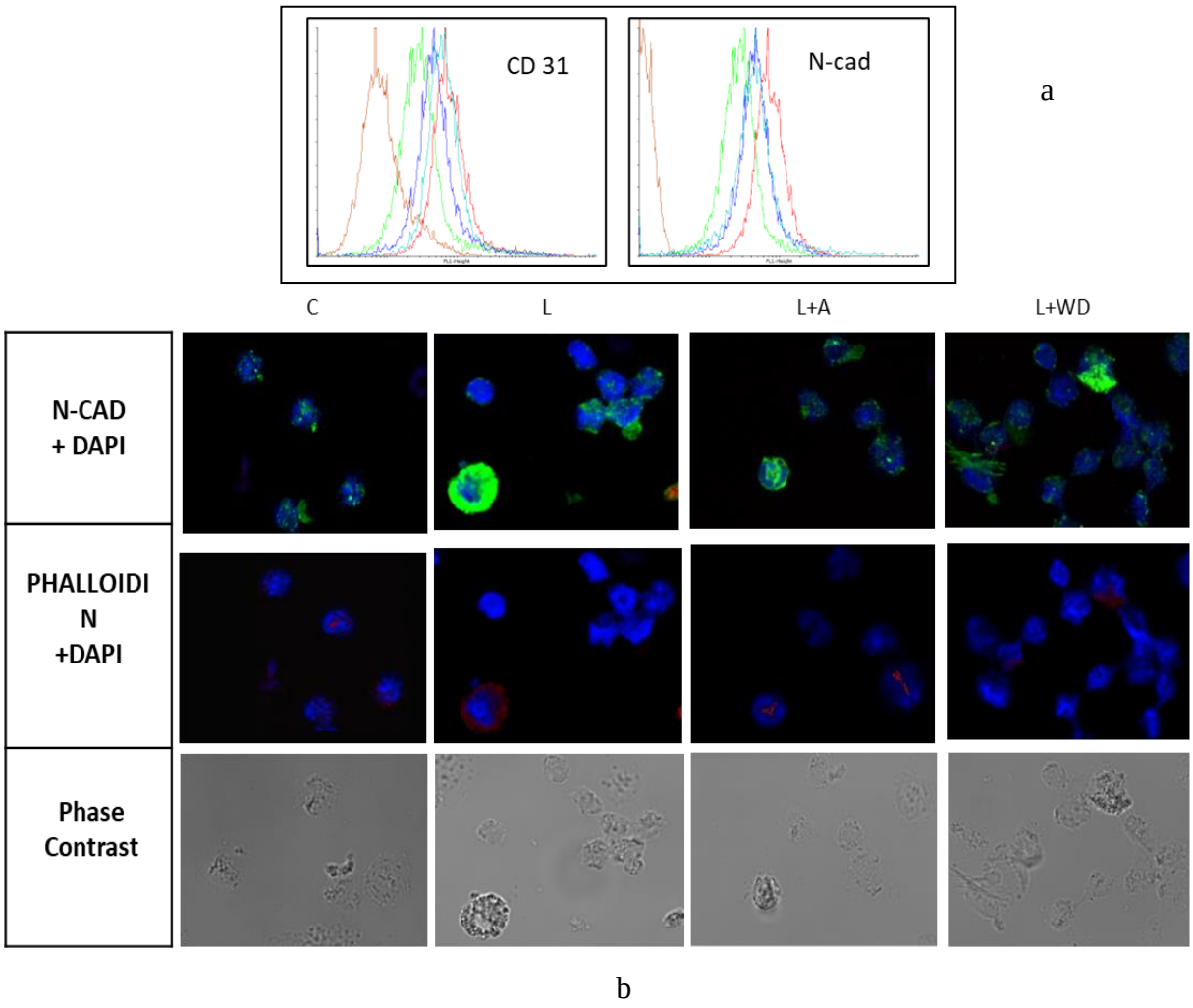


Figure 2. a. Histogram overlay plots of CD31and N-CADHERIN, depicting expression of the protein across all four experimental groups. **b.** Left column: Confocal images of N-CAD and DAPI. Middle column: Confocal images of PHALLOIDIN and DAPI. Right column: Phase contrast image of bone marrow cells.

6.3.2. Reduction in Cellular proliferation is noted due to Ashwagandha and Withanolide D administration

Cellular proliferation indicates the formation of new cells. Cyclin D1 is a major protein that facilitates the transition from the G1 phase to the S phase during the progression of the cell

cycle. It has oncogenic potential and is highly expressed in various human malignancies (Fernandes et al., 2018). In our experiment, the expression level of Cyclin D1 was significantly heightened (MFI 286.93±5.23) in leukemic conditions compared to the control group (MFI 139.39±3.12). The elevated level of Cyclin D1 clearly indicated the progression of leukemic cells. We further examined cellular proliferation using the MTT assay. The leukemic samples showed higher OD values (1.09±0.56) compared to the control group (0.725±0.33). These higher OD values can be interpreted as an indication of the presence of a greater number of proliferating cells that formed more formazan crystals.

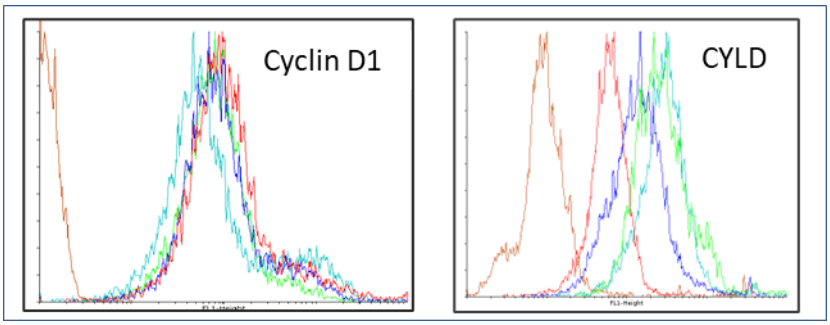
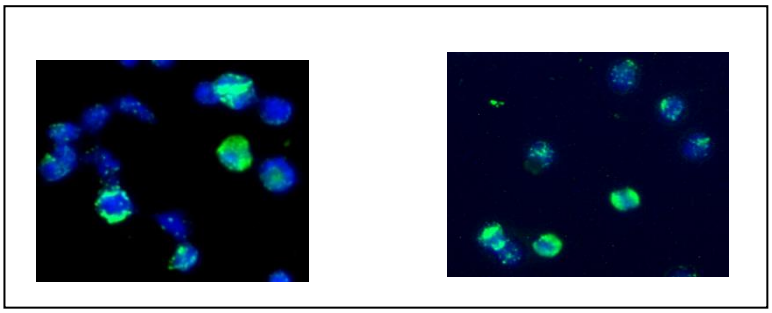


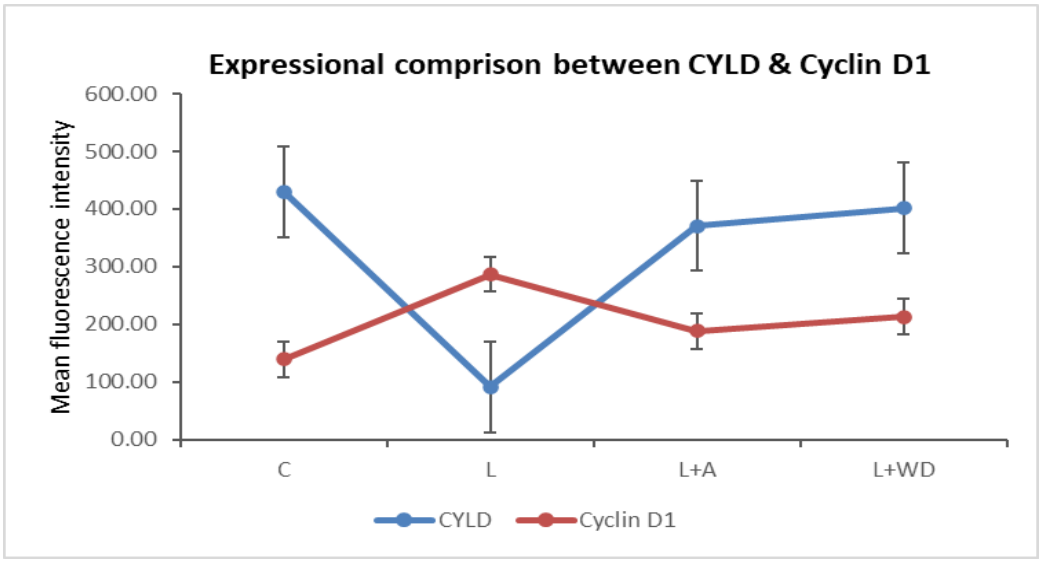
Figure 3. Histogram overlay plots of Cyclin D1, and CYLD depicting expression of the protein across all four experimental groups.

In the groups treated with Ashwagandha and Withanolide D, the expression of Cyclin D1 began to shift (L+A MFI 188.35±4.11 & L+WD MFI 213.54±2.54) towards normal values. This change indicated a cessation in the proliferative mechanism in leukemic cells influenced by Ashwagandha and Withanolide D. The MTT assay additionally verified the aforementioned findings as there was a modification in the optical density value in the treated groups (L+A MFI 0.875±1.33 & L+WD MFI 0.931±0.31).

CYLD plays a crucial role in the process of osteoclast formation, the maintenance of bone equilibrium, spermatogenesis, the progression of the cell cycle, cell migration, and acts as a suppressor of tumors in various malignancies (Arora et al., 2015; Lin et al., 2016; van Andel et al., 2017; Verhoeft et al., 2016; Yang et al., 2015). The reduction in CYLD levels leads to the development of tumors. In our experiment, the expression of CYLD in the leukemic group showed a notable decrease (MFI 91.52±1.06) compared to the control group (MFI 429.81±4.21).



a



b

Figure 4. a. Representative immune fluorescence image of the Cyclin D1(left), and CYLD (right). Nuclei stained with DAPI (blue) and proteins are stained with Alexa 488(green). **b.** Graph portraying expressional comparison between Cyclin D1, and CYLD.

The decline in the expression of the machinery responsible for suppressing tumor growth ultimately facilitated in the disease progression. the expression began to increase (L+A MFI 370.71 ±2.71 & L+WD MFI 401.55±5.14) following the administration of Ashwagandha and Withanolide D, indicative of the shift towards normal values. (Figure 3, 4a, b).

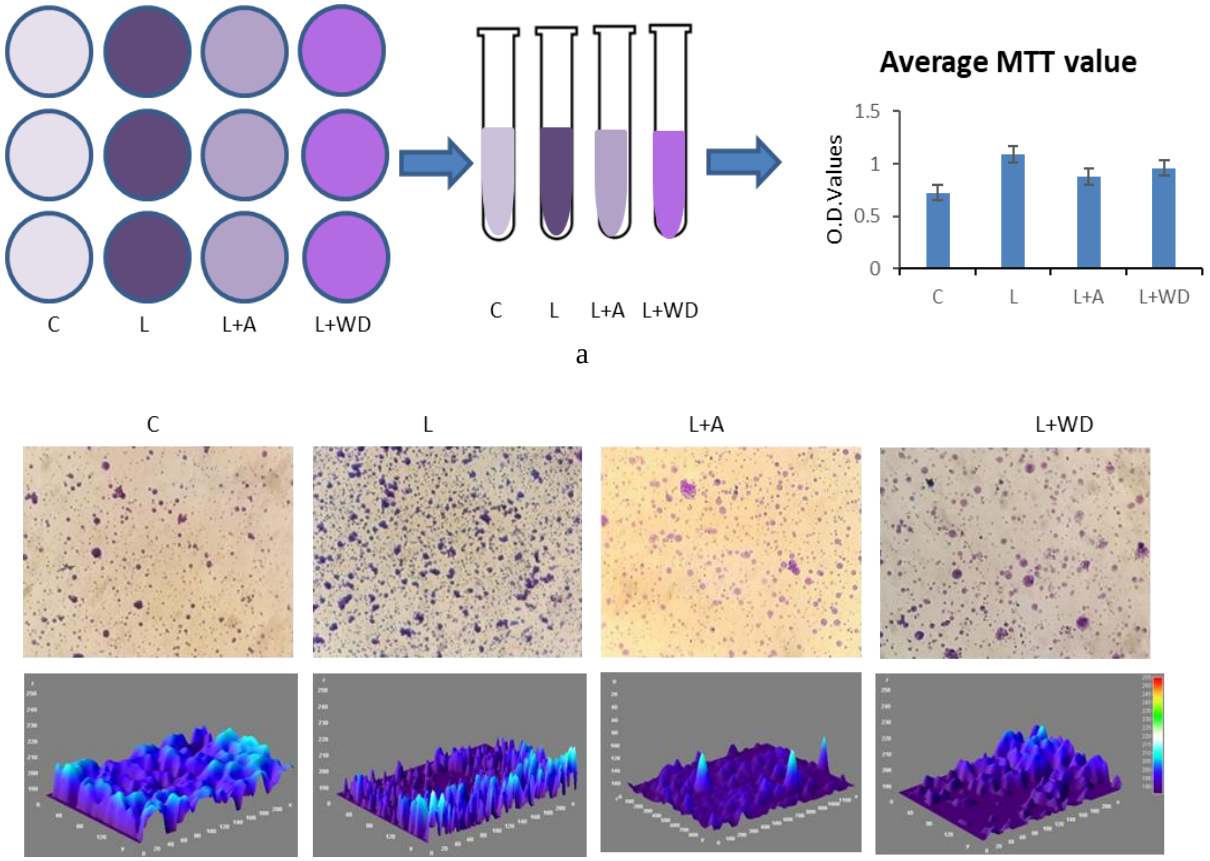


Figure 5. a. Left: graphical representation of 24 well culture plate showing formation of formazan crystals in the experimental design. Middle: representative image of cuvette containing solutions before O.D. measurement. Right graph depicting average MTT values in the three experimental groups**. b.** Image of the cells with formazan crystals in the four experimental groups. **c.** 3D interactive plot depicting cells with formazan crystals in the MTT assay.

6.3.4. Ashwagandha and Withanolide D hinders Cellular migration in leukaemic cells

Bone marrow explants from all four groups were assessed. The leukemic groups exhibited a greater number of cellular emanations from the explants compared to the control groups. Modifications in the bone marrow milieu resulted in the propagation of aberrant cellular lineages. Consequently, a larger quantity of cells emerged from the leukemic bone marrow explants in contrast to the control explants.

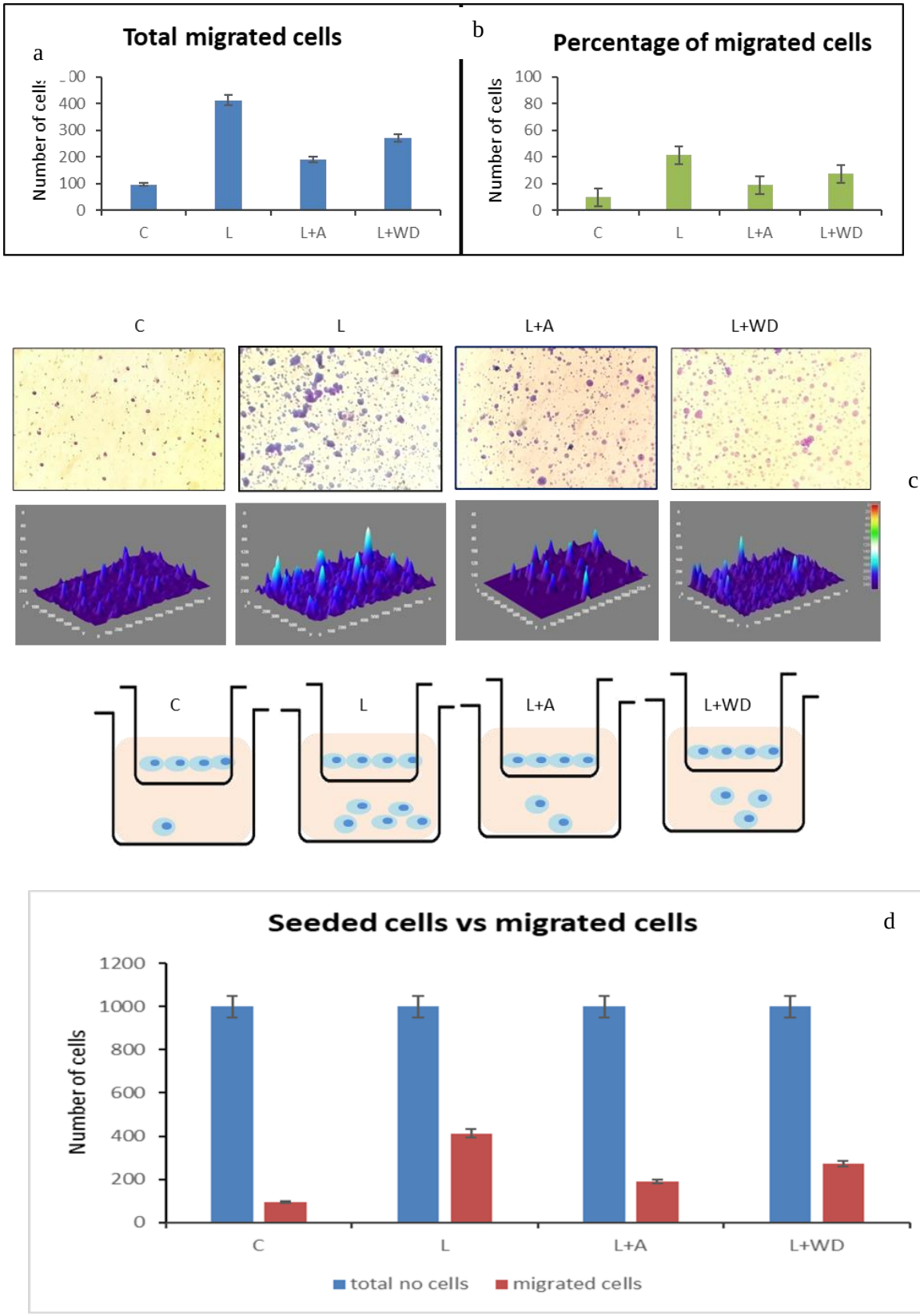
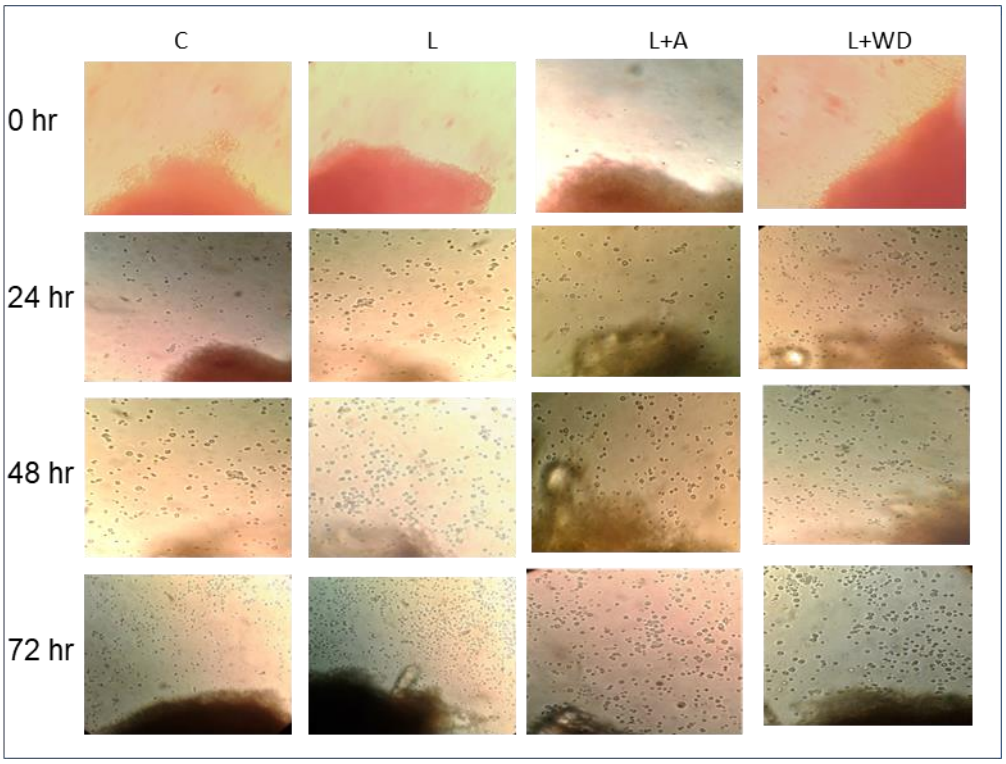


Figure 6. a. Graph depicting total migrated cells in the experimental set up. **b.** Graph depicting percentage of total migrated cells. **c.** Upper row depicts migrated cells stained with Cresyl violet in four experimental groups. The bottom row depicts 3D interactive plots of the aforementioned migrated cells. **d.** Graphical representation of

the cellular migration via transwell plates in the experimental set up. **e.** Bar graph showing total seeded or plated cells versus total migrated cells.

In the groups treated with Ashwagandha and Withanolide D, the emanation of cells from the explants was lesser to that of the leukemic explants. This clearly indicated that alterations have occurred in the bone marrow microenvironment as a consequence of Ashwagandha and Withanolide D's administration. Consequently, the propagation of abnormal cellular lineages has been significantly diminished. Concurrently, the findings from transwell migration assays corroborated the aforementioned observations. In the leukemic group, a greater number of cells (412.4± 3.05) migrated to the lower compartment in comparison to the control cells (95.6±4.97). In the wells treated with Ashwagandha and Withanolide D, the migration of cells was markedly diminished (L+A 189.1±4.13 & L+WD 270.8±5.72) (figure 7a).

A scanning electron micrograph affirmed the presence of elongated projections in the leukemic cells. The movement and spread of leukemic cells were facilitated by these projections. Only a minimal number of projections were discernible in the control cells. Reductions in the number of cellular projections were observed in the cells treated with Ashwagandha and Withanolide D (figure 7 b).



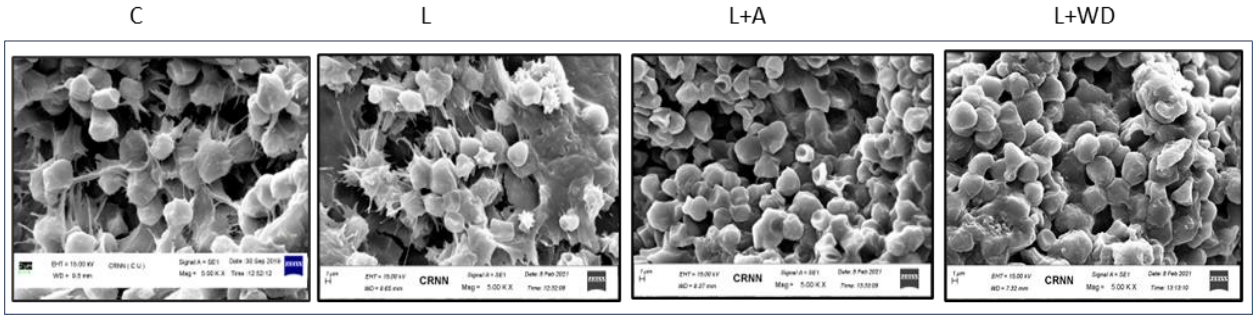


Figure 7.a. Images depicting cellular migration from bone marrow explants. **b.** Scanning electron micrograph of bone marrow of control, leukaemic and leukaemic treated with Ashwagandha and Withanolide D.

6.3.5. Expression of cytoskeletal proteins was altered in the presence of Ashwagandha and Withanolide D

Various components of cytoskeletal proteins were assessed. The expression of β-actin in the leukemic cells showed an increase of (MFI 185.3±1.55) in comparison to the control (MFI 113.18±2.12). Similarly, β-tubulin expression in leukemic bone marrow cells, which is another vital component of the cytoskeleton, demonstrated an increase of (MFI 276.52±2.51) in contrast to control bone marrow cells (MFI 167.75±2.45). Vimentin expression witnessed an elevation in leukemic cells (MFI 328.54±1.98) as opposed to control cells (MFI 83.71±2.38).

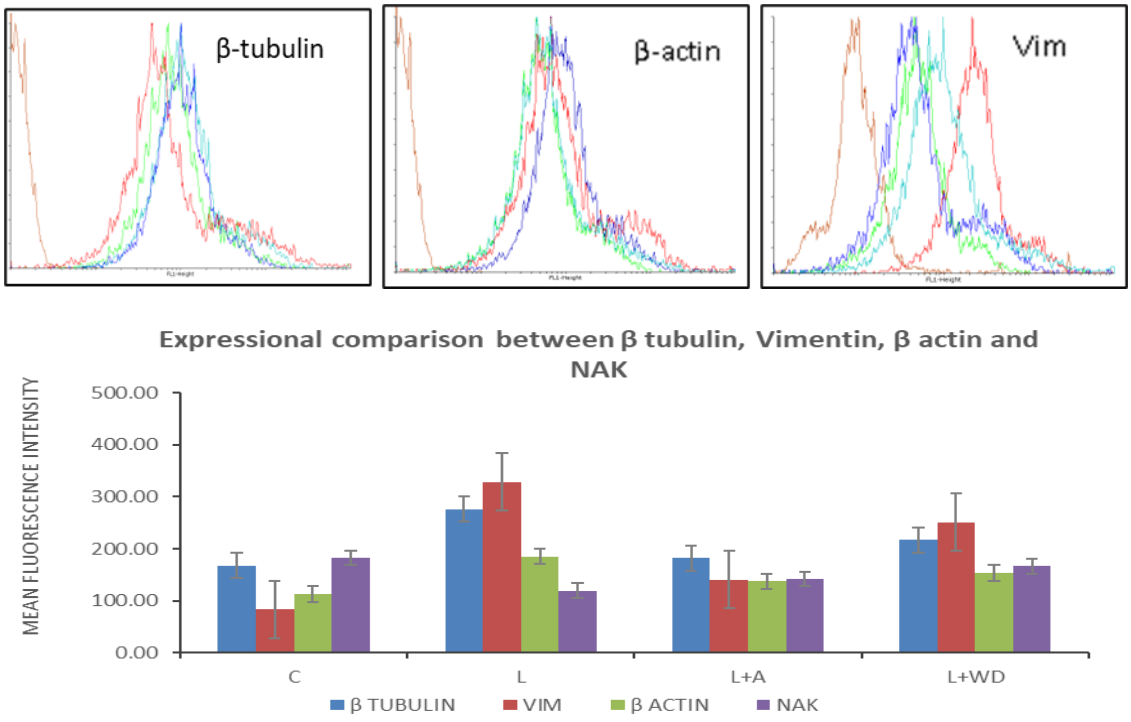


Figure 8. a. Histogram overlay plots of β-TUBULIN, β-ACTIN and VIMENTIN depicting expression of the protein across all four experimental groups. **b.** Bar graph shows casing expressions of β-TUBULIN, β-ACTIN VIMENTIN and NAK in all three groups.

β-actin expression (L+A MFI 137.55± 3.4 & L+WD MFI 153.11±1.83), β-tubulin expression (L+A MFI 181.99±3.11, L+WD MFI 216.67±3.41), and Vimentin expression (L+A MFI 140.79±1.97 & L+WD MFI 251.05±3.31) exhibited a shift towards the normal values following the administration of Ashwagandha and Withanolide D.

Phalloidin is a toxic substance derived from Amanita phalloides which is commonly referred to as the "death cap" mushroom, exhibits a binding affinity towards F actin molecules, thereby effectively hindering the depolymerization process of actin fibers. In our experiment, the expression of phalloidin was considerably higher in comparison to the control cells, indicating the presence of activated actin structures within the cellular milieu. Conversely, in the treated groups, a decline was observed in the aforementioned expression.

Another vital element, NAK plays a significant role in the initiation of cytoskeletal protein expression. NAK expression experienced a significant decrease (MFI 119.73±3.22) when contrasted against the control (MFI 182.59±2.71), in conditions characterized by leukemic manifestations. Conversely, in the group treated with Ashwagandha and Withanolide D, NAK expression began to demonstrate a shift towards normalcy (L+A MFI 141.98±4.31 & L+WD MFI 166.98±1.93).

The findings elucidated above were further corroborated by confocal imaging, immunofluorescence imaging, and phase contrast microscopy (Figure 3a-d).

6.4. DISCUSSION

The importance of the cytoskeleton in the context of leukemia is vast. It comprises microtubules, microfilaments, and intermediate filaments. Microtubules play a vital role in cellular growth, movement, and important signaling events. Microtubules form the spindle that facilitates accurate chromosomal segregation in the process of mitosis. In light of this observation, we have discovered a major increase in the expression of tubulins in leukemic conditions. Concurrently, there is also a notable increase in the expression of Cyclin D1 in

leukemic cells. It can be argued that the elevated expression of Cyclin D1 assists the cells in initiating the proliferation process. Meanwhile, tubulins regulate spindle dynamics and protein-protein interactions, leading to the movement of chromosomes to their designated locations. Collectively, both tubulins and Cyclin D1 contribute to the progression of the disease. Another vital element is CYLD, which typically functions as a tumor suppressor, cell cycle regulator; experienced a significant decrease in leukemic bone marrow cells. In a previous study, we have demonstrated that the expression of BCL2 was upregulated in leukemic cells, thus leading to the disease proliferation by inhibiting the apoptotic mechanism. A decrease in CYLD resulted in an inability to target the expression of BCL2, causing an increase in BCl2 expression. Consequently, the apoptotic pathway was inactivated, promoting the abnormal cells' proliferation and leukemogenesis. Our findings align with those of previous studies conducted by Lin et al. and Andel et al. (Lin et al., 2016; van Andel et al., 2017).

Metastasis that is the spread of cancer to secondary tumor sites, is a prominent concern due to its association with approximately 90% of cancer-related deaths. This process includes the occurrence of epithelial-to-mesenchymal transition and its reversal, mesenchymal-to-epithelial transition. A crucial aspect of achieving migratory potential is the alteration of the cellular phenotype. Specifically, lamellipodia, invadopodia, and filopodia are formed to facilitate cellular movement, and the reorganization of the actin cytoskeleton plays a pivotal role in this phenomenon. The acquisition of motor skills necessary for invasiveness relies on the continuous polymerization and depolymerization of actin molecules.

The adhesion on the surface of anchor proteins also engages in communication with the structural proteins present in the cytosol. Cadherins establish communication with catenin, actin, and various other cytoarchitectural proteins. Within our experimental design, N CAD established communication with VIM and β actin. Consequently, there was a transformation in the cytoarchitecture of leukemic cells, leading them to shift towards an environment rich in nutrients and oxygen. In our experiment, the expressions of two crucial markers for epithelial-mesenchymal transition (EMT), namely N-cadherin and Vimentin, were significantly heightened in leukemic cells found in the bone marrow. The cells experienced a loss of polarity and junctions as a result of this, while simultaneously acquiring migratory potential and invasiveness. Further, morphological changes were also observed due to alterations in the expression of cytoskeletal proteins.

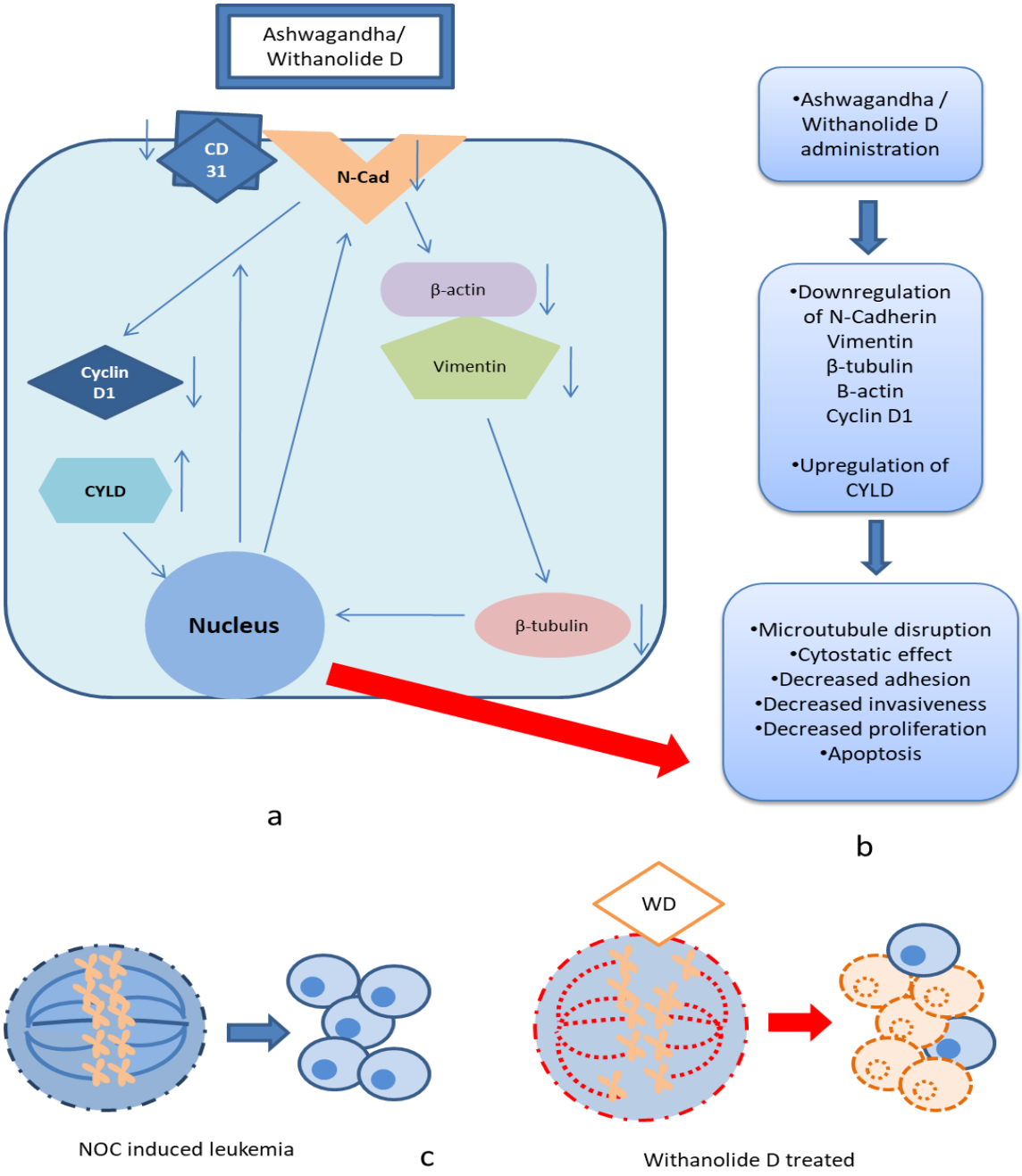


Figure 9. **a.** Graphical representation of Ashwagandha and Withanolide D: it downregulates expression of Cyclin D1, CD31, N-CADHERIN, β-tubulin, β-actin, and VIMENTIN and upregulates expression of CYLD in leukaemic cells. **b**. A flow diagram depicts the effects of Ashwagandha and Withanolide D on the expression of the cytoskeleton and adhesion proteins and their effects on cellular functions. **c**. Left: in the absence of WD, leukaemic cells reproliferate rapidly and interfere with the progression of the disease. Right: In the presence of WD microtubule structures re disrupted resulting in obstructed chromosomal migration and hindered cellular proliferation.

Elevated expression of CD31 or PECAM 1 in the leukemic cells hindered mitochondrial apoptosis and anchorage-independent growth. Further, through the stimulation of microvessel growth it facilitated metastasis. The aberrant cells managed to evade the process of programmed cell death due to the elevated levels of epithelial-mesenchymal transition (EMT) markers, diminished CYLD expression, as well as increased expression of CD31 or PECAM 1. These alterations ultimately contributed to the disease progression. Conversely, overexpression of CD31 initiated a homotypic interaction with endothelial cells in the bone marrow, resulting in an increased ability to migrate across the endothelium. Consequently, a higher count of CD31 could potentially be correlated with an increased count of white blood cells in the peripheral blood (as shown in figure 4a).

Ashwagandha and Withanolide D administration had a multifaceted impact on the arrangement of cells, the dynamics of the cell structure, and the growth of the leukemic cells. Initially, the interaction between Ashwagandha and Withanolide D and tubulin proteins caused a decrease in the movement of microtubules. As the expression of tubulin declined, the structures of microtubules began to separate. As a result, the transport of proteins and organelles within the cell declined, along with a decrease in the movement of motor proteins through the microtubule network. Simultaneously, the dynamics of spindle fibres were suppressed, which caused cell division to stop and ultimately resulted in the cell death that were rapidly. Secondly, an increase in the expression of CYLD removed the suppression over the cellular machinery that triggers programmed cell death and increased interaction with important components of various other pathways that directly or indirectly suppressed the growth of the abnormal cell lineage. A decline in the expression of Cyclin D1 further slowed down the growth of leukemic cells.

Thirdly, a reduction in the expression of CD31 and N-CAD upon the addition of Ashwagandha and Withanolide D. This decline in expression had an impact on the attachment points on the cell surface and also impeded the communication between molecules on the cell surface and molecules within the cell architecture. Consequently, in leukemic cells induced by NOC, the number of cellular processes decreased resulting in a less spiky appearance of the cells. Ashwagandha and Withanolide D also caused Vimentin and β actin expression started returning to their normal levels. The Vimentin filaments began to disassemble as a result. The disassembly of these filaments had a notable effect on the movement of cells and their ability to invade, while increasing the rate of cell death in leukemic cells (figure 4b).

In view of the present investigation, it can be argued that Ashwagandha and Withanolide D functions as a powerful regulator of cytoskeletal proteins and significantly influences the growth, movement, and attachment of leukemic bone marrow cells induced by NOC. Ashwagandha and Withanolide D may offer potential avenues for improved therapeutic control and intervention of the pathophysiology associated with NOC-induced leukemia.

Chapter 07

Ashwagandha and Withanolide D modulated RNAi machinery and activated autophagy pathway in leukemic bone marrow cells.

CHAPTER SUMMARY

The current chapter addresses the efficacy of Ashwagandha and Withanolide D and its interactions with the RNAi machinery components (DICER, DROSHA, AGO1, and AGO2) in modifying other cellular processes, including DNA damage repair and autophagy machinery, in leukaemia induced by NOC. To examine the components of the aforementioned pathways, various cytological, immunocytological, phase contrast microscopic, confocal microscopic, flow cytometric, etc. approaches were applied. Ashwagandha and Withanolide D altered decreased oncogenic expression, improved DNA repair mechanisms, deregulated RNAi machinery, and activated autophagy mechanisms in leukaemia, promising to open up new methods in the therapeutic management of leukaemia.

7.1. INTRODUCTION

RNA interference, or RNAi, refers to a short 21–23 nucleotide-long sequence of RNA that pairs with and targets endogenous mRNAs (Grishok, 2005). Craig Mello and Andrew Fire discovered this unique biological phenomenon in 1998 (MacFarlane & R. Murphy, 2010; Mohr et al., 2010). Endogenously formed RNAi modulates numerous cellular functions. Short, double-stranded RNAs, or microRNAs (miRNA), are formed from larger RNA transcripts by RNA polymerase II. (Ali Syeda et al., 2020; Xin et al., 2017) These initially larger transcripts are known as pri-miRNA. The RNase III enzyme Drosha and its cofactor DiGeorge syndrome critical region gene 8 (DGCR8) process the pri-miRNA into hairpin precursors known as pre-miRNA. After that, Dicer, along with TRBP, cleave the pre-miRNA hairpin. This complex separates the hairpin loop from the double-stranded structure and forms a miRNA duplex. Argonaut (AGO) proteins joined with Dicer and the pre-miRNA complex and formed a ternary complex. Now this double-stranded RNA is unwounded and turned into active single-stranded miRNA by the RNA-induced silencing complex (RISC). This complex is guided by binding to the regions that have partial sequence similarity within the 3’ untranslated region (UTR) of target messenger RNAs (mRNA) (Casey et al., 2019; Hammond, 2001; Müller et al., 2020). Upon binding, miRNA plays a crucial role in a wide range of biological processes. Cellular proliferation, differentiation, stress response, pathway activation, apoptosis, and autophagy are greatly modulated by miRNAs. A single miRNA can

simultaneously modulate a variety of cellular processes by targeting multiple targets (Morgens et al., 2016; Saema et al., 2015).

Various diseases, including cancer, are the outcomes of miRNA involvement. In cancer, differential expression of miRNAs was observed. They acted both as tumour suppressors and activators. A critical involvement in disease initiation, development, metastasis, and treatment sensitivity was also identified. (Campbell & Choy, n.d.; Das et al., 2019; Scherr et al., 2004). In haematological malignancies, miRNAs play a very decisive role. Surprisingly, the first concrete evidence of miRNA involvement in cancer was found in the chronic lympholytic leukaemia (CLL) pathogenesis (Gómez-Gómez et al., 2019; Landry et al., 2015; Tyner et al., n.d.; Yendamuri & Calin, 2009).

Different miRNAs were shown to be involved in various neoplastic malignancies. In acute lymphoblastic leukaemia (ALL), miR-128-8b, miR181b-1, miR204, and miR218 were upregulated and contributed to the disease progression. Downregulated let-7 and miR-181a, along with upregulated miR155, were noted in CLL. Involvement of upregulated miR-127, miR-154, miR-323, and miR-299 greatly aids in acute myeloid leukaemia (AML) pathogenesis. Chronic myelogenous leukaemia (CML) pathophysiology showed modulation of the miR217-92 region (Allegra et al., 2014; Bai et al., 2013; Gazon et al., 2016; Gelmez et al., 2017; Pandita et al., 2019).

LIN28, a highly conserved RNA-binding protein, was first identified in *Caenorhabditis elegans*. LIN28 is present in almost all mammals and includes two homologous members, LIN28A and LIN28B. Both have similar domain structures, with an N-terminal cold-shock domain (CSD) and two C-terminal CCHC zinc finger domains and functions. LIN28 blocks the let-7 group of miRNA expression by binding to the pre-let-7 loops. On the other hand, let-7 itself binds to the 3' UTR region of LIN28 mRNA and negatively regulates LIN28 expression. LIN28 is predominantly found in the cytoplasm and actively participates in let-7-dependent and let-7-independent mechanisms of protein processing. In various cancers, LIN28 is often found upregulated and aids in the progression of the disease (Balzeau et al., 2017; Chen et al., 2019; Ustianenko et al., 2018, p. 28; Wang et al., 2015, p. 2).

In the Ayurvedic system of treatment, Ashwagandha, or *Withaniania somnifera,* is used for its' anti-tumour, anti-depressant, anti-inflammatory, and neuroprotective effects. Recent studies revealed numerous active compounds, mostly steroidal lactones, are present in

Ashwagandha (Dutta et al., 2019; Egbuna et al., 2019). The anti-cancer effect of Ashwagandha and its isolated bioactive compounds were also studied in detail. Previously, the effect of Withanolide D, an Ashwagandha derivative, on RNAi machinery in a leukemic context had not been studied. This study will shed light on the effect of the bioactive molecule on epigenetic regulation of leukemic pathophysiology induced by NOCs.

7.2. MATERIALS AND METHODS

7.2.1. Animal development and maintenance

Three groups of Swiss albino mice (*Mus musculas*) were taken for the experimental purpose. Group I considered a control group designated as C. Group II was the leukemic group and was termed L, and the third group, or Group III, is denominated L+WD. 10–14-day-old Swiss albino mouse pups were administered with 80 mg/kg body weight of ENU intraperitoneally. A similar amount of sterile PBS was administered to the control group of animals. 4-6 months later, ENU-administered pups developed into leukemic animals. Post-disease development, one group of animals was administered with Withanolide D extract, and another group was administered with an equal volume of distilled water.

All the animals were caged in the Central Animal Facility following Institutional Animal Ethical Committee (IAEC) guidelines. Animals were supplemented with a normal diet and water *ad libitum* along with 12 hours of light and dark cycles during the experimental period.

7.2.2. Administration of Withanolide D

Animals of Group III/L+A a received Ashwagandha extract at a dose of 600mg/kg bodyweight and Group IV/L+WD was administered with Withanolide D at a dose of 37.5mg/kg bodyweight via oral route for 30 days. An equal amount of distilled water was administered by oral route in the animals of group I/C and group II/L

7.2.3. Confocal microscopy

The bone marrow cells of all four groups were fixed and permeabilized. Fixed cells were treated with an anti-ATG12 primary antibody and incubated for 45 minutes at 37ºC. Post-incubation, excess primary antibody was rinsed off and followed by an Alexa 488 secondary

stain. Finally, cells were placed on a slide and covered with DAPI shield. Slides were analysed using a Zeiss LSM700 confocal microscope.

7.2.4. Assessment of RNAi machinery

Bone marrow cells from all three groups (i.e., control, leukemic, leukemic treated with Ashwagandha and Withanolide D-treated) were fixed with 1.5% PFA. Post fixation cells were washed with 1X PBS and then permeabilized with 90% chilled methanol. Finally, cells were washed with PBS and resuspended in FACS fluid. 2µl of anti-DICER, anti-DROSHA, anti-AGO1, and anti-AGO2 antibodies (Cell Signalling Technology, USA) were added per 2 X 10^6 cells in each tube and incubated at 37ºC for 0 minutes. Post-incubation cells were washed, and Alexa Fluor 488 (Invitrogen, USA) was added to each tube, followed by 30 minutes of incubation in the dark at 37ºC. Finally, the mean fluorescence intensity (MFI) of immunostained DICER, DROSHA, AGO1, and AGO2 was determined by the flowcytometric method.

7.2.5. Study of autophagy mechanisms

Bone marrow cells from control, leukemic, and leukemic patients treated with Ashwagandha and Withanolide D were subjected to TRAF2 and P62 immunostaining to determine their levels. 2µl of anti-TRAF2 and anti-P62 (Cell Signalling Technology, USA) antibodies were added to each tube containing PFA-fixed and permeabilized 2 x 10^6 cells. After the addition of antibodies, cells were incubated at 37 ºC for 30 minutes. After that, cells were added with 2µl of Alexa Fluor 488 (Invitrogen, USA)-tagged secondary antibodies, followed by incubation at 37ºC. Finally, cells were washed and analysed with the BD FACS Calibur (Becton-Dickinson, USA).

7.2.6. Flow cytometric evaluation of proliferation

Flow cytometric studies were taken into account and thoroughly discussed in our prior research articles. Briefly, 1.5% PFA-fixed bone marrow cells were permeabilized with 90% chilled methanol, followed by a PBS wash and resuspension in FACS fluid. Each tube was added with the respective antibodies (anti-BMI-1, anti-LIN28A, and anti-N-MYC from Cell Signalling Technology, USA) and incubated at 37ºC for 30 minutes. Thereafter, each tube was added with fluorochrome-labelled secondary antibodies (Alexa Fluor 488, Invitrogen,

USA) at similar conditions. Finally, cells were analysed using BD FACS Calibur (Becton-Dickinson, USA) and Cell Quest Pro software.

7.2.7. Immunofluorescence

Expression patterns of TRAF2, P62, N-MYC, and LIN28A were accessed by immunofluorescence (IF) analysis from four different groups: control, leukemic, leukemic treated with Ashwagandha and Withanolide D-treated bone marrow cell samples with primary antibody (1:200 dilution) and secondary antibody (1:600 dilution). Nuclei were stained using DAPI shield (Sigma-Aldrich, USA). Slides were examined under a fluorescence microscope (Axio Scope A1, Zeiss, Germany).

7.2.8. Evaluation of DNA breakage by comet assay

A comet assay or single-cell gel electrophoresis was performed to analyse DNA damage assessment. Briefly, bone marrow cells from all three groups were suspended in low-melting agarose (1%) (SRL, India) at 37°C and then spread on slides thinly coated with 1% normal agarose (SRL, India). After that, cells were covered with cover glass and kept at 4°C for 1 h for the solidification of agarose. Following agarose solidification, coverslips were removed, and cells were dipped in lysis buffer at 4°C for 1 hour. Then the slides were placed in the electrophoresis chamber filled with electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH 13). Electrophoresis was performed for 40 minutes. Post-electrophoresis slides were placed in neutralisation buffer (0.4M Tris-HCI, pH 7.5), and after that, they were washed with water. All the steps were performed in the dark to prevent further DNA damage. Finally, cells were stained with DAPI (Sigma-Aldrich, USA) and photographed under a fluorescence microscope (Axio Scope.A1, Zeiss, Germany). All images were analysed with Comet Score (version 2.0.0.8, USA).

7.2.9. Statistical analysis

Data from all the groups are presented as mean ± standard deviation. Intergroup comparisons were evaluated using the Students t test, and one-way analysis of variance (ANOVA) was used for multiple comparisons. A probability of ≤ 0.05 was considered statistically significant.

7.3. RESULTS

7.3.1. The activation of the deregulated RNAi machinery in leukemic bone marrow cells mediated by Ashwagandha and Withanolide D.

Components of RNAi machinery have a crucial role in the regulation of mRNA processing. Our study showed a major overexpression of DICER in leukemic mice (MFI 356.09±1.33) compared to the control (MFI 171.55±2.96). Similarly, DROSHA (MFI 351.23±1.57) exhibited heightened expression in leukemia than its control counterpart (MFI 136.3+0.98). The progression of the disease was due to the augmented expression of DICER and DROSHA in leukemia. AGO1 and AGO2 were evaluated in all four groups. The expression of AGO1 (MFI 418.89+2.13) was lower than that of AGO2 (MFI 501.59±1.78) in the leukemic groups. However, AGO1 (MFI 200.39±3.11) was expressed at a higher level than AGO2 (MFI 125.81±2.01) in the control group. In the leukemic groups, both AGO1 and AGO2 were found to be overexpressed compared to the control (Figures 1a–c). The overexpression of the RNAi components played major role in the progression of the disease.

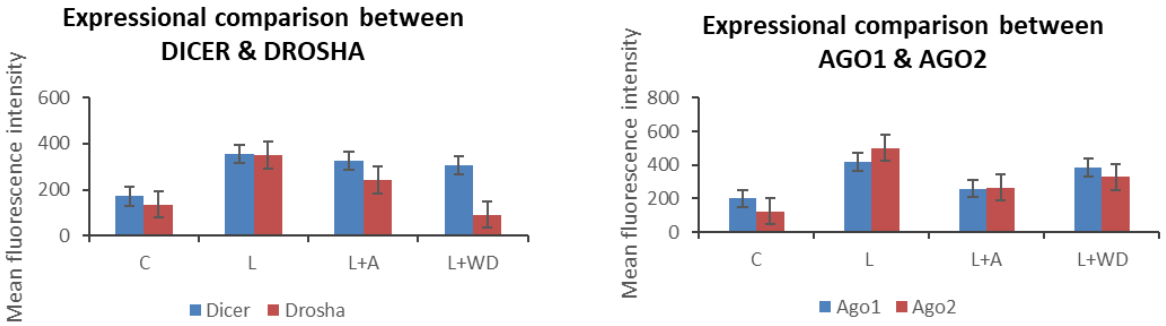
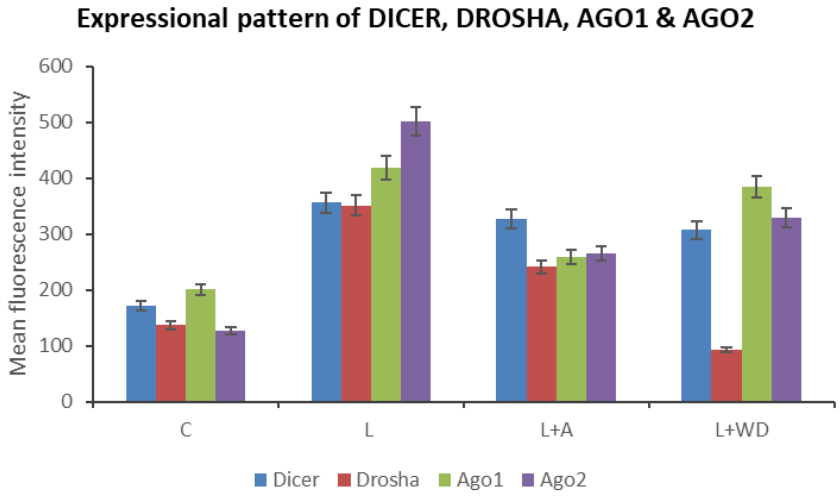


Figure 1. a. Histogram overlay of RNAi machinery components, DICER, DROSHA, AGO1 and AGO2. **b.** Bar graph depicting expressional comparison between DICER and DROSHA. **c**. Bar graph depicting expressional comparison between AGO1 and AGO2

The expressions of DICER (L+A MFI 326.57±3.42 , L+WD MFI 306.85±2.45), DROSHA (L+A MFI 241.16±3.11, L+WD MFI 92.32±2.78), AGO1 (L+A MFI 259.24±4.12, L+WD MFI 384.3±3.07), and AGO2 (L+A MFI 265.07±2.62, L+A MFI 328.54±1.98) were found to be significantly reduced than their leukemic counterpart after the administration of Ashwagandha and Withanolide D, along with a shift towards the control parameter (Figure

2a-d). The shift in the expressions of the RNAi machinery components helped in improving disease condition.

a



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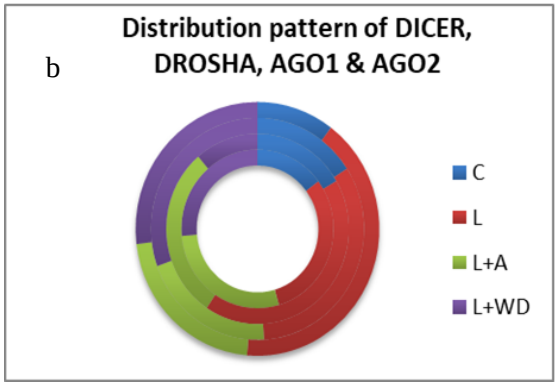
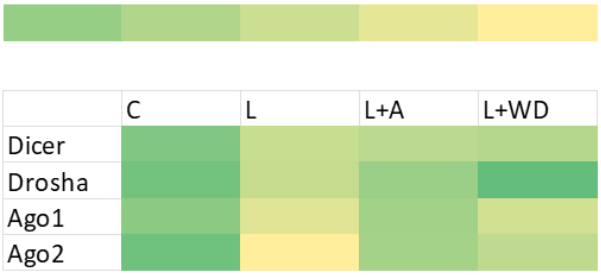




c

Figure 2. a..Bar graph depicting expression pattern of DICER, DROSHA, AGO1 and AGO2 in four groups (e.g. C, L, L+A & L+WD) **b**. Distribution pattern of DICER, DROSHA, AGO1 and AGO2 in all four groups (e.g., C, L, L+A & L+WD). **c**. Heat map of DICER, DROSHA, AGO1 and AGO2 expression pattern

7.3.2. Ashwagandha and Withanolide D induced modulation of autophagy pathway in leukemic cells

The modulation of RNA interference (RNAi) exerts an influence over the autophagy process, which is characterized by high precision and is strongly influenced by microRNA (miRNA) and RNAi pathways. Any disruption in the RNAi machinery has a profound impact on this exquisitely regulated process. Cells exhibit a reluctance to activate or forcibly suppress the autophagy machinery under leukemic conditions. Cells persistently generate or form aberrant proteins upon the cessation of this machinery. DNA repair mechanism is harmed due to dysregulated autophagy machinery, leading to the accumulation of DNA damage in cells

(Frankel & Lund, 2012; Jing et al., 2015; Lin et al., 2015). Moreover, the mRNAs responsible for conveying the signal for the principal components of the autophagy pathway undergo considerable processing by the same RNAi machinery. Our findings have unveiled that the expression of TRAF2 in leukemic cells was markedly higher (MFI 373.38± 2.23) in comparison to control cells (MFI 174.44 ± 4.11). This augmentation exerted a stimulatory effect on another important protein expression, that is P62, whose expression was significantly lower in leukemic cells (MFI 97.51 ± 1.65) as compared to control expression (MFI 269.23 ± 2.17).

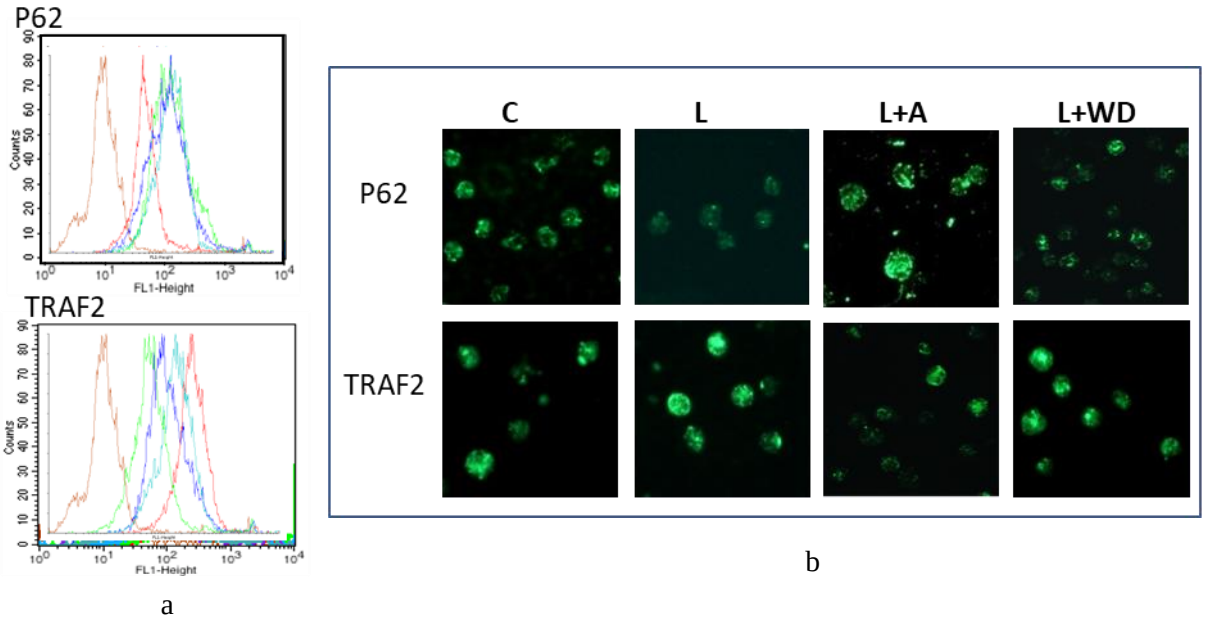


Figure 3. a. Histogram overlay of autophagy machinery components, TRAF2 and P62. **b**. Fluorescence microscopic image of TRAF2 and P62 in bone marrow cells of all three groups (e.g., C, L L+A & L+WD).

The regulation of RNA interference (RNAi) machinery and autophagy pathways was expertly coordinated by LIN28A, a molecule that governs a cluster of microRNAs. Significantly, the expression of LIN28A in leukemic cells was higher (MFI 89.15 ± 2.13) and in control cells it was significantly declined (MFI 47.17±3.23) (Figure 3a & b).

Administration of Ashwagandha and Withanolide D resulted in significant alterations in the expressions of TRAF2 (L+A MFI 235.29±3.22, L+WD MFI 296.33±5.04) and P62 (L+A MFI 228.81±1.33, L+WD MFI 169.39±3.01) when compared to their leukemic counterparts, causing a shift towards normal values. A similar observation was made in the expression of

LIN28A (L+A MFI 55.66 ± 3.44, L+WD MFI 73.6±2.51), indicating a shift towards control expressional values from leukemic values. Furthermore, fluorescence imaging of the aforementioned molecules, along with confocal and phase contrast imaging of ATG12, a key molecule in the autophagy pathway, confirmed the activation of the autophagy pathway in coordination with the modulation of the RNAi machinery (Figure 4 a & b).

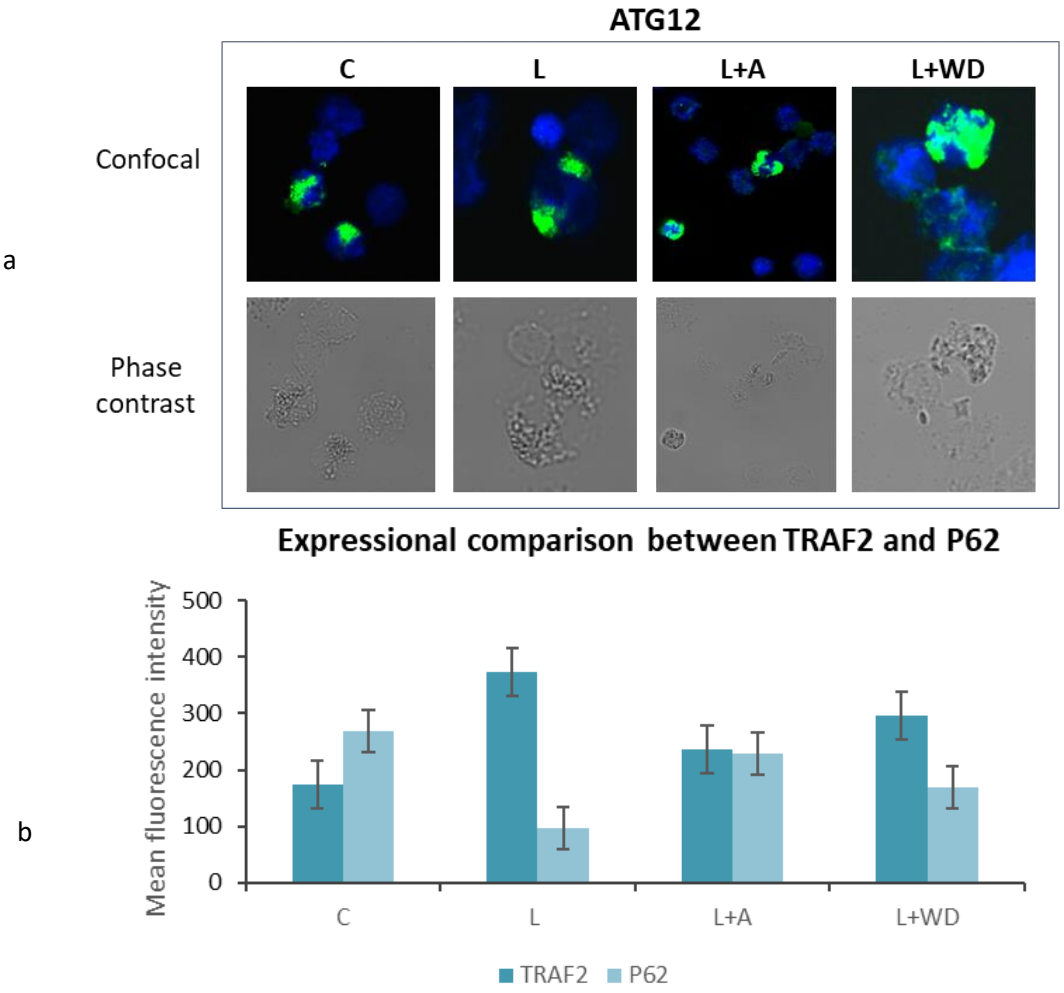
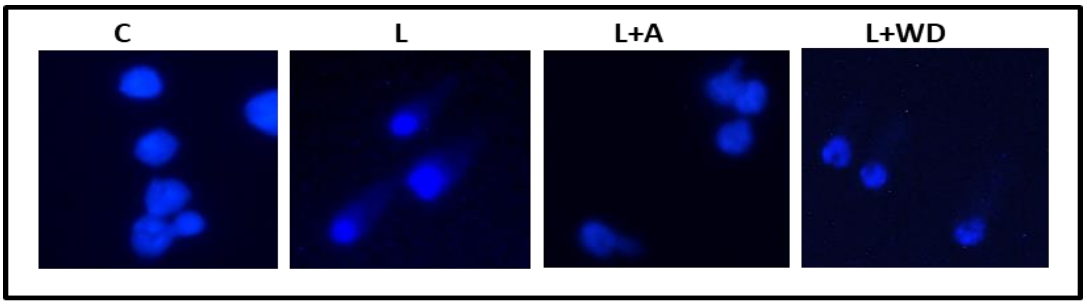


Figure 4. **a.** Upper row: Confocal microscopic image of ATG12 of control, leukemic and leukemic treated with Ashwagandha and Withanolide D bone marrow cells. Bottom row: Phase contrast microscopic image of control, leukemic and leukemic treated with Ashwagandha and Withanolide D bone marrow cells. **b.** Bar graph describing expressional pattern of TRAF2 and P62.

7.3.3. Ashwagandha and Withanolide D induced DNA breakage and halts progression of the disease.

Bone marrow cells were examined for the presence of DNA fragmentation. Briefly, in control cells insignificant amount of DNA damage was found, as evidenced by the absence of broken DNA tails. In cases of leukemia, the occurrence of genetic abnormalities and improper CpG methylation is common; as a result, the extent of DNA breakage is significantly elevated. Remarkably, substantial DNA damage repair in leukemic cells was noted upon treatment with Ashwagandha and Withanolide D. Our findings demonstrated that the amount of DNA in the form of broken tails was considerably higher in leukemic cells (37.89%±2.55) compared to their Ashwagandha (9.91%±5.22) and Withanolide D (21.79±3.2) -treated counterparts. Furthermore, the leukemic cells exhibited 3.82 times more DNA damage. The higher proportion of fragmented DNA in the leukemic cells suggests abnormal genetic stability and condition in the diseased leukemic cells. This decreased level of DNA breakage subsequent to treatment initiated cellular genetic repairing, causing a decline in abnormal cellular lineage and alleviation in disease progression. (Figure 5. a-h).



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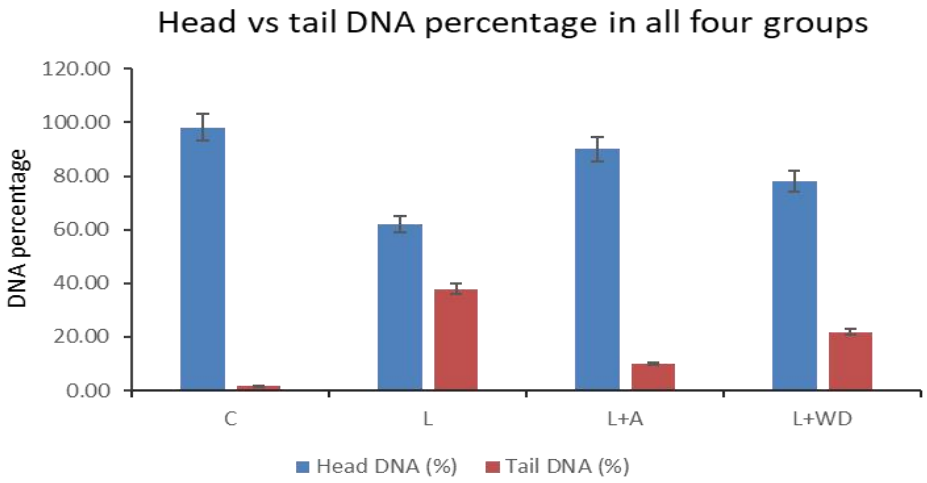
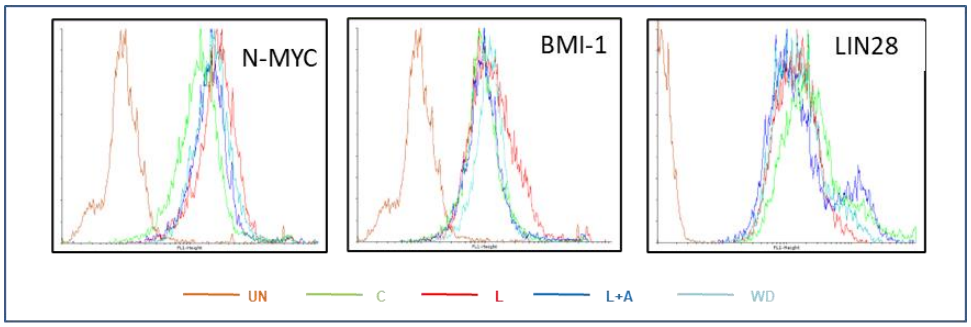


b

Figure 5. a. DAPI stained bone marrow cells of C, L L+A & L+WD groups depicting DNA damage in comet assay. **b**. Bar graph depicting head DNA percentage vs tail DNA percentage in all four groups (e.g., C, L, L+A & L+WD).

Further investigation of two crucial molecules, namely BMI-1 and N-MYC was conducted. BMI-1 serves as a pivotal constituent of polycomb group complex-1 (PRC-1). This intricate complex holds a significant role in the process of chromatin remodeling and, in turn, exerts epigenetic control over multiple genes expression that regulate the self-renewal of somatic stem cells. Moreover, it plays an important role in the mechanism of DNA repair and provides assistance in developing resistance against chemotherapeutic agents. Conversely, N-MYC is associated with hematological malignancies and various tumors.



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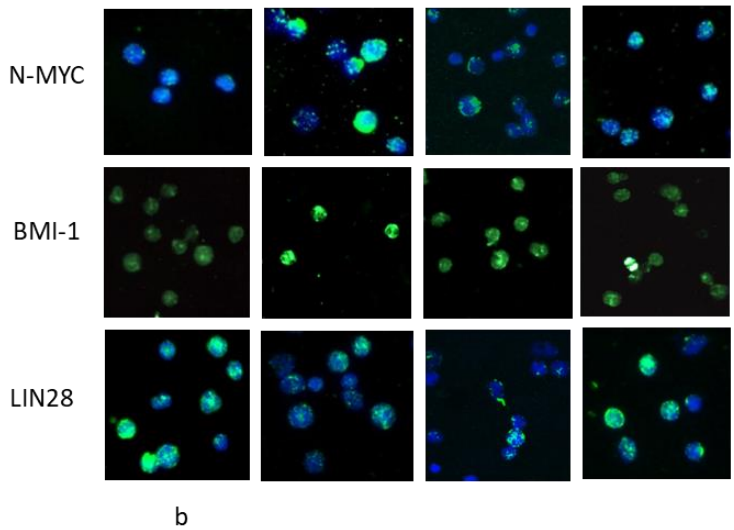

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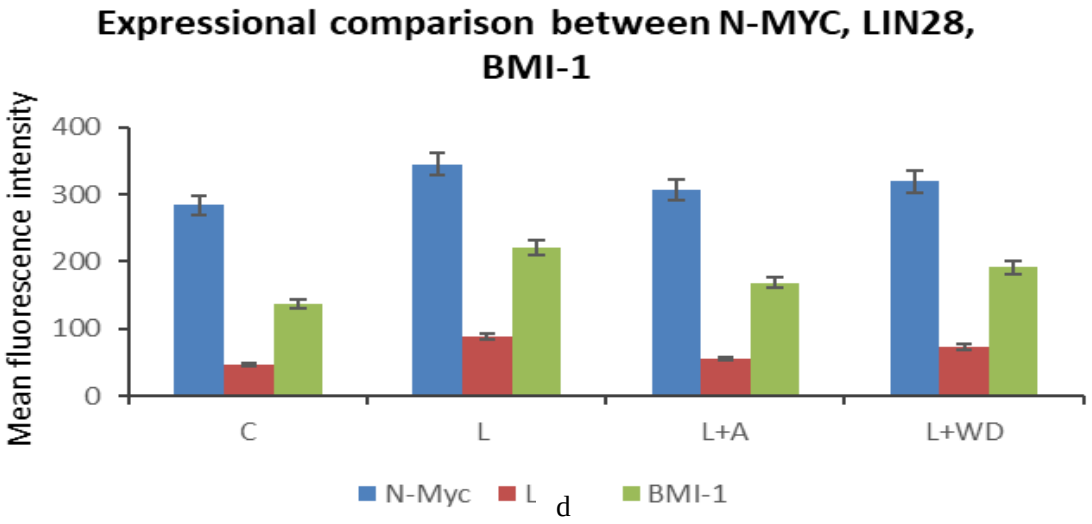

Figure 6. a. Histogram overlay of LIN28, N-MYC and BMI-1. **b**. Immunofluorescence images of LIN28, N-MYC and BMI-1 in four experimental groups (e.g., C, L, L+A and L+WD). **c.** Expressional comparison between LIN28, BMI-1 and N-MYC in all four experimental groups.

Our findings suggested that the overexpression of N-MYC (MFI 345.15± 3.21) in leukemic cells was more prominent compared to their control counterparts (MFI 284.38±2.43). Further, the expression of BMI-1 in leukemic MFI cells (221.31±2.33) indicates a greater amount of DNA damage when compared to control MFI cells (137.54±1.89). Significant reductions were observed in the expression of BMI-1 (L+A MFI 168.79±1.78, L+WD MFI, 191.55±3.71) and N-MYC (L+A MFI 307.85±3.71, L+WD MFI 319.4±2.67) following the administration of Ashwagandha and Withanolide D. This decrease in expression was evidently associated with an escalation in DNA damage, subsequently impeding the advancement of the disease (Figure 5. a &b, 6.a -c).

7.4. DISCUSSION

The examination of DICER, AGO, and DROSHA was conducted in multiple malignancies and found to be vital for disease initiation. In our investigation, enhanced quantities of DICER, DROSHA, AGO1, and AGO2 denoted active engagement and involvement of the RNA interference apparatus. As a result of the genomic insult caused by ENU, the constituents of the RNA interference apparatus commenced expressing at heightened levels. Elevated levels of RNA interference apparatus proteins assist in the processing and upregulation of oncogenes by repressing the oncogenic suppressor. A feedback loop was established among the overexpressed oncogenes and the genes implicated in the advancement of the ailment, which also facilitated the activation of the RNA interference apparatus. A bidirectional pressure on the miRNA processing mechanism and ensuing silencing of suppressor genes and other suppressor miRNAs significantly contributed to the progression of the ailment (Figure 7).

We have observed elevated levels of LIN28 in our experimental configuration. The repression of LIN28 is a common occurrence, resulting in the Let-7 miRNA cluster deterioration (Gozuacik et al., 2017; Liu et al., 2015). The Let-7 miRNAs interact with the 3' UTR region and regulate the synthesis of numerous oncogenes. One such oncogene is N-MYC, which we have identified to have increased expression in cases of leukemia. The heightened levels of LIN28 could have diminished the let-7 cluster of miRNAs, thereby removing the regulation of the oncogene N-MYC in leukemia. The removal of N-MYC repression activated another vital protein, BMI-1, which participates in cancer cell proliferation, responsiveness to chemotherapy, metastasis, invasion, and DNA damage repair. DNA damage is prevalent in leukemic cells. The increased levels of BMI-1 facilitated DNA damage repair in the leukemic state, thereby contributing to disease progression. (Fig. 7a)

The role of the autophagy pathway in the context of leukemia was of great significance. Within our model of leukemia, the expression of P62 was observed to be downregulated. This decline in P62 expression severely hinders the transportation of ubiquitinated cargo to the autophagosome. Concurrently, the reduced expression of ATG12 contributes to a decline in autophagosome formation. The process of autophagy is substantially suppressed due to the diminished traffic and production of autophagosomes. Downregulation causing impairment of the autophagy system negatively affects the mechanism of DNA repair, leading to erroneous DNA repair and the non-homologous end-joining of double-strand breaks. These

cells lacking autophagy, accumulate DNA damage and consequently contribute to the disease progression (Limanaqi et al., 2019).

Ashwagandha and Withanolide D possess the potential to combat cancer in various blood-related disorders, such as leukemia according to extensive investigation. The results obtained from our research can be organized systematically as: The interaction of Ashwagandha and Withanolide D to the genes responsible for regulating the components of the RNA interference (RNAi) machinery leads to a reduction in the expression levels of DICER, DROSHA, AGO1, and AGO2. This decrease in expression undeniably hampers the processing of microRNAs (miRNAs). Consequently, a limited quantity of processed miRNAs adheres to a smaller pool of messenger RNAs (mRNAs) in their 5' untranslated region (5'UTR), thereby hindering the processing of oncogenic suppressor proteins. As a result, the gradual increase in suppressor proteins and miRNAs exerts feedback pressure on the RNAi machinery, eventually leading to its diminished activation. This reduced activation level subsequently results in a decreased amount of miRNA processing. Further, the silencing of suppressor miRNAs and mRNAs contributes to shifting the disease scenario towards normalcy. (See Figure 8 for a visual representation of this process).

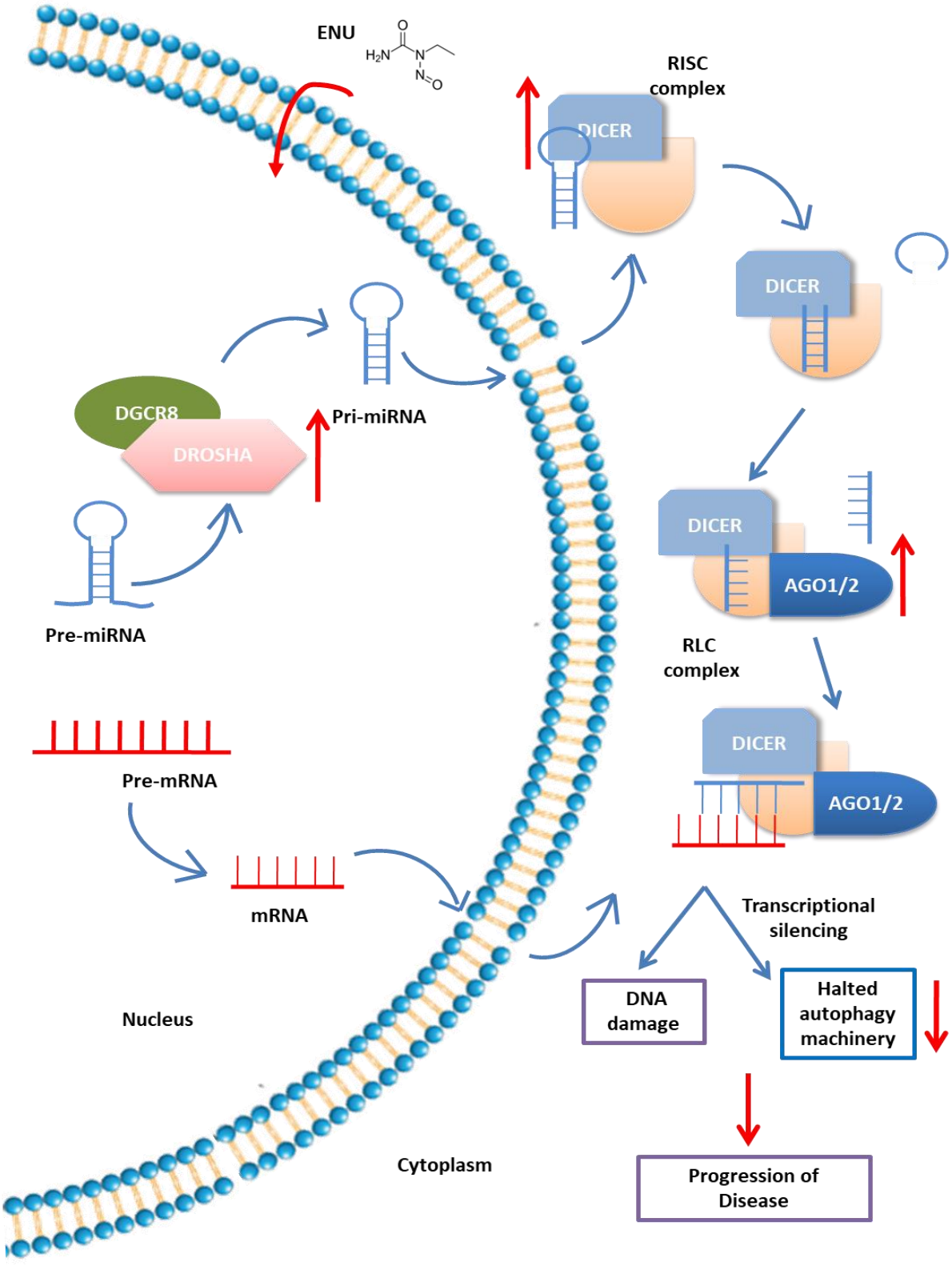

Figure 7. Graphical representation of modulation of RNAi components and their effects in NOC induced leukemic bone marrow cells.

Ashwagandha and Withanolide D , both exerted their impact on the leukemic cells through a dual process. Cells that exceeded their reparative capacity were compelled towards their demise, whereas cells with potential for repair were prompted to commence the restoration process. Ashwagandha and Withanolide D effectively intervened in the excessive expression of LIN28, thereby alleviating the repression exerted on the Let-7 family of miRNAs. Consequently, the Let-7 miRNA family orchestrated a two-fold mechanism: first, the repression of the oncogene N-MYC, and second, the feedback repression of LIN28. This bidirectional pressure played a significant role in mitigating the progression of the disease.

In our study, the utilization of Ashwagandha and Withanolide D facilitated the reinstatement of the autophagy apparatus. The elevated P62 levels in the treated cells indicated the movement of ubiquitin-tagged cargo traffic towards the autophagosome. Moreover, the formation of autophagosomes, which was previously been hindered due to the downregulation of ATG12 in leukemic cells, commenced as a result of the increased concentrations of ATG12, subsequent to the administration of Ashwagandha and Withanolide D. The activation of the autophagy machinery contributed to the elimination and eradication of misfolded proteins and also resulted in an augmentation of DNA damage repair. (Figure 8).

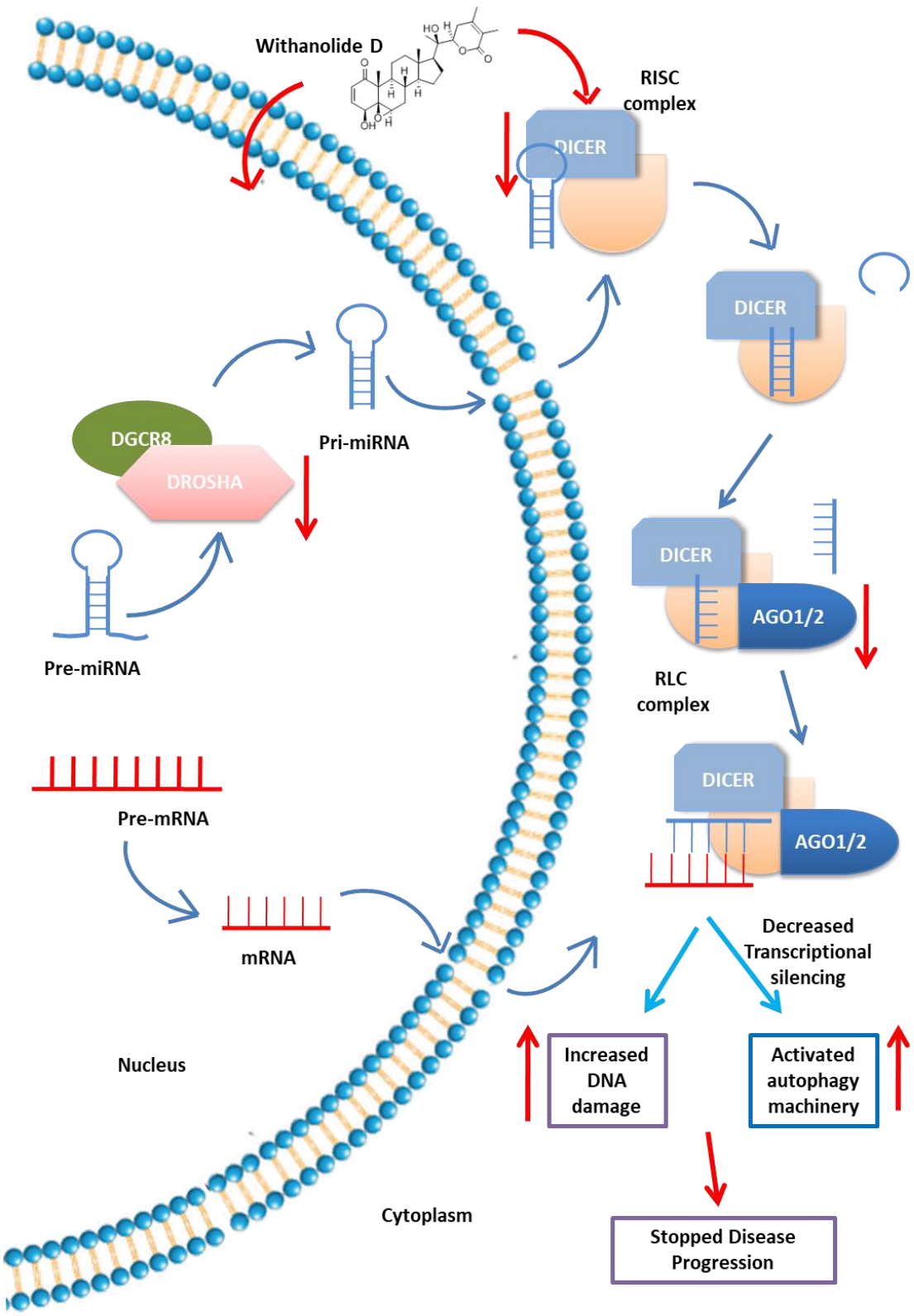

Figure 8. Graphical Pictorial representation of modulation of RNAi components and their effects in Ashwagandha and Withanolide D treated NOC induced leukemic bone marrow cells.

Collectively, it can be conceived that the modulatory effects of Ashwagandha and Withanolide D on RNA interference present considerable potential in the realm of therapeutic interventions pertaining to leukemia, as well as various other hematological malignancies, Ashwagandha and Withanolide D exerted its impact on the leukemic cells through a dual process. Cells that exceeded their reparative capacity were compelled to death, whereas cells having the potential to repair were prompted to commence the restoration process. Ashwagandha and Withanolide D, both effectively intervened in the excessive expression of LIN28, thereby mitigating the repression exerted on the Let-7 family of miRNAs. Consequently, the Let-7 miRNA family orchestrated a two-fold mechanism: first, the repression of the oncogene N-MYC, and second, the feedback repression of LIN28. This bidirectional pressure played a pivotal role in alleviating the progression of the disease (Figure 9).

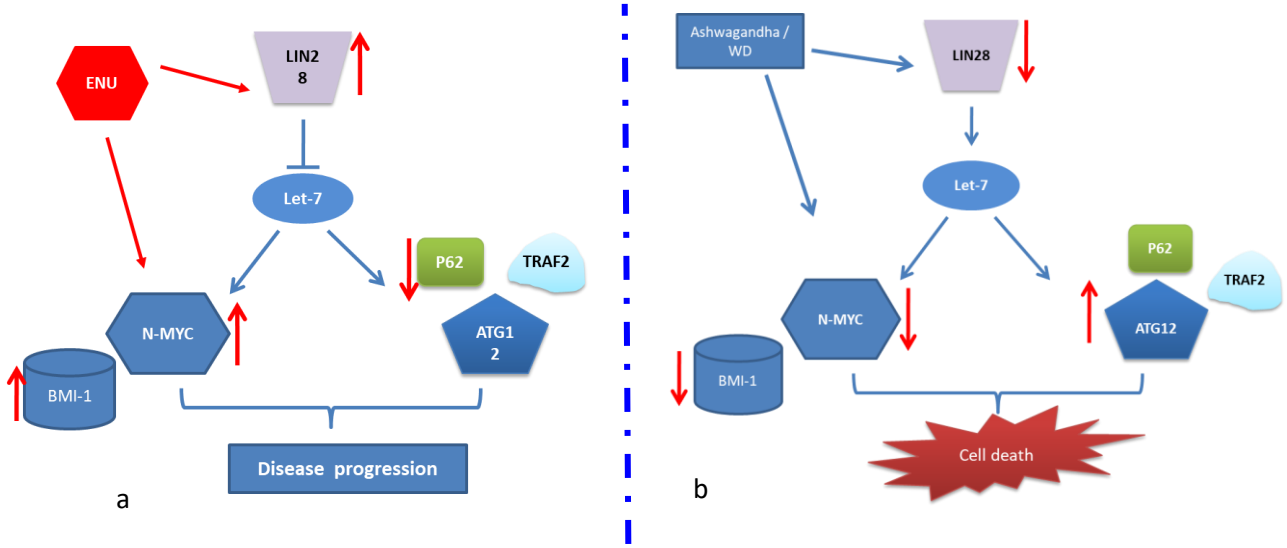

Figure 9. Diagrammatic representation of modulation of autophagy machinery and DNA damage scenario in NOC induced leukemic and in Ashwagandha and Withanolide D treated bone marrow cells.

a. In NOC induced leukemic condition elevated LIN28 expression suppressed Let-7 group of miRNAs. Suppression of Let-7 miRNAs increased N-MYC expression and simultaneously suppressed autophagy pathway components e.g. TRAF2, P62 and ATG12. **b.** In Ashwagandha and Withanolide D treated bone marrow cells LIN28 expression was decreased. Decreased LIN28 expression removed suppression on Let-7 group of miRNAs. Removal of suppression activated autophagy machinery, increased DNA damage and decreased N-MYC expression and disease progression is halted.

Chapter 08

Conclusion

Conclusion

Our study revealed significant anti-leukemic therapeutic potential of Ashwagandha as a whole as well as the extracted Withanolide D using ENU induced mouse model. In the context of the deregulated hedgehog singling pathway, both forms of the phytochemicals were found to be associated with the hindrance of the activation of the PTCH, resulting in the disruption of the communication between PTCH and SMO. This might have resulted in the decreased cytoplasmic internalisation of the SMO. As a result, the communication between SMO and SuFu got reduced which can be correlated with the decreased nuclear translocation of GLI-1. Thus, a significant association between the therapeutic administration of Ashwagandha and Withanolide D and disruption of pathological activation of hedgehog signalling pathway has been established in the murine model of leukaemia.

At the same time, in the presence of Ashwagandha and Withanolide D, dissociation of NRF2 from KEAP1 was noted. Simultaneous degradation of ubiquitinated NRF2 obstructed it from translocating into the nucleus hindering the activation of key downstream factors. The down-regulation of NRF2/KEAP1 axis also led to decline in the SHH expression together with the disruption of the Hedgehog signalling cascade. Thus, Ashwagandha and Withanolide D is found to affect disease related activation of both canonical and non-cannonical hedgehog signalling pathways.

In case of apoptotic mechanism, Ashwagandha and Withanolide D helped to increase the expression of PUMA, CD11b, and Caspase 3, pro-apoptotic proteins, while simultaneously decreasing the expression of BCL2, TERT, and anti-apoptotic proteins. As a result of this expressional shift, the repression of the apoptotic machinery was removed, and abnormal cells that initially escaped cellular death and helped in the progression of the disease were now systematically eliminated.

Elevated P62 in the phytochemical treated cells indicated the movement of ubiquitin-tagged cargo traffic towards the autophagosome. Autophagosome formation, which was very much obstructed due to ATG12 down-regulation in leukemic cells, got re-started due to the ameliorating effect of Ashwagandha as well as Withanolide D as depicted by the elevated levels of ATG12. Activation of autophagy machinery not only helped in the clearance and removal of misfolded proteins but also increased DNA damage repair.

Ashwagandha and Withanolide D restricted the proliferation of malignancy associated abnormal clones by modulating microtubular disruption along with mitotic arrest of cells, which finally led to the apoptosis. Simultaneously, the expressional decline of the key proliferative proteins, viz; CYLD and N-MYC also contributed to restricting the abnormal growth of leukaemic cells. Decline in the expression patterns of N-CAD, CD31, Vim, β-actin, and β–tubulin in the Ashwagandha and Withanolide D treated groups, signifies the phytochemical associated disruption of cell-cell as well as cell-matrix adhesion properties in leukemic cells together with filament disassembly and decline in cellular migration and invasion.

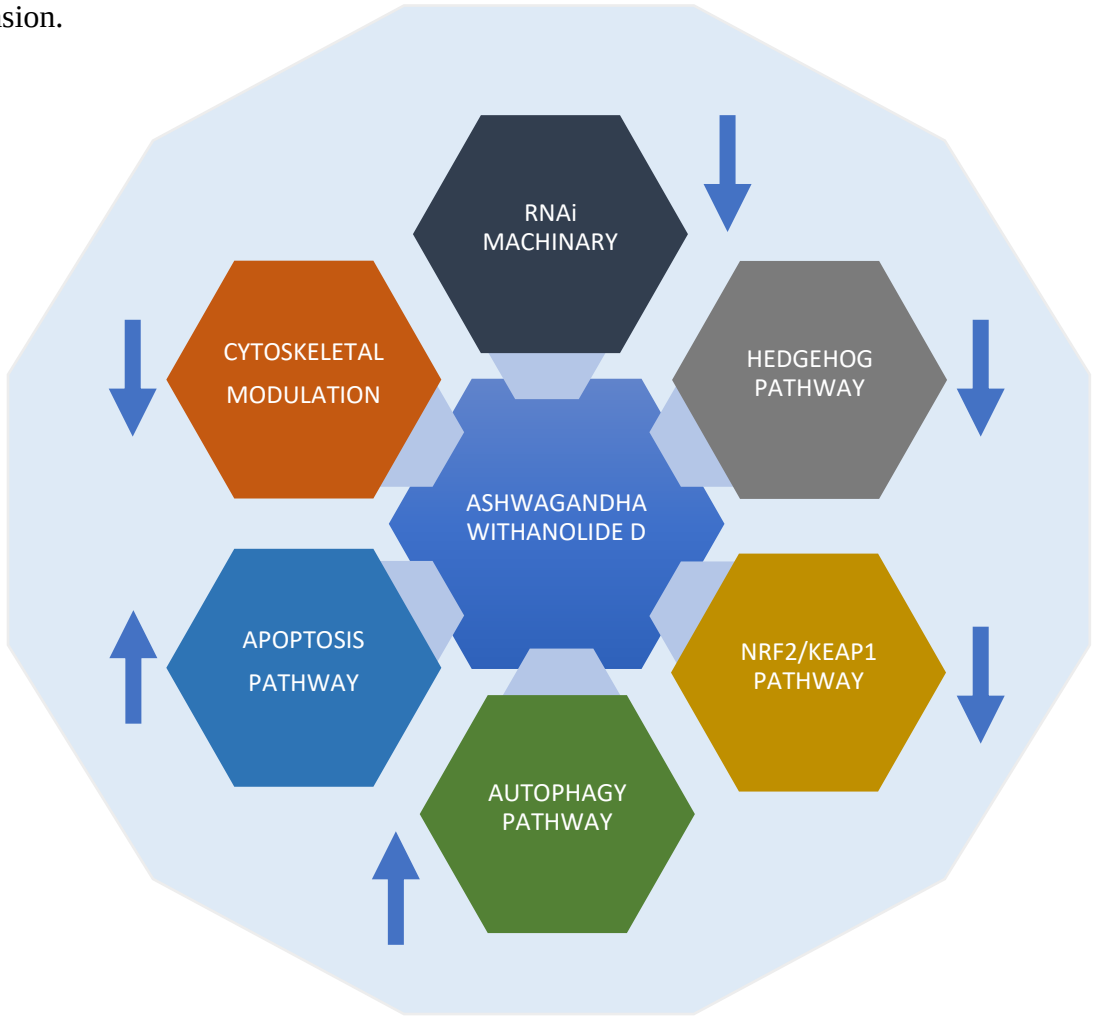

Figure 1: Graphical representation of cumulative effects of Ashwagandha and Withanolide D on the downregulation of aberrantly active Hedgehog pathway, NRF2/KEAP1 pathway, RNAi machinery, cytoskeletal modulatory proteins and upregulation of suppressed autophagy and apoptosis pathways.

Administration of Ashwagandha and Withanolide D in the leukemic mice was also found to be associated with the disruption of RNAi machinery, as depicted by the decreased expression patterns of DICER, DROSHA, AGO1, and AGO2, which hinted towards the deterioration in miRNA processing mechanism. Decline of the miRNA processing with the effect of phytochemicals, can be a possible mechanism for the decrease of the abnormal expression of tumorogenic proteins.

Collectively, it can be concluded that Ashwagandha and its active compound, Withanolide D, mediates the restoration of the deregulated Hedgehog signalling pathway, NRF2/KEAP1axis, activation of apoptotic machinery, decline of abnormal cellular growth, reduced cellular adhesion, reduction in metastasis and invasiveness, RNA interference modulation, restoration of hindered autophagy machinery, and enhanced DNA damage repair activity in leukemic bone marrow cells. It is anticipated that the present study may emerge as a promising approach in the therapeutic domain of environmental toxicant mediated leukaemia.

Chapter 09

Statistics

Statistics

Locomotor activity

Locomotor activity	**Control (X±SD)**	**Leukemic (X± SD)**	**L+A (X±SD)**	**Leukemic+WD (X±SD)**	***P* value**
	68.5± 2.74	41.3± 3.62	56.2± 2.9	50.1± 1.4	**≤0.001**

Bodyweight

Bodyweight	**Control (X±SD)**	**Leukemic (X± SD)**	**L+A (X±SD)**	**Leukemic+WD (X±SD)**	***P* value**
	35.5±3	22±2.20	31.2±1.9	29.5± 1.2	**≤0.001**

Table XI. Hematological parameters in the four experimental groups (C=control ,L=leukaemic, L+A=leukaemic treated with Ashwagandha, Leukemic+WD =Leukemic treated with Withanolide D)

Parameters	**Control (X±SD)**	**Leukemic (X± SD)**	**L+A (X±SD)**	**Leukemic+WD (X±SD)**	***P* value**
Hemoglobin (g/ dL)	12.87 ± 0.26	16.6± 1.97	14.0 ± 0.47	14.1± 0.32	**≤0.001**
(WBC) (X 10^3/µl)	6.3 ± 1.28	35.3 ± 2.57	19.4 ± 0.68	26.2± 0.72	**≤0.001**
(RBC) (X 10^6/ µl)	8.43 ± 0.32	12.31 ± 0.23	9.19± 0.86	9.0± 0. 95	**≤0.001**
Reticulocyte (%)	0.81 ± 0.15	1.53 ± 0.09	0.94 ± 0.07	1.12± 0.08	**≤0.001**
Neutrophil (%)	22.55 ± 2.21	17.32 ± 2.21	19.75 ± 2.75	17.93± 2. 54	**≤0.001**
Blast (%)	-	31.25 ± 6.65	21.33± 4.38	27.21±1. 41	**≤0.001**
Lymphocyte (%)	71.8 ± 2.16	53 ± 2.54	64.25 ± 7.63	58.44±5. 74	**≤0.001**

Assessment of cellular viability

Parameters	Control (X±SD)	Leukemic (X± SD)	L+A (X±SD)	Leukemic+WD (X±SD)	*P* value
Live cells	91± 0.85	97.78±1.12	92.87±1.12	94.15±1.33	**≤0.0001**
Dead cells	9±1.04	2.22±0.95	7.13± 0.71	5.85±0.63	**≤0.0001**

Expression of hedgehog signaling pathway components

Parameter	Control (X±SD)	Leukemic (X± SD)	L+A (X±SD)	Leukemic+WD (X±SD)	*P* value
SHH	36.07±3.2	62.55±2.11	47.62±2.01	56.34±2.11	**≤0.001**
HHIP	72.03± 1.92	23.77±2.01	54.83±2.14	43.27±1.87	**≤0.001**
PTCH	42.51±2.41	87.18±2.33	61.32±1.93	74.59±4.33	**≤0.001**
SMO	78.69±3.1	109.57±2.43	88.28±1.98	96.76 ±2.61	**≤0.001**
SUFU	39.56 ±2.6	54.75±3.01	67.87±2.09	53.77±2.10	**≤0.001**
GLI 3	80.44±1.93	55.22± 2.01	71.16±2.63	61.91±2.62	**≤0.001**
GLI1	63.96±2.21	103.62±2.13	81.11±2.44	94.21±1.87	**≤0.001**

Expressional pattern of NRF 2 keap 1 axis components

Parameter	Control (X±SD)	Leukemic (X± SD)	L+A (X±SD)	Leukemic+WD (X±SD)	*P* value
NRF2	64.97±1.75	263.79±2.33	154.31 ± 2.71	180.02±2.47	**≤0.001**
KEAP 1	113.52±3.21	157.52±1.56	124.77±1.67	141.3±3.45	**≤0.001**
GSK3β	48.47±0.98	118.74±2.43	75.3±1.88	98.6±1.61	**≤0.001**
β-TrCP	58.33±0.99	109.7±1.67	71.98±2.07	89.63±3.01	**≤0.001**

Apoptosis pathway components

Parameter	Control (X±SD)	Leukemic (X± SD)	L+A (X±SD)	Leukaemic+WD (X±SD)	*P* value
BCL2	184.95±2.42	323.66±1.36	226.38 ±2.55	265.33±1.98	**≤0.001**
PUMA	297.16±2.01	138.09±1.32	248.88±4.11	229.66±2.11	**≤0.001**
CASP3	123.23±3.11	90.74±3.93	111.23±2.49	102.27±1.97	**≤0.001**

NAI and LAI values

Parameter	Control (X±SD)	Leukemic (X± SD)	L+A (X±SD)	Leukaemic+WD (X±SD)	*P* value
NAI	70.81±0.87	95.7±0.38	81.2±1.32	87.7±1.95	**≤0.0001**
LAI	29.19±1.34	4.93±0.56	18.8±1.44	12.3±1.76	**≤0.0001**

Expressional pattern of NCAD and CD31

Parameter	Control (X±SD)	Leukemic (X± SD)	L+A (X±SD)	Leukaemic+WD (X±SD)	*P* value
CD31	32.91±2.35	90.43±3.11	58.3±1.34	76.39±3.52	**≤0.0001**
N-CAD	48.39±1.78	82.58±2.10	61.45±3.18	72.13±2.54	**≤0.0001**

Expressional pattern of proliferative markers

Parameter	Control (X±SD)	Leukemic (X± SD)	L+A (X±SD)	Leukaemic+WD (X±SD)	*P* value
CYCLIN D1	139.39±3.12	286.93±5.23	188.35±4.11	213.54±2.54	**≤0.0001**
CYLD	429.81±4.21	91.52±1.06	370.71 ±2.71	401.55±5.14	**≤0.0001**

MTT

OD values	Control (X±SD)	Leukemic (X± SD)	L+A (X±SD)	Leukaemic+WD (X±SD)	*P* value
	0.725±0.33	1.09±0.56	0.875±1.33	0.931±0.31	**≤0.05**

Migration of cells

Average cell numbers	Control (X±SD)	Leukemic (X± SD)	L+A (X±SD)	Leukaemic+WD (X±SD)	*P* value
	95.6±4.97	412.4± 3.05	189.1±4.13	270.8±5.72	**≤0.0001**

Expressional comparison between cytoskeletal proteins

Parameter	Control (X±SD)	Leukemic (X± SD)	L+A (X±SD)	Leukaemic+WD (X±SD)	*P* value
NAK	182.59±2.71	119.73±3.22	141.98±4.31	166.98±1.93	**≤0.0001**
β TUBULIN	167.75±2.45	276.52±2.51	181.99±3.11	216.67±3.41	**≤0.0001**
VIM	83.71±2.38	328.54±1.98	140.79±1.97	251.05±3.31	**≤0.0001**
β ACTIN	113.18±2.12	185.3±1.55	137.55± 3.4	153.11±1.83	**≤0.0001**

Expressional pattern of RNAi machinery

Parameter	Control (X±SD)	Leukemic (X± SD)	L+A (X±SD)	Leukaemic+WD (X±SD)	*P* value
DICER	171.55±2.96	356.09±1.33	326.57±3.42	306.85±2.45	**≤0.0001**
DROSHA	136.3+0.98	351.23±1.57	241.16±3.11	92.32±2.78	**≤0.0001**
AGO1	200.39±3.11	418.89+2.13	259.24±4.12	384.3±3.07	**≤0.0001**
AGO2	125.81±2.01	501.59±1.78	265.07±2.62	328.54±1.98	**≤0.0001**

Expressional pattern of N-MYC and BMI-1

Parameter	Control (X±SD)	Leukemic (X± SD)	L+A (X±SD)	Leukaemic+WD (X±SD)	*P* value
N MYC	284.38±2.43	345.15± 3.21	307.85±3.71	319.4±2.67	**≤0.0001**
BMI-1	137.54±1.89	221.31±2.33	168.79±1.78	191.55±3.71	**≤0.0001**
LIN28A	47.17±3.23	89.15 ± 2.13	55.66 ± 3.44	73.6±2.51	**≤0.0001**

Expressional pattern of Autophagy machinery

Parameter	Control (X±SD)	Leukemic (X± SD)	L+A (X±SD)	Leukaemic+WD (X±SD)	*P* value
P62	269.23 ± 2.17	97.51 ± 1.65	228.81±1.33	169.39±3.01	**≤0.0001**
TRAF2	174.44 ± 4.11	373.38± 2.23	235.29±3.22	296.33±5.04	**≤0.0001**

DNA damage estimation

Parameter	Control (X±SD)	Leukemic (X± SD)	L+A (X±SD)	Leukaemic+WD (X±SD)	*P* value
	1.79±3.52	37.89%±2.55	9.91%±5.22	21.79±3.2	**≤0.0001**

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