Pathophysiological alterations in experimentally induced leukemic bone marrow and therapeutic immune-modulation by *Eclipta alba* involving NF-kB signaling

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By

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University of Jadavpur

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled "Pathophysiological alterations in experimentally induced leukemic bone marrow and therapeutic immune-modulation by *Eclipta alba* involving NF-kB signaling", submitted by Smt. **Subhashree Bhattacharyya** who got her name registered on 17.09.2019 for the award of Ph. D. (Science) degree of Jadavpur University, is absolutely based upon her own work under the supervision of Dr. Sujata Law and that neither this thesis nor any part of it has been submitted for either any degree / diploma or any other academic award anywhere before.

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Dedications

I dedicate this thesis to my parents, Champa Bhattacharyya & Subhasish Bhattacharyya and to my husband Prithijit Majumdar, for the constant support, motivation and encouragement.

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In the entire tenure of my research work, there are many individuals and organizations I have received immense help and support from. I would like to take this opportunity to devote a few words of thanks to them.

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Subhashree Bhattacharyya

Abstract

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The increase in civilization and mankind is a reason for the growing industrialization and hence portrays to be a leading source of many carcinogenic chemicals to the environment. N-nitroso compounds (NOCs) are such carcinogens that are widespread in our environment through various food products, tobacco, pesticides, and rubber making factories, etc. In our study, we have used one such NOC called N-N' ethylnitrosourea (ENU) to induce leukemia in the Swiss albino mice model. Leukemia is a heterogeneous malignancy of the blood, and it is highly prevalent in young adults and teens. Although, there has been enormous advancement in leukemic chemotherapy to date, there are still many toxic side effects. Hence, the exploration for natural anti-cancer products from Mother Nature is ongoing in the scientific community, as it is readily available, less toxic and, economical.

In this research, we have explored the anti-leukemic effects of the age-old Ayurvedic herb *Eclipta alba* and its isolated active compound wedelolactone on the bone marrow cells of ENU-induced leukemic mice. The underlying anti-inflammatory mechanism of the herbal extract and wedelolactone via the down-regulation of NF-kB signaling pathway, inhibition of COX-2 activation, and suppression of the NLRP3 inflammasome in the bone marrow cells of leukemia has been reported in this research. Furthermore, *Eclipta alba* and wedelolactone showed pro-apoptotic effects via the mitochondria-mediated cytochrome c pathway and hence portrayed its anti-proliferative role on the bone marrow of ENU-induced leukemia.

Taken together, it can be concluded that even though both *Eclipta alba* and wedelolactone individually can be considered as potential candidates, for anti-leukemic research but in comparison the herbal extract containing a concoction of many bioactive compounds, portrays better ameliorating properties as compared to the active compound treatment alone.

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Abbreviation

αMEM: *Alpha-Minimum Essential Medium*

ALL: *Acute lymphoblastic leukemia*

AML: *Acute myeloblastic leukemia*

APC: *Antigen presenting cells*

ASC: *Apoptosis-associated speck like protein*

ASK1: *Apoptosis signal-regulating kinase 1*

AgNOR: *Agryophilic nucleolar organiser region*

Bcl2: *B-cell leukemia/lymphoma 2 protein*

Bax: *Bcl-2-associated X protein*

BcR: *B-cell receptor*

BSA: *Bovine serum albumin*

BMNC: *Bone marrow mono-nuclear cells*

CEPBA: *CCAAT-enhancing binding protein alpha*

CLL: *Chronic lymphoblastic leukemia*

CML: *Chronic myelogenous leukemia*

CXCR4: *C-X-C motif chemokine receptor-4*

CTL: *Cytotoxic T-lymphocytes*

CCL4: *CC Chemokine ligand 4*

CCL3: *CC Chemokine ligand 3*

COX-2: *Cyclooxygenase 2*

CARD: *Caspase Recruitment Domain*

CAF: *Cancer associated fibroblasts*

DPX: *Dibutylphthalate polystyrene xylene*

DSS: *Dextran sulphate sodium*

ENU: *N'-N'ethyl nitrosurea*

EBV: *Epstein Barr virus*

ECAD: *Epithelial cadherin*

EP: *Eclipta prostrata*

EA: *Eclipta alba*

FBS: *Fetal bovine serum*

FACS: *Fluorescence activated cell sorting*

GATA2: *GATA binding protein 2*

GSK-3: *Glycogen synthase kinase-3*

GM-CSF: *Granulocyte macrophage colony stimulating factor*

hTERT: *Human telomerase reverse transcriptase*

HTLV-1*: Human T-cell leukemia virus 1*

HSPC: *Human stem and progenitor cells*

HepG2: *Hepatoblastoma cell line*

H&E: *Hematoxylin & Eosin*

IL-15: *Inter-leukin 15*

IP: *Intra-peritoneal*

IL-1α: *Inter-leukin 1-alpha*

IL-1β: *Inter-leukein 1-beta*

IFN-γ: *Interferon gamma*

IL-1R: *Inter-leukin 1 receptor*

IKKα: *Inhibitory-kB kinase alpha*

IKKβ: *Inhibitory-kB kinase beta*

IKKγ: *Inhibitory-kB kinase gamma*

$\boldsymbol{I_kB_{\alpha}}$*: Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha*

iNOS: *Induclible Nitric oxide synthase*

JGB: *Janus green B*

JNK: *Jun-N-terminal kinase*

LRR: *Leucine-rich repat*

MTT: *(3*-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide)

MMP: *Matric metalloproteinase*

MPAL: *Mixed-phenotype Acute Leukemia*

MHC: *Major histocompatibility complex*

Mir155: *MicroRNA 155*

MPO: *Myeloperoxidase*

MFI: *Mean fluorescence Intensity*

MSC: *Mesenchymal stem cells*

NBT: Nitroblue tetrazolium

NMU: N-**Nitroso**-N-methylurea

NBU: 1-Butyl-1-nitrosourea

NDMA: N-Nitrosodimethylamine

NDEA*:* N-Nitrosodiethylamine

NAD$^+$/H: *N*icotinamide adenine dinucleotide (NAD) + hydrogen

NOC: *N-nitroso compound*

NF-kB: nuclear factor kappa light chain enhancer of activated B cells

NLRP3: nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3

NEMO: *NF-kappa-B essential modulator*

NLS*: Nuclear localization signals*

NLR: *NOD-like" receptor*

NOD: *Nucleotide oligomerization domain*

NSE*: Non specific esterase*

NASID*: Non-steroidal anti-inflammatory drugs*

NO: *Nitric oxide*

PBS*:* Phosphate-buffered saline

PI3K/AKT: *phosphoinositide 3-kinase*

PBMC: *Peripheral blood mononuclear cells*

PYD: *pyrin* domain

PGE2: *Prostaglandin E2*

PGA2: *Prostaglandin A2*

PGG2: *Prostaglandin G2*

PGH2: *Prostaglandin H2*

PGJ2: *Prostaglandin H2*

PFA: *Paraformaldehyde*

ROS: *Reactive oxygen species*

RPMI: *Roswell Park Memorial Institute*

RBC: *Red blood cells*

SBB: *Sudan black B*

TKI: *Tyrosine kinase inhibitor*

TNF-α: *Tumor necrosis factor alpha*

***TCL*-1:** *T-Cell Leukemia/Lymphoma 1*

TLR: *Toll like receptor*

TNFR: *Tumor necrosis factor receptors*

VEGF: *Vascular endothelial growth factor*

VEGFR: *Vascular endothelial growth factor receptor*

WBC: *White blood cells*

WDL: *Wedelolactone*

XIAP: *X-linked inhibitor of apoptosis protein*

Aims and objectives

The aim of the present thesis is to unearth the therapeutic effects of *Eclipta alba* and wedelolactone on ENU-induced Swiss albino mice model. The objectives of the study are as followed:

- To develop and maintain ENU-induced leukemic animal model.
- To prepare the alcoholic herbal extract of *Eclipta alba* and to carry out its chemical characterization.
- To isolate the active compound from the herbal extract.
- To understand the preliminary changes on survivability, body weight and peripheral blood post treatment with *Eclipta alba*.
- To study the proliferative capacity of the bone marrow cells pre-and post-treatment.
- To isolate bone marrow cells from experimental groups and elucidate the therapeutic effect of whole extract and active compound on ENU- induced bone marrow cells via immune-modulation of NF-kB signaling pathway.
- To delineate the potentials of *Eclipta alba* and wedelolactone treatment on NLRP3 inflammasome pathway and explore its anti-proliferative role in marrow cells of leukemia.
- To understand the underlying anti-inflammatory (via COX-2 pathway); anti-apoptotic (via mitochondria mediated apoptosis) and anti-angiogenic mechanism of the herb and its isolated compound on ENU-induced bone marrow cells.

Chapter 1

Chapter 1.

1. Introduction

1.1. Background

Leukemia is a heterogeneous haematological malignancy leading to aberrant proliferation of abnormal white blood cells in the bone marrow, while replacing the healthy blood cells (Figure 1). Leukemia was first recognized in the year 1845, when a research was published about a patient who died due to an excessively high count of blood cells. Leukemia can be classified as acute or chronic, depending on the rapidity of proliferation of leukemic cells. Acute leukemia develops faster and results in an increased population of abnormal and undifferentiated hematopoietic cells, whereas the number of erythrocytes and platelets in peripheral blood are reduced (Tebbi, 2021). Comparatively, chronic leukemia develops slowly and involves the uncontrolled proliferation of mature and differentiated hematopoietic cells. Leukemia is classified as lymphoid, myeloid, mixed or undifferentiated based on the origin of the abnormal hematopoietic cell (Kasteng et al., 2007).

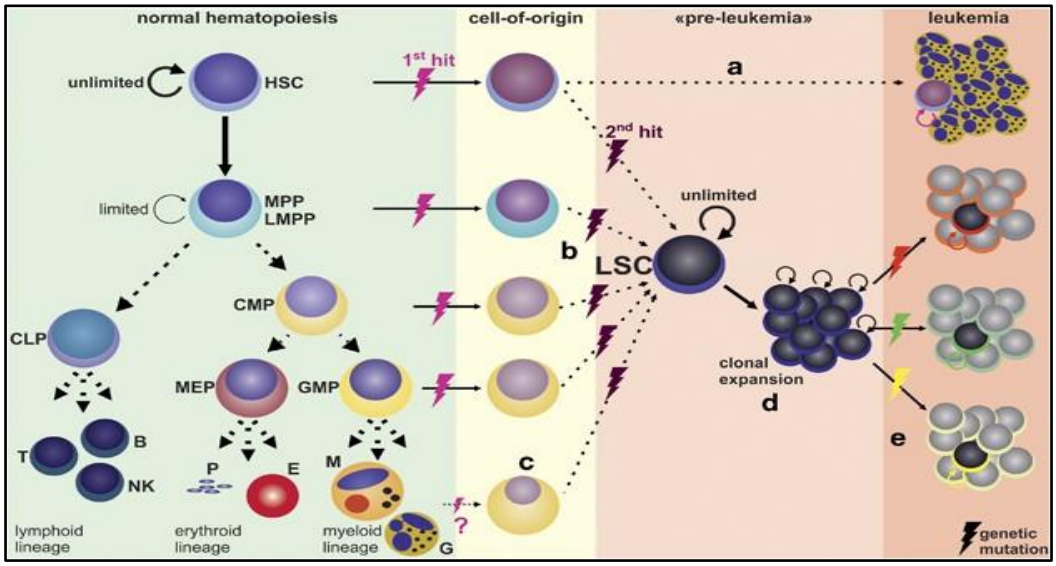


Figure 1. Regulation of normal and leukemic hematopoiesis (Adapted from Riether et al., 2015)

1.1.1. Acute lymphoblastic leukemia (ALL)

ALL is a haematological malignancy originating from B-cell or T-cell haematological precursor. ALL develops for recurrent chromosomal mutations, mostly translocations on the genes encoding transcription factors in control of B- or T-cell differentiation. Majority of ALL (80-85%) involves B-cell precursor, whereas only 15-20% are of T-cell lineage (Graux, 2011). ALL is one of the most common type of leukemia affecting children and teens below 20 years of age group. Childhood ALL mostly affects boys rather than girls (male: female ratio, 55%-45%). Advances in chemotherapeutic regimens have increased the survival rate from 10% in 1960s to 90% today (O'Brien et al., 2018).

1.1.2. Chronic lymphoblastic leukemia (CLL)

CLL is a haematological malignancy arising from the accumulation of immune-incompetent, mature, minimally self-renewing, monoclonal B-cells that evades apoptotic mechanism (Chiorazzi et al., 2005). CLL is more prevalent in western countries as compared to Asia and in adults above 55 years, which suggests that both environmental and genetic factors increases the predisposition of the disease. Usually men are more affected by CLL than females and it is considered as an indolent disease, in which asymptomatic people may never need the treatment (Rozman and Monserrat, 1995).

1.1.3. Acute myelogenous leukemia (AML)

AML is a genetically heterogeneous malignant disorder that involves uncontrolled proliferation of myeloid blasts. It is most aggressive cancer and the most common symptoms are bleeding, anaemia, infectious complications leading to hospitalization and death (Boyd et al., 2017). AML mostly affects older adults, generally above 60 years with lower survival rate with increasing age (Klepin and Balducci, 2009).

1.1.4. Chronic myelogenous leukemia (CML)

CML involves proliferation of transformed hematopoietic progenitor cells like myeloid, monocytic, erythroid, megakaryocytic, B-lymphoid and also T-lymphoid lineages. CML is specifically characterized by an abnormality of the karyotype involving the reciprocal chromosomal translocation t(9;22) and fusion of BCR gene on chromosome 22 and ABL1 gene on chromosome 9, leading to a dysregulated tyrosine kinase on chromosome 22, known as Philadelphia (Ph) chromosome (Deininger et al., 2000).

1.1.5. Mixed phenotype acute leukemia (MPAL)

MPAL is a rare and acute, comprising 2-5% of all leukemia. It is also referred to mixed lineage leukemia, bilineal leukemia, hybrid leukemia, and biphenotypic leukemia. It is a heterogeneous disorder comprising both myeloid and lymphoid blasts or a single blast cell population co-expressing lymphoid and myeloid markers (Weinberg and Arber, 2010). Due to its rarity (0.35 cases per 1,000,000 person-years) and uncommon diagnosis, chemotherapeutic treatment is relied on ALL-like regimen or hematopoietic stem cell transplant. The incidence of MPAL is higher in males and the median age of diagnosis is around 50 years (Khan et al., 2018).

1.2. Etiology of leukemia

1.2.1. Genetic factors

Patients with DNA repair disorders, chromosomal anomalies and genetic synfromes like Down syndrome, Li Fraumeni syndrome, Klinefelter syndrome, Fanconi's anaemia, Shwachman syndrome, Bloom syndrome are predisposed to AML and ALL. Immunodeficiency syndromes including Wiskott-Aldrich and Bruton agammaglobulinemia, increases susceptibility to leukemogenesis. People affected with myelodysplasia, primary thrombocythemia, polycythemia vera are associated with high risk to develop leukemia (Miranda-Filho et al.,

2018). Mutations inherited by autosomal dominant fashion on CEPBA, RUNX1 and GATA2 are potential cause of leukemia (Stieglitz and Loh, 2013).

1.2.2. Environmental and occupational exposure

Environmental exposures to different carcinogens like chemicals, radiations and infections can be a potential cause of leukemic development (Stewart, 1956; Zeeb and Blettner, 1998). Occupations handling with hydrocarbon compounds like benzene, gasoline and trichloroethylene have higher risk of developing leukemia (Rinsky et al., 1981). Certain occupations dealing with petrochemicals, pesticides and fertilizers increases risk of leukemia (Descatha et al., 2005; Lindquist et al., 1991; Wong Otto et al., 2010). Occupations like textile manufacturers and semiconductor workers, handling alkylating agents like formaldehyde are susceptible to leukemia (Goldstein, 2011).

1.2.3. Exposure to radiations

Leukemogenesis in the survivors of Hiroshima atomic bomb explosion leading to radiation exposure is well established (Hsu et al., 2013). Ionizing radiation at various phases of life like preconception, in utero and post natal exposures increases the risk of different subtypes of leukemia (Kheifets et al., 2017; M. Cuttler and S. Welsh, 2015). Low UVB exposures in few countries have been reported to have higher incidence of leukemia (Mohr et al., 2011).

1.2.4. Chemotherapy

Cancer patients who have received chemotherapeutic treatments, with specially alkylating agents and topoisomerase II inhibitors have higher risk of acute leukemia. Dexrazoxane, a topoisomerase II inhibitor used for chemotherapy, increases the risk of AML (Tebbi et al., 2007). According to a study, chemotherapy using dexamethasone or daunorubicin, induces stiffness in leukemic cells leading to vascular complications (Lam et al., 2007).

1.2.5. Viral infections

Human T-cell leukemia virus type I (HTLV-1) increases the risk of leukemia, mainly subtypes of ALL (Matsuoka, 2003). Epstein-Barr virus (EBV), COVID-19 and severe acute respiratory syndrome is associated with leukemic development (Li et al., 2007; Martín-Lorenzo et al., 2015; Taub et al., 2020).

1.3. Symptoms of leukemia

Usual clinical symptoms of leukemia are fever, fatigue, repeated infections and joint pain. For older adults above 65 years, CLL includes lymphadenopathy, splenomegaly, repeated infections, weight loss and anorexia (Friese et al., 2011). Apart from these there are certain specific symptoms for each subtype of leukemia, like chest pain in acute leukemia, haemorrhage, bone pain and shortness of breath is common for AML and abnormal sweating in chronic subtype (Howell et al., 2013). According to a study, 95% of children with ALL is marked with infiltration of blasts in peripheral blood and enlarged lymph nodes and/or spleen (Bernbeck et al., 2009).

1.4. Epidemiology of leukemia

During 2018 leukemia was 15th most common cancer as declared by GLOBACON (Global Cancer Observatory), and 11th most leading cause of cancer mortality, with 4,37,033 cancer cases and 309,006 mortality cases. It was reported around the same time that 29% of all childhood cancer is leukemia. Incidence rate (6.1 per 1,00,000 in males and 4.3 per 1,00,000 in females) and mortality rate (4.2 per 1,00,000 in males and 2.8 per 1,00,000 in females) was higher in males compared to females (Baeker Bispo et al., 2020). According to the American Cancer Society, around 59,610 cases of leukemia and 23,710 cases of mortality from leukemia is reported in United States for 2023. In 2022, leukemia was accounted for 2.5% of all cancers and 3.1% of mortality related to cancer (Huang et al., 2022).

1.5. Conventional chemotherapeutic treatments of leukemia

1.5.1. Chemotherapeutic drugs against AML

The well-established chemotherapy regimen for AML includes a 7-days continuous infusion course of Cytarabine with a 3-days course of Anthracycline drug like daunorubicin (Döhner et al., 2017). Apart from this, common chemotherapeutic drugs for AML includes: Cladribine (2-CdA), Fludarabine, Mitoxantrone, Etoposide (VP-16), 6-thioguanine (6-TG), Hydroxyurea, etc.

1.5.2. Chemotherapeutic drugs against ALL

Chemotherapy using oral tyrosine kinase inhibitor (TKI) like dasatinib, ponatinib, bosutinib, nilotinib, and imatinib are used for ALL treatment (Slayton et al., 2018). Hyper-fractionated CVAD (Cyclophosphamide, vincristine sulfate, doxorubicin, and dexamethasone), interchanging with methotrexate and cytarabine are used for adults with ALL below 65 years old (Foà et al., 2020).

1.5.3. Chemotherapeutic drugs against CML

CML falls under one of the cancer with target oriented therapy with the target being BCR-ABL tyrosine kinase enzyme. TKIs like imatinib, nilotinib, dasatinib, ponatinib and bosutinib are used as first line therapy against CML (Yun et al., 2016).

1.5.4. Chemotherapeutic drugs against CLL

CLL is an indolent disease hence patients don't need a chemotherapy until they meet the criteria to receive it. Major types of chemotherapeutic drug against CLL includes fludarabine, cyclophosphamide and rituximab (called as FCR in combination) or bendamustine, rituximab (BR) (Eichhorst et al., 2016; Fischer et al., 2016).

1.5.5. Chemotherapeutic drugs against MPAL

MPAL is based on ALL-like regimen as it is rare with only 2-5% of all leukemia and with an uncommon diagnosis because lack of trial data and poor characterization of this subtype (Khan et al., 2018).

1.6. Clinical side effects of chemotherapy

Tumor lysis syndrome takes place when many cancer cells die within a short span and they release their contents in blood which includes large amount of potassium, phosphate and uric acids. This leads to serious metabolic disorders like hyperkalemia, hyperphosphatemia, hypocalcemia, and hyperuricemia that can eventually damage organs (Figure 2). It is very common in haematological malignancies usually AML, either spontaneously or after receiving the chemotherapy (Howard et al., 2016; Wierda et al., 2019).

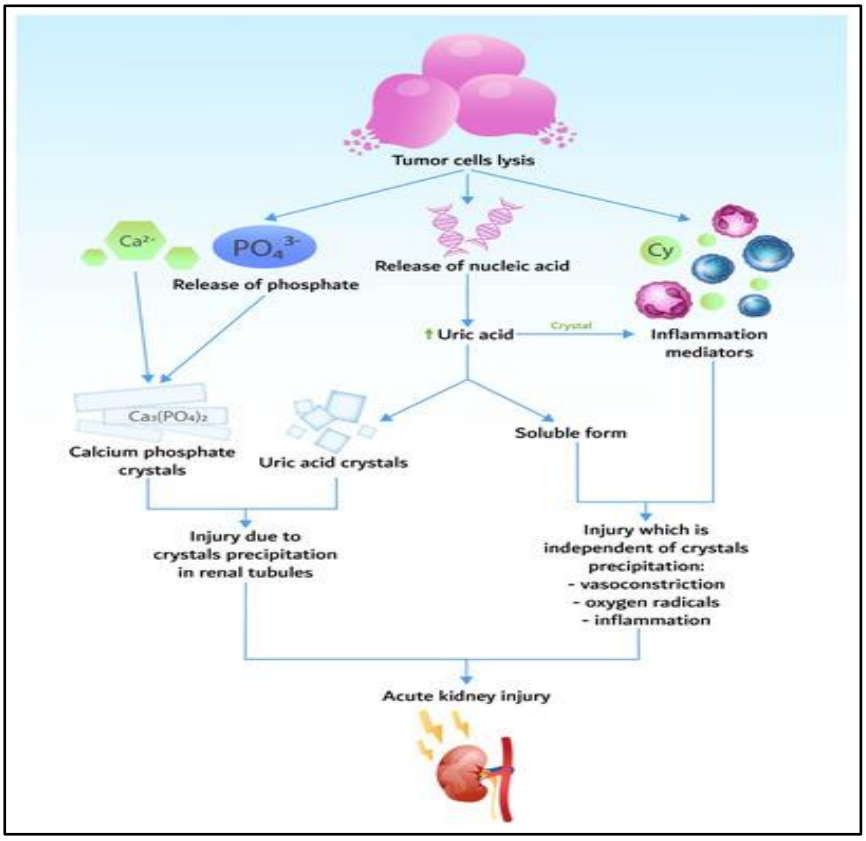


Figure 2. Tumor lysis syndrome leading to renal injury (Adapted from Lupușoru et al., 2022)

One of the vital side effect of chemotherapy in acute leukemic patients is neutropenia, or the decrease of neutrophil cells in blood which increases vulnerability towards infections (Ahmadzadeh et al., 2013; Freifeld et al., 2011). Immunosuppression post-chemotherapy is one of the potential reason for risks from dangerous infections. Acute leukemic patients undergoing chemotherapy also gets prone to infections with Human herpesvirus (HHVs) (Handous et al., 2020). Patients with acute promyelocytic leukemia suffers from Disseminated Intravascular Coagulation (DIC) in which the process for clotting of blood is hampered and leads to thrombosis and haemorrhage (Chojnowski, 2015; Ikezoe, 2014).

1.7. Effects of herbs and active compounds against leukemia

80% of global population is dependent on herbal therapy because of its minimal side-effects and easy accessibility. Use of herbal therapy against leukemia is prominent in developing countries.

1.7.1. Anti-leukemic effect of Scutellaria baicalensis and its active compounds Baicalin and Wogonin

Scutellaria baicalensis (SB) is a golden herb used in Chinese medicine for over thousand years because of its anti-tumor effect on various cancers, particularly leukemia (Motaez et al., 2015; Özmen et al., 2010; Shieh et al., 2006; Wang et al., 2014; Xiang et al., 2022; Ye et al., 2007) (Figure 3). The herbal extract reported to inhibit Blin-1 ALL cells by showing apoptotic and anti-proliferative effect by inducing mitochondrial damage and modulating Bcl-2 and CDK genes. Treatment with SB also decreased c-myc expression in Blin-1 leukemic cells, which can regulate apoptosis (Kumagai et al., 2007). Ethanolic extract of *Scutellaria baicalensis* roots inhibits expression of phosphatidyl inositol 3-kinase (PI3K)/Akt signaling in U937 leukemic cells and induces apoptosis by modulation of Bcl-2/Bax/Caspase-9 and -3 (Choi et al., 2019).

Figure 3. (a) *Scutellaria baicalensis* plant. (b) Dried root of plant. (c) Hand drawn image of thr plant (Adapted from Zhao et al., 2016).

Baicalin is an active compound isolated from the root extract of SB and it is reported to induce anti-tumor effect against acute leukemia, especially with Mixed-lineage leukemia1 and PBX1 gene rearrangements (Figure 4).

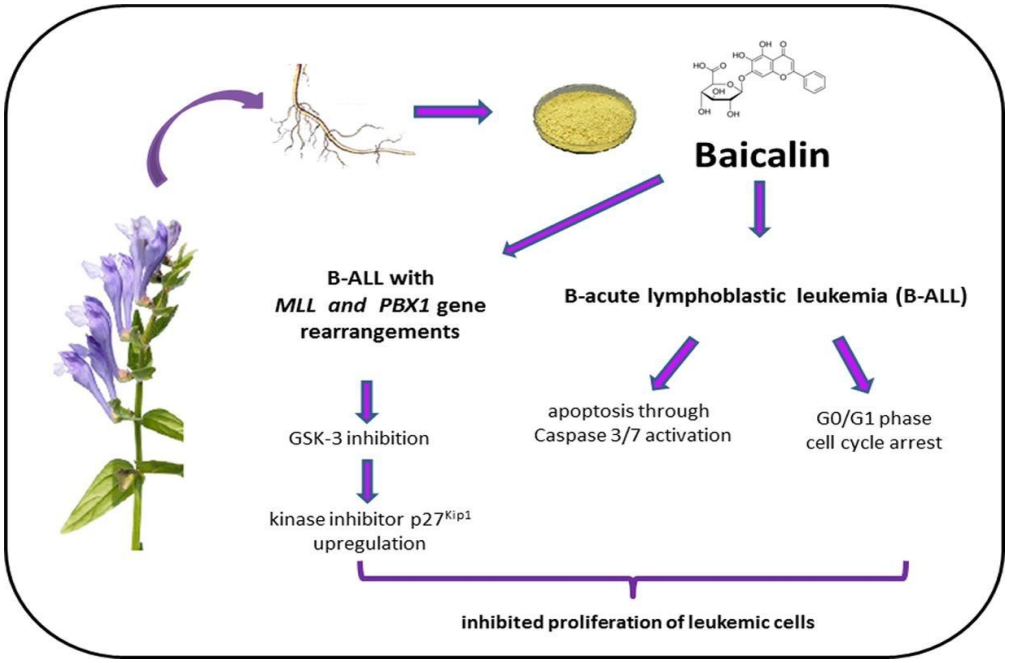


Figure 4. Anti-proliferative effect of Baicalin against acute leukemia (Adapted from Orzechowska et al., 2020)**.**

Baicalin inhibits GSK-3 by up-regulating the downstream molecule p27 ^{Kip1} to mediate its anti-proliferative effects on leukemic cells (Orzechowska et al., 2020). Baicalin also inhibits the proliferation of megakaryoblastic leukemic cells by arresting the cell cycle in G1 phase, both in cell lines and mouse model (Yu et al., 2016).

Wogonin, the main active compound of SB root extract is reported for it anti-cancer effect on human myelogenous leukemia HL-60 cells by modulation of Bax/Bcl-2 mediated apoptosis and via reducing telomerase activity by inhibition of hTERT promoter of c-myc gene (Huang et al., 2010; Huynh et al., 2017; Pei et al., 2022). Wogonin increases the transcription of phospholipid scramblase 1 (PLSCR1), leading to cell-cycle arrest in AML cells (U937 and HL-60 cell line) (Chen et al., 2013). It is also reported to suppress STAT3 to exert anti-proliferation in ALL cells (Xiao et al., 2017).

1.7.2. Anti-leukemic effect of Panax Ginseng and its active ingredient ginsenoside

Root of *Panax ginseng* is traditionally used in East Asian countries and it is well documented to exhibit anti-leukemic effect (Cho et al., 2009; Jia et al., 2009; Kang et al., 2005; Tung et al., 2010; Xiong et al., 2017). *Ginseng* extract is documented to induce apoptosis in U937 leukemic cells, via Bcl2, Bcl-xl and caspase modulation. It also decreases COX-2 and inducible nitric oxide synthase expression, hence targets the inflammatory response in leukemic cells. *Ginseng* extract is reported to down-regulate the expression of human telomerase reverse transcriptase gene that influences and decreases the expression of c-myc in leukemia (Park et al., 2009). In a study by Jo et al., *Ginseng* extract inhibits proliferation of NB-4 acute pro-myelocytic leukemia cells, and modifies the MYC-SKP2-CDKN1B pathway as a molecular mechanism of P/D transition in leukemia (Figure 5).

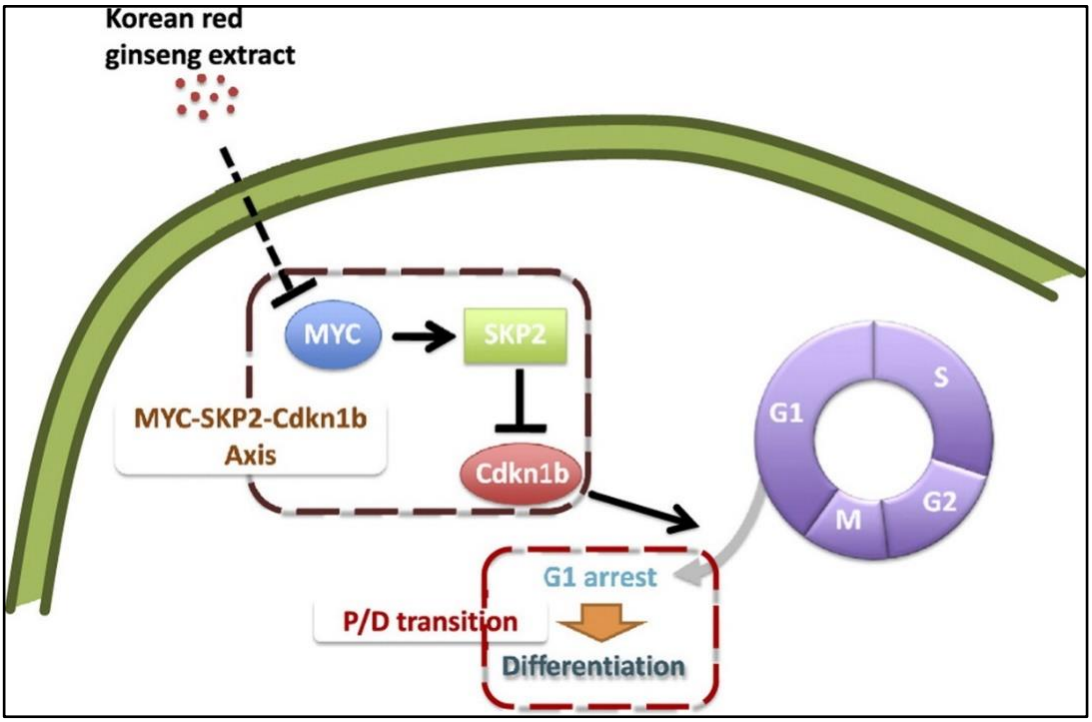


Figure 5. Anti-leukemic effect of *Ginseng* extract on acute leukemia via MYC-SKP2-CDKN1B axis (Adapted from Jo et al., 2013)

Ginsenoside is one of the main active compound of *Ginseng* root extract and it is reported to supress the invasion and migration of THP-1 leukemic cells by inactivating the MAPK signaling pathway (Choi et al., 2011). Ginsenoside inhibits growth of HL-60 leukemic cells by mediating apoptosis via TNF-α signaling (Huang et al., 2016).

1.7.3. Anti-leukemic effect of Typhonium flagelliforme

Typhonium flagelliforme is common in Southeast Asian countries and traditionally used by the ethnic population of Malaysia for its anti-leukemic effect. It is reported to supress WEHI-3 leukemic cell growth by inducing apoptosis and proliferation both in vitro and in leukemic mice model, as well as shows cytotoxicity towards P388 leukemic cells (Choo et al., 2001; Mohan et al., 2010a). It shows apoptotic effect on leukemic cells via activating the mitochondrial pathway leading to the activation of caspase-9, cleavage of PARP and subsequently release of cytochrome c (Mohan et al., 2010b).

Apart from these, there are various other herbs which shows anti-leukemic effect, as listed below:

Hibiscus cannabinus (Hassan et al., 2016; Tseng et al., 2000; Zangeneh and Zangeneh, 2020)*;* ***Euphorbia formosana*** (Cruz et al., 2020; Hsieh et al., 2013; Tesfaye et al., 2020)*;* ***Vernonia amygdalina*** (Khalafalla et al., 2009)*;* ***Achillea fragrantissima*** (Alenad et al., 2013)*;* ***Ganoderma lucidum*** (Claudia et al., 2006)*;* ***Berberis vulgaris*** (Kuo et al., 2004)*;* ***Moringa oleifera*** (Akanni et al., 2014; Dilworth et al., 2020)*;* ***Camellia sinensis*** (Ahmeda et al., 2020; Borutinskaitė et al., 2018; Ghosh et al., 2006; Othman et al., 2020; Yusoff et al., 2022).

1.8. An overview on inflammation and cancer

The critical inter-relationship between cancer and inflammation is well established. In the year of 1863, Rudolf Virchow was the first scientist to establish the co-relation between cancer and inflammation. He suggested that it's the inflammatory microenvironment which supports malignant tissues and the infiltration of lymphocytes marks the origin of cancer. The prognosis of inflammation involves the migration of leukocytes and very importantly tissue mast cells towards the site of wound. Recruitment of the inflammatory cells towards the site of wound allows the proliferation of fibroblasts and endothelial cells which further triggers adhesion and leukocyte activation (Hussain and Harris, 2007). Cancer cells secretes various chemokines and cytokines which attracts leukocytes and hence any neoplasm consists of inflammatory components like neutrophils, eosinophils, basophils, macrophages, lymphocytes and mast cells. These cells further produces cytokines like TNF-α, MMPs, interferons and interleukins which leads to chronic inflammation. Neoplastic tissues are mostly characterized by Tumour-associated macrophages (TAMs) which can furthermore secrete various angiogenic growth factors that allows cancer progression and proliferation (Misra et al., 2018).

Chapter 2.

Chapter 2.

2. Review of literature

With the increasing civilization, the exposure to carcinogenic pollutants from industries like factories have significantly increased. This has led to several harsh impacts on humans, one being the increasing incidence of cancer. According to reports, the rate of cancer will be doubled in next 20 to 30 years and by 2030, there will be 26 million new cancer cases and 17 million cancer deaths worldwide (Mistry et al., 2011; Quante et al., 2016; Thun et al., 2009).

2.1. N-nitroso compounds (NOCs) and its various sources

Human population is exposed to nitrates through majorly through chemicals in environment, food and drinking water. In mammals, nitrates can be converted into N-nitroso compounds (NOCs) by reacting with amines, amides and amino acids (Badawi, 2000). Tobacco, smoked meat, pesticides and rubber making factories are few of the various sources of NOC exposure to humans (Figure 6) (Eichholzer and Gutzwiller, 1998; Jain et al., 2020; Lijinsky, 1999)

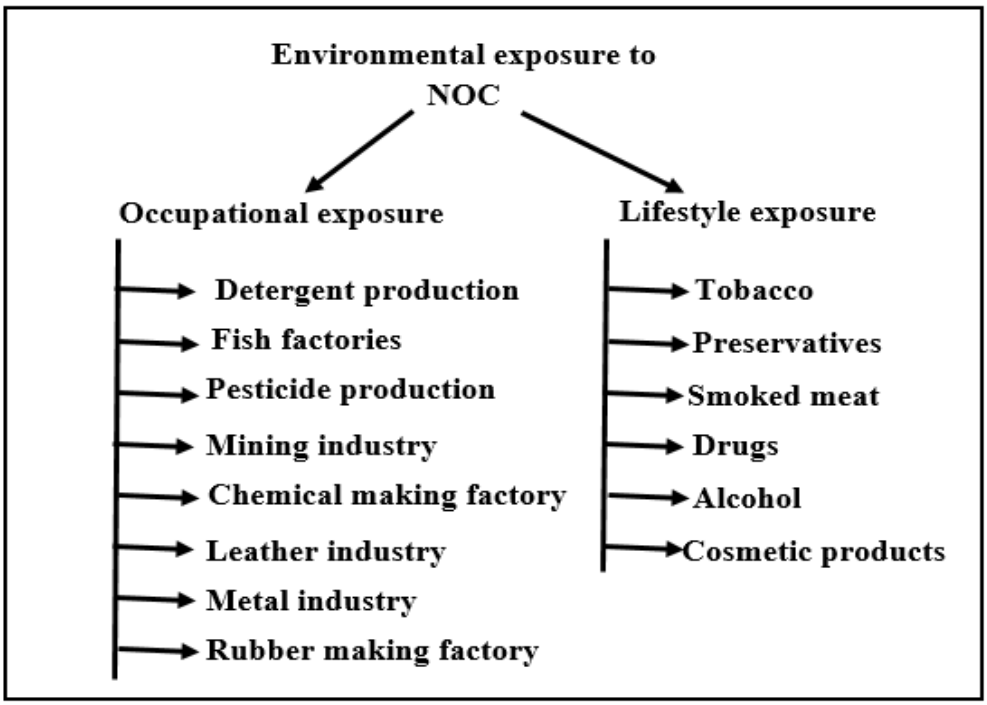


Figure 6. Human exposure to environmental NOC

The two major classification of NOCs are N-nitrosamines and N-nitrosamides. Both are composed of a nitroso group (-N=O) attached to a nitrogen atom (-N-N=O). In the year of 1964, occurrence of N-nitrosamines was first reported in a fish meal which was cured in nitrite. This led to more research on NOCs in nitrite cured food products (Originalmitteilungen, 1964; Topps and Elliott, 1965). The presence of NOCs in tobacco and cigarette smoke was first established by Druckrey and Preussmann (Druckrey et al., 1967). Presence of nitrate compounds in drinking water is also one of the environmental risk factor and can cause methemoglobinemia (Fewtrell, 2004). Analytical methods detected the presence of volatile NOCs in urban air in the year 1976 (Fine et al., 1976). The presence of NOCs in cutting fluids used in metal cutting and grinding industry have also been studied ("N-Nitrosodiethanolamine in Synthetic Cutting Fluids : A Part-Per-Hundred Impurity Perception of Impossible Limb Positions Induced by Tendon Vibration," 1976).

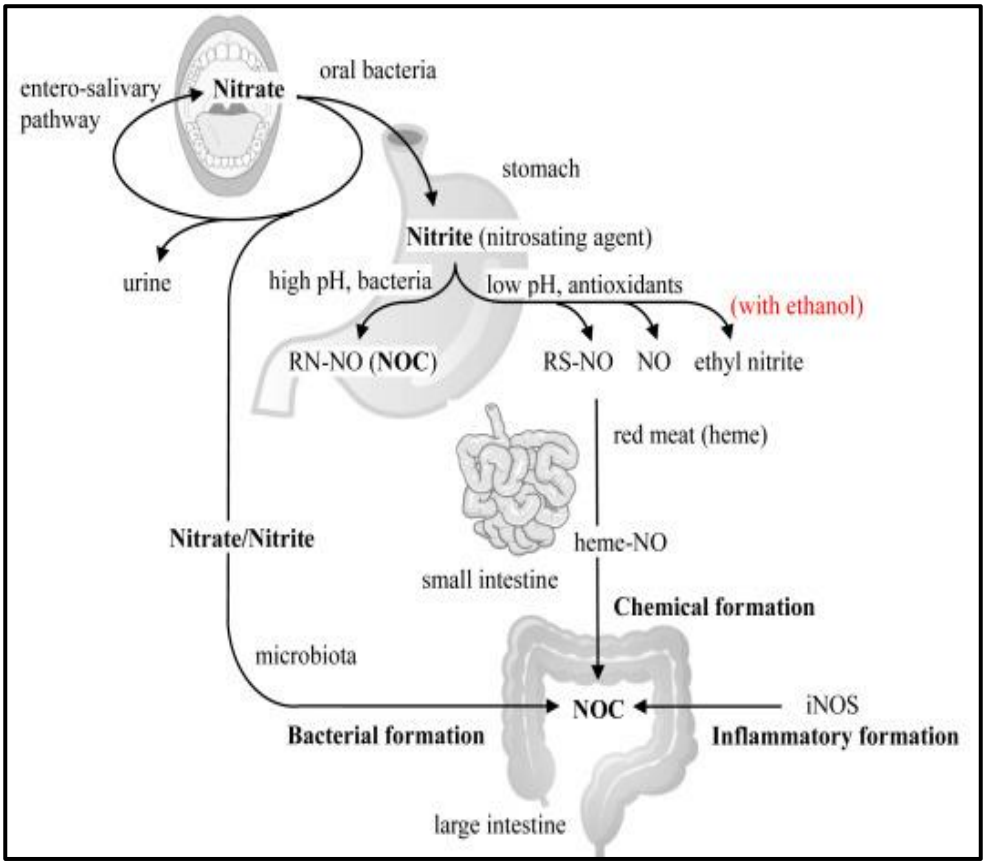


Figure 7. Endogenous formation of NOC (Adapted from Kobayashi 2018)

NOCs can also be formed endogenously in the gastro-intestinal tract through dietary nitrates and nitrites. Other factors like gastric pH, microbial flora and food products also influences the NOC formation (Figure 7). Apart from food sources nitrate is accumulated in water due to over use of nitrogen fertilizers and hence in United States there is a set limit of 10mg/L nitrate-nitrogen (N) in drinking water.

2.1.1. Carcinogenic effects of NOCs in humans

Apart from posing toxicological risks, the major issue is the potent carcinogenic effects of NOC (Figure 8) (Odashima, 1980). Stomach nitrates and nitrites are converted to nitrous acid that is reactive with amines and amides to lead to the formation of nitrosamines and nitrosamides respectively, which are carcinogenic to animals. These compounds are reported to cause cancer in almost 40 different species and can effect multiple organs (Loh et al., 2011).

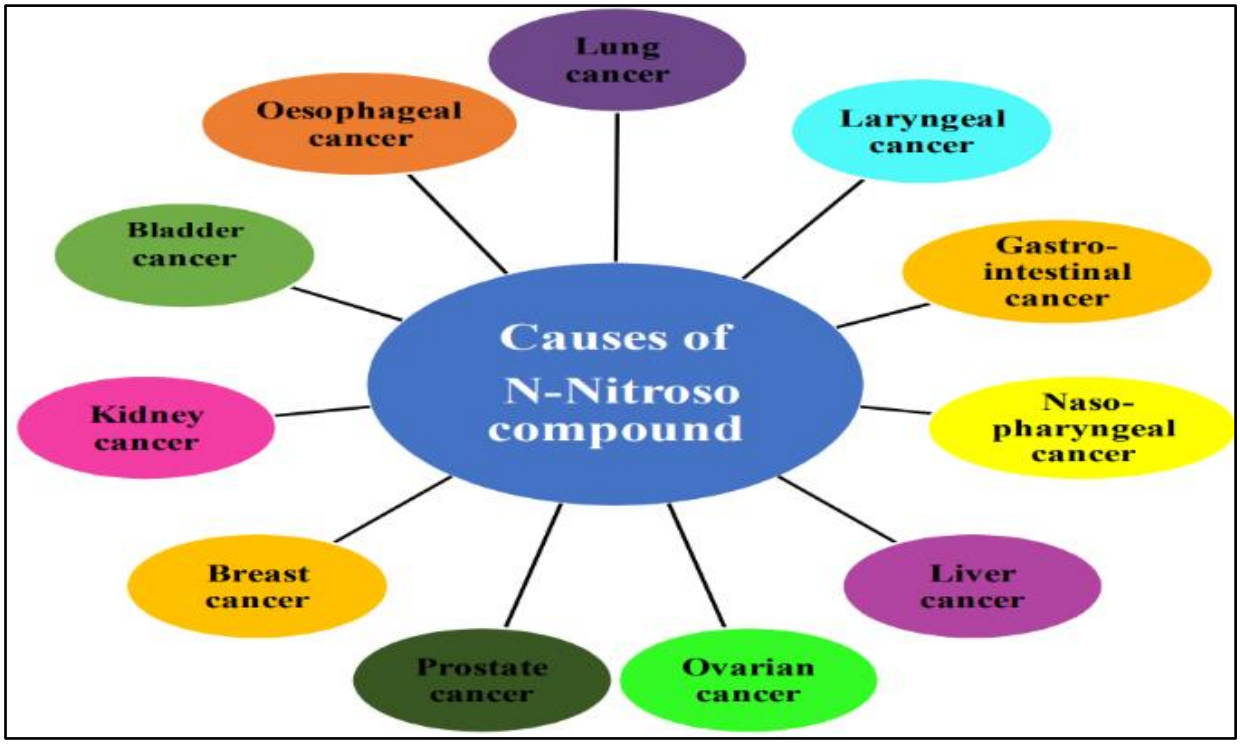


Figure 8. Carcinogenic effects of NOC (Adapted from Jain et al. 2020)

Barnes and Magee in the year of 1954 established that dimethylnitrosamine (DMN) a NOC, causes severe liver necrosis and malignant tumor in rats, when administered orally or parenterally (Magee and Barnes, 1956). According to case control studies NOC exposure through diet can lead to gastric, stomach, oesophagus and nasopharynx cancers (Keszei et al., 2013; Mirvish, 1995; Pignatelli et al., 1993; Tricker and Preussmann, 1991; Ward and Weisburger, 1975). N-nitrosodimethylamine (NDMA) a NOC, is present in some food products, also categorized as "probably carcinogenic to humans" and its intake is related to gastrointestinal, mainly rectal cancer (Grosse et al., 2006; Loh et al., 2011). High fat and cholesterol diet with nitrite cured meat and fishes mostly includes NOCs like dimethylnitrosamines and are associated with an increased risk of lung cancer. Red meat or processed meat contains high amount of NOCs and according to a case study it is supposed to cause colorectal carcinoma in humans (Zhu et al., 2014). Endogenously formed NOCs contributes to 45 to 75% of exposure and it is correlated to a significant increase in non-cardia gastric cancer cases (Jakszyn et al., 2006). A case control study in China revealed the association of urinary levels of NOCs, due to exposure to nitrites and the risk of gastric cancer, post *H.pylori* bacterial infection (Xu et al., 2015). Also, pancreatic cell explants exposed to NOCs leads to uncontrolled proliferation and mitotic activation as well as forms tumour when injected into nude mice. Dietary NOCs like N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA) present in processed meat, cheese and red meat plays an etiological role in pancreatic cancer progression (Rhim et al., 1997; Risch, 2003; Rohrmann et al., 2007; Zheng et al., 2019). During pregnancy, maternal exposure to dietary NOCs through consumption of cured meat products is well associated with the development of brain tumour in the child. A case-control study held in Los Angeles hypothesized that *in utero* exposure of NOCs and its precursors can lead to brain tumours and it is considered as the most carcinogenic

compound to the nervous system in experimental animals (Dietrich et al., 2005; Preston-Martin et al., 1982).

2.1.2. Role of NOCs in leukemia and lymphoma

Intake of higher dietary nitrate is associated with an increased risk for developing non-Hodgkin lymphoma by 40% (Aschebrook-Kilfoy et al., 2012). Dietary NOCs also increases the risk for follicular and T-cell lymphoma, as reported in a population based case-control study in Connecticut women (Börzsönyi et al., 1976; Kilfoy et al., 2010).

N-methyl-N-nitrosourea (NMU) is an alkylating agent with carcinogenic properties and it belongs to the group of NOC, which is widely used for breast cancer development in animal models as well as leads to colon cancer, pancreatic cancer, liver cancer, brain cancer, etc. (Boileau et al., 2003; García-González et al., 2000; Gullino et al., 1975; Russo and Russo, 1996). It is well established that NMU significantly induces leukemia in Sprague-Dawley rats and thymic lymphoma and myelogenous leukemia (Chang et al., 2012; da Silva Franchi, C.A., Bacchi, M.M., Padovani, C.R., & de Camargo, 2003; Huggins et al., 1982; Newcomb, 1997; P. R., 1985). N-nitroso-N-butylurea (NBU), a NOC is frequently used for leukemic induction in mice and it leads to chromosomal changes in leukemic rats (Bauer et al., 1973; Kozu et al., 1978; Martelli et al., 1988; Osaka et al., 1998; Tsujimura et al., 2004; Uenaka et al., 1978). NOC when induced intragastrically leads to malignant lymphomas in Swiss mice (Börzsönyi and Csik, 1975).

2.2. N-N’ ethylnitrosourea (ENU) and its carcinogenic potentials

N-N’ ethylnitrosourea (ENU) is a chemical, regarded as a “biological hazard” and it is classified as a NOC (Figure 9). It is a DNA alkylating agent that can lead to DNA mutations, over-expression of oncogenes and activation of the signaling pathways of cancer (Sareddy et al., 2009).

ENU is neurotoxic to stem cells and its prenatal exposure can lead to malignant gliomas, brain tumors and several other cancers (Briançon-Marjollet et al., 2010; Capilla-Gonzalez et al., 2010; Esapa et al., 2015; Janke et al., 2013; Searle and Thomas, 1973; Slikker et al., 2004).

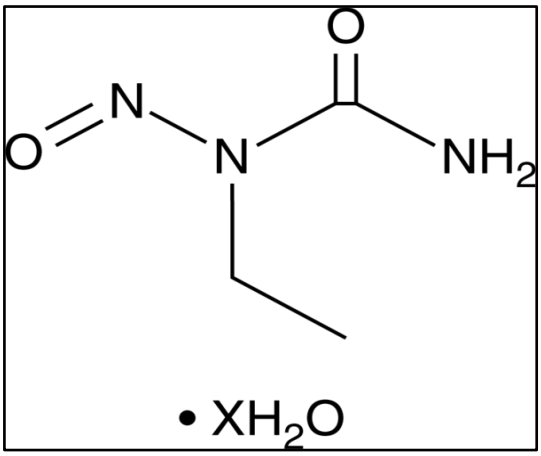


Figure 9. Chemical structure of N-N' ethylnitrosourea (ENU)

A study demonstrated ENU as a potent mammary carcinogen that induces solid papillary adenocarcinomas in pituitary-isografted mouse (Swanson et al., 1996).

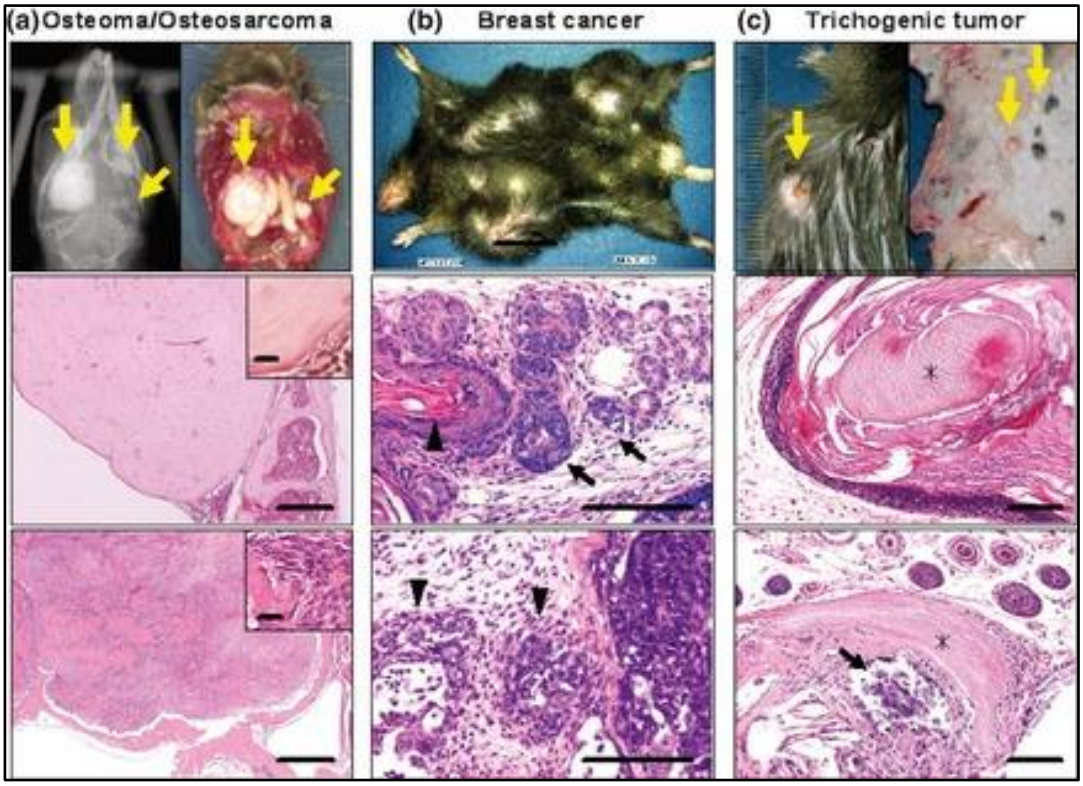


Figure 10. ENU induced novel mice models (Adapted from Toki et al. 2013)

Two intra-peritoneal doses of ENU after 9th and 11th week of birth leads to lung cancer in mouse model (Herzog et al., 2004; Horlings and Demant, 2005). A study shows, an intra-peritoneal dose of ENU at 85 to 100 mg/kg body weight (weekly twice) to B6 male mice, which were further mated with D2 female mice led to a F1 progeny of offspring that derived osteoma, trichogenic tumor and breast cancer (Figure 10) (Toki et al., 2013).Exposure to ENU at different phases of pregnancy leads to intracranial tumours and peripheral nerve sheath tumours (PNTs) in the offspring (Figure 11). Not merely the direct prenatal exposure but also prenatal progenitor's exposure to ENU is a potential risk to develop brain tumours (Bulnes et al., 2021).

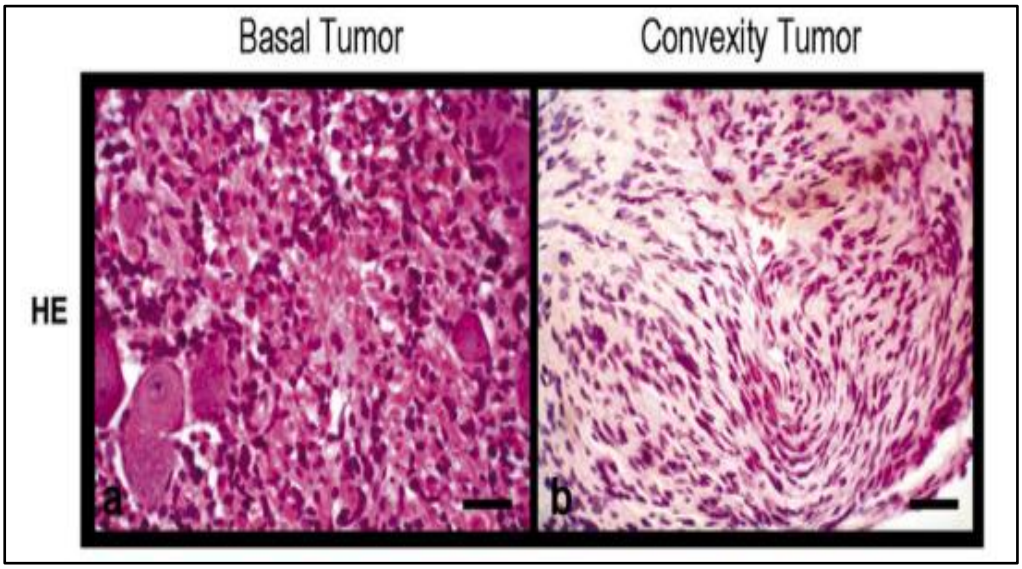


Figure 11. ENU-induced tumours in the nerve sheath (Adapted from Bulnes et al. 2021)

Female Sprague-Dawley rats around a month old, were intra-peritoneally injected with 165mg/kg of ENU leading to mammary tumours of sizes ranging between 8-18mm in diameter within around 9 and 21 weeks (Vohra et al., 2021). Another frequently studied ENU induced mice model is of multiple intestinal neoplasia, due to a mutation on the *Apc* (Adenomatous polyposis coli) gene. It is reported that both intestinal and mammary cancers arises due to the mutations caused by ENU induction (Moser et al., 1995). Induction of glioblastoma multiforme tumours in the central nervous system was carried out using ENU intravenously at a dose of

50mg/kg on the 19th day of gestation in rats (Hervouet et al., 2013). Intra-peritoneal or transplacental induction of ENU to Sprague-Dawley and Berlin-Druckrey IV rats leads to an unusual ovarian tumour consisting of Sertoli's like cells (Stoica and Capen, 1985). A study revealed the liver of neonatal Big Blue transgenic mice are more sensitive to mutations on *cII* gene induced by ENU compared to adult mice, hence suggesting age-dependent sensitivity towards ENU by liver cells (Mei et al., 2005). A research was conducted in which out of 240 ENU-injected mice, 140 of them developed tumours of many varieties like sarcoma, lymphoma and carcinomas, whereas the most common was thymic lymphoma (Esapa et al., 2015; Timothy S Fenske et al., 2006) (Figure 12).

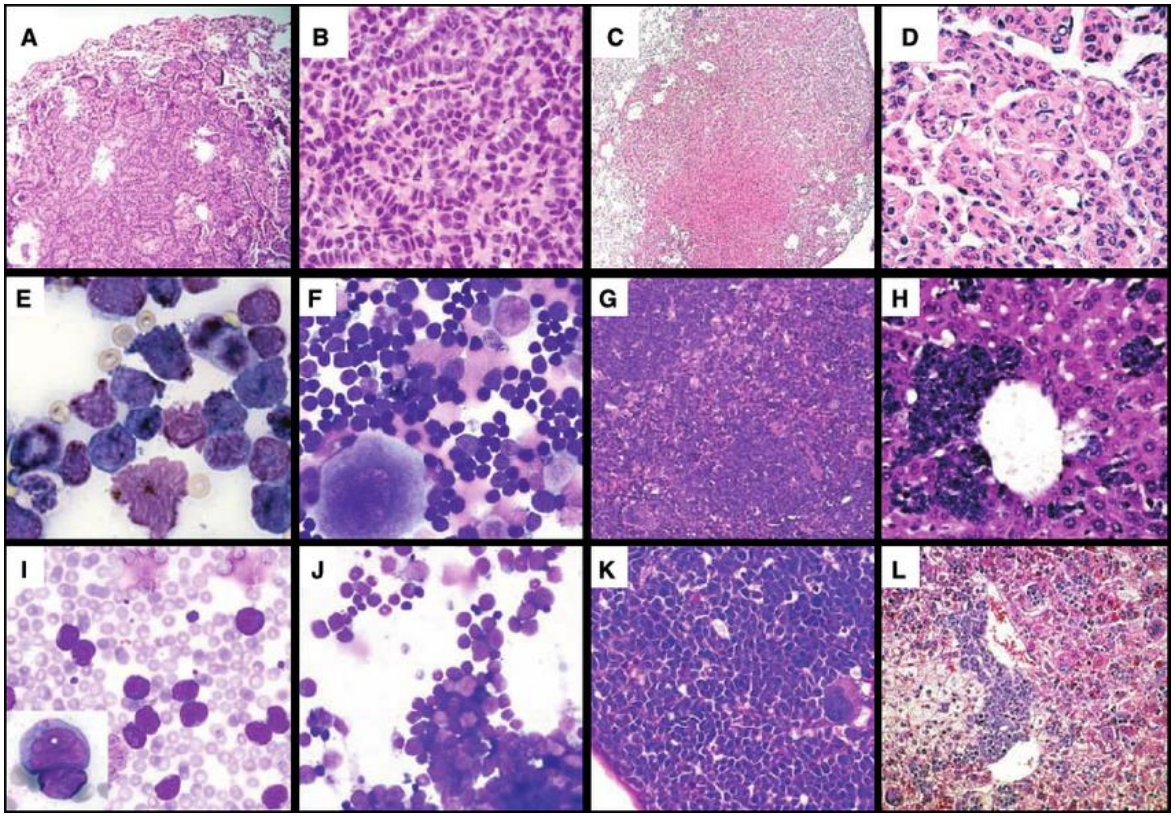


Figure 12. ENU induced tumours (Adapted from Fenske et al. 2006))

2.2.1. ENU induced leukemia

ENU is an alkylating agent that mutates the DNA by transferring an alkyl group to a nucleobase leading to leukemogenesis (Aliyu et al., 2019; Dexter et al., 1974; Gerson et al., 1986; Justice

et al., 1999). ENU induces a mutation in every 700 loci and 80mg/kg intra-peritoneal dose of ENU induces leukemia in mice while up-regulating angiogenesis via immune-modulation of Vascular Endothelial Growth Factor (VEGF) and inhibition of apoptosis through the anti-apoptotic proteins Bcl-2 and Bax. Excessive infiltration of neoplastic lymphocytes is reported in the kidneys and lungs and increased blast population in the peripheral blood of ENU-induced leukemia (Figure 13) (Aliyu et al., 2020).

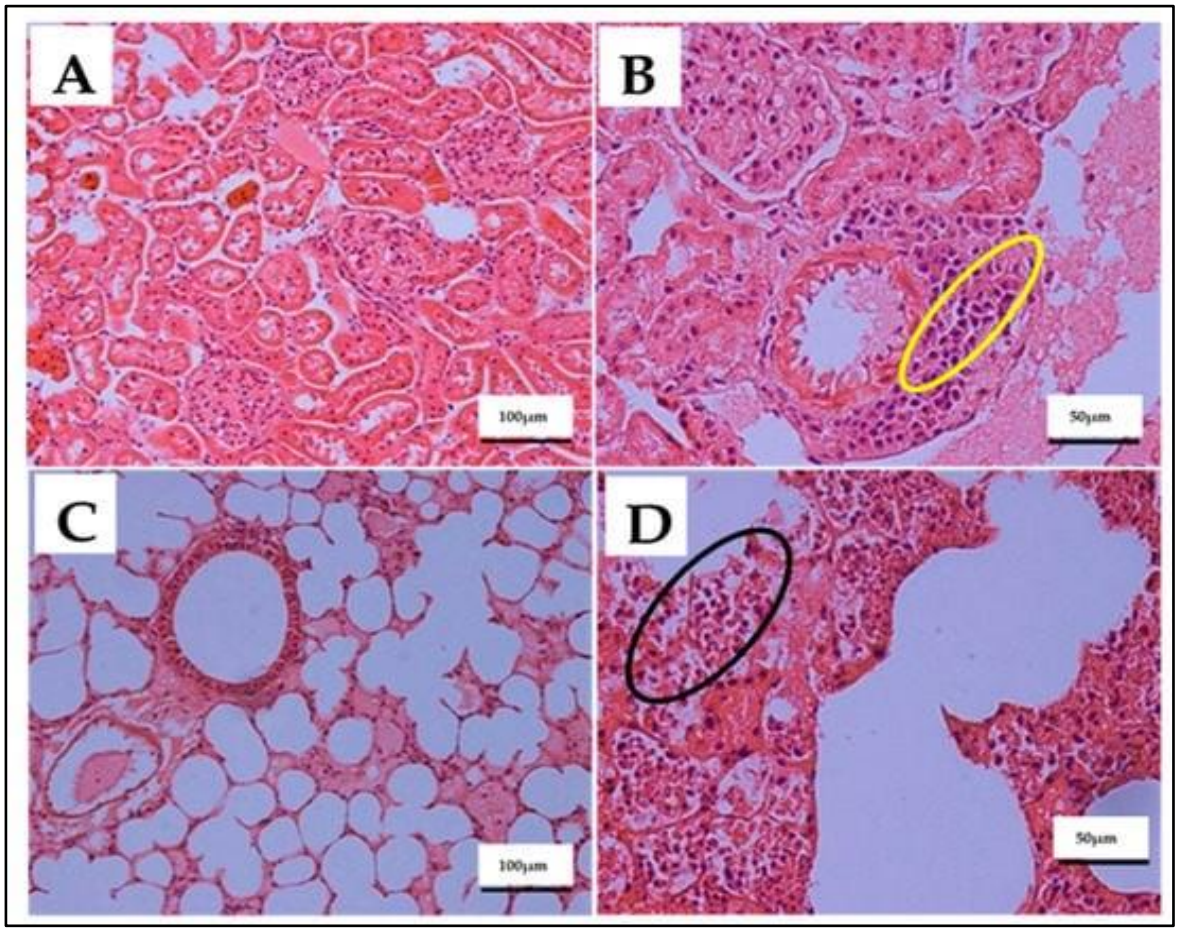


Figure 13. A comparison of kidney sections from (A) control and (B) ENU-induced leukemia and lung sections of (C) control and (D) ENU-induced leukemic mice (Adapted from Aliyu et al. 2020)

ENU induces myeloid malignancies by creating sporadic mutations in mice genome and ENU induced leukemia is an useful model organism for AML research (Higuchi et al., 2002; Yuan et al., 2001). A study by Hadjiolov et al., 50 mg ENU induced myeloid leukemia and thymic

lymphoma whereas myeloid cells were more predisposed to the carcinogenic action of ENU than lymphoid progenitor (Hadjiolov, 1972).

Intra-peritoneal injection of ENU at a dose of 80mg/kg body weight induces leukemia within a span of 6 to 8 months. According to the study by Ritam et al., deregulation of the classical p53 signaling pathway contributes to leukemogenesis post ENU-induction leading to a decline in p21 fluorescence expression and down-regulation of p53-Ndn-Gfi axis (Chatterjee et al., 2016a). Leukemic condition was confirmed by the sharp incline in population of blasts in marrow and excessive WBCs in peripheral blood (Figure 14).

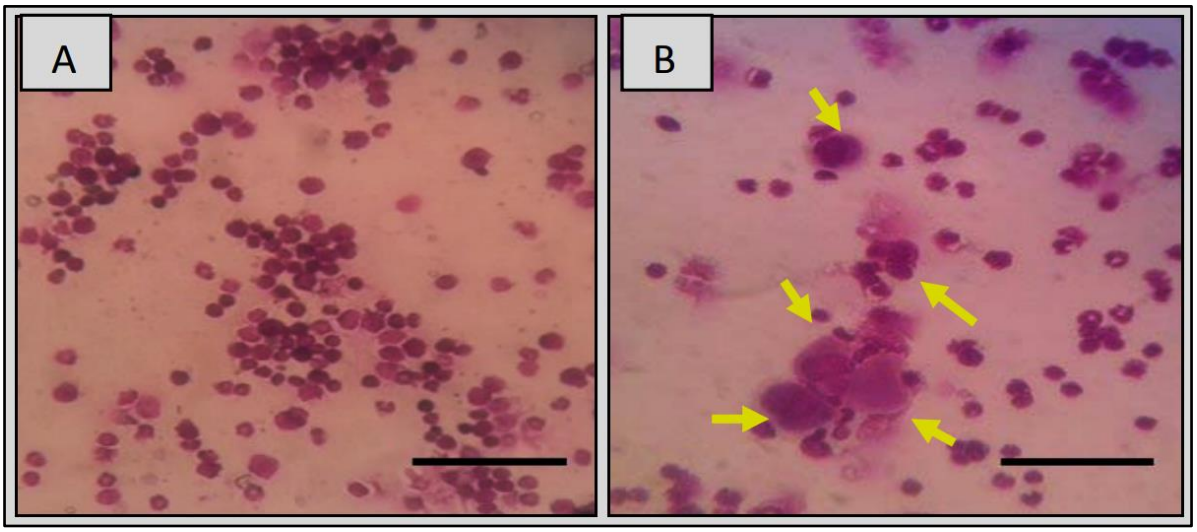


Figure 14. Geimsa stained bone marrow of control and ENU-induced leukemia showing blast burden in leukemic condition (Adapted from Chatterjee et al., 2016)

Prosun et al., conducted a long-term bone marrow cell culture study on 80mg/kg body weight of ENU induced leukemic mice. Long and elongated stromal fibroblast cells were identified along with excessive proliferation of leukemic marrow. These stromal fibroblast cells are meant to provide support and maintenance to the leukemic bone marrow cells in a hyper-proliferative disease condition (Figure 15). Moreover, the expression of VEGFR was increased in leukemic condition, in account of the increased stromal endothelial cells in the bone marrow microenvironment (Basak et al., 2010).

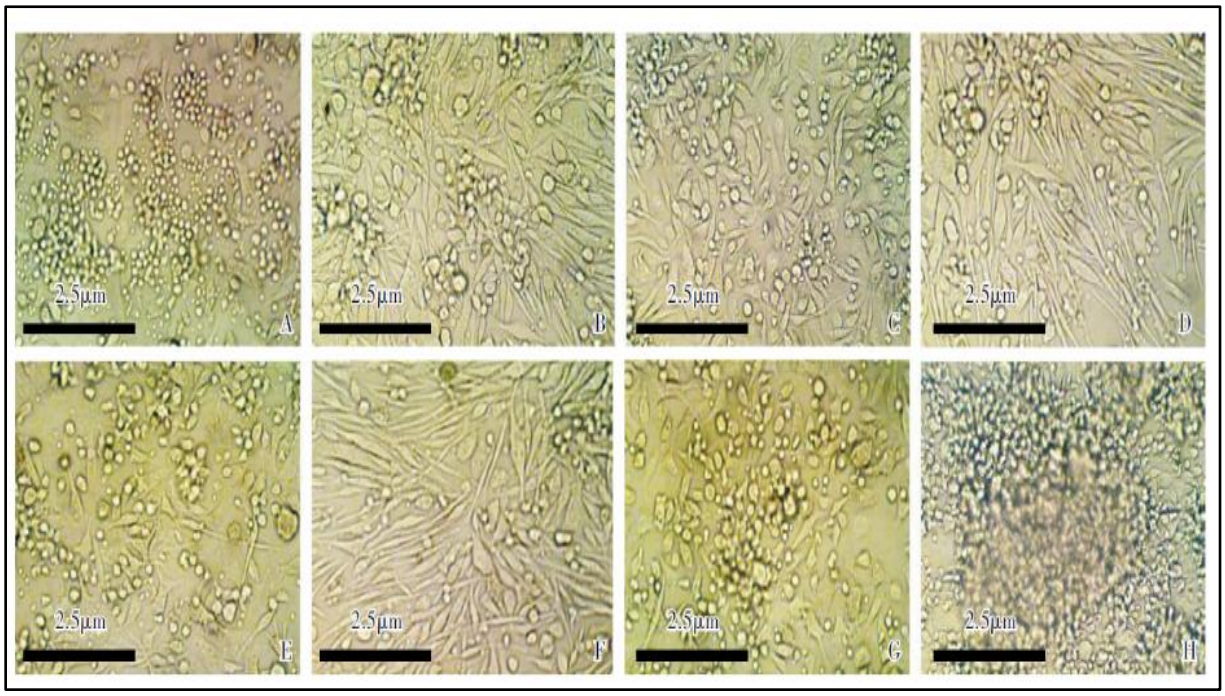


Figure 15. Long-term bone marrow cell culture study of ENU-induced leukemia showing increased stromal fibroblast cells (Adapted from Basak et al., 2010)

In a comparative hematopoietic research the bone marrow cells of ENU-induced leukemia were tested for mitochondrial membrane stability by Janus Green B staining (JGB). No mitochondrial damage was observed in ENU-induced leukemic mice which signifies less or no apoptosis of the leukemic proliferating cancer cells. Mallory's trichrome staining of ENU-induced leukemic bone showed infiltrating stromal fibroblasts supporting the maintenance of leukemic cells (Chatterjee et al., 2016b).

In a study conducted by Sukalpa et al., canonical Wnt signaling pathway is reported to be deregulated in the bone marrow cells of ENU-induced leukemic mice (Chattopadhyay et al., 2019). Fluorescence expression of proteins belonging to the Wnt pathway like Wnt3a, Fzd7, β-catenin and cyclinD1 are increased and expression of Dkk1, Wnt antagonistic protein decreased due to ENU-induction. The leukemic group expressed a mixed phenotype consisting of both lymphoid and myeloid lineage as confirmed by blood profile (Figure 16).

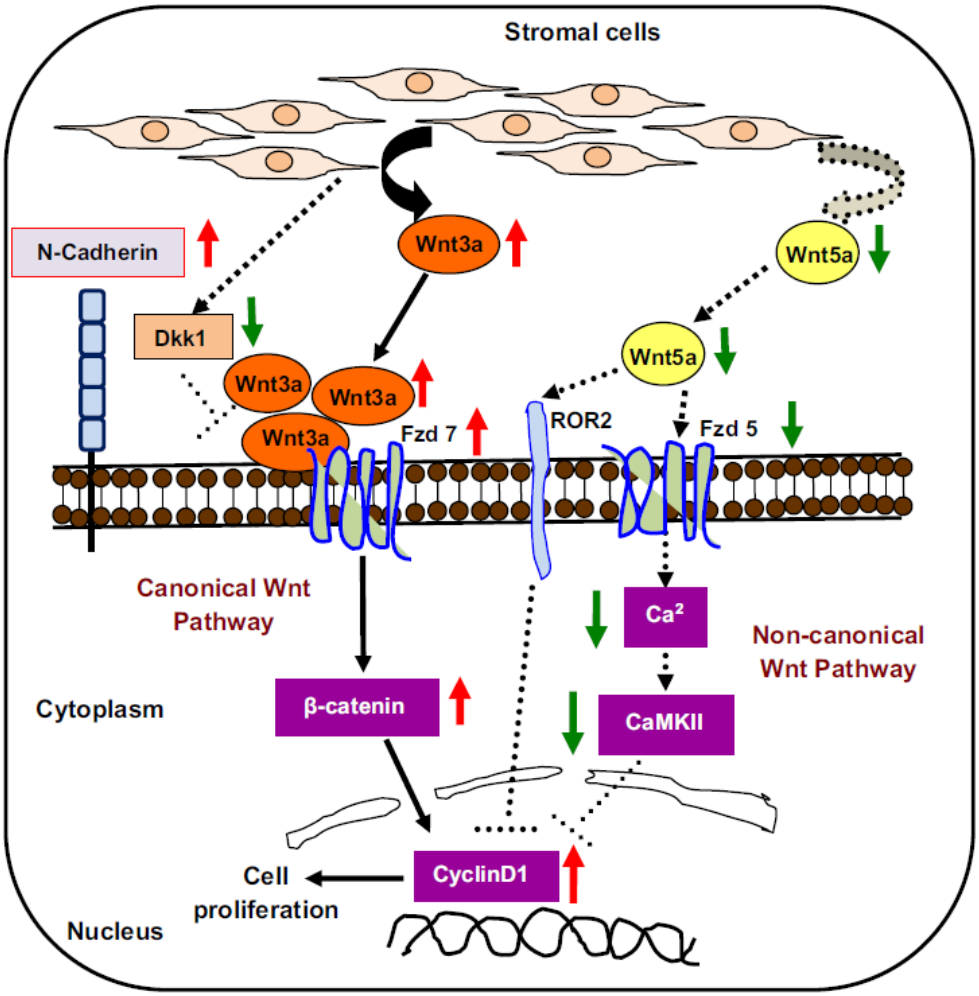


Figure 16. Deregulation of the Wnt signaling pathway in ENU-induced marrow cells (Adapted from Chattopadhyay et al., 2019)

2.3. Treatments of ENU-induced leukemia

2.3.1. Treatment with Niacin on ENU-induced leukemia

Cancer patients usually becomes nutritionally compromised mostly due to the toxic side effects of chemotherapy. Niacin or vitamin B3 is necessary for the production of nicotinamide adenine dinucleotide (NAD^+/H) and NAD phosphate whereas its deficiency is common in patients who underwent chemotherapy. A research by Barleman et al., revealed that Niacin supplementation to ENU-induced leukemic mice can significantly reduce the incidence of leukemia (Bartleman et al., 2008).

2.3.2. Cytokine therapy on ENU-induced leukemia

Intra-peritoneal treatment with 10µg/kg/day of IL-15 and 5µg/kg/day of GM-CSF significantly up-regulates macrophage cells and their chemotactic movement in ENU-induced leukemic mice model. Furthermore, the expressional profile of pro-inflammatory cytokines and tumoricidal molecules were restored by the combined cytokine therapy on leukemic mice (Singha and Maiti, 2019). Another study reported the cytokine therapy using IL-3 and GM-CSF in combination increased the cytotoxic T-lymphocyte (CTL) activity, hence increased perforin and granzyme B activities in ENU-induced leukemia (Singha et al., 2015).

2.3.3. Blue light exposure treatment on ENU-induced leukemia

Photodynamic therapy has been extensively used in recent years for cancer treatment. Blue light exposure was carried out for 3h around 9 to 16 weeks on ENU-induced leukemic animal model. From the peripheral blood of leukemic rats, lymphocyte cells were isolated and incubated with blue-light exposure for 7 days. Lymphocyte cells remained unaffected but the growth of leukemic cells were inhibited post blue-light therapy (Ohara et al., 2002).

2.3.4. Olive leaf extract and IL-28B combination therapy

Ethanolic olive leaves extract (EOLE) and IL-28B cytokine combination therapy was studied on ENU-induced leukemic Swiss albino mice. Post leukemic development EOLE treatment was continued for 4 weeks and IL-28B therapy for one week, which significantly declined blast cell population in peripheral blood. Apart from stabilizing the immune status, also the natural killer cell activity was increased post treatment and hence the levels of perforin and granzyme was inclined in ENU-induced leukemia (Debasish Maiti et al., 2022).

2.4. The Ayurvedic herb *Eclipta alba*

Eclipta alba (L.) L. (Syn.: Eclipta prostrata (L.) Hassak, Family: Asteraceae) is an annual herbaceous plant and commonly referred to as false daisy in English, Bhringaraj in Bengali and Han Lian Cao in Mandarin.

Figure 17. *Eclipta alba* plant *(*Adapted from Timalsina and Neupane, 2021)

It is considered as a weed of ethnomedicinal significance, commonly used for anti-microbial and anti-oxidant effects, as well as for liver, skin, respiratory, gastrointestinal and heart diseases (Feng et al., 2019; Jahan et al., 2014; Panghal et al., 2011; Rangineni et al., 2007; Singh et al., 2001, 1993; Udayashankar et al., 2016; Uddin et al., 2010). This species of herb grows in tropical warm climate and distributed all over in India, Nepal, China, Brazil, Thailand and other parts of the world (Figure 17).

2.4.1. Traditional uses of Eclipta alba

Eclipta alba is well used for its many ethnomedicinal values and 80% of Nepalese population depends on herbal plants for disease remedies and because of the sub-tropical climate Nepal is a source of many medicinal herbs (Figure 18). In local Nepali language, *Eclipta alba* is referred

to as *Nash jhar*. The aerial parts of the herb, shoot and leaf is used for cuts, wounds and for jaundice (Adhikari et al., 2019; Gautam, 2013; Rai, 1970). The use of the extract to treat snake bite is well known in China, Brazil and some parts of India (Diogo et al., 2009; Gupta and Peshin, 2012; Jeeva et al., 2006; Pithayanukul et al., 2004).

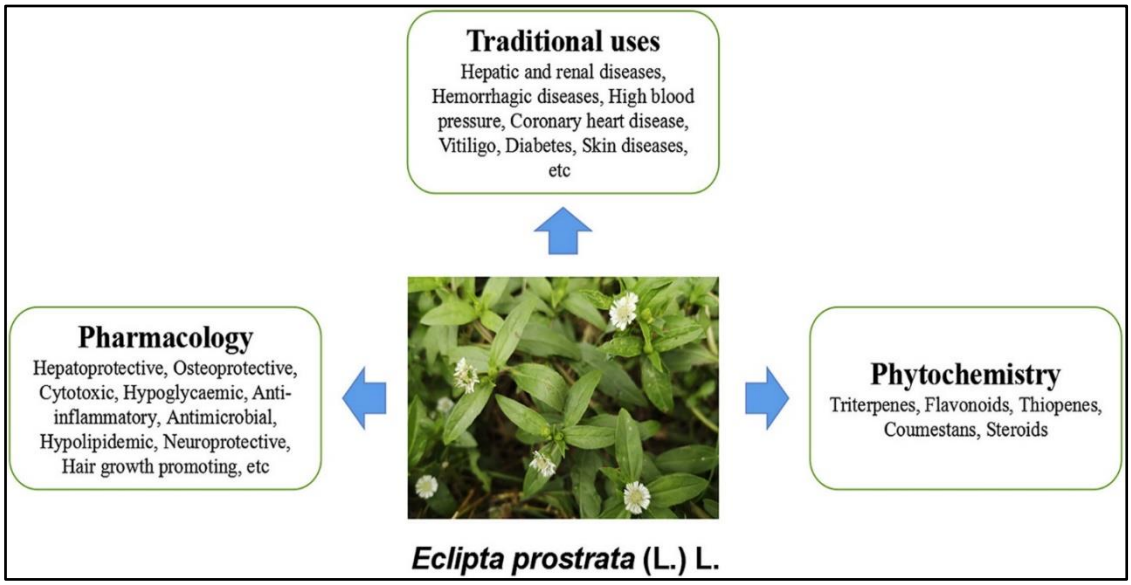


Figure 18. Ethnomedicinal uses of *Eclipta alba* (Adapted from Feng et al., 2019)

A study was conducted on a population of Uttar Pradesh, India which reported the ethnomedicinal effect of the plant extract on haemorrhoids, high blood pressure, jaundice and liver enlargement (Khan and Khan, 2008). The herbal juice is also used as a potent hair-promoting agent in various parts of India (Begum et al., 2014; Datta et al., 2009). Apart from these several other reports suggests that the herbal extract is known for its ethnomedicinal values against gastrointestinal disorders, asthma, fever, spleen and liver enlargement (Jahan et al., 2014).

2.4.2. Effects of Eclipta alba on various cancer cells

The juice of *Eclipta alba* shows anti-migratory and anti-angiogenic properties on various cancer cells (Lirdprapamongkol et al., 2008).

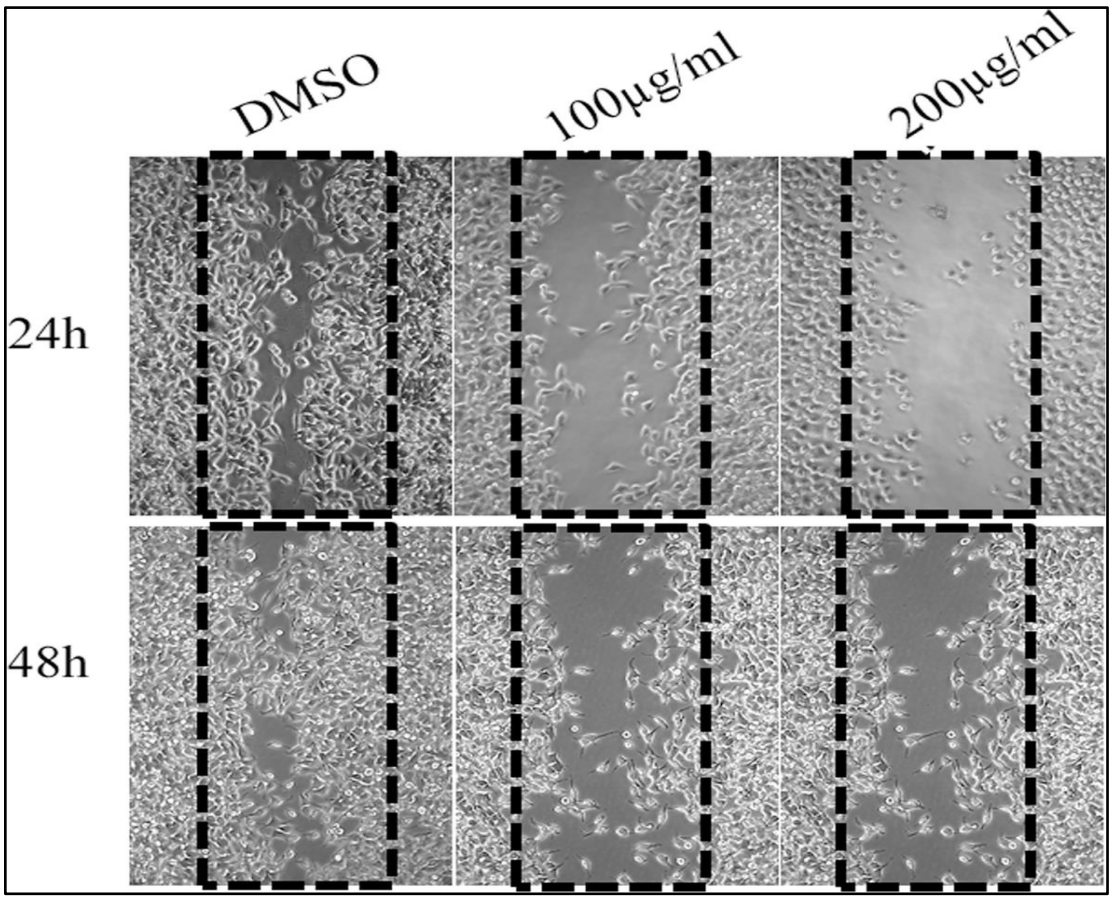


Figure 19. Phase contrast image of methanolic *Eclipta alba* extract suppressing migration of HCT-116 colon cancer cells (Adapted from Nelson et al., 2020).

Methanolic extract of *Eclipta alba* is reported to show anti-tumor effects against colorectal cancer cells HCT-116, as well as inhibits the migration of HCT-116 cells at a dose of 200 µg/ml (Nelson et al., 2020) (Figure 19).

Hydro-alcoholic extract of *Eclipta alba* inhibited cell proliferation, induces apoptosis and anti-invasive effects in HepG2, A498 and C6 glioma cell lines in a dose-dependent manner (Figure 20). The extract is considered as potent multi-drug resistant agent and an adjunct novel therapeutic for hepatocellular carcinoma via down-regulation of MMP and NF-kB signaling pathway (Chaudhary et al., 2014).

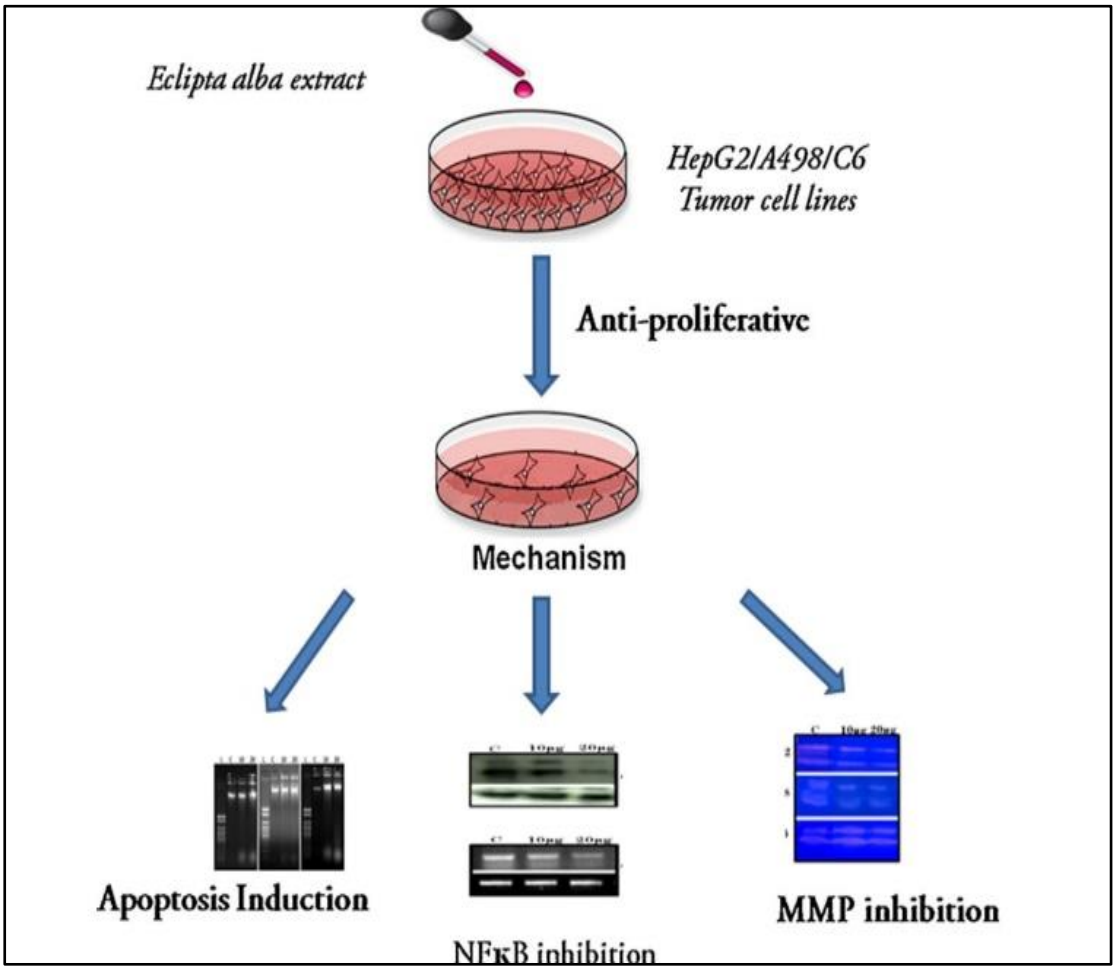


Figure 20. Anti-tumor effect of *Eclipta alba* on cancer cell lines (Adapted from Chaudhary et al., 2011)

Eclipta alba extract is reported for its anti-cancer effect on oral cancer cells (Jayaraman et al., 2022). The extract inhibits migration of oral cancer cells SCC-9, HSC-3 and TW2.6 by the down-regulation of matrix- metalloproteinase (MMP) enzyme and levels of phosphorylated extracellular signal-regulated kinase 1/2 (Liao et al., 2018). The whole plant extract demonstrated apoptosis and growth inhibition on HCC-827, i.e. Human lung epithelial adenocarcinoma cell line (N. et al., 2012).

Alcoholic extract of *Eclipta alba* induces apoptosis via disrupting mitochondrial membrane and DNA damage on MCF 7 and MDA-MB-231 breast cancer cells in a dose-dependent manner (Yadav et al., 2017). Another study reported, the chloroform fraction of *Eclipta alba* induces cytotoxicity against breast cancer cells by up regulation of Hsp60, hence disrupts

mitochondrial potential and down regulating anti-apoptotic protein XIAP both *in-vivo* and *in-vitro* (Arya et al., 2015).

2.4.3. Phytochemical constituents of Eclipta alba

Active principles of extract includes coumestans, alkaloids, flavonoids, alkaloids, sterols, terpenoids, fatty alcohols, volatile oils, saponins, sesquiterpene lactones, thiophenes, phenolic acids and polyacetylenic compounds (Table 1) (Feng et al., 2019).

S.No.	Parts	Chemical constituents
1	Leaves	Wedelolactone[1.6%], Desmethylwedelolactone, Desmethyl-wedelolactone-7-glucoside, stigmasterol
2	Roots	Hentriacontanol, Heptacosanol & Stigmasterol, Ecliptal, Eclalbatin.
3	Aerial parts	β-amyrin & Luteolin-7-0-glucoside, Apigenin, Cinnaroside, Sulphur compounds, Eclalbasaponins I-VI
4	Stems	Wedelolactone
5	Seeds	Sterols, Ecliptalbine (alkaloid)
6	Whole plant	Resin, Ecliptine, Reducing sugar, Nicotine, Stigmasterol, Triterpene saponin, Eclalbatin,Ursolic acid, Oleanolic acid.

Table 1. Phytochemical constituents of *Eclipta alba* (Adapted from Jaglan and Gill, 2013)

2.5. Wedelolactone: The active coumestan *of Eclipta alba*

Coumestan is coumarin derivatives and an organic compound, and mostly found as a central core in many natural metabolites found in plant products. Wedelolactone, demethylwedelolactone and demethylwedelolactone-7-glucoside are the major coumestans found in the extract, reported for their anti-cancer, anti-bacterial, anti-hepatotoxic and trypsin inhibitory effects (Jadhav et al., 2009; Uddin et al., 2010). The chemical structure of wedelolactone is composed of three hydroxyl groups at carbons C-1, C-8 and C-9 and one methoxy group at C-3 carbon (Figure 21) (Pôças et al., 2006).

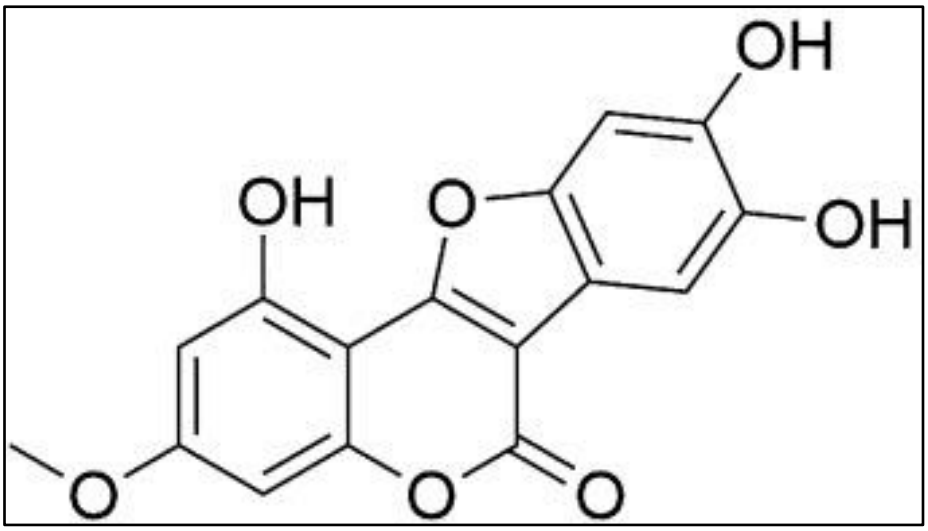


Figure 21. Chemical structure of wedelolactone (Adapted from Zhu et al., 2019)

2.5.1. Pharmacological effects of Wedelolactone

Wedelolactone is a multi-target active compound that has cytotoxic potency towards cancer cells. It has various pharmacological effects like anti-cancer, anti-hepatotoxic, anti-inflammatory, anti-phospholipase, etc. According to a recent study, wedelolactone acts as a proteasome inhibitor in breast cancer cells (Nehybová et al., 2017). Proteases like chymotrypsin and trypsin are involved in various diseases like cancer. Wedelolactone and demthylwedelolactone, the coumestan of *Eclipta alba* are reported as potent trypsin inhibitors (Syed et al., 2003). Anti-microbial properties of wedelolactone is also reported in which wedelolactone significantly inhibited the growth of *Staphylococcus epidermidis* and *Salmonella typhimurium* (Dalal et al., 2012). The excessive production of free radicals like reactive oxygen and nitrogen species (ROS/NOS) are the major cause of development of different diseases, including cancer. Wedelolactone is proved as a powerful anti-oxidant which can guard mesenchymal stem cells from ROS induced damage (Li et al., 2020).

2.6. The inter-relation between inflammation and leukemia

Inflammation contributes to the production of angiogenic factors that in return allows proliferation of cancer cells along with the suppression of cell death mechanisms (Grivennikov et al., 2011; Puissant and Medyouf, 2022; Rashighi and Harris, 2017).The inter-link between leukemia and inflammation has been well studied (Récher, 2021). Infiltration of B-lymphocytes in the bone marrow and lymph nodes is one of the major cause of accumulation of CLL cells leading to the establishment of chronic leukemia. Inflammatory chemokines are secreted by inflamed tissues that leads to the migration and invasion of neutrophils and monocytes, which further creates a microenvironment for the trafficking of lymphocytes (Bleul et al., 1996). Chemokines like CCL3 and CCL4 are produced by mature hematopoietic cells which acts as a chemoattractant for inflammatory cells like neutrophils, mast cells and macrophages (Menten et al., 2002). Furthermore, SDF-1 chemokine signals via CXCR4 that leads to B-lymphopoeisis (Nagasawa et al., 1994). Blood obtained from CLL patients revealed an increased population of Tie-2 expressing monocytes with altered genes involved in inflammation and phagocytosis (Maffei et al., 2013). A study reported that the blood plasma of patients with childhood CLL shows evidence of chronic inflammation with a significant increase in IL-2, IL-10 and IL-17a (Ariffin et al., 2017).

2.6.1. Role of inflammatory cytokines in the pathophysiology of leukemia

Inflammatory cytokine TNF-α activates the pro-inflammatory regulator NF-kB, while the anti-inflammatory cytokine IL-10 down-regulates NF-kB (Raza and Shata, 2013). Increase in TNF-α also increases the micro-RNA *mir-155* in the marrow cells of leukemic patients which causes the B-cell hyperproliferation (Chen et al., 2010; Costinean et al., 2006). AML cells are reported to release various cytokines like IL-1β, IL-3, IL-1α, TNF-α and GM-CSF that further allows the progression of tumours (Figure 22).

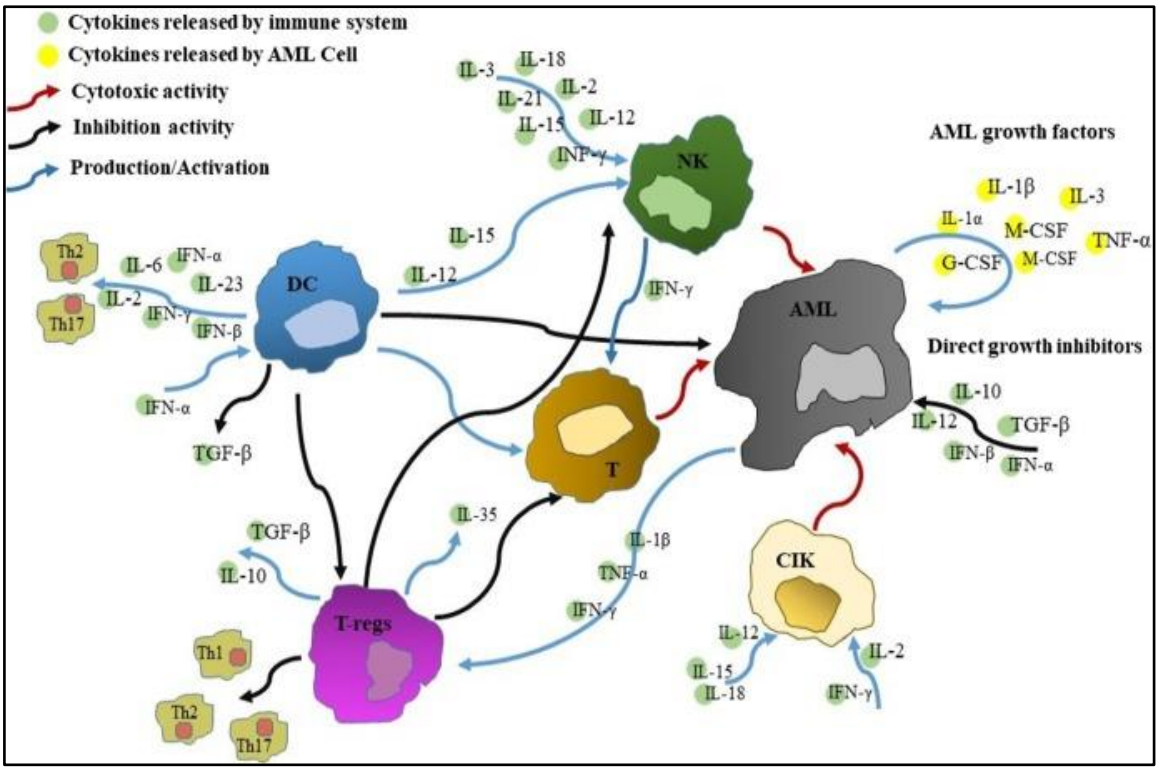


Figure 22. Inflammatory cytokines released by AML cells (Adapted from (Karimdadi Sariani et al., 2021)

2.7. NF-kB signaling pathway: A hallmark of cancer

NF-kB pathway is composed of both canonical and non-canonical signaling pathways.

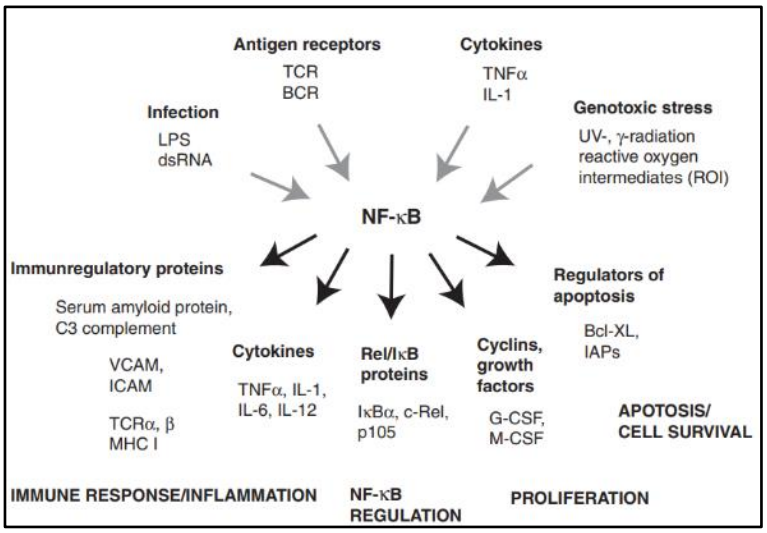


Figure 23. Different stimuli and targets of NF-kB gene (Adapted from Oeckinghaus and Ghosh, 2009)

The classical canonical pathway is regulated by cell surface receptors like the TLR-family, BcR, TNFR, RANK, CD-30L and IL-1R; cytokines like TNF-α and IL-1 (Oeckinghaus et al., 2011) (Figure 23). The targets of NF-kB are key players of inflammation and immune response like MHC molecules. The target genes also includes anti-apoptotic proteins like Bcl family as well as proteins that regulate proliferation like cyclins, hence ensuring survivability and proliferation of cancer cells. The NF-kB transcription factor family is made of five major proteins: p65 (RelA), RelB, c-Rel, NF-kB1 (p105/p50 dimer) and NF-kB2 (p100/p52 dimer) (Bonizzi and Karin, 2004).

Activation of this pathway is dependent on the IKK family that consists of IKKα, IKKβ and IKKγ or NEMO (Gasparini et al., 2014). During the activation of IKK complex, the downstream protein IkBα gets phosphorylated and goes through ubiquitin-mediated proteosomal degradation (Figure 24) (Oeckinghaus and Ghosh, 2009). Post activation, the homo-or hetero-dimers translocate to the nucleus, for transcription to begin on the target genes.

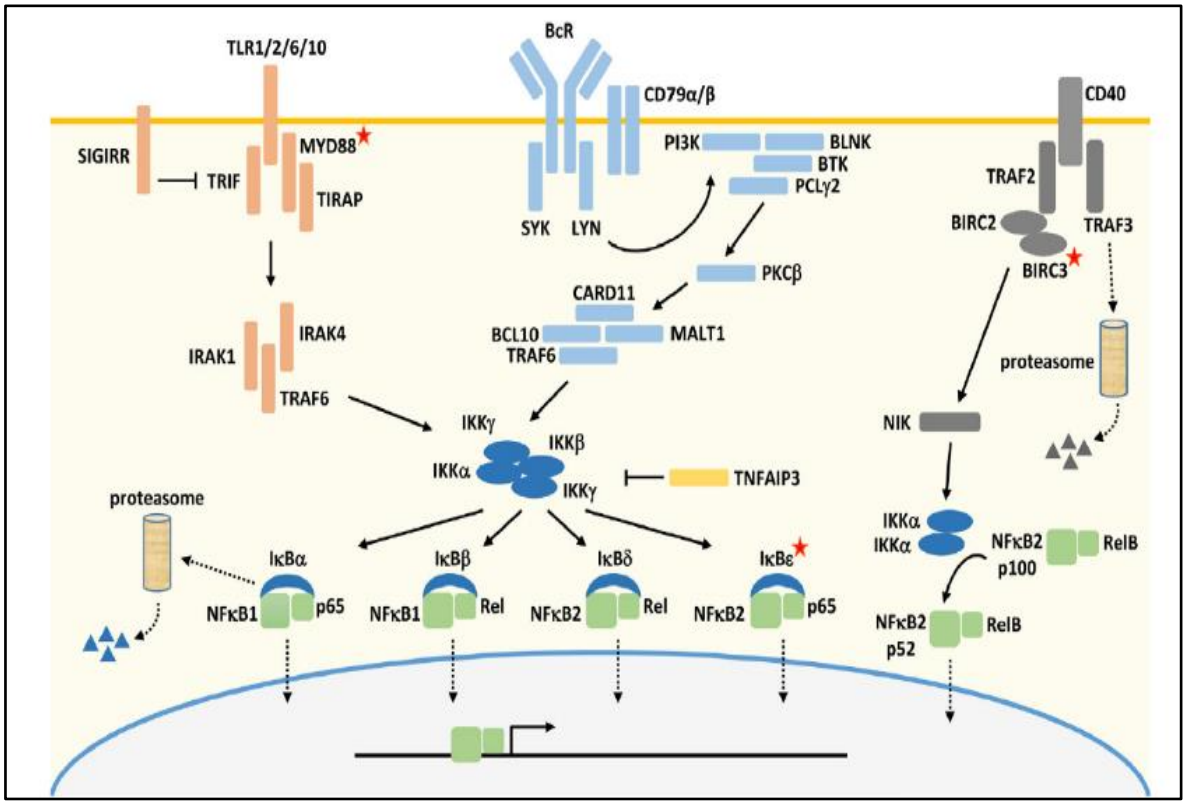


Figure 24. Graphical representation of NF-kB pathway (Adapted from Mansouri et al., 2016)

In unstimulated condition, the homo-or hetero-dimers are sequestered in the cytosol as IkBα masks the nuclear localization signal (NLS) of the transcription factors. NF-kB over-expression stimulates the increase in expression of adhesion molecules like E-selectin, VCAM-1 and ICAM-1 that increases leukocyte transmigration. It also further elevates the expression of the inflammatory molecules COX-2 and iNOS (Ghosh and Hayden, 2008).

2.7.1. Role of NF-kB signaling pathway in leukemic pathophysiology

NF-kB signaling pathway is considered to be a hallmark of cancer as it plays a major role in cancer cell survival, proliferation as well as inflammation (Breccia and Alimena, 2010; Hanahan and Weinberg, 2011; Mantovani et al., 2008).

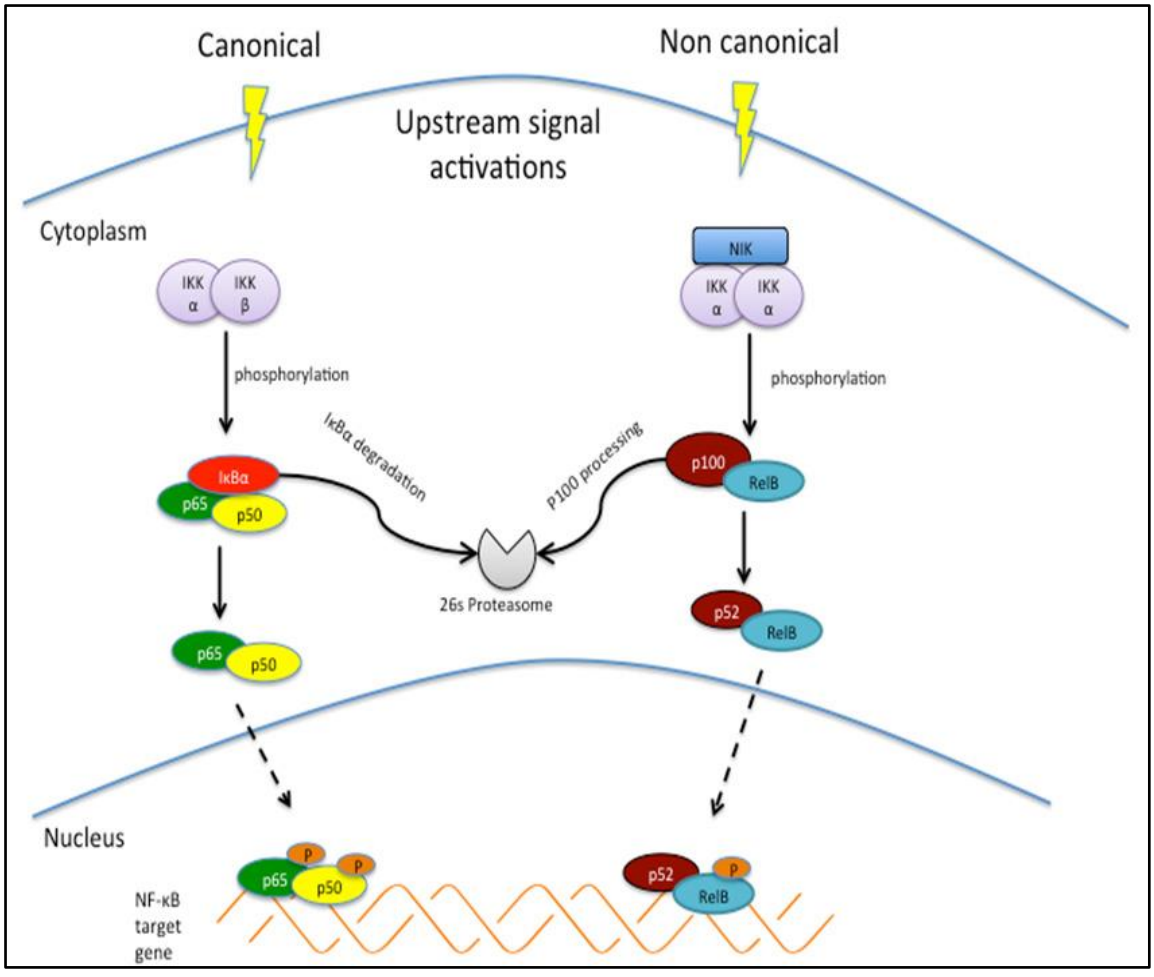


Figure 25. Canonical and non-canonical NF-kB signaling pathway (Adapted from Zhou et al., 2015)

Over-expression of NF-kB pathway with TCL1 oncogene is reported to be responsible for aggressive CLL, as studied in *Eμ-TCL1* mice (Costinean et al., 2006). Hence, aberrantly expressed NF-kB pathway plays a major role in the initiation and promotion of various cancers, like leukemia and (Rodríguez-Vicente et al., 2017; Staudt, 2010; Villamor et al., 2011). According to a study, over-expression of NF-kB could be a critical role in the development of childhood ALL as hyper-phosphorylated form of IkBα was observed leading to its subsequent degradation and activation of NF-Kb in the bone marrow samples. NF-kB acts as a switch which ensures the survivability of ALL cells by suppressing cellular death and enhancing cell proliferation (Kordes et al., 2000; Kumar et al., 2014). 40% cases of AML reported aberrantly activated NF-kB pathway in the marrow cells due to constant activation of IKK leading to IkBα degradation (Figure 25). Various nurse cells supports the microenvironment of CLL by activation of different receptors like CD40 and its overexpression leads to aberrant canonical and non-canonical NF-kB activation (Donnell et al., 2023; Pekarsky et al., 2010). Hence, enough reports suggests NF-kB pathway can act as a selective leukemic therapeutic target for the amelioration of leukemia (Acute et al., 2013; Garding et al., 2013; Pan et al., 2017).

2.7.2. Natural therapeutics that targets NF-kB pathway in leukemia

There are over 750 inhibitors of NF-kB pathway including both synthetic and natural, as it is an excellent therapeutic target for various diseases as well as cancer. Exisulind is a derivative of the natural Sulindac, which is reported to inhibit IKK phosphorylation and activation (Bremner and Heinrich, 2002; Gilmore and Herscovitch, 2006; Nam, 2006). Flavopiridol is a herbal medicine used in India which acts as NF-kB inhibitor by modulating the activation of IKK and promoting cell death in AML (Amir Fathi, 2011). It has been used in Phase II trial and reported to decrease blast population in AML by over 80% (Karp et al., 2007). According to a study, combination therapy of curcumin and carnosic acid can lead to apoptotic cell death in human leukemic cells (Pesakhov et al., 2010). Genistein is a natural polyphenol and an

inhibitor of protein tyrosine kinases, induces apoptosis and cell cycle arrest in leukemic cells (Shen et al., 2007).

2.7.3. Inhibitory effect of Eclipta alba and wedelolactone on NF-kB pathway in cancer

As reported by Harshita et al., ethanolic *Eclipta alba* extract at 500mg/kg suppressed NF-kB pathway as well as ROS levels in liver cancer animal model. Inhibition of the NF-kB pathway showed an increase in apoptosis and anti-cancer effects in the animal model (Chaudhary et al., 2014, 2011)

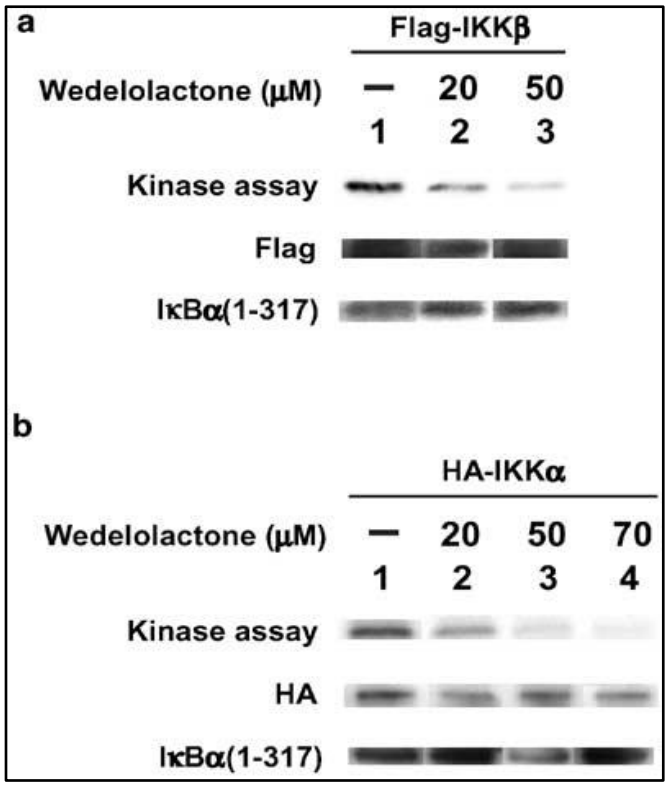


Figure 26. Inhibitory effect of wedelolactone on NF-kB molecules (Adapted from Kobori et al., 2004)

Wedelolactone is reported to suppress NF-kB pathway in various cancer models as anti-NF-kB translocation and apoptotic agent (Liu et al., 2016; Yuan et al., 2013; Zhu et al., 2019b). Wedelolactone is reported as a "potent IKK inhibitor" in cultured cells, which supress the NF-kB pathway and in turn inhibits LPS-inhibits caspase-11 expression (Figure 26) (Kobori et al.,

2004; Li et al., 2003). A study confirmed that wedelolactone is efficient in regulating IKK in NF-kB pathway and ameliorate inflammation and oxidative stress in MPC-5 cells (Figure 27) (Zhu et al., 2019a).

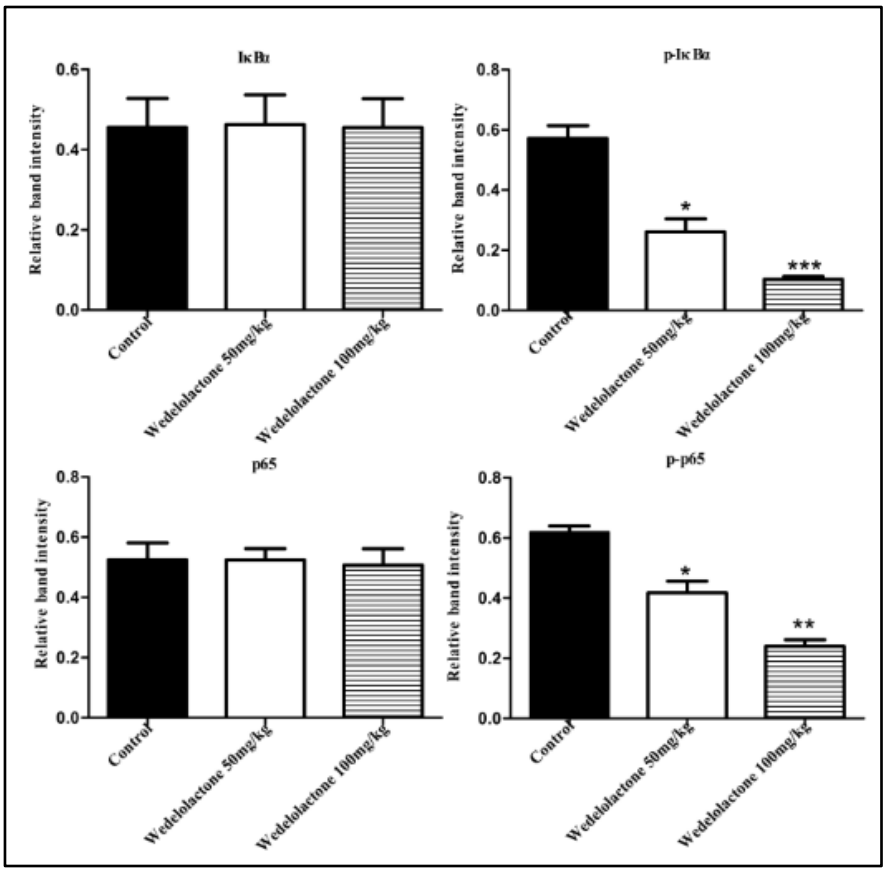


Figure 27. Effect of wedelolactone at 50mg/kg and 100mg/kg dose on NF-kB molecules (Adapted from Luo et al., 2018)

2.8. The NLRP3 inflammasome: Its structure and function

In the year 2002, inflammasome was first identified by Dr. Tschopp in which he demonstrated it as “caspase-activating complex” (Martinon et al., 2002). Inflammasomes are multi-protein complexes formed in the cytosol as it mediates inflammatory and immune response. The different categories of nucleosomes are NLRP1, NLRP3, NLRC4, NLRP6 and AIM2 inflammasomes.

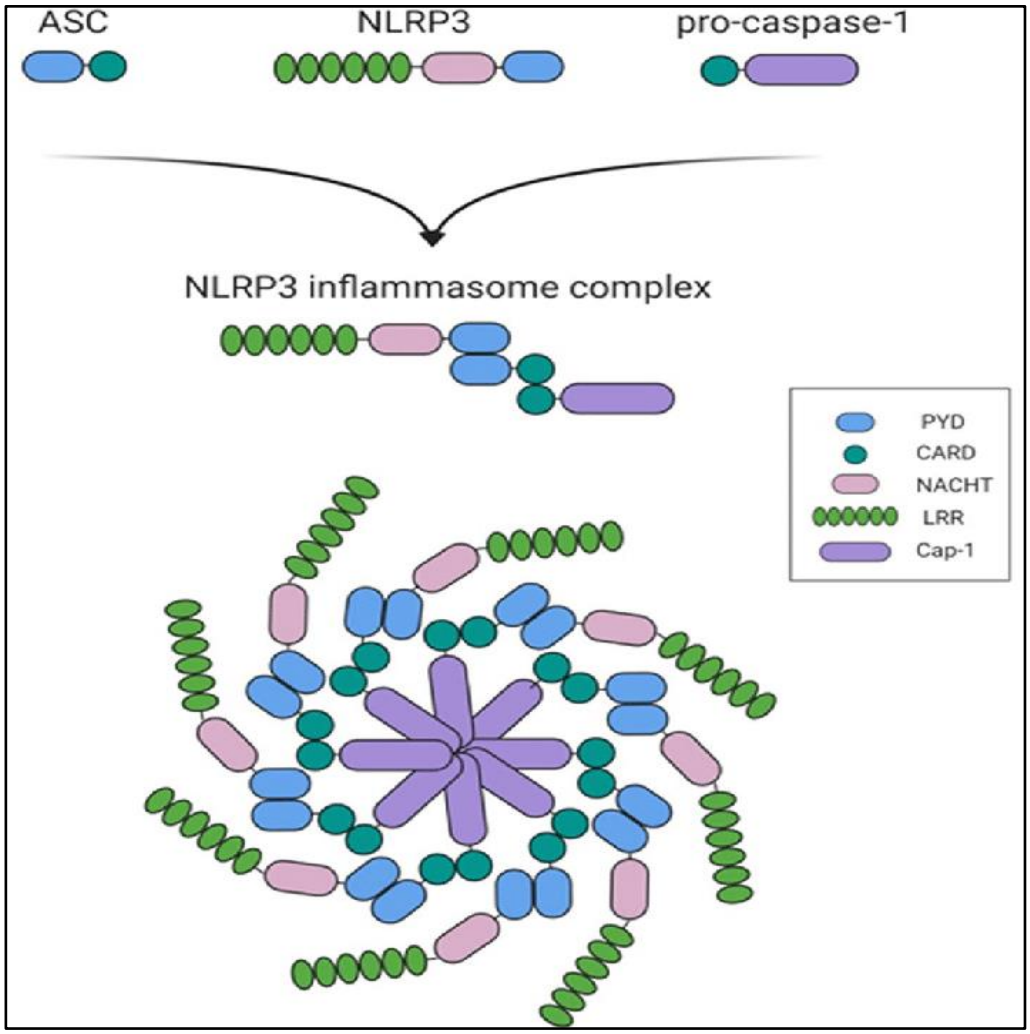


Figure 28. Schematic representation of NLRP3 inflammasome (Adapted from Kyung et al., 2021)

The NOD-like receptor protein 3 (NLRP3) is one of the well-studied inflammasome and it is present in an inactivated state in cytosol (Dagenais et al., 2012; Mariathasan et al., 2004). Inflammasomes are made of NOD-like receptors (NLR); apoptosis-associated speck like protein (ASC) and caspase-1 (Figure 28) (Xu et al., 2019). NLRP3 consists of an amino terminal pyrin domain (PYD), leucine-rich repeat (LRR) domain in the carboxyl terminal and nucleotide-binding and oligomerization domain in centre (NACHT). The adaptor protein ASC is made of two domains: PYD domain in amino terminal and carboxy-terminal caspase recruitment domain (CARD) and pro-caspase1. Once stimulated the NACHT domain is

involved in ATPase activity needed for oligomerization and the PYD domain interacts with the PYD domain of ASC and together forms the NLRP3 complex (Figure 29) (Duncan et al., 2007; Hafner-bratkovi et al., 2018; Razmara et al., 2002).

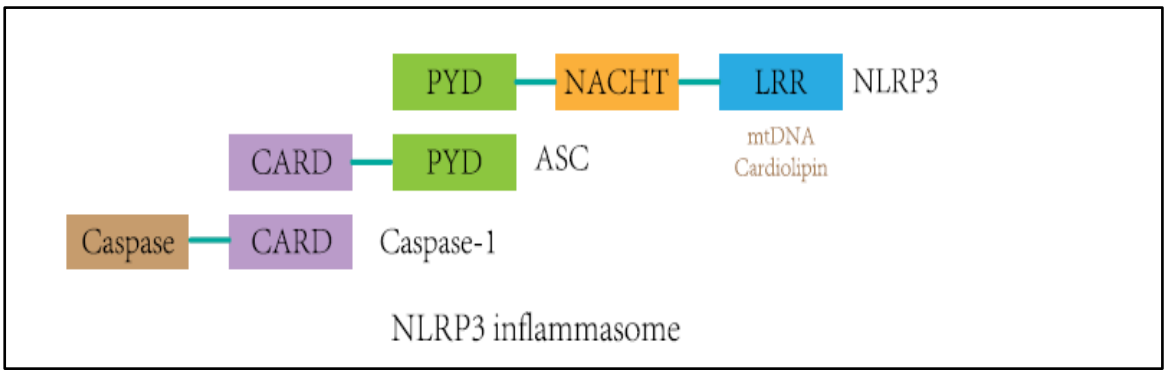


Figure 29. Domains of NLRP3 inflammasome complex (Adapted from Xu et al., 2019)

Inflammasomes are the key regulators of inflammation and it can moreover activate the pro-inflammatory cytokines IL-1β and IL-18. NLRP3 inflammasome is mostly expressed in inflammatory cells like macrophage, neutrophils and dendritic cells. Activation of NLRP3 can be carried out by the auto-phosphorylation of TLR which leads to NF-kB activation and further cleavage of procaspase-1 into caspase-1 to lead to maturation and secretion of IL-1β and IL18 (Jo et al., 2015; Lamkanfi and Dixit, 2009; Place and Kanneganti, 2017).

2.8.1. Role of NLRP3 inflammasome in leukemia

NLRP3 inflammasome is considered as a "rising star" for the studies in regards of haematological malignancies as according to evidences, NLRP3 plays an important role in the expansion of HSPCs (Ratajczak et al., 2020). Persistent activation of NLRP3 leads to over-expression of IL-1β, which on the other hand increases inflammation and that can result to carcinogenesis (Drexler et al., 2012; Fink and Cookson, 2005; Guarda et al., 2011; He et al., 2017; Li et al., 2017). Leukemia is an inflammation-driven carcinogenesis and the over-expression of NLRP3 leads to high levels of pro-inflammatory cytokines like IL-1β and IL-18 that in turn stimulates leukemic cells for the secretion of IL-1β (Figure 30) (Urwanisch et al., 2021).

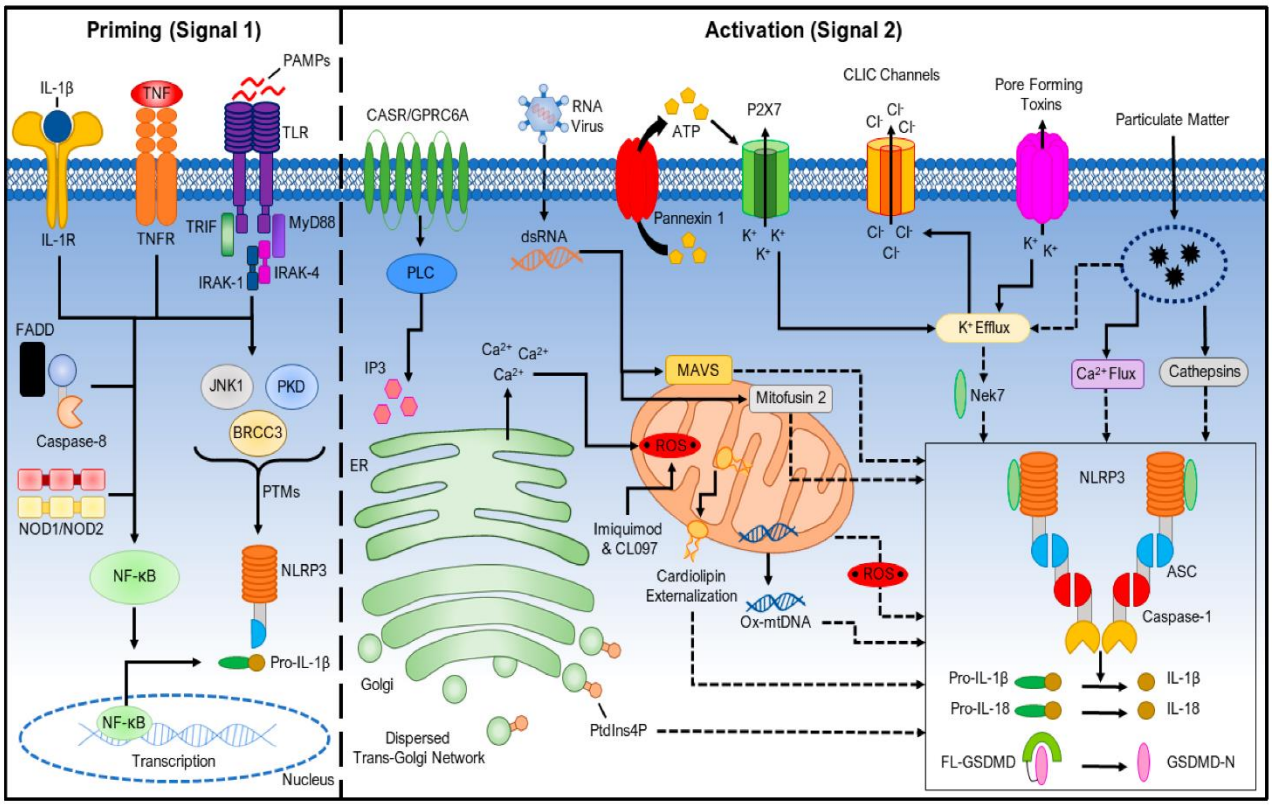


Figure 30. NLRP3 inflammasome signaling pathway and its activation (Adapted from Kelley et al., 2019)

A study reported an increase in NLRP3 expression in the bone marrow mononuclear cells (BMNC) and peripheral blood mononuclear cells (PBMC) in AML, involved in expansion of Th cell subset (Azizi et al., 2015; Hamarsheh et al., 2020; Jia et al., 2017). Mutations on oncogenic *KRas* is highly common in leukemia and according to a report it induces NLRP3 activation as well as increases ROS production in leukemia (Hamarsheh et al., 2020).

2.8.2. Inhibitory role of wedelolactone on NLRP3 inflammasome

Different reports claims the inhibitory effect of herbs and various active compounds on regulating NLRP3 inflammasome in different disease models (Pan et al., 2016; Yoon et al., 2015). Hao et al., reported in a study that wedelolactone can supress NLRP3 inflammasome by phosphorylating Ser/Thr domain and inhibit downstream caspase-1 and IL-1β production by blocking both ASC oligomerization and speck formation (Figure 31) (Pan et al., 2020).

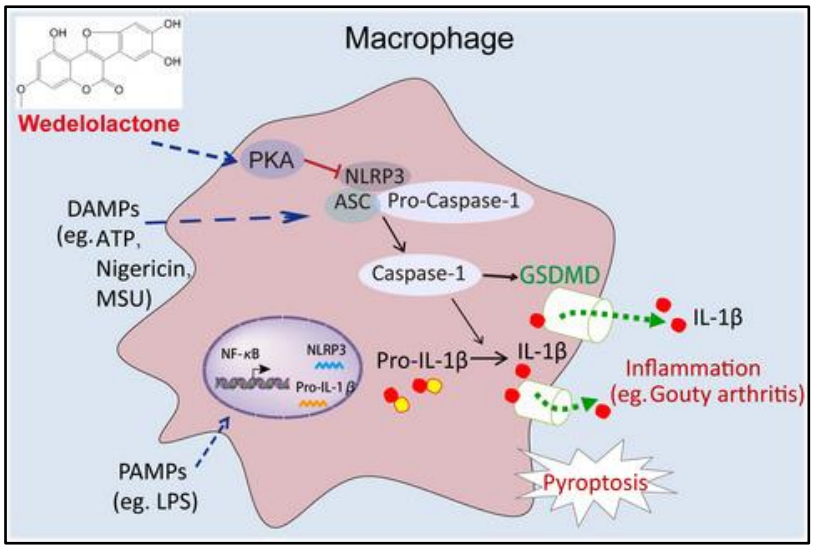


Figure 31. Suppressive role of wedelolactone on NLRP3 inflammasome (Adapted from Pan et al., 2020)

At a dose of 50mg/kg wedelolactone supressed NLRP3 inflammasome activation as well as inhibited the downstream caspase-1 activation and production of pro-inflammatory IL-1β in dextran sodium sulphate (DSS) induced mice model (Figure 32) (Lin et al., 2018; Wei et al., 2017).

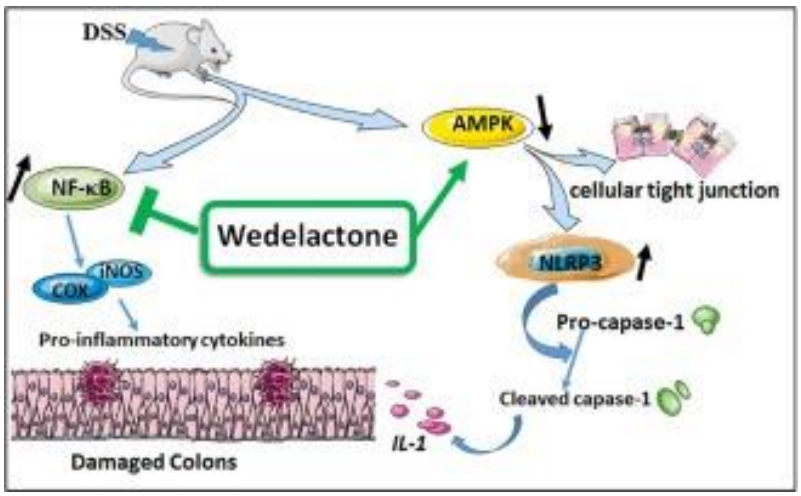


Figure 32. Protective effect of wedelolactone on DSS mice via NLRP3 pathway (Adapted from Wei et al., 2017)

Nai et., stated in his research that wedelolactone is a "caspase-11 inhibitor" and when used at a dose of 30mg/kg for 7 days it led to the reduction of caspase-1 activation and IL-1β maturation in renal fibrosis (Miao et al., 2019).

2.9. The inflammatory COX-2/iNOS/PGE2 pathway

Cyclooxygenases (COXs) are group of enzymes which catalyzes the conversion of arachidonic acids to prostaglandins. This occurs step by step, as firstly arachidonic acid is converted to prostaglandin G2 (PGG2) by phospholipase A2 (PLA2). Secondly, PGG2 is converted into prostaglanding H2 (PGH2) by cyclooxygenase activity. Further PGH2 is converted to different prostaglandins like PGE2, PGD2, PGJ2 and thromboxanes (Figure 33). Prostaglandin E2 (PGE2) mainly plays a critical role in vasodilation, inflammation, pain and fever which leads to pro-carcinogenic effect (Chandrasekharan and Simmons, 2004). COX-2 is located on human chromosome 1q25.2-q25.3 and its promoter displays NF-kB and IL-6 response element (Hashemi Goradel et al., 2019).

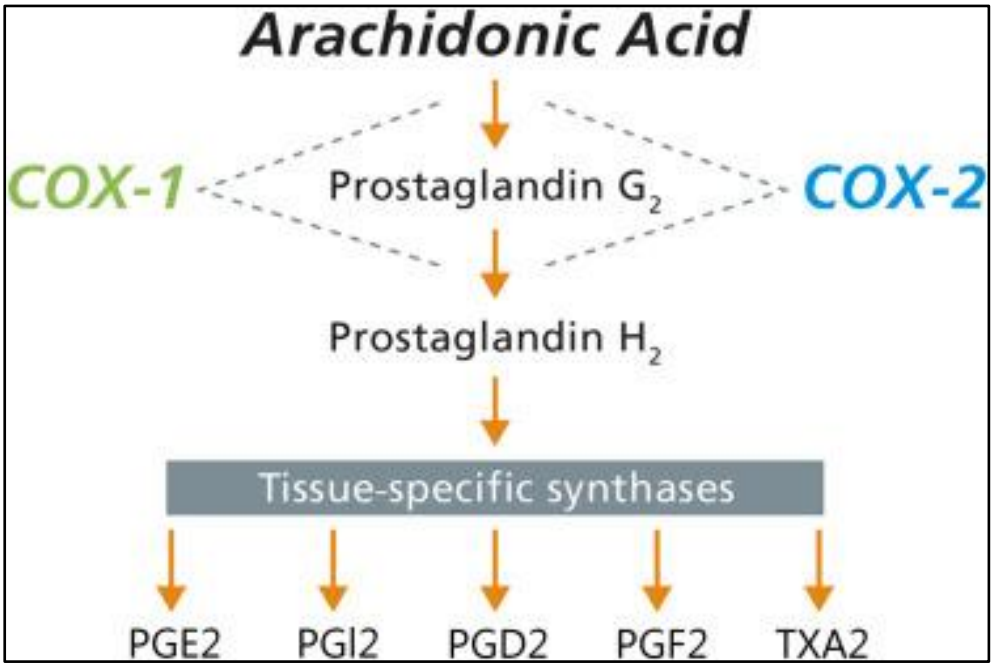


Figure 33. The COX pathway (Adapted from Nørregaard et al., 2015)

2.9.1. Role of COX-2 in cancer progression and leukemia

COX-2 is over-expressed in various cancers like breast, colorectal, pancreas and haematological malignancies (Dubois et al., 2018; Ristima et al., 2002). COX-2 influences the pathophysiology of cancer as it promotes cell proliferation, angiogenesis, inflammation, invasion, metastasis and suppresses apoptosis (Diederich et al., 2010; Sobolewski et al., 2010).

COX-2 is secreted by cancer cells in the tumour microenvironment, cancer associated fibroblasts (CAF) and macrophage type 2 (M2) cells (Figure 34). NF-kB activation plays the role of an upstream modulator of COX-2 in cancer (Evans and Kargman, 2004; Hashemi Goradel et al., 2019; Wang et al., 2007). According to reports, NSAIDs that blocks the COX-2 expression can induce cell death via mitochondrial cytochrome-c release in esophageal cancer cells (Li et al., 2001; Rottey et al., 2006).

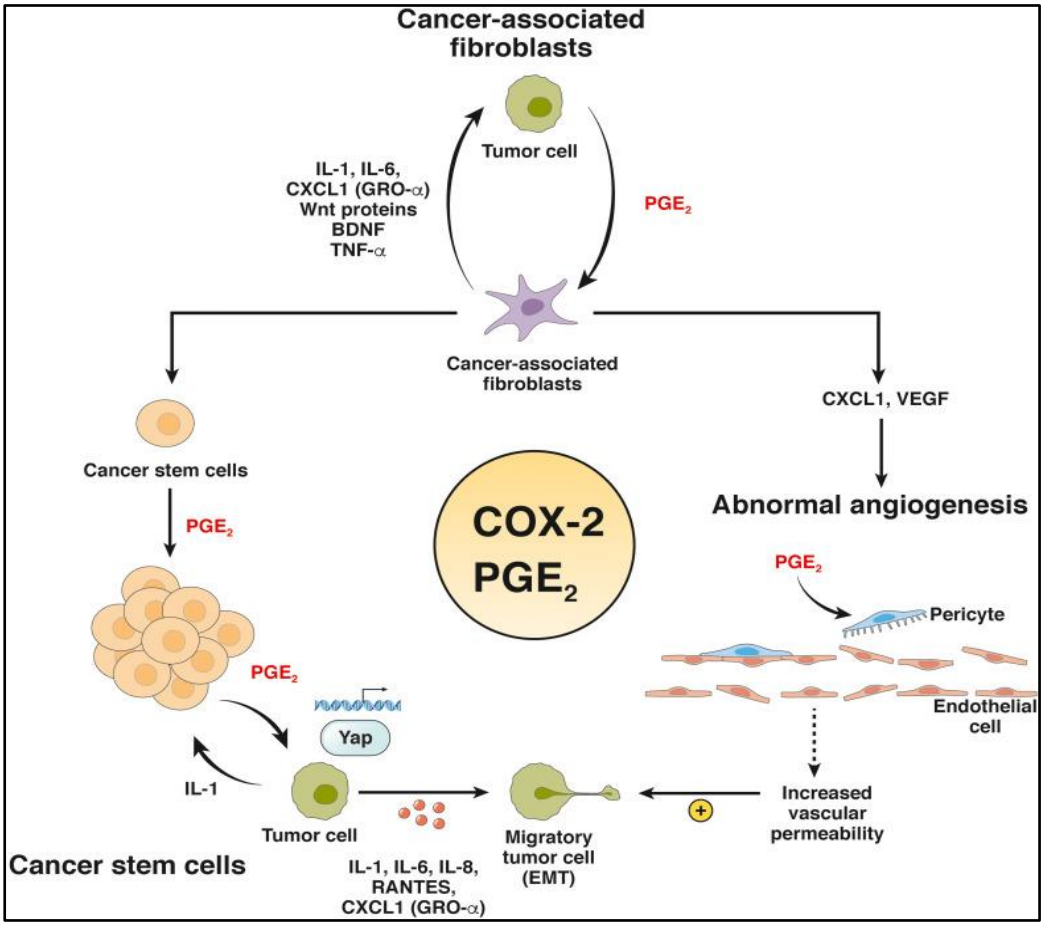


Figure 34. Role of COX-2 and PGE2 in maintaining TME (Adapted from Wang et al., 2021)

Hematopoietic malignancies like leukemia shows constitutive activation of COX-2 which in turn allows cancer cell survival and proliferation (Bernard et al., 2008; Ohsawa et al., 2006). Elizabeth et al., reported an increased expression of COX-2 and prostaglandins in CLL cells possibly promoting CLL cell expansion (Figure 35) (Ryan et al., 2006).

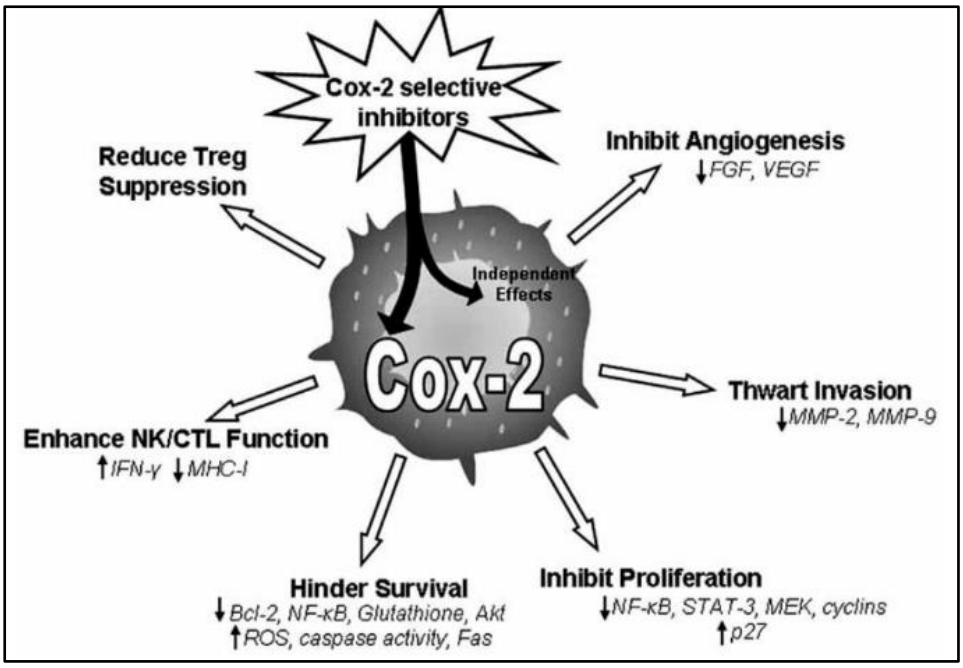


Figure 35. COX-2 in haematological malignancies

Release of IL-1β by cancer cells induces the mesenchymal stromal cells (MSC) co-cultured with AML cells to express high levels of COX-2 and PGE2 which contributes to the TME and microenvironment-mediated chemoresistance in leukemia (Carter et al., 2019). Paola et al., reported an increase of COX-2 mRNA expression by 38-fold in B-CLL cells as compared to normal $CD19^{+}$ B-cells while providing resistance to cell death (Secchiero et al., 2005). High levels of COX-2 is reported in the bone marrow cells of CML, which also significantly co-relates to lesser survivability of leukemic patients (Giles et al., 2002).

2.9.2. Role of PGE2 in regulating apoptosis and angiogenesis

PGE2 can be considered as the key player in cell proliferation being the down-stream molecule of COX-2 (Nakanishi and Rosenberg, 2013; Rama pai et al., 2002). Invasion and metastasis of cancer cells are regulated by prostaglandins, MMPs and other cytokines (Grivennikov et al., 2011). PGE2 regulates cell death by inhibition of mitochondrial cytochrome-c release and increased expression on anti-apoptotic protein bcl-2 as well as via inhibition of Fas-mediated apoptosis (Cells et al., 2001; Wang et al., 2005). Angiogenesis or blood vessel formation is an important step in carcinogenesis and VEGF plays an important role as a pro-angiogenic

molecule. PGE2 increases angiogenesis by stimulating the production of VEGF whereas VEGF stimulates production of COX-2 as a positive feedback mechanism (Pelus and Hoggatt, 2011; Rottey et al., 2006; Zarghi and Arfaei, 2011).

2.9.3. Suppressive effect of Eclipta alba and wedelolactone on COX-2 pathway

Whole plant extract of *Eclipta alba* showed significant anti-inflammatory effect while down-regulating the mRNA expression of iNOS and COX-2 in RAW 264.7 (Bov et al., 2021; Kim et al., 2017; Ryu et al., 2013; Tewtrakul et al., 2011) Fang et al., reported that wedelolactone significantly reduces the expression of COX-2 and its downstream effector molecules PGE2 and iNOS in RAW 264.7 cells, which makes them potential anti-inflammatory targets for cancer therapy (Yuan et al., 2013).

2.10. Hypothesis of the thesis

Increasing human population is one of the major cause for the severe increase in environmental pollutants and carcinogens due to various human occupational and food habits. Conventional chemotherapy is expensive for a mass of population mostly in developing or under-developed countries and it has severe toxic side effects on human body. Mother Nature has been an armamentarium of herbal products, which are a boon to the modern drug inventions and research. Keeping these few points in mind, our work was hypothesized to elucidate the therapeutic potentials of *Eclipta alba* an its active compound wedelolactone on ENU-induced leukemia. ENU is a carcinogen which is classified as NOC, and that can significantly cause leukemic development when induced in mice pups. *Eclipta alba* and wedelolactone has been reported in various cancer and disease models for therapeutic effects but no work has been conducted till date on a leukemic animal model. Hence, through this work we tried to unveil the action of these herbal products on ENU-induced leukemia in Swiss albino mice model.

Chapter 3.

Chapter 3.

Materials and methods

3.1. Chemicals and stains used in the research

- Acetonitrile (HPLC grade) Merck, Germany
- Acetone Merck, USA
- α-napthyl acetate Sigma-Aldrich, USA
- β-mercaptoethanol Himedia, India
- Brilliant Cresyl Blue Stain Sigma-Aldrich, USA
- Buffered phenol Sidma-Aldrich, USA
- Bovine Serum Albumin Sigma, Germany
- Cedarwood oil Merck, Germany
- Citric acid Merck, India
- Crystal violet stain Sigma-Aldrich, USA
- DPX Sigma, Germany
- DAPI shield Sigma-Aldrich, USA
- Drabkin's reagent Organo Biotech Lab, India
- Dextrose Merck, Germany
- Eosin Sigma-Aldrich, USA
- Ethanol Bengal chemicals, India
- ENU Sigma, USA
- FACS fluid Becton Dickinson and Company, USA
- Formic acid Merck, India
- Formaldehyde Merck, India
- Geimsa Himedia, India

- Glycerol — Merck, India
- Gelatin — Sigma-Aldrich, USA
- Glutaraldehyde — Lancaster, UK
- Glacial Acetic Acid — Merck, India
- Hydrogen peroxide — Sigma, Germany
- Haematoxylin — Sigma,-Aldrich, USA
- Heparin — Braun, USA
- Isopropyl alcohol — Merck, India
- Leucine Enkephalin — Sigma-Aldrich, USA
- Leishman stain — Sigma-Aldrich, USA
- Myeloperoxidase stain — Sigma-Aldrich, USA
- Methanol — Bengal chemicals, India
- Methylene Blue stain — Merck, India
- MTT — Sigma-Aldrich, USA
- NBT — Sigma, Germany
- Pararosaniline-sodium nitrite — Sigma-Aldrich, USA
- Paraffin wax — Merck, India
- Paraformaldehyde — Sigma, Germany
- Potassium Chloride — Merck, India
- Perl's Prussian Blue stain — Sigma-Aldrich, USA
- RBC-diluting fluid — International Biological Lab, India
- Schiff's reagent — Sigma-Aldrich, USA
- Silver Nitrate — Merck, India
- Sudan black B stain — Sigma-Aldrich, USA
- Sodium thiosulfate — Merck, India

- Sodium Chloride — Merck, India
- Sodium hydroxide — Merck, India
- Sorenson's phosphate buffer — Merck, India
- Toluidine Blue stain — Sigma, USA
- Tween-20 — Sigma, Germany
- Trypan blue — Merck, USA
- Wedelolactone — Sigma-Aldrich, USA
- WBC-diluting fluid — International Biological Lab, India
- Xylene — Merck, India

3.2. Plastic and Glassware used in research

- Beaker — US Plastic Corporation, USA
- Conical flask — Borosil, India
- Cell strainer (100µm) — Sigma, USA
- Cover glass — Blue Star, India
- Centrifuge tube — Borosil, India
- Cryo Box — Eppendorf, Germany
- Coplin jar — Sigma, USA
- FACS tube — Becton, Dickinson and Company, USA
- Funnel — Borosil, India
- Falcon tube — Falcon, USA
- Glass slides — Blue Star, India
- Gloves — Sigma, USA
- Mice water bottle — Sigma, USA
- Micro-centrifuge rack — Tesca Technologies, India

- Measuring cylinder Borosil, India
- Measuring flask Borosil, India
- Oral gavage needle (20g) Sigma, USA
- Pasteur pipettes Borosil, India
- Pipettes (all sizes) Thermo Fisher Scientific, USA
- Pipette tips Eppendorf, Germany
- Petri plate Borosil, India
- Polystyrene membrane filter unit Millipore, USA
- Syringe (26 gauge) Dispovan, India
- Squeeze bottles US Plastic corporation, USA
- Transwell membrane plate Corning, USA
- Test tube rack Naugra export, India
- Test tube Borosil, India
- Tissue culture flask Corning, USA
- Watch glass Borosil, India
- Whatman filter paper(25mm Grade1) Sigma, USA
- 75-mm culture plate Corning, USA
- 35-mm cell culture plate Corning, USA
- 60-mm cell culture plate Corning, USA

3.3. Equipment used for research

- CO2 incubator Heal Force HF212, Germany
- Centrifuge Remi, India
- Cell culture hood Yorko, USA
- Distillation plant (water) Borosil, India

- Fluorescence microscope Leica DM8
- Electronic weighing machine Wensar, India
- ESI-MS-Direct Infusion Waters, USA
- HPLC machine Shimadzu Prominence, Japan
- Haemocytometer chamber Rohem, India
- Flowcytometer Becton Dickinson and company, USA
- Freezer (-80˚C and -20˚C) Vestfrost, Denmark
- Hot Air Oven Biological Enterprises, India
- Inverted microscope Olympus, Japan
- Laminar Air Flow Ultra Biotech, India
- Light microscope Olympus, India
- Microwave LG, India
- Phase contrast microscope Dewinter, Italy
- Rotary Evaporator Sigma, USA
- Spectrophotometer Chemito Instruments, India
- Scanning electron microscope Hitachi, Japan
- Table-top centrifuge Hettich, Germany
- Vortex Accurate Scientific Instruments, India
- Water bath Bio Technics, India

3.4. Media and its chemical components

- α-MEM Invitrogen, USA
- Bovine Serum Albumin Invitrogen, USA
- Fetal Bovine Serum Lonza, USA
- L-Glutamine Invitrogen, USA

- Penicillin Sigma, USA
- RPMI-1640 Sigma, USA
- Streptomycin Sigma, USA

3.5. Antibodies used in research

- p65 Cell Signaling Technologies, USA
- cRel Cell Signaling Technologies, USA
- p50 Cell Signaling Technologies, USA
- IKBα Cell Signaling Technologies, USA
- IKKβ Cell Signaling Technologies, USA
- IKKγ Cell Signaling Technologies, USA
- IKKα Cell Signaling Technologies, USA
- IL-1β Cell Signaling Technologies, USA
- RelB Cell Signaling Technologies, USA
- NF-kB1 Cell Signaling Technologies, USA
- SOD2 Cell Signaling Technologies, USA
- NIK Cell Signaling Technologies, USA
- NRF2 Cell Signaling Technologies, USA
- NLRP3 Cell Signaling Technologies, USA
- ASC Cell Signaling Technologies, USA
- Cleaved caspase-1 Cell Signaling Technologies, USA
- Cleaved IL-1β Cell Signaling Technologies, USA
- GM-CSF Santa Cruz Biotechnology, USA
- CD 11b Cell Signaling Technologies, USA
- Caspase-3 Cell Signaling Technologies, USA

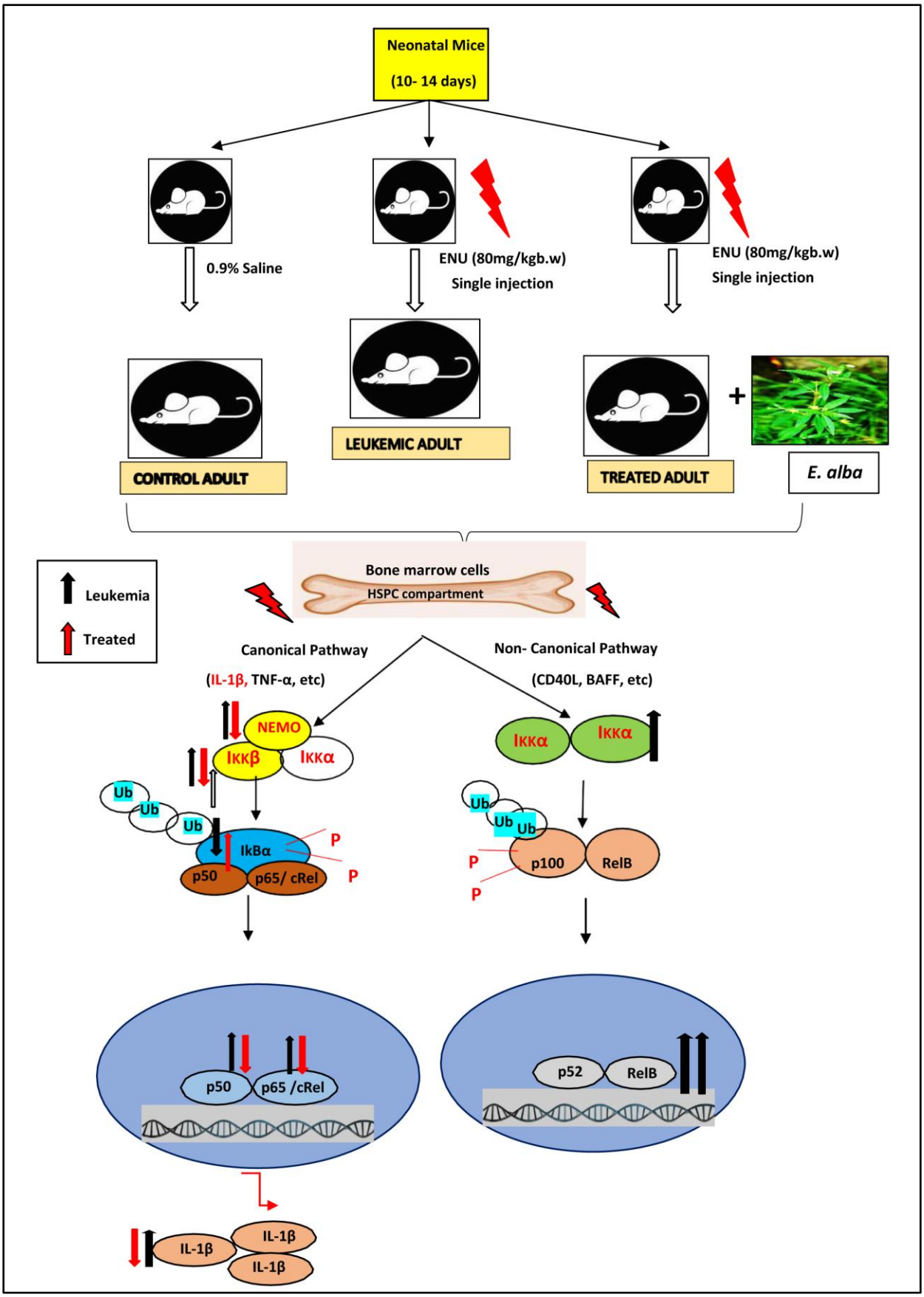
- ASK-1 Cell Signaling Technologies, USA
- JNK Cell Signaling Technologies, USA
- Bax Cell Signaling Technologies, USA
- Bcl-xl Cell Signaling Technologies, USA
- Cytochrome c Santa Cruz Biotechnology
- VEGF Cell Signaling Technologies, USA
- COX-2 Cell Signaling Technologies, USA
- iNOS Cell Signaling Technologies, USA
- PGE2 Cell Signaling Technologies, USA
- E-cadherin Santa Cruz Biotechnology
- Cyclin-D1 BD Biosciences, USA
- Alexa Fluor 488 Invitrogen, USA

3.6. Software used in research

- Graph Pad Prism USA
- Cell Quest Pro Becton Dickinson and company, USA
- Image J USA
- Adobe Photoshop USA
- Mass Lynx 4.1 USA
- Excel Microsoft, USA

ALL PROTOCOLS AND DETAILED METHODS ARE EXPLAINED IN DETAIL IN THE FOLLOWING CHAPTERS.

Chapter 4.


Neonatal Mice
(10- 14 days)
0.9% Saline
ENU (80mg/kgb.w)
Single injection
ENU (80mg/kgb.w)
Single injection
CONTROL ADULT
LEUKEMIC ADULT
TREATED ADULT
E. alba
Leukemia
Treated
Bone marrow cells
HSPC compartment
Canonical Pathway
(IL-1β, TNF-α, etc)
Non- Canonical Pathway
(CD40L, BAFF, etc)
NEMO
IKKβ
IKKα
IKKα
Ub
IkBα
P
p50
p65/ cRel
p100
RelB
p52
IL-1β

Chapter 4.

Over-expression of NF-kB signaling pathway in the bone marrow cells of ENU-induced leukemic animal model and its amelioration by *Eclipta alba* and wedelolactone

4.1. Chapter summary

This present chapter contains the research based on the immune-modulating potentials of *Eclipta alba* and wedelolactone on the NF-kB pathway in the bone marrow cells of ENU-induced leukemia. Body weight, survivability, peripheral blood hemogram, bone marrow cytology, histology, and cell culture studies were done both pre-and post-treatment to understand the anti-cancer effect of whole extract. Physical appearance of leukemic mice became better and body weight was increased significantly, post-treatment. Rate of survivability was increased after *Eclipta alba* treatment, signifying no toxic side effects of the herb. Significant decrease in leucocytosis was observed in peripheral blood and bone marrow sections in treated group compared to leukemia. Moreover, treated marrow cells revealed a decrease in SBB and MPO positive cells which signifies the anti-leukemic effect of the herb. NF-kB signaling molecules were studied in the isolated bone marrow cells of the experimental groups, by flow-cytometry and immunofluorescence assay. Significant changes were observed in the canonical pathway post treatment with extract and isolated compound, with decreased expression of NF-kB and its downstream pro-inflammatory molecule IL-1β. No significant changes were observed in the non-canonical NF-kB pathway. Therefore, it can be concluded that *Eclipta alba* and the isolated wedelolactone is a novel the therapeutic candidate which can significantly target NF-kB pathway and IL-1β, one of the hallmarks of cancer progression.

4.2. Introduction

NOCs are potent carcinogenic chemicals which are present in the environment in various food products like smoked and nitrate cured meat as well through occupational exposures like rubber and pesticide-making industries (Haorah et al. 2001). ENU belongs to NOC family and its prenatal exposure causes leukemia in mice (Basak, Chatterjee, P. Das, et al. 2010; Capilla-Gonzalez et al. 2010; Chatterjee, Chattopadhyay, and Law 2016a).

Over 300,000 cases of leukemia per year is reported globally and it is a type of blood cancer in which healthy blood cells are replaced with leukemic cells, with heterogeneous population of neoplasms (Chatterjee, Chattopadhyay, Sanyal, et al. 2016; Chattopadhyay et al. 2019).

NF-kB signaling pathway is considered as a "hallmark of cancer" and it regulates inflammation, cancer cell proliferation and cancer cell survival by evading apoptosis. Over-expression and continuous stimulation of the NF-kB signaling pathway is a cause of leukemogenesis (Zhou et al. 2015; Mansouri et al. 2016; Carter et al. 2019; Donnell et al. 2023).

Over millions of years, Indian Ayurveda has exploited Mother Nature for being an armamentarium of medicinal plants and herbal based anti-cancer drugs. (Saedi et al. 2014). Various medicinal herbs are known for their anti-cancer properties, because of the concoctions of physiologically active compounds present in them (Huang et al. 2005; Khalafalla et al. 2009; Jayaraman et al. 2022). There are abundant reports which suggests NF-kB can be modulated by several medicinal herbs on various cancer models (Bremner and Heinrich 2002).

Eclipta alba is one such Ayurvedic herb which has been reported for its anti-cancer property on different types of cancer. Wedelolactone is one of its major active compound, which is also recognized as potent "IKK inhibitor", one of the important regulator of NF-kB pathway as well as reported to degrade IkBα in BALBc cells (Kobori et al. 2004; Chaudhary et al. 2011; Chaudhary et al. 2014; Zhong et al. 2019).

It is referred as “the weed of ethnomedicinal significance” because of its immune-modulatory, anti-inflammatory and anti-tumour effects (Leal et al. 2000; Rangineni et al. 2007; Lirdprapamongkol et al. 2008; Jahan et al. 2014; Arya et al. 2015; Kim et al. 2017).

Furthermore, this is the first study to report the effects of *Eclipta alba* and wedelolactone on a haematological malignant model. Hence, through the present study we aimed to observe the expressional profile of all the NF-kB molecules in the marrow cells of ENU-induced leukemia.

4.3. Methods and materials

4.3.1. Plant material

Fresh and dried leaves of *Eclipta alba* was procured from the Medicinal Plant Garden, located in the Narendrapur Ramakrishna Mission Ashrama on July’2019.

After the authentication of the sample by Prof Jukta Adhikari (Department of Botany, Barasat Government College, India), the specimen was deposited at Calcutta School of Tropical Medicine, Kolkata, India The voucher no. of the deposited plant sample is 756870.

4.3.2. Eclipta alba extract preparation

A coarse powder was made by crushing the dried leaves using an electric blender and it was suspended in 80% ethanol. The solution was kept in room temperature for 24 hours. The crude extract was collected by maintaining sterile conditions, using the double filtration method using Grade 1 Whatman filter paper, 25 mm (Sigma, USA). The filtration process was repeated 5 times to make sure no powder particle was present in the extract solution.

The extract was concentrated by rotary evaporator by reduced pressure at 50˚C and the solvent was removed thoroughly. The residue was reconstituted in double-distilled water and stored at -20˚C (Yadav et al. 2017a).

4.3.3. Chemical characterization of Eclipta alba using chromatography

The crude extract of *Eclipta alba* was characterized chemically using HPLC method, using the standard wedelolactone (WDL, purity ≥98%, CAS No: 524-12-9; Sigma-Aldrich). HPLC instrument used was Shimadzu Prominence HPLC (Model No. CBM-20A, Japan) which was equipped with Guard column (Security Guard Cartridge system, Phenomenex, USA) LC-20AT pump, SIL-20AT autosampler, and SPD-M20A detector and Discovery C18 column (ODS, 25 cm × 4.6 mm, 5 μm, Supelco, USA). HPLC-grade acetonitrile (ACN) was purchased from Merck, Germany and deionized water by Milli-Q system (Millipore, USA). The mobile phase was composed of acetonitrile: water (ACN%, v/v) at a flow rate of 1 ml/min and programmed as followed: 20–45% (for 15 min); 45–100% (for 30 min), and 100 (for 40 min). The injection volume was 10 μl at a detection wavelength of 365 nm. The yield of WDL obtained was 12.05 mg/ml.

4.3.4. ESI-MS Direct Infusion technique

The isolated compound by HPLC (wedelolactone) went through qualitative analysis by ESI-MS Direct Infusion technique. The instrument used for this method was Xevo-G2-XS-QTof-MS (Waters) and for its calibration leucine enkephalin (mass 555.62 g/mol) was used. With an injection volume of 10 μl at a flow of 5 μl/min the acquisition was done, using a positive ionization mode with electrospray capillary voltage of 3.0 kV. The cone gas flow at 30 L/h with source and desolvation temperature of 100˚C and 250˚ C, respectively. Mass Lynx 4.1 software was used to analyse the data.

4.3.5. Maintenance of the animals

Inbred and healthy Swiss albino mice (*Mus musculus*) aging 12 weeks approximately with a weight around 32 grams of both sexes were maintained at the animal house of Calcutta School of Tropical Medicine, India. They were provided with a standard diet and water *ad libitum* and

kept under a strictly controlled room temperature of 22 ± 2˚C with a 12h light-dark cycle with controlled humidity. The animals were maintained with full consent and by following all the authorized norms of the Institutional Animal Ethical Committee (IAEC), EU Directive 2010/63/EU and also adhering by the guidelines of "The Committee for the Purpose of Control and Supervision of Experiments on Animals", Government of India ((Law et al. 2001; Basak, Chatterjee, M. Das, et al. 2010; Chatterjee, Chattopadhyay, and Law 2016b; Daw et al. 2016; Law et al. 2018; Daw and Law 2021a).

4.3.6. Development of ENU-induced leukemic mice model

ENU (Sigma, USA) is a highly reactive compound as it decomposes above pH 7.0 in aqueous media. ENU was prevented to decompose from light by keeping its prepared solution in freezer until further use. Swiss albino litter pups aging between 10 to 14 days with a weighing around 4 to 5 grams were injected with a single intra-peritoneal dose of ENU at a dose of 80 mg/kg body weight. After 6 to 8 months of ENU induction, leukemic development was observed and the clinical features of leukemia started to show, which was confirmed by routine peripheral blood smear studies (Law et al. 2001; Basak, Chatterjee, M. Das, et al. 2010; Basak, Chatterjee, P. Das, et al. 2010; Chatterjee, Chattopadhyay, Sanyal, et al. 2016; Chatterjee, Chattopadhyay, and Law 2016b; Chattopadhyay et al. 2019; Bhattacharyya and Law 2021; Bhattacharyya and Law 2022). The control group received 0.9% saline intraperitoneally and all the experimental groups were provided with standard diet and water *ad libitum*.

4.3.7. Therapeutic dosimetry

No acute toxicity of the crude extract was observed even up to 2000 mg/kg body weight as according to various (Singh et al. 1993; Singh et al. 2001; Jahan et al. 2014; Singh et al. 2014; Yadav et al. 2017b). According to another study, 10.4 mg/kg of alcoholic extract for 14 days showed no signs of mortality (Zhao et al. 2015).

We carried out a preliminary test on the basis of prior literature reviews, and tested the efficacy of the crude extract on leukemia via oral gavage at various doses at 1000 mg/kg; 1100 mg/kg; 1200 mg/kg; 1300 mg/kg, and 1400 mg/kg and the normal group were provided with a standard diet. Post the pilot study, the oral dosage was fixed at 1200 mg/kg body weight regularly for 4 weeks.

Wedelolactone at a dose of 50 mg/kg immune-modulated NF-kB signaling pathway and significantly ameliorated liver damage (Luo et al. 2018; Feng et al. 2019). Another report suggests, the dose 50 mg/kg body weight of wedelolactone is reported to significantly down-regulate NLRP3 inflammasome and its downstream caspase-1 activation in DSS-induced colon damage (Wei et al. 2017). 50 mg/kg of wedelolactone was reported to down-regulate IL-6/STAT3 axis and show an anti-inflammatory response in ulcerative colitis (Tigari 2022).

On the basis of data, a dose of 50 mg/kg body weight wedelolactone was standardized via oral gavage for 4 weeks regularly.

4.3.8. Experimental groups

According to the experimental plan, four experimental groups were formed, with 30 mice per group (n=30). The groups were as followed:

- Leukemic group (ENU induced);
- Leukemia (ENU induced) + *Eclipta alba* extract treated (1200 mg/kg via daily oral gavage for 4 weeks);
- Leukemia (ENU induced) + wedelolactone treated (50 mg/kg via daily oral gavage for 4 weeks);
- Control group of mice received equal volume of saline in similar condition.

4.3.9. Assessment of survivability and body weight post-treatment

A preliminary assessment was carried out on the survivability and physical appearance, like body weight of the mice among different experimental groups. An increase in survivability in the leukemic mice group, post treatment with crude extract is a pointer towards its anti-cancer efficacy. The number of survival days post-treatment was compared with leukemic and control mice. To understand the efficacy of the treatment on physical appearance, the body weights of each experimental group's pre-and post-treatment were recorded on a daily basis.

4.3.10. Isolation of the bone marrow cells

With full consent from the institutional animal house ethical committee, the mice from each experimental groups were sacrificed by cervical dislocation and their femur and tibia (long bones) were surgically removed. A cut was made on both ends of the bones by sterilized scissor and the red-pulp marrow region was flushed out in RPMI-1640 media (Sigma, USA) using a 26 gauge sterile needle. Some intact marrow sections were used to make bone marrow smears and rest for different protocols. The marrow cells were constantly passed through 100 µm cell strainer by repeated pipetting and washed thoroughly in chilled media to make sure there is a single cell suspension without debris (Law et al. 2001; Basak, Chatterjee, M. Das, et al. 2010; Chatterjee, Chattopadhyay, and Law 2016b; Chatterjee, Chattopadhyay, and Law 2016a; Bhattacharyya and Law 2021; Daw and Law 2021b; Bhattacharyya and Law 2022).

4.3.11. Characterization of leukemia pre-and post-treatment

4.3.11.1. Peripheral blood collection by tail-vein puncture

The one thing to keep in mind while collecting blood quickly is to make sure there is minimum tissue trauma put on the mice. The mice were anesthetized were placed in a ventral position to easily identify its dorsal lateral vein. Tail tip was rubbed with warm water to make sure there is enough blood flow and sterility is maintained. Using a 25-gauge sterile needle a puncture

was made on the tail. The blood was drawn out in a small capillary tube. After blood collection, the site of puncture was rubbed with alcohol to avoid further infections.

4.3.11.2. Total WBC count, total RBC count, differential WBC count, reticulocyte count, and haemoglobin estimation

10µl of blood was mixed with 190µl of WBC diluting fluid (Turk's fluid) in a ratio of 1:20 and incubated for 10 minutes in room temperature. 150 µl of solution was charged into the Neubauer chamber (Rohem, India) keeping the pipette at a 45˚ angle. The chamber was covered with a glass coverslip and the solution filled through the counting chamber under the cover slip while making sure no air-bubbles were trapped in between. After sometime, the chamber was placed on the microscope stage.

For RBC count, the blood was diluted 200 times using RBC diluting fluid (Dacie's fluid) . 10µl of blood sample was mixed with 1990µl of RBC diluting fluid and left at room temperature for 10 minutes. Around 150µl of solution was charged into the chamber to calculate the total number of RBC.

To carry out differential WBC count, fresh blood smears were made by using a drop of blood on a clean grease free glass slide and air-dried. The slide was stained for 2 minutes and double amount of water was re-added to slide for 6 minutes. The slides were washed thoroughly using tap water and kept on microscope stage.

Reticulocytes are immature RBCs with RNA. They are stained using brilliant cresyl blue to observe the erythropoietic development of marrow. The blood sample and stain was mixed in 1:1 ratio and kept aside for 15 to 20 minutes at 37˚C. The solution was pipetted repeatedly and taken onto a grease free glass slide to form a smear which was allowed to air dry. The number of reticulocytes were calculated under the microscope.

For haemoglobin estimation, Drabkin's solution was collected in two test-tubes. One test-tube was kept blank and in one test-tube, 10 µl of blood was mixed with 2.5ml of Drabkin's solution and pipetted several times. The solutions were kept in room temperature for 10 minutes till the development of colour. The optical density (OD) was calculated using a colorimeter at 540 nm against the blank Drabkin's solution.

4.3.11.3. Staining techniques for neutrophils and blast identification

Both peripheral blood smear and bone marrow smears were collected to observe neutrophils and blast population. Leishman staining was carried out by staining the smears for 2 minutes by keeping the slides completely covered. Post that, double the volume of distilled water was used to cover up the slides and kept for around 6 minutes. Later, the slides were washed in running tap water and the excess water was absorbed with a tissue paper. The marrow and blood smears were also stained with Geimsa by keeping the slides to stain for 2 to 3 minutes and washed off by distilled water (Gajendra et al. 2015). The polymorphic nucleus of the neutrophils appeared dark purple with a light pink cytoplasm and likewise they were counted. The blasts appeared large with undefined nucleus (Meisner and Johnson 2008; Chatterjee, Chattopadhyay, and Law 2016b; Bhattacharyya and Law 2021).

4.3.12. Myeloperoxidase staining of bone marrow cells

Myeloperoxidase (MPO) staining technique allows us to identify the enzymes in the cytosol of specific blood cells that characterizes immature blasts. MPO staining can detect myeloblasts and immature myeloid cells present in leukemia.

From each experimental groups bone marrow smears were prepared and air dried for 5 minutes. The slides were immersed in a solution of Buffered formal acetone (pH 7.4) for 20 seconds at room temperature for fixation and washed under running tap water to be air dried. A working substrate solution was made by mixing 30mg DAB with 60ml of Sorenson's buffer with 120µl

H_2O_2. The slides were immersed in the working substrate solution for 20 minutes, rinsed and air dried again. The slides were counter-stained with haematoxylin for 2 minutes and rinsed under running water and air-dried. After mounting the slides, they were placed on microscope stage to be visualized under light microscope (Olympus CH20i, Japan) (Grootjans et al. 2013).

4.3.13. Sudan Black B staining of bone marrow cells

Bone marrow smears were made on clean and grease free glass slides from control, leukemic and treated groups. The slides were fixed in the vapour of formaldehyde for 15 minutes approximately. A working solution was made of Sudan Black B (SBB) using SBB and phenol buffer. The slides were kept in the working solution for an hour and washed by 70% alcohol with three changes and then rinsed with tap water. Using Leishman stain the slides were counter-stained, mounted with cover slip and visualized under light microscope (Olympus CH20i, Japan) (Methods et al. 1990).

4.3.14. Perl's staining of bone marrow cells

Fixation of the bone marrow smears from each experimental groups were carried out in 10% formalin. The marrow smears were immersed in a working solution containing equal volumes of 4% aqueous potassium ferrocyanide and 4% aqueous hydrochloric acid to form 2% aqueous potassium ferrocyanide hydrochloric acid. The slides were washed using distilled water and counter-stained with eosin for 2 minutes. The smears were dehydrated using 70%, 95%, and 100% increasing grade of ethanol for 10 seconds each. The slides were immersed in oil and observed under light microscope (Santambrogio et al. 2022) (Olympus CH20i, Japan).

4.3.15. Identification of splenomegaly

The spleen were removed surgically from each experimental group and measured to understand the change in its appearance after the treatment.

4.3.16. Hematoxylin and Eosin staining of the long bone

The femur bone was decalcified and the bone marrow was prepared in a wax block using the routine method (Dancey et al. 1976). The sections from each experimental animals were cut and placed on a grease free glass slide and deparaffinized using xylene. The slides were made to pass through downgrades of alcohol using 100%, 90%, 75%, 50% and 30% alcohol, each for 5 minutes. Post this, the slides were immersed in distilled water for 2 minutes for re-hydration of the bone marrow sections. Each slides were immersed in the hematoxylin stain for a minute and then washed under running water. Counter-stain was carried out using eosin for 30 seconds. The slides were again dehydrated using an increasing grade of alcohol (70%, 95%, and 100%) for 2 seconds each and the mounted slides were observed under light microscope (Olympus, Ch20i, Japan) (Fischer et al. 2012).

4.3.17. Short term culture of bone marrow cells

Single cell suspension of marrow cells (1 x 10 cells/ml) were suspended in a media containing 4ml of RPMI-1640 (Sigma, USA) added with 30% FBS in 75 mm culture plates (Corning, New York). To the cells, 100 µl.ml of penicillin and streptomycin respectively were added. The cells were maintained at a constant 37˚C in a 4% CO_2 humidified chamber. The cells were observed at different time intervals of 0h, 24h and 72h under the inverted microscope.

4.3.18. Flow-cytometric analysis of NF-kB protein expression

The bone marrow cells from different experimental groups were fixed using 1.5% paraformaldehyde (PFA) solution for 15 minutes in dark, at 37˚C controlled temperature. Post that, the cells were counted (2 x 10^6 cells/ml); washed in PBS and suspended in FACS fluid. For staining the internal proteins the cells were vortexed and treated with chilled methanol drop by drop for 30 minutes. The bone marrow cells were washed using PBS and suspended again in the FACS fluid. Cells were counted and pipetted in FACS tubes and stained with 2µl of

primary antibodies of p50, p65, RelB, cRel, IKKα, IKKβ, IKKγ, IKBα, IKKβ, IKKγ, IL-1B, NF-kB1 and NIK (all from Cell Signaling Technology, USA). The cells were incubated in the primary antibodies respectively for 30 minutes at 37°C temperature. The counterstaining of the bone marrow cells were carried out using the secondary antibody conjugated with Alexa flour 488 (Invitrogen, USA) and the marrow cells were again kept in the dark at 37°C for next 30 minutes. The marrow cells of each experimental groups were washed using PBS and their protein expression analyzed using the BD FACS Calibur flowcytometer machine (Becton Dickinson, USA) using the CellQuest Pro software (v9.1 Becton Dickinson).

4.3.19. Immunofluorescence imaging of the marrow cells

The bone marrow cells of each experimental group, tagged with the respective primary antibodies p50, p65, RelB, cRel, IKKα, IKKβ, IKKγ, IKBα, IKKβ, IKKγ, and IL-1B (all from Cell Signaling Technology, USA) for FACS analysis were smeared on a clean grease free slide and mounted with DAPI shield (Sigma, USA) in dark. The fluorescence expression were observed using a fluorescence microscope, Leica DM8 and photographs of protein expressions were analyzed by Leica DX software.

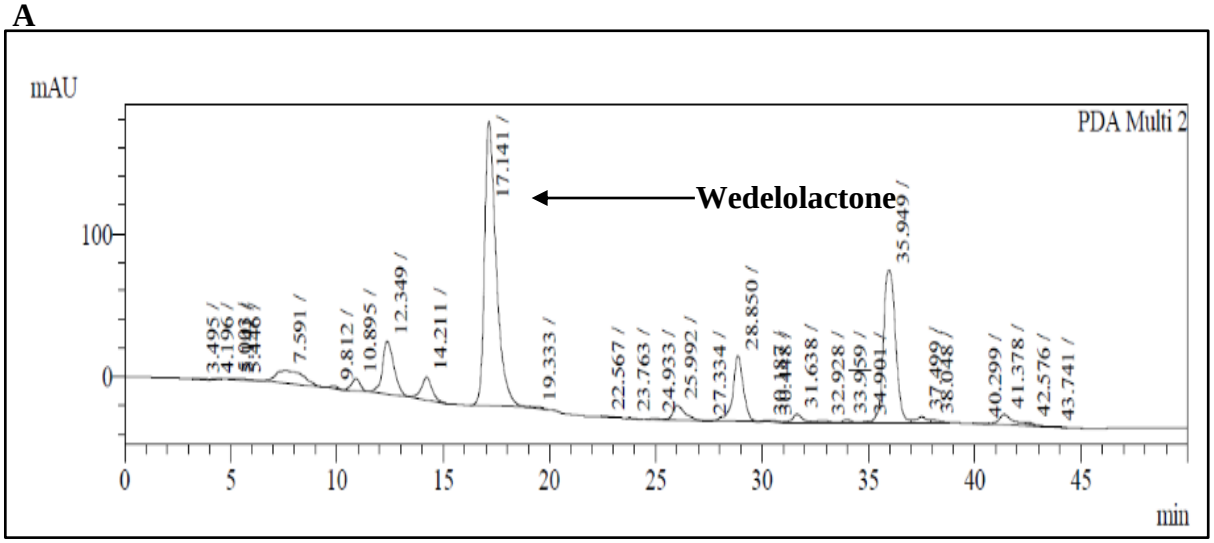
4.4. Statistics

Statistical analysis were performed using unpaired Student's t-test and One-way ANOVA by Tukey's Test (post- hoc testing). All the quantitative data were shown as Mean ± Standard deviation (SD). Each experiment was repeated thrice and for every comparison, $P \leq 0.05$ was considered significantly significant.

4.5. Results

4.5.1. Identification of wedelolactone and chemical characterization of the extract using HPLC

A



B

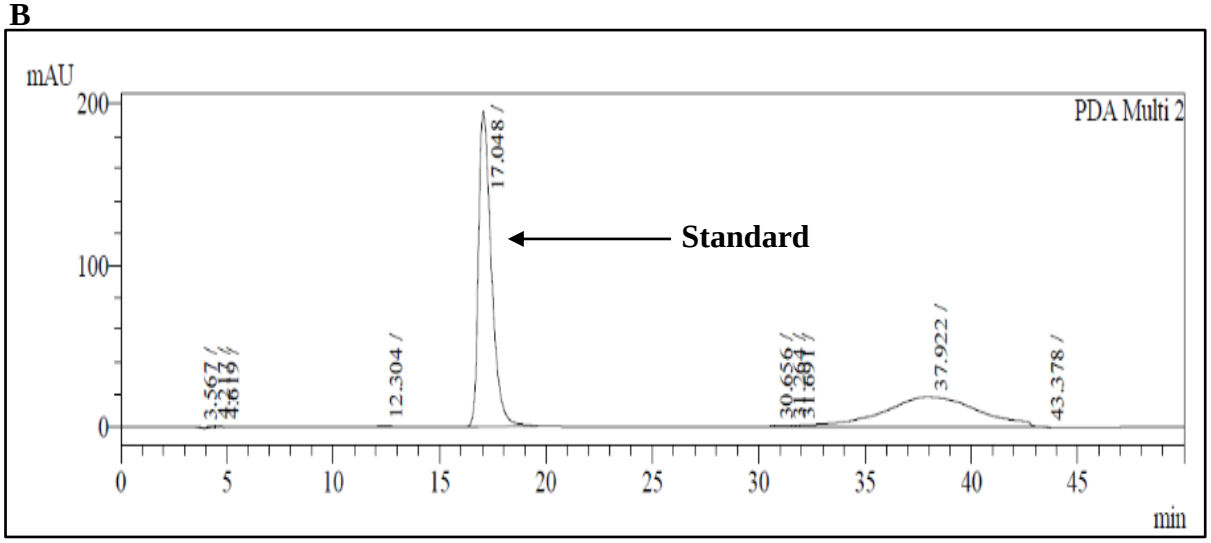


Figure 36. Chromatogram analysis (A) HPLC of the whole extract reporting wedelolactone at the retention time 17.141. (B) HPLC of standard wedelolactone at retention time of 17.048

Wedelolactone is one of the major active compound found in *Eclipta alba* ethanolic extract. The chromatogram analysis was carried out using HPLC at 365 nm which revealed multiple peaks of biochemical compounds present in the extract. Wedelolcatone was isolated which was

recorded to be present at a retention time of 17.141 in the extract as compared with standard wedelolactone (Sigma, USA) at a retention time of 17.048 (Figure 36. A, B).

4.5.2. Characterization of isolated wedelolactone using ESI-MS technique

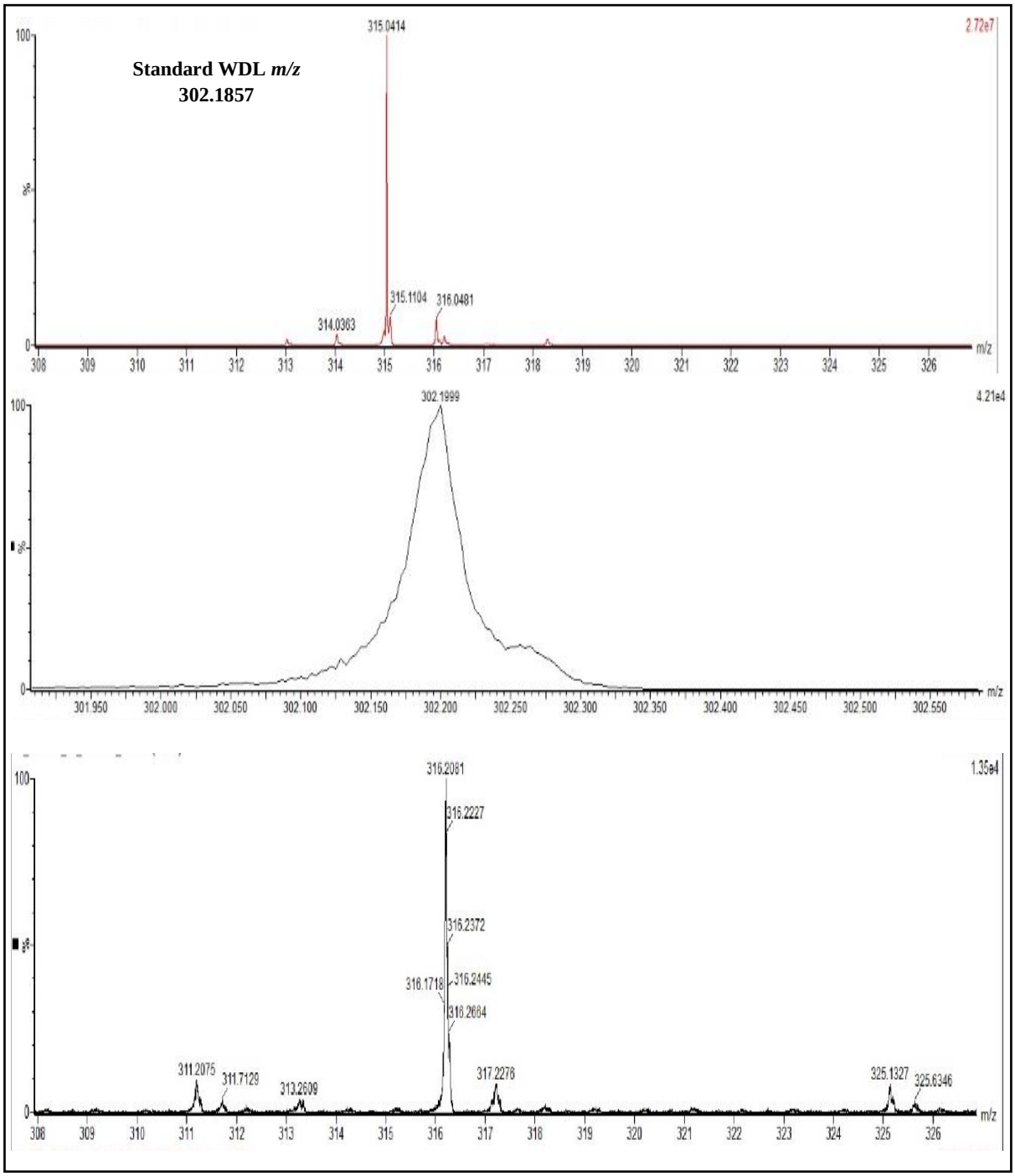


Figure 37. ESI-MS-Direct Infusion analysis of standard wedelolactone with *m/z* 302.1857

ESI-MS Direct infusion technique was carried out to characterize the isolated wedelolactone by comparing it to the standard compound. The isolated wedelolactone was approximately identified at TOF MS ES+ 4.21 e4 in protonated form in the negative-ion mode. The standard wedelolactone peak was at m/z 316.2081 and another fragment ion with m/z 302.19 was detected, preferably indicating the methyl group (-CH_3) of wedelolactone (Figure 37, 38).

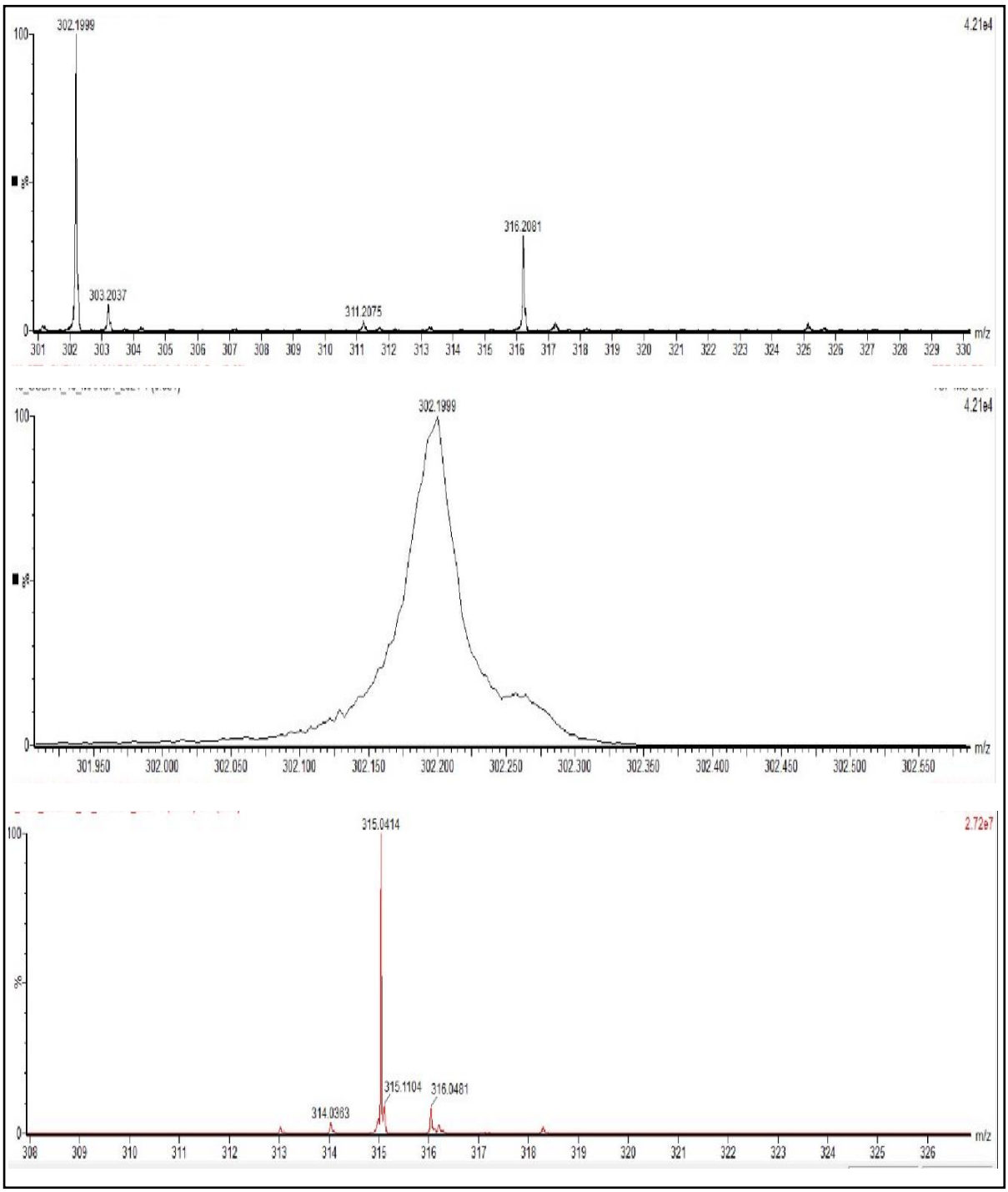


Figure 38. ESI-MS-Direct Infusion analysis of isolated wedelolactone m/z 302.1999

4.5.3. Preliminary assessment of the therapeutic dosimetry

According to the literature review, up to 2000 mg/kg body weight no signs of toxicity was observed in leukemia. A preliminary test was carried out to assess the dosage of the extract and accordingly we observed the lethal concentration or LD50 of the extract to be 2250 mg/kg body weight (Figure 39. A) as 50% mortality rate was observed in 6 mice each among 8 dose groups. The treatment dose or EC50 was estimated at 1200 mg/kg body weight (Figure 39. B) as 50% effective response was reported. EC50 was considerably lesser compared to previous reports.

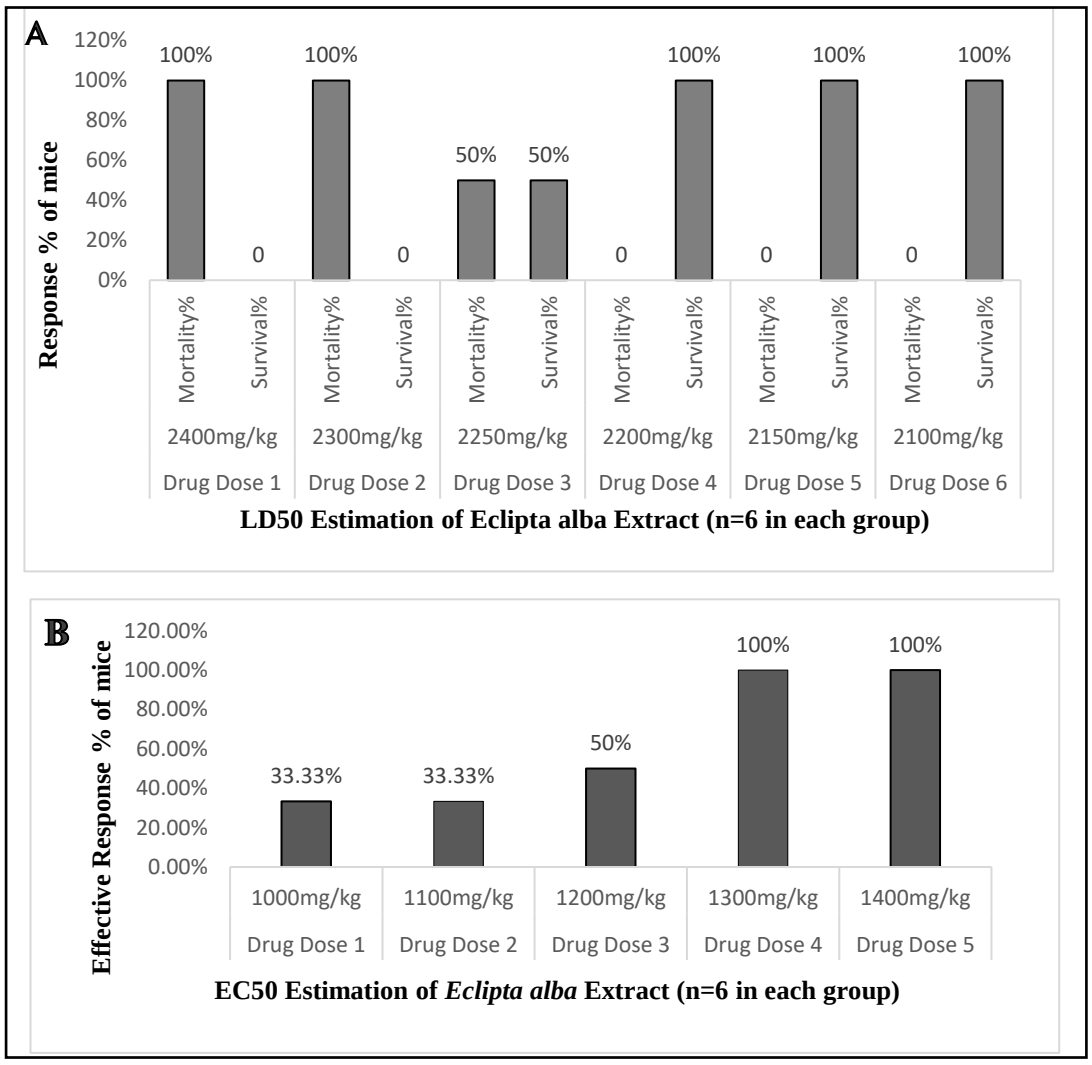


Figure 39. Therapeutic dosimetry study

4.5.4. Ameliorating effect of herbal extract on body weight and survivability of leukemic mice

Along with leukemic development, the physical state of the mice deteriorates which mostly effects the body weight and finally their rate of survivability. ENU portrays a massive effect on the body weight of the leukemic mice as it decreased significantly (11.33 ± 0.763 g) as compared to the healthy group 31.66 ± 1.04 g; P = 0.001). Interestingly, the group which received extract treatment (16.15 ± 1.20 g; P = 0.06) showed a slight amelioration in contrast to ENU-induced leukemia (Figure 40).

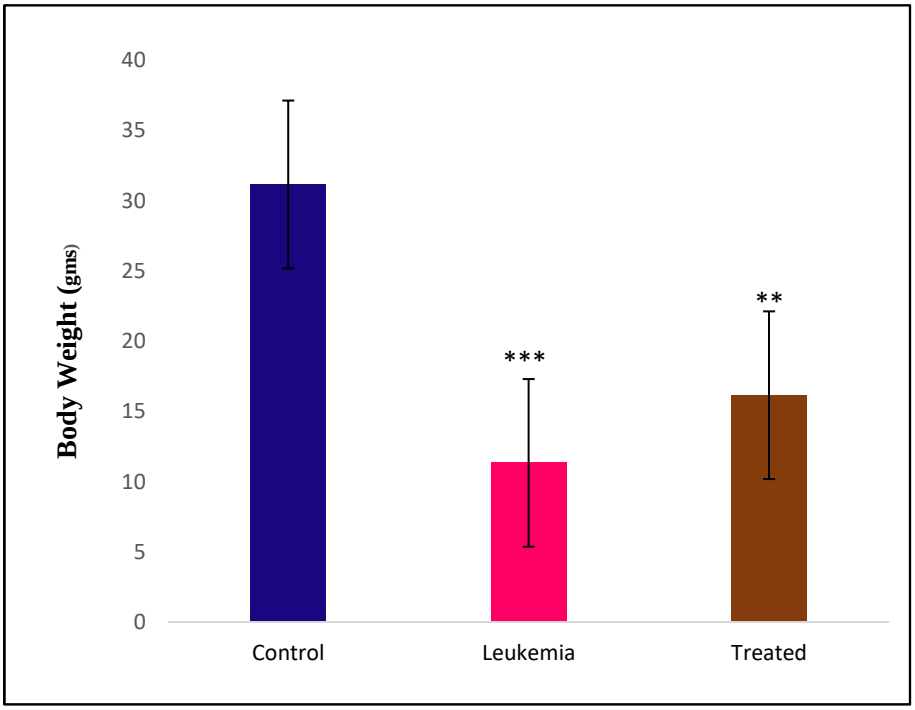


Figure 40. Graph representation of changes in body weight pre-and post-treatment with the whole extract (***P < 0.001; Values are Mean ± SEM, t-test).

Moreover, the treated group showed a significant increase in their rate of survivability (Leukemia Vs Treated: 15.5 ± 1.29 Vs 23 ± 2.16; P < 0.001) as compared to the diseased state,

proving that the extract plays a beneficiary role in combating the first signs of leukemia (Figure 41).

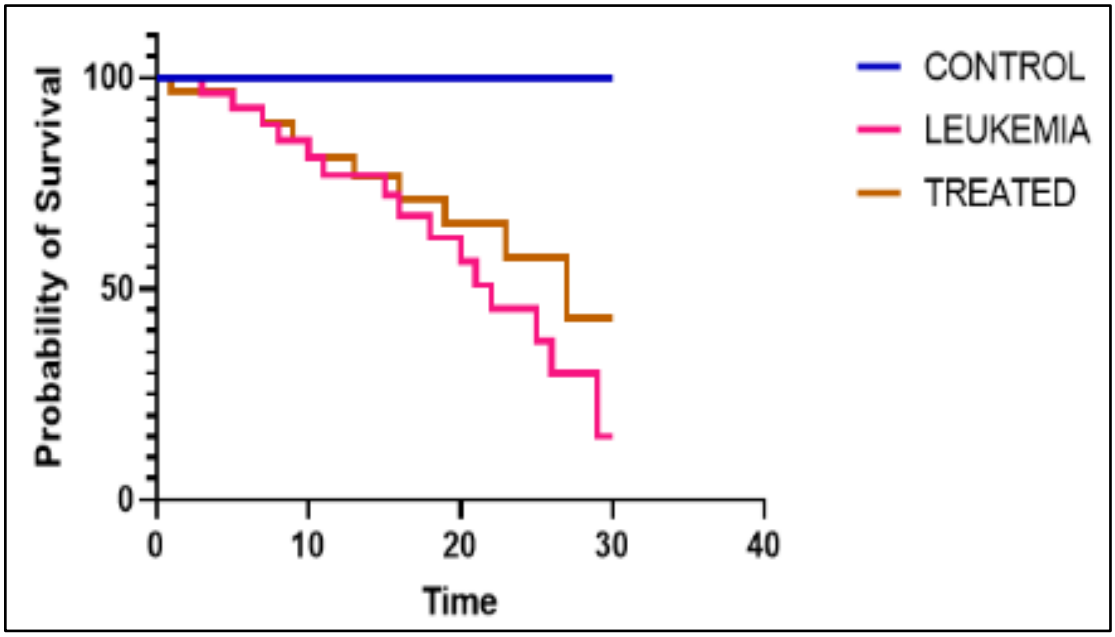


Figure 41. Kaplan- Meier curve representing the changes in the rate of survivability after treatment with herbal extract

4.5.5. Effect of the leaf extract and active compound on peripheral blood hemogram

The peripheral blood hemogram was carried to understand the leukemic pathophysiology post-treatment (Table 2). A significant increase in the WBCs were observed in the leukemic group (36.17 ± 0.60 _ 103 cells/mm3, p < .001) as compared to healthy control (7.58 ± 0.54 _ 103 cells/mm3). A decrease of leucocytosis by around 1.4 and 1.1-folds (25.49 ± 1.25 _ 103 cells/mm3, p < .001 and 30.40 ± .41 _ 103 cells/mm3, p < .001) were observed extract and active compound treated groups respectively, therefore showing an amelioration on ENU-induced leukemia (Figure 42). A considerable increase in the number of red blood cells (RBC) and reticulocyte count (10.94 ± 0.30 _ 103 cells/mm3, p < .001 and 1.93% ± 0.031%, p < .001) were noted in the ENU-induced leukemic group, as compared to control (6.89 ± 0.78 _ 103 cells/mm3 and 10.94% ± 0.30%). A significant decline of RBC count by 1.1 and 1.0-folds in the crude extract treated group (9.58 ± 0.42 _ 103 cells/mm3, p = .003) and wedelolactone

(10.37 ± 0.32 _ 103 cells/mm3, p = .05) treated groups were observed. The reticulocyte count was decreased by 1.1 and 1.0-folds in the extract (1.71% ± 0.04%, p = .05) and wedelolactone (1.86% ± 0.01%, p = .028) treated groups.

The concentration of haemoglobin in peripheral blood was increased in diseased group (19.42 ± 0.38 g/dl; $P < 0.001$) as compared to control (16.16 ± 0.52 g/dl). The treated group (18.21 ± 0.20 g/dl; 19 ± 0.66 g/dl; $P = 0.022$) showed some changes in the blood haemoglobin concentration.

Parameters	**Control (X ± SD)**	**Leukemia (X ± SD)**	**Extract (X ± SD)**	**Wedelolactone (X ± SD)**
WBC (x 10^3/mm^3)	7.58 ± 0.54	36.17 ± 0.60***	25.49 ± 1.25***	30.40 ± 0.41***
RBC (×10^6/ mm^3)	6.89 ± 0.78	10.94 ± 0.30***	9.58 ± 0.42##	10.57 ± 0.32
Reticulocyte (%)	0.54 ± 0.18	1.93 ± 0.031***	1.71 ± 0.04 **	1.86 ± 0.018*
Hemoglobin	16.16 ± 0.52	19.42 ± 0.38**	18.21 ± 0.20 *	19.0 ± 0.66
Neutrophils (%)	16.37 ± 1.10	44.87 ± 0.85***	36.12 ± 0.92***	40.43 ± 0.42***
Blasts (%)	----	37.225 ± 0.85	31.25 ± 1.70***	34.13 ± 0.31##

Table 2. Peripheral hemogram profile of the experimental groups **(***Values are mean ± SEM for 6 animals in each observation. *P=0.028, **P=0.005, ***P<0.001, ## P=0.006, ##P=0.005)*

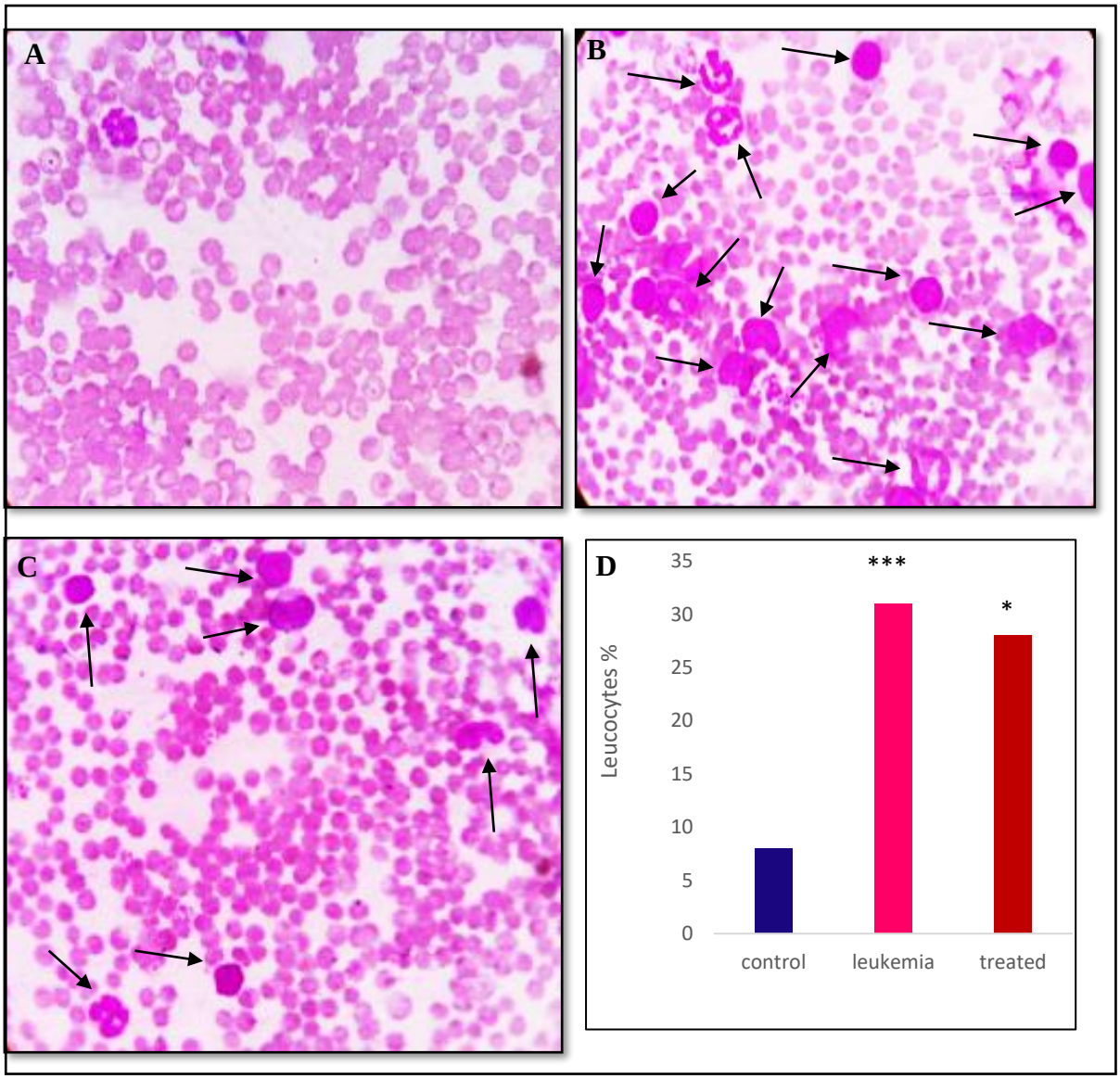


Figure 42. WBC population in the peripheral blood of (A) control, (B) leukemia (marked with black arrows), (C) treated. (D) Representative graph showing the difference in WBC population among the three groups.

4.5.6. Identifying neutrophils and blast cell population pre-and post-treatment

A significant decline in the number of blast cells were observed in both the treated groups by 1.1 (31.25% ± 1.70%; $p < .001$) and 1.0-folds (34.13% ± 0.31%, $p = .006$) after extract and wedelolactone treatments respectively.

In ENU-induced leukemic group (37.22% ± 0.85%) the blast cell population was drastically high, which is one of the factors of leukemia. Excessive infiltration of neutrophil population was observed in leukemic group (44.87 ± 0.85% ; $p < 0.001$) in contrast to control (16.37 ± 1.10), which was reduced post-treatment with extract (36.12 ± 0.92; $p < 0.001$) and active compound (40.43 ± 0.42; $p < 0.001$) (Table 2).

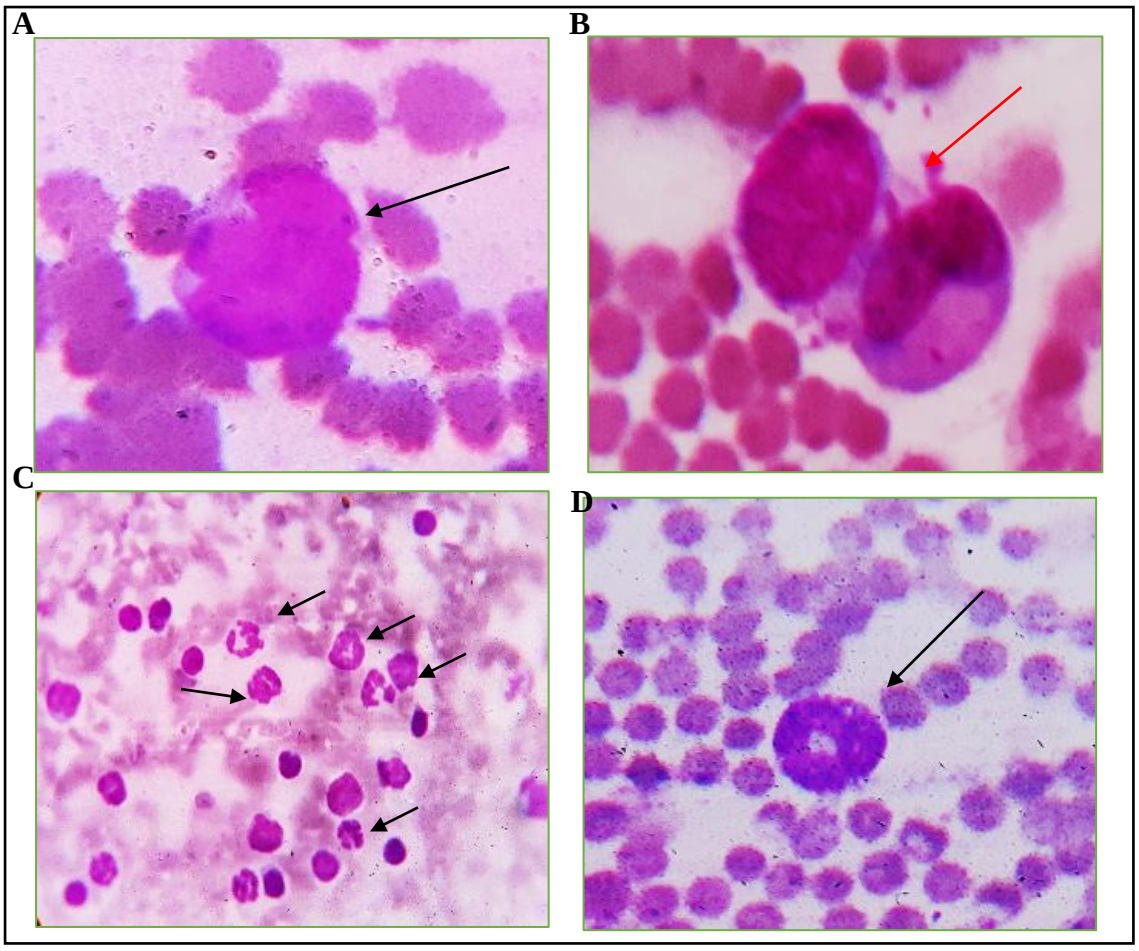


Figure 43. Peripheral blood smear from ENU induced leukemia as identified by Leishman stain. (A) Highly immature polymorphic blast (black arrow), (B) abnormal myeloblast (red arrow), (C) significant leukocytosis and excessive infiltration of dysplastic neutrophils (black arrow) and (D) immature hypo-lobulated ring neutrophil (black arrow).

Cytological study revealed the presence of numerous abnormal blasts, hypo-lobulated ring and dysplastic neutrophils in ENU-induced leukemic blood (Figure 43).

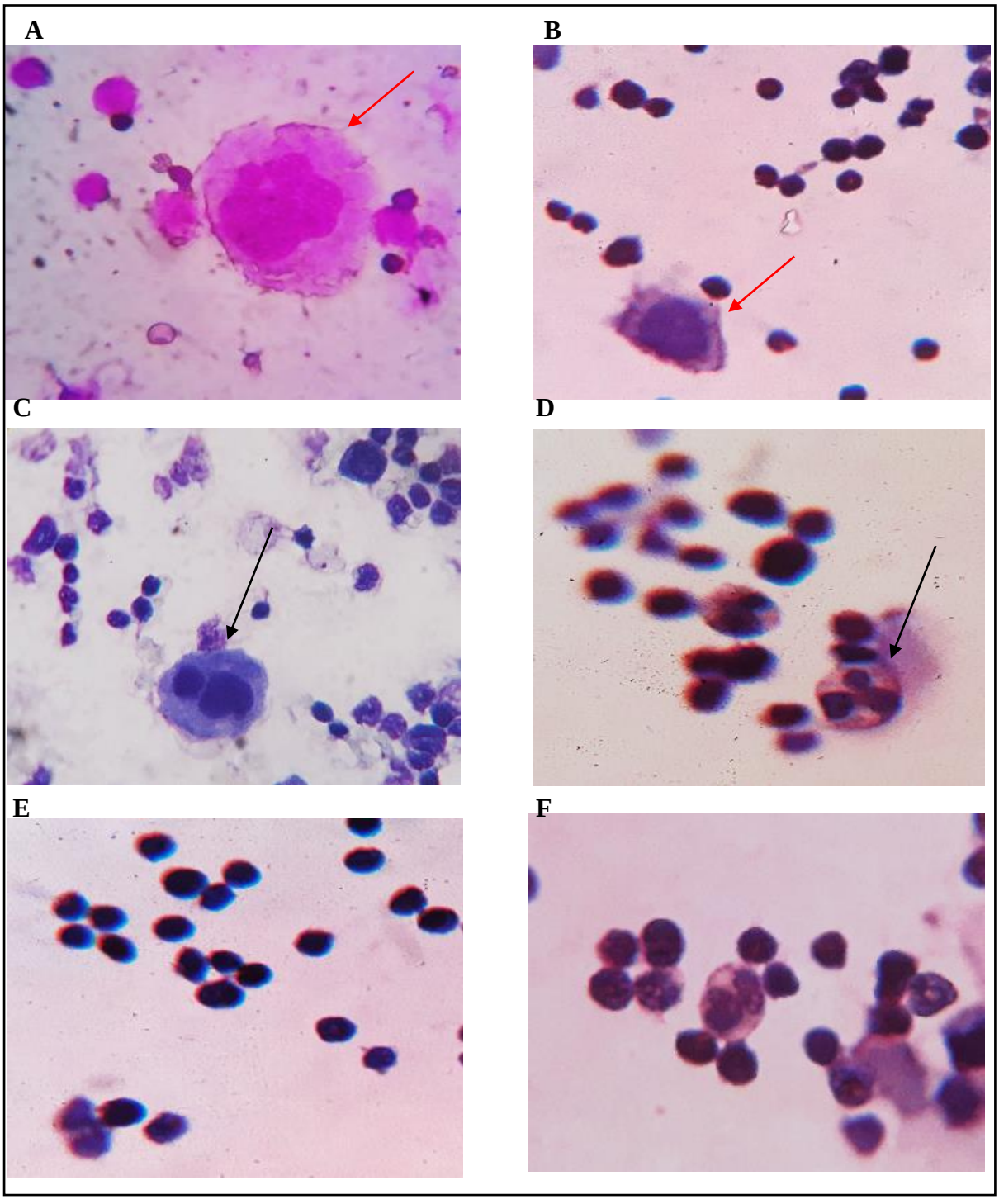
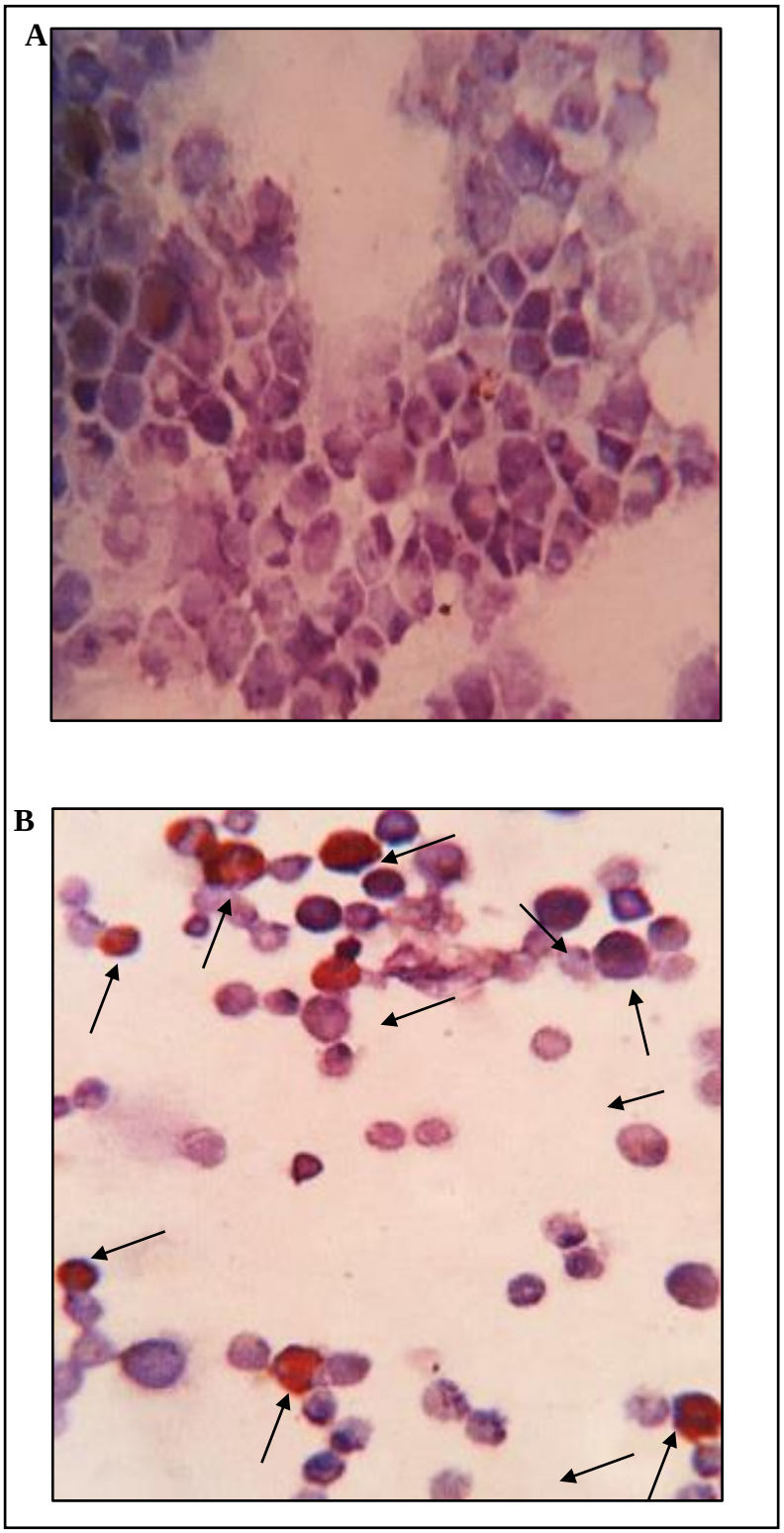


Figure 44. Geimsa stained leukemic marrow smear (A, B) abnormal megakaryoblast (red arrow); (C) hypo-lobulated megakaryocyte (black arrow); (D) fan shaped nuclei (black arrow) and (E, F) high blast cell population.

The bone marrow cells stained by Geimsa from diseased group revealed an excessive infiltration of various kind of blast cells (Figure 44).

4.5.7. Effect of the treatments on MPO activity in bone marrow cells



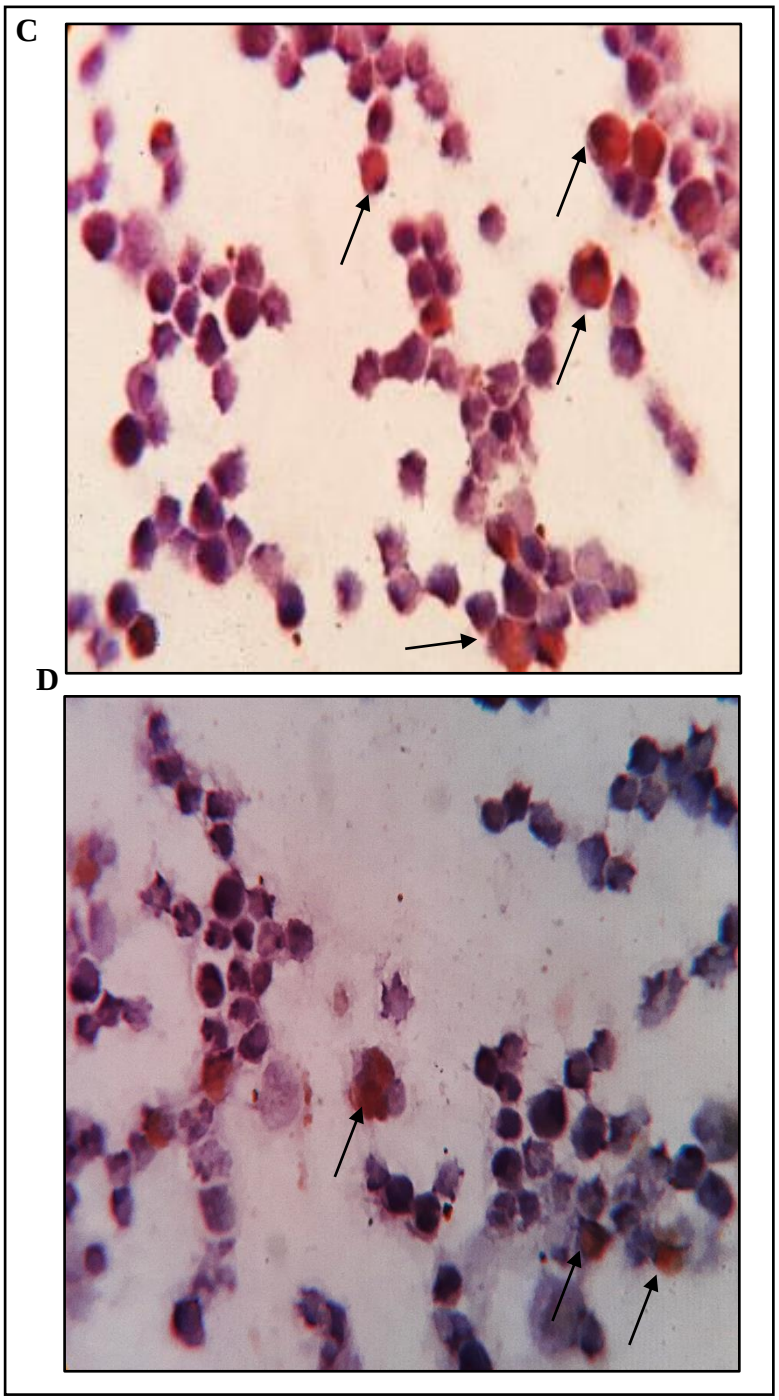
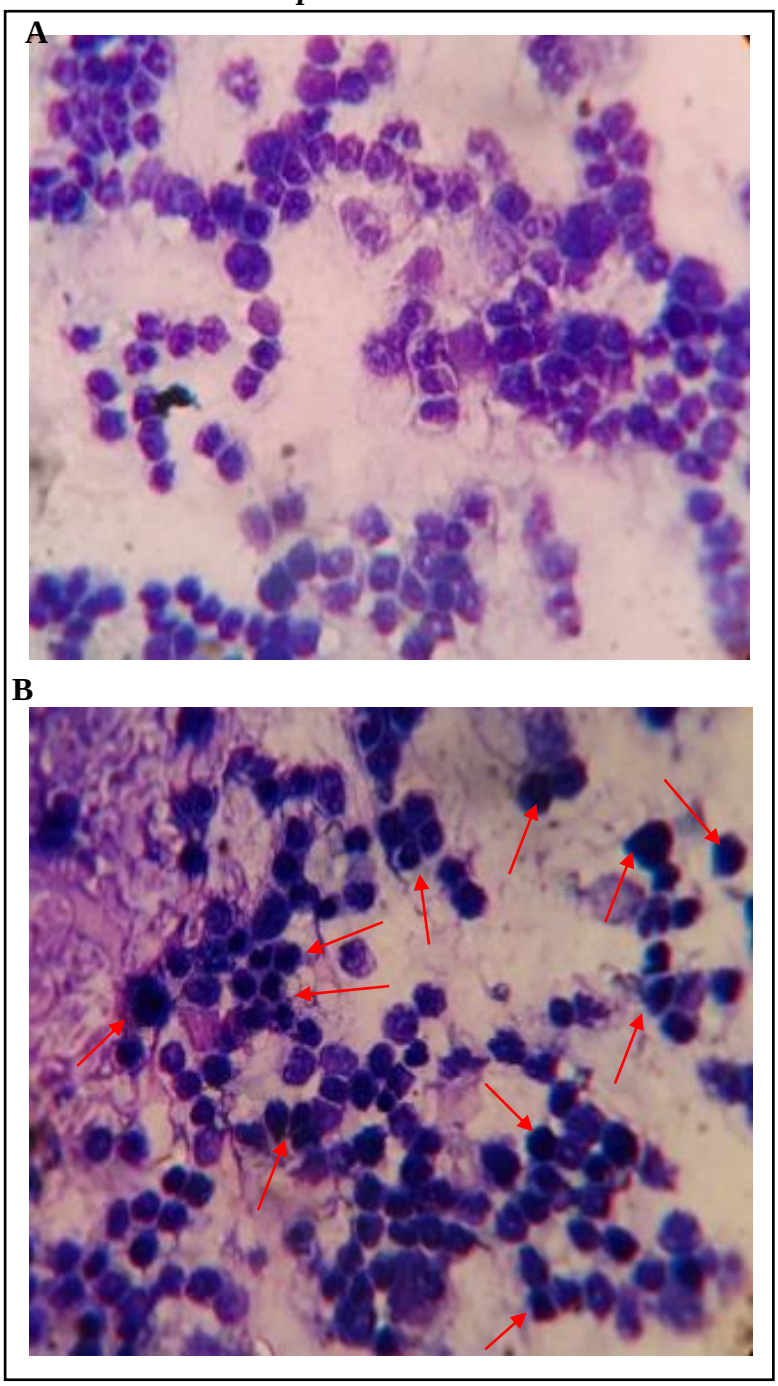


Figure 45. Cytochemical staining using MPO stain on the bone marrow cells from experimental groups. (A) Control, (B) Leukemic marrow cells shows an increase in dark brown stained MPO positive cells (black arrow). (C, D) Lower population of MPO positive cells revealed in the treated marrow cells.

MPO staining of the bone marrow cells reveals the increase in MPO positive cells which contains excessive azurophilic granules and myeloblasts containing auer rods in ENU- induced leukemic condition in contrast to control. Significant decrease in MPO positive population (Leukemia versus Treated = 66.67 ± 3.21 versus 57.67 ± 2.52; p = 0.018) was observed post treatment (Figure 45).

4.5.8. Effect of the treatments on SBB positive cells in bone marrow cells



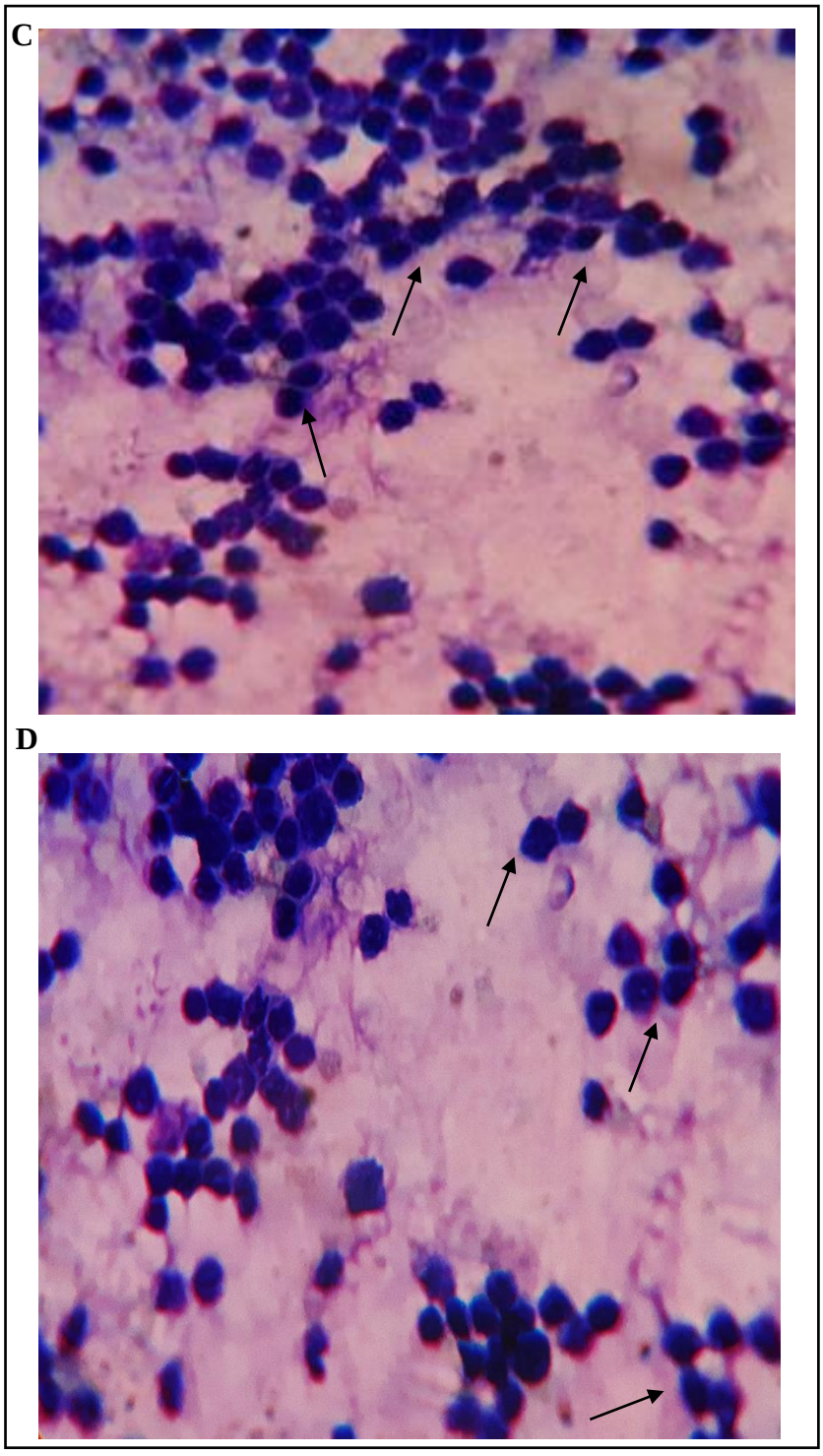
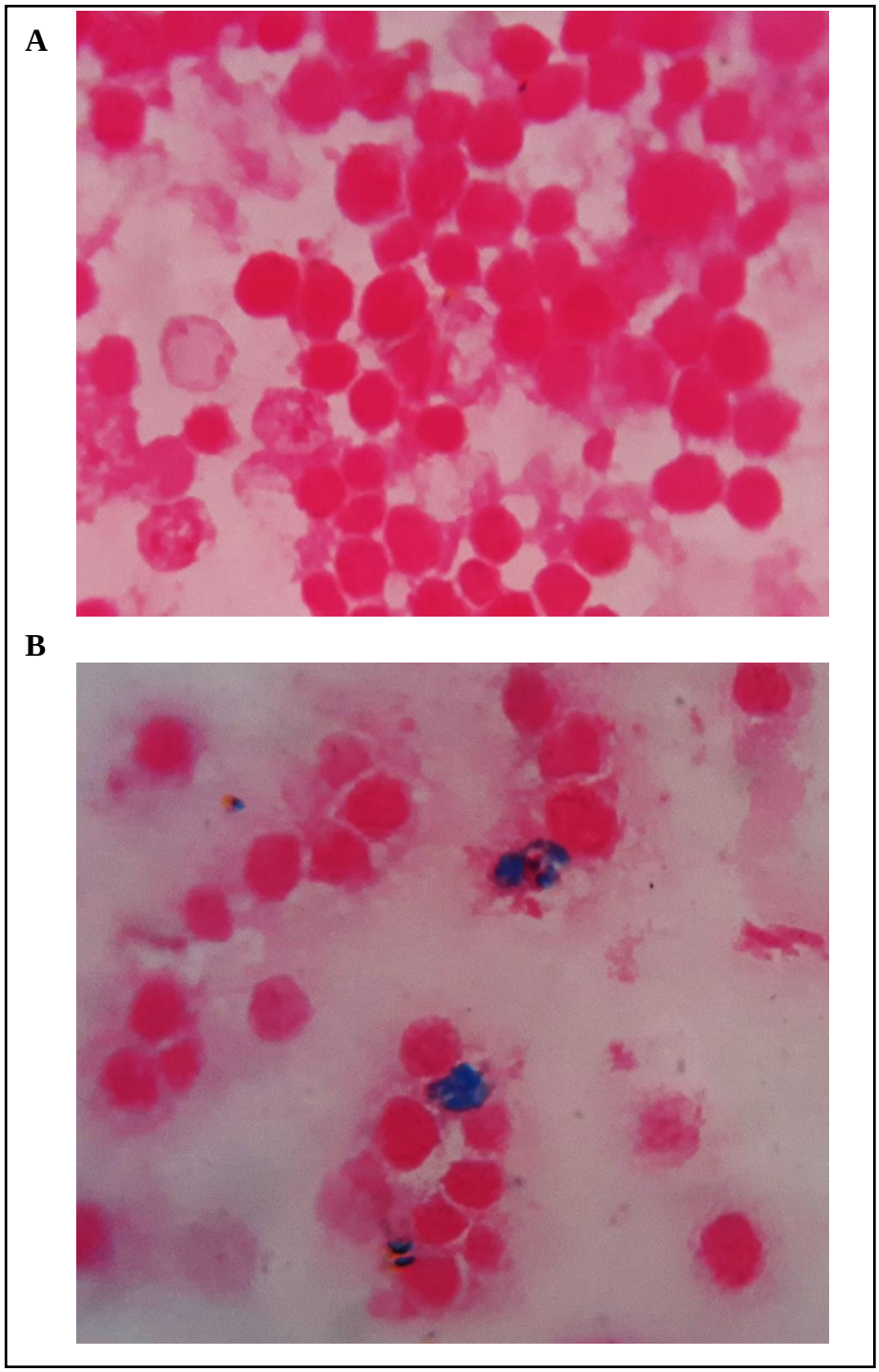


Figure 46. Sudan black B (SBB) staining of the bone marrow smear. (A) Control, (B) Leukemic marrow cells revealed high number of azurophilic SBB positive cells, (C and D) Extract treated and active compound treated marrow cells reveals comparatively lesser number of SBB positive cells.

Bone marrow cells isolated from the diseased groups showed the presence of abnormal myeoblasts which are SBB positive cells (Figure 46). No such immature myeoblasts were present in the bone marrow cells of the normal group. In treated group a significant decrease in SBB positive cells were observed post treatment as compared to leukemia (Leukemia versus Treated = 68.67 ± 3.06 versus 55.33 ± 5.51; p = 0.02).

4.5.9. Cytological changes as observed by Perl's stain on bone marrow cells after treatments



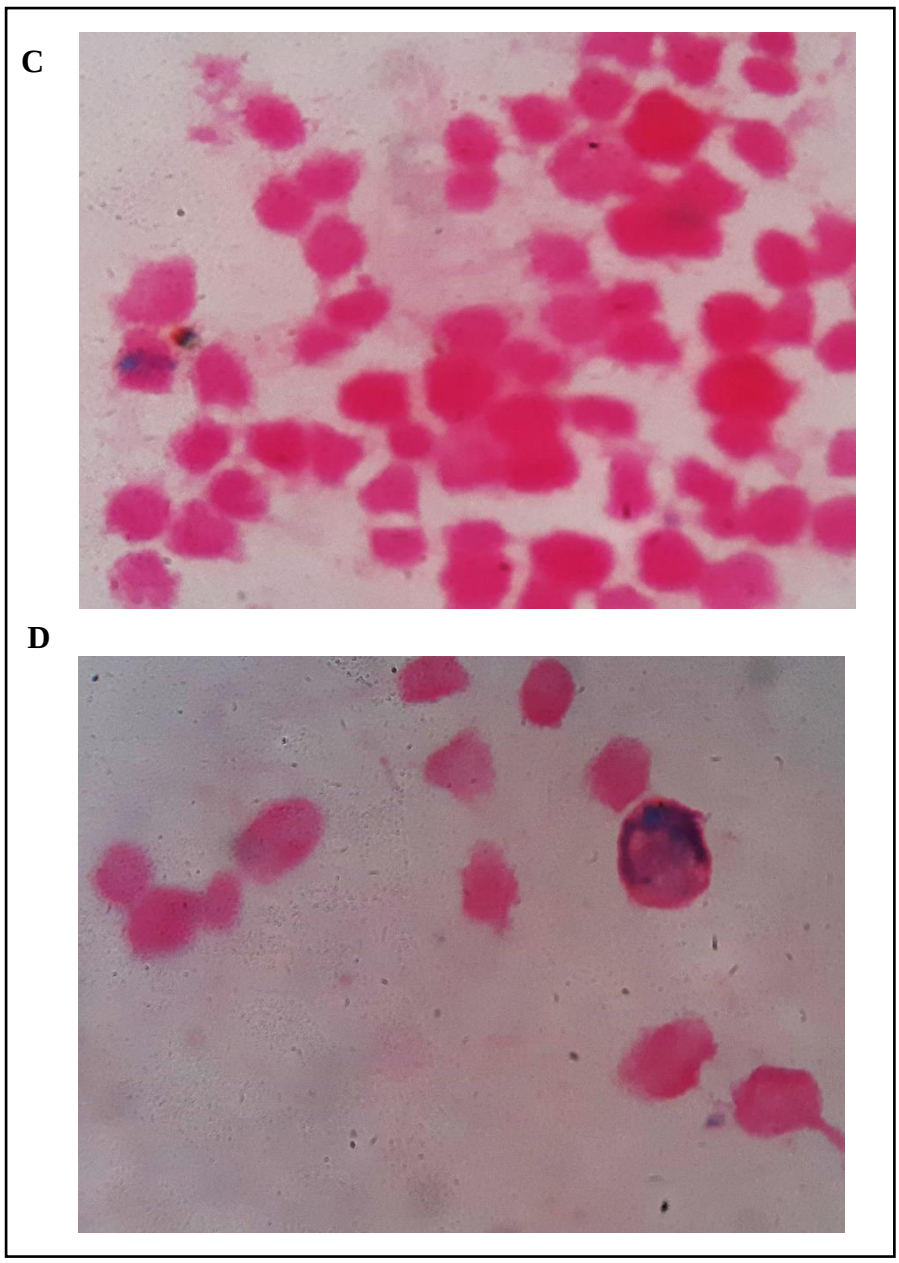


Figure 47. Perl's staining of the bone marrow smear. (A) Control, (B) Leukemic marrow cells showing an increased number of siderocytes and sideroblasts, (C and D) Extract treated and active compound treated marrow cells shows a decreased number of Perl's positive marrow cells.

Bone marrow cells from leukemic group reveals the presence of sideroblasts and siderocytes showing macrophagic iron stored in marrow (Figure 47). No sideroblasts were observed in control group. Post-treatment the number of sideroblasts were reduced as compared to

leukemia (Leukemia versus Treated = 23.00 ± 5.09 versus 13.43 ± 2.21; p = 0.01) (Gfhc et al. 2022).

4.5.10. Effect of treatments on splenomegaly

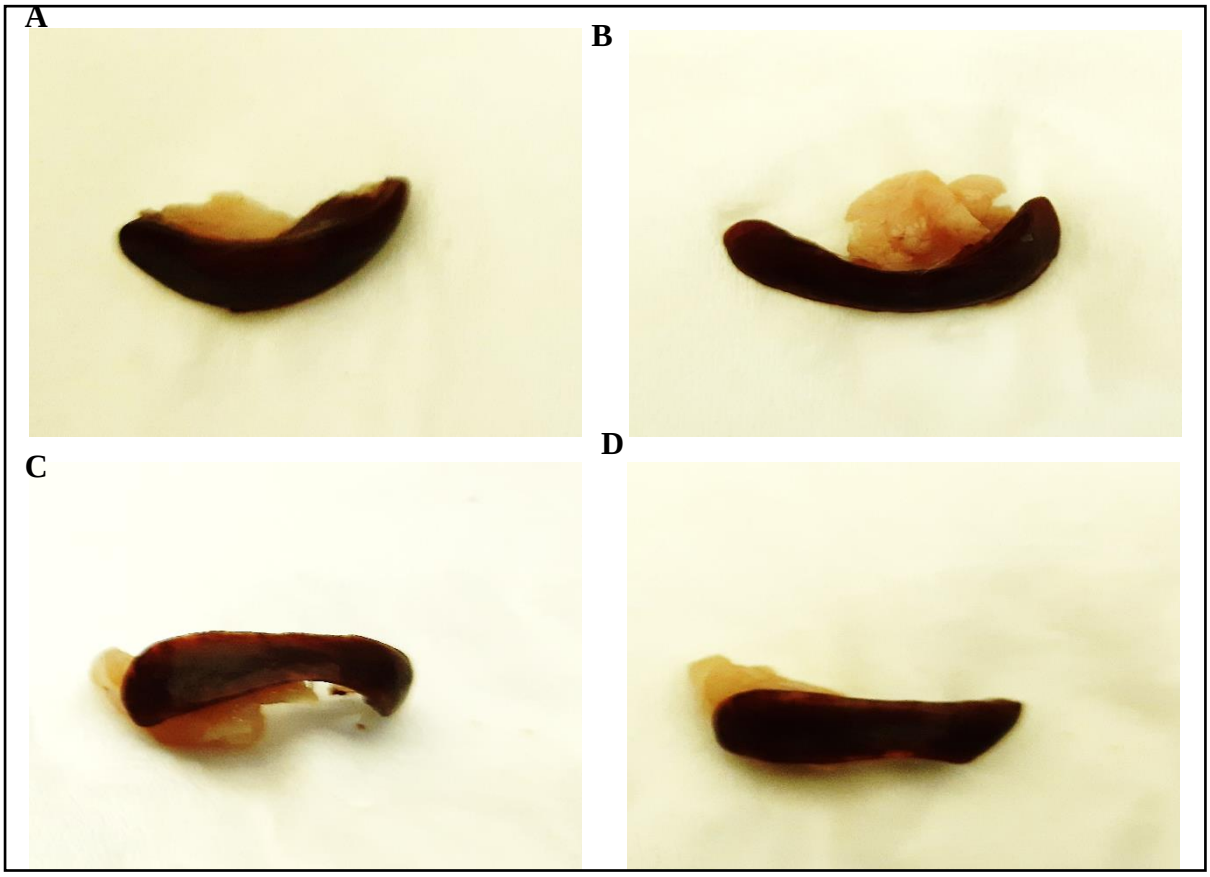


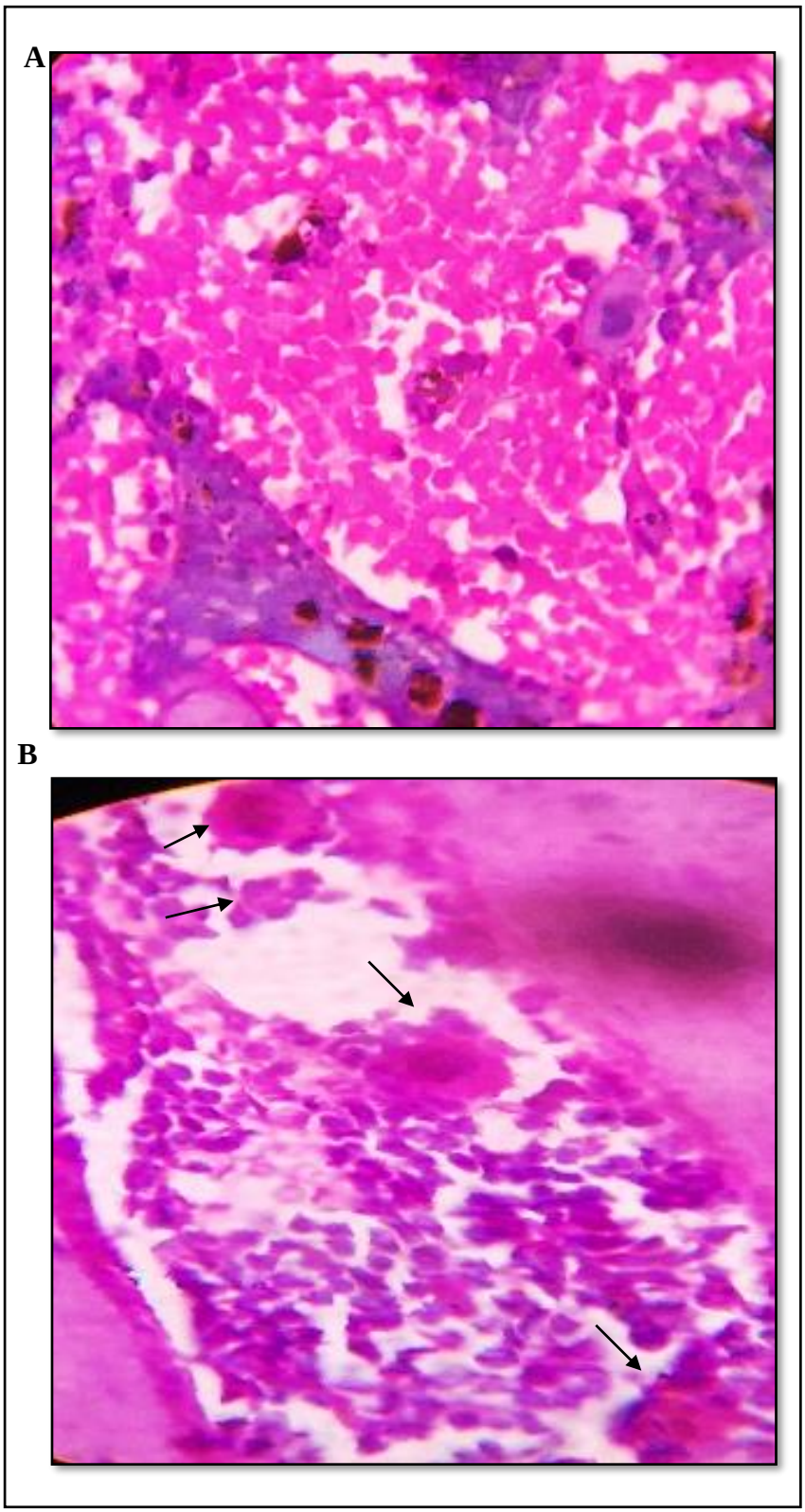
Figure 48. Isolation of spleen from experimental groups. (A) Control, (B) Leukemia, (C) Extract treated group and (D) Wedelolactone treated group.

The spleen was enlarged massively in ENU-treated leukemic group with fat accumulation (Figure. 48) as compared to control. Spleen isolated from treated groups were not much reduced in size but significant difference in fat accumulation was noted.

4.5.11. Bone marrow histology before and after treatment

Bone marrow sections from each experimental groups were stained with H&E stain to identify blast cell population. Marrow section from leukemic group showed an excessive infiltration of blast cells with abnormal localization of immature precursors of blasts and an abnormal

architecture of the bone marrow in contrast to control. No such significant changes in marrow architecture was observed in the treated groups but blast burden was fairly reduced (Figure 49).



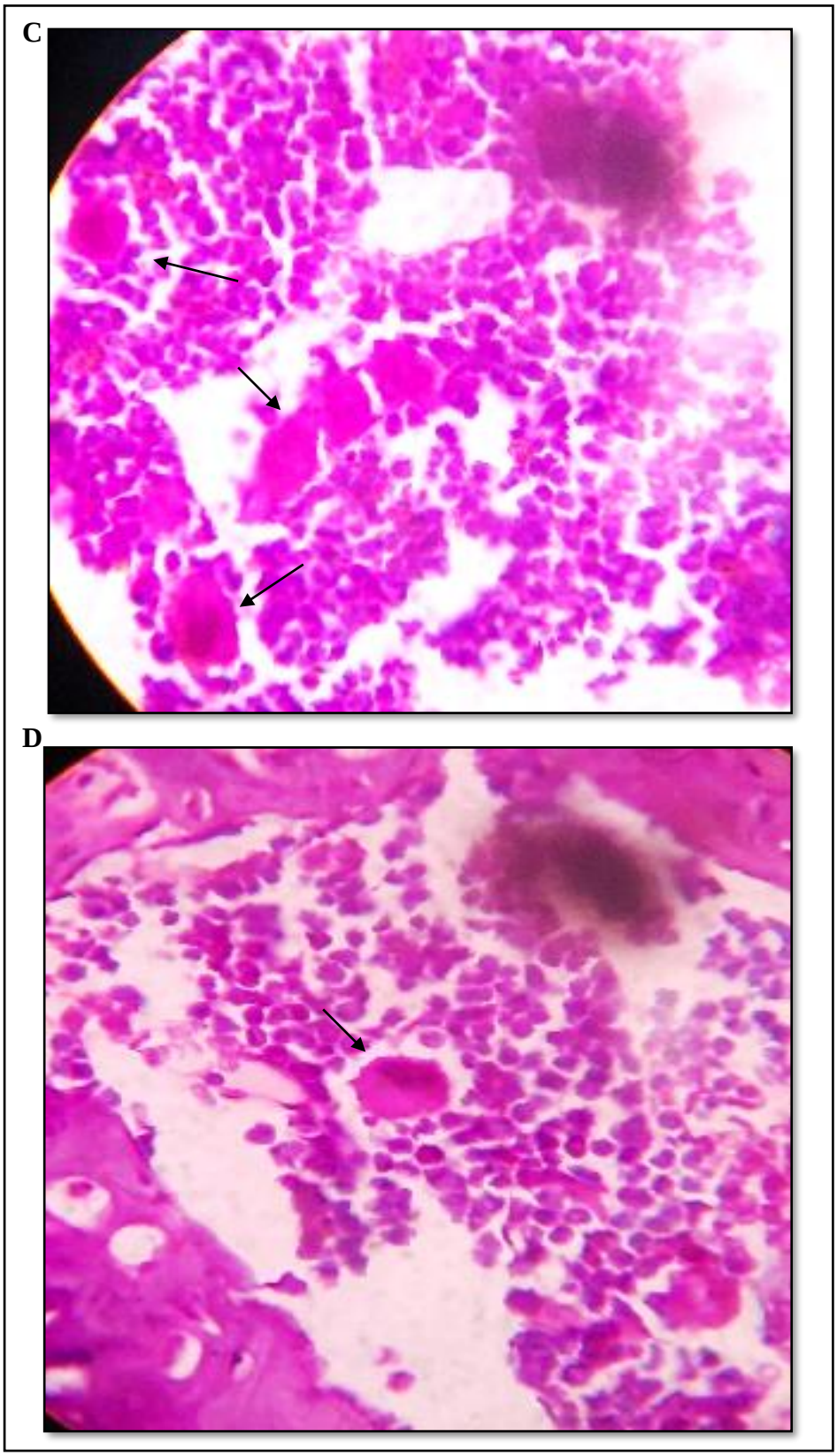
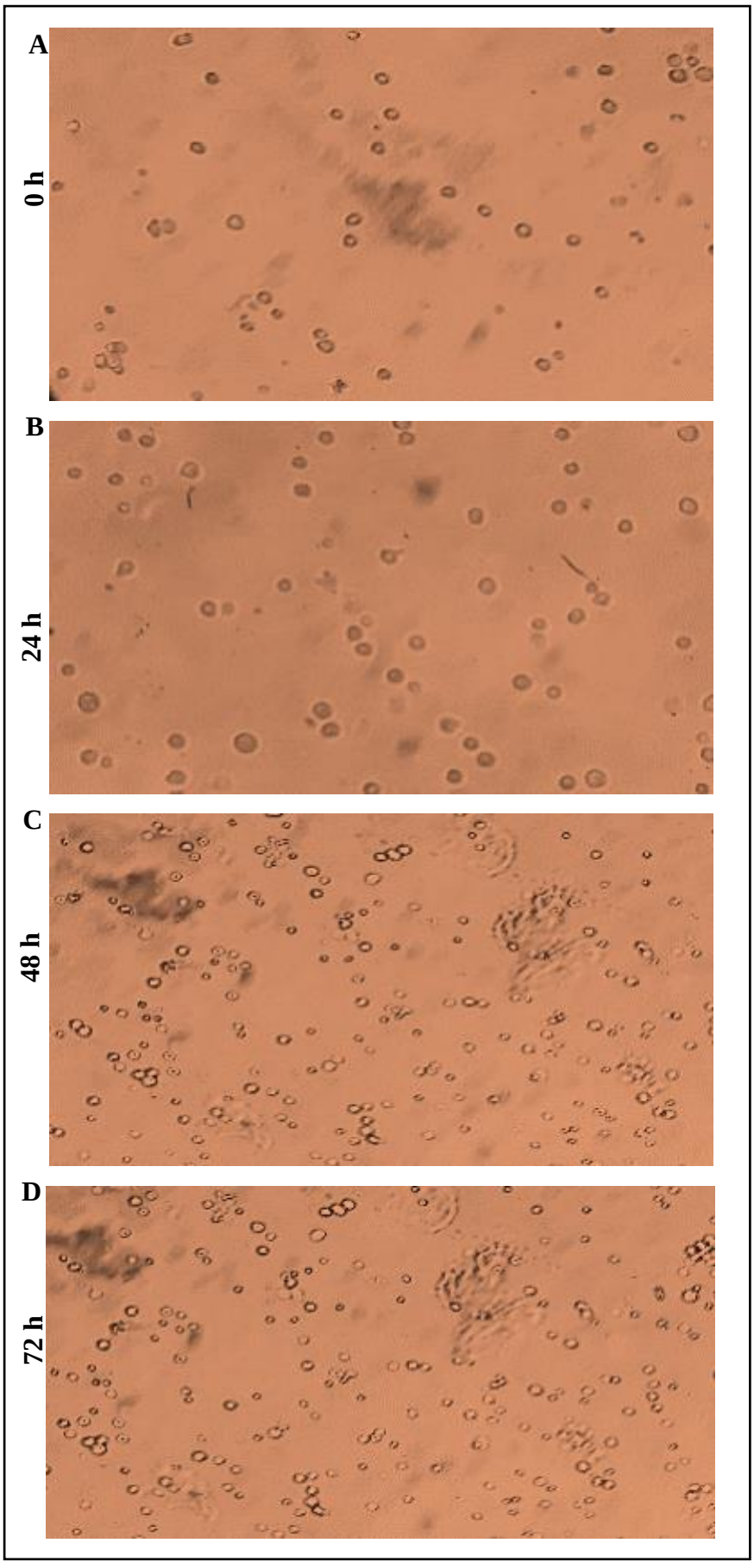
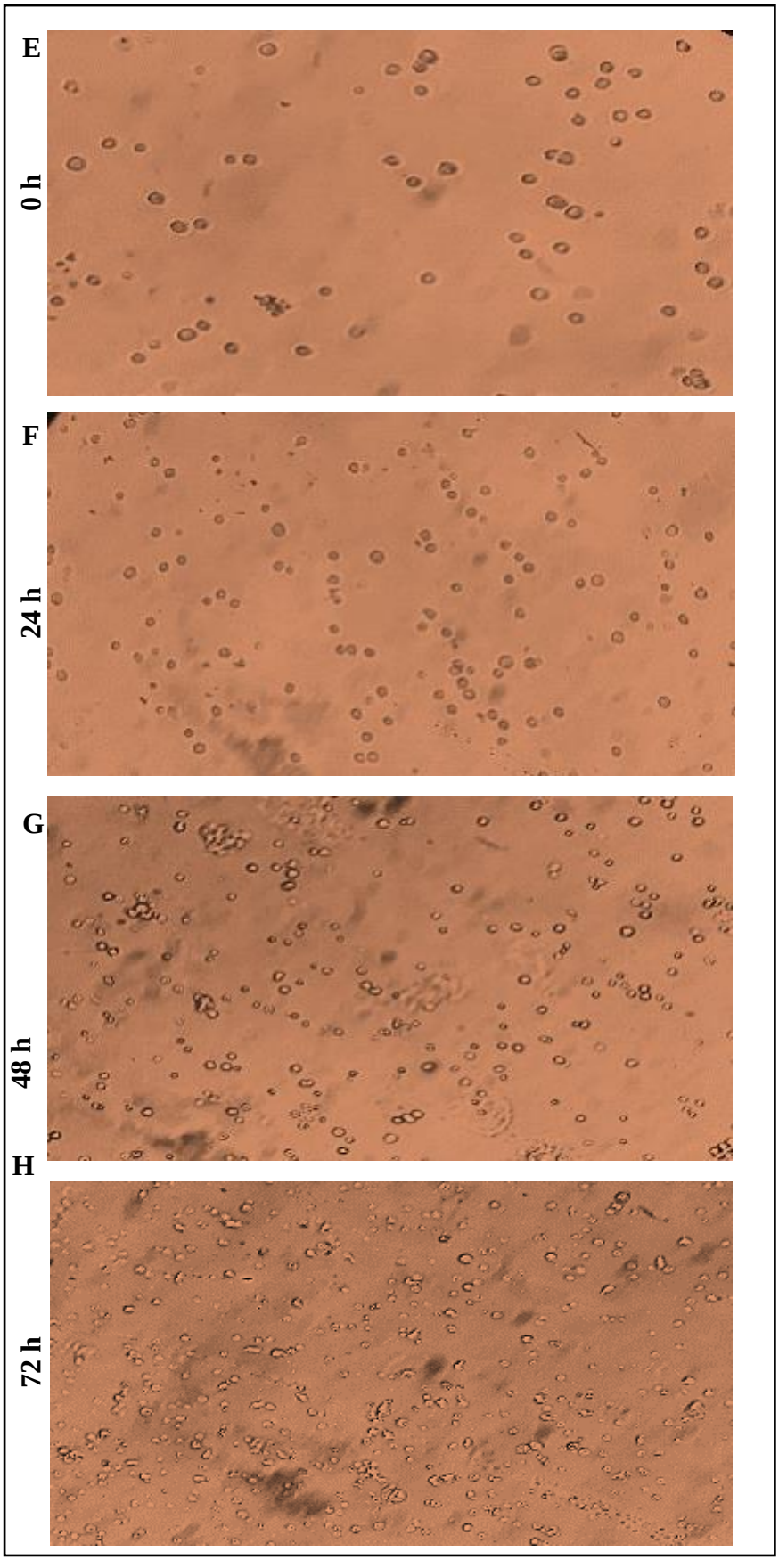


Figure 49. Bone marrow sections of (A) Control, (B) Leukemia and (C, D) Treated groups stained with H&E stain showing the infiltration of blasts cells, marked with black arrows

4.5.12. Effect of treatment on short-term bone marrow cell culture




E
0 h
F
24 h
G
48 h
H
72 h

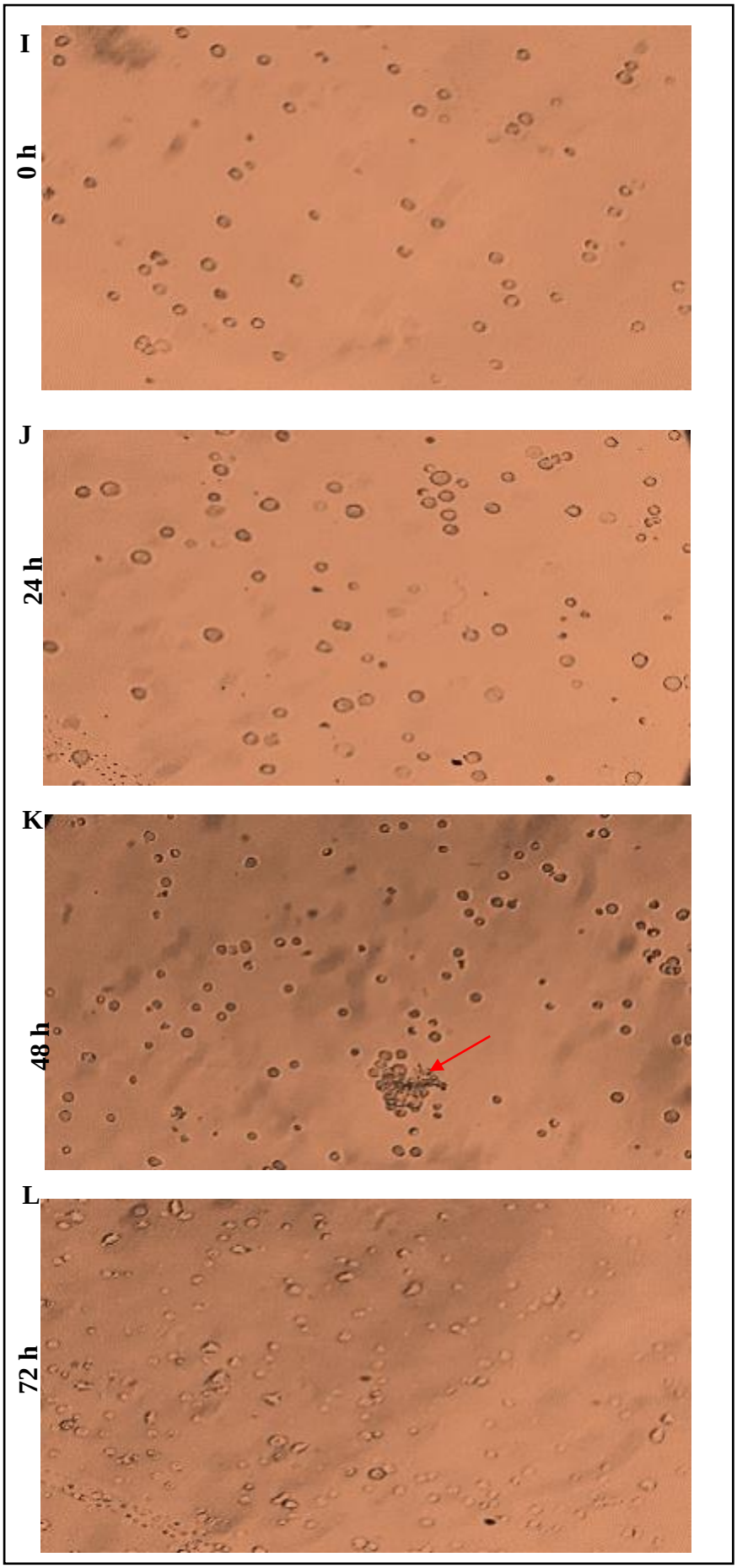
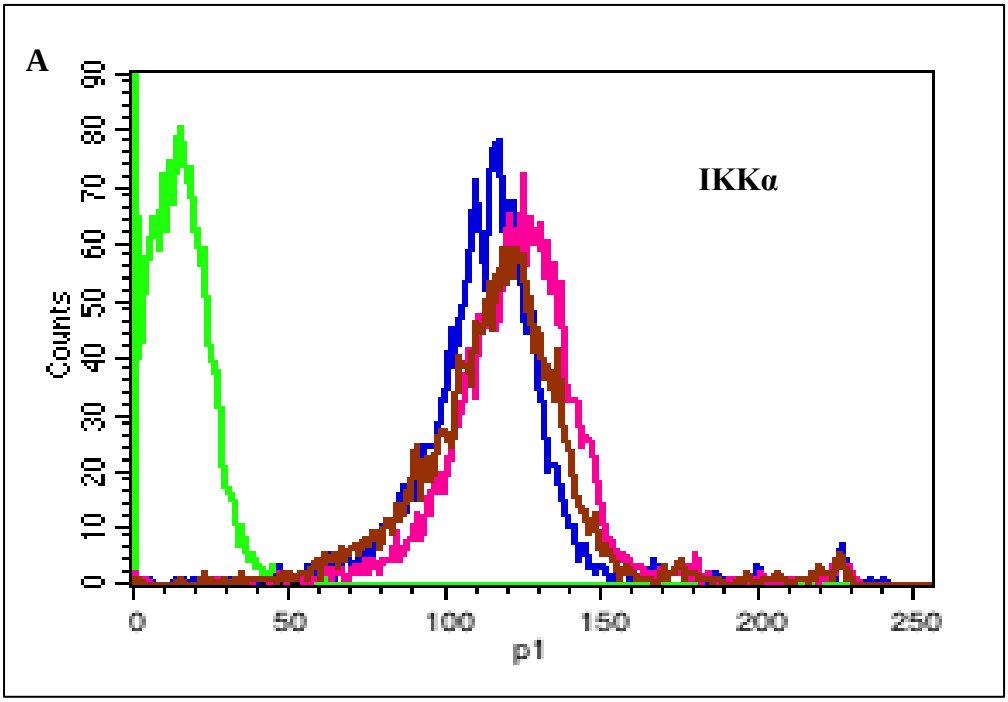
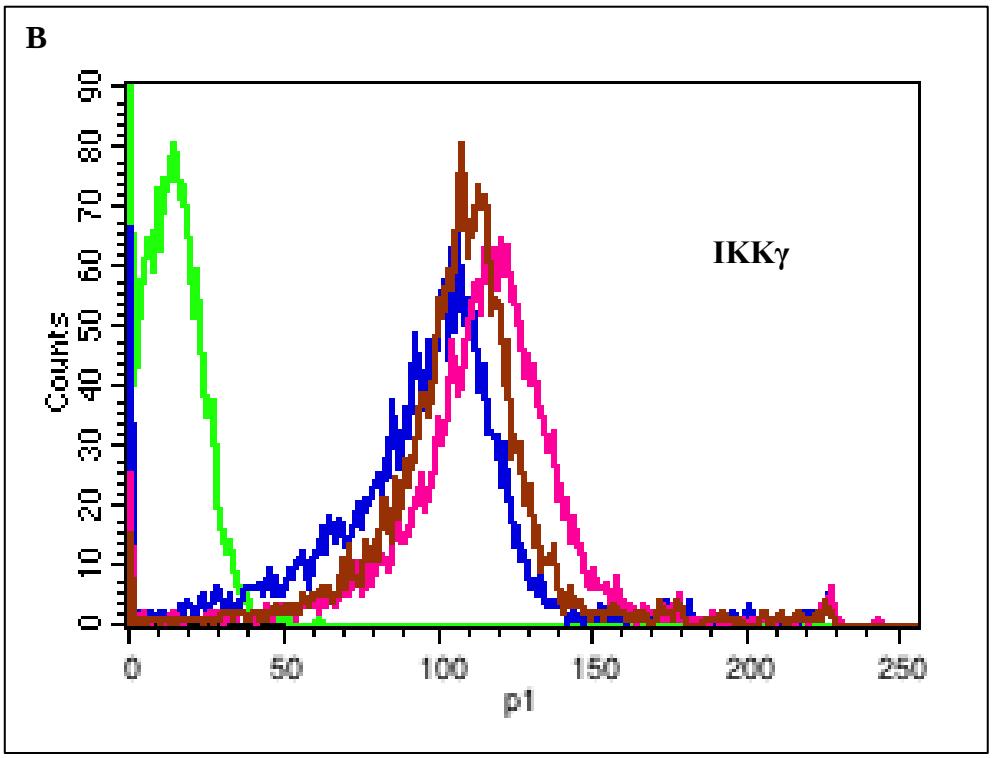

I
0 h
J
24 h
K
48 h
L
72 h

Figure 50. Short term culture of bone marrow cells. Bone marrow cell culture from ENU-induced leukemia (E; 0 h, F; 24 h, G; 48 h. H; 72 h) shows excessive marrow cell proliferation at 48 h, in contrast with control (A; 0 h, B; 24 h, C; 48 h, D; 72 h) b. In extract treated group, fair decrease in cell growth along with the presence of apoptotic bodies (marked in red arrow) has been observed (I; 0 h, J; 24 h, K; 48 h, L; 72 h).

A fair decrease in the bone marrow cell proliferation were observed post treatment with *Eclipta alba* extract, with the evidence of apoptotic bodies. The leukemic group expressed massive proliferation of the bone marrow cells especially at 48h, along with the formation of spindle shaped stromal precursor (Figure 50).

4.5.13. Immunomodulation of the NF-kB pathway by Eclipta alba and wedelolactone




B
IKKγ
Counts
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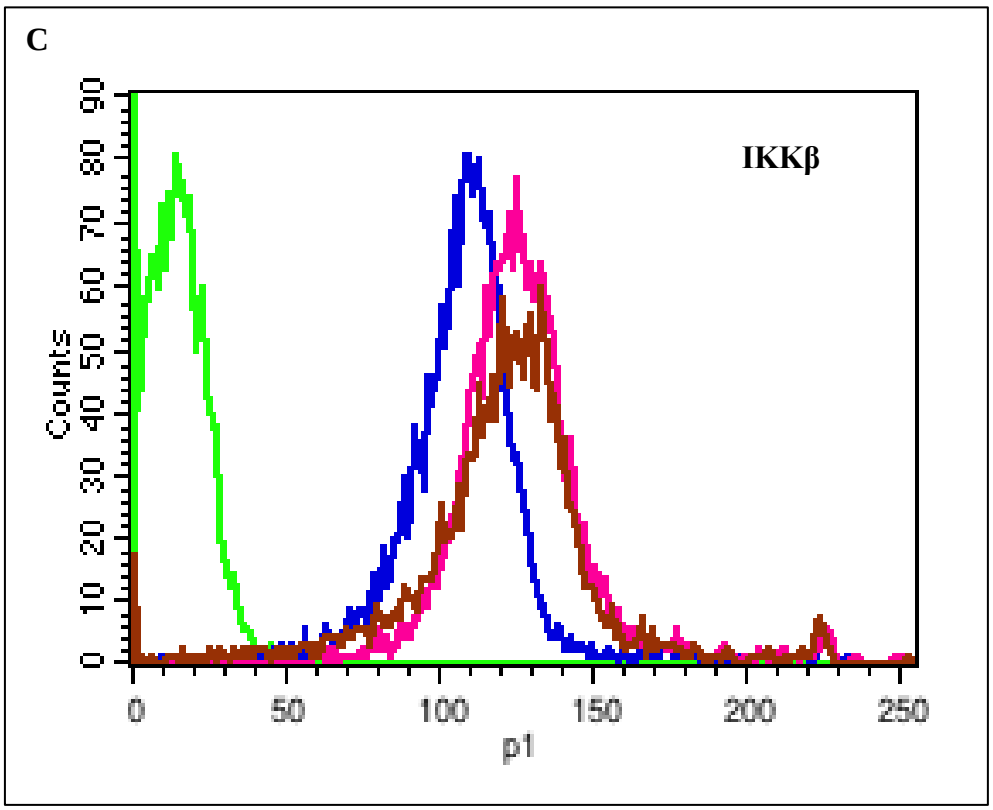
10
20
30
40
50
60
70
80
90
0
50
100
150
200
250
p1


C
IKKβ
Counts
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10
20
30
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50
60
70
80
90
0
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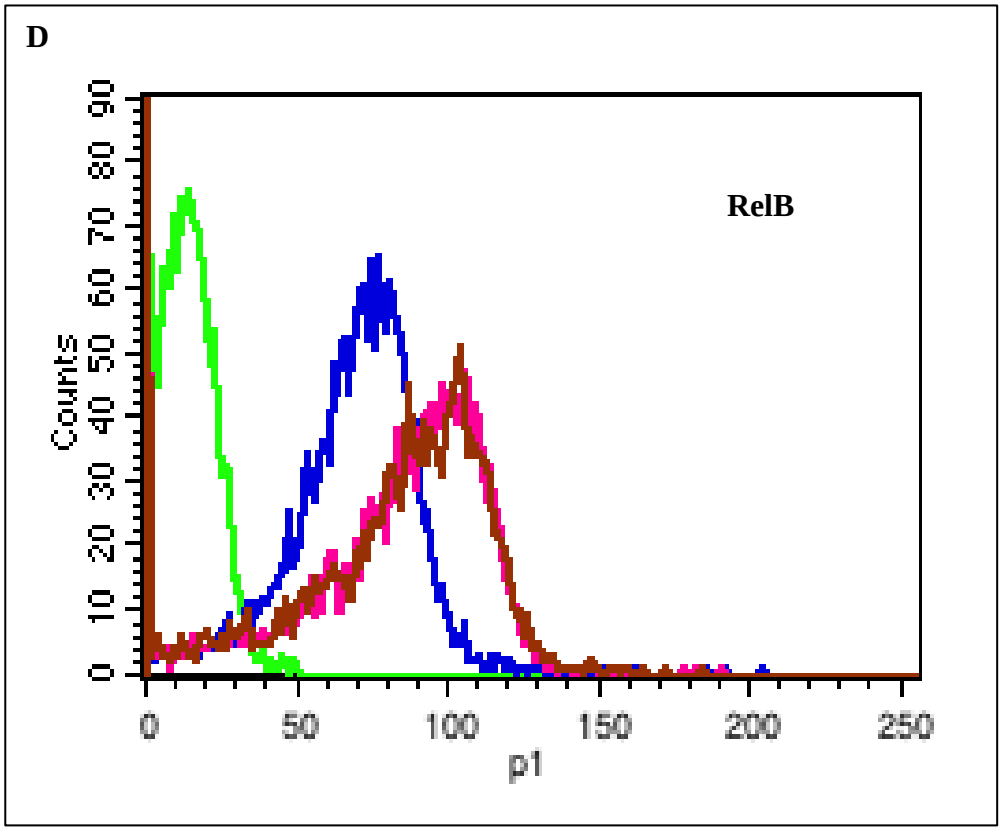
100
150
200
250
p1


D
RelB
Counts
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40
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80
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0
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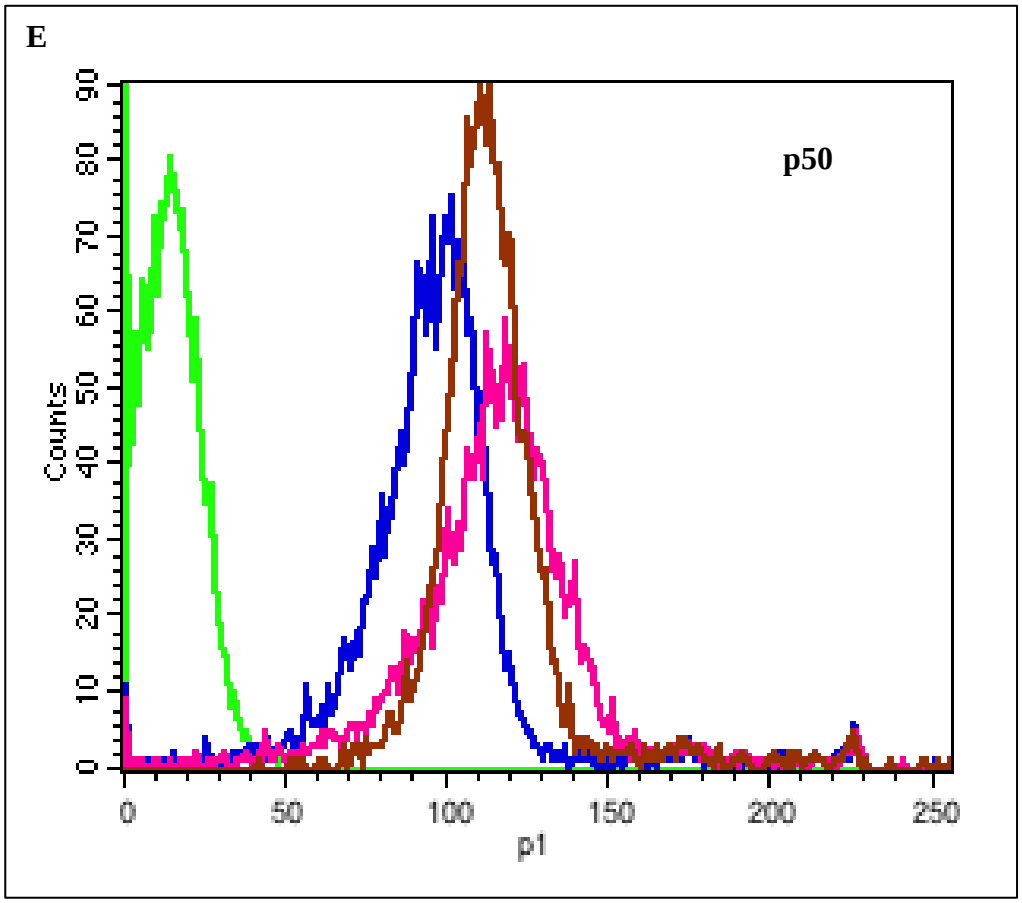
p1


E
p50
Counts
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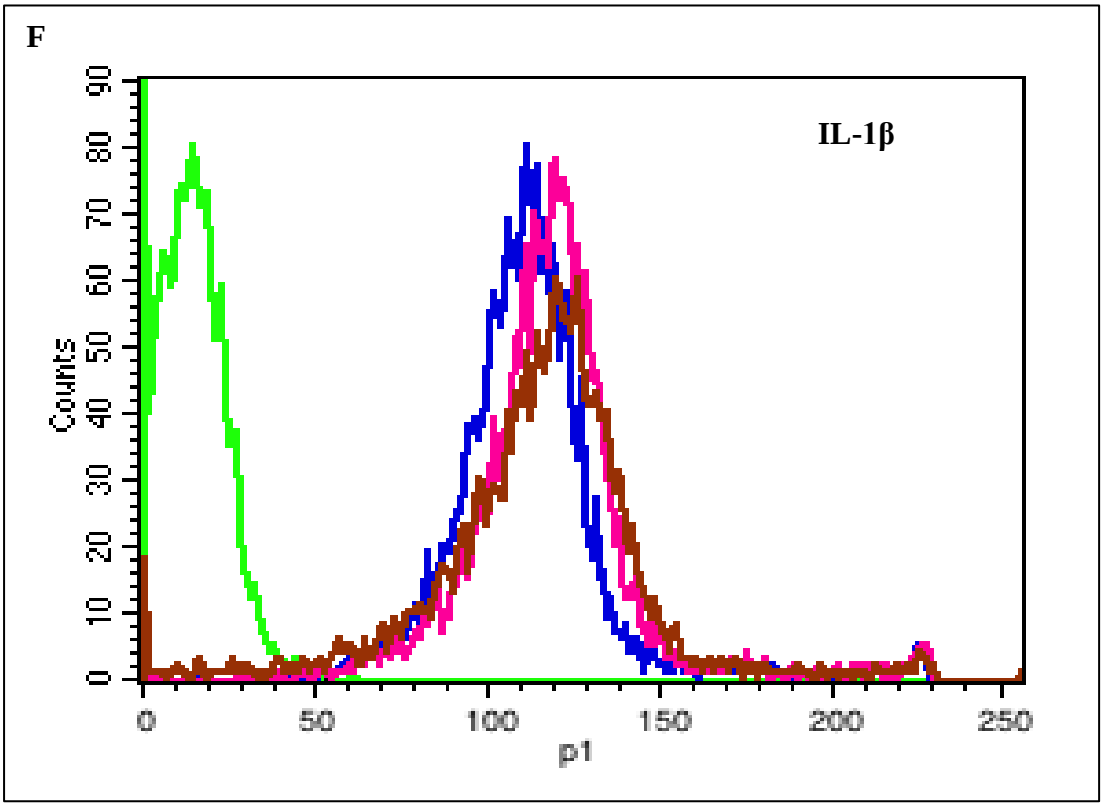
p1

F


IL-1β
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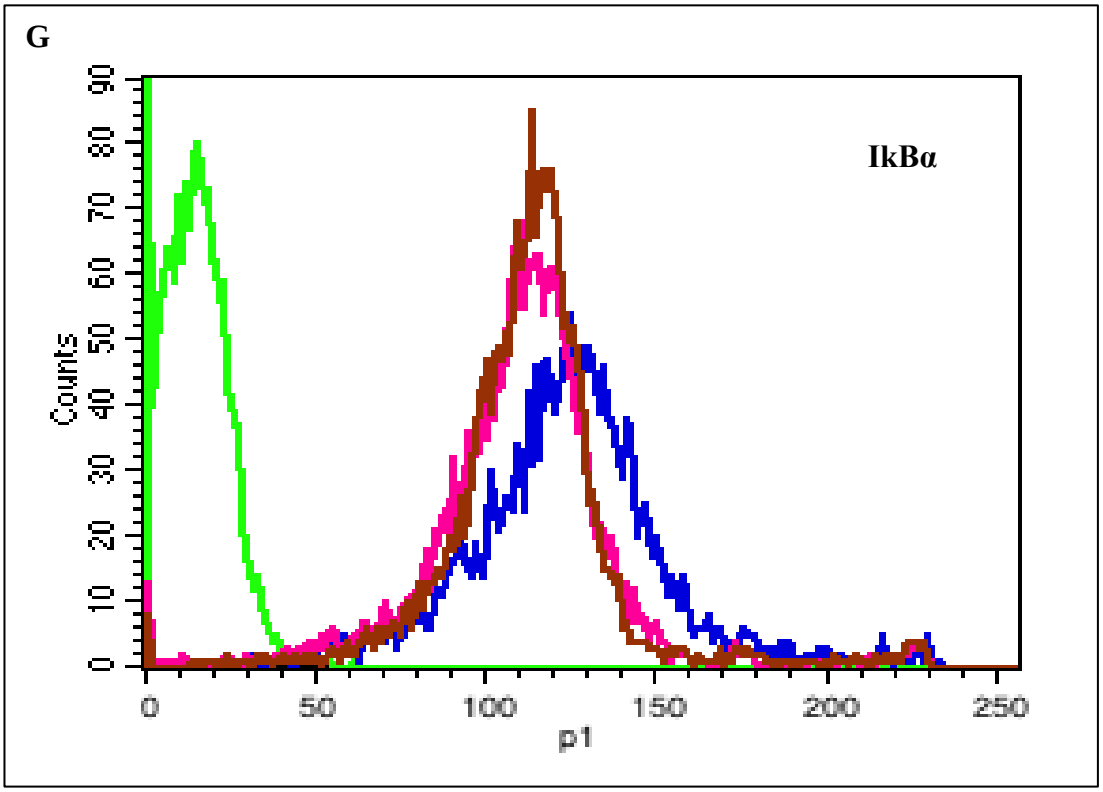
p1

G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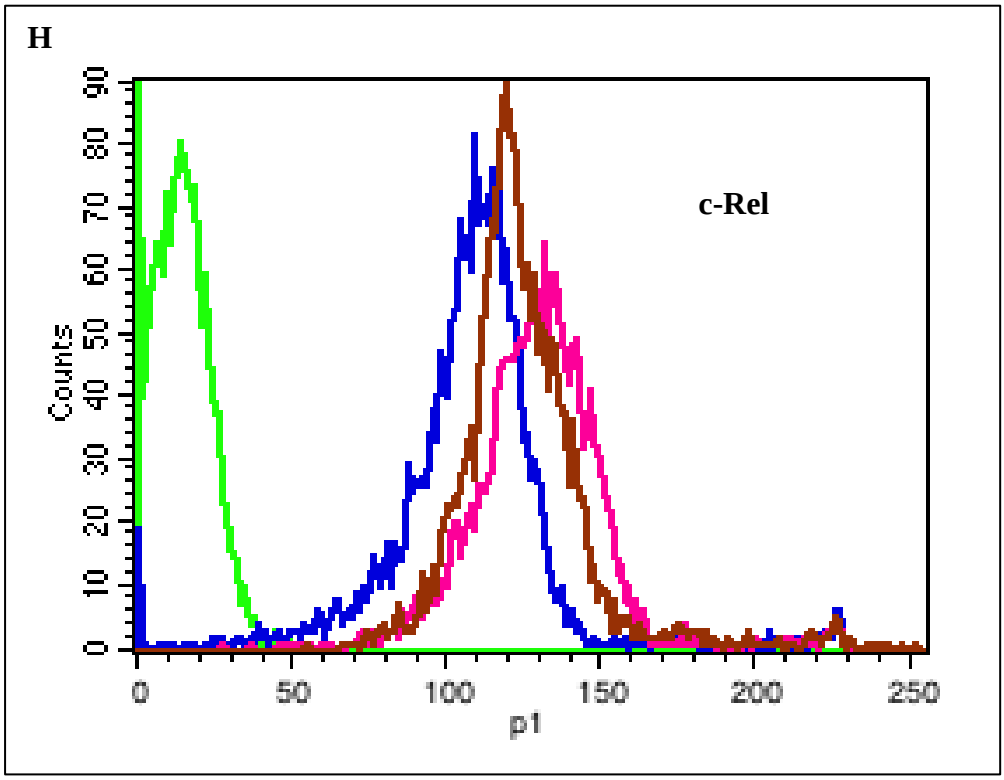
p1

H


c-Rel
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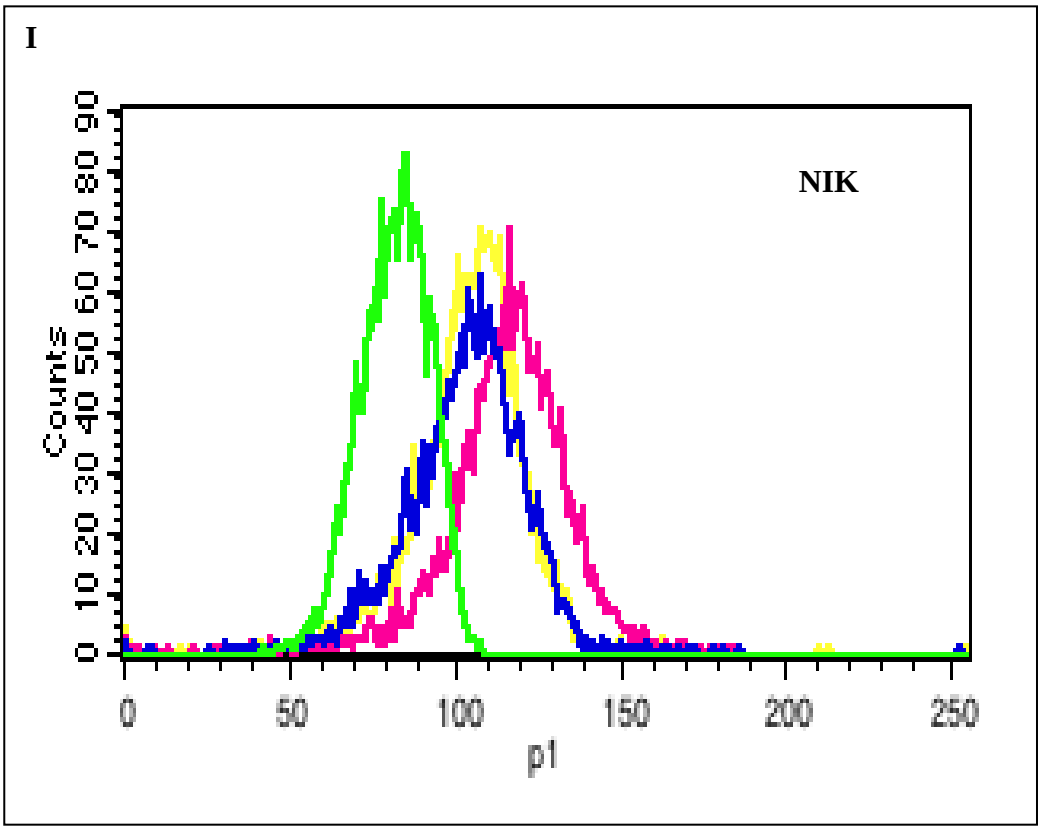
200
250
p1

I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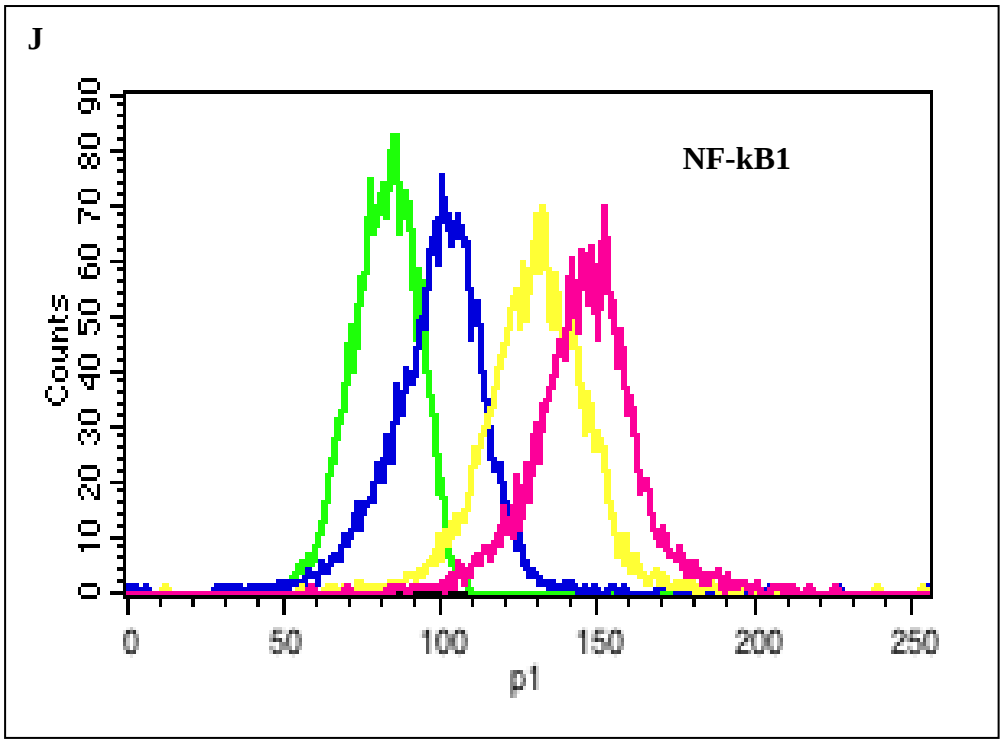
200
250
p1


J
NF-kB1
Counts
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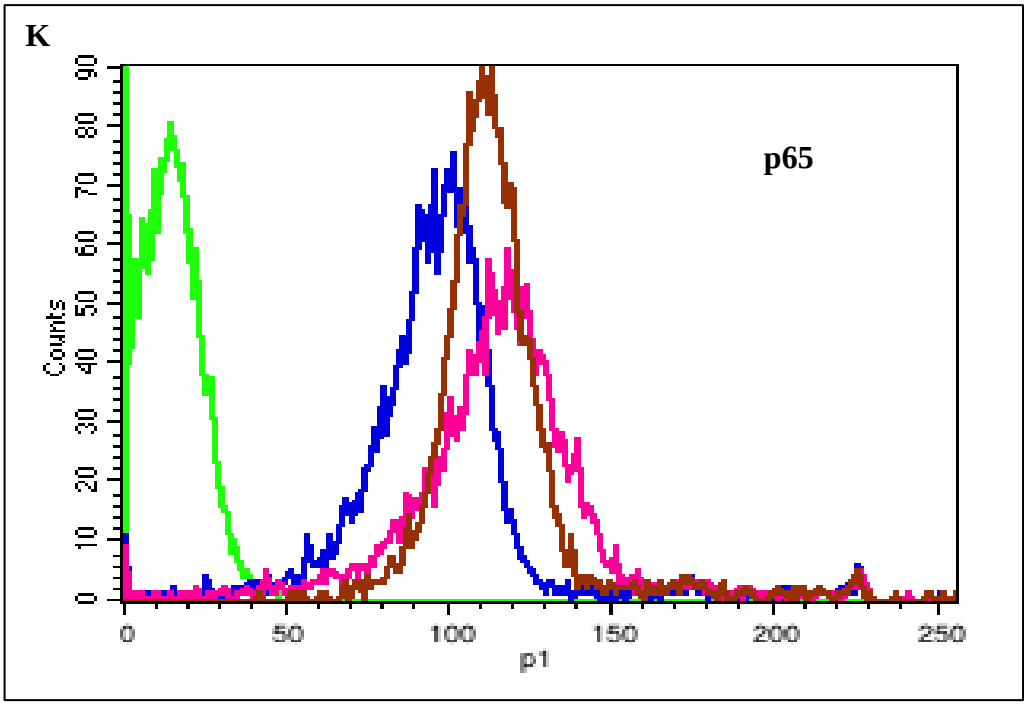
150
200
250
p1


K
p65
Counts
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90
0
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200
250
p1

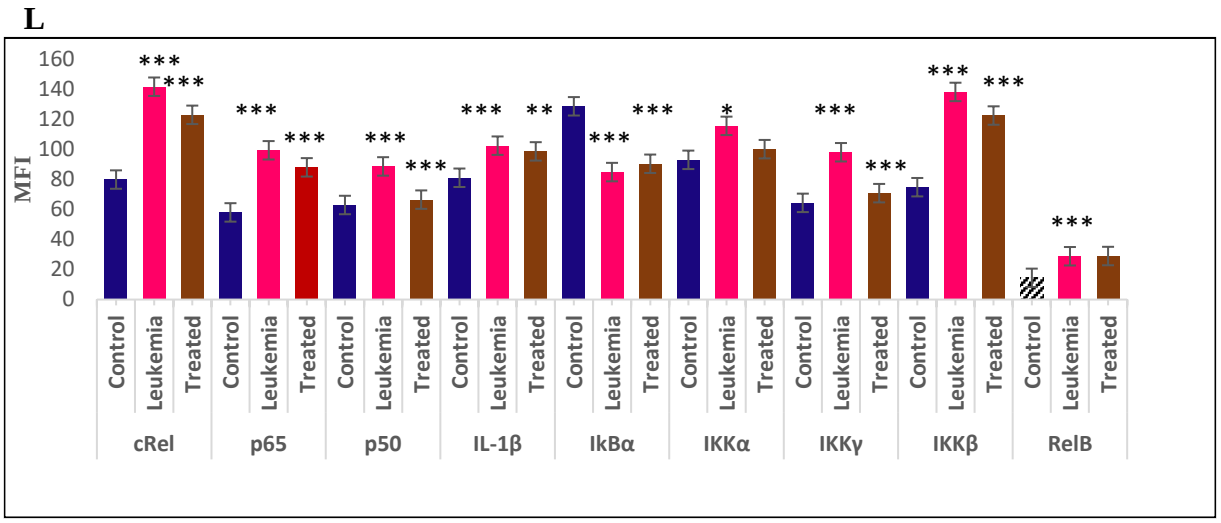


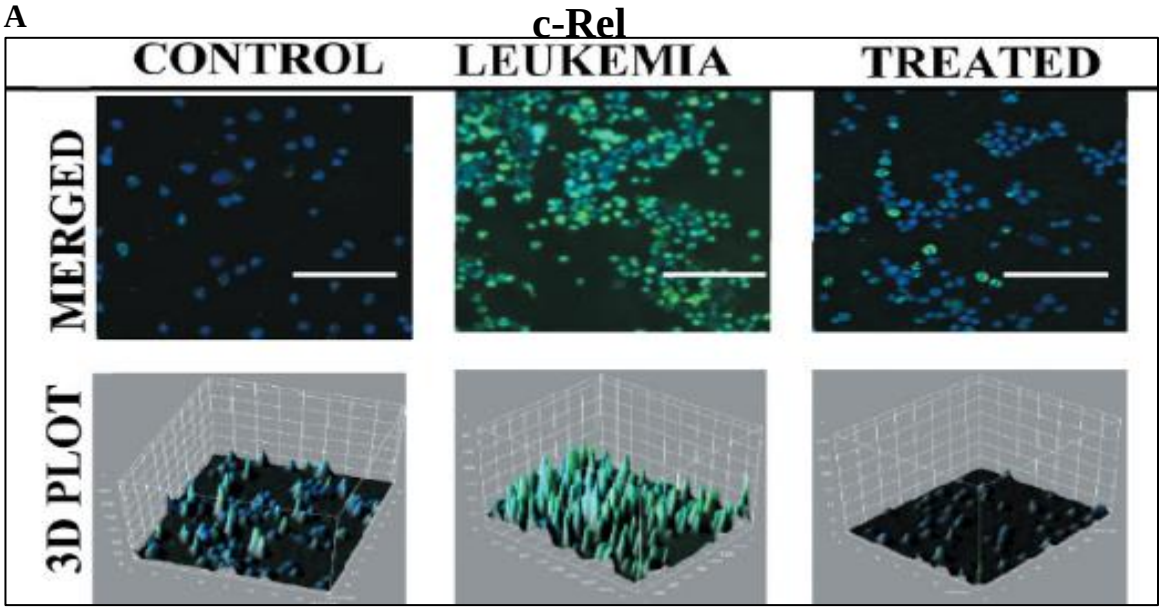
Figure 51. Flowcytometric expression to evaluate the efficacy of *Eclipta alba* to modulate the NF-kB signaling pathway in the bone marrow. (A – K) Representative histogram overlay revealed significant down-regulation of IKKα, IKKγ. cRel, p65, p50, IL-1β, and up regulation of IkBα in post treatment groups compared to leukemic mice. No significant changes were observed in IKKβ and RelB post *Eclipta alba* treatment. (L) Graphical representation of the Mean Fluorescence Intensity (MFI) values of the molecules (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; Values are Mean ± SEM; One-way ANOVA by Tukey's Test).

According to the flowcytometric expression, significant (P < 0.001) increase of c-Rel (Figure 51. H) in the bone marrow cells of leukemic mice were observed (MFI; 141.48 ± 0.5) by 1.4-fold in contrast to control (MFI; 79.77 ± 0.68). Significant (P < 0.001) decrease of c-Rel by 1.1-fold (MFI; 122.79 ± 0.71) compared to leukemic group was noted after *Eclipta alba* treatment. p65 expressional profile (Figure 51. K) was significantly (P < 0.001) increased in the marrow cells of disease group (MFI; 99.22 ± 0.69) by 1.7-fold compared to control (MFI; 57.93 ± 0.90). Significant decline in the (P < 0.001) p65 expression (MFI; 87.89 ± 0.84) by 1.1-fold was observed in extract treated group as compared to ENU induced leukemia. Furthermore, significant (P < 0.001) increase of p50 expression (Figure 51. E) and IL-1β (Figure 51. F) was seen in diseased marrow cells (MFI; 88.50 ± 0.50 and 102.30 ± 0.60,

respectively) compared to normal (MFI; 62.86 ± 0.80 and 80.96 ± 0.94, respectively) by 1.4 and 1.2-folds. Significant decrease of p50 and IL-1β ($P < 0.001$ and $P = 0.008$) was revealed post treatment (MFI; 66.43 ± 0.61 and 98.49 ± 1.31) compared to leukemia. Drastic down regulation ($P < 0.001$) of IkBα (Figure 51. G) by 1.5-fold was revealed in diseased marrow cells (MFI; 84.74 ± 0.65) compared to healthy group (MFI; 128.46 ± 0.5). Fair increase ($P < 0.001$) of IkBα expression (MFI; 90.26 ± 0.64) by 1.0-fold was noted in treated group. IKKγ or NEMO (Figure 51. B) and IKKβ (Figure 51. C) revealed a significant increase in its expression ($P < 0.001$) in diseased state (MFI; 97.92 ± 0.07 and 138.01 ± 0.07, respectively) compared to normal (MFI; 64.28 ± 0.28 and 74.69 ± 0.59 respectively) by 1.5 and 1.8-fold. Massive decline ($P < 0.001$) of IKKγ and IKKβ expression (MFI; 70.73 ± 0.64 and 122.32 ± 0.32, respectively) was seen in treated mice by 1.3 and 1.1-fold compared to diseased state.

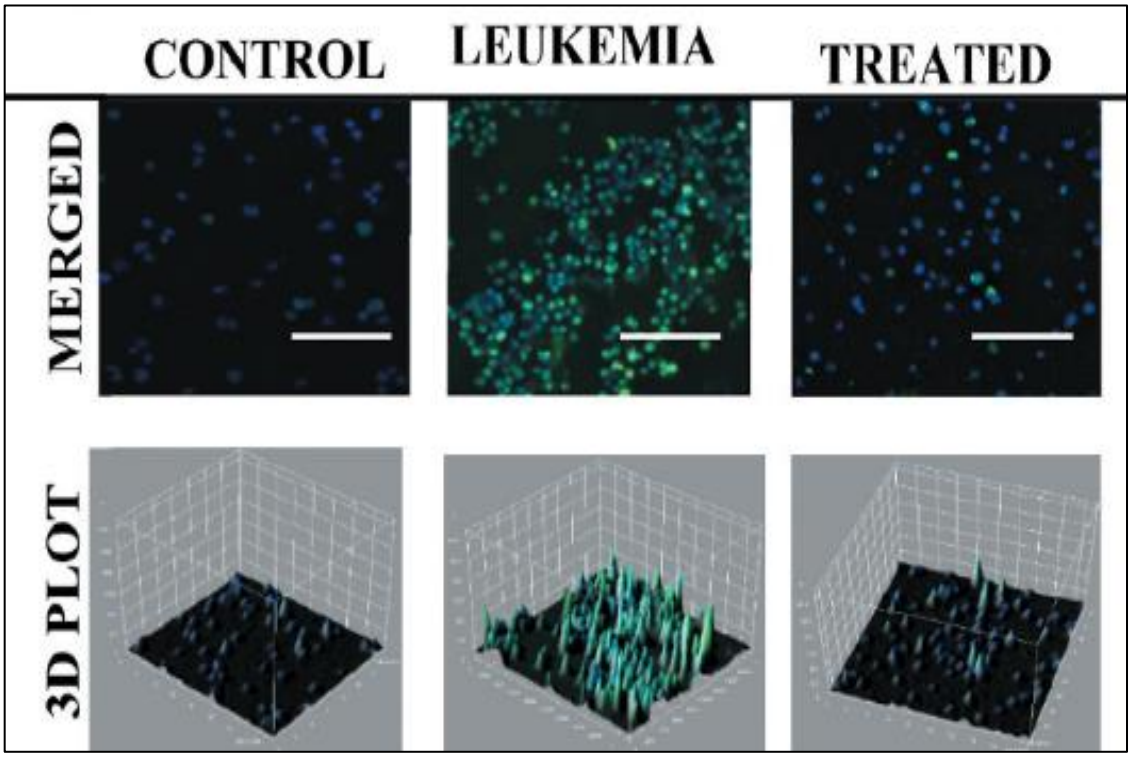
Significant ($P = 0.022$ and $P < 0.001$) incline in IKKα expression (Figure 51. A; Table 1) and RelB expression (Figure 51. D) in diseased state (MFI; 115.46 ± 0.46 and 28.80 ± 0.25) was observed in contrast to healthy control (MFI; 92.88 ± 0.83 and 14.44 ± 0.41). No effect of treatment was observed on IKKα and RelB of treated group (MFI; 99.7 ± 0.95 and 28.94 ± 0.05).

4.5.14. NF-kB modulation as studied by immune-fluorescence



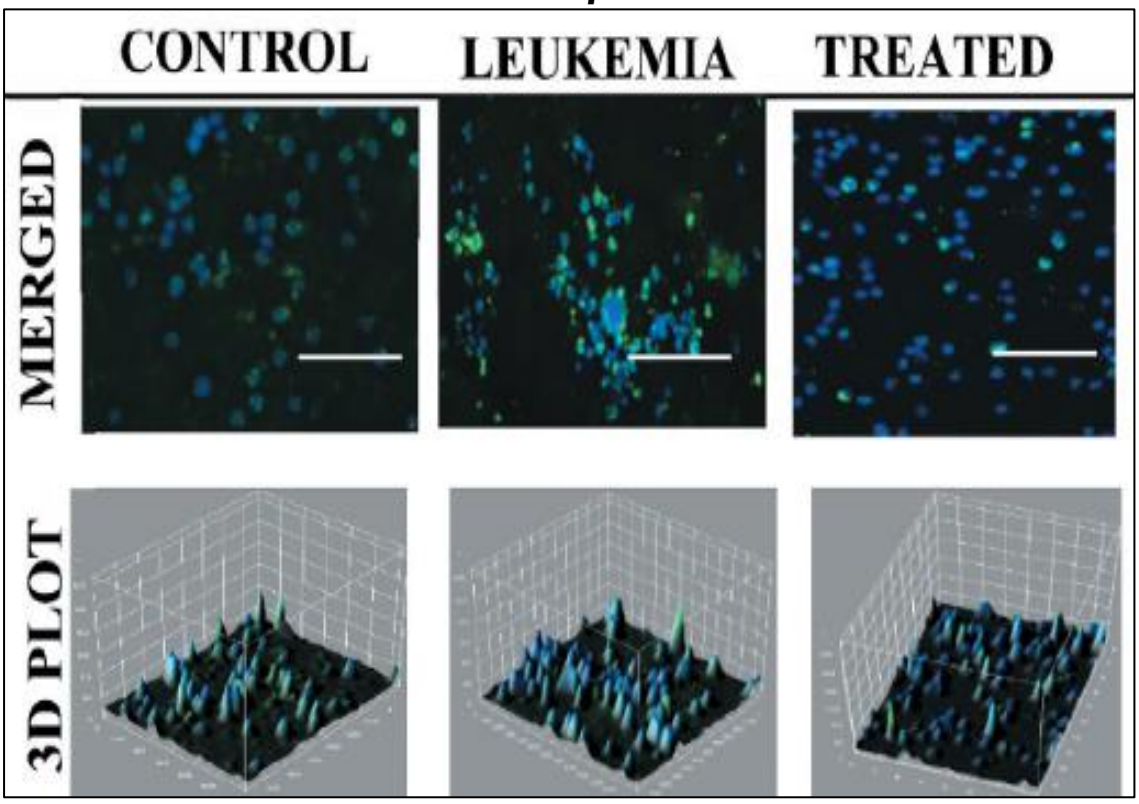
B

p65


CONTROL
LEUKEMIA
TREATED
MERGED
3D PLOT

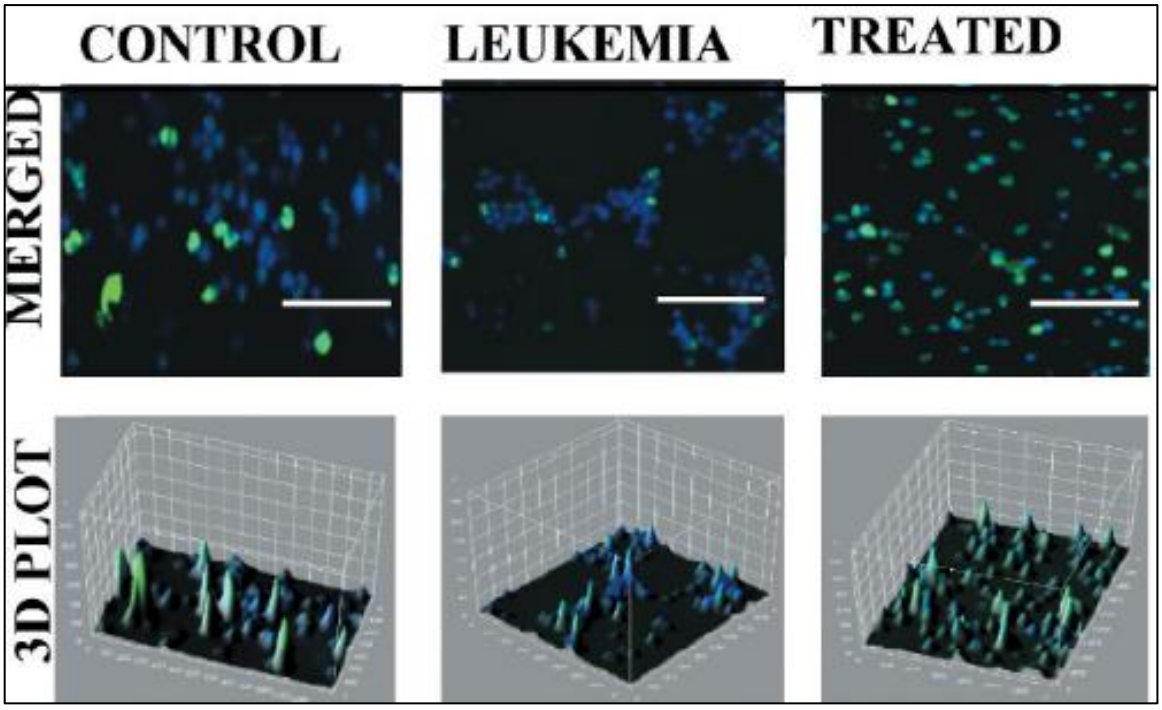
C

IL-1β


CONTROL
LEUKEMIA
TREATED
MERGED
3D PLOT

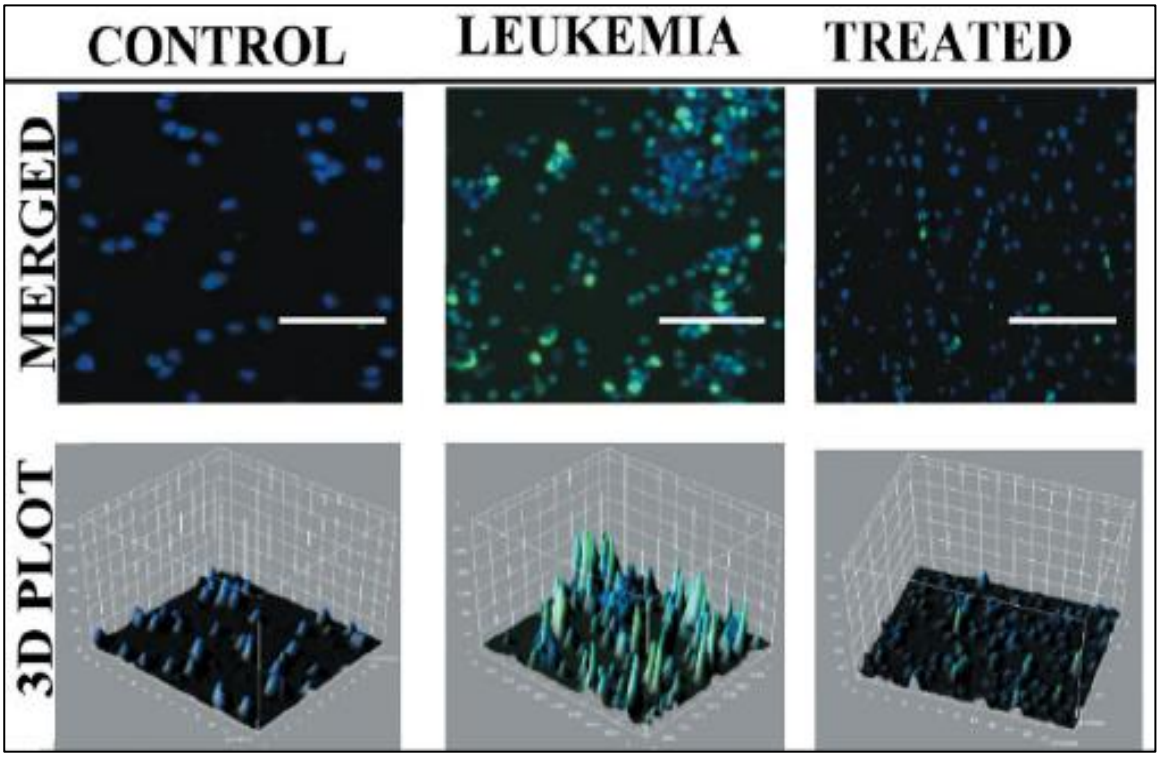
D

$I_kB\alpha$



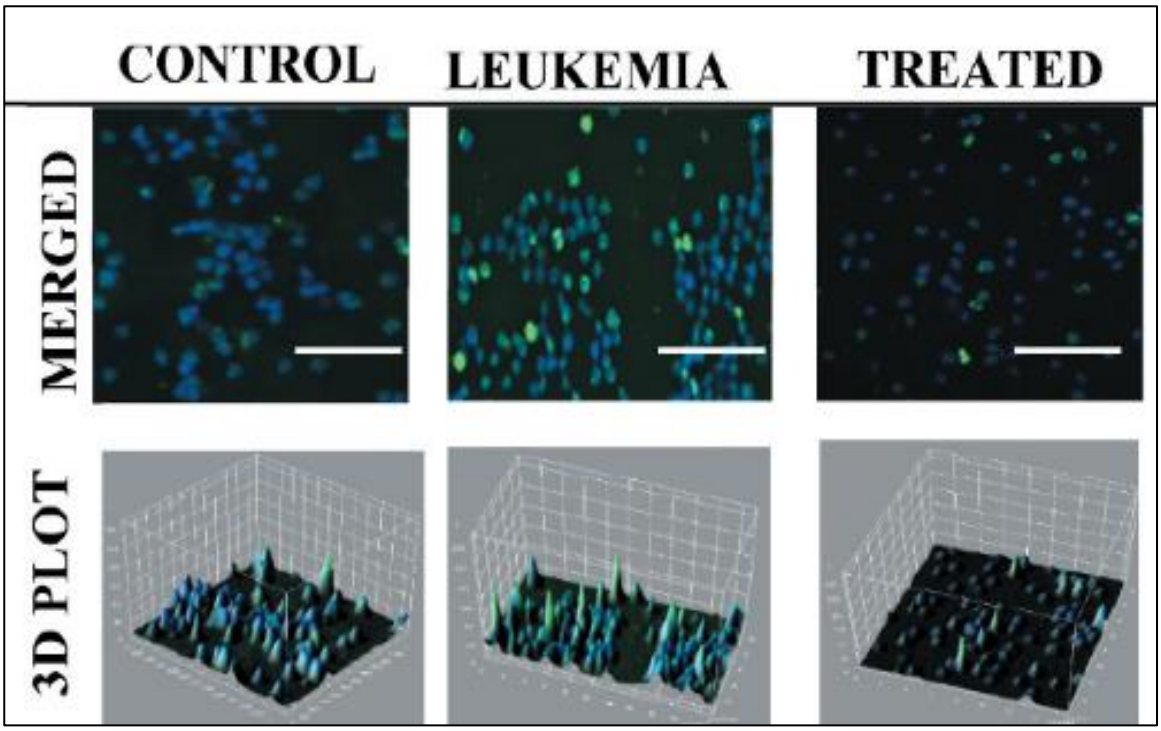
E

p50



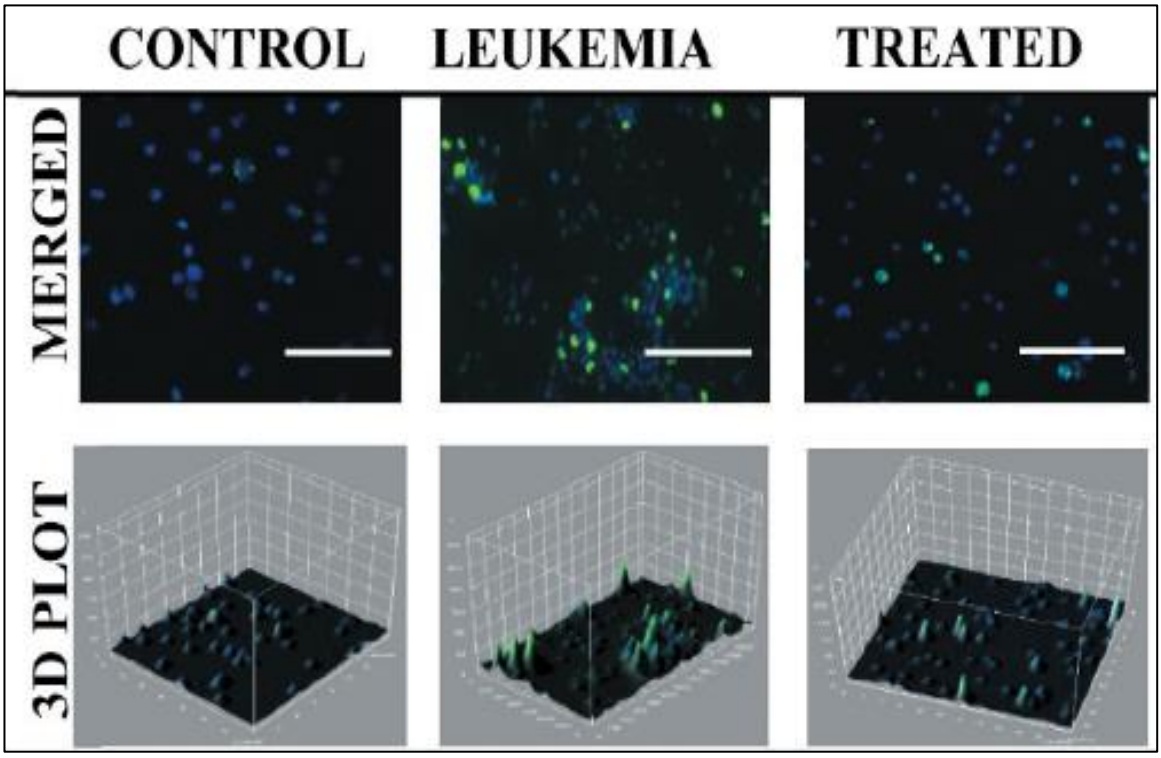
F

IKKα

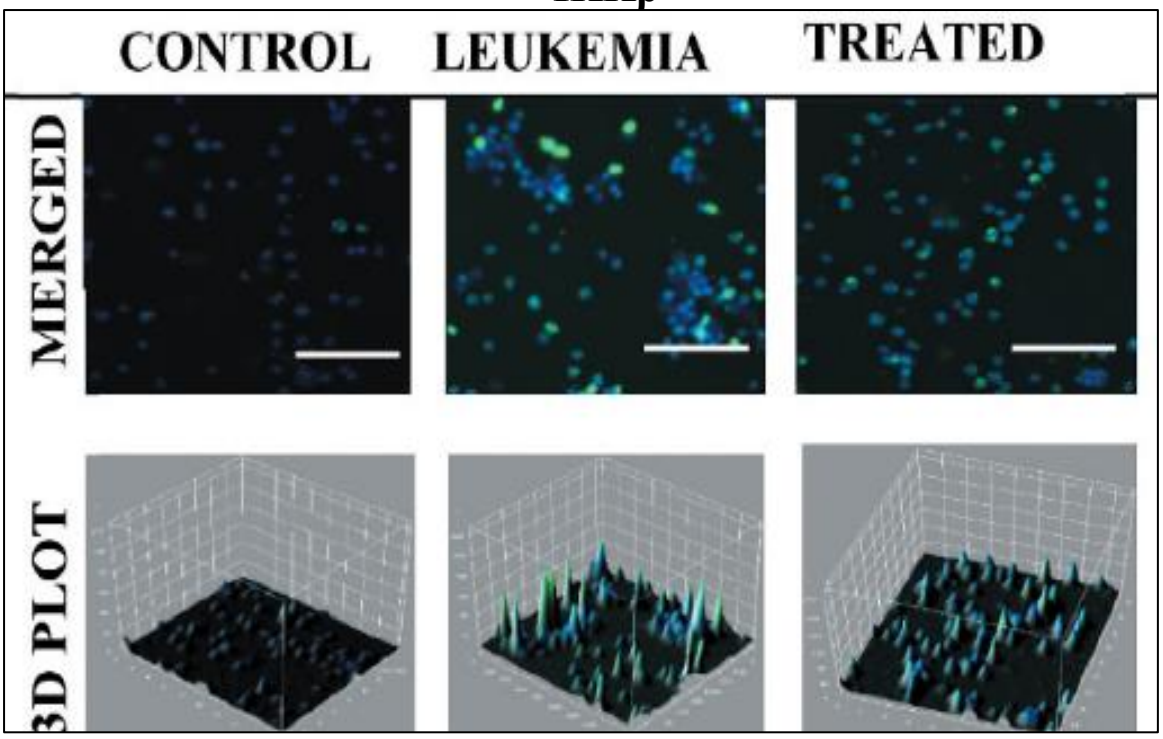

CONTROL
LEUKEMIA
TREATED
MERGED
3D PLOT

G

IKKγ


CONTROL
LEUKEMIA
TREATED
MERGED
3D PLOT

H


IKKβ
CONTROL
LEUKEMIA
TREATED
MERGED
3D PLOT

I

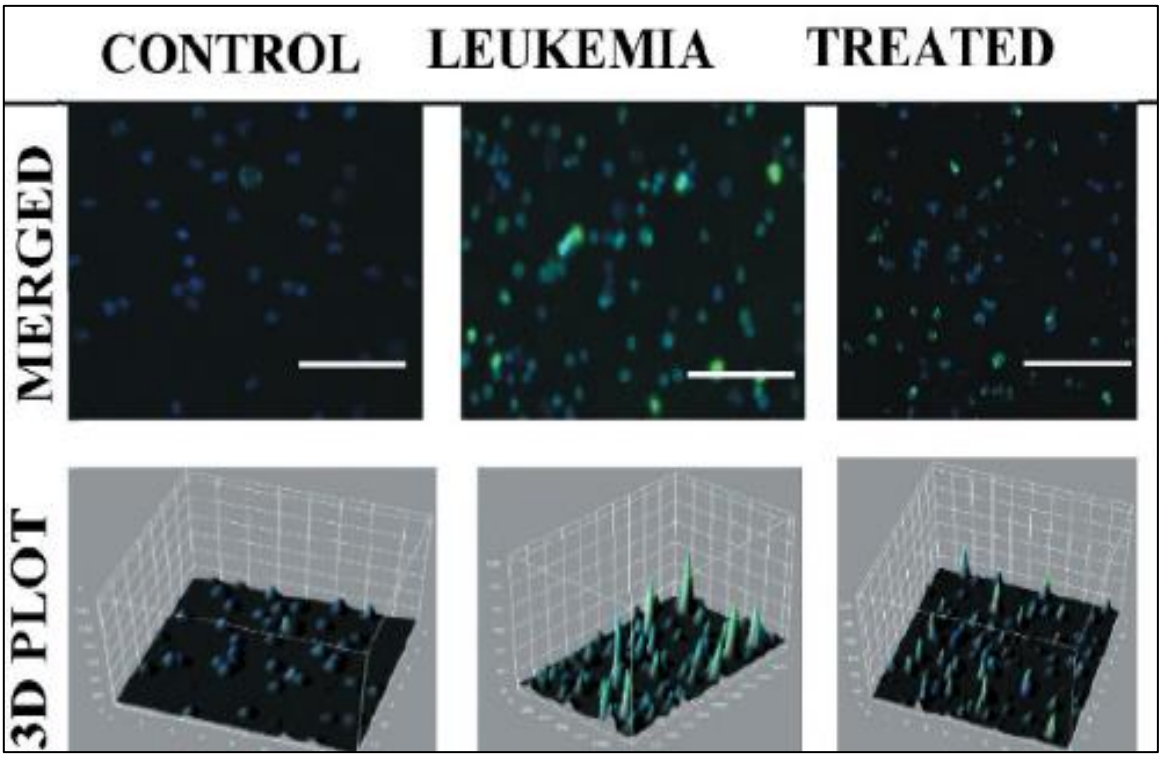

RelB
CONTROL
LEUKEMIA
TREATED
MERGED
3D PLOT

Figure 52. Fluorescence microscopy of marrow cells and the 3D plots (A–I) of control, leukemia and treated group. The representative images were analyzed by 3D interactive surface plot (ImageJ) *(Scale bar 100 μm)*.

For further confirmation on the fluorescence expression of NF-kB proteins like c-Rel, p65, p50, IL-1β, IkBα, IKKγ and IKKβ in the bone marrow cells of experimental groups, the fluorescence intensity signals were recorded by immunocytochemistry (Figure 52) and using the ImageJ software 3D surface plots were generated which resembled to flowcytometric evaluation.

Table 3. Mean Fluorescence Intensity (MFI) represents expression patterns of proteins of NF-kB pathway.

Proteins	MFI of control (X ± SD)	MFI of Leukemia (X ± SD)	MFI of Treated (X ± SD)
cRel	79.77 ± 0.68	141.48 ± 0.50***	122.79 ± 0.71***
p65	57.93 ± 0.90	99.22 ± 0.69***	87.89 ± 0.84***
p50	62.86 ± 0.80	88.50 ± 0.50***	66.43 ± 0.51***
IL-1β	80.96 ± 0.94	102.30 ± 0.60***	98.49 ± 1.31**
IkBα	128.46 ± 0.50	84.74 ± 0.65***	90.26 ± 0.64***
IKKα	92.88 ± 0.83	115.46 ± 0.46*	99.70 ± 0.95#
IKKγ	64.28 ± 0.28	97.92 ± 0.07***	70.73 ± 0.64***
IKKβ	74.69 ± 0.59	138.01 ± 0.07***	122.32 ± 0.32***
RelB	14.44 ± 0.41	28.80 ± 0.25***	28.94 ± 0.05#

Values are mean ± SEM for 6 animals in each observation. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, as compared with control group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, as compared with Leukemia group. Values marked as (#) are not significant.

4.6. Discussion

NOC and its exposure through environment is already a reason for several human cancers. Through our study we tried to delineate the ameliorating property of *Eclipta alba*, an age old

Ayurvedic medidicnal herb on the leukemia inducing carcinogenic NOC, N-N-ethylnitrosourea (ENU).

Present leukemic conventional chemotherapy has increased the survival rate, but the major disadvantages like aplastic anaemia, bone marrow failure, chemotherapeutic resistance, immunosuppression, cellular toxicity, etc still remains as a massive drawback. The use of medicinal herbs as a therapy for leukemia is quite well recognized in developing countries. This research revealed that *Eclipta alba* has novel potentials to complement the traditional chemotherapy, as it is economical with lesser side-effects as well as potential anti-leukemic drug.

High levels of WBCs and blast cell population along with an increase in other blood parameters like RBC, reticulocytes, haemoglobin in the peripheral blood is a foremost indication of the onset of leukemia. After the therapy, the first signs of amelioration were the significant reduction of these blood parameters. The role of the herb in amelioration of the diseased state was then confirmed by cytological staining procedures like MPO and SBB on the selective myeoblast lineage. A significant reduction in the myeoblast population (MPO-positive and SBB-positive) of the treated group were observed post-treatment. As studied by H&E staining protocol, there was a significant reduction in the blast cells population in the marrow section of the treated group but no architectural changes were observed compared to leukemia. Significant betterment of the physical parameters like an increased survivability and body weight of the ENU-induced group post treatment, marked towards the therapeutic efficacy of the herb.

Uncontrolled stimulation of NF-kB signaling pathway in leukemia is well studied, hence confirming as a potential therapeutic target (Zhou et al. 2015). The increase in the blast cells and leukocytes in the hematopoietic population is possibly linked with the highly stimulated NF-kB pathway and leukemogenesis (Udayashankar et al. 2016). Significant decline in the

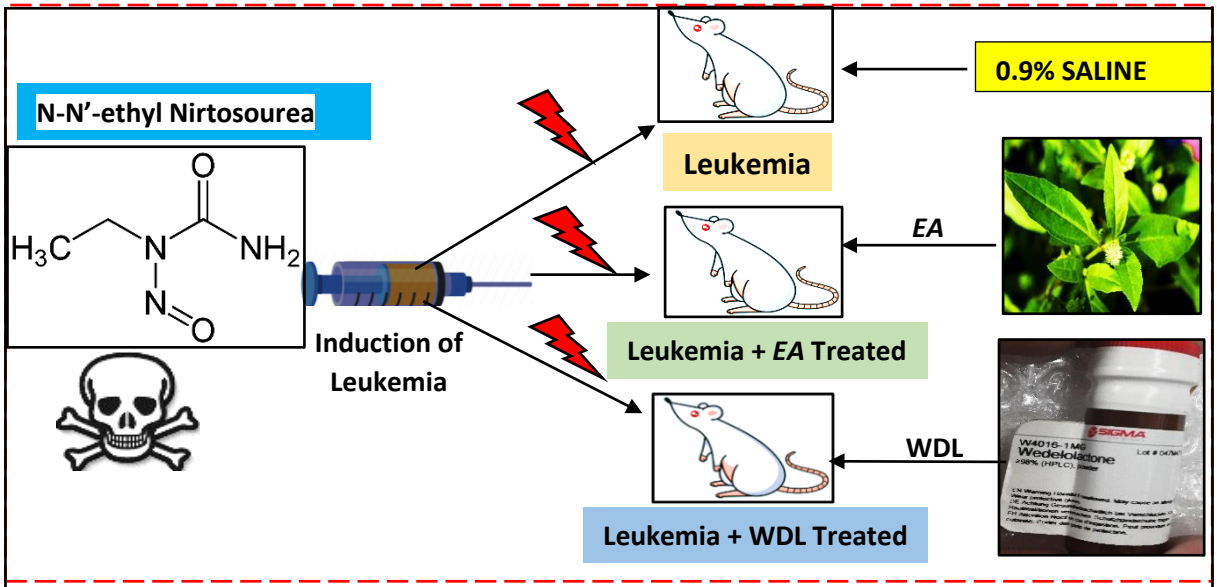
blasts and leukocyte cell population, as well as reduction in leukemic cell proliferation post-treatment with *Eclipta alba confirms it as an anti-cancer agent.*

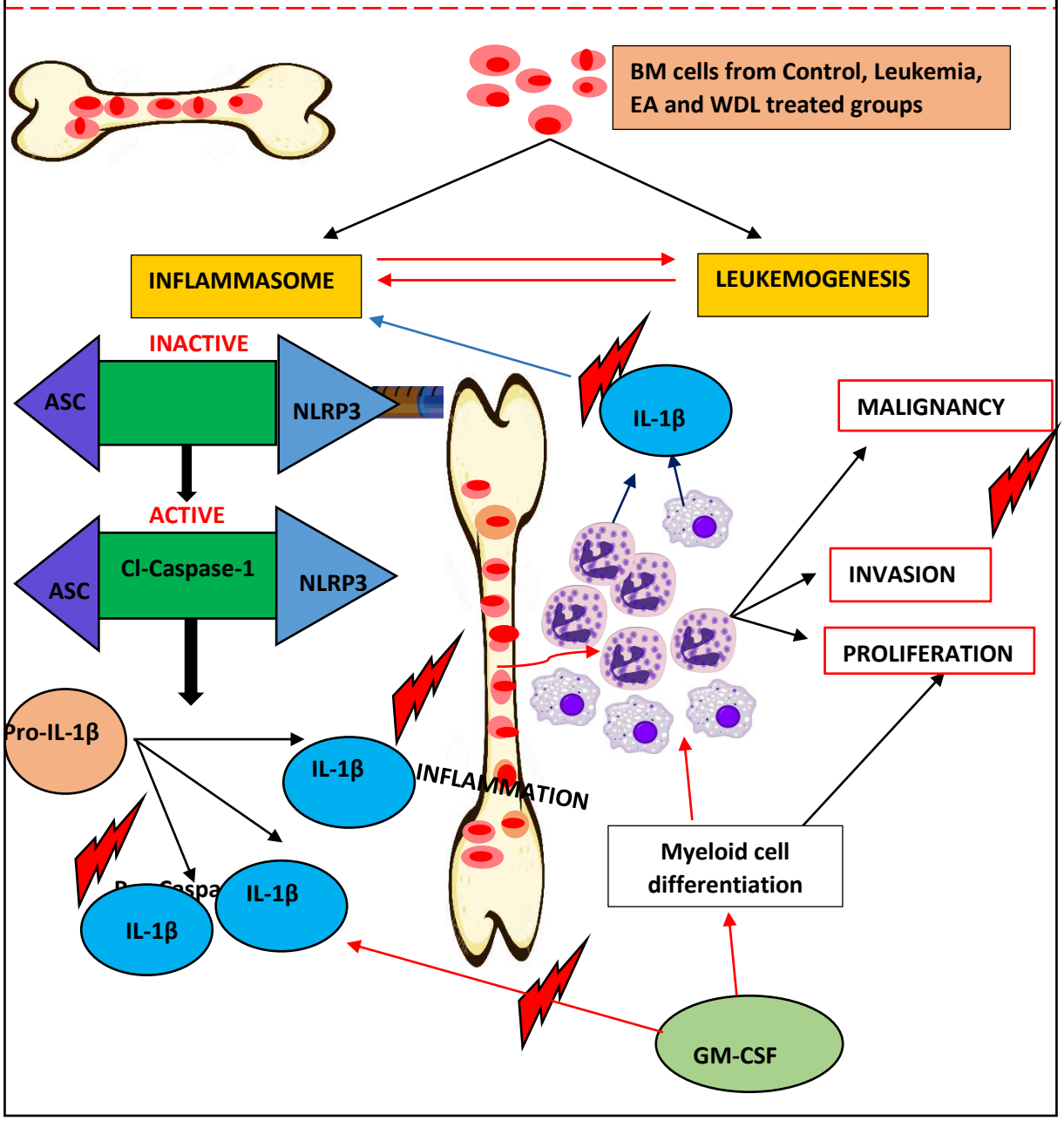
When the NF-kB pathway is activated, IKK plays a role of "central regulator" and in our experimental endeavour we delineated that IKKβ and IKKγ which were up-regulated in leukemia was significantly down-regulated post treatment with extract hence it could be concluded that the extract can immune-modulate the canonical NF-kB pathway. Activated IKK leads to the degradation of the phosphorylated IkBα or "super repressor" (Cilloni et al. 2007). In our study we found a significant decrease in the IkBα expression in leukemic marrow cells, co-relating to its rapid proteosomal degradation because of IKK activation (Kobori et al. 2004). Significant incline in its activation was observed post-treatment, hence once again proving it as an anti-cancer extract. IkBα phosphorylation and ubiquitin mediated degradation leads to the activation of the down-stream molecules like c-Rel, p50, p65 in canonical pathway and RelB, p52 in non-canonical pathway. The fluorescence expression of these subunits were increased in leukemic group. Post-treatment with extract the expression of canonical NF-kB subunits were reduced but the non-canonical subunits remained unaffected. Activation of NF-kB further stimulates IL-1β activation, the major pro-inflammatory cytokine. Leukemia is an inflammation-driven cancer and bone marrow cells revealed a high expression of IL-1β, which was significantly reduced post-treatment.

4.7. Conclusion

It can be hence concluded that ENU is an environmental pollutant and its induction leads to the development of leukemic mouse model. ENU-induced leukemia reveals a highly activated and stimulated expression of the NF-kB signaling pathway and the treatment with extract can effectively reduce the aberrant activation, hence proving to be a novel therapeutic for leukemic treatment.

Chapter 5.


N-N'-ethyl Nirtosourea
Induction of Leukemia
0.9% SALINE
Leukemia
EA
Leukemia + EA Treated
WDL
Leukemia + WDL Treated


BM cells from Control, Leukemia, EA and WDL treated groups
INFLAMMASOME
LEUKEMOGENESIS
INACTIVE
ASC
NLRP3
ACTIVE
Cl-Caspase-1
Pro-IL-1β
IL-1β
INFLAMMATION
MALIGNANCY
INVASION
PROLIFERATION
Myeloid cell differentiation
GM-CSF

Chapter 5.

Activation of the NLRP3 inflammasome pathway in ENU-induced leukemia and its treatment using *Eclipta alba* and its active coumestan wedelolactone

5.1. Chapter summary

NOCs are present around us through various sources like cigarette smoke, chemicals from rubber, pesticides and fertilizer making factories. Increased exposures to these NOCs are a potential reason for the increased number of cancers among human population. Prenatal exposure to ENU (belongs to the NOC family) with a single intra-peritoneal dose, induces leukemia in the animal model. The constant search for anti-cancer drugs has lately increased because of the increasing incidence of cancer and through this, the lime-light has shifted on the medicinal plant based drugs for their promising anti-cancer effects. *Eclipta alba* is an age old Ayurvedic plant well known for its anti-inflammatory effect on various diseases and anti-cancer effect on several cancers, although not yet studied on any haematological malignant model. The major active compound of the extract is wedelolactone, which is considered as "IKK-inhibitor", that is a principal regulator of the famous inflammatory NF-kB pathway, also considered as a "hallmark of cancer". Leukemia is reported to be inflammation-driven, and that leads to stimulated inflammasome. In our study we tested the efficacy of *Eclipta alba* to down-regulate the NLRP3 inflammasome, at a dose of 1200 mg/kg b.w and wedelolactone at 50 mg/kg b.w by oral gavage for 4 weeks through various experimental endeavours. It could be concluded that although both the treatments can be considered as novel therapeutics to reduce NLRP3 inflammasome activation in the bone marrow cells of leukemia**,** but the extract holds better ameliorating properties against leukemia, as compared to the active compound alone.

5.2. Introduction

Environmental exposures to NOCs through nitrate cured food products like smoked meat; tobacco smoke; chemicals from plastic making factories and fertilisers plays a very major role in the increase in cancer development of humans (Grosse et al., 2006; Haorah et al., 2001; Mirvish, 1995). Intra-peritoneal injection of a single dose of ENU (which belongs to the NOC family) leads to leukemogenesis in the Swiss albino mice model (Basak et al., 2010b, 2010a; Capilla-Gonzalez et al., 2012).

Inflammasome is a multi-protein complex which usually remains inactivated in the cytosol, but stimulated during inflammation and its prolonged activation is a potential cause of cancer (Di Virgilio, 2013).

The NLRP3 inflammasome is present in the cytosol and it consists of 3 domains: NLRP3 protein; apoptosis- associated speck like protein (ASC) and procaspase-1. When the inflammasome is activated, it forms several NLRP3 speck complexes and the inactive procaspase-1 gets cleaved into its activated form of caspase-1 (Ratajczak et al., 2020). Cleaved caspase-1 in its active form further stimulates the production of the two major pro-inflammatory cytokines IL-1β and IL-18, which together promotes cancer. NLRP3 is considered as a "rising star" for being a major cause of haematological malignancies like leukemia (Hemmati et al., 2017). Leukemia is a heterogenic malignancy which develops from hematopoietic stem/ progenitor cells (HSPCs) and 2.8% among all the cancer cases are reported as leukemia in the world. According to literature review, there is an interlink between NLRP3 inflammasome over-expression and leukemic progression and hence targeting the NLRP3 inflammasome pathway is considered as a therapeutic potential (Jo et al., 2015).

Various plant-based medicines is reported to ameliorate NLRP3 inflammasome pathway in different disease models (Budai et al., 2013; Ekor, 2014; Hu et al., 2012; Kim et al., 2014; Pan

et al., 2016, 2020; Tozsér and Benko, 2016; Yoon et al., 2015; Zhong et al., 2019). *Eclipta alba* is one such Ayurvedic medicinal herb well known for its anti-inflammatory and anti-cancer efficacy on various disease models (Chaudhary et al., 2014; Lirdprapamongkol et al., 2008; Nelson et al., 2020; Yadav et al., 2017a). The principal active compound in *Eclipta alba* extract is wedelolactone and it is observed to down-regulate the NLRP3 inflammasome aberrant activation in different cancers (Hsieh et al., 2015; Kobori et al., 2004; Lin et al., 2018; Liu et al., 2016; Nehybová et al., 2017; Pan et al., 2020; Sarveswaran et al., 2012; Wei et al., 2017; Yuan et al., 2013; Zhu et al., 2019). Even though many medicinal herbs have been studied to regulate the NLRP3 pathway, very few data is present on natural products immune-modulating NLRP3 inflammasome in haematological malignancies. Through this study we aimed to delineate the efficacy of the whole extract and wedelolactone on regulating the NLRP3 inflammasome activation on the ENU-induced bone marrow cells.

5.3. Materials and methods

5.3.1. Plant material

Fresh and dried leaves of *Eclipta alba* was procured from the Medicinal Plant Garden, located in the Narendrapur Ramakrishna Mission Ashrama on July'2019.

After the authentication of the sample by Prof Jukta Adhikari (Department of Botany, Barasat Government College, India), the specimen was deposited at Calcutta School of Tropical Medicine, Kolkata, India The voucher no. of the deposited plant sample is 756870.

5.3.2 Eclipta alba extract preparation

A coarse powder was made by crushing the dried leaves using an electric blender and it was suspended in 80% ethanol. The solution was kept in room temperature for 24 hours. The crude extract was collected by maintaining sterile conditions, using the double filtration method using

Grade 1 Whatman filter paper, 25 mm (Sigma, USA). The filtration process was repeated 5 times to make sure no powder particle was present in the extract solution.

The extract was concentrated by rotary evaporator by reduced pressure at 50˚C and the solvent was removed thoroughly. The residue was reconstituted in double-distilled water and stored at -20˚C (Yadav et al., 2017b).

5.3.3. Chemical characterization of Eclipta alba using chromatography

The crude extract of *Eclipta alba* was characterized chemically using HPLC method, using the standard wedelolactone (WDL, purity ≥98%, CAS No: 524-12-9; Sigma-Aldrich). HPLC instrument used was Shimadzu Prominence HPLC (Model No. CBM-20A, Japan) which was equipped with Guard column (Security Guard Cartridge system, Phenomenex, USA) LC-20AT pump, SIL-20AT autosampler, and SPD-M20A detector and Discovery C18 column (ODS, 25 cm × 4.6 mm, 5 µm, Supelco, USA). HPLC-grade acetonitrile (ACN) was purchased from Merck, Germany and deionized water by Milli-Q system (Millipore, USA). The mobile phase was composed of acetonitrile: water (ACN%, v/v) at a flow rate of 1 ml/min and programmed as followed: 20–45% (for 15 min); 45–100% (for 30 min), and 100 (for 40 min). The injection volume was 10 µl at a detection wavelength of 365 nm. The yield of WDL obtained was 12.05 mg/ml.

5.3.4. ESI-MS Direct Infusion technique

The isolated compound by HPLC (wedelolactone) went through qualitative analysis by ESI-MS Direct Infusion technique. The instrument used for this method was Xevo-G2-XS-QTof-MS (Waters) and for its calibration leucine enkephalin (mass 555.62 g/mol) was used. With an injection volume of 10 µl at a flow of 5 µl/min the acquisition was done, using a positive ionization mode with electrospray capillary voltage of 3.0 kV. The cone gas flow at 30 L/h

with source and desolvation temperature of 100˚C and 250˚ C, respectively. Mass Lynx 4.1 software was used to analyse the data.

5.3.5. Maintenance of the animals

Inbred and healthy Swiss albino mice (*Mus musculus*) aging 12 weeks approximately with a weight around 32 grams of both sexes were maintained at the animal house of Calcutta School of Tropical Medicine, India. They were provided with a standard diet and water *ad libitum* and kept under a strictly controlled room temperature of 22 ± 2˚C with a 12h light-dark cycle with controlled humidity. The animals were maintained with full consent and by following all the authorized norms of the Institutional Animal Ethical Committee (IAEC), EU Directive 2010/63/EU and also adhering by the guidelines of "The Committee for the Purpose of Control and Supervision of Experiments on Animals", Government of India ((Basak et al., 2010a; Chatterjee et al., 2016a; Daw et al., 2016; Daw and Law, 2021a; Law et al., 2001, 2018).

5.3.6. Development of ENU-induced leukemic mice model

ENU (Sigma, USA) is a highly reactive compound as it decomposes above pH 7.0 in aqueous media. ENU was prevented to decompose from light by keeping its prepared solution in freezer until further use. Swiss albino litter pups aging between 10 to 14 days with a weighing around 4 to 5 grams were injected with a single intra-peritoneal dose of ENU at a dose of 80 mg/kg body weight. After 6 to 8 months of ENU induction, leukemic development was observed and the clinical features of leukemia started to show, which was confirmed by routine peripheral blood smear studies (Basak et al., 2010a, 2010b; Bhattacharyya and Law, 2021, 2022; Chatterjee et al., 2016c, 2016a; Chattopadhyay et al., 2019; Law et al., 2001). The control group received 0.9% saline intraperitoneally and all the experimental groups were provided with standard diet and water *ad libitum*.

5.3.7. Therapeutic dosimetry

No acute toxicity of the crude extract was observed even up to 2000 mg/kg body weight as according to various (Jahan et al., 2014; Singh et al., 2001, 1993, 2014; Yadav et al., 2017a). According to another study, 10.4 mg/kg of alcoholic extract for 14 days showed no signs of mortality (Zhao et al., 2015).

We carried out a preliminary test on the basis of prior literature reviews, and tested the efficacy of the crude extract on leukemia via oral gavage at various doses at 1000 mg/kg; 1100 mg/kg; 1200 mg/kg; 1300 mg/kg, and 1400 mg/kg and the normal group were provided with a standard diet. Post the pilot study, the oral dosage was fixed at 1200 mg/kg body weight regularly for 4 weeks.

Wedelolactone at a dose of 50 mg/kg immune-modulated NF-kB signaling pathway and significantly ameliorated liver damage (Feng et al., 2019; Luo et al., 2018). Another report suggests, the dose 50 mg/kg body weight of wedelolactone is reported to significantly down-regulate NLRP3 inflammasome and its downstream caspase-1 activation in DSS-induced colon damage (Wei et al., 2017). 50 mg/kg of wedelolactone was reported to down-regulate IL-6/STAT3 axis and show an anti-inflammatory response in ulcerative colitis (Tigari, 2022).

On the basis of data, a dose of 50 mg/kg body weight wedelolactone was standardized via oral gavage for 4 weeks regularly.

5.3.8. Experimental groups

According to the experimental plan, four experimental groups were formed, with 30 mice per group (n=30). The groups were as followed:

- Leukemic group (ENU induced);

- Leukemia (ENU induced) + *Eclipta alba* extract treated (1200 mg/kg via daily oral gavage for 4 weeks);
- Leukemia (ENU induced) + wedelolactone treated (50 mg/kg via daily oral gavage for 4 weeks);
- Control group of mice received equal volume of saline in similar condition.

5.3.9. Isolation of the bone marrow cells

With full consent from the institutional animal house ethical committee, the mice from each experimental groups were sacrificed by cervical dislocation and their femur and tibia (long bones) were surgically removed. A cut was made on both ends of the bones by sterilized scissor and the red-pulp marrow region was flushed out in RPMI-1640 media (Sigma, USA) using a 26 gauge sterile needle. Some intact marrow sections were used to make bone marrow smears and rest for different protocols. The marrow cells were constantly passed through 100 µm cell strainer by repeated pipetting and washed thoroughly in chilled media to make sure there is a single cell suspension without debris (Basak et al., 2010a; Bhattacharyya and Law, 2022, 2021; Chatterjee et al., 2016a, 2016b; Daw and Law, 2021b; Law et al., 2001).

5.3.10. Study to determine survivability and bone marrow parameters post-treatment with extract and active compound

The bone marrow cells from leukemic group of mice were stained using Geimsa stain to study the onset of the disease. Post-treatment with both whole extract and active compound wedelolactone separately, the difference in the number of blast cells were counted by Light microscope. Rate of survivability was compared among both the treatment groups as well as diseased and control (Chatterjee et al., 2016c; Daw et al., 2019; Law et al., 2018).

5.3.11. Hematoxylin and Eosin staining of the long bone

The femur bone was decalcified and the bone marrow was prepared in a wax block using the routine method (Dancey et al., 1976). The sections from each experimental animals were cut and placed on a grease free glass slide and deparaffinized using xylene. The slides were made to pass through downgrades of alcohol using 100%, 90%, 75%, 50% and 30% alcohol, each for 5 minutes. Post this, the slides were immersed in distilled water for 2 minutes for re-hydration of the bone marrow sections. Each slides were immersed in the hematoxylin stain for a minute and then washed under running water. Counter-stain was carried out using eosin for 30 seconds. The slides were again dehydrated using an increasing grade of alcohol (70%, 95%, and 100%) for 2 seconds each and the mounted slides were observed under light microscope (Olympus, Ch20i, Japan) (Fischer et al., 2012).

5.3.12. Silver nitrate staining of the bone marrow cells

Two working solutions were made to carry out the staining procedure. For the first solution, 2% of gelatin was mixed in 1% formic acid solution. A second solution was made bydissolving silver nitrate in distilled water. 1 part of the first solution was mixed with 2 parts of the second working solution to make a silver nitrate (AgNOR) working solution. Bone marrow cells from different experimental groups were smeared on grease free glass slides. In dark and humidified chamber, at room temperature the bone marrow smears were dipped in coplin jars containing the silver nitrate working solution for 30 minutes. The slides were then washed thoroughly with distilled water and again dipped in a coplin jar containing 5% sodium thiosulfate solution (Merck) till next 2 to 3 minutes. The slides were washed using running tap water and the bone marrow cells were observed using a light microscope (Olympus CH20i,Japan), while the number of AgNOR dots were visualized and counted (Daw et al., 2019).

5.3.13. Bone marrow cell viability assay using MTT

To carry out this assay, the MTT (3,4,5 dimethylthiazol 2 yl)-2,5 dipenyl tetrazolium bromide) dye was used, as based on the principle of the reduction of MTT by NADP(H) dependent cellular oxidoreductase enzyme to convert MTT into the insoluble formazan. The bone marrow cells isolated from each experimental groups were put in cell culture and at different time intervals of 0h, 24h, 48h and 72h they were tested using MTT (Daw et al., 2019). The appearance of formazan crystals were of purple colour (different shades among each experimental sample) and it was dissolved using isopropanol. Using a colorimeter (EES-DIGI-8 F, India) the OD of each sample was calculated at 540nm.

5.3.14. Invasion and trans-well migration assay of bone marrow cells

To study the rate of migration and invasion capability of the leukemic bone marrow cells pre- and post-treatments as compared to the healthy group, the bone marrow cells were plated in a double-chambered transwell membrane plate coated with matrigel (Corning). Bone marrow cells in a concentration of 3 x 10^6 cells in a single cell suspension was plated in the upper chamber of the transwell membrane plate in 200 µl serum free DMEM media. In the lower chamber of the transwell plate a chemoattractant was coated using a solution of 20% FBS (Fetal Bovine Serum) in 500 µl DMEM. The transwell plate was kept at 37˚C room temperature for two consecutive nights at 5% CO_2. Following to incubation the transwell plate was immersed in 4% PFA solution was 5 minutes and washed with PBS for the fixation of bone marrow cells. The bone marrow cells present in both the upper and lower chambers were then stained with 0.1% crystal violet solution for 5 minutes and then washed with distilled water. The bone marrow cells which migrated from the upper to the lower chamber were identified and were counted using a light microscope (Olympus CH20i, Japan).

5.3.15. Long term BM cell culture

Single cell suspension of bone marrow cells (2 x 10^6 cells/ ml) from each experimental groups were plated with RPMI-1640 media enriched with 30% FBS, 1% bovine serum albumin (BSA) and 0.02% of β- mercaptoethanol. The cell culture plates were maintained at a constant temperature of 37˚C in 5% CO_2. The media was discarded and freshly replenished after every 2 days. Bone marrow cells were observed under inverted microscope at regular intervals (Dewinter) (Basak et al., 2010a, 2010b; Bhattacharyya and Law, 2022).

5.3.16. Flow-cytometry to study NLRP3 inflammasome pathway

From each of the experimental groups, bone marrow cells were isolated and in dark at 37˚C for 15 minutes they were fixed using 1.5% paraformaldehyde (PFA) solution. After this the bone marrow cells at a concentration of 2 x 10^6 cells/ml in the cell suspension were washed in PBS and diluted in FACS fluid. The cells were vortexed to internalize the membrane by treating with chilled methanol drop by drop for 20 minutes. The marrow cells were washed and diluted in FACS fluid and finally distributes in the FACS tubes. The cells were then stained with 2 µl of primary antibodies (anti-NLRP3, anti-ASC, anti-cleaved caspase1 and anti-cleaved IL-1β) of Mouse Reactive Inflammasome antibody kit (Cell Signalling Technology) and anti-GM-CSF (Santa Cruz biotechnology) and they were incubated at 37˚ C for 30 min. Then, they were counter-stained using the secondary antibody combined with Alexa flour 488 (Invitrogen, USA) and incubated at 37˚ C for next 30 min. The bone marrow cells were washed using PBS and analyzed by flow-cytomety. The Mean Intensity Fluorescence (MFI) was calculated by BD FACS Calibur flowcytometer (Becton Dickinson, USA) and the data was collected using CellQuest Pro software (v9.1 Becton Dickinson).

5.3.17. Immuno-cytochemistry of the bone marrow cells using fluorescence microscope

From the previous flow-cytometry experiment, bone marrow cells tagged with the NLRP3 Inflammasome antibody molecules (anti-NLRP3, anti-ASC, anti-cleaved caspase1, anti-IL-1β; Cell Signaling Technology) and anti-GM-CSF (Santa Cruz biotechnology) were smeared on a glass slide and mounted by DAPI shield (Sigma, USA). The fluorescence expression of each protein was observed by the Zeiss LSM 800 confocal laser scanning microscope.

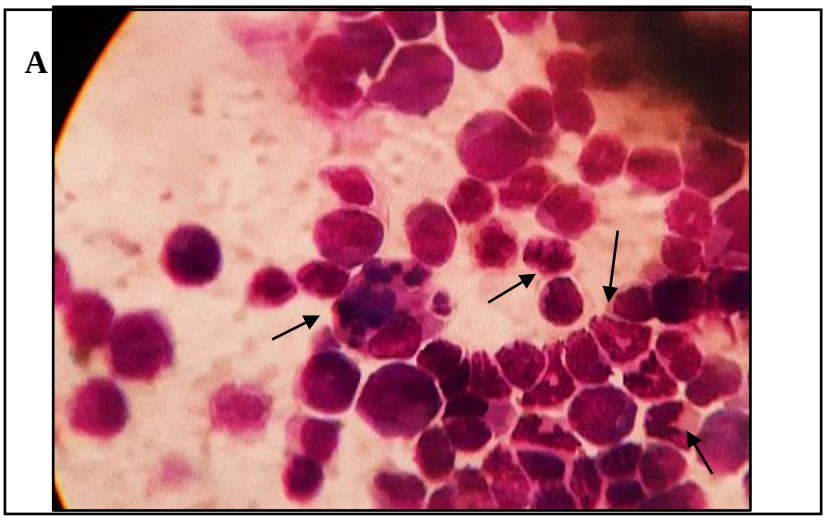
5.4. Statistics

Unpaired Student's t-test and One-way ANOVA by post-hoc testing (Tukey's Test) was performed for statistical analysis. Every quantitative data were represented as Mean ± Standard deviation (SD). For every comparison, $P \leq 0.05$ was considered as significantly significant. All he experiments were repeated thrice.

5.5. Results

5.5.1. Amelioration of the bone marrow status post-treatments

Cytological staining of the bone marrow cells using Geimsa, revealed the hyper-proliferative production of abnormal blast cells and doughnut shaped neutrophils in the diseased condition, pointing towards the inflammation-driven malignancy in the leukemic microenvironment (Figure 53 A - D).



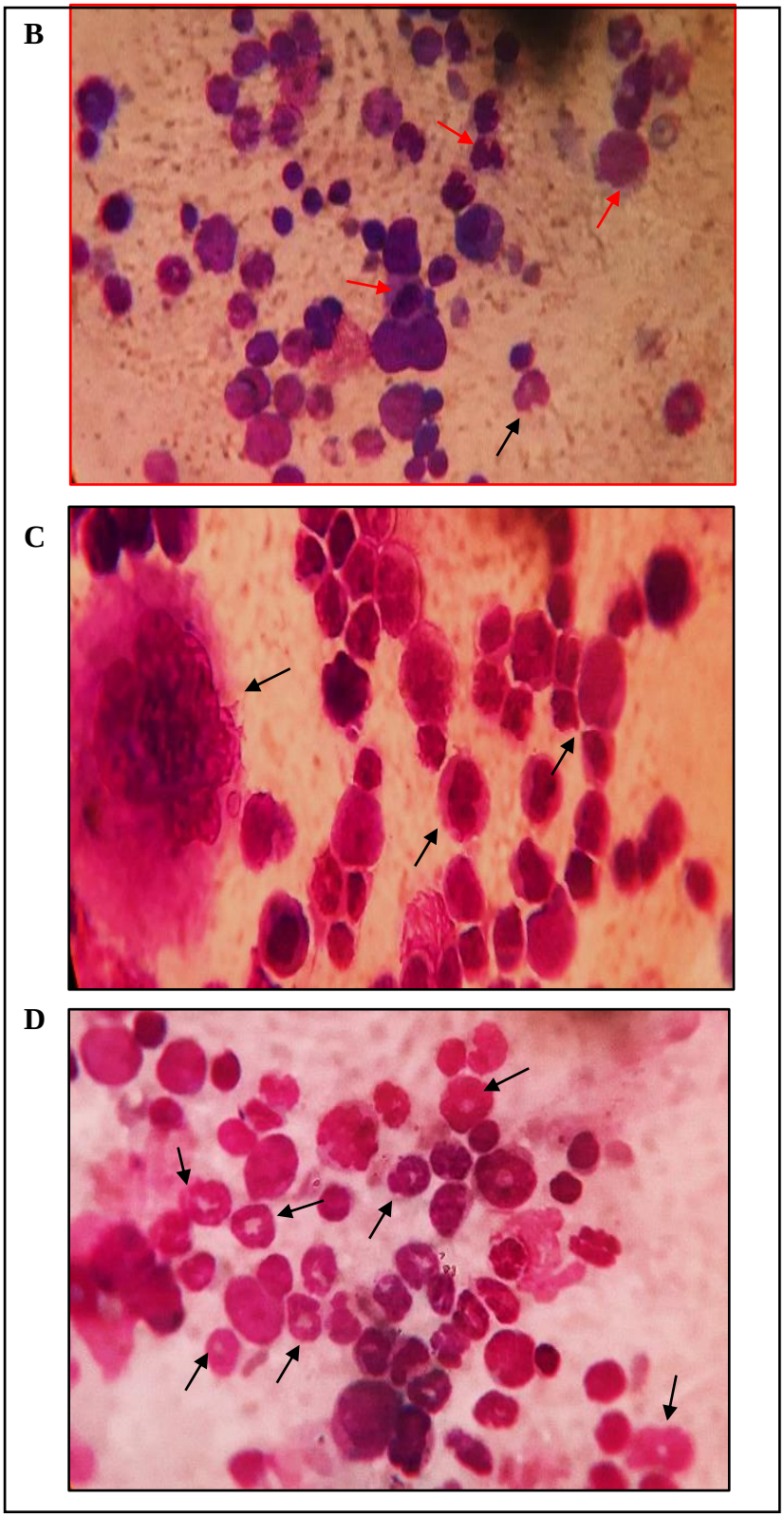
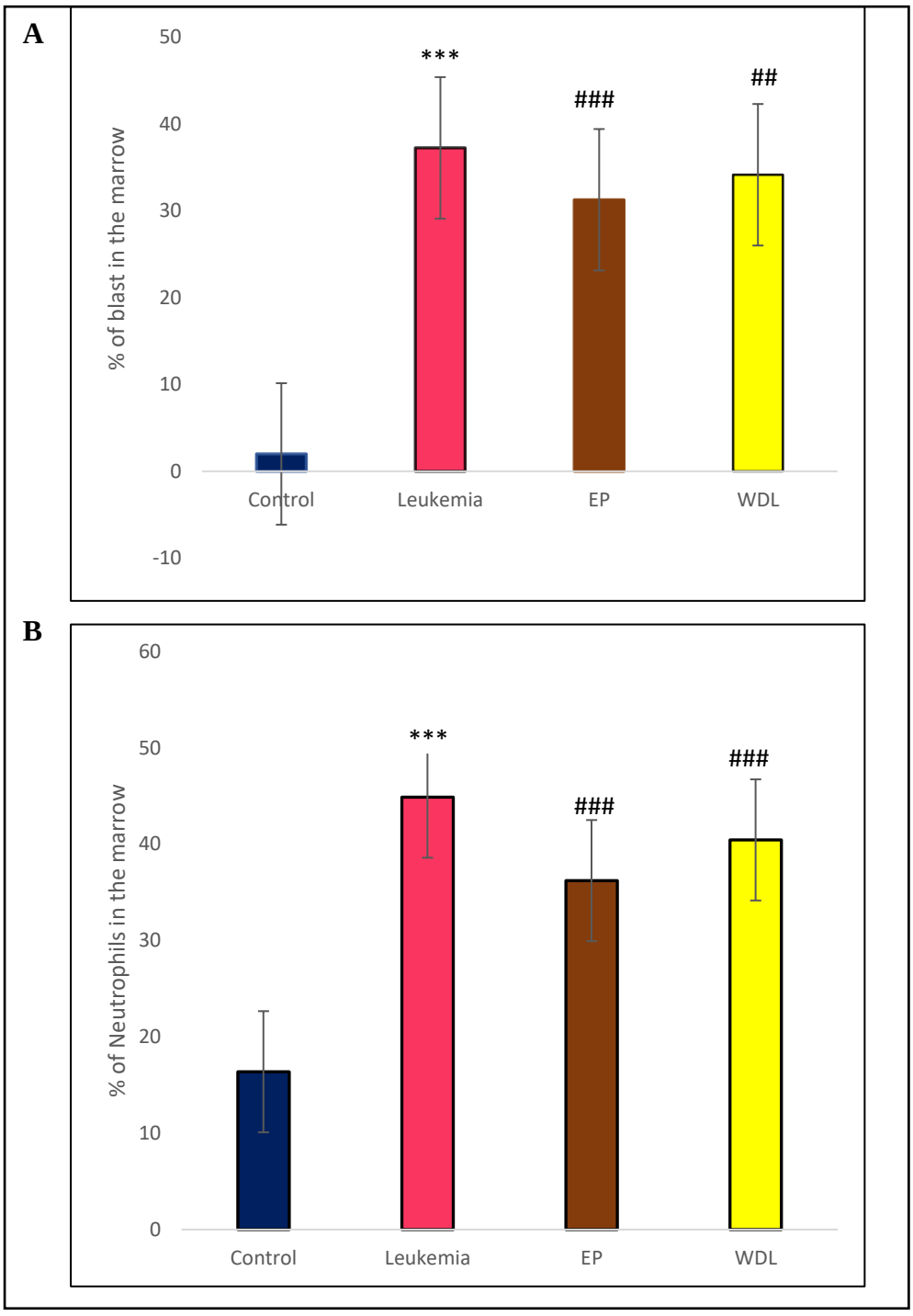


Figure 53. ENU-induced leukemic bone marrow cells and its morphological changes. (A) Heterogenous blast cell (black arrow), (B) polymorphic myeloblast (red arrow), (C) hypo-lobulated promegakaryocyte (black arrow) and (D) donought shaped or ring neutrophils (black arrow).

After the treatment with the whole extract and wedelolactone separately, the number of abnormal immature blasts as well as neutrophils were significantly reduced. Although both the treatment showed fair amelioration on the survivability rate, the crude extract showed better ameliorating potential compared to the active compound alone (Figure 54 A - C).



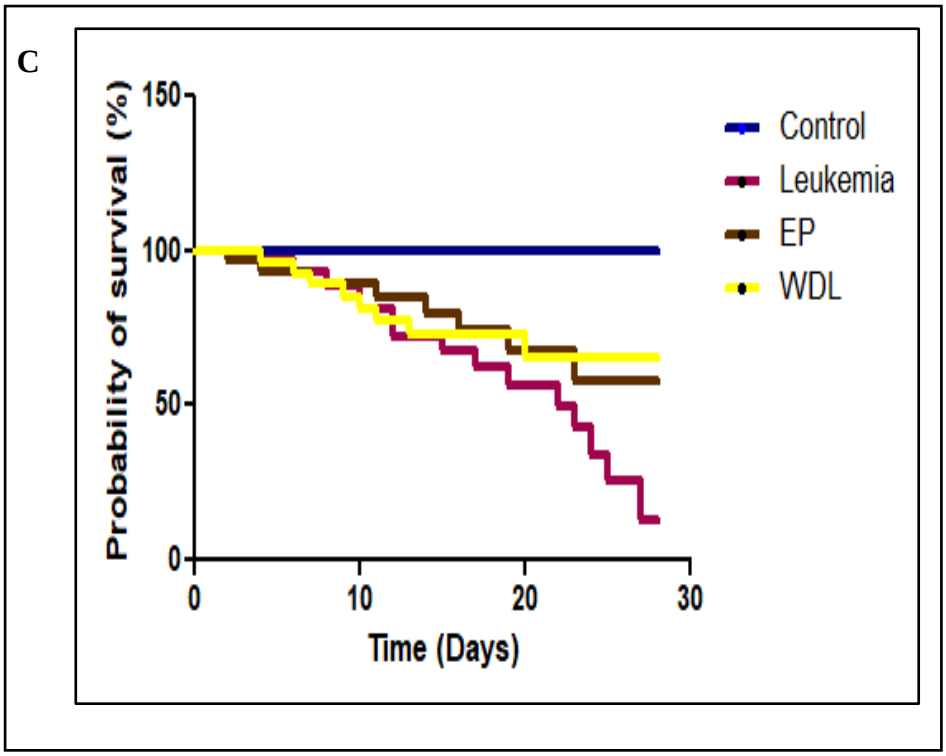


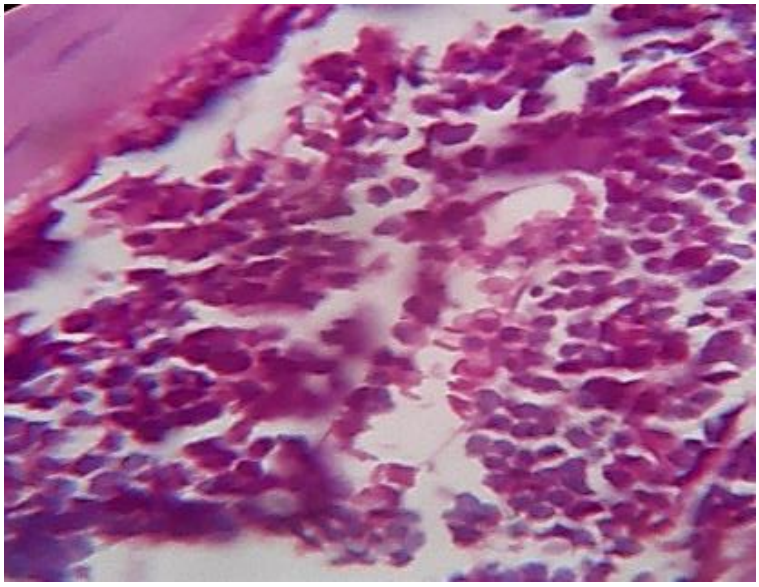
Figure 54. Representative graphs. (A) Graphical comparison of the number of blast cell population before and after treatments, (B) Graphical comparison of the number of neutrophil population before and after treatments and (C) Kaplan-meier curve of survivability representing each experimental group.

5.5.2. Tracking of leucocytes in the bone marrow sections by H&E staining

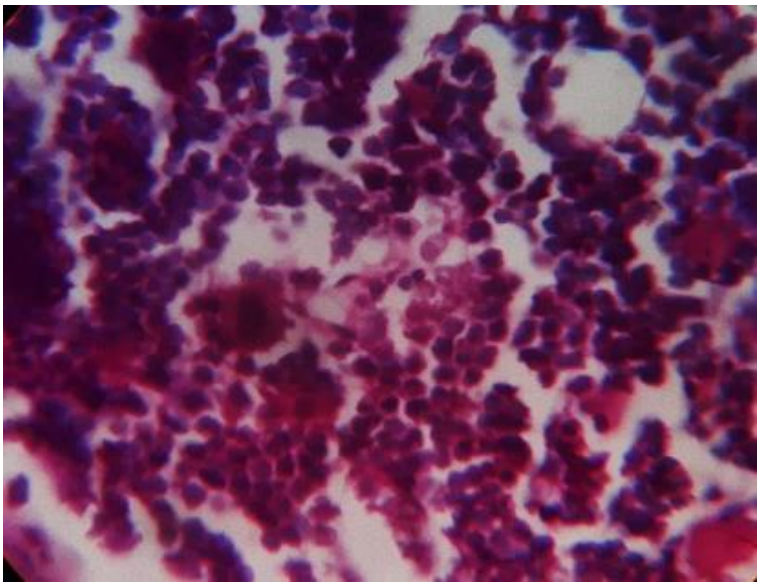
Increased population of leucocytes hints towards the inflammatory driven malignant condition in the bone marrow. Post-treatment with extract and wedelolactone the leucocyte infiltration reduces in the marrow, which clearly hints towards the anti-leukemic potentials of both the treatments.

Highly increased population of leucocytes were observed in the bone marrow section of leukemia (86 ± 2.94%, $P<0.001$), as compared to the normal group (14.25 ± 4.64%), pointing towards its inflammation-driven malignancy. In the bone marrow section of extract and wedelolactone treated groups (70 ± 6.58%, $P=0.004$; 75.5 ± 3.87%, $P=0.013$) significant reduction of leucocytes were observed as compared to leukemia (Figure 55 A - E).

A



B



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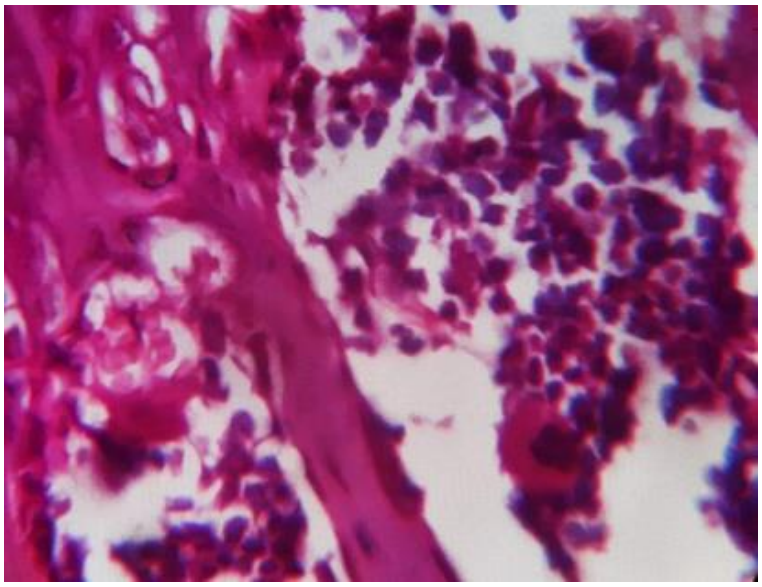


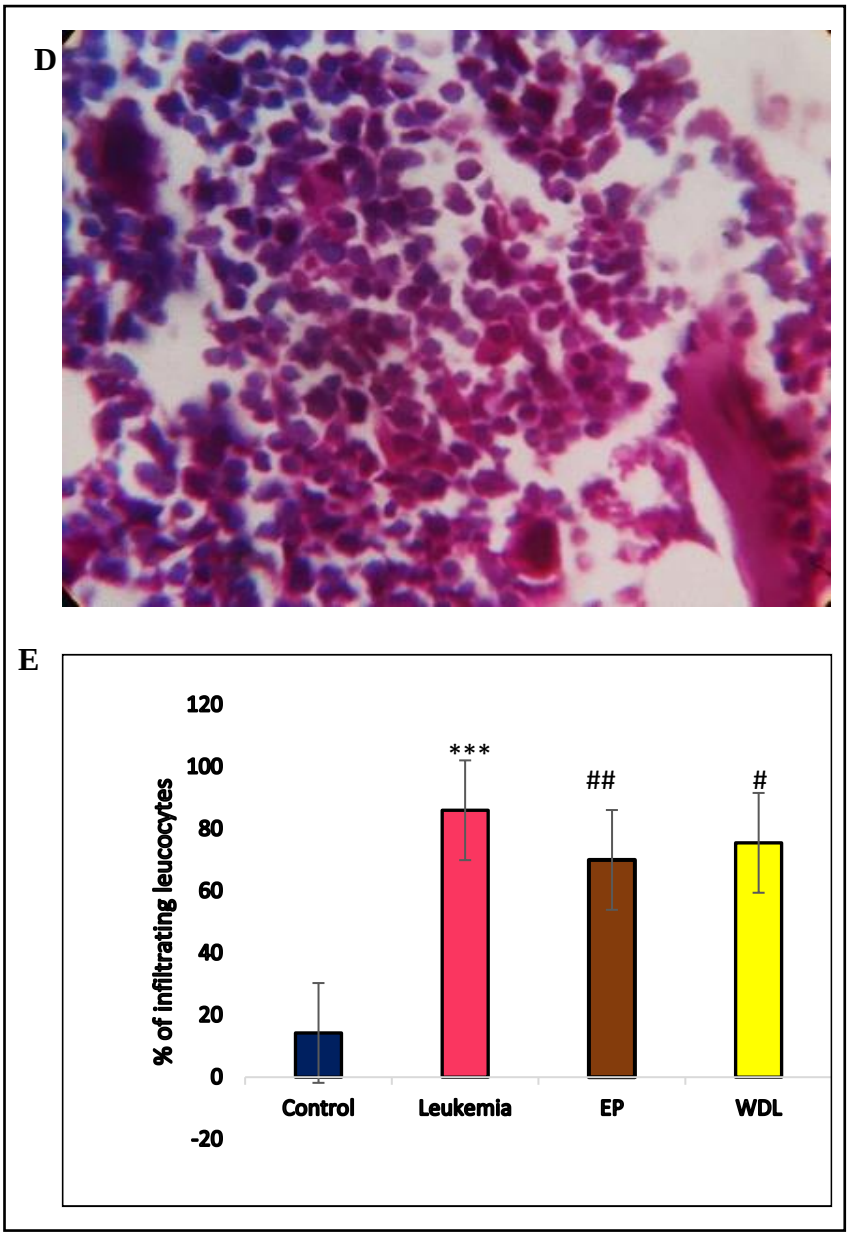
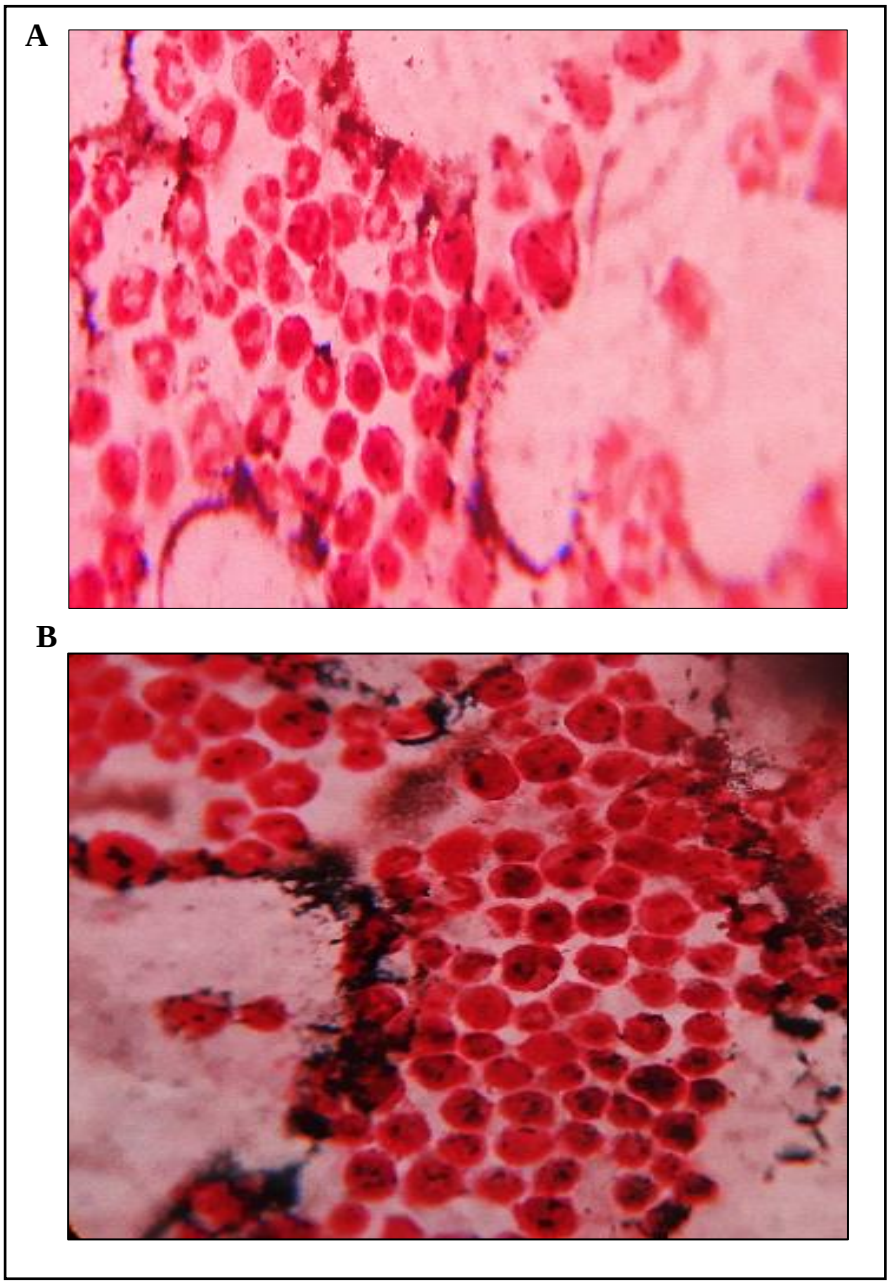


Figure 55. Infiltration of leucocytes in the bone marrow section. (A) Bone marrow section from control, (B) highly infiltrated leucocytes in the leukemic bone marrow, (C) extract treated marrow section, (D) wedelolactone treated group, and (E) Graphical depiction of mean ± SD values of leucocyte infiltration in marrow cells pre-and post-treatment [*P<0.05, **P<0.01, ***P<0.001 significance of leukemia as compared to control. #P<0.05, ##P<0.01, ###P<0.001 significance of EP and WDL treatment in comparison to leukemia].

5.5.3. Anti-proliferative effect of the herb and active compound on leukemic cells

AgNOR staining revealed black dots which signifies the proliferative capability of the cells. In the leukemic bone marrow cells many NOR were black dots revealed which indicates excessive proliferation as compared to healthy controls, in which no such dots were shown. After treatment with extract and wedelolactone, the number of black dots were reduced as compared to leukemia (Figure 56).



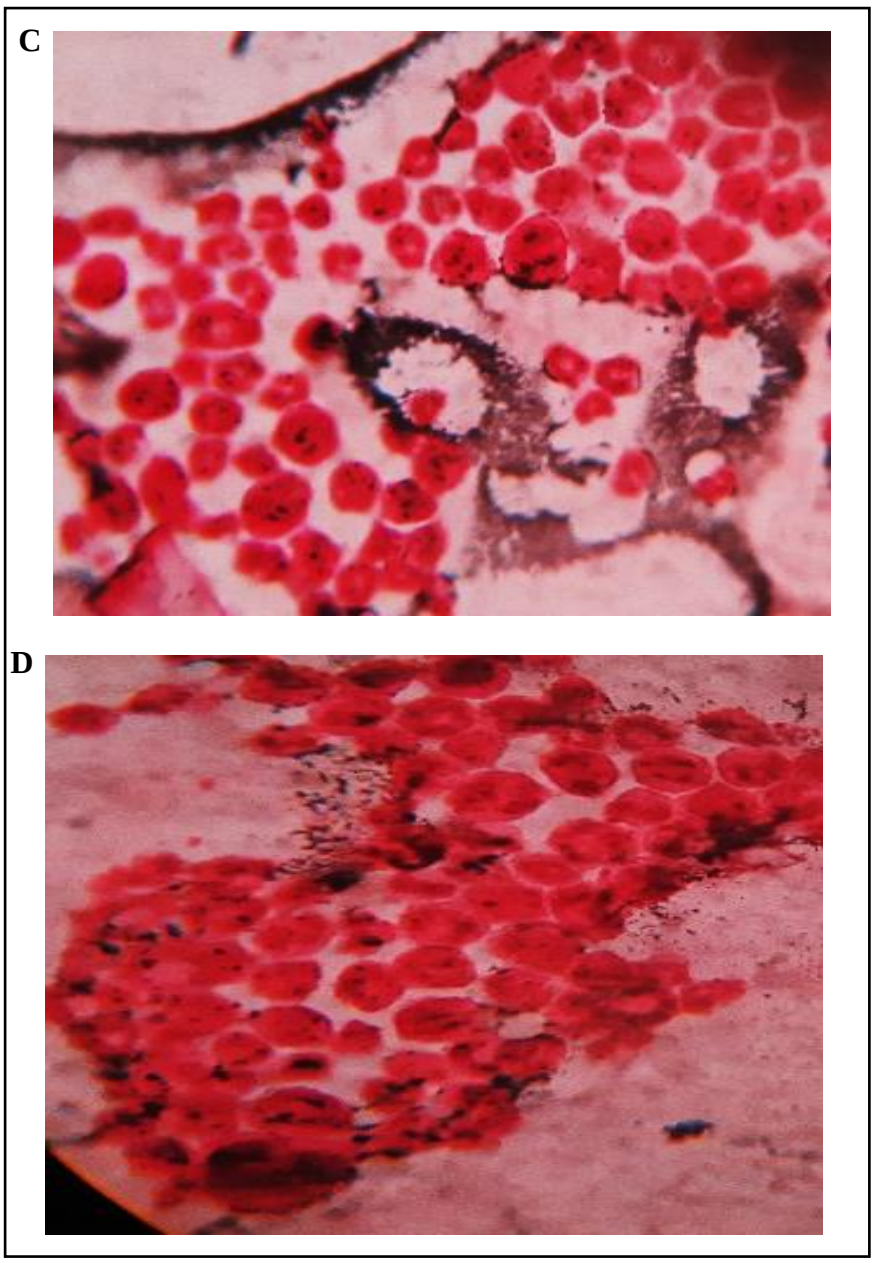


Figure 56. Silver nitrate staining of bone marrow cells revealed no malignancy in (A) healthy group, in contrast to leukemia, (B) an increase in malignancy with excessive dots in diseased group, (C) lesser malignant marrow cells in extract treated and (D) wedelolactone treated group were observed

5.5.4. Effect of treatment on the rate of proliferation of leukemic cells

Through MTT assay the rate of proliferation in the bone marrow cells were calculated by assessment of the O.D values. In the diseased group, a bright purple colour was obtained which

signified the higher rate of proliferation hence an increased formation of formazan crystals. After the treatment with extract and wedelolactone, the bone marrow cells revealed a decrease in the proliferative capacity as compared to leukemic condition (Figure 57).

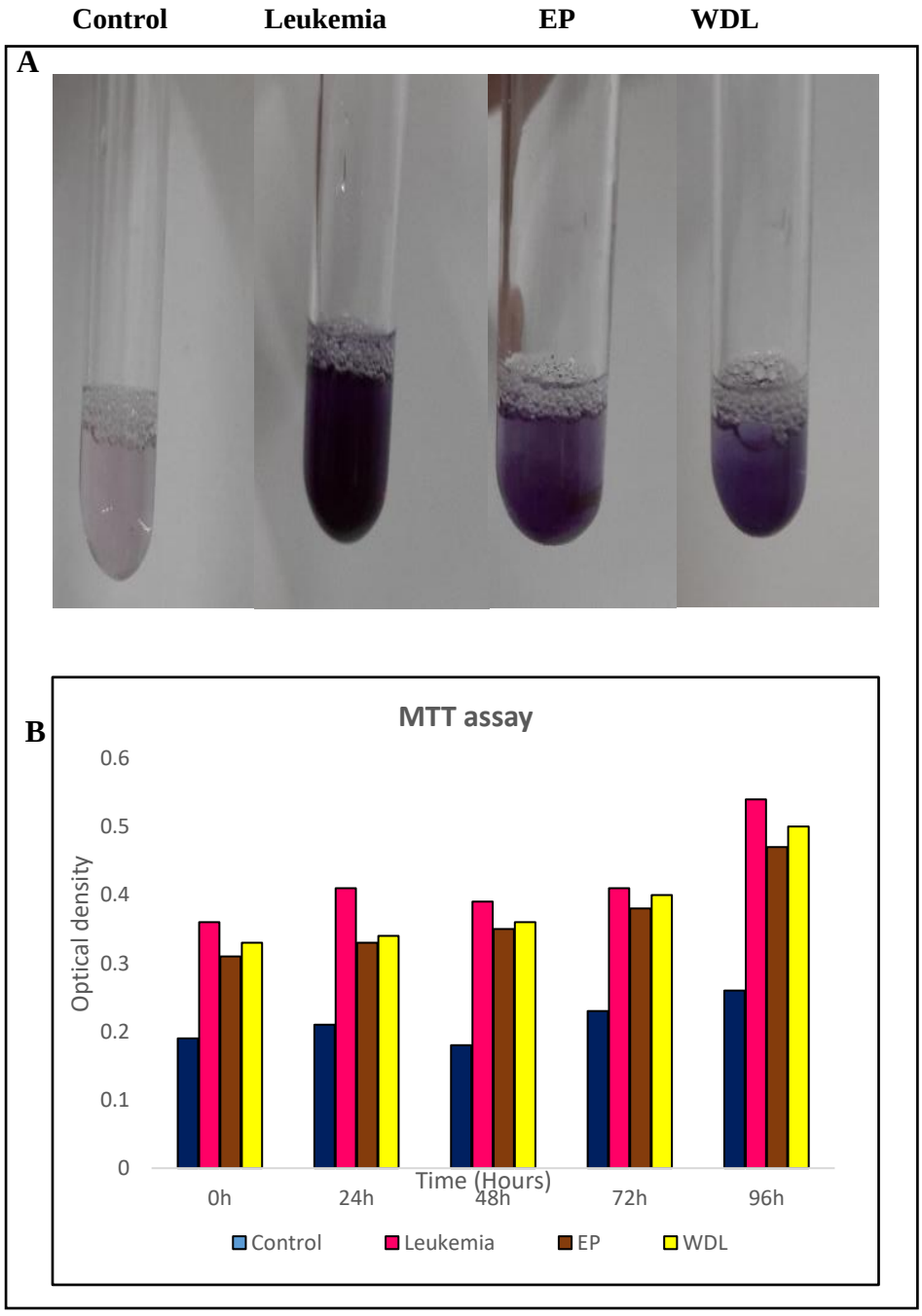
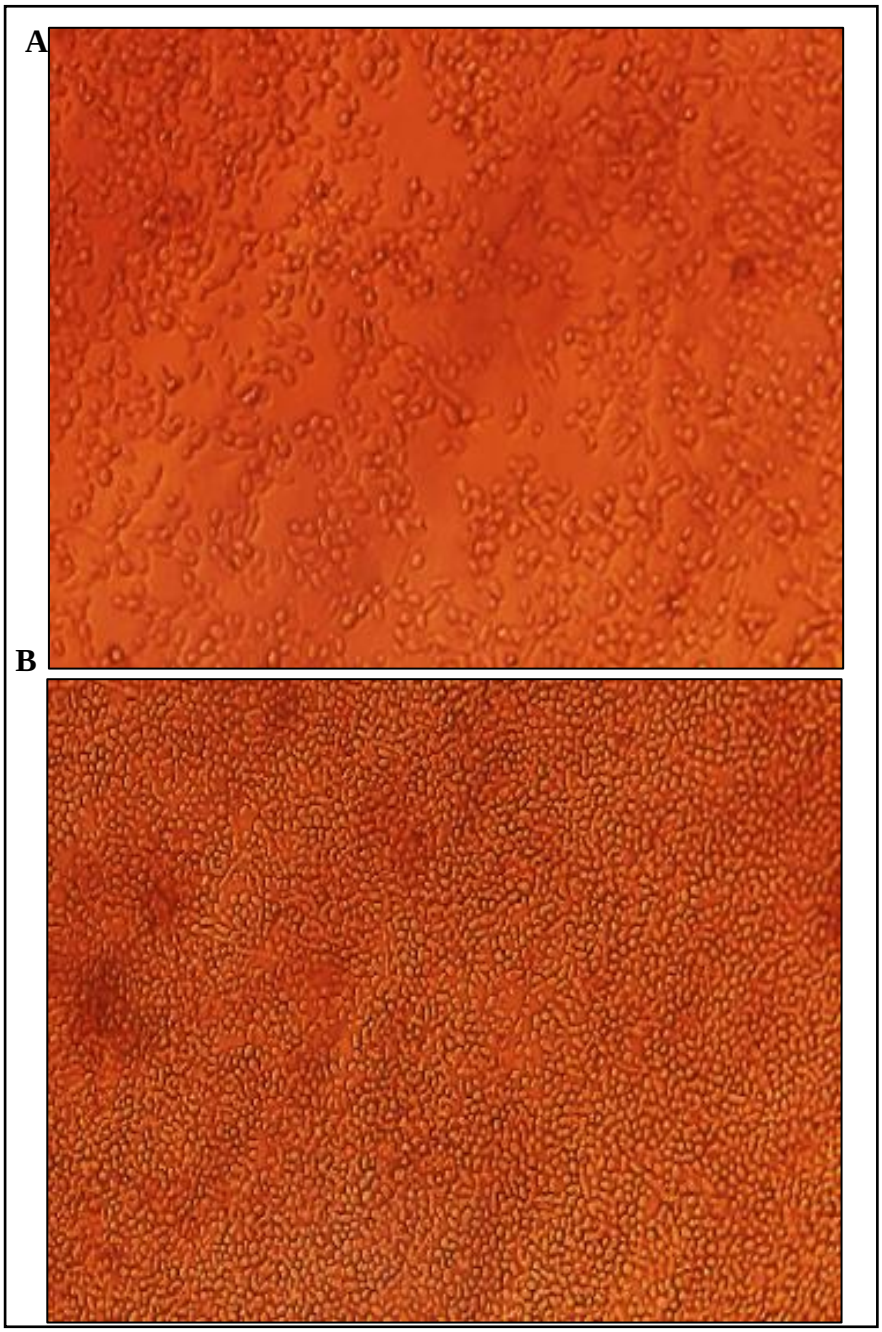


Figure 57. Proliferative assessment by MTT assay. (A) Glass tubes containing cells from each experimental groups at 96h intervals, revealing the formation of purple colour due to the presence of formazan crystals dissolved in isopropanol after the MTT experiment, (B)

representative graph showing the OD's of bone marrow cell growth kinetics at an interval of 0h, 24h, 48h, 72h and 96h.

5.5.5. Effects of the treatments on the long-term leukemic bone marrow cell culture

A cell culture was performed on the bone marrow cells for long-term to study the proliferative capacity of the leukemic cells before and after treatment. Within 96h of culture, an excessive increase in the number of bone marrow cells were observed in leukemia with a typically longer cells which resembled stromal cells.



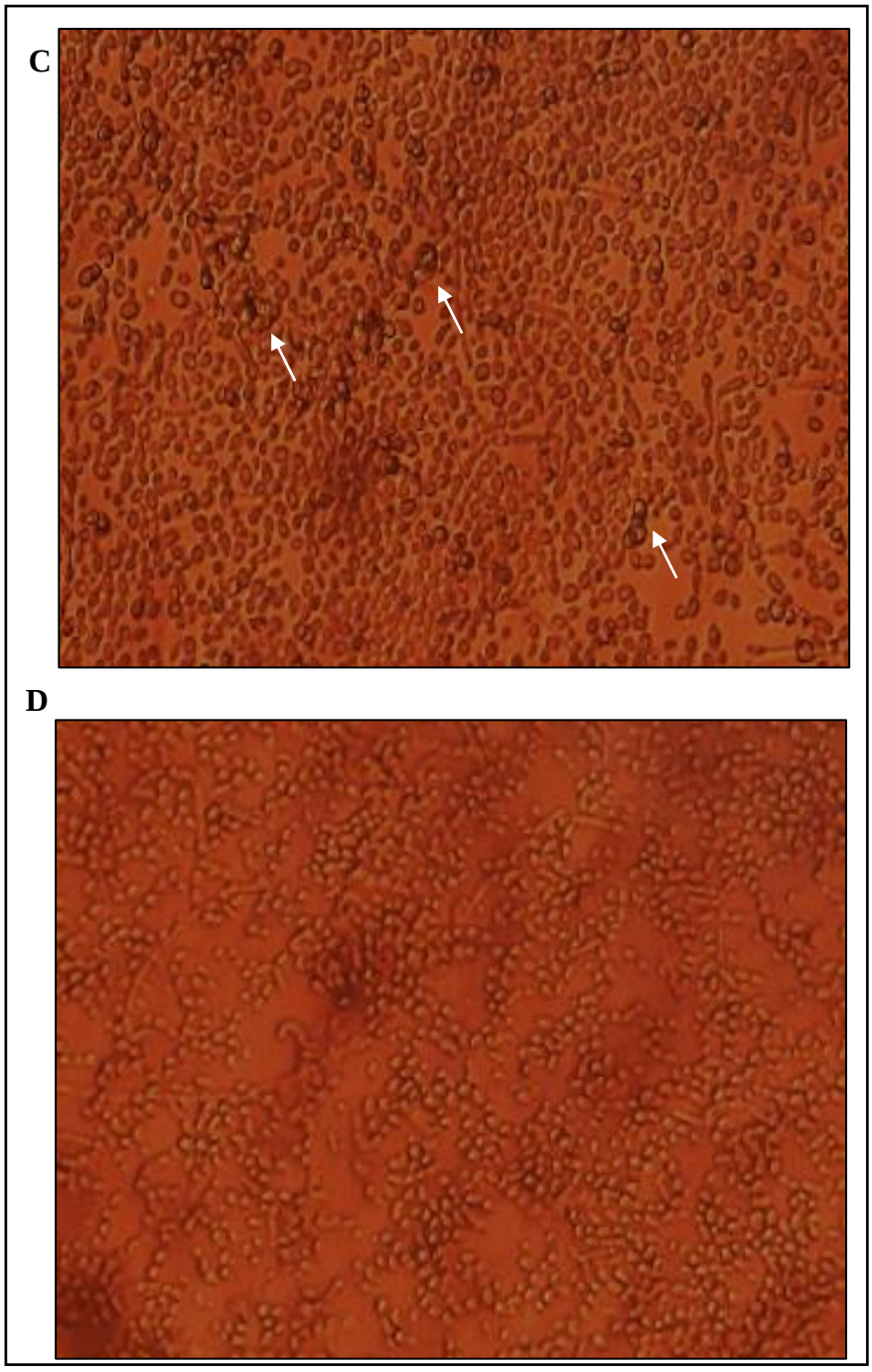
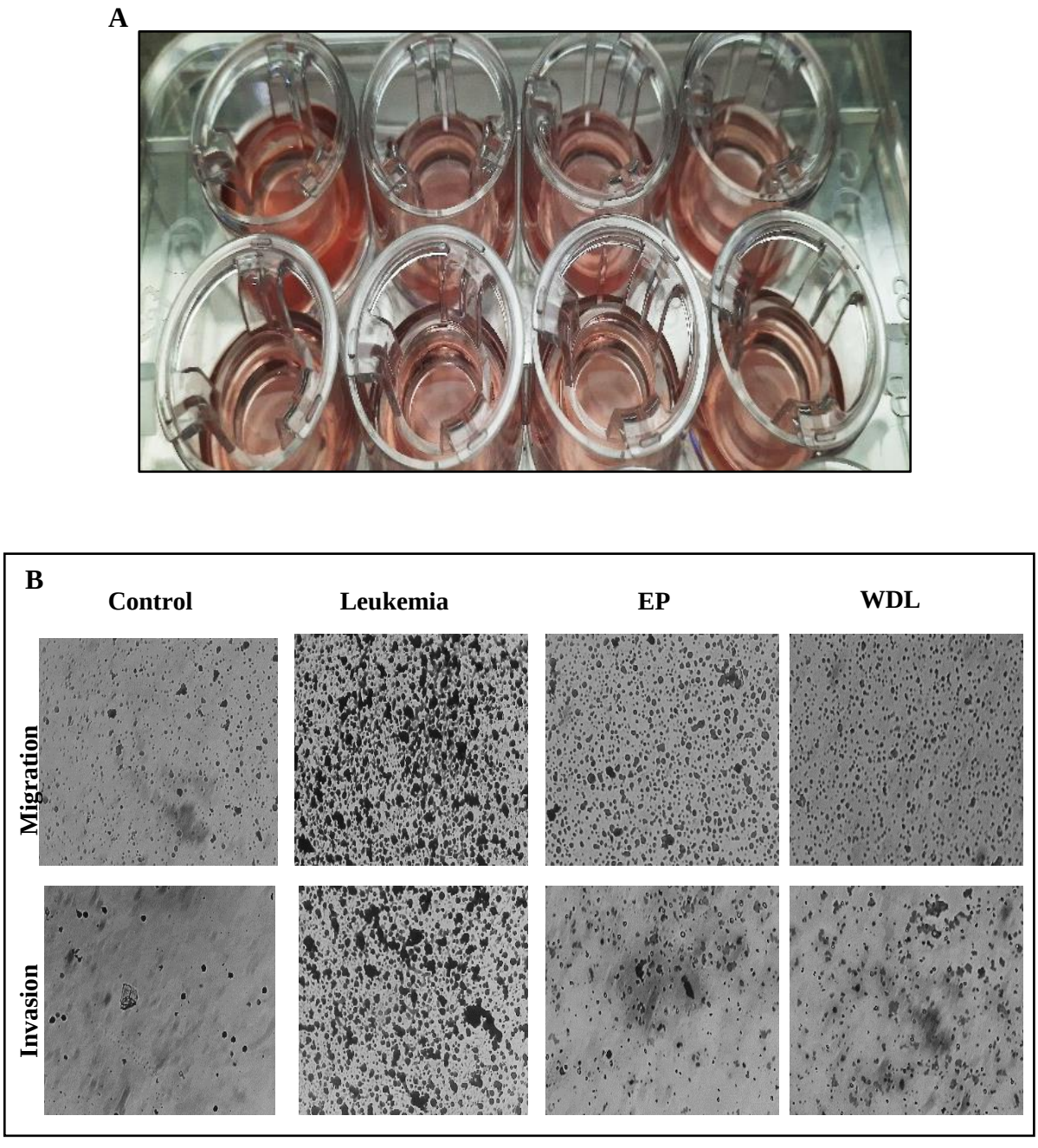


Figure 58. Long-term bone marrow cell culture at 96h. (A) Control, (B) excessive infiltration of marrow cells in leukemic group revealing stromal cell like appearance, (C) apoptotic bodies in the extract treated (white arrow) and (D) decreased proliferation in the wedelolactone treated group.

The proliferation of bone marrow cells from the extract and active compound treated group were fairly reduced but the stromal cells were observed in both (Figure 58).

5.5.6. Effects of EP and WDL on the invasion and migration capacity of marrow cells

The invasive and migratory potentials of the bone marrow cells were observed by plating them on the transmembrane plate.The ENU-induced diseased group showed a significant increase in the migration capacity of the bone marrow cells in contrast to control. After the treatments with extract and wedelolactone the invasive capacity was fairly decreased in comparison to leukemia (Figure 59).



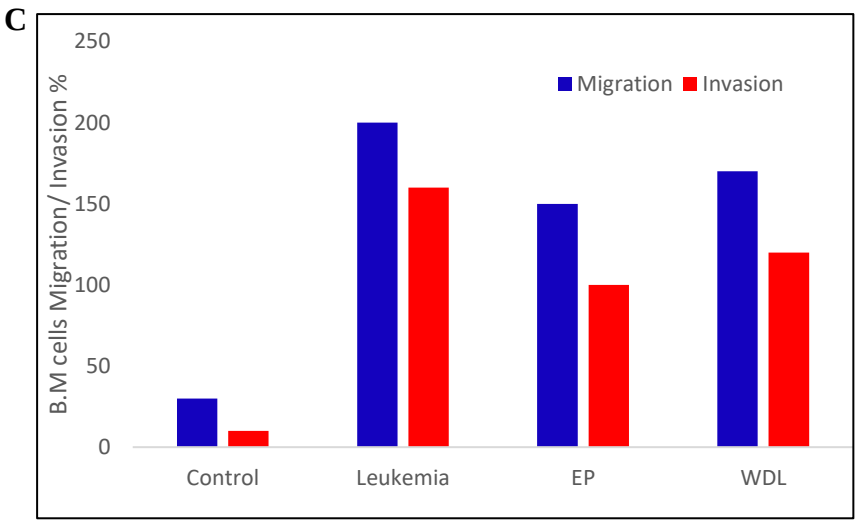
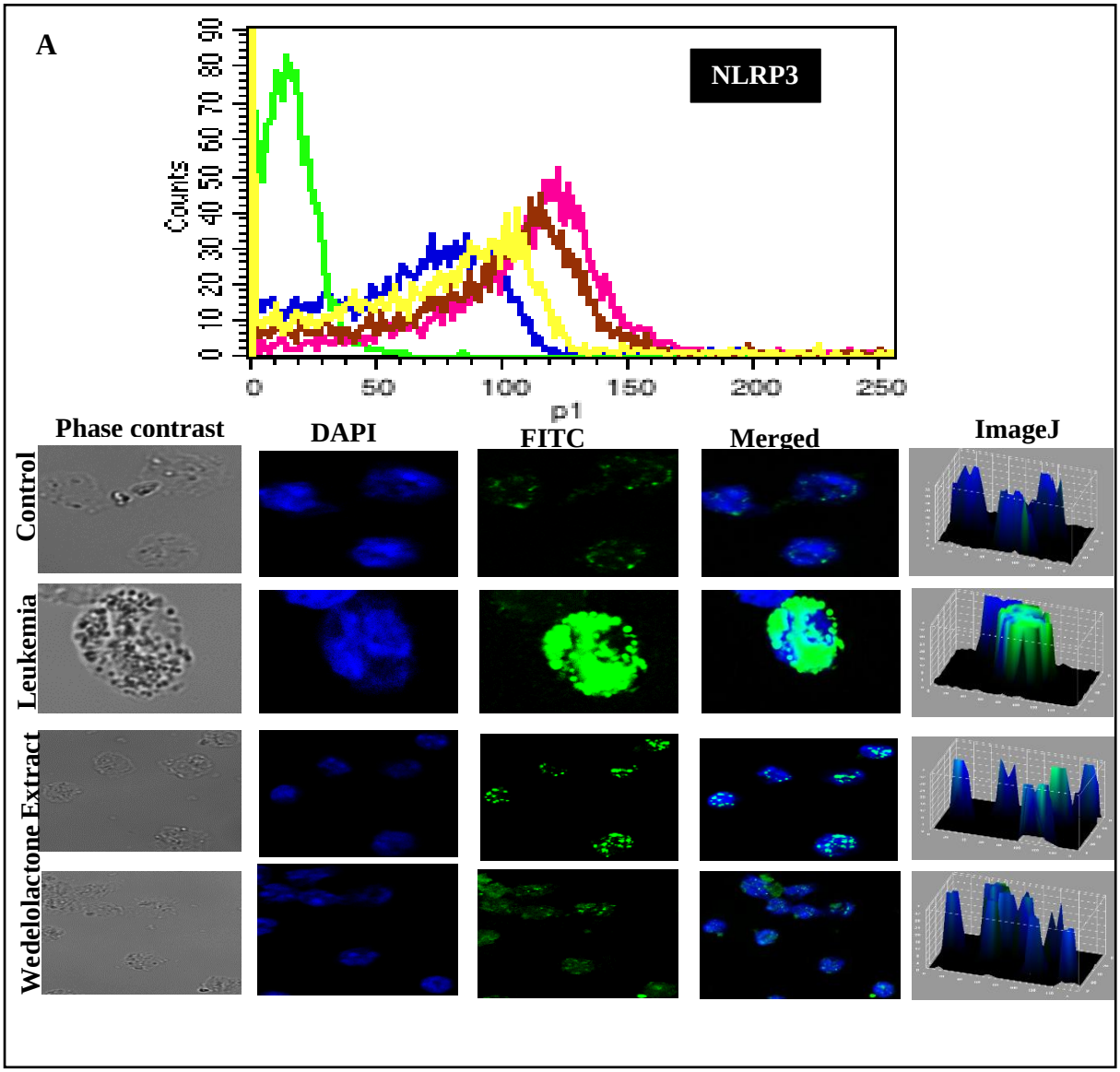


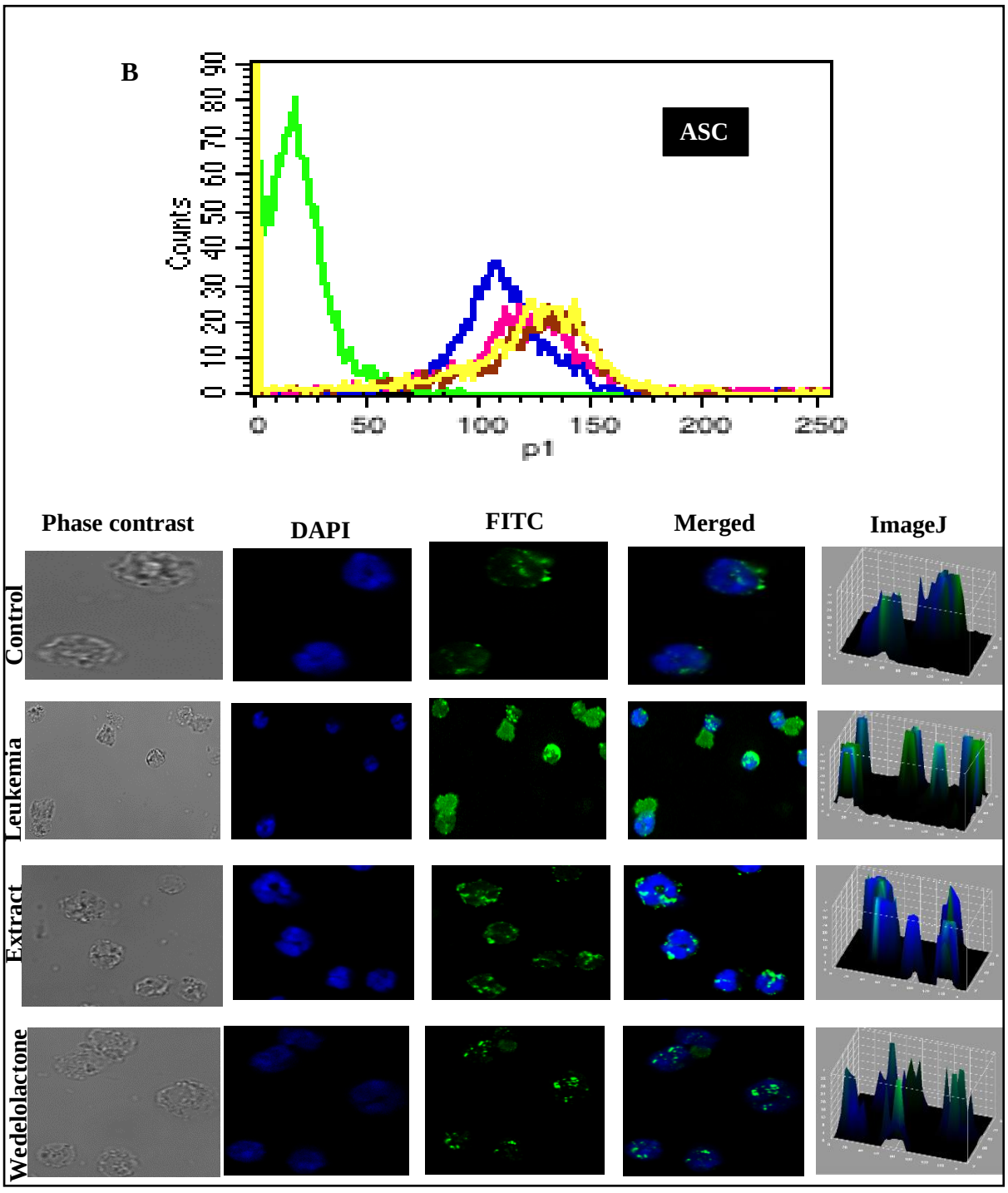
Figure 59. Migrating capacity of the bone marrow cells before and after treatment as studied by the transwell assay. (A) Images of the transwell experimental plates, (B) marrow cells from each experimental groups after 48h of invasion and migration on transwell plates, and (C) graphical representation of the invasive capacity of marrow cells pre-and post-treatment.

5.5.7. Inhibitory role of Eclipta alba and wedelolactone on the NLRP3 inflammasome in bone marrow cells of leukemia

The expressional profile of NLRP3 inflammasome molecule revealed an increase of NLRP3 expression by 3.9-folds in leukemia (MFI; 81.00 ± 0.66, P<0.001) as compared to control (MFI; 20.40 ± 0.90) (Figure 60 A). NLRP3 expression was down-regulated in both the extract and active compound treated groups by 1.2 and 2.6-folds respectively (MFI; 64.22 ± 0.58, P<0.001; MFI; 30.45 ± 0.75, P<0.001), in comparison with diseased. The expression of the speck-like protein, ASC was up-regulated in diseased condition (MFI; 125.79 ± 1.58, P<0.001) by 2.14-folds in contrast with the healthy control (MFI; 58.24 ± 1.97, P<0.001) (Figure 60 B). Significant down-regulation of the protein was observed after extract treatment (MFI; 116.30 ± 0.85, P<0.001) and active compound treatment (MFI; 118.56 ± 1.25, P=0.001) by 1.08 and 1.06-folds respectively. The extract and wedelolactone treated group (MFI; 52.5 ± 1.80, P<0.001 and MFI; 62.92 ± 1.015, P=0.002) showed a significant decline in the expression of

cleaved caspase-1, the down-stream molecule of NLRP3 inflammasome pathway by 1.3 and 1.0-folds in contrast to leukemia (MFI; 68.54 ± 0.50) (Figure 60 E). Control group expressed a reduced profile of cleaved caspase-1 by around 1.8-folds (MFI; 36.34 ± 0.95, P<0.001). An increased expression of the pro-inflammatory cytokine cleaved IL-1β was seen in the leukemic condition by 3.21-folds (MFI; 143.09 ± 0.86, P<0.001) as compared to normal (MFI; 44.53 ± 0.68). Extract (MFI; 77.73 ± 0.92, P<0.001) and wedelolactone treatment (MFI; 87.15 ± 1.23, P<0.001) ameliorated the expression of the cytokine and treated groups by 1.8 and 1.6-folds respectively (Figure 60 C). The study by fluorescence microscopy revealed the same expressional profiles of the molecules.




B
ASC
Counts
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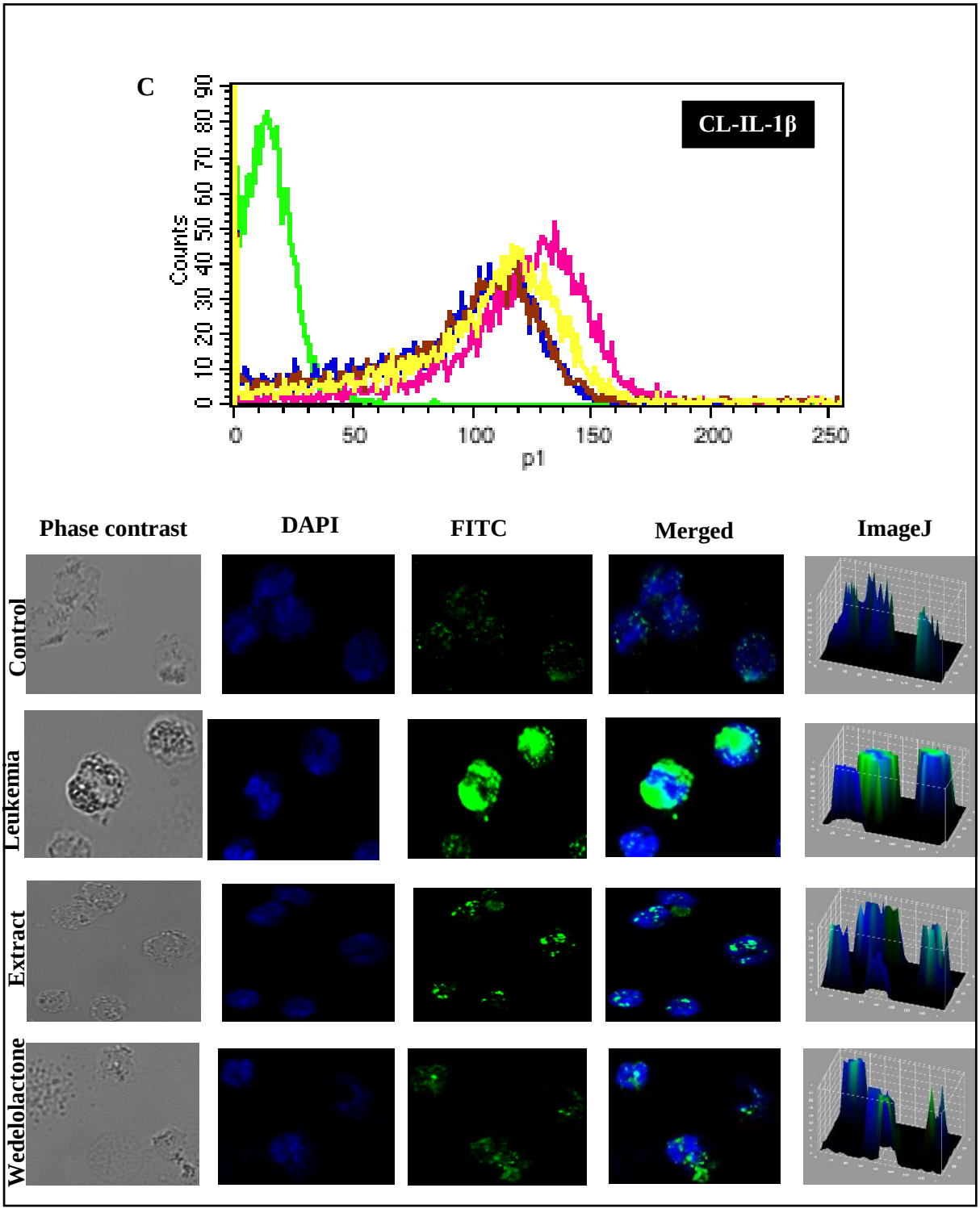
Leukemia
Extract
Wedelolactone


C
CL-IL-1β
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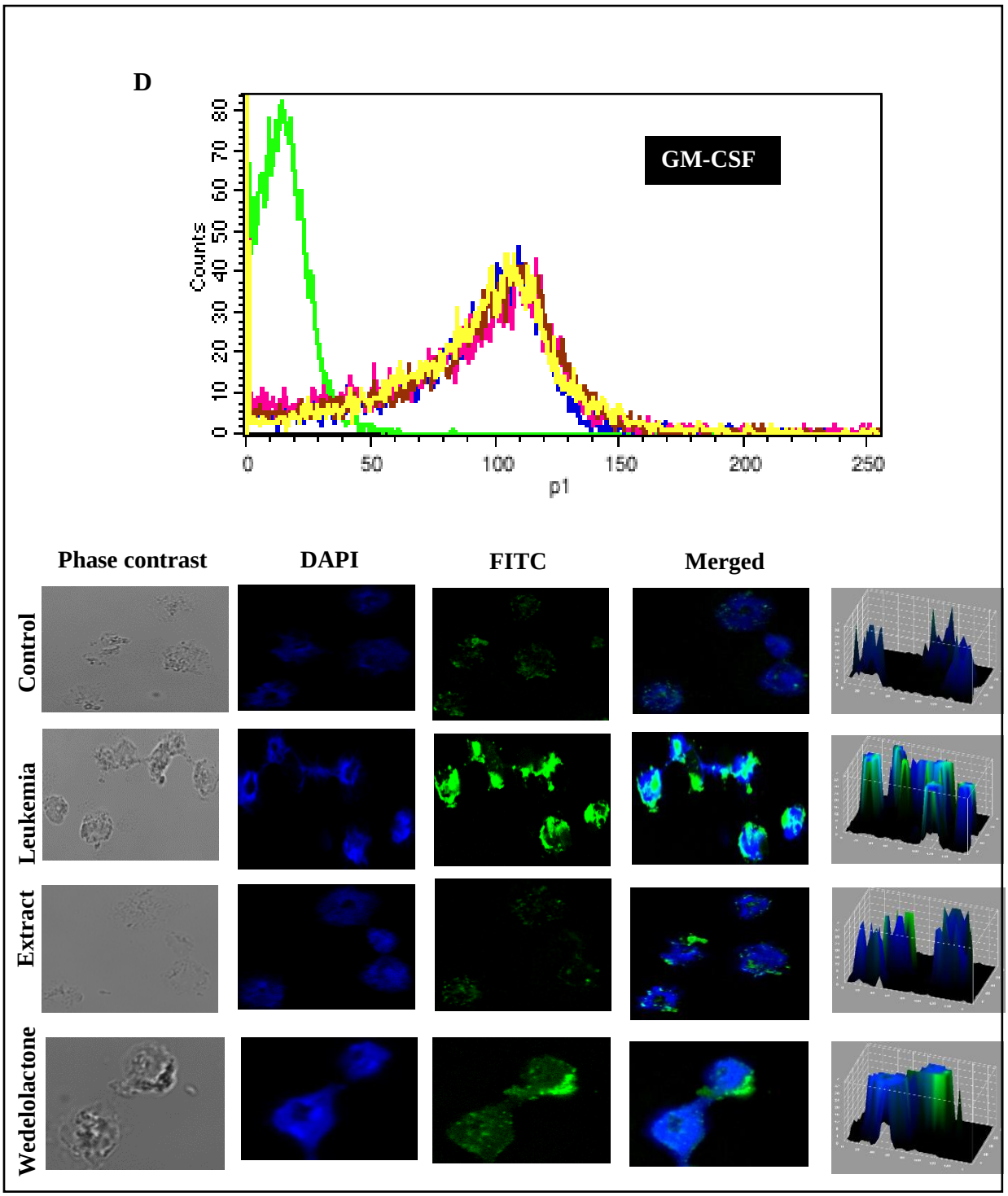
Extract
Wedelolactone


D
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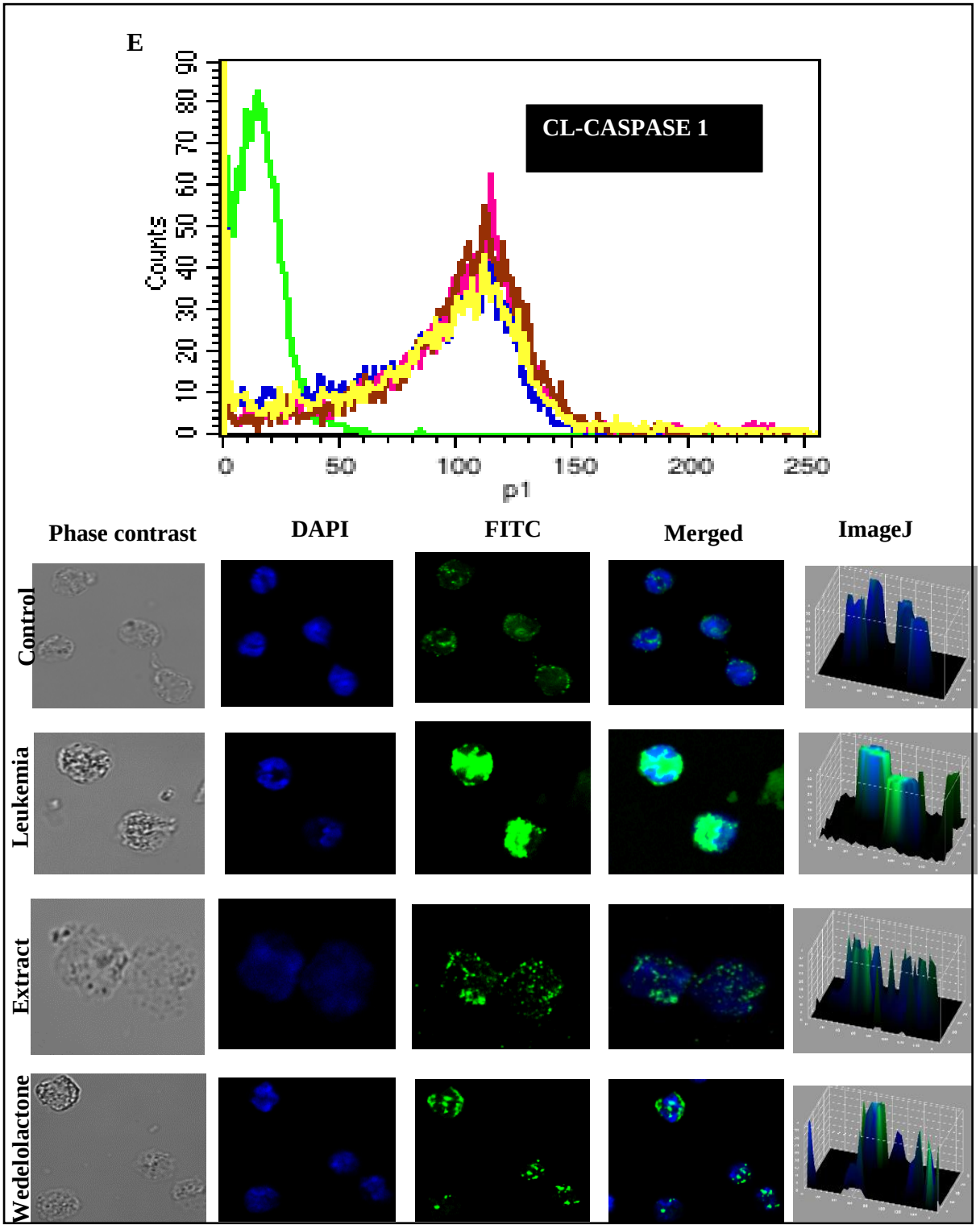


Figure 60. Graphical representative of histogram overlays showing the MFI values of (A) NLRP3, (B) ASC, (C) Cleaved-IL-1β, (D) GM-CSF and (E) Cleaved-Caspase-1. Confocal

fluorescence and phase contrast images of the bone marrow cells from experimental groups and the Image J analytical surface 3D interactive plots.

GM-CSF is a marker to study the proliferative potentials of the cell. As studied by flow-cytometry and fluorescence microscopy, GM-CSF was significantly increased by 1.48-folds in leukemia bone marrow cells (MFI; 62.19 ± 0.73, P<0.001) as compared to healthy group (MFI; 41.92 ± 0.96). In extract treated group (MFI; 49.80 ± 0.88, P<0.001) and wedelolactone (MFI; 51.19 ±1.30, P<0.001) treated groups GM-CSF expression was down-regulated by 1.2–folds in comparison to leukemia (Figure 60 D).

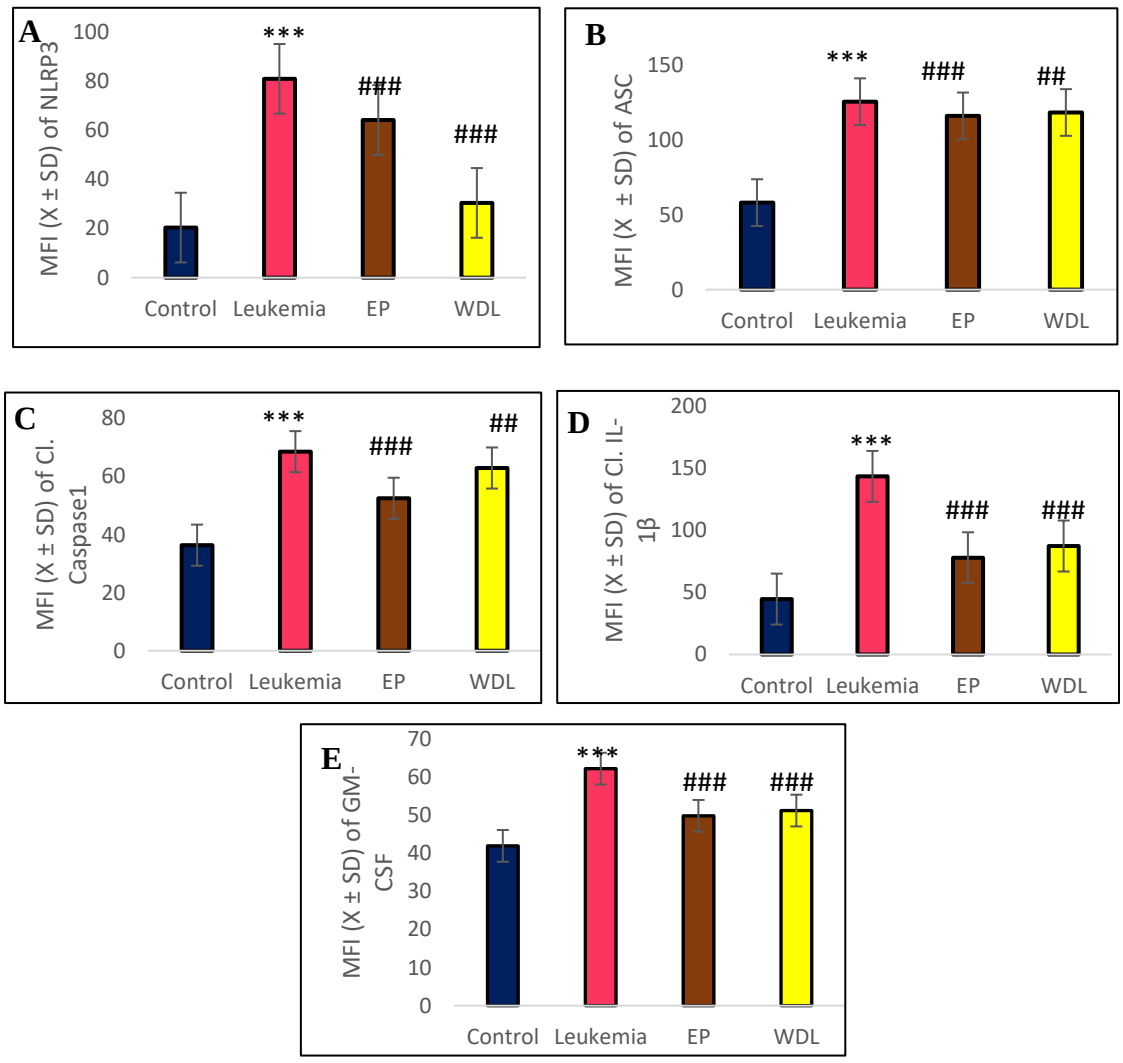


Figure 61. Graphical representation of MFI values depicting Mean ± SD of: (A) NLRP3, (B) ASC, (C) CL-Caspase1, (D) CL-IL1β, and (E) GM-CSF expression in the bone marrow cells

of each group (***P<0.001 significance of leukemia as compared to control. ##P<0.01, ###P<0.001 significance of EP and WDL treatment in comparison to leukemia).

Table 4. Mean Fluorescence Intensity (MFI) represents expression patterns of inflammasome proteins

Proteins	MFI of control (X ± SD)	MFI of Leukemia (X ± SD)	MFI of EA Treated (X ± SD)	MFI of WDL Treated (X ±SD)
NLRP3	20.40 ± 0.90	81.00 ± 0.66***	64.22 ± 0.58***	30.45 ± 0.75***
ASC	58.24 ± 1.97	125.79 ± 1.58***	116.30 ± 0.85***	118.56 ± 1.25**
Cl-caspase-1	36.34 ± 0.95	68.54 ± 0.50***	52.5 ± 1.80***	62.92 ± 1.015##
Cl-IL-1β	44.53 ± 0.68	143.09 ± 0.86***	77.73 ± 0.92***	87.15 ± 1.23***
GM-CSF	41.92 ± 0.96	62.19 ± 0.73***	49.80 ± 0.88***	51.19 ± 1.30***

*Values are mean ± SEM for 6 animals in each observation. ##P = 0.02, ∗∗P = 0.001, ***p<0.001*

5.6. Discussion

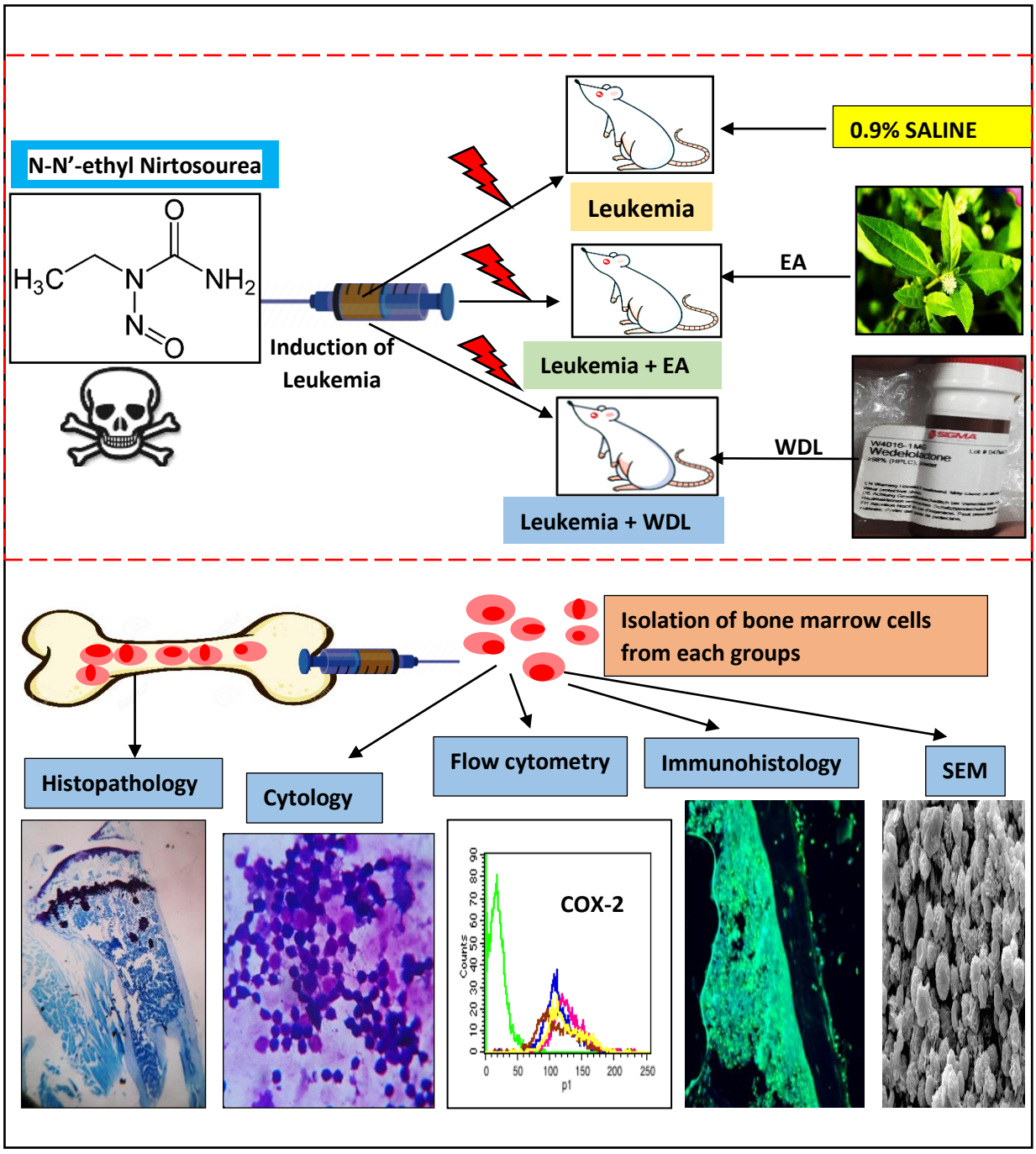
Increasing civilization leads to the ever increasing industrialization that in turn is responsible for the production of different carcinogens to the environment like NOCs. We are exposed to such NOCs from different food products like cured meat and smoked fish, tobacco smoke and also through occupational exposures like chemicals emitted from pesticide making factories (Herzog et al., 2004; Kobayashi, 2018).In our study we have explored the leukemogenic potentials of one such NOC, called ENU and aimed to study the ameliorating property of *Eclipta alba* and wedelolactone on the immune-modulation of NLRP3 pathway and anti-proliferative role in bone marrow cells of leukemia. Persistent inflammation is a key role player of continuous carcinogenesis that meanwhile activates NLRP3 inflammasome in bone marrow

cells (Dagenais et al., 2012; Kyung et al., 2021). Hence, research on inflammasome inhibitors plays a potential role on cancer research. NLRP3 inflammasome is aberrantly expressed in the marrow cells of ENU-induced leukemia, which leads to the over-expression of the down-stream speck like adaptor protein ASC. In the extract and active compound treated groups, NLRP3 and ASC protein were down-regulated significantly, hence confirming the anti-cancer potentials (Kelley et al., 2019). The higher expression of the downstream molecules cleaved caspase-1 and inflammatory cytokine IL-1β in leukemia was reduced post-treatments (Puissant and Medyouf, 2022). To study the anti-cancer effect of the treatment, we studied its anti-malignant and anti-proliferative role along with the anti-inflammasome effect. Silver nitrate staining protocol confirmed the anti-malignant role of *Eclipta alba* and wedelolactone on bone marrow cells of leukemia. The transwell assay and long-term bone marrow culture revealed the excessive invasion and proliferation of ENU-induced leukemia, which was also confirmed by the GM-CSF expressional profile (Carter et al., 2019). GM-CSF plays a dual role on promoting differentiation, mobilization and maturation of myeloid cells, in addition to being the key regulator of IL-1β production (Costinean et al., 2006). Both the treatments significantly decreased the GM-CSF expression as well as the proliferation capability as observed by MTT, transwell assay and long-term culture study.

5.7. Conclusion

It can be concluded that the herb and its active compound plays a major role in the inhibition of NLRP3 expression in the bone marrow, along with playing an anti-proliferative role in ENU-induced leukemia.

Chapter 6.


N-N'-ethyl Nirtosourea
Induction of Leukemia
Leukemia
0.9% SALINE
EA
Leukemia + EA
WDL
Leukemia + WDL
Isolation of bone marrow cells from each groups
Histopathology
Cytology
Flow cytometry
Immunohistology
SEM
COX-2
Counts
p1

Chapter 6.

Immunomodulation of COX-2 and cytochrome-c by environmental pollutant N- N' ethylnitrosourea in bone marrow cells of leukemic mice and its treatment by *Eclipta alba* and wedelolactone

6.1. Chapter summary

In the present chapter, the underlying anti-inflammatory mechanism of *Eclipta alba* and wedelolactone via COX-2 pathway and their pro-apoptotic role by initiating mitochondria-mediated apoptosis on the bone marrow cells of ENU-induced leukemic mice has been explained. Human exposure to NOCs are abundant in the environment, most evident at tyre and rubber making industry, metal cutting shops and through consumption of smoked meat. In this study we have used one such ENU to induce leukemia in mice. Leukemia is not just a chronic disease but most common cancer among children and teens. Literature review suggests leukemia can be driven by persistent inflammation and inflammasome activation. In ENU-induced leukemic animal model, an excessive inflammatory scenario was observed in the bone marrow of mice. Significant reduction in the inflammatory cells were confirmed by cytological staining of marrow and a decrease in the released inflammatory mediators like COX-2, PGE2 and iNOS were observed after treatment with extract and active compound. Reduced population of leukemic cells with polymorphic surface ridges were observed post-treatment by surface electron microscopy, which was also confirmed by the reduction of cytochrome c release in the marrow hence pointing towards the pro-apoptotic role mediated by mitochondria. The study suggests *Eclipta alba* and wedelolactone to be novel complementary therapy for the amelioration of ENU induced leukemogenesis.

6.2. Introduction

The occurrence ofNOCs in the environment through cured meat, tobacco products, rubber-making factories, pesticides and industrial situations is a possible cause of human cancer (Mirvish, 1995). Prenatal exposure to ENU, a carcinogen that belongs to the family of NOC causes leukemia in mice (Capilla-Gonzalez et al., 2012; Chatterjee et al., 2016a; Chattopadhyay et al., 2019). Leukemia is a haematological malignant disorder characterized with low survival rates as constant stimulation of oncogenic pathways leads to drug resistance and complete remission is rarely achieved (Bhatnagar et al., 2021). The critical role of COX-2/PGE2/IL1β in leukemic growth and its microenvironment by aggravating the inflammatory immune response is recently reported (Carter et al., 2019; Giles et al., 2002; Secchiero et al., 2005). The connection between the sites of chronic inflammation with the origin of cancer was hypothesized by Rudolf Virchow back in 1863. Cyclooxygenase-2 (COX-2) and its enzymatic product prostaglandin E2 (PGE2) are the lead players of inflammation, whereas well documented in cancer progression by promoting invasion, angiogenesis, metastasis and inhibiting apoptosis (Rizzo, 2011).

Worldwide 80% of the people are dependent on medicinal plant based Cancer treatment since it is easily accessible, less toxic and cost-effective (Ekor, 2014). *Eclipta alba* is one such Ayurvedic herb known for its anti-inflammatory, anti-proliferative and anti-cancer potentials (Arya et al., 2015; Chaudhary et al., 2014; Kim et al., 2015; Liao et al., 2018; Lirdprapamongkol et al., 2008). The herbal extract is reported to inhibit PGE2 release by down-regulating iNOS and COX-2 expression in RAW264.7 macrophage cells (Tewtrakul et al., 2011). A study showed, the extract has major anti-angiogenic effects on *in vivo* cancer cells (Lirdprapamongkol et al., 2008). Wedelolactone is a multi-target bioactive compound of *Eclipta alba* extract, reported for exhibiting cytotoxicity towards various cancer cells and holds great promise as a potential anti-cancer treatment (Nehybová et al., 2017; Tewtrakul et al.,

2011). Wedelolactone is reported to induce caspase-dependent apoptosis and severely down-regulate the expression of an oncogene *c-Myc* in prostate cancer cells (Sarveswaran et al., 2016, 2012).

Although there are several reports on the anti-cancer mechanism of *Eclipta alba* extract and isolated active compound on various type of cancer cells, none is reported on any haematological malignant in vivo model. Our last study was the first to establish the ameliorating properties of the extract and its active compound wedelolactone on leukemic mice via NLRP3 inflammasome signaling pathway (Bhattacharyya and Law, 2022).This study was conducted as a continuation of the promising research work, in which we considered the phyto-therapeutic treatment of *Eclipta alba* and its active compound wedelolactone on ENU induced leukemic mice model to study the anti-inflammatory mechanism via COX-2/PGE2 modulation and its pro-apoptotic role in the bone marrow cells.

6.3. Materials and methods

6.3.1. Plant material extract preparation and chemicals

The dried leaves of *Eclipta alba* were procured from the Medicinal Plant Garden of Narendrapur Ramakrishna Mission Ashrama, Kolkata, India. The leaf powder was extracted in ethanol for 24h followed by double filtration, maintaining a sterile condition. After the complete removal of solvent, the extracts were concentrated at 50˚C by rotary evaporator. The filtrate was reconstituted in double-distilled water and stored at -20˚C until further use (Yadav et al., 2017). The presence of the major active compound wedelolactone in the extract (12.5 mg/ml of wedelolactone in 1200 mg/ml of extract) was carried out with the reference standard (Wedelolactone, purity ≥ 98%, CAS No: 524-12-9; Sigma-Aldrich, USA) by HPLC method and the chemical-characterization of the isolated wedelolactone was confirmed by ESI-MS-

direct infusion method. The protocol for HPLC analysis and ESI-MS-direct infusion methods are shown in our previous research methodologies (Bhattacharyya and Law, 2021).

6.3.2. Animal maintenance

Inbred Swiss albino mice were maintained at the animal house of the Calcutta School of Tropical Medicine. The mice were fed a standard diet and water *ad libitum*, under sterile conditions with a 12-h light-dark period under controlled room temperature (22 ± 2)°C. All the procedures were in consent with the authorized guidelines of the Institutional Animal Ethical Committee (IAEC); EU Directive 2010/63/EU and also according to The Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India (Registration number: 681/GO/Re/S/02/CPCSEA).

6.3.3. Leukemic induction by ENU

A single intra-peritoneal injection of N-N' ethylnitrosourea (ENU, Sigma, USA; 80mg/kg) was administered to litter pups aging 10-14 days and weighing 4-5gm, to induce leukemic condition within 6-8 months (Basak et al., 2010; Chatterjee et al., 2016a, 2016c, 2016b).

6.3.4. Therapeutic dose and animal grouping

As per our previous study, the dose for extract and wedelolactone via oral gavage was fixed at 1200 mg/kg and 50 mg/kg body weight respectively for 4 weeks after leukemic onset (Bhattacharyya and Law, 2022). The study was carried out among 4 different experimental groups as followed:

Experimental groups (n = 6 mice per group):

Group I: Control group;

Group II: Leukemic group (6 months after ENU induction);

Group III: Leukemia + extract treated group (6 months after ENU induction + 4 weeks oral gavage of extract);

Group IV: Leukemia + wedelolcatone treated group (6 months after ENU induction + 4 weeks oral gavage of wedelolactone).

6.3.5. Bone marrow isolation

The bone marrow cells from femur bones were flushed out using sterile 26 gauge needle. Marrow cells were passed through a 100 µm mesh and washed thrice in chilled RPMI-1640 media (Sigma, USA) to remove debris for the procurement of single cell suspension.

6.3.6. Periodic Acid Schiff staining of bone marrow cells

Bone marrow smears from the experimental groups were fixed in formaldehyde and ethanol solution and thoroughly washed under running tap water. The slides were dipped in periodic acid solution for 5 min, washed in distilled water and thereafter immersed in the Schiff's reagent for 10 min followed by a wash under tap water. Cells were counter stained using light green solution, mounted and observed under light microscope (Olympus, Ch20i, Japan) (Daw et al., 2016).

6.3.7. Nonspecific- esterase staining of bone marrow cells

Bone marrow smears from the experimental groups were immersed in a pararosaniline-sodium nitrite and α-napthyl acetate solution for 20 min. The slides were counter stained with Hematoxylin and observed under light microscope (Olympus, Ch20i, Japan) (Daw and Law, 2021).

6.3.8. Bone marrow histology

The long bones from the experimental groups were surgically removed and fixed in 10% neutral buffered formalin for 24h. Followed by decalcification using 10% formic acid, the

bones were passed through an increasing concentration of alcohol and the tissues were embedded in paraffin. Bone marrow sections were cut and stained with routine Haematoxylin and Eosin (H&E) to be observed under light microscope (Olympus, Ch20i, Japan) (Bhattacharyya and Law, 2021).

6.3.9. Toluidine blue staining of bone marrow

The bone marrow sections from the experimental groups were deparaffinised, hydrated with distilled water and stained with Toluidine blue for 2 min. The stained sections were rinsed thrice in distilled water and dehydrated with increasing concentration of alcohol. The slides were mounted and observed under light microscope (Olympus, Ch20i, Japan) (Ali et al., 2014).

6.3.10. Scanning electron microscopy

Bone marrow tissues from the experimental groups were fixed in 2.5 % glutaraldehyde solution for overnight and the tissues were dehydrated by passing them through the downgrades of alcohol. Marrow tissues were dried and coated with gold in vacuum and observed under the scanning electron microscope (Zeiss, Oxford instruments).

6.3.11. Flow-cytometry

Single cell suspensions (approximately 1×10^6 cells/ml) of bone marrow from each experimental groups were fixed in 1.5 % paraformaldehyde (PFA) solution, washed with PBS and suspended in FACS sheath fluid (Becton Dickinson, USA). The cells were stained with 2µl of FITC-tagged CD 11b primary antibody (Cell Signaling Technologies, USA) and incubated at 37˚C for 30 min in dark. Simultaneously to study the internalized proteins, PFA fixed unstained cells from each group were treated with chilled methanol for 30mins, washed with PBS and suspended in FACS fluid. The cells were stained with the respective primary antibodies: anti- mouse cleaved Caspase-3, ASK-1, JNK, Bax, Bcl-xl, Cytochrome c, VEGF, COX-2, iNOS, PGE2 (all from Cell Signaling Technologies, USA); anti-cytochrome c and E-

cadherin (both from Santa Cruz Biotechnology, USA). The stained marrow cells were incubated for 30 min at 37˚ C and tagged with goat anti-rabbit secondary antibody conjugated with Alexa Fluor-488 (Invitrogen, USA) and incubated at 37˚ C for next 30 min. Bone marrow cells were washed and analyzed (10,000 events) by BD FACS Calibur (Becton– Dickinson, USA) using Cell Quest Pro software (Becton–Dickinson, USA).

6.3.12. Immunofluorescence of bone marrow

The bone marrow sections were deparaffinised in xylene and rehydrated with descending concentrations of alcohol. The sections were treated with antigen retrieval buffer solution at 85 °C for 15 min, followed by a wash and then immersed in blocking solution at 37 °C for 30 min. Marrow sections from each experimental group were individually incubated with primary antibodies (Cell Signaling Technologies, USA): anti COX-2 (1:1000 dilution in PBS) at 37 °C for 2–3 h. The sections were washed in PBS, followed by incubation with secondary antibody conjugated to Alexa Fluor 488 (Invitrogen, USA) for 1–2 h, washed with PBS and the slides were mounted with DAPI shield (Sigma, USA). The signals were recorded using a fluorescence microscope, Leica DM8 and the data was analyzed by Leica DX software.

6.4. Statistics

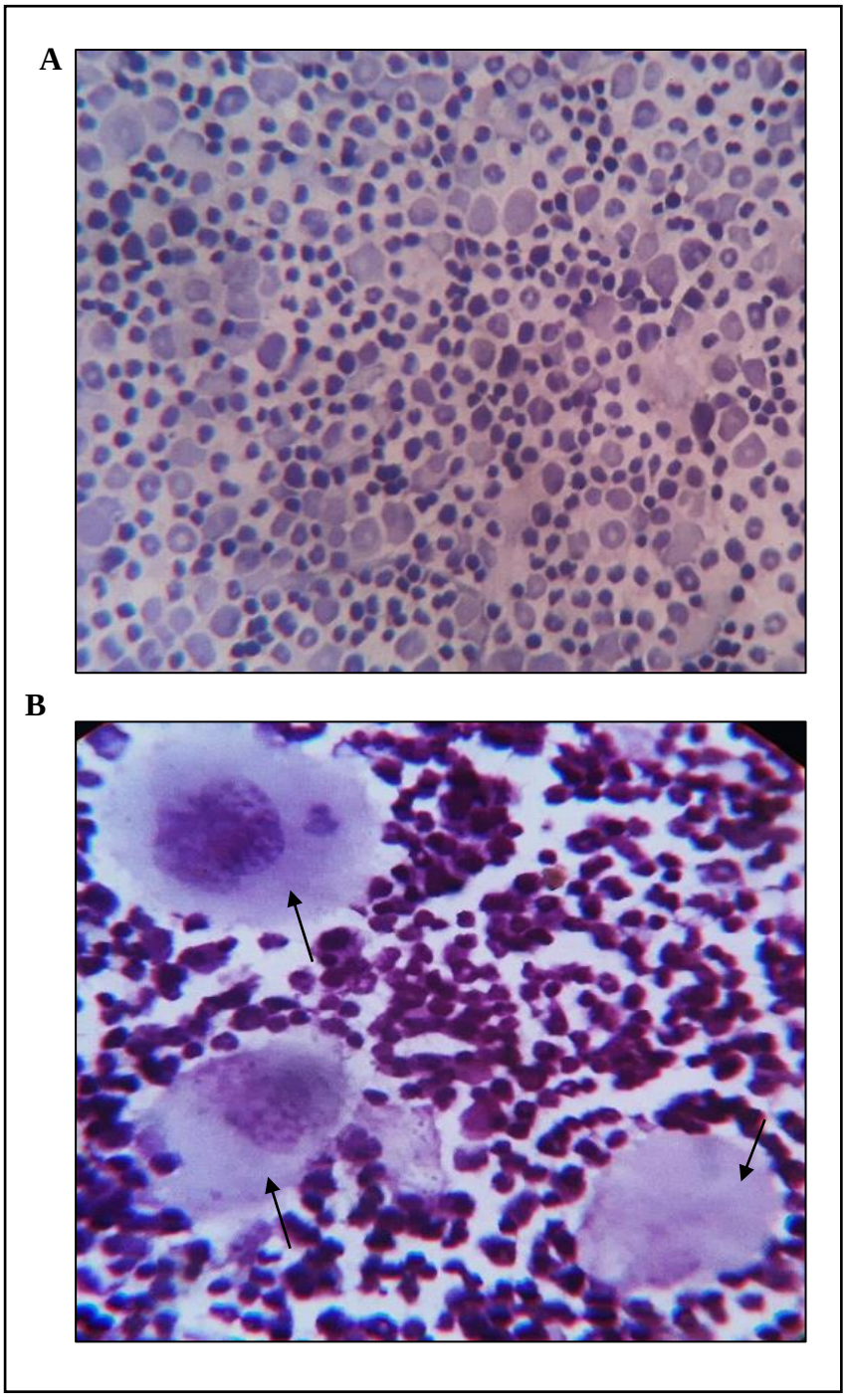
Every quantitative data were represented as Mean ± SD. For every comparison, $p \leq .05$ was considered as significantly significant. Unpaired Student's t-test and One-way ANOVA by post hoc testing (Tukey's test) was performed for statistical analysis. All the experiments were repeated thrice

6.5. Results

6.5.1. Cytopathological assessment of leukemogenesis before and after treatment

The bone marrow smear of Leukemic animals (20% ± 4.31%, $p < 0.001$) stained with PAS (Periodic Acid Schiff) revealed an increased number of PAS positive population and abnormal

megakaryoblast as compared to control (5% ± 0.08%, $p < 0.001$) (Figure 61 A - D). Treatment with extract (17% ± 3.82%, $p = 0.008$) and active compound (12% ± 6.52%, $p < 0.001$) showed significant reduction in PAS stained cells as compared to leukemia (Figure 62).



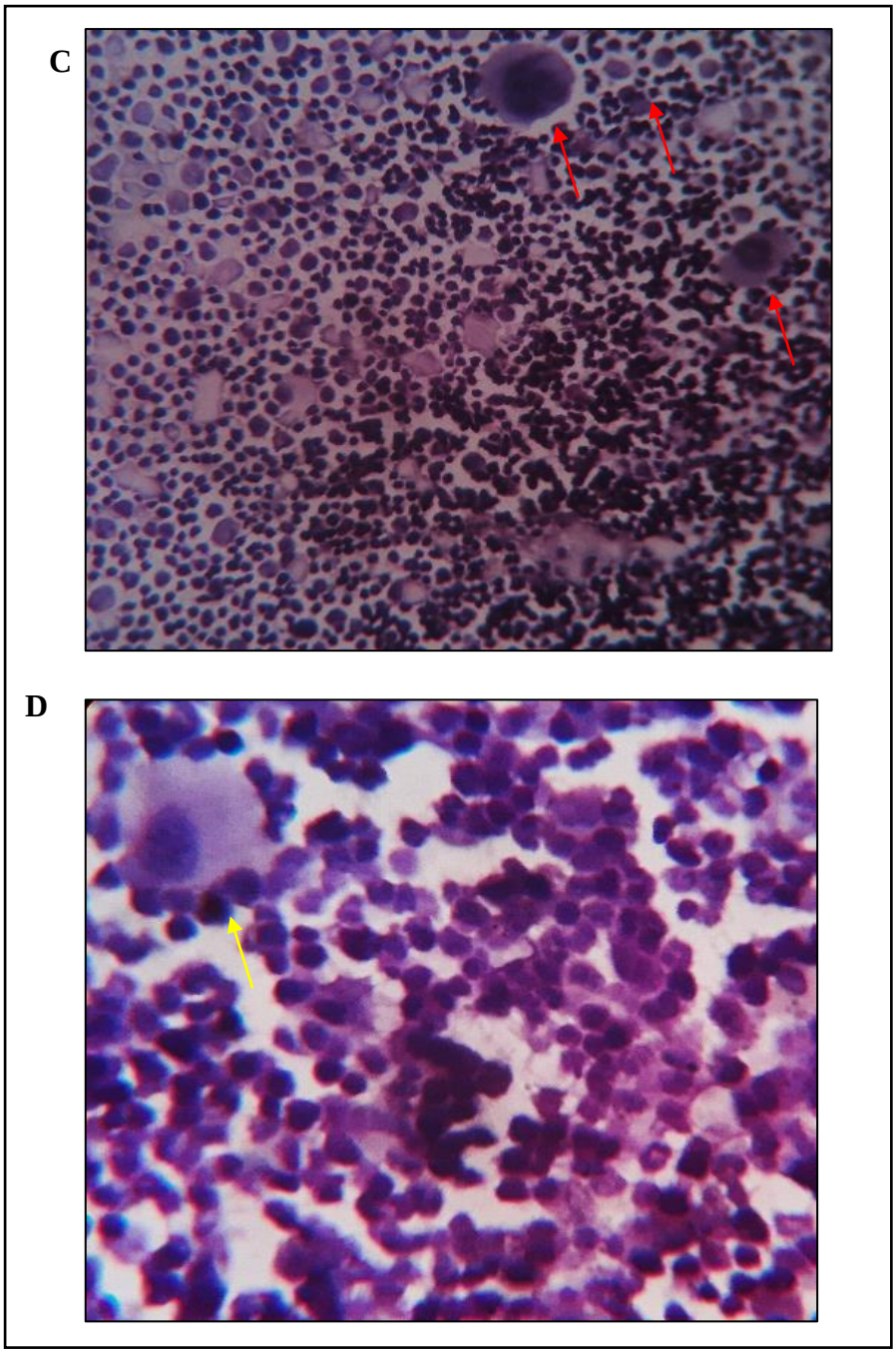


Figure 61. Cytological study of bone marrow to evaluate the ameliorating effects of treatment. PAS staining of marrow cells to identify megakaryoblast: (A) control, (B) leukemia (black arrow), (C) Extract treated (red arrow), (D) WDL treated (yellow arrow)

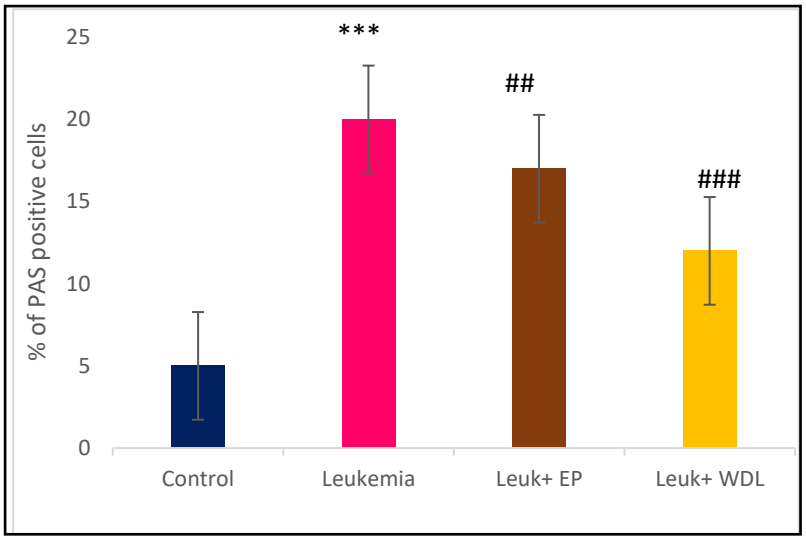
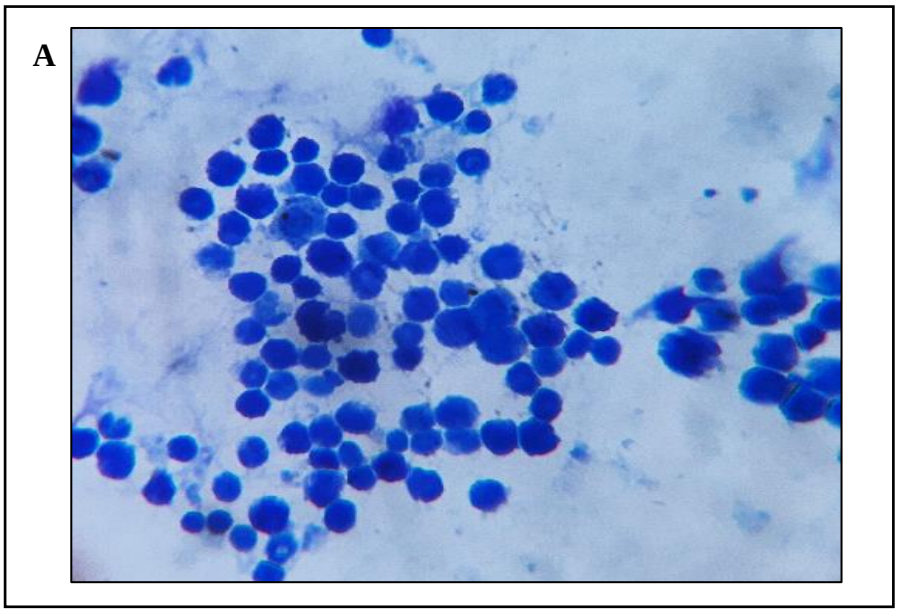
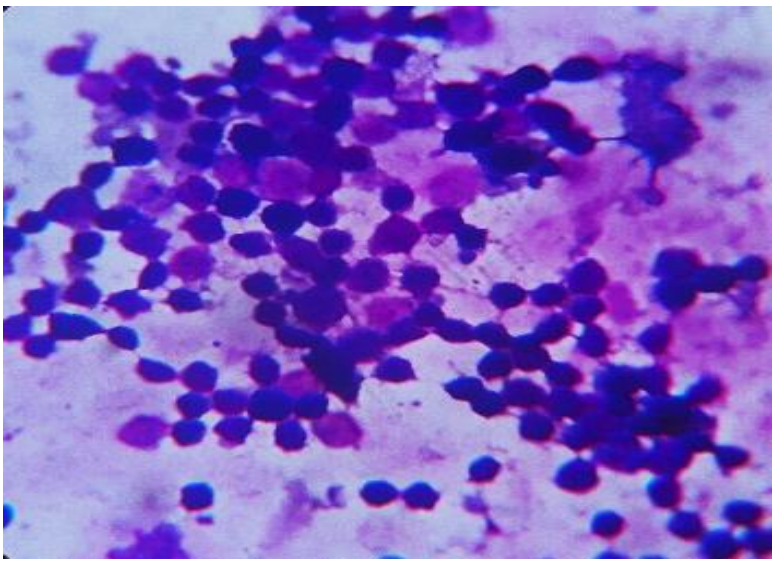


Figure 62. Graphical depiction of mean ± SD values of PAS positive cells infiltrating in marrow pre-and post-treatment [###P=0.001, ##P=0.008, ***P<0.001].

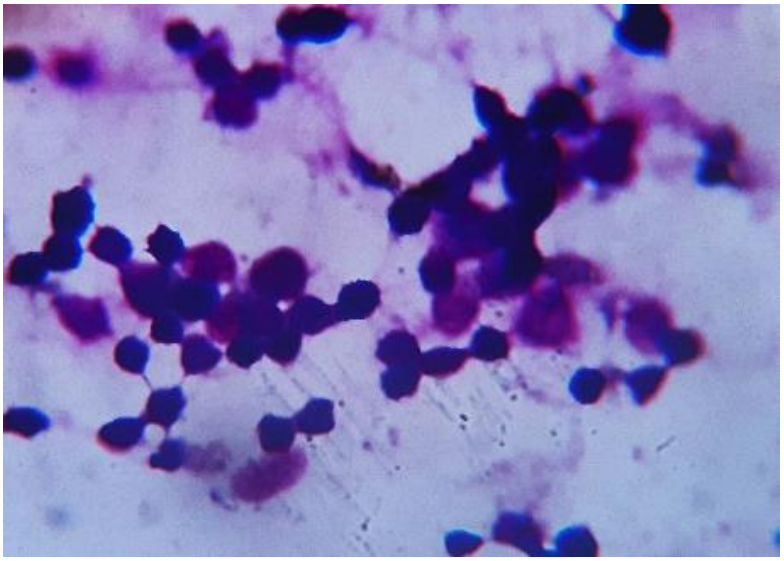
Myeoblasts and mononuclear phagocytes were identified by NSE (Nonspecific- esterase) as membrane bound enzymes present in monocytes. Diseased group showed a highly elevated population of monocytes (27.3% ± 1.30%, p <0.001) compared to control (2.33% ± 0.04%, p < 0.001). Treatment with extract (21.6% ± 0.12%, p = 0.02) and wedelolactone (19% ± 1.22%, p = 0.01) showed a moderately significant decrease in monocyte population (Figure 63 A - E).



B



C



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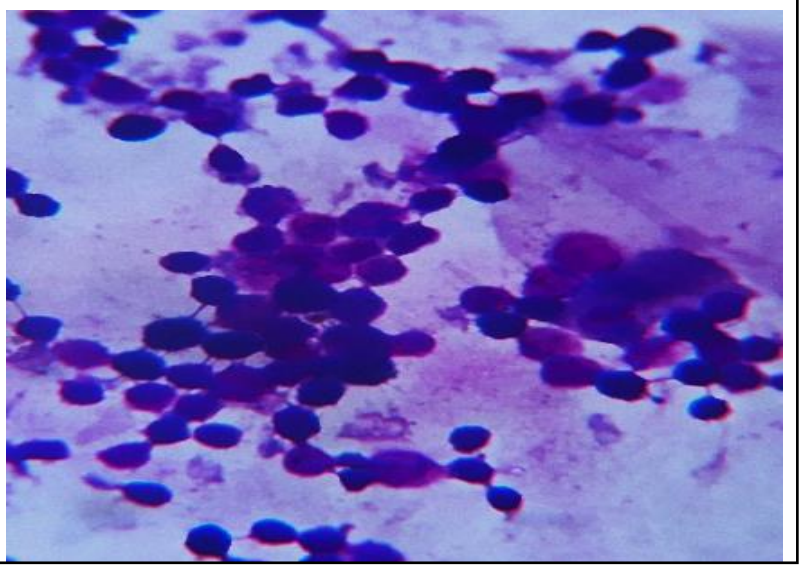


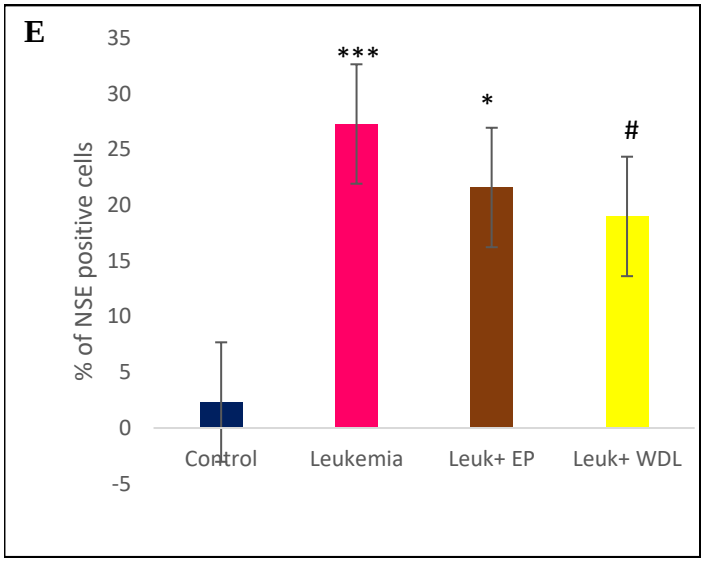
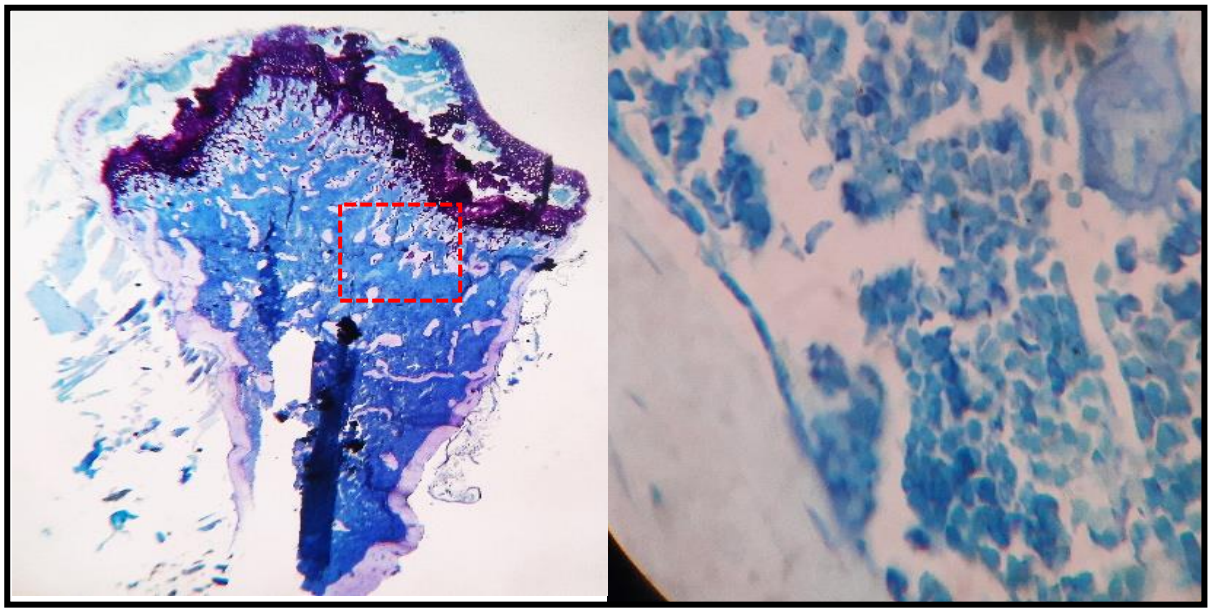


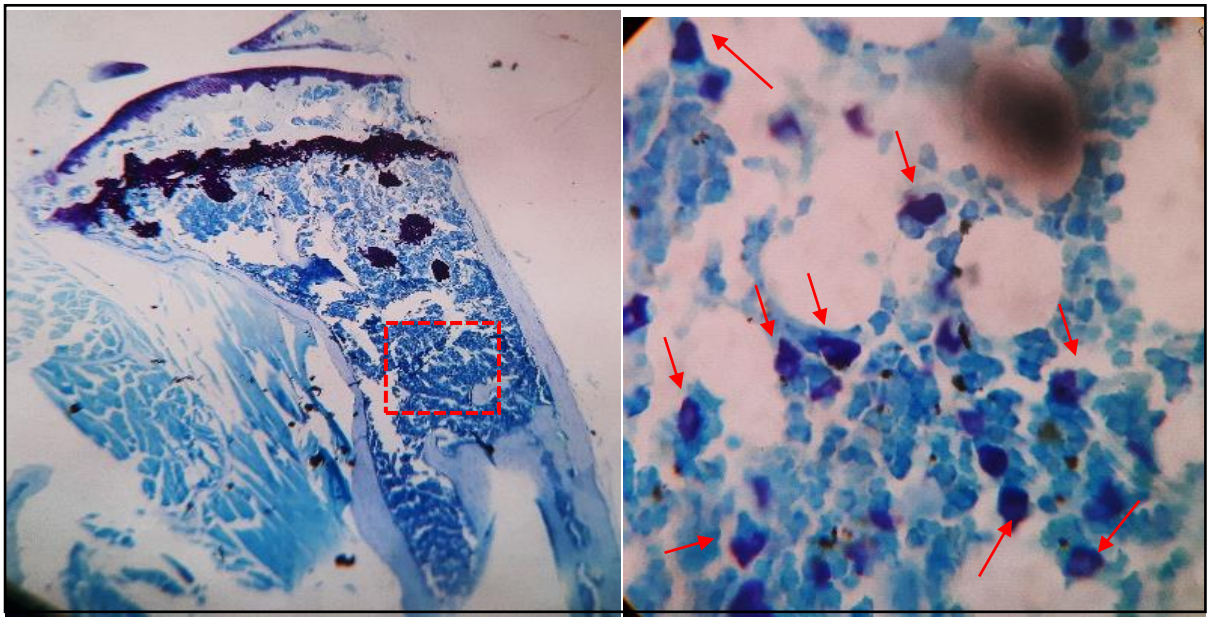
Figure 63. NSE staining to identify mononuclear phagocytes: (A) control, (B) leukemia ,(C) Extract treated, (D) wedelolactone treated, and (E) graphical depiction of mean ± SD values of NSE positive cells in marrow of experimental groups [*P= 0.02, #P= 0.01, *** P< 0.001].

6.5.2. *Role of extract and wedelolactone on mast cell and macrophage mediated inflammation in leukemic marrow*

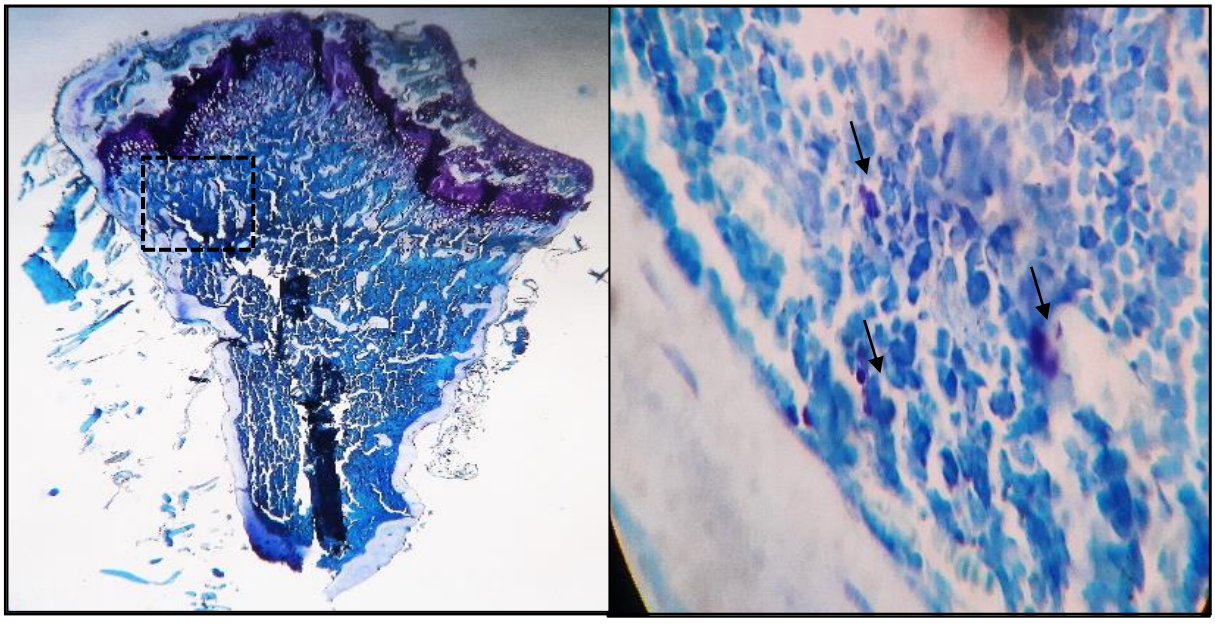
A



B



C



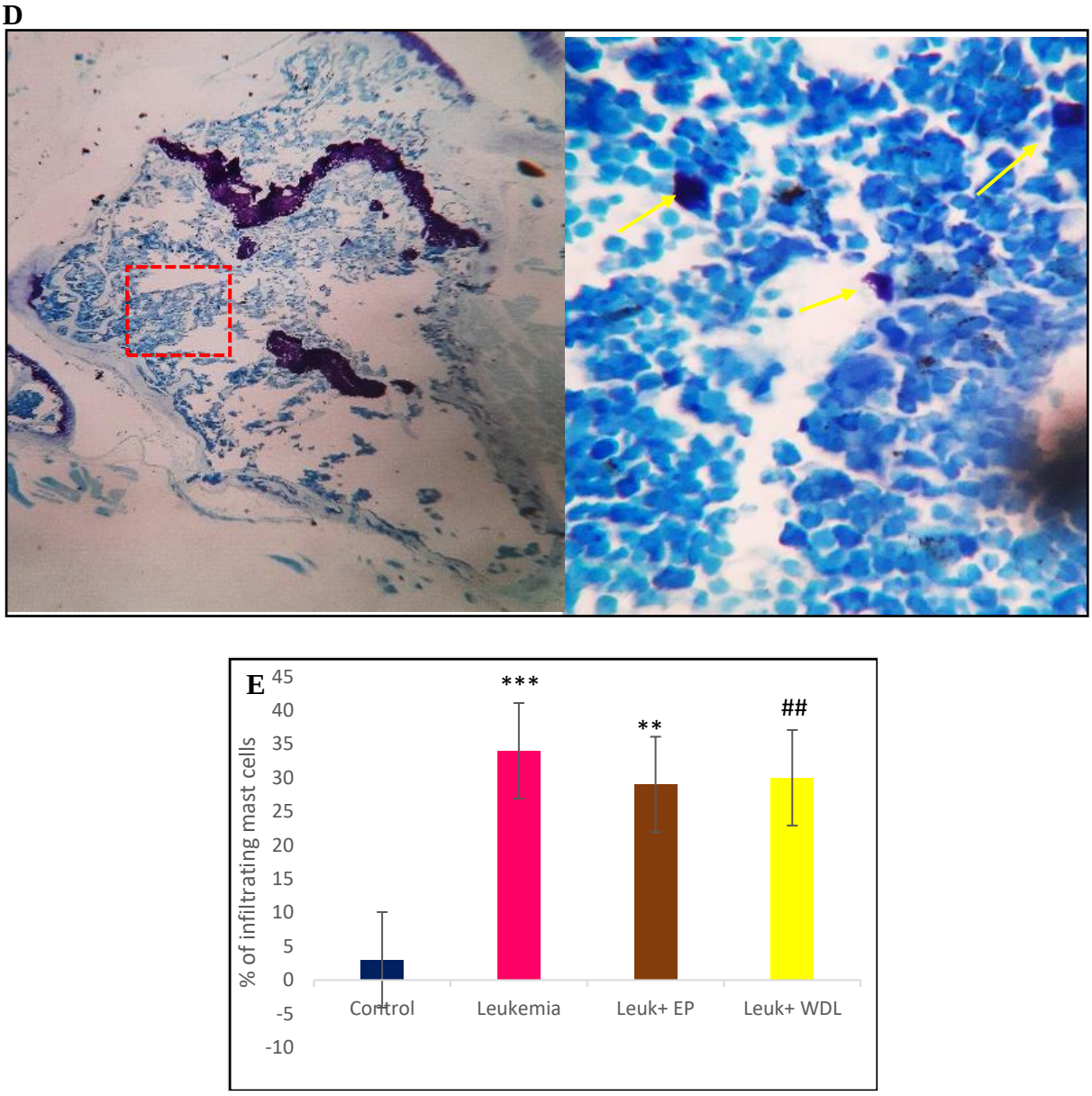
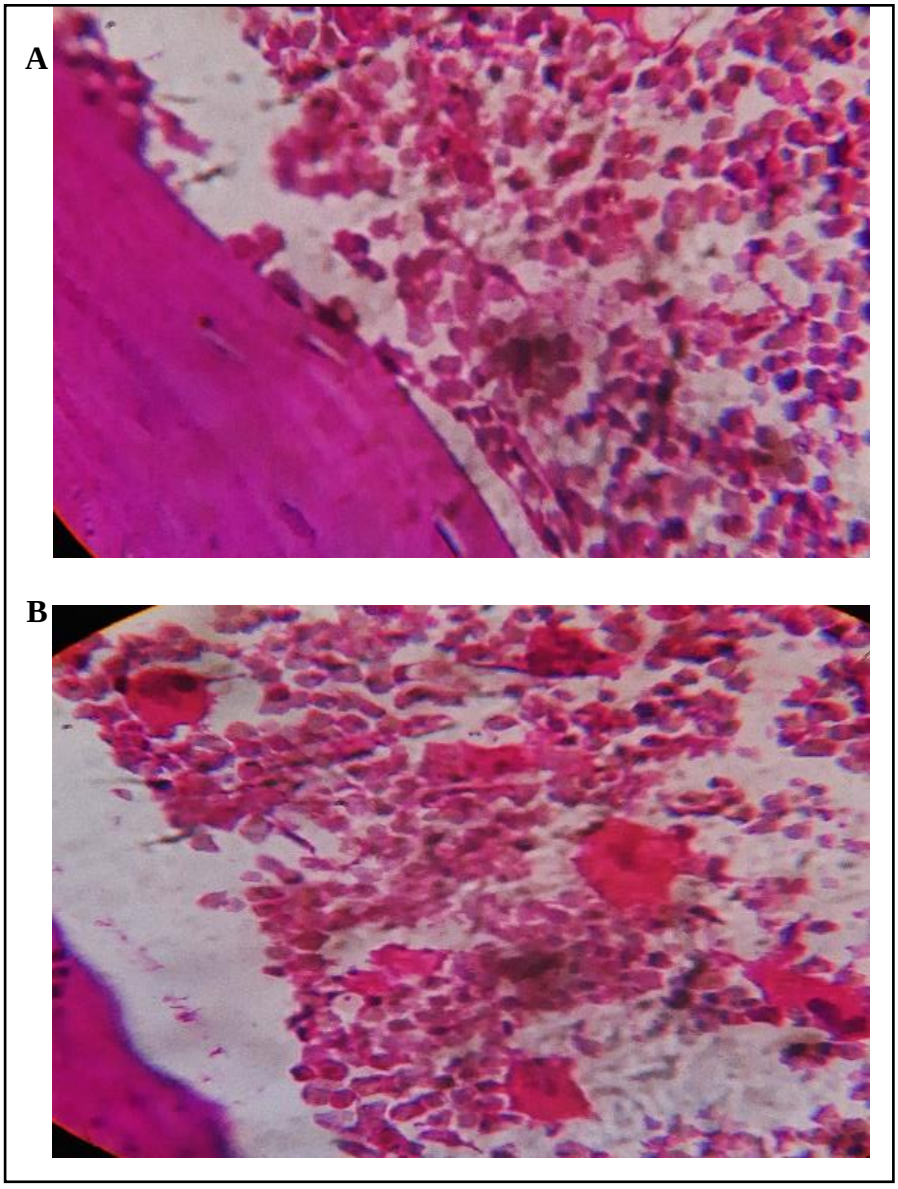


Figure 64. Histological assessment to identify inflammatory cells infiltrating in bone marrow. Toluidine Blue staining shows mast cell trafficking in the marrow section: (A) control, (B) leukemia (red arrow), (C) Extract treated (black arrow), (D) Wedelolactone treated (yellow arrow), and (E) graphical depiction of mean ± SD values of infiltrating mast cells [**P=0.003, ##P=0.008, ***P< 0.001].

Histological study was done on the bone marrow section by Toluidine blue staining. Leukemic section showed a significant increase in infiltrating mast cells (34% ± 0.03%, $p < 0.001$) as compared to control (3% ± 1.20%, $p < 0.001$). In contrast to leukemic section, treatment with extract (29% ± 2.30%, $p = 0.003$%) and wedelolactone (30% ± 0.20%, $p = 0.008$) showed moderate decline in the mast cell population (Figure 64 A - E). Highly proliferating macrophage population in leukemic marrow section (87% ± 0.57%, $p < 0.001$, compared to control: 11% ± 0.21%, $p < 0.001$) was spotted by regular H&E staining. Extract (81% ± 3.34%, $p < 0.001$) and wedelolactone (83% ± 0.77%, $p = 0.008$) treated groups showed significantly reduced macrophage cells (Figure 65 A – E).



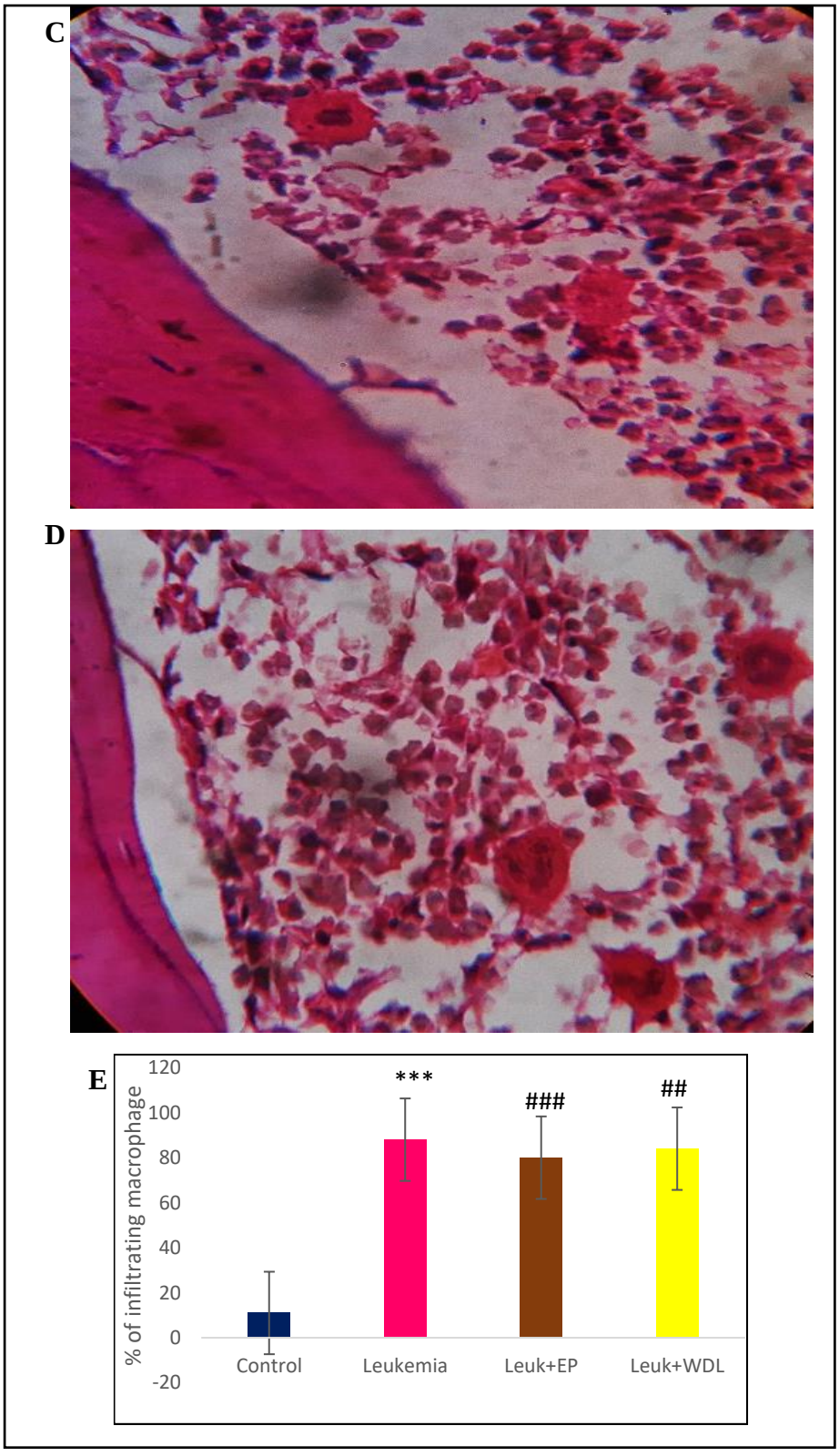


Figure 65. H&E staining shows infiltrating macrophage: (A) control, (B) leukemia, (C) Extract treated, (D) wedelolactone treated, and (H) graphical depiction of mean ± SD values of NSE positive cells in marrow of experimental groups [##P= 0.008, ***P< 0.001, ### P<0.001].

6.5.3. Eclipta alba and wedelolactone mediated inhibition on fluorescence expression of inflammatory pathway

Flowcytometric analysis confirmed an increase in the fluorescence expression of COX-2 by 1.87-folds in diseased group (MFI; 124.22 ± 0.69, $p < 0.001$) as compared with control (MFI; 66.13 ± 1.20) (Figure 66).

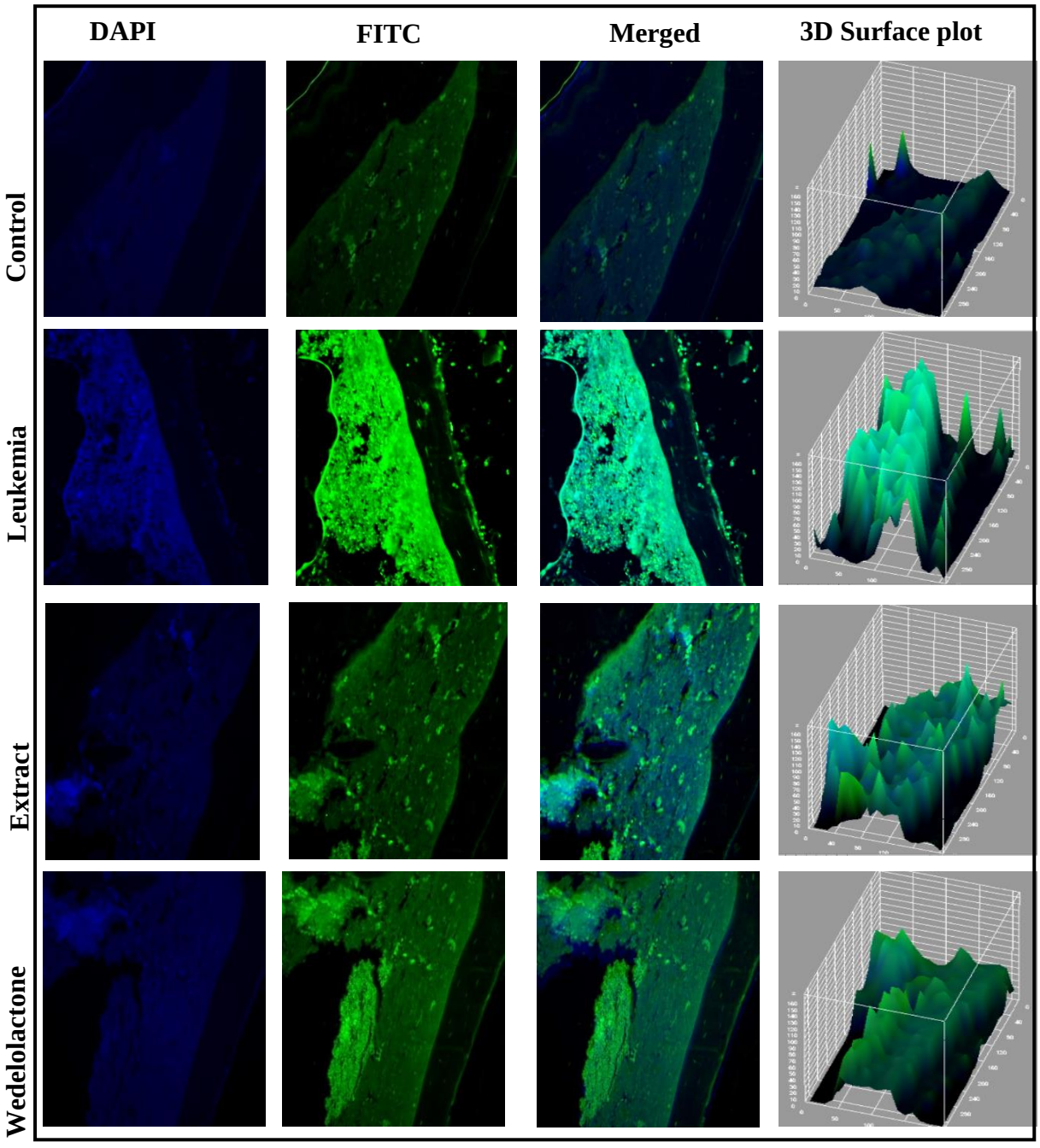


Figure 66. COX-2 expression in bone marrow cells. Fluorescence microscopy: Analysis of immunofluorescence of the bone marrow cells stained with anti-COX-2 antibody, cytoplasm

stained with FITC and nucleus is stained with DAPI of: (A) control, (B) leukemia, (C) extract treated, and (D) wedelolactone treated. The representative images were analyzed by 3D interactive surface plots (ImageJ) (Scale bar 100 µm) (n = 20).

Table 5. Mean Fluorescence Intensity (MFI) represents expression patterns of inflammatory target proteins

Proteins	MFI of control (X ± SD)	MFI of Leukemia (X ± SD)	MFI of Leukemia+ EA (X ± SD)	MFI of Leukemia+ WDL (X ±SD)
COX2	66.13± 1.20	124.22 ± 0.69***	69.07 ± 1.11###	112.0 ± 1.01###
iNOS	34.26 ± 1.40	629.32 ± 1.50***	104.24 ± 0.66###	176.11 ± 0.84###
PGE2	116.12 ± 1.18	275.32 ± 0.58***	118.13 ± 1.02###	121.18 ± 1.28###
CD11b	131 ± 0.68	656 ± 1.0***	133 ± 0.92###	117.23 ± 0.68###

*Note: Values are mean ± SEM for six animals in each observation, repeated thrice. ***p<0.001 significance of leukemia compared to control. ### p<0.001 significance of treated groups compared to leukemia*.

Target Proteins	**Control**	**Leukemia**	**Extract**	**Wedelolactone**
COX2				
iNOS				
PGE2				
CD11b				

Treatment with plant extract revealed a significant decline by 1.79-folds (MFI; 69.07 ± 1.11, $p < 0.001$), whereas COX-2 expression in wedelolactone treated group (MFI; 112 ± 1.001, $p < 0.001$) was down-regulated by 1.1-folds as compared to leukemia.

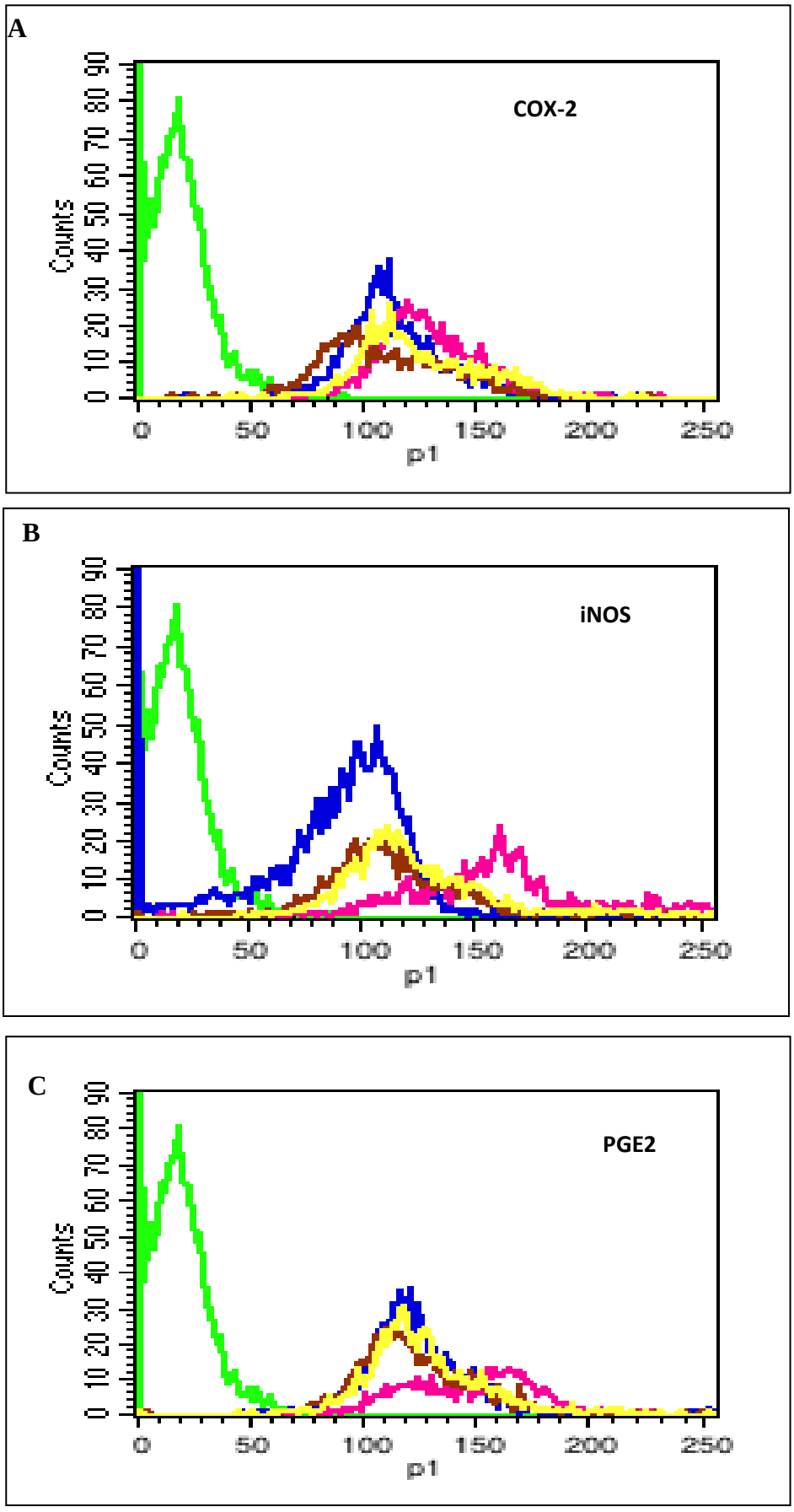
The immuno-histological study with COX-2 on bone marrow sections depicted significant difference in fluorescence intensity between leukemic and treated profiles (Figure 67 A). Fluorescence expression of iNOS was drastically high in leukemic condition (MFI; 629.32 ± 1.50, $p < 0.001$) by 18.36-folds compared to control (MFI; 34.26 ± 1.40).

Treatment with extract and wedelolactone significantly reduced iNOS expression by 6.03-folds (MFI; 104.24 ± 0.66, $p < 0.001$) and 3.57-folds (MFI; 176.11 ± 0.814, $p < 0.001$) respectively compared to leukemia (Figure 67 B).

PGE2 expression in leuekmia (MFI; 275.32 ± 0.58, $p < 0.001$) was increased by 2.37-folds compared to control (MFI; 116.12 ± 1.18, $p < 0.001$). Treatment with plant extract (MFI; 118.13 ± 1.02, $p < 0.001$) and wedelolactone (MFI; 121.18 ± 1.28, $p < 0.001$) significantly reduced PGE2 expression compared to diseased state by 2.33 and 2.27-folds respectively (Figure 67 C).

The expressional profile of CD11b maker showed an increase of 5-folds in leukemia (MFI; 656 ± 1, $p < 0.001$) compared to control (MFI; 131 ± 0.68).

Significant reduction of extract treated (MFI; 133 ± 0.92, $p < 0.001$) and wedelolactone group (MFI; 117.23 ± 0.68, $p < 0.001$) by 4.9 and 5.6-folds were observed (Figure 67 D).


A
COX-2
Counts
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0 50 100 150 200 250
p1
B
iNOS
Counts
0 10 20 30 40 50 60 70 80 90
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p1
C
PGE2
Counts
0 10 20 30 40 50 60 70 80 90
0 50 100 150 200 250
p1

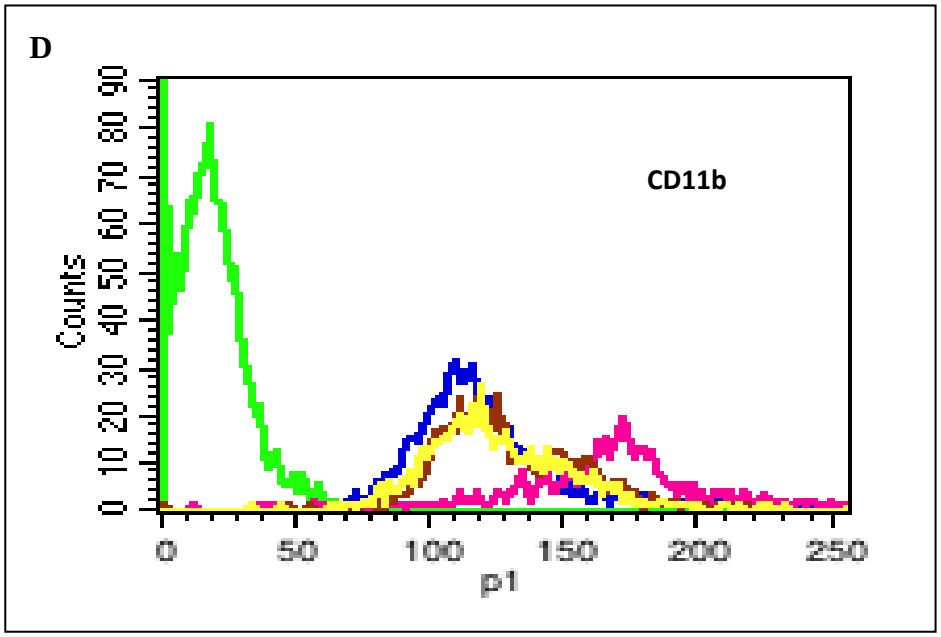
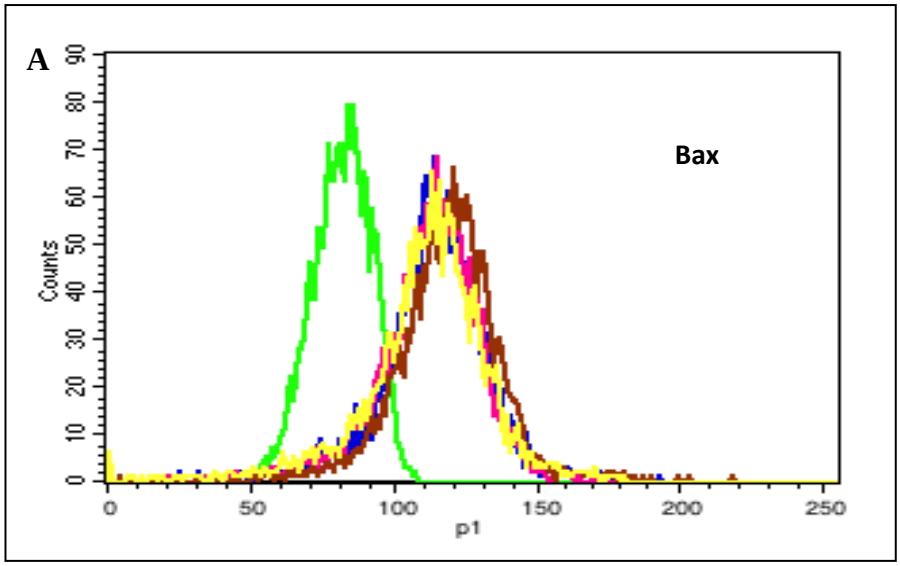


Figure 67. Immunomodulation of inflammatory molecules post-treatment with extract treated and wedelolactone treated group. Assessment of fluorescence expression by flowcytometry: Histogram overlays depicting MFI values of: (A) Cox-2, (B) iNOS, (C) PGE2, (D) CD-11b,

6.5.4. Assessment of extract and wedelolactone on apoptotic and anti-angiogenesis regulation of bone marrow cells

Fluorescence expression of Bax was down-regulated by 1.09-folds in leukemic condition (MFI; 64.09 ± 1.137, $p < 0.001$) as compared to control (MFI; 70.02 ± 0.97). Extract treated group showed an incline of 1.29-folds (83.01 ± 0.98, $p < 0.001$) and wedelolactone treated group showed an average increase in Bax expression by 0.82-folds (MFI; 68.14 ± 0.79, $p = 0.007$) compared to leukemia (Figure 68 A). Cytochrome c expression in leuekmia was down-regulated by 2.10-folds (MFI; 193.26 ± 1.40, $p < 0.001$) compared to control (MFI; 407.20 ± 1.06). Treatment with extract showed an increase of 4.22-folds (MFI; 816.20 ± 1.31, $p < 0.001$) and expression of wedelolactone group (MFI; 541.89 ± 1.64, $p < 0.001$) increased with 2.80-folds compared to leuekmia. (Figure 68 B). ASK1 expression was decreased in leukemia by

1.3-folds (MFI; 52.183 ± 1.28, p <0.001) in respect to control (MFI; 71.27 ± 0.63). Extract (MFI; 98.03 ± 1, p < 0.001) and wedelolactone treated group (MFI; 80.98 ± 0.97, p < 0.001) showed an increase by 1.8 and 1.5-folds respectively (Figure 68 C). Fluorescence expression of JNK in diseased condition was declined by 1.5-folds (MFI; 37.03 ± 1.06, p < 0.001) compared to control. An increase by 2.9-folds in extract treated group (MFI; 109.22 ± 0.69, p < 0.001) and by 2.8-folds in wedelolactone treated group (MFI; 107.30 ± 1.48, p < 0.001) was observed compared to leukemia (Figure 68 D). Bcl-xl was increased in leuekmia by 1.9-folds (MFI; 178.25 ± 0.65, P< 0.001) compared to control (MFI; 89.15 ± 1.23). Extract and wedelolactone groups showed a decreased expression by 1.6 (MFI; 106.09 ± 1.01, p < 0.001) and 1.5-folds (MFI; 118 ± 1; p < 0.001) respectively compared to leukemia (Figure 68 E. Caspase 3 expression in leukemic condition (MFI; 57.16 ± 1.25, p < 0.001) was declined by 1.23-folds compared to control (MFI; 70.31 ± 1.49). Extract treatment revealed an increase by 1.8-folds (MFI; 104.30 ± 1.47, p <0.001) and an increase by 1.29-folds in active compound treated group (74.29 ± 1.46, p < 0.001) compared to leukemia (Figure 68 F 2). E-cadherin fluorescence was decreased in leukemia (MFI; 43.27 ± 1.43, p < 0.001) by 1.5-folds as compared to control (MFI; 65.14 ± 0.79). *Eclipta alba* treated group (MFI; 78.13 ± 1.02, p < 0.001) revealed an increase by 1.8-folds and wedelolactone treatment (MFI; 46 ± 0.99) showed no significant change as compared to leuekmia (Figure 68 G). VEGF expression in leukemia (MFI; 164.16 ± 0.77, p <0.001) was up-regulated by 4.1-folds compared to control (MFI; 39.2 ± 1.05, p < 0.001) and decreased in extract treated (MFI; 61.21 ± 0.7, p <0.001) and wedelolactone treated group (MFI; 119.2 ± 1.42, p < 0.001) by 2.6-fold and 1.3-folds respectively (Figure 68 H).


A
Bax
Counts
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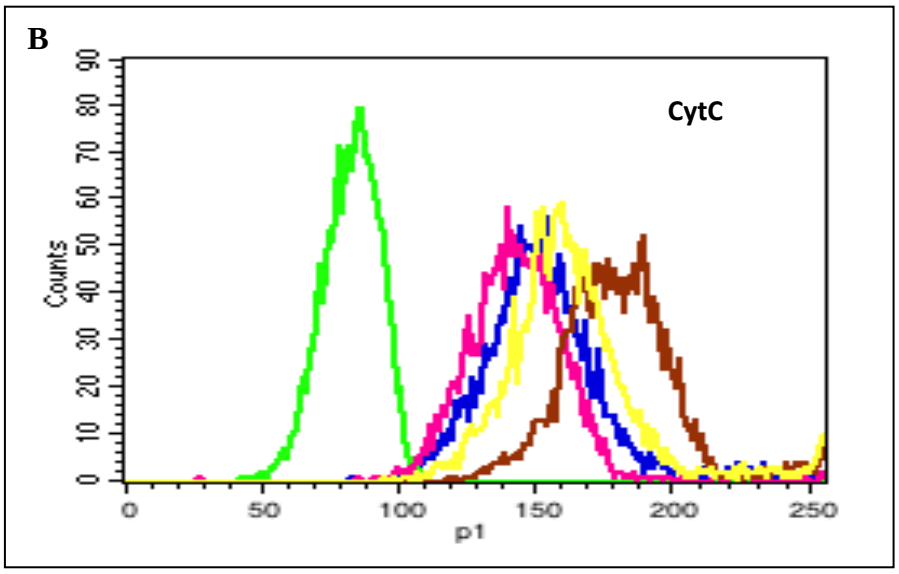
10
20
30
40
50
60
70
80
90
0
50
100
150
200
250
p1


B
CytC
Counts
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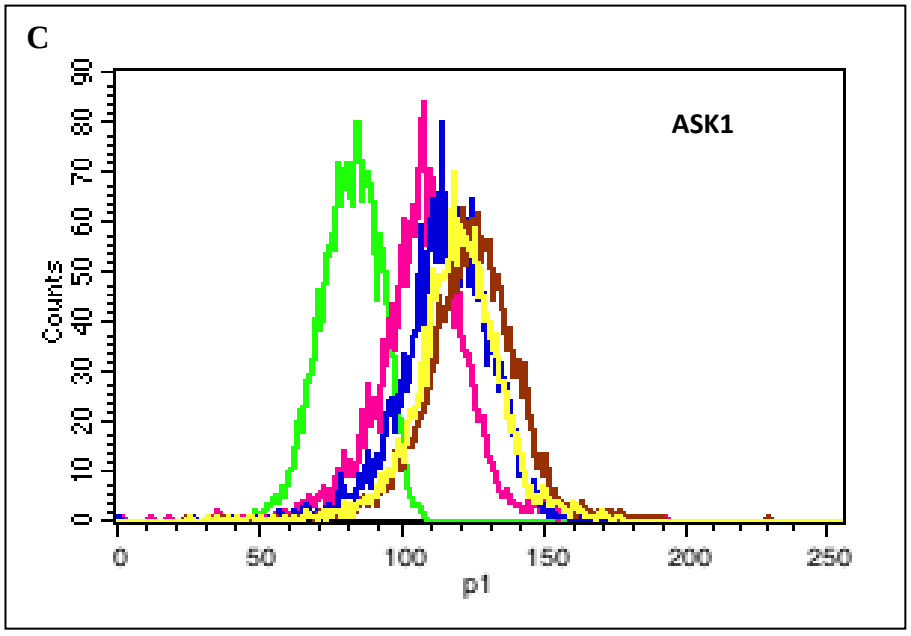
p1


C
ASK1
Counts
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20
30
40
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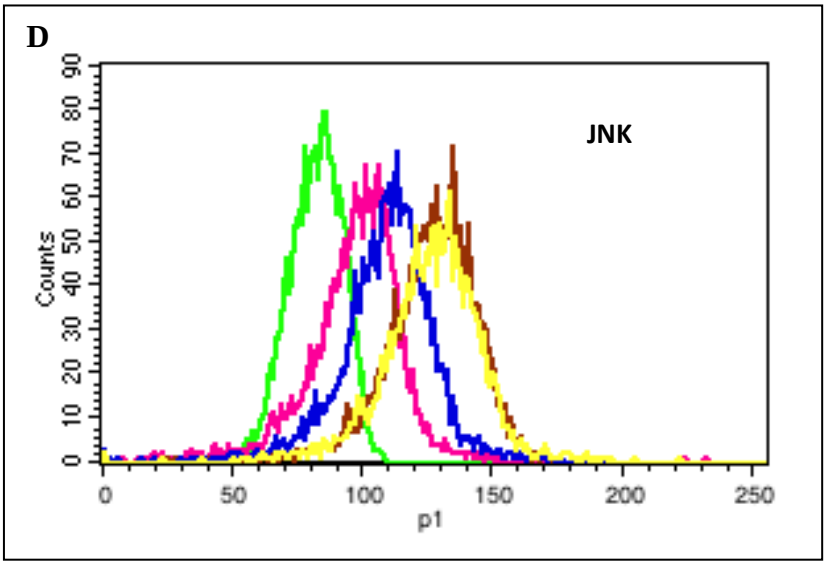
60
70
80
90
0
50
100
150
200
250
p1


D
JNK
Counts
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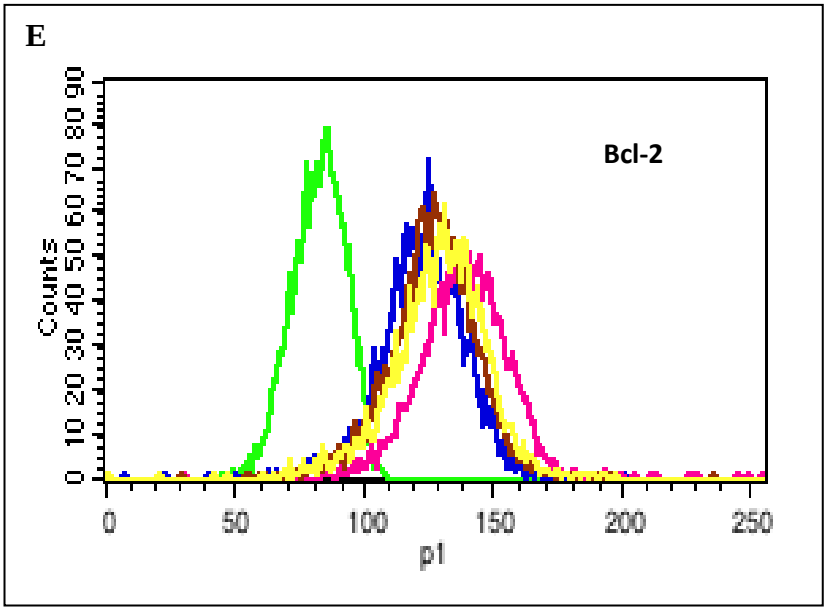
0 50 100 150 200 250
p1


E
Bcl-2
Counts
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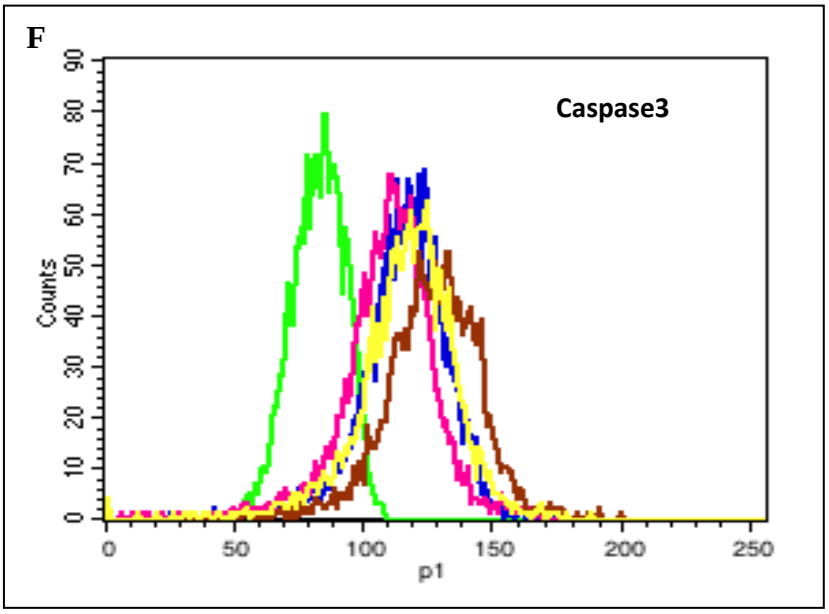
p1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Caspase3
Counts
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0 50 100 150 200 250
p1

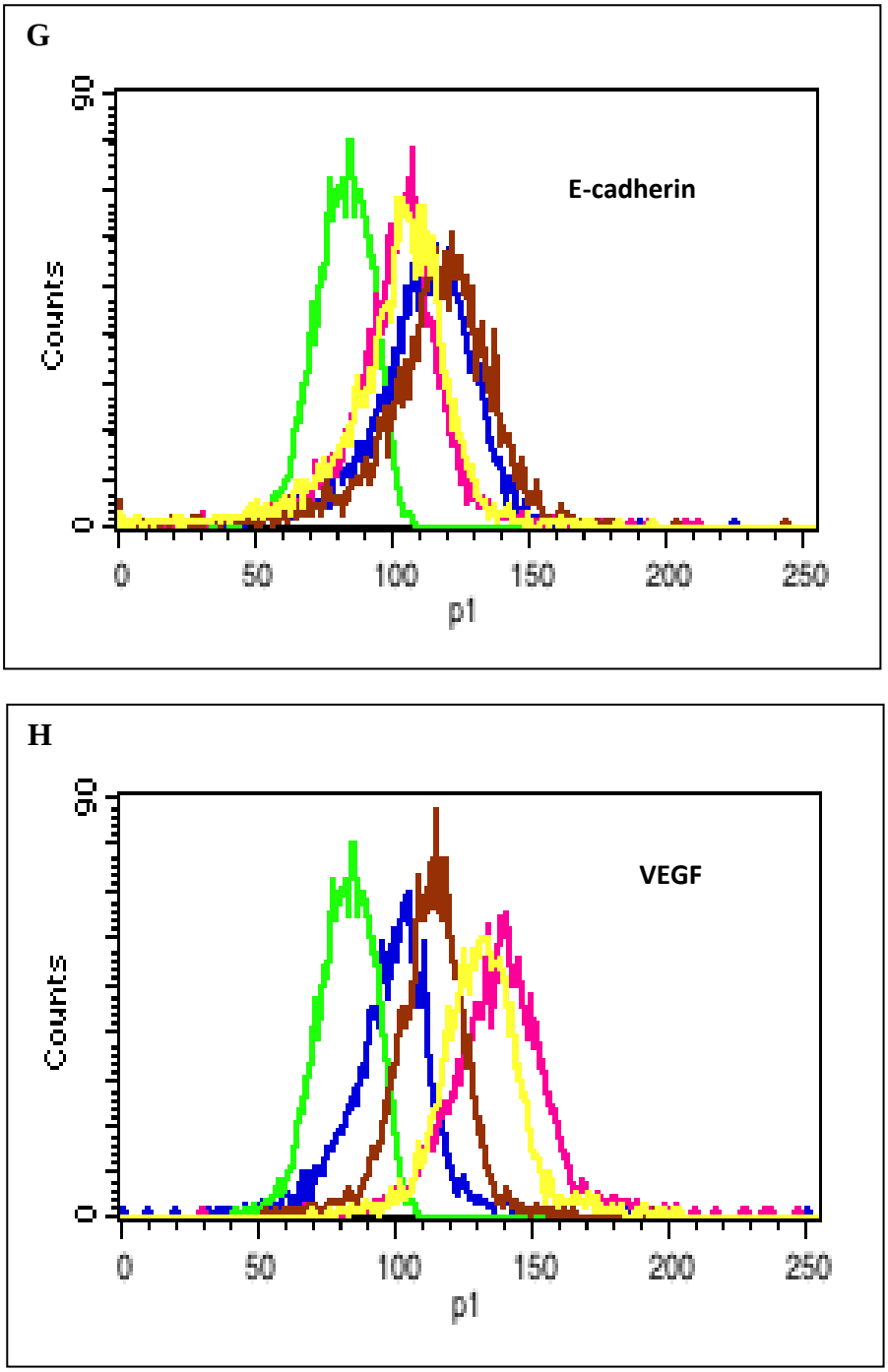


Figure 68. Immunomodulation of apoptotic and angiogenic molecules post-treatment with extract and wedelolactone. Assessment of fluorescence expression by flowcytometry: Histogram overlays depicting MFI values of: (A) Bax, (B) Cytochrome-c, (C) ASK1, (D) JNK, (E) Bcl-2, (F) Caspase-3, (G) E-cadherin, and (H) VEGF.

Table 6. Mean Fluorescence Intensity (MFI) represents expression patterns of apoptotic target proteins

Proteins	MFI of control (X ± SD)	MFI of Leukemia (X ± SD)	MFI of Leukemia + EP (X ± SD)	MFI of Leuekmia + WDL (X ± SD)
Bax	70.02 ± 0.97	64.09 ± 1.13***	83.01 ± 0.98###	68.14 ± 0.79##
Cytochrome c	407.20 ± 1.06	193.26 ± 1.40***	816.20± 1.31###	541.89 ± 1.64###
JNK	58.2 ± 0.72	37.07 ± 1.06***	109.22 ± 0.69###	107.30 ± 1.48###
ASK1	71.27 ± 0.63	52.18 ± 1.28***	98.03 ± 1###	80.98 ± 0.97###
Caspase 3	70.31 ± 1.49	57.16 ± 1.25***	104.3 ± 1.47###	74.29 ± 1.46###
Bcl-xl	89.15 ± 1.23	178.25 ± 0.65***	106.09 ± 1.01###	118 ± 1###

*Note: Values are mean ± SEM for six animals in each observation, repeated thrice. ***p<0.001 significance of leukemia compared to control. ### p<0.001, ##p= 0.0071 significance treated group as compared to leukemia.*

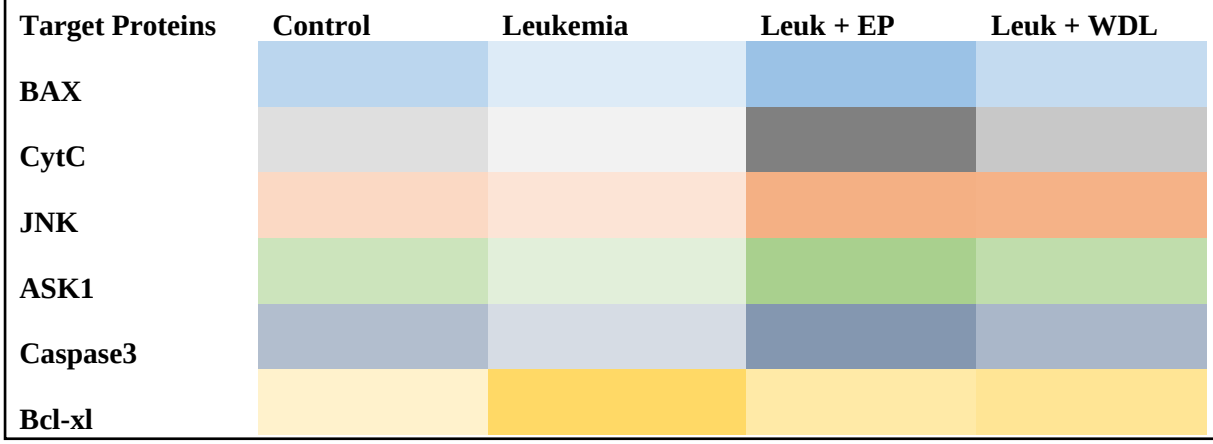
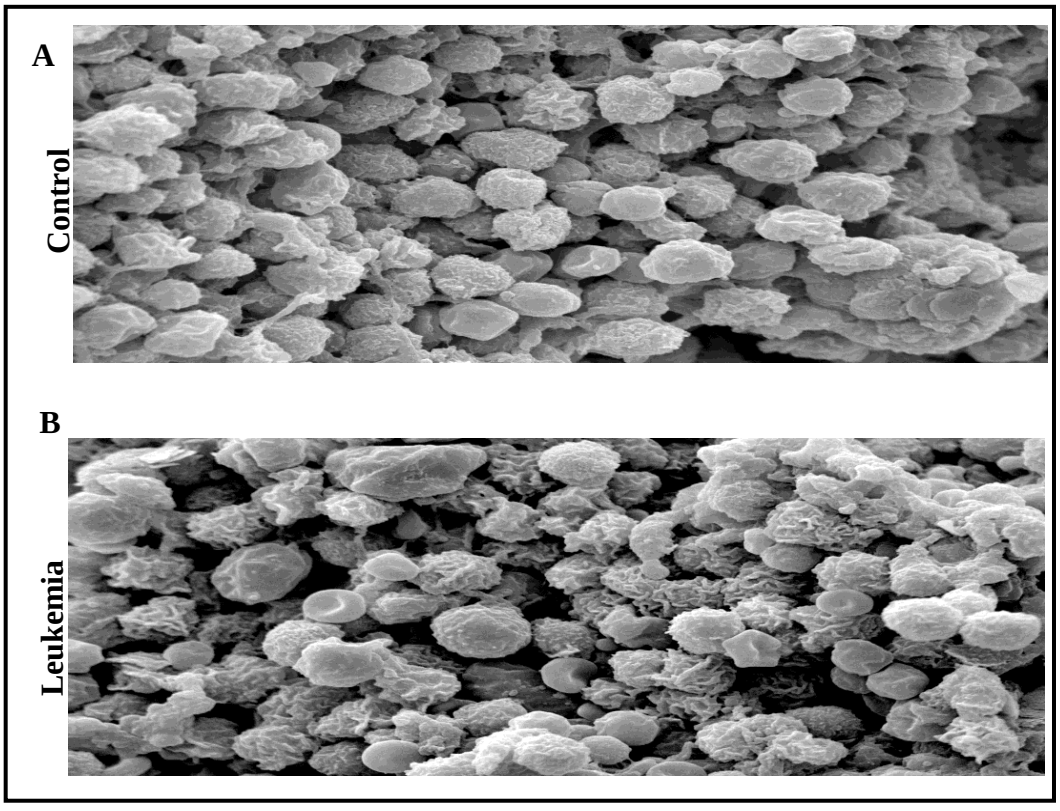


Table 7. Mean Fluorescence Intensity (MFI) represents expression patterns of angiogenic target proteins

Proteins	MFI of control (X ± SD)	MFI of Leukemia (X ± SD)	MFI of Leukemia+ EP (X ± SD)	MFI of Leukemia+ WDL (X ±SD)
E-CAD	65.14± 0.79	43.27 ± 1.43***	78.13 ± 1.02###	46 ± 0.99
VEGF	39.2 ± 1.05	164.16 ± 0.77***	61.21 ± 0.70###	119.2 ± 1.42###

*Note: Values are mean ± SEM for six animals in each observation, repeated thrice. ***p<0.001 significance of leukemia compared to control. ### p<0.001 significance of treated groups compared to leukemia*.

6.5.5. Effect of treatment on the changes of stromal microenvironment in leukemia



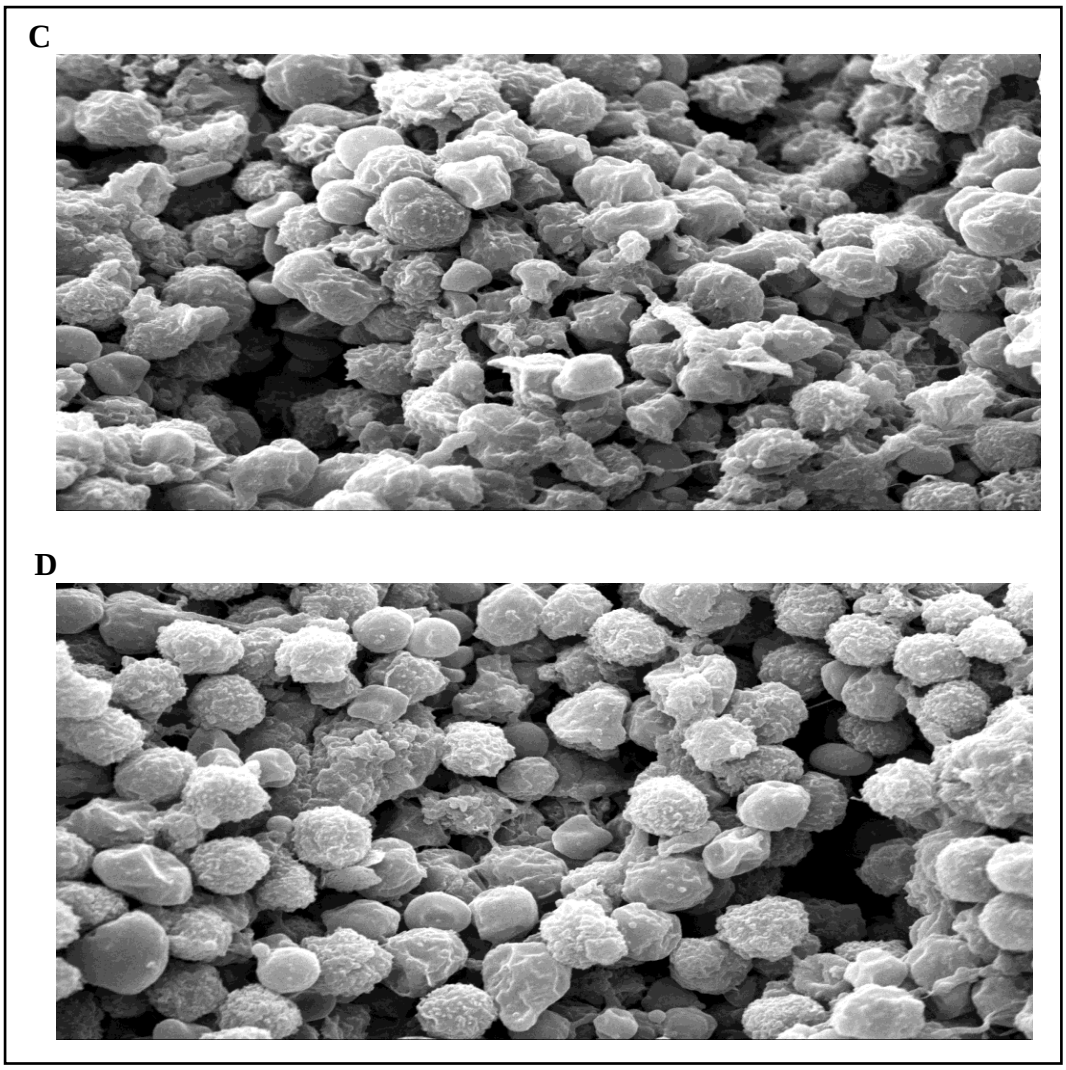


Figure 69. Scanning electron microscopy: The scanning electron microscopic picture of (A) control, (B) leukemia, (C) extract treated, and (D) wedelolactone treated groups depicted the micro-environmental scenario of marrow cells with increased surface ridges and ruffles. (Mag: 30KX, Scale bar 200 nm) (n = 20).

The scanning electron micrographs revealed the microenvironmental scenario of the marrow cells from experimental groups (Figure 69 A-D). An increase in the surface ridges and ruffles on abnormal cells were observed in the leuekemic group. The marrow cells from ENU-treated leukemic group showed discontinuous stromal adherence compared to treated groups. The population of abnormal cells with discontinuous surfaces was moderately decreased in *Eclipta alba* and wedelolactone treatment groups.

6.6. Discussion

Environmental exposures to NOC's can lead to several human cancers and in the current study we have used ENU, a chemical belonging to the NOC family, for inducing leukemia in mice model. Leukemia is the most common type of cancer worldwide and the use of natural compounds that shows anti-cancer properties have been the backbone of cancer research for past 30 years (Chandramohan Reddy et al., 2012; Saedi et al., 2014; Subhashini et al., 2004). Our previous findings delineated that *Eclipta alba* and wedelolactone can ameliorate leukemic condition by its anti-proliferative effects; down-regulating NLRP3 and NFkB expression in the bone marrow cells, suggesting it as a potential anti-inflammatory therapy (Bhattacharyya and Law, 2022, 2021). However, the complete mechanism underlying the anti-inflammatory effect have not been fully studied. Thus, through our present investigation we aimed to elucidate the underlying anti-inflammatory mechanism of extract and wedelolactone and its apoptotic role on the bone marrow cells of ENU- induced leukemic mice.

Growing evidences reveals that macrophage infiltrates the leukemic inflammatory microenvironment, participates in leukemogenesis by gaining certain phenotypes and correlates with poor prognosis. Corresponding to which, we observed a sharp increase in mononuclear phagocyte and myeoblast population in ENU-induced leuekemia and its treatment with active compound showed better amelioration than crude extract. Supporting the evidence, a significant decrease in myeloid cells expressing CD11b marker was observed post both the treatment.

The connection of macrophage with inflammation is well known as its aberrant activation leads to an overproduction of the inflammatory mediators, nitric oxide (NO) and PGE2 (Nakagawa et al., 2012; Yu et al., 2012). Hence to establish an anti-inflammatory agent, it is necessary to study the suppression of NO and PGE2 via iNOS and COX-2 respectively. Although the

inhibitory role of *Eclipta alba* and wedelolactone on iNOS/COX-2 mediated PGE2 expression is reported in RAW 264.7 cell line, its anti-inflammatory mechanism in haematological malignancy has never been explored (Ryu et al., 2013; Tewtrakul et al., 2011; Yuan et al., 2013). In line with the evidence, we observed an increased expression of COX-2, PGE2 and iNOS in leukemia (Bernard et al., 2008; Giles et al., 2002). High levels of Cox-2 and PGE2 enhances angiogenesis and increasing evidences suggests the co-dependency of inflammation and angiogenesis are the leading cause of cancer progression (Pelus and Hoggatt, 2011; Rizzo, 2011). The over-expression of the potent angiogenic mediator VEGF, in diseased state corresponds to vascular hyper-permeability by allowing the inflammatory cells to be recruited to the site of cancer by promoting angiogenesis (Song et al., 2012). Although both the treated group showed significant decrease in the expressional profile of these major inflammatory mediator and VEGF, we noted a compelling effect of extract treated group in comparison to wedelolactone. Post-treatment with *Eclipta alba* significantly up-regulated E-cadherin expression, the key player of metastasis suppression, although wedelolactone showed no significant difference. The treatments were also effective in mitigating mast cell trafficking in bone marrow tissue.

Natural compounds are well reported to induce apoptosis by exhibiting cytotoxicity specifically towards cancer cells, which provides novel opportunities for cancer drug development (Fulda, 2010). Leukemic marrow cells with pleomorphic microvilli on surface were detected by SEM and we have observed a slight decrease in the population of cells with extensive surface projections post-treatment. Cytochrome c not only serves as a tumor marker for leukemia but also monitors the potency of an anti-cancer drug (Osaka et al., 2006). ASK 1 activates JNK which signals the pro-apoptotic member Bax, to induce apoptosis by the mitochondrial release of cytochrome c that consecutively activates caspase-3 cascade, whereas the anti-apoptotic member Bcl-xl prevents the same (Cory and Adams, 2002). In the current study, we observed

Eclipta alba and wedelolactone are able to activate ASK 1 induced apoptosis by significantly up-regulating downstream proteins JNK, Bax and caspase-3 and down-regulating Bcl-xl fluorescence expression hence modulating the release of cytochrome c. Taken together, our study could elaborate the underlying anti-inflammatory mechanism and pro-apoptotic effects of *Eclipta alba* and wedelolactone on leukemia.

6.7. Conclusion

In conclusion, our results demonstrated that both extract and active compund showed anti-inflammatory role by down-regulation of COX-2/PGE2/iNOS pathway and pro-apoptotic role via mitochondrial-mediated pathway by the up-regulation of cytochrome c release in the bone marrow cells of ENU-induced leukemic mice.

General Discussion

General discussion & conclusion

Environmental exposure to various carcinogenic compounds has increased with the increasing civilization**.** N-nitroso compounds (NOCs) are such carcinogens present in our environment through various food sources like nitrate cured meat and smoked fish, known to cause different types of human cancers (Mirvish 1995; Eichholzer and Gutzwiller 1998; Badawi 2000; Keszei et al. 2013; Jain et al. 2020) Apart from food sources, NOCs are also found in the environment through tobacco smoke and various manufacturing industries of rubber, pesticides, fertilizers, and tyres (Haorah et al. 2001; Descatha et al. 2005). There are several reports of NOCs causing different kinds of cancer among the human population like pancreatic cancer, gastric cancer, and gastrointestinal cancer (Pignatelli et al. 1993; Zheng et al. 2019).

N-N' ethylnitrosourea (ENU) is a potentially carcinogenic chemical, which belongs to the family of NOC and it is reported for the development of different animal models of cancers like lymphoma, brain cancer, and breast cancer (Hadjiolov 1972; Moser et al. 1995; Slikker et al. 2004). In this research, a single intra-peritoneal injection of ENU *(80 mg/kg body weight)* has been induced on Swiss albino litter pups aging between 10 to 11 days, to develop leukemia in around 6 to 8 months (Basak, Chatterjee, P. Das, et al. 2010; Basak, Chatterjee, M. Das, et al. 2010; Chatterjee, Chattopadhyay, and Law 2016; Chatterjee, Chattopadhyay, Sanyal, et al. 2016; Chattopadhyay et al. 2019; Bhattacharyya and Law 2022). Leukemia is a type of blood cancer that is marked by a heterogeneous population of neoplasms, with a higher incidence mostly in young adults (Chang et al. 2012; Ariffin et al. 2017; Bawazir et al. 2019). In this present research, the increase in the leucocyte and blast cell population in the peripheral blood, as well as bone marrow of leukemic mice group has been documented, which correlates with the increase in inflammation in the bone marrow microenvironment. Carcinogenesis and inflammation is tightly interlinked as it supports one another and leukemia is reported as an

"inflammation-driven-malignancy" under various circumstances (Grivennikov et al. 2011; Maffei et al. 2013; Récher 2021; Puissant and Medyouf 2022). In this research, the increased expression of various inflammatory molecules in the bone marrow cells of ENU-induced leukemic mice model has been explored, which correlates with enhanced leukemogenesis (Bernard et al. 2008; Mansouri et al. 2016; Jia et al. 2017).

Although several chemotherapeutic drugs and anti-leukemic therapy has advanced by modern medicine, still the toxic side effects remain the major drawback. Hence, one of the potential worldwide scientific research is to explore natural anti-cancer drugs specifically through plant-based products, because of their lesser side effects, lesser toxicity, more availability, and cheaper price (Nam 2006; Fulda 2010; Cragg and Newman 2013). In several developing and under-developed countries there is still a practice of using herbal therapy for leukemia (Saedi et al. 2014). In this research, an Ayurvedic medicinal plant called *Eclipta alba* is explored for its ameliorating anti-cancer potentials on the bone marrow of ENU-induced leukemia. *Eclipta alba* is reported for its anti-inflammatory and anti-cancer potentials on different disease models and no study was previously carried out to find its effect on any haematological model (Rama Parmar et al. 2010; Chaudhary et al. 2011; Chaudhary et al. 2014; Liao et al. 2018). The principal active compound present in the alcoholic extract of the herb is wedelolactone, which is reported as a "IKK-inhibitor", which suppresses the activation of the NF-kB inflammatory pathway, a hallmark of cancer (Bremner and Heinrich 2002; Gilmore and Herscovitch 2006; Idris et al. 2009). Although this is a novel and first study on the efficacy of wedelolactone on leukemia, but literature review suggests wedelolactone as an anti-cancer and anti-inflammatory compound in different cancers (Liu et al. 2014; Chen et al. 2015; Feng et al. 2019). In the present study, wedelolactone was successfully isolated from an ethanolic extract of *Eclipta alba* using chromatography and ESI-MS direct infusion technique. The leaf extract (*1200*

mg/kg body weight) and wedelolactone (*50 mg/kg body weight*) were individually used as therapeutic treatments on ENU-induced leukemic mice, via oral gavage for constant 4 months.

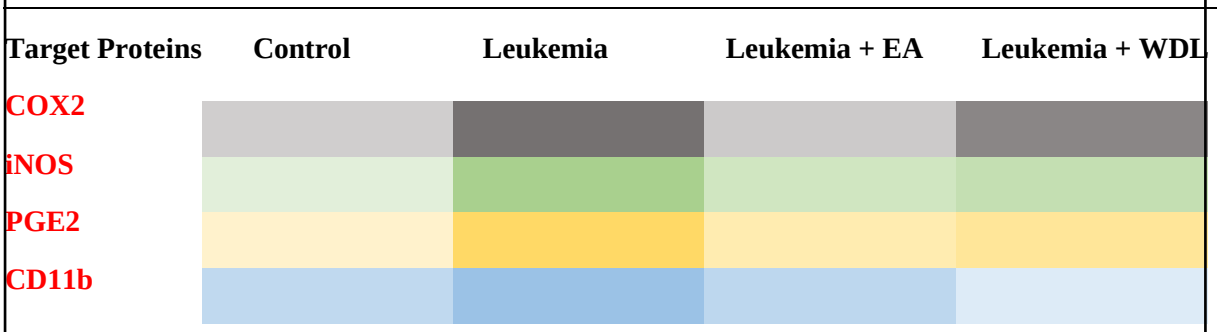
The bone marrow cells from the control group, leukemic group, *Eclipta alba* extract treated group and wedelolactone treated group were isolated and the expression of different inflammatory molecules were studied. NF-kB is one of the major inflammatory signaling pathway which increases the expression of IL-1β, a pro-inflammatory cytokine and its aberrant stimulation allows the pathogenesis and maintenance of leukemic blood cells, hence establishing NF-kB as an interesting therapeutic target (Mansouri et al. 2016; Hemmati et al. 2017; Carter et al. 2019). In the basic preliminary research, *Eclipta alba* and wedelolactone not only significantly ameliorated the physical characteristics of leukemia like survivability and body weight but also reduced the excessive infiltration of inflammatory markers like ring shaped neutrophils, mast cells, and leucocytes from bone marrow as well as peripheral blood. Different cytological staining confirmed the decrease in blast cells post treatment with extract and active compound. Detailed study delineated that *Eclipta alba* and wedelolactone decreased the expression of the proteins in the canonical NF-kB signaling pathway. Constant activation of the NF-kB signaling acts as a stimulus for the expression of the underlying inflammatory COX-2 expression (Evans and Kargman 2004). In the bone marrow cells of ENU-induced leukemia, COX-2/iNOS/PGE2 signaling axis was up-regulated, due to the high levels of NF-kB. *Eclipta alba* and wedelolactone treatment significantly reduced the expression of COX-2 pathway in the leukemic bone marrow cells, hence once again delineating its anti-inflammatory properties. Increased inflammation is a cause of NLRP3 inflammasome complex activation, which or else remains in its inactivated state in the cytosol (Jo et al. 2015; Kyung et al. 2021). Once stimulated the NLRP3 forms an activated complex which in turn activates caspase-1 that further activates the down-stream IL-1β. In ENU-induced leukemia, the expression of NLRP3 inflammasome and hence IL-1β is excessively and uncontrollably higher but after the

therapeutic treatments, a significant reduction in the NLRP3 inflammasome complex has been observed. Apart from the inflammatory scenario, the ENU-induced diseased bone marrow cells revealed excessive proliferation which is also a common feature of leukemia. The treatments with herbal extract and active compound expressed anti-proliferative potency on the bone marrow, which was confirmed by trans-membrane assay, long-term bone marrow cell culture and GM-CSF expression in the marrow cells. Proliferation of abnormal bone marrow cells are because of loss of cell death, and post-treatment by *Eclipta alba* and wedelolactone it was confirmed that they shows a pro-apoptotic role by inducing cell death through mitochondrial pathway mediated through cytochrome c release.

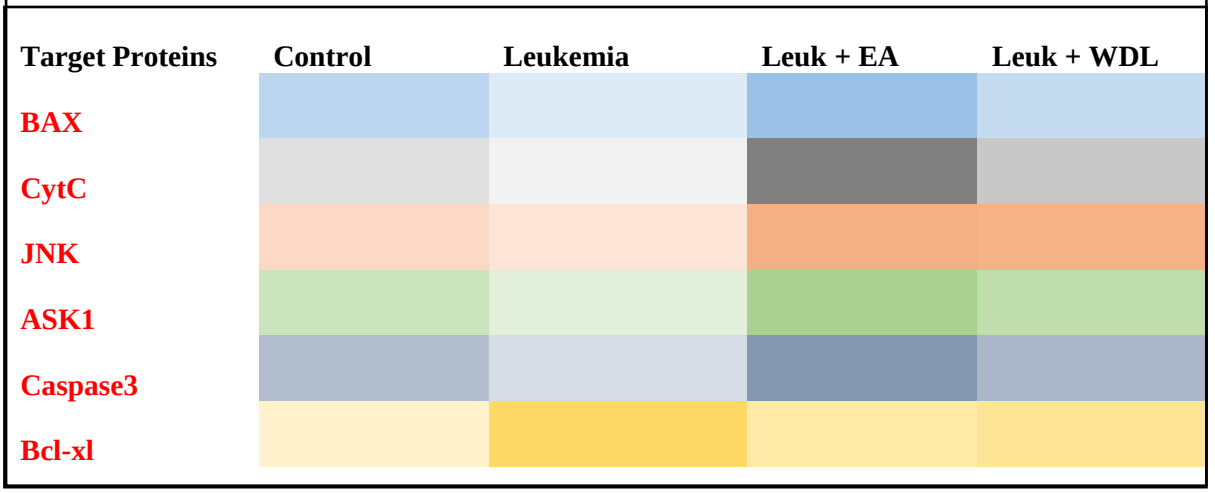
In conclusion, it can be stated that *Eclipta alba* ethanolic extract and its isolated active compound wedelolactone plays an ameliorating role in the bone marrow cells of ENU-induced leukemia by down-regulation of NF-kB signaling pathway, COX-2 inflammatory pathway, and NLRP3 inflammasome pathway. The individual treatments also plays a major anti-proliferative role on leukemic cells which confirms both of them as potential therapeutic candidates for leukemic research. Although taken together, the data suggests the whole extract to have higher ameliorating effects on the bone marrow cells of ENU-induced leukemia as compared to the active compound treatment alone, probably due to the presence of a concoction consisting the benefits of various active compounds in the whole extract.

Statistics

Proteins	MFI of control (X ± SD)	MFI of Leukemia (X ± SD)	MFI of Leukemia+ EA (X ± SD)	MFI of Leukemia+ WDL (X ±SD)
COX2	66.13± 1.20	124.22 ± 0.69 **(p<0.001)**	69.07 ± 1.11**(p<0.001)**	112.0 ± 1.01**(p<0.001)**
iNOS	34.26 ± 1.40	629.32 ± 1.50 **(p<0.001)**	104.24 ± 0.66**(p<0.001)**	176.11 ± 0.84**(p<0.001)**
PGE2	116.12 ± 1.18	275.32 ± 0.58 **(p<0.001)**	118.13 ± 1.02**(p<0.001)**	121.18 ± 1.28**(p<0.001)**
CD11b	131 ± 0.68	656 ± 1.0 **(p<0.001)**	133 ± 0.92**(p<0.001)**	117.23 ± 0.68**(p<0.001)**

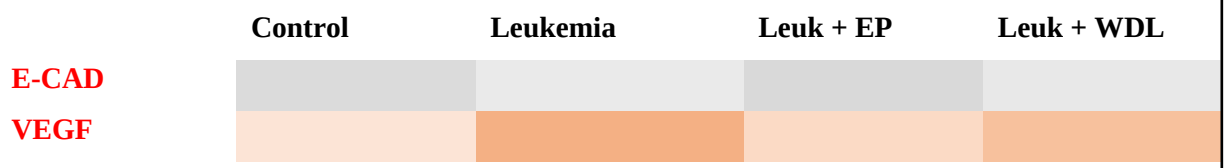


Proteins	MFI of control (X ± SD)	MFI of Leukemia (X ± SD)	MFI of Leukemia + EA (X ± SD)	MFI of Leuekmia + WDL (X ± SD)
Bax	70.02 ± 0.97	64.09 ± 1.13**(p<0.001)**	83.01 ± 0.98**(p<0.001)**	68.14 ± 0.79**(p=0.0071)**
Cytochrome c	407.20 ± 1.06	193.26 ± 1.40**(p<0.001)**	816.20± 1.31**(p<0.001)**	541.89 ± 1.64**(p<0.001)**
JNK	58.2 ± 0.72	37.07 ± 1.06**(p<0.001)**	109.22 ± 0.69**(p<0.001)**	107.30 ± 1.48**(p<0.001)**
ASK1	71.27 ± 0.63	52.18 ± 1.28**(p<0.001)**	98.03 ± 1**(p<0.001)**	80.98 ± 0.97**(p<0.001)**
Caspase 3	70.31 ± 1.49	57.16 ± 1.25**(p<0.001)**	104.3 ± 1.47**(p<0.001)**	74.29 ± 1.46**(p<0.001)**
Bcl-xl	89.15 ± 1.23	178.25 ± 0.65**(p<0.001)**	106.09 ± 1.01**(p<0.001)**	118 ± 1**(p<0.001)**



Proteins	MFI of control (X ± SD)	MFI of Leukemia (X ± SD)	MFI of Leukemia+ EA (X ± SD)	MFI of Leukemia+ WDL (X ±SD)
E-CAD	65.14± 0.79	43.27 ± 1.43**(p<0.001)**	78.13 ± 1.02**(p<0.001)**	46 ± 0.99(**NS**)
VEGF	39.2 ± 1.05	164.16 ± 0.77**(p<0.001)**	61.21 ± 0.70**(p<0.001)**	119.2 ± 1.42**(p<0.001)**

Note: NS= Non-significant



Note: Each experiment repeated 3 times, EA=Eclipta alba, WDL= Wedelolactone, NS=Non-significant.

Proteins	MFI of control (X ± SD)	MFI of Leukemia (X ± SD)	MFI of EA Treated (X ± SD)	MFI of WDL Treated (X ±SD)
NLRP3	20.40 ± 0.90	81.00 ± 0.66**(p<0.001)**	64.22 ± 0.58**(p<0.001)**	30.45 ± 0.75**(p<0.001)**
ASC	58.24 ± 1.97	125.79 ± 1.58**(p<0.001)**	116.30 ± 0.85**(p<0.001)**	118.56 ± 1.25**(p=0.001)**
Cl-caspase-1	36.34 ± 0.95	68.54 ± 0.50**(p<0.001)**	52.5 ± 1.80**(p<0.001)**	62.92 ± 1.015**(p=0.02)**
Cl-IL-1β	44.53 ± 0.68	143.09 ± 0.86**(p<0.001)**	77.73 ± 0.92**(p<0.001)**	87.15 ± 1.23**(p<0.001)**
GM-CSF	41.92 ± 0.96	62.19 ± 0.73**(p<0.001)**	49.80 ± 0.88**(p<0.001)**	51.19 ± 1.30**(p<0.001)**

Proteins	MFI of control (X ± SD)	MFI of Leukemia (X ± SD)	MFI of EA (X ± SD)	MFI of WDL (X ± SD)
cRel	79.77 ± 0.68	141.48 ± 0.50(**p<0.001)**	122.79 ± 0.71(**p<0.001)**	134 ± 0.91(**p<0.001)**
p65	57.93 ± 0.90	99.22 ± 0.69(**p<0.001)**	87.89 ± 0.84(**p<0.001)**	90.44 ± 0.66(**p<0.01)**
p50	62.86 ± 0.80	88.50 ± 0.50(**p<0.001)**	66.43 ± 0.51(**p<0.001)**	75 ± 0.87(**p<0.001)**
IL-1β	80.96 ± 0.94	102.30 ± 0.60(**p<0.001)**	98.49 ± 1.31**(p<0.001)**	98.8 ± 0.45(**p<0.001)**
IkBα	128.46 ± 0.50	84.74 ± 0.65(**p<0.001)**	90.26 ± 0.64(**p<0.001)**	88.7 ± 0.56(**p=0.02)**
IKKα	92.88 ± 0.83	115.46 ± 0.46**(p<0.05)**	99.70 ± 0.95(N.S)	101.4 ± 0.89(**p<0.001)**
IKKγ	64.28 ± 0.28	97.92 ± 0.07(**p<0.001)**	70.73 ± 0.64(**p<0.001)**	87.88 ± 0.23(**p<0.001)**
IKKβ	74.69 ± 0.59	138.01 ± 0.07(**p<0.001)**	122.32 ± 0.32(**p<0.001)**	129.45± 0.23(**p<0.05)**
RelB	14.44 ± 0.41	28.80 ± 0.25(**p<0.001)**	28.94 ± 0.05(N.S)	28.0 ± 0.44(**p<0.001)**

Parameters	**Control (X ± SD)**	**Leukemia (X ± SD)**	**EA Treated (X ± SD)**	**WDL Treated (X ± SD)**
WBC (x 10^3/mm^3)	7.58 ± 0.54	36.17 ± 0.60**(p<0.001)**	25.49 ± 1.25**(p<0.001)**	30.40 ± 0.41**(p<0.001)**
RBC (×10^6/ mm^3)	6.89 ± 0.78	10.94 ± 0.30**(p<0.001)**	9.58 ± 0.42**(p=0.006)**	10.57 ± 0.32**(p<0.001)**
Neutrophils (%)	16.37 ± 1.10	44.87 ± 0.85**(p<0.001)**	36.12 ± 0.92**(p<0.001)**	40.43 ± 0.42**(p<0.001)**
Reticulocyte (%)	0.54 ± 0.18	1.93 ± 0.031**(p<0.001)**	1.71 ± 0.04**(p=0.005)**	1.86 ± 0.018**(p=0.028)**
Blasts (%)	----	37.225 ± 0.85**(p<0.001)**	31.25 ± 1.70**(p<0.001)**	34.13 ± 0.31**(p=0.005)**

Note: Each experiment repeated 3 times, EA=Eclipta alba, WDL= Wedelolactone, NS=Non-significant.

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List of publications

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Environmental pollutant ENU induced leukemic NF-kB signaling amelioration by *Eclipta alba* in murine model

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ABSTRACT

Exposure to N-nitroso compounds (NOCs) in our environment via pesticides, tobacco, and smoked meat can be potentially carcinogenic. The induction of N-N′ ethylnitrosourea (ENU), a genotoxic NOC, leads to leukemogenesis. The study aimed to explore the ameliorating effect of the Ayurvedic herb *Eclipta alba* on the bone marrow cells of ENU-induced leukemic mice. *Eclipta alba* is investigated for its anti-cancer effect on various cell lines, but never on haematological malignant models. Theefficacy of the extract was explored on leukemia by changes in body weight, survivability, peripheral blood hemogram, bone marrow cytological, histological, and cell culture studies pre-and post-treatment. The treated group revealed significant immunomodulation of the expressional profile of NF-kB family and IL-1β in marrow cells, by flow-cytometry, and immunofluorescence study. Through our experimental endeavour we depicted the cellular mechanism, signaling modality and tried to establish the anti-cancer potency of *Eclipta alba* on ENU-induced leukemia.

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N-N′ ethylnitrosourea; leukemia; *Eclipta alba*; ayurveda

Introduction

The carcinogenic potentials of N-nitroso compounds (NOCs) and their primary exposure from the environment via tobacco, pesticides and smoked meat are well established (Haorah et al. 2001). Neonatal exposure to N-N' ethylnitrosourea (ENU) a NOC leads to carcinogenesis and leukemic progression (Capilla-Gonzalez et al. 2012; Chattopadhyay et al. 2019). Approximately, 300,000 cases of leukemia are diagnosed every year globally and it is characterized by a heterogeneous population of neoplasm arising from the malignant transformation of the hematopoietic stem/progenitor cells (HSPCs) (Chattopadhyay et al. 2019). In Leukaemia, the bone marrow niches mainly supported leukemogenesis instead of normal hematopoiesis (Basak et al. 2010a). The nuclear factor kappa B (NF-kB) family members play a major role in regulating carcinogenesis and inflammatory response. Mutations resulting in the aberrant expression of NF-kB pathway contribute towards leukemic transformation, hence, supporting the role of NF-kB as a 'hallmark of cancer' (Zhou et al. 2015). Various medicinal plants have been mentioned as inhibitors of NF-kB activation, portraying its multi-disciplinary approach (Bremner and Heinrich 2002).

Medicinal plants contain physiologically active phytochemicals that have been exploited for their therapeutic benefits against cancer, in Indian Ayurveda and Traditional medicine (Yadav et al. 2017a). For millennia, Mother Nature has been the armamentarium of traditional plant-derived chemotherapeutic drugs. Epidemiological studies have shown ample evidences of one of the traditional ethnomedicinal herb *Eclipta alba*, as an anti-cancer agent on several types of

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cancer (Chaudhary et al. 2014; Arya et al. 2015a; Yadav et al. 2017a). *Eclipta alba* (L.) (syn. *Eclipta prostrata* (L.) L.Hassk.) of the family Compositae is an annual herbaceous plant and is regarded as a weed of ethnomedicinal significance. It is a traditional folk medicinal plant, commonly known as Bhringaraja or Kesharaja in Ayurveda, Kadim-el-bint in Arabian medicine (Chaudhary et al. 2011), and Mo-Han-Lian in traditional Chinese medicine (Liu et al. 2014). *Eclipta alba* shows immunomodulatory, anti-cancer (Lirdprapamongkol et al. 2008; Arya et al. 2015b; Yadav et al. 2017a) anti-inflammatory and antitumor properties (Jahan et al. 2014). *Eclipta alba* has been evaluated in *in-vivo* hepatocellular carcinoma model, to suppress NF-kB as a potent multidrug resistance (MDR) agent (Chaudhary et al. 2014). Further, *Eclipta alba* is reported for its anti-proliferative effects by inhibition of NF-kB on HepG2, A498, and C6 cell lines (Chaudhary et al. 2011). The major active compound present, wedelocatone inhibits NF-kB pathway in RAW 264.7 cells (Yuan et al. 2013) and is responsible to suppress osteoclastogenesis by modulating NF-kB/c-fos/NFATc1 pathway (Liu et al. 2016) and also reported to directly degrade the $I_kB\alpha$ complex in BALBc/3T3 cells (Kobori et al. 2004).

Although various studies suggest the convincing potential of *Ecliptaalba* to be an adjunctive agent for chemotherapy, its possibilities on modulating NF-kB pathway in the bone marrow have not been investigated yet. So, the present study aimed to analyse the role of *Eclipta alba* to modulate NF-kB pathway and inhibit leukemogenesis in the bone marrow of ENU-induced leukemic mice.

Materials and methods

Plant Material

Freshly dried *Eclipta alba* leaves were obtained from the Narendrapur Ramakrishna Mission Ashrama, Medicinal Plant Garden, India in July 2019 and the sample was authenticated by Prof Jukta Adhikari, Professor of Botany Department, Barasat Government College, West Bengal, India. Specimen sample has been submitted at Calcutta School of Tropical Medicine, Kolkata, India with voucher no. 756870.

Preparation of Eclipta alba *extract*

The dried leaves were crushed into a coarse powder and extracted in ethanol for 24 h followed by double filtration using 25 mm Grade 1 Whatman filter paper (Sigma, USA) under sterile conditions. The solvent was thoroughly removed and the extracts were concentrated at 50°C by rotary evaporator. The filtrate was reconstituted in double-distilled water and stored at −20°C until further use (Yadav et al. 2017a).

Characterization of Eclipta alba *extract*

HPLC-grade acetonitrile was purchased from Merck (Germany), deionized water was purified by Milli-Q system (Millipore, USA) and the standard wedelolactone was purchased from Sigma-Aldrich (USA), with purity over 98%. Chromatographic analysis was performed using Shimadzu Prominence HPLC (Model No. CBM-20A, Japan) with LC-20AT pump, SIL-20AT autosampler, and SPD-M20A detector and equipped with Discovery C18 column (ODS, 25 cm × 4.6 mm, 5 µm, Supelco, USA) and Guard column (Security Guard Cartridge system, Phenomenex, USA). The mobile phase was composed of acetonitrile: water (ACN%, v/v) at a flow rate of 1 ml/min and programmed as followed: 20–45% (for 15 min); 45–100% (for 30 min), and 100 (for 40 min). The injection volume was 10 µl at a detection wavelength of 365 nm.

Animal maintenance

Inbred Swiss albino mice were maintained under controlled room temperature (22 ± 2)°C at the animal house of the Calcutta School of Tropical Medicine (India). The animals were fed a standard diet and water *ad libitum*, under sterile conditions with a 12-h light-dark period in consent with the authorized guidelines of the Institutional Animal Ethical Committee (IAEC), and EU Directive 2010/63/EU also according to The Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India (Registration number: 681/GO/Re/S/02/CPCSEA).

ENU-induced leukemia

Litter pups aging 10–14 days weighing 4–5 g were prepared with a single intra-peritoneal injection of N-N' ethylnitrosourea (ENU, Sigma, USA; 80 mg/kg) to develop the leukemic condition in 6–8 months and confirmed by blood hemogram as described previously (Law et al. 2001, 2003; Basak et al. 2010a, 2010a; Chatterjee et al. 2016, 2016a; Chattopadhyay et al. 2019).

Therapeutic dosimetry and animal grouping

In a study by Yadav et al., alcoholic extract of *Eclipta alba* was reported for its anti-cancer activity and showed no toxicity up to 2000 mg/kg body weight (Yadav et al. 2017b). Similarly, Singh et al. twice reported that no acute toxicity of *Eclipta alba* was observed up to 2000 mg/kg (Singh et al. 2013, 2014). Furthermore, another study reported no acute toxicity of the alcoholic extract at dose 2000 mg/kg orally for 48 h on CCl4 induced mice model (Singh et al. 1993).

Following the previous literature surveys, we tested the effect of *Eclipta alba* extract on the experimental mice model at different doses of 1000 mg/kg; 1100 mg/kg; 1200 mg/kg; 1300 mg/kg, and 1400 mg/kg via oral gavage and the control group received a standard diet. Based on the pilot study, the treatment dose was standardised at 1200 mg/kg body weight via oral gavage for 4 weeks (Jahan et al. 2014). According to the experimental plan, three groups (n = 30) of animals were maintained: Group I: Leukemic mice; Group II: Leukemic mice treated with *Eclipta alba* extract (*1200 mg/kg*) via oral gavage for 4 weeks; Group III: Control group of mice received an equal volume of saline in similar condition.

Survivability and body weight

Survivability of leukemic mice, post treatment with *Eclipta alba* is an indicator of anti-cancer activity of the extract. This was carried out by comparing the number of days the animals survived during the treatment, as compared to the diseased and control groups. Furthermore, to assess the effect of the treatment on animal body weight, the final weights of the mice were estimated.

Bone marrow isolation and single cell preparation

The long bones were dissected aseptically and bone marrow cells were flushed out into RPMI-1640 media (Sigma, USA) using a sterile 26 gauge needle. Followed by repeated pipetting, cells were made to pass through a 100 µm cell strainer and washed thrice in chilled RPMI-1640 media to remove debris and to procure single cell suspension as described previously (Chatterjee et al. 2016b).

Disease characterization before and after drug administration

Three hundred microliter of peripheral blood was obtained by tail-vein puncture and differential leukocyte count (DC), total leukocyte count (TC), reticulocyte count and haemoglobin estimation were performed. Blood and bone marrow smears were also stained with Leishman (Himedia, India)

to observe changes in cellular morphology (Daw et al. 2019). Marrow smears were examined by Myeloperoxidase staining (MPO) after fixation in Buffered Formal Acetone and treatment with Sorenson buffer for 20–25 minutes (Chatterjee et al. 2016a). Smears were counterstained with Giemsa and photographed under light microscope (Olympus, Ch20i, Japan). Sudan black B (SBB) staining was also performed by placing fixed marrow smears in the working solution and phenol buffer for 1 h, followed by three changes in 70% alcohol and a final tap water wash. Smears were counterstained with Leishman stain and observed under light microscope (Daw et al. 2016).

Histology of femur

From the experimental groups, femurs were surgically isolated and fixed in 10% neutral buffered formalin for 24 h. After the decalcification of the bones in 10% formic acid, it was gradually passed through the ascending grades of alcohol and finally embedded in paraffin. Five micrometer bone marrow sections were cut and stained with routine Hematoxylin and Eosin (HE), and observed for the histopathological changes under the light microscope (Olympus CH20i, Japan) (Chatterjee et al. 2016b).

Short term bone marrow culture

Bone marrow cells (1×10^6 cells/ml) were suspended in 75 mm culture plates (Corning, New York) with 4 ml of RPMI-1640 (Sigma, USA) media, supplemented with 30% FBS (Fetal Bovine Serum, Lonza, USA) and maintained at 37°C in 4% CO_2 humidified chamber. The cultures were observed at 0 h, 24 h, 48 h, and 72 h under inverted microscope at 40X magnification as described previously (Basak et al. 2010a).

Flowcytometric analysis

For the analysis of intra-cellular proteins, bone marrow cells (2×10^6 cells/ml) from control, diseased and treated groups were fixed with 1.5% paraformaldehyde (PFA) for 15 minutes at 37°C in dark. Cells were washed twice and treated with chilled methanol for membrane internalization at 4°C for 20 minutes. Cells were distributed in polystyrene tubes and stained with 2 µL of primary antibodies (Cell signalling technology, USA) against p65, cRel, p50, I_KBα, IKKβ, IKKγ, IKKα, IL-1B and RelB and were incubated for 30 minutes at 37°C. Finally, cells were counterstained with secondary antibody conjugated with Alexa flour 488 (Invitrogen, USA) and tubes were then again incubated in the dark at 37°C for 30 min. Finally, the cells were washed in PBS and analyzed with a BD FACS Calibur flowcytometer (Becton Dickinson, USA) using CellQuest Pro software (v9.1 Becton Dickinson).

Fluorescence imaging

Bone marrow cells prepared for flowcytometric analysis of anti p65, anti cRel, anti p50, anti I_KBα, anti IKKβ, anti IKKγ, anti IKKα, anti IL-1B and anti RelB proteins (Cell Signaling Technologies, USA) were smeared on slide and mounted using DAPI shield (Sigma, USA). The signals were recorded photographically using a fluorescence microscope, Leica DM8 and photographs were analyzed by Leica DX software.

Statistical Analysis

Statistical analysis was performed using unpaired Student's t-test and One-way ANOVA by post-hoc testing (Tukey's Test). All the quantitative data were shown as Mean ± Standard deviation (SD). For every comparison, $P \leq 0.05$ was considered significantly significant. Each experiment was repeated thrice.

Results

HPLC analysis of Eclipta alba *extract and identification of active compound*

Wedelolactone is one of the principal constituents of *Eclipta alba* and also the presence of other phytochemicals like dimethyl-wedelolactone, desmethyl-wedelolactone-7 glucoside, and saponins are majorly responsible for the antitumor effects of the extract (Jadhav et al. 2009; Chaudhary et al. 2011; Han et al. 2015). HPLC analysis was performed to illustrate the ethanol extract of *Eclipta alba* (Figure 1A). The chromatogram recorded at 365 nm revealed several peaks of which wedelolactone was identified at the retention time of 17.141.

Effects of Eclipta alba *on body weight, survivability and peripheral blood hemogram on ENU induced leukemic mice*

In leukemic group, an excessive decrease in body weight (11.33 ± 0.763 g) and physical appearance was noticed compared to control (31.66 ± 1.04 g; $P = 0.001$), hence indicating a strong side effect of the ENU induced leukemic mice. Conversely, slight positive changes in the body weight and physical condition was observed post treatment (16.15 ± 1.20 g; $P = 0.06$; Figure 1B). Increased survivability (Leukemia s Treated: 15.5 ± 1.29 vs 23 ± 2.16; $P < 0.001$) of treated group, as compared to the leukemic group hinted towards the preliminary signs of the anti-cancer effect of the extract (Figure 1C). Blood hemogram depicted significant leucocytosis in leukemic mice (32.14 ± 0.79 × 10^3 cells/mm^3, $P < 0.001$) compared to control (7.66 ± 0.60 × 10^3 cells/mm^3). Marked decrease by 1.3-fold in WBC count of treated group (24.06 ± 2.53 × 10^3 cells/mm^3; $P = 0.002$) compared to leukemic mice was observed while confirming the preliminary effects of *Eclipta alba* (Figure 2C i–iv). Red blood cell (RBC) count and reticulocyte count was significantly elevated in leukemic group (11.08 ± 0.62 × 10^6 cells/mm^3; $P < 0.001$ and 1.94 ± 0.03%; $P < 0.001$) compared to control (7.71 ± 0.33 × 10^6 cells/mm^3 and 0.573 ± 0 .219%). Moreover, *Eclipta alba* significantly decreased both RBC and reticulocyte count (9.58 ± 0.52 × 10^6 cells/mm^3; $P = 0.026$ and 1.58 ± 0.07%; $P = 0.043$) compared to leukemic group by 1.1 and 1.2-fold, respectively. Hemoglobin content was increased in Leukemic group (19.42 ± 0.38 g/dl; $P < 0.001$) compared to control (16.16 ± 0.52 g/dl). Significant decrease was observed in treated group (18.21 ± 0.20 g/dl; $P = 0.022$) compared to leukemic mice. Furthermore, a sharp rise in blast percentage was depicted in the blood film study of leukemic group (36.83 ± 1.607%), which significantly declined by 1.2-fold in treated group (30.66 ± 1.527%; $P = 0.009$), hence reinforcing the therapeutic potential of *Eclipta alba* extract. Hematological analysis revealed the occurrence of abnormal blasts, hypo-lobulated ring neutrophils and infiltration of dysplastic neutrophils in leukemic blood smear (Figure 2A i–v) indicative of leukemic onset. Peripheral blood count was regarded as the initial predictive tool to ascertain leukemic progression post treatment with *Eclipta alba*.

Hematopoietic changes in leukemic condition induced by Eclipta alba *extract*

Bone marrow and peripheral blood scenarios depicted the aggressive assault on normal hematopoietic physiology in leukemic condition. Marked increase of blast burden in leukemic marrow smears was confirmed by cytopathological study (Figure 2B i–vi). The bone marrow sections of leukemic animals showed typical infiltration of blast cells and abnormality in the bone marrow architecture as compared to control (Figure 2D i,ii). Leukemic bone marrow section revealed an unusual positioning of blast cells, also termed as Abnormal localization of immature precursors (ALIP) (Figure 2D iii). No exclusive improvement was observed in the architecture of the marrow sections, but a considerable decrease of blasts was observed in the treated group (Figure 2D iv). Cytochemical analysis with MPO staining of marrow cells was indicative of the excessive increment in MPO positive cells (azurophilic granules and auer

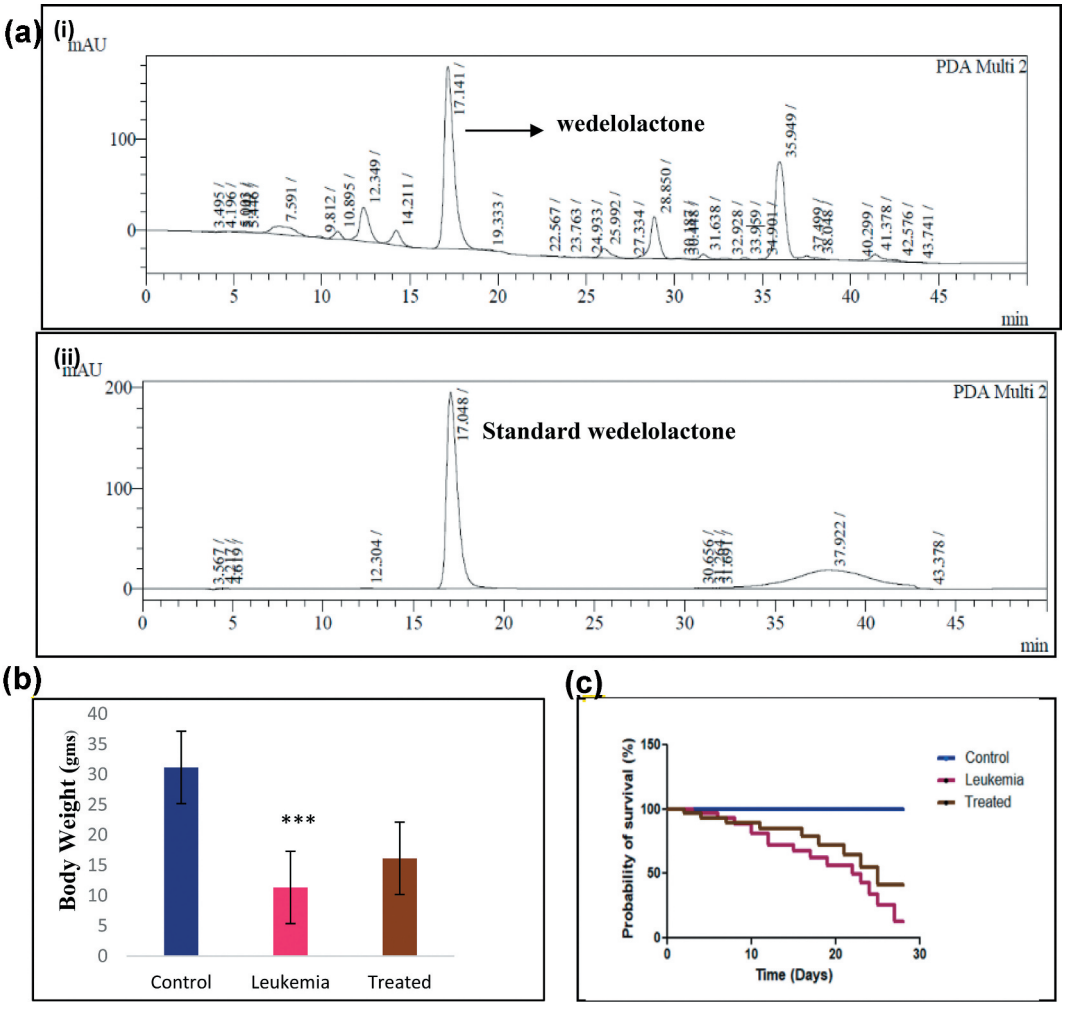

Figure 1. (A) Characterization of *Eclipta alba*: (i) HPLC chromatogram of *Eclipta alba* extract at 365 nm, (ii) Standard Wedelolactone. (B) Effect of *Eclipta alba* on bodyweight: The graph depicts significant changes in body weight pre and posttreatment with extract (***$P < 0.001$; Values are Mean ± SEM, t-test). (C) Effect of extract on the survivability: Kaplan-Meier curve comparing the survivability post treatment of experimental animals.

rods containing myeloblasts) in leukemic condition (Figure 3A ii, iv) compared to control (Figure 3A i). Notable decline in MPO positive population (Leukemia versus Treated = 66.67 ± 3.21 versus 57.67 ± 2.52; $P = 0.018$) was revealed post treatment with *Eclipta alba* (Figure 3A iii, v). Leukemic cells also revealed strongly SBB positive cells which further confirmed the presence of abnormal myeoblasts (Figure 3B ii) compared to control (Figure 3B i). Significant decrease of SBB stained population (Leukemia versus Treated = 68.67 ± 3.06 versus 55.33 ± 5.51; P = 0.02) was studied in treated group (Figure 3B iii, iv). Furthermore, *in vitro* short-term bone marrow cell culture study was conducted in our endeavours to study the efficacy of *Eclipta alba* on hematopoietic cell proliferation and treated cells proliferated at a significantly slower rate compared to leukemic group which showed rapid cell growth at 48 h (Figure 3C xi). By 72 h, the bone marrow culture of the leukemic population showed excessive proliferation and a clear indication of spindle shaped stromal precursor generation, which was considerably less in the treated population (Figure 3C viii,xii).

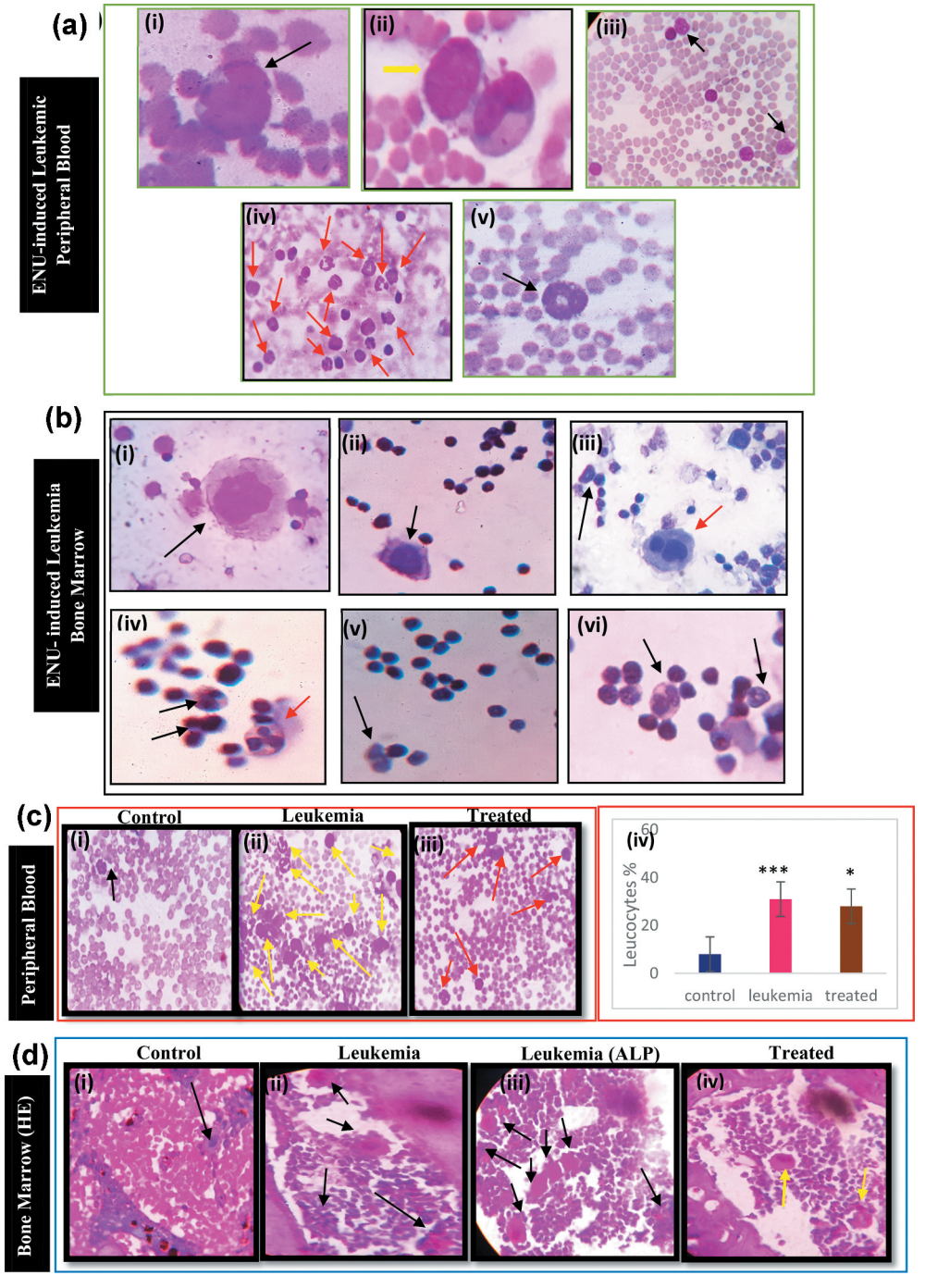

Figure 2. Pathological features of experimental leukemic mice before and after treatment with *Eclipta alba* extract. (A) Peripheral blood smear before treatment: Morphologically distinct leukemic blasts were identified in the (i–v) peripheral blood smear (Leishman stained). (i) Highly immature polymorphic blast (black arrow), (ii) abnormal myeloblast (yellow arrow), (iii) abnormal lymphoblast (black arrow), (iv) significant leukocytosis and excessive infiltration of dysplastic neutrophils (red arrow) and (v) immature hypo-lobulated ring neutrophil (black arrow). (B) Bone Marrow smear before treatment: Giemsa stained leukemic marrow smear revealed (i, ii) abnormal megakaryoblast (black arrow), (iii) hypo-lobulated megakaryocyte (red arrow), (iv) fan shaped nuclei (red arrow) and (v, vi) increase in blast burden. (C) Representative comparison of peripheral blood smear of pre and post *Eclipta alba* treatment.(i) Control (black arrow), (ii) Leukemic peripheral blood (yellow arrow) showed significant leukocytosis neutrophil infiltration as compared to (iii) treated mice (red arrow). (iv) Graphical representation of the decrease in leukocytosis after treatment with *Eclipta alba*. (D) Hematoxylin and Eosin stain: Histopathological study revealed normal cellular architecture of marrow cells in (i) control and (ii) leukemic condition depicted infiltration of blasts and (iii) ALIP. (iv) Treated marrow showed decline in blast population. [$^{**}P = 0.002$, $^{***}P < 0.001$, Values are Mean ± SEM,t-test]. [Mag: (a i,ii,iv,v) and (b i–vi and d i–iv) 1,000 x, (a iii and c i–iii) 400x]. (Color figure online).

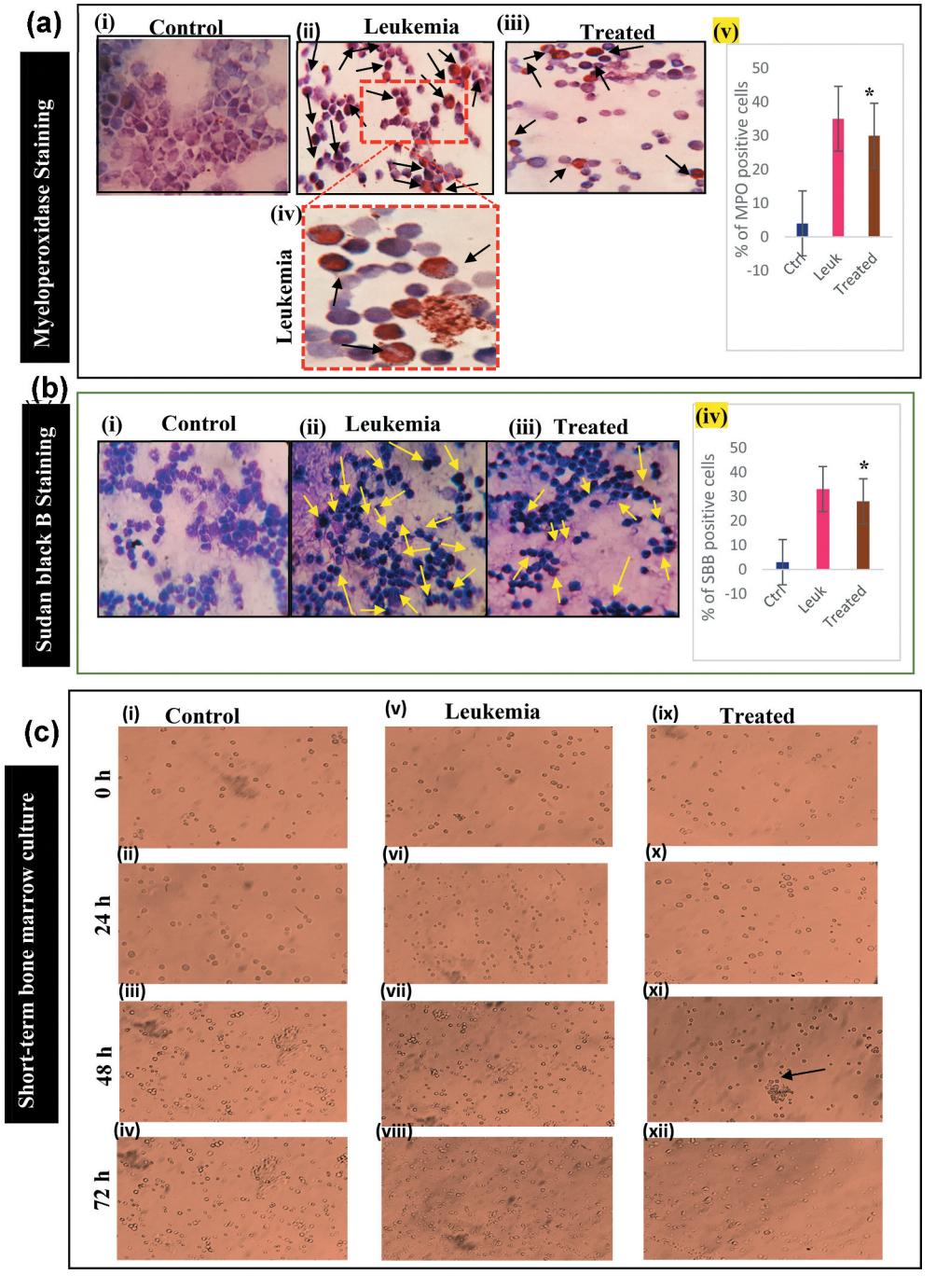

Figure 3. Cytochemical staining and short term bone marrow cell culture from control, leukemic and treated groups. (A) Myeloperoxidase (MPO) staining: (i) Control, (ii) Sharp rise in the number of dark brown stained MPO positive cells were identified in leukemic marrow (black arrow). (iii) Decrease in the number of MPO positive cells revealed in the treated bone marrow group. (iv) Presence of azurophilic granules and auer rods (black arrow) containing abnormal blast cells in leukemic marrow. (v) Graph depicts notable decrease of MPO positive cells in treated group (*0.018; Values are Mean ± SEM, t-test) compared to leukemia (B) Sudan black B (SBB) staining: (i) Control, (ii) SBB staining revealed a large number of darkly stained cells indicative of the increment in typical azurophilic SBB positive cells (yellow arrow) of leukemic marrow compared to (iii) treated group. (iv) Graph depicts significant decrease in SBB positive cells in treated group (*$P = 0.02$; Values are Mean ± SEM, t-test) (C) Short term Bone marrow cell culture: In contrast with control (i; 0 h, ii; 24 h, iii; 48 h, iv; 72 h) bone marrow cell culture from leukemic mice group (v; 0 h, vi; 24 h, vii; 48 h. viii; 72 h) revealed rapid cellular proliferation mainly at 48 h. Retardation in cellular proliferation post treatment observed in treated group (ix; 0 h, x; 24 h, xi; 48 h, xii; 72 h). Apoptotic bodies (black arrow) were observed in treated group at 48 h (xi). [Mag: (a i–iii), (b i–iv) 1,000x, (c i–xii), 400x]. (Color figure online).

Eclipta alba *confers alterations on the expression profile of canonical NF-kB pathway in the leukemic marrow*

Flowcytometric analysis revealed significant ($P < 0.001$) expressional upregulation of c-Rel (Figure 4A; Table 1) in hematopoietic population of leukemic mice (MFI; 141.48 ± 0.5) by 1.4-fold compared to control (MFI; 79.77 ± 0.68). Significant ($P < 0.001$) expressional decline of c-Rel by 1.1-fold (MFI; 122.79 ± 0.71) compared to leukemic group was found post treatment with *Eclipta alba*. Expression of p65 (Figure 4B) was significantly ($P < 0.001$) higher in leukemic bone marrow (MFI; 99.22 ± 0.69) by 1.7-fold compared to control (MFI; 57.93 ± 0.90). Drastic decrease ($P < 0.001$) of p65 expression (MFI; 87.89 ± 0.84) by 1.1-fold was observed in treated group compared to leukemic mice. Moreover, significant ($P < 0.001$) expressional increase of p50 (Figure 4C) and IL-1β (Figure 4D) was observed in leukemic marrow (MFI; 88.50 ± 0.50 and 102.30 ± 0.60, respectively) compared to control (MFI; 62.86 ± 0.80 and 80.96 ± 0.94, respectively) by 1.4 and 1.2-folds. Marked decrease of p50 and IL-1β expression ($P < 0.001$ and $P = 0.008$) was observed post treatment with extract (MFI; 66.43 ± 0.61 and 98.49 ± 1.31) compared to diseased group. Significant down regulation ($P < 0.001$) of IkBα (Figure 4E) by 1.5-fold was observed in leukemic marrow (MFI; 84.74 ± 0.65) compared to control (MFI; 128.46 ± 0.5). Notable increase ($P < 0.001$) of IkBα expression (MFI; 90.26 ± 0.64) by 1.0-fold was observed in treated group in comparison to diseased. IKKγ or NEMO (Figure 5B) and IKKβ (Figure 5C) showed drastic expressional increase ($P < 0.001$) in leukemic marrow cells (MFI; 97.92 ± 0.07 and 138.01 ± 0.07, respectively) compared to control (MFI; 64.28 ± 0.28 and 74.69 ± 0.59 respectively) by 1.5 and 1.8-fold. Concomitant decline ($P < 0.001$) of IKKγ and IKKβ expression (MFI; 70.73 ± 0.64 and 122.32 ± 0.32, respectively) was revealed in post treatment group by 1.3 and 1.1-fold compared to leukemic mice. To further confirm the expression of c-Rel, p65, p50, IL-1β, IkBα, IKKγ and IKKβ in control, leukemic and treated group, fluorescence intensity signals were studied by immunocytochemistry (Figure 4 A–E; Figure 5B,C,E) and 3D plots were generated by ImageJ software which corresponded to ample evidence to flowcytometric evaluation.

Effect of Eclipta alba *on the major transcription factor of non-canonical NF-kB pathway: RelB expression in the leukemic bone marrow*

Significant ($P = 0.022$ and $P < 0.001$) increase in IKKα (Figure 5A; Table 1) and RelB (Figure 5D) in leukemic group (MFI; 115.46 ± 0.46 and 28.80 ± 0.25) was analysed compared to control (MFI; 92.88 ± 0.83 and 14.44 ± 0.41). *Eclipta alba* extract did not show any adverse effect on IKKα and RelB of treated group (MFI; 99.7 ± 0.95 and 28.94 ± 0.05) which is also confirmed by 3D plots of fluorescence signals for both IKKα and RelB (Figure 5A, D).

Discussion

Exposure to NOCs, an established environmental pollutant is involved in the etiology of several human cancers. Through our experimental endeavour, we wanted to explore the amelioration of cell signaling modality by age-old herb, *Eclipta alba* on the leukemogenic effects of a potent NOC, N-N-ethylnitrosourea (ENU). Although the present synthetic treatments for leukemia have prolonged the survival rate of patients, the major constraint remains the adverse side effects, such as aplastic anaemia, bone marrow failure, chemotherapeutic resistance, immunosuppression, cellular toxicity, etc. (Saedi et al. 2014). For that reason, the application of medicinal plants as curative agent against leukemia is prominent in developing countries (Saedi et al. 2014). Our study on the traditional herb *Eclipta alba* portrayed its promising potentials to function as a sensitizer to complement the approach of conventional chemotherapy, mainly for patients with poor socio-economic background as it is cheap, readily available and nontoxic within referred dosimetry.

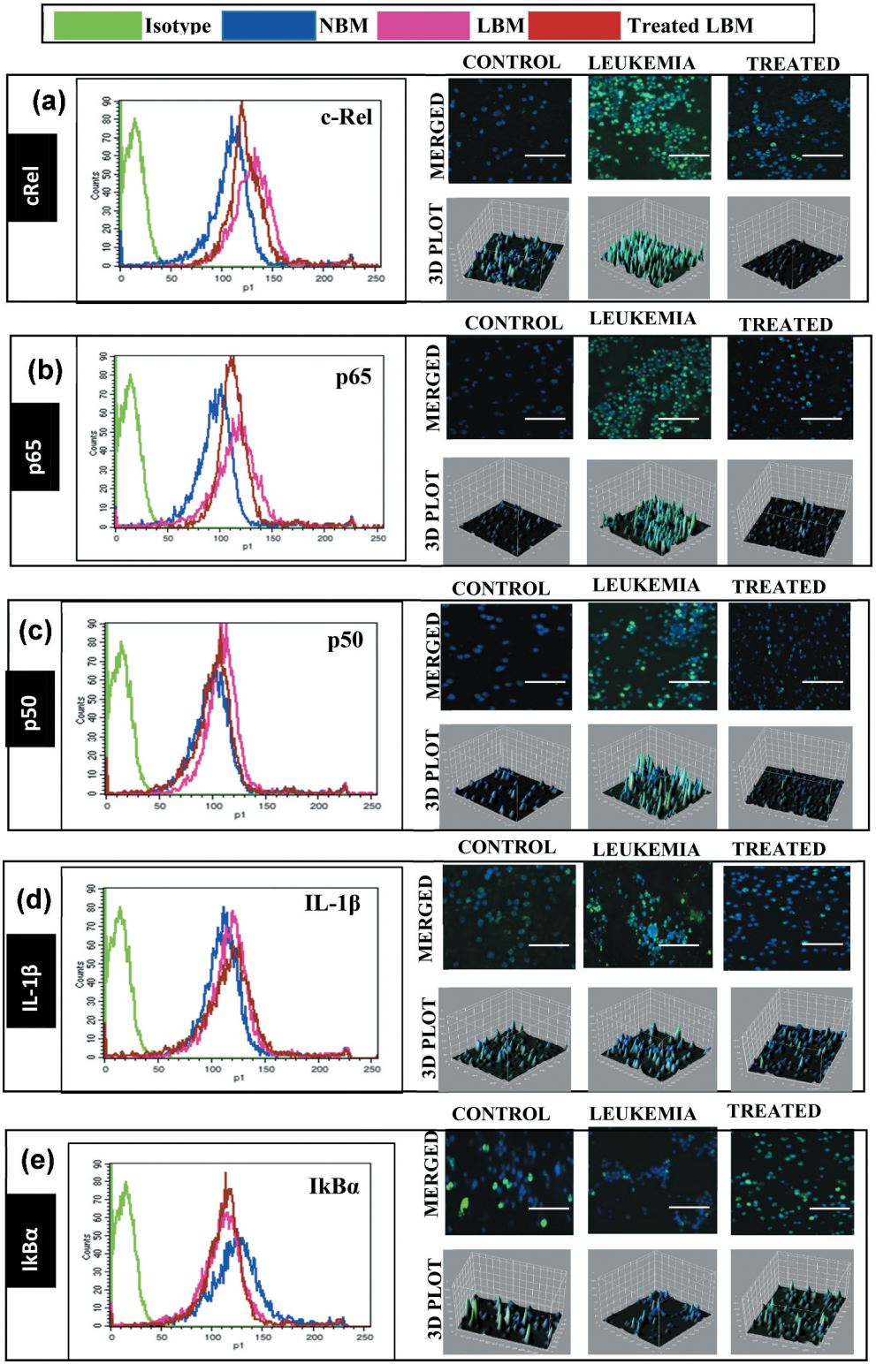

Figure 4. Flowcytometric and immunofluorescence expression. Evaluation of *Eclipta alba* extract on modulating the status of NF-kBsignaling pathway in the bone marrow. Representative histogram overlay revealed significant down-regulation of (A) cRel, (B) p65, (C) p50, (D) IL-1β, and up regulation of (E) IkBα in post treatment groups compared to leukemic mice. Immunofluorescence of bone marrow cells along with their analytical 3D plots showed in panel (A – D) of control, leukemia and treated group. The representative images were analyzed by 3D interactive surface plot (ImageJ) *(Scale bar 100 μm)*. (Color figure online).

Table 1. Mean Fluorescence Intensity (MFI) represents expression patterns of proteins of NF-kB pathway.

Proteins	MFI of control	MFI of Leukemia	MFI of Treated
	(X ± SD)	(X ± SD)	(X ± SD)
cRel	79.77 ± 0.68	141.48 ± 0.50***	122.79 ± 0.71***
p65	57.93 ± 0.90	99.22 ± 0.69***	87.89 ± 0.84***
p50	62.86 ± 0.80	88.50 ± 0.50***	66.43 ± 0.51***
IL-1β	80.96 ± 0.94	102.30 ± 0.60***	98.49 ± 1.31**
IkBα	128.46 ± 0.50	84.74 ± 0.65***	90.26 ± 0.64***
IKKα	92.88 ± 0.83	115.46 ± 0.46*	99.70 ± 0.95#
IKKγ	64.28 ± 0.28	97.92 ± 0.07***	70.73 ± 0.64***
IKKβ	74.69 ± 0.59	138.01 ± 0.07***	122.32 ± 0.32***
RelB	14.44 ± 0.41	28.80 ± 0.25***	28.94 ± 0.05#

*Values are Mean ± SD; One-way ANOVA by Tukey's Test. *P < 0.05, **P < 0.01, ***P < 0.001, as compared with control group. *P < 0.05, **P < 0.01, ***P < 0.001, as compared with Leukemia group. Values marked as (#) are not significant.*

Extreme leucocytosis and mobilization of leukemic blasts in the peripheral circulation was the foremost indication of leukemic onset in our model as evidenced by enhanced values of the vital haematological parameters like RBC, reticulocytes, haemoglobin, etc. The preliminary sign post-herbal treatment was the significant decrease of these blood parameters, which hinted towards recovery to some extent. The effectiveness of *Eclipta alba* on alteration of hematopoietic condition was further confirmed by cytochemical staining of the selective myeoblast lineage by MPO as well as SBB where significant decline of myeoblast population in the treated group was observed. The histopathological status of bone marrow pre and post treatment as studied by routine HE stain showed considerable decrease in blast population in comparison to control. Apart from changes in **haematological** and **histological** parameters, significant changes in the body weight and increased survivability of treated group hinted towards amelioration of the physical condition from leukemic state.

Aberrant activation of NF-kB pathway in leukemia has been reported by various studies, hence presenting it as an attractive therapeutic target (Zhou et al. 2015). Clinical features related to sharp rise in leukocytes and immature blast cells has a possible link between overexpression of NF-kB signaling and leukemic cell proliferation (Udayashankar et al. 2016). We observed significant reduction in the proliferation rate of bone marrow cells from treated group that once again proves the efficacy of *Eclipta alba* as an anti-neoplastic agent.

In stimulated cells, IKK complex is the potential 'central regulator' of NF-kB activation. IKKβ and IKKγ has been reported to be essential to the canonical pathway, while IKKα is involved in non-canonical activation. Our experiment revealed significant increase of IKKβ, and IKKγ (NEMO) in leukemic bone marrow cells. Expression of IKKβ and IKKγ was down regulated after treatment with *Eclipta alba*, which supported our hypothesis of the extract as an effective modulator of NF-kB signaling via canonical pathway in murine marrow cells. The IKKα expressional profile remained unaffected by the extract. Activation of IKK complex triggers the phosphorylation-induced degradation of its downstream molecule IkBα or 'the super repressor'. Defective or lower level of IkBα correlated well with uncontrolled NF-kB expression (Cilloni et al. 2007). Interestingly, we observed a significant expressional decline of IkBα in leukemic marrow cells as IkBα undergoes rapid proteosomal degradation in response to NF-kB activation (Kobori et al. 2004). Simultaneously, significant increase in IkBα was revealed in treated marrow population, which throws a ray of hope for the promising anti-cancer effect of *Eclipta alba*.

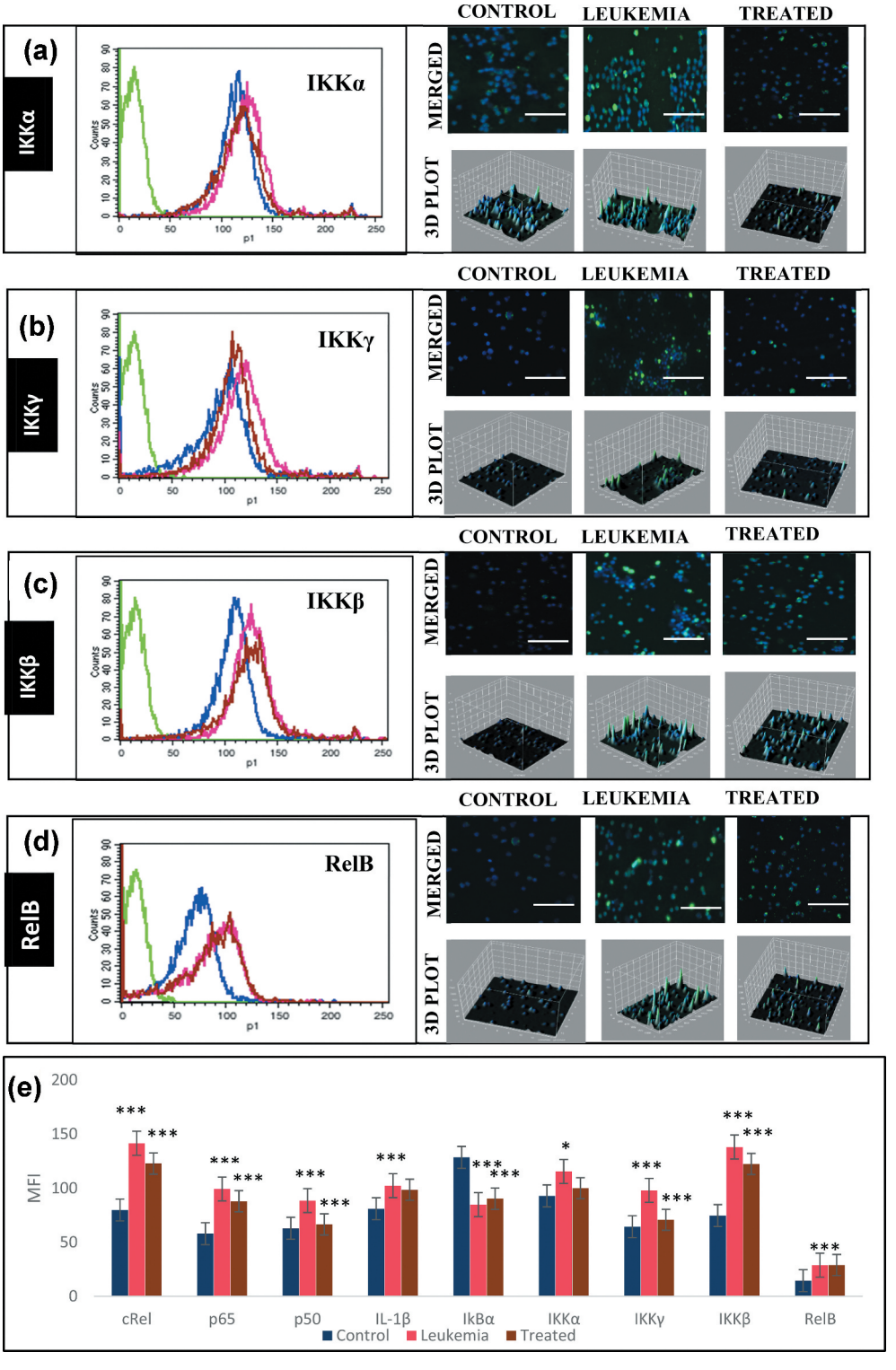

Figure 5. Flowcytometry and immunofluorescence. Assessment of *Eclipta alba* extract on inhibition of NF-kB signaling pathway in leukemic marrow. Representative histogram overlay revealed significant down-regulation of (A) IKKα, (B) IKKγ. No significant changes were observed in (C) IKKβ and (D) RelB post *Eclipta alba* treatment. (E) Graphical representation of the Mean Fluorescence Intensity (MFI) values of the molecules (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; Values are Mean ± SEM; One-way ANOVA by Tukey's Test). Immunofluorescence of bone marrow cells along with their analytical 3D plots showed in panel (A–D) of control, leukemia and treated group. The representative images were analyzed by 3D interactive surface plot (ImageJ) *(Scale bar 100 μm)*. (Color figure online).

Degradation of the IkBα suppressor frees the downstream molecules or the NF-kB transcription factor subunits: c-Rel, p50 and p65 in the form of homo or heterodimers in case of canonical and RelB and p52 for non-canonical activation (Cilloni et al. 2007). In our experiment, constitutive over expression of the subunits was observed in leukemic bone marrow cells which were significantly declined in the treated group. Unchanged RelB expression post-treatment, once again highlighted the efficacy of herbal extract in modulation of NF-kB via canonical pathway. For further confirmation, we examined the expressional profile of pro-inflammatory cytokine IL-1β which is reported to be highly overexpressed in leukemic conditions and acts as a stimulator of NF-kB pathway (Zhou et al. 2015). The treated bone marrow cells showed a significant reduction in IL-1β expression, which was up regulated in leukemic state, hence confirming the activation of NF-kB in our ENU induced mouse model and also explaining the potency of the extract in modulating the pathway.

Limitations of the study

In our experimental endeavour, we have intricately delineated the cellular mechanism and signaling modality but the detailed genetic molecular analysis concerning various genes are yet to be done and is the limitation of the study, which we shall definitely try to invade in our future experimental design.

Conclusion

We can conclude that the environmental pollutant ENU-induced leukemic mouse model revealed significant leukemogenesis, dysregulation and over expression of the NF-kB signaling pathway where *Eclipta alba* extract effectively combated the expression of NF-kB canonical family signaling molecules, and hence can be a promising target for amelioration of leukemic condition.

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Disclosure Statement

All authors declared no potential conflict of interest.

Declaration of interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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RESEARCH ARTICLE

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Environmental pollutant N-N′ethylnitrosourea-induced leukemic NLRP3 inflammasome activation and its amelioration by *Eclipta prostrata* and its active compound wedelolactone

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Abstract

Environmental exposure of N-nitroso compounds (NOCs) from various sources like tobacco smoke, pesticides, smoked meat, and rubber manufacturing industries has been an alarming cause of carcinogenesis. Neonatal exposure to the carcinogenic N-N′ethylnitrosourea (ENU), a NOC has been established to cause leukemogenesis. Our world is constantly battling against cancer with consistent investigations of new anti-cancer therapeutics. Plant derived compounds have grasped worldwide attention of researchers for their promising anti-cancer potentials. *Eclipta prostrata* is one such ayurvedic herb, renowned for its anti-inflammatory properties. Currently, it has been explored in various cancer cell lines to establish its anti-cancer effect, but rarely in in-vivo cancer models. Wedelolactone (WDL), the major coumestan of *E. prostrata* is recognized as an inhibitor of IKK, a master regulator of the NF-kB inflammatory pathway. As persistent inflammation and activated inflammasome contribute to leukemogenesis, we tried to observe anti-leukemogenic efficacy of *E. prostrata* and its active compound WDL on the marrow cells of ENU induced experimental leukemic mice. Treatment groups were administered an oral gavage at a dose of 1200 mg/kg and 50 mg/kg b.w of crude extract and WDL respectively for 4 weeks. Various parameters like hemogram, survivability, cytological and histological investigations, migration assay, cell culture, flowcytometry and confocal microscopy were taken into consideration pre- and post-treatment. Interestingly, the plant concoction portrayed maximum effects in comparison to WDL alone. The study suggests *E. prostrata* and WDL as vital complementary adjuncts for anti-inflammasome mechanism in ENU-induced leukemia.

KEYWORDS
bone marrow, *Eclipta prostrata*, inflammasome, leukemia, N-N′ ethylnitrosourea, wedelolactone

1 | INTRODUCTION

Epidemiological evidence has established the pivotal role of N-nitroso compounds (NOCs) in the etiology of carcinogenesis.[1] Primary exposure of NOCs from various sources like tobacco smoke, chewing tobacco, smoked meat and industrial manufacturing exposure of pesticides, rubber, and tire products are detrimental for our environment.[2] Prenatal exposure to N-N′ ethylnitrosourea (ENU) a NOC, induces carcinogenesis and eventually results in leukemic pathophysiology.[3–5]

Leukemia is a heterogenic neoplasm arising from the malignancy of hematopoietic stem/ progenitor cells (HSPCs) and contributes to 2.8% among all the cancer cases globally.[5] Inflammasome is a multi-protein complex that plays the central responsibility in the pathogenesis of the multifaceted and heterogenic disease called cancer.[6] The inactive complex comprising of NLRP3 protein; apoptosis-associated speck like protein (ASC) and procaspase-1 resides in the cytoplasm, which upon activation, accumulates to form several NLRP3 speck complexes and the procaspase-1 gets cleaved into its functional form to activate the pro-inflammatory cytokines IL-1β and IL-18, whose pleiotropic effects on hematopoiesis promotes carcinogenesis.[7] Recent investigations have designated NLRP3 inflammasome as the "rising star" for hematological pathologies like eukemia, which is an inflammation-driven malignancy.[8,9] The tight interlink among NLRP3 inflammasome and carcinogenesis has attracted global attention towards inflammasome inhibitors for potential anticancer approaches.[10]

Use of herbal medicines as a source of new drugs and promising integrative therapy; has been embraced worldwide due to its extensive benefits and lesser side effects.[11] Herbal extracts and their active compounds have been reported to counteract NLRP3 inflammasome on various disease pathogenesis.[12–18] One such ethnomedicinal herb *Eclipta prostrata* (EP) also known as Bhringaraj in Ayurveda, is established for its anti-inflammatory and anticancer properties.[19–24] Wedelolactone (WDL), the major coumestan derivative of this herb, is exclusively reported for anticancer effects and has been investigated to hinder the overexpression of NLRP3 inflammasome.[25–34]

Although, there is an emerging research on plant-based pharmaceuticals as a complementary approach for targeting NLRP3 inflammasome, there is an urgent need of investigation regarding the role of natural compounds on reducing the over-expression of inflammasomes resulting to hematological malignancies. This study concentrates on the ameliorating effects of EP and its active compound WDL on leukemogenesis via NLRP3 inflammasome activation in the bone marrow (BM) of ENU-induced leukemia.

2 | MATERIALS AND METHODS

2.1 | Plant material and preparation

EP was procured from the Medicinal Plant Garden of Narendrapur Ramakrishna Mission Ashrama, Kolkata, India. Plant material was authenticated by Prof. Jukta Adhikari, Professor of Botany Department, Barasat Government College, West Bengal, India in July 2019. Specimen sample has been submitted at Calcutta School of Tropical Medicine, Kolkata, India with the voucher no. 756870. The dried leaf powder was extracted in ethanol for 24 h. Following double filtration under sterile condition, the solvent was thoroughly removed and the extracts were concentrated at 50°C by rotary evaporator. The filtrate was reconstituted in double distilled water and stored at −20°C until further use.[40,41]

2.2 | Chemical characterization by ESI-MS-Direct infusion

Chromatographic analysis of plant extract was performed using Shimadzu Prominence HPLC (Model No. CBM-20A, Japan) with LC-20AT pump, SIL-20AT autosampler and SPD-M20A detector and equipped with Discovery C18 column (ODS, 25 cm × 4.6 mm, 5 μm, Supelco, USA) and Guard column (Security Guard Cartridge system). Composition of mobile phase was acetonitrile: water (ACN%, v/v) at a flow rate of 1 ml/min and programmed at 20%–45% (for 15 min); 45%–100% (for 30 min) and 100 (for 40 min). WDL was obtained at a yield of 12.05 mg/ml and its isolation was carried out by HPLC with the reference standard (WDL, purity ≥98%, CAS No: 524-12-9; Sigma-Aldrich). For further confirmation, qualitative analysis of the isolated compound was performed by ESI-MS-Direct Infusion method using Xevo-G2-XS-QTof-MS (Waters) instrument. The acquisition was done with an injection volume of 10 μl at a flow of 5 μl/min in a positive ionization mode with electrospray capillary voltage of 3.0 kV. The cone gas flow at 30 L/h with source and desolvation temperature of 100 and 250°C, respectively. Data were analyzed using Mass Lynx 4.1 software. Calibration of instrument was done with Leucine encephalin (mass 555.62 g/mol).

2.3 | Animals

Inbred Swiss albino mice of both sexes were maintained at the animal house of the Calcutta School of Tropical Medicine under controlled room temperature (22 ± 2)°C. The animals were fed a standard diet and water ad libitum, under sterile conditions with a 12-h light–dark period. All the procedures were in consent with the authorized guidelines of the Institutional Animal Ethical Committee (IAEC); EU Directive 2010/63/EU and also according to The Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India (Registration number: 681/GO/Re/S/02/CPCSEA).

2.4 | Induction of leukemia by N-N′ethylnitrosourea

A single intra-peritoneal injection of N-N′ ethylnitrosourea (ENU, Sigma; 80 mg/kg) was administered to litter pups aging 10–14 days and weighing 4–5 gm, were administered to induce leukemic condition within 6–8 months, as demonstrated earlier.[4,5,35–39]

2.5 | Therapeutic dosimetry and animal grouping

Previous studies reported no acute toxicity of EP aqueous extract up to 2000 mg/kg body weight.[40,42,43] Furthermore, Zhao et al., reported no mortality induced by EP alcoholic extract up to 10.4 mg/kg for 14 days.[44] In a report by Wei et al., an oral dose of 50 mg/kg of WDL, significantly ameliorated DSS-induced colon damage by inhibiting NLRP3 inflammasome and its downstream caspase-1 phosphorylation.[34] Study

by Luo et al., delineated the dose of 50 mg/kg of WDL reasonably inhibited immune-mediated liver damage and fairly modulated the NF-kB signaling pathway.[45]

The dose of EP and WDL in our study was standardized at 1200 and 50 mg/kg body weight respectively for 4 weeks via oral gavage after the complete leukemic development (6 months) of ENU-induced mice.

Experimental plan ($n = 6$ mice per group):

Group I: ENU-induced Leukemic mice;

Group II: ENU-induced Leukemic mice + EP whole extract (1200 mg/kg via daily oral gavage for 4 weeks);

Group III: ENU-induced Leukemic mice treated with isolated WDL (50 mg/kg via daily oral gavage for 4 weeks);

Group IV: Control group of mice received equal volume of saline in similar condition.

2.6 | Isolation and single cell preparation of bone marrow cells

The experimental animals were sacrificed by cervical dislocation and the long bones (femur, tibia) were surgically removed. Using a sterile 26 gauge needle, the BM cells were flushed out into RPMI-1640 media (Sigma). The cells were passed through a 100 μm cell strainer and washed thrice in the chilled media to acquire a single cell suspension without debris.[46]

2.7 | Assessment of peripheral blood hemogram and survivability

Disease development and post treatment effects were confirmed by performing peripheral blood hemogram.[36,39,46–48] Total RBC and WBC counts were determined by standard laboratory protocol, using hemocytometer chamber (Rohem, India). Differential leukocyte counts (DC) were carried out by Leishman staining. Reticulocyte count was determined using brilliant cresyl blue staining and hemoglobin estimation was studied using Drabkin's reagent (Stanbio reagent, India). To observe changes in the cellular morphological patterns, marrow smears were also stained with Giemsa (Himedia, India). Survivability of pre and post treated groups was monitored.

2.8 | Histological investigation of bone marrow

To observe changes in the histopathological scenario, the BM sections from experimental groups were stained with the routine hematoxylin & eosin (H&E) procedure and observed under light microscope Olympus CH20i, Japan).

2.9 | Silver nitrate nucleolar organizer regions staining

Nucleolar organizer regions (NORs) are DNA segments of nucleoli containing coding genes for ribosomal RNA and contribute to cellular synthesis. Silver nitrate (AgNOR) staining is a suitable method for identification of the NORs, which are detectable as black dots by the light microscope and corresponds to demonstrate the proliferative rate and degree of malignancy in different cancers. The rise in AgNOR is a pre-malignant feature, demonstrating increased proliferative rate. 1 part of 2% gelatin in 1% formic acid and 2 part of 50% silver nitrate (Merck) in distilled water was mixed to prepare a silver nitrate solution. For 30 min, the marrow cell smears were immersed in the working solution inside a dark humified chamber, followed by a distilled water wash and immersion in 5% sodium thiosulfate (Merck) solution. The AgNOR dots in the marrow cells were visualized using light microscope (Olympus CH20i, Japan).[49]

2.10 | MTT assay

Present number of viable cells can be estimated by the NADP(H) dependent cellular oxido-reductase enzyme mediated reduction of the tetrazolium dye MTT (3,4,5 dimethylthiazol 2 yl)-2,5 dipenyl tetrazolium bromide) into an insoluble formazan.[49] 1×10^6 bone marrow cells/ml were seeded in 12-well plates with 3 replicate wells with RPMI-1640 medium, without color indicator phenol red (Thermo Fisher Scientific), supplemented with 20% FBS and maintained at 37°C. At 0, 24, 48 and 72 h the marrow cells of the experimental groups from culture were tested by MTT. Formazon (purple color) was dissolved in isopropanol and optical density (OD) was taken at 540 nm by colorimeter (EES-DIGI-8F, India).

2.11 | Transwell migration and invasion assay

The migration and invasion capability of the BM cells from the experimental groups were measured using Transwell membrane plate (Corning) coated with matrigel. In the upper chamber, single cell suspension of marrow cells (3×10^6 cells) in 200 μl serum free DMEM media was placed. 20% FBS with 500 μl of DMEM was added in the lower chamber to work as a chemoattractant. After 48 h incubation in 5% CO_2 at 37°C, the migrated cells were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet. Migrated marrow cells in five random fields of each well were counted at 100× magnification under light microscope (Olympus CH20i, Japan).

2.12 | Long term bone marrow cell culture

BM cells (2×10^6 cells/ml) were plated on 35 mm-cell culture plates (Corning), in a concoction of RPMI-1640 (Sigma-Aldrich) media added with 30% FBS (Lonza), 1% bovine serum albumin (Sigma-Aldrich) and 0.02% β-mercaptoethanol (Himedia), which was drained and replenished every 48 h. The plates were maintained in 5% CO_2 at 37°C. Culture plates were inspected under inverted microscope (Dewinter).

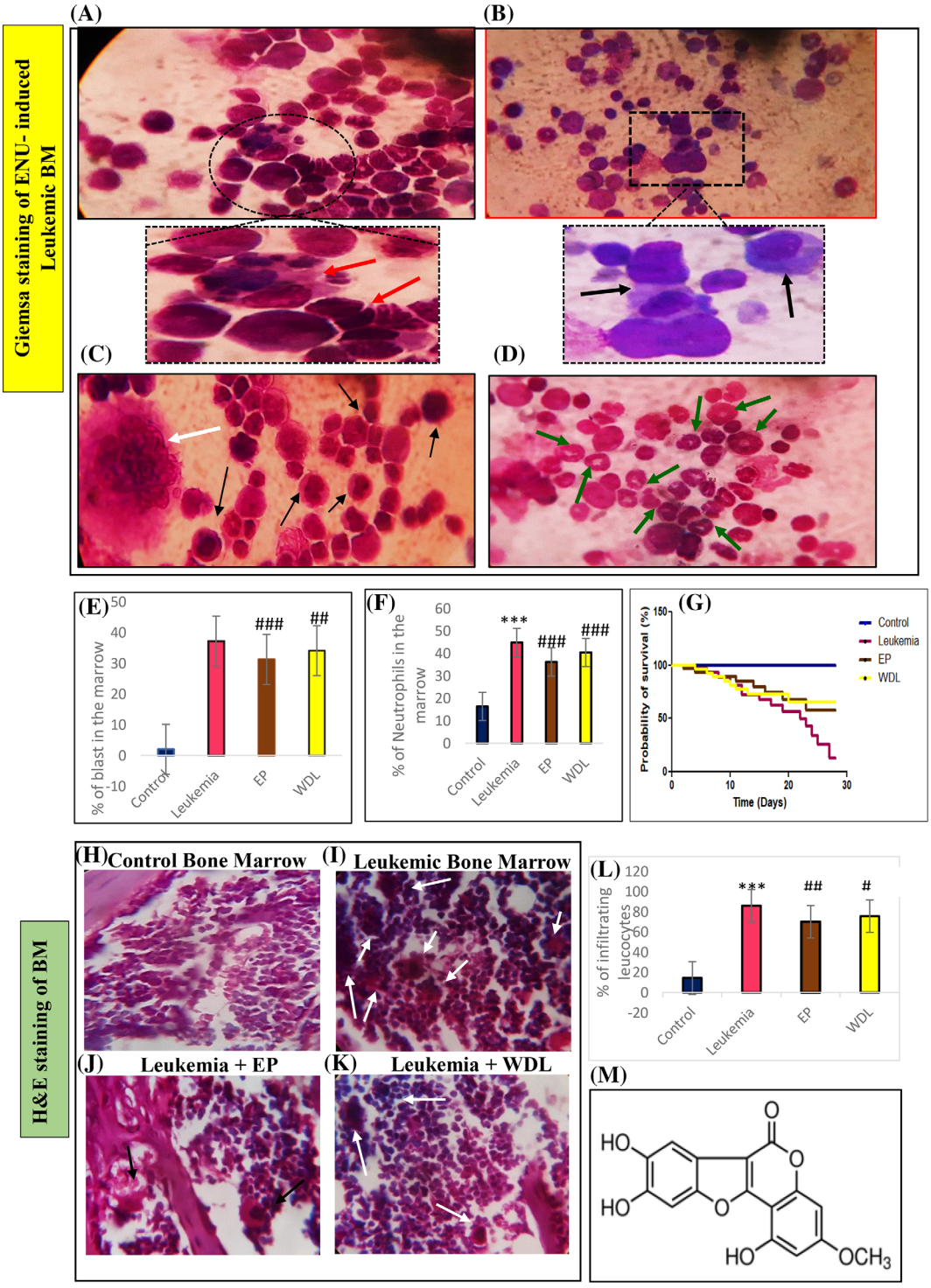

FIGURE 1 Morphological features of N-N′ethylnitrosourea (ENU)-induced leukemic bone marrow cells. (A) Anomalous leukemic blast cell (red arrow) identified in marrow smear (Leishman stained), (B) abnormal myeloblast (black arrow), (C) hypo-lobulated promegakaryocyte (white arrow) and typical blast cells (black arrow) and (D) infiltrated donought neutrophils (green arrow). Graphical representation of marrow status pre- and post-treatment. (E) Percentage of blast in the marrow of experimental groups, and (F) percentage of infiltrated neutrophils in the marrow, before and after treatment. Survival analysis: (G) comparison of survivability pre- and post-treatment by Kaplan–Meir curve. Bone marrow section histology: identification of leucocytes (H&E staining) in the bone marrow sections revealed (I) a sharp rise in numbers in leukemic group (white arrow) as compared to (H) control, and significant amelioration of leucocyte infiltration in both (J) EP (black arrow) and (K) WDL treated group (white arrow). (L) Graphical depiction of mean ± SD values of leucocyte infiltration in marrow cells pre- and post-treatment (*p < .05, **p < .01, ***p < .001 significance of leukemia as compared to control. #p < .05, ##p < .01, and ###p < .001 significance of EP and WDL treatment in comparison to leukemia). (M) Chemical structure of wedelolactone

TABLE 1 Comparative hemogram profile of experimental groups

Parameters	Control (*X* ± SD)	Leukemia (*X* ± SD)	EP treated (*X* ± SD)	WDL treated (*X* ± SD)
WBC ($\times 10^3/mm^3$)	7.58 ± 0.54	36.17 ± 0.60***	25.49 ± 1.25###	30.40 ± 0.41###
RBC ($\times 10^6/ mm^3$)	6.89 ± 0.78	10.94 ± 0.30***	9.58 ± 0.42##	10.57 ± 0.32
Neutrophils (%)	16.37 ± 1.10	44.87 ± 0.85***	36.12 ± 0.92###	40.43 ± 0.42###
Reticulocyte (%)	0.54 ± 0.18	1.93 ± 0.031***	1.71 ± 0.04##	1.86 ± 0.018#
Blasts (%)	–	37.225 ± 0.85	31.25 ± 1.70###	34.13 ± 0.31##

Note: Values are mean ± SEM for six animals in each observation, repeated thrice. ***p < .001 significance of leukemia as compared to control. #p < .05, ##p < .01, ###p < .001 significance of *Eclipta prostrata* (EP) and wedelolactone (WDL) treatment in comparison to leukemia.

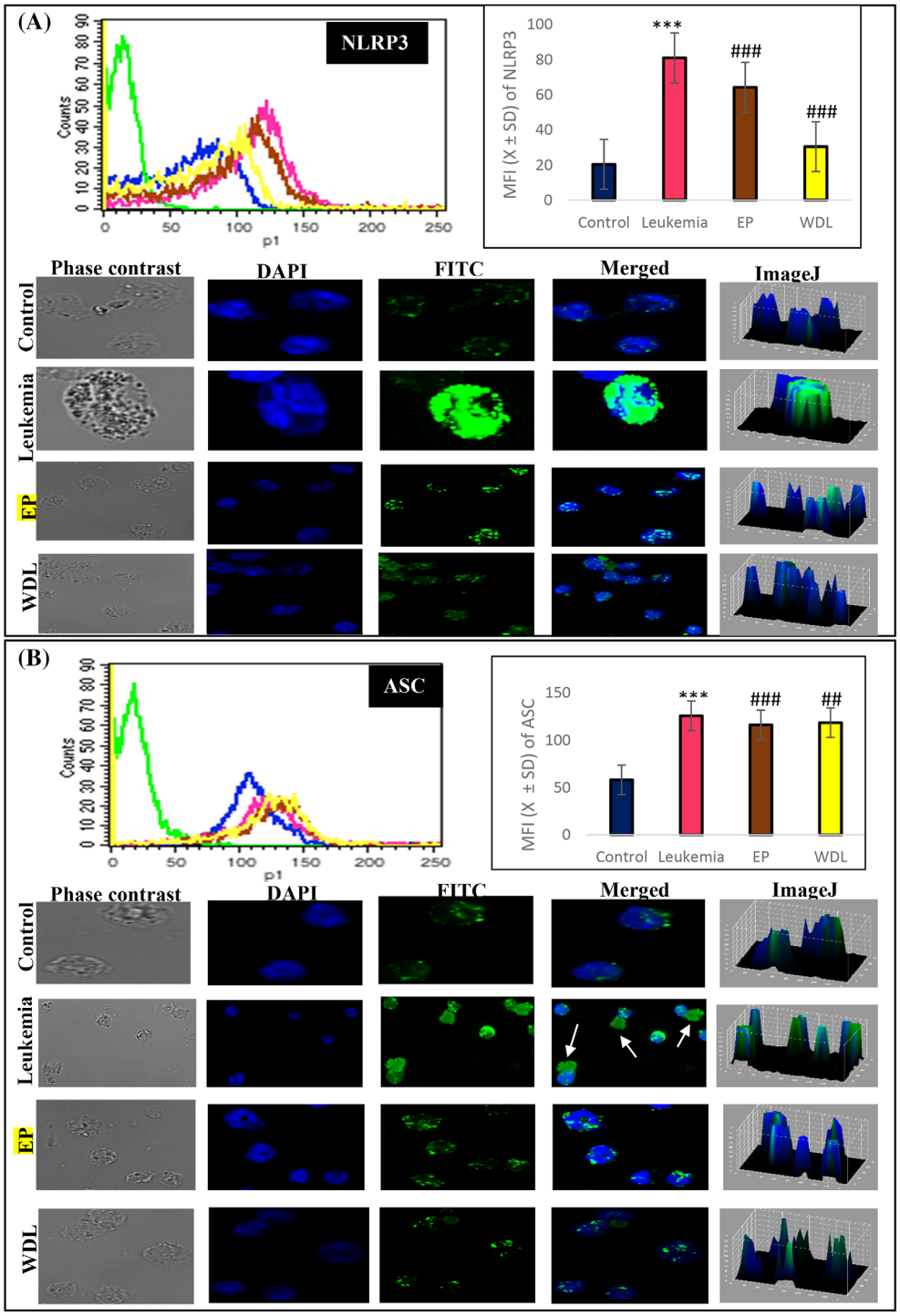

FIGURE 2 Assessment of fluorescence expression by flowcytometry and confocal microscopy. Attenuating effects of *Eclipta prostrata* (EP) and wedelolactone (WDL) treatment on the NLRP3 inflammasome activation of N-N′ethylnitrosourea-induced leukemic marrow cells. Representative histogram overlays and graphical illustration of MFI values depicting mean ± SD (***p < .001 significance of leukemia as compared to control. ##p < .01, ###p < .001 significance of EP and WDL treatment in comparison to leukemia): (A) NLRP3, (B) apoptosis-associated speck like protein. Confocal images of marrow cells from experimental groups and the analytical 3D plots showed in panel (A,B). The representative images were analyzed by 3D interactive surface plot (ImageJ)

2.13 | Flowcytometric analysis of signaling molecules

Marrow cells (2×10^6 cells/ml) from experimental groups were fixed with 1.5% paraformaldehyde (PFA) for 15 min at 37°C, washed and treated with chilled methanol for 20 min. Cells were distributed and stained with primary antibodies (anti-NLRP3, anti-ASC, anti-cleaved caspase1 and anti-cleaved IL-1β) of Mouse Reactive Inflammasome antibody kit (Cell Signaling Technology) and anti-GM-CSF (Santa Cruz biotechnology) were incubated for 30 min at 37°C. The marrow cells were counter stained and incubated with secondary antibody combined with Alexa flour 488 (Invitrogen) at 37°C for another 30 min, followed by PBS wash. Mean intensity fluorescence (MFI) data was analyzed by BD FACS Calibur flowcytometer (Becton Dickinson) using CellQuest Pro software (v9.1 Becton Dickinson).

2.14 | Immunofluorescence

Slides smeared with marrow cells tagged with Inflammasome antibody molecules (anti-NLRP3, anti-ASC, anti-cleaved caspase1, anti-IL-1β; Cell Signaling Technology) and anti-GM-CSF (Santa Cruz biotechnology) were mounted by DAPI shield (Sigma). Fluorescence images were obtained by Zeiss LSM 800 confocal laser scanning microscope.

2.15 | Statistical analysis

Unpaired Student's *t*-test and One-way ANOVA by post hoc testing (Tukey's test) was performed for statistical analysis. Every quantitative data were represented as Mean ± SD. For every comparison, $p \leq .05$ was considered as significantly significant. All he experiments were repeated thrice.

3 | RESULTS

3.1 | Identification of wedelolactone

Multiple compounds present in the whole ethanol extract of EP were separated and WDL was identified and isolated using HPLC, followed by ESI-MS-Direct infusion for further definitive identification of WDL. At TOF MS ES+ 4.21 e4, isolated compound was tentatively identified as WDL in protonated form in the negative-ion mode, with *m/z* 316.2081 in comparison with authentic WDL standard. Another fragment ion with *m/z* 302.19 was detected, probably indicating the methyl group of WDL (Figure **S1**).

3.2 | Effects of *Eclipta prostrata* and wedelolactone on the leukemic prognosis

Hematological investigations of the leukemic BM cells, unveiled the overproduction of abnormal blast cells hence marking the leukemic onset and excessive infiltration of ring-shaped nuclei neutrophils or doughnut cells, indicating the inflammatory microenvironment (Figure 1A–D). The EP and WDL treated groups showed reasonable decline of the blast and neutrophil percentage in the marrow cells (Figure 1E,F). Furthermore, treated groups showed fairly increase in survivability compared to leukemic group, therefore validating the curative potentials of EP and WDL (Figure 1G). Peripheral hemogram was conducted for the corroboration of leukemia pathogenesis and leukemogenesis post-treatment with EA and WDL (Table 1). Striking increase of leukocytosis was confirmed in the leukemic group ($36.17 \pm 0.60 \times 10^3$ cells/mm^3, $p < .001$) compared to control ($7.58 \pm 0.54 \times 10^3$ cells/mm^3). Considerable decrease of leukocytosis by 1.4 and 1.1-folds ($25.49 \pm 1.25 \times 10^3$ cells/mm^3, $p < .001$ and $30.40 \pm .41 \times 10^3$ cells/mm^3, $p < .001$) were noticed in EP and WDL treated groups respectively, hence approving their preliminary effects on leukemic pathogenesis. Significant increase in red blood cells (RBC) and reticulocyte count ($10.94 \pm 0.30 \times 10^3$ cells/mm^3, $p < .001$ and 1.93% ± 0.031%, $p < .001$) of leukemic group was observed, as compared to control ($6.89 \pm 0.78 \times 10^3$ cells/mm^3 and 10.94% ± 0.30%). Decrease of the RBC count by 1.1 and 1.0-folds in the EP ($9.58 \pm 0.42 \times 10^3$ cells/mm^3, $p = .003$) and WDL ($10.37 \pm 0.32 \times 10^3$ cells/mm^3, $p = .05$) treated groups respectively; and significant decline of reticulocyte count by 1.1 and 1.0-folds in the EP (1.71% ± 0.04%, $p = .05$) and WDL (1.86% ± 0.01%, $p = .028$) treatment groups were observed. Furthermore, a significant decrease in the blast percentage of both the treated groups were noticed, by 1.1 (31.25% ± 1.70%; $p < .001$) and 1.0-folds (34.13% ± 0.31%, $p = .006$) in EP and WDL groups respectively as compared to leukemic group (37.22% ± 0.85%) hence fortifying the therapeutic virtue of EP and WDL.

3.3 | Identification of leucocytes by hematoxylin & eosin

Analysis by H&E staining revealed the extreme infiltration of leucocytes in the BM sections of diseased group (86% ± 2.94%, $p < .001$) in contrast with the control (14.25% ± 4.64%), hence highlighting the inflammatory microenvironment in the leukemic BM. Marrow sections from EP and WDL treated groups (70% ± 6.58%, $p = .004$; 75.5% ± 3.87%, $p = .013$) showed moderate alleviation with lesser infiltrated leucocytes, as compared to leukemic condition (Figure 1H–L).

3.4 | *Eclipta prostrata* and wedelolactone treatment confers immunomodulation on the expressional profile of inflammasome complex

Flowcytometric analysis validated a sharp incline by 3.9-folds, in the expressional profile of NLRP3 inflammasome molecule in the marrow population of leukemic group (MFI; 81.00 ± 0.66, $p < .001$) as compared with control (MFI; 20.40 ± 0.90) (Figure 2A, Table 2). Significant

TABLE 2 Mean fluorescence intensity (MFI) represents expression patterns of proteins

Proteins	MFI of control (*X* ± SD)	MFI of leukemia (*X* ± SD)	MFI of EP treated (*X* ± SD)	MFI of WDL treated (*X* ± SD)
NLRP3	20.40 ± 0.90	81.00 ± 0.66***	64.22 ± 0.58###	30.45 ± 0.75###
ASC	58.24 ± 1.97	125.79 ± 1.58***	116.30 ± 0.85###	118.56 ± 1.25##
Cl-caspase-1	36.34 ± 0.95	68.54 ± 0.50***	52.5 ± 1.80###	62.92 ± 1.015##
Cl-IL-1	44.53 ± 0.68	143.09 ± 0.86***	77.73 ± 0.92###	87.15 ± 1.23###
GM-CSF	41.92 ± 0.96	62.19 ± 0.73***	49.80 ± 0.88###	51.19 ± 1.30###

Note: Values are mean ± SEM for six animals in each observation, repeated thrice. ***p < .001 significance of leukemia as compared to control. ##p < .01, ###p < .001 significance of *Eclipta prostrata* (EP) and wedelolactone (WDL) treatment in comparison to leukemia.

down-regulation in NLRP3 expression was observed in both EP and WDL treated groups by 1.2 and 2.6-folds respectively (MFI; 64.22 ± 0.58, p < .001; MFI; 30.45 ± 0.75, p < .001), in contrast with leukemia. Expression of ASC protein was drastically up-regulated in the diseased group (MFI; 125.79 ± 1.58, p < .001) by 2.14-folds in comparison with the control (MFI; 58.24 ± 1.97, p < .001. Interestingly, significant decrease in ASC expression of EP (MFI; 116.30 ± 0.85, p < .001) and WDL (MFI; 118.56 ± 1.25, $p = .001$) by 1.08 and 1.06-folds respectively were observed among the treated groups (Figure 2B, Table 2). Furthermore, EP and WDL treatment (MFI; 52.5 ± 1.80, p < .001 and MFI; 62.92 ± 1.015, $p = .002$) could significantly decrease the expression of the downstream cleaved caspase-1 by 1.3 and 1.0-folds in comparison with the diseased state (MFI; 68.54 ± 0.50). In contrast with the diseased state, control group showed significantly low expression of the cleaved caspase-1 by around 1.8-folds (MFI; 36.34 ± 0.95, p < .001) (Figure 3A, Table 2). The active pro-inflammatory cytokine, cleaved IL-1 ± β was exceedingly over-expressed in the leukemic condition by 3.21-folds (MFI; 143.09 ± 0.86, p < .001) as compared to control (MFI; 44.53 ± 0.68). Excellent amelioration on the expressional level of cleaved IL-1β was analyzed in EP (MFI; 77.73 ± 0.92, p < .001) and WDL (MFI; 87.15 ± 1.23, p < .001) treated groups by 1.8 and 1.6-folds respectively (Figure 3B, Table 2). Furthermore, to authenticate the data, expressional analysis of the fluorescent intensity signals on the NLRP3, ASC, Cleaved Caspase-1 and Cleaved IL-1β molecules in BM cells of each experimental groups were done by confocal microscopy.

3.5 | Analysis of fluorescence expression in GM-CSF profile post treatment with *Eclipta prostrata* and wedelolactone in marrow cells

Flowcytometric expression of GM-CSF was significantly higher by 1.48-folds in ENU-induced leukemia (MFI; 62.19 ± 0.73, p < .001) as compared to control (MFI; 41.92 ± 0.96). However, GM-CSF expression was significantly curtailed in EP (MFI; 49.80 ± 0.88, p < .001) and WDL (MFI; 51.19 ± 1.30, p < .001) treated groups by 1.2-folds in contrast to diseased state. Furthermore, the data was validated by confocal microscopy (Figure 4D).

3.6 | Effects of *Eclipta prostrata* and wedelolactone on the invasion and migration capacity of marrow cells

The transwell assay delineated a sharp rise in migration and invasion potentiality of ENU-induced leukemic marrow cells in contrast with control. Treatment with EP and WDL could markedly reduce the migration capacity of the marrow cells (Figure 4A–C). The percentage calculation of migration/invasion by bone marrow cells from experimental groups was based on the formula as followed:

$$\%\text{Magnification/invasion} = (\text{mean score treatment group/mean score of leukemia group} \times 100\%).$$

3.7 | Evaluation of malignancy and proliferative indices in the bone marrow cells, pre- and post-treatment

The leukemic marrow cells (Figure 5B) revealed an excessive number of black dots (NORs) as compared to control (Figure 5A), hence indicating an increased proliferation and malignancy. EP and WDL treated groups showed moderately less number of NORs in the marrow population, as compared with the diseased condition (Figure 5C,D).

The rate of proliferation of the BM cells from the experimental groups were evaluated by MTT assay and their growth kinetics were observed by reporting the OD values at separate time intervals of 0, 24, 48, 72, and 96 h from marrow cell culture. Leukemic group showed a sharp incline in the proliferation rate in comparison with control and an increase in the formation of formazan produced a darker purple color (Figure 5I,J). Post treatment groups of EP and WDL marrow cells showed a somewhat lesser proliferative tendency as compared to diseased state.

3.8 | Effects of *Eclipta prostrata* and wedelolactone treatment on the proliferation of bone marrow cells in long-term culture

Long-term cell culture was further performed to analyze the rate of proliferation and growth pattern of the BM cells from experimental

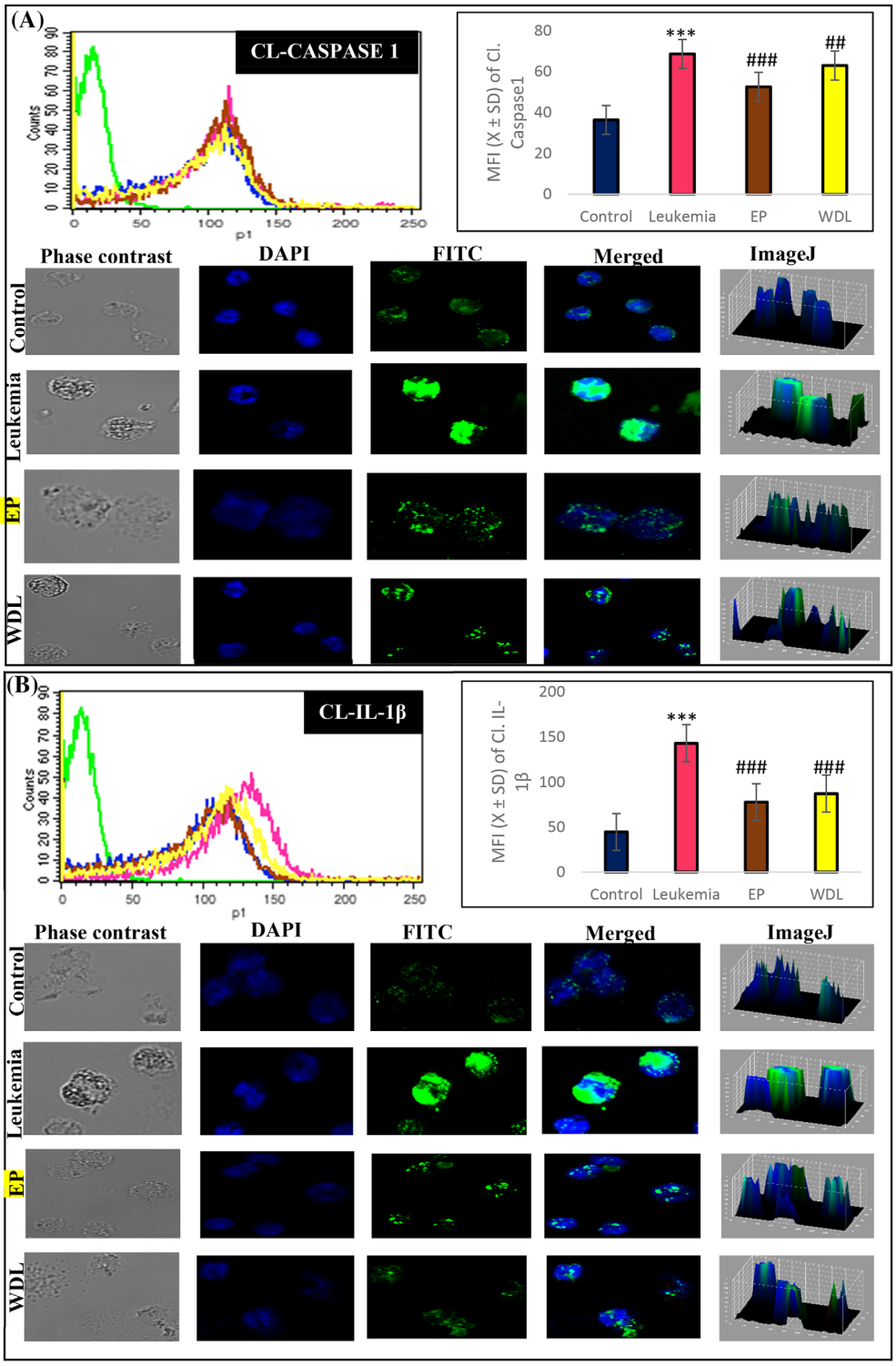

FIGURE 3 *Eclipta prostrata* (EP) and wedelolactone (WDL) treatment attenuates the over-expression of Cleaved Caspase-1 and Cleaved IL-1β in N-N′ethylnitrosourea-induced leukemia. Histogram plots of flowcytometry and the illustrative graphical mean intensity fluorescence values depicting mean ± SD of (***$p < .001$ significance of leukemia as compared to control. ##$p < .01$, ###$p < .001$ significance of EP and WDL treatment in comparison to leukemia). (A) Cleaved caspase-1, (B) cleaved IL-1β. Pre- and post-treatment status of the molecules in bone marrow cells depicted by images of confocal microscopy and 3D interactive surface plots (ImageJ) showed in panels (A,B)

groups. Leukemic marrow cells depicted a strikingly elevated rate of proliferation with the formation of anomalous cells presumably leukemic cells and prominent spindle shaped stromal cells, as compared to control within 96 h (Figure 5E,F). Marrow cells from EP and WDL treatment groups also indicated similar stromal cell formations, but the proliferative rate was lesser as compared to leukemic group (Figure 5G,H).

4 | DISCUSSION

The civilization of mankind is both a boon and bane to our environment. An increase in world industrialization leading to environmental pollutants has been the potential cause of carcinogenesis since decades. N-nitroso compounds (NOC) are one such major carcinogen environmentally present from tobacco, pesticides, cured meats,

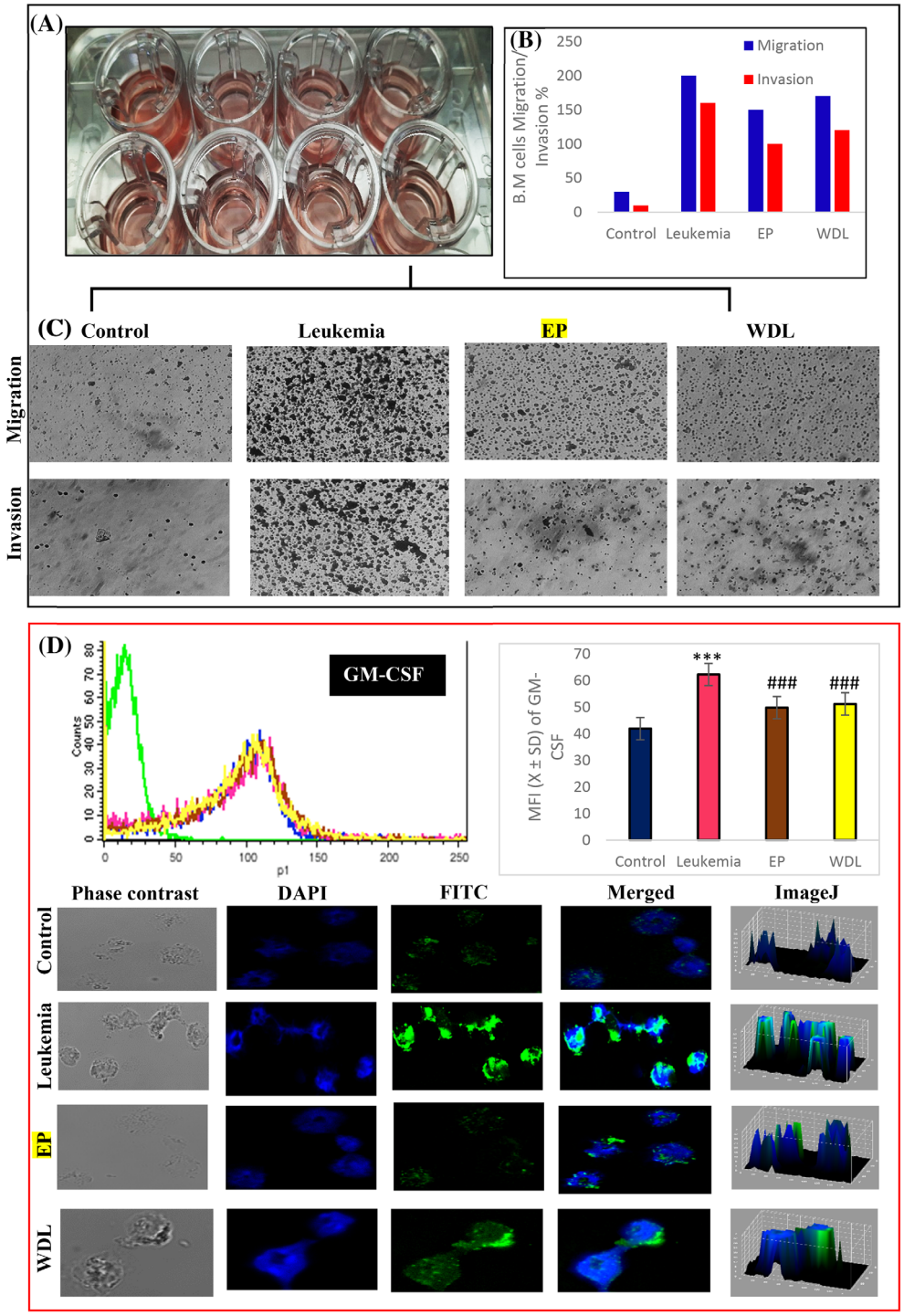

FIGURE 4 Invasion and migration capacity of bone marrow of experimental groups. Depictive images of transwell migration. (A) Representative image of transwell experimental plates. (B) Graphical demonstration of the invasive and migratory capacity before and after treatment and (C) bone marrow cells from experimental groups allowed to migrate and invade for 48 h in transwell plates. Fluorescence analysis of GM-CSF. (D) histogram overlay of flowcytometric profile and representative graphical mean intensity fluorescence (MFI) values depicting mean ± SD of GM-CSF expression in the marrow cells of experimental groups (***p < .001 significance of leukemia as compared to control. ###p < .001 significance of *Eclipta prostrata* and wedelolactone treatment in comparison to leukemia). Confocal images and 3D surface interactive plots (ImageJ) showed in same panel

rubber making industries, etc. In the recent years, traditional plants and their active components have grabbed the world's attention for serving as excellent anti-cancer leads. Through our study, we aimed to explore the leukemogenic potentials of ENU and its amelioration via NLRP3 inflammasome activation by the whole concoction of the ayurvedic herb EP as well as its active compound WDL separately on the bone marrow of ENU-induced leukemic mice model.

Preliminarily, we examined the effects of EP and WDL on the leukemic condition, by complete hemogram and survivability studies and interestingly, both the treatments mitigated the indications of the initial leukemic onset. The induction of persistent inflammatory microenvironment via inflammasome has long been established to play the role of a key element in carcinogenesis, hence putting the limelight on inflammasome inhibitors as prospective anti-cancer therapeutics.[10,50,51] Current reports of the aberrant expression of NLRP3 inflammasome in the marrow cells have shown NOCs to be a catalyst for the pathogenicity of leukemia.[9,52] In resemblance with the reports on the status of NLRP3 in leukemia, we observed an over

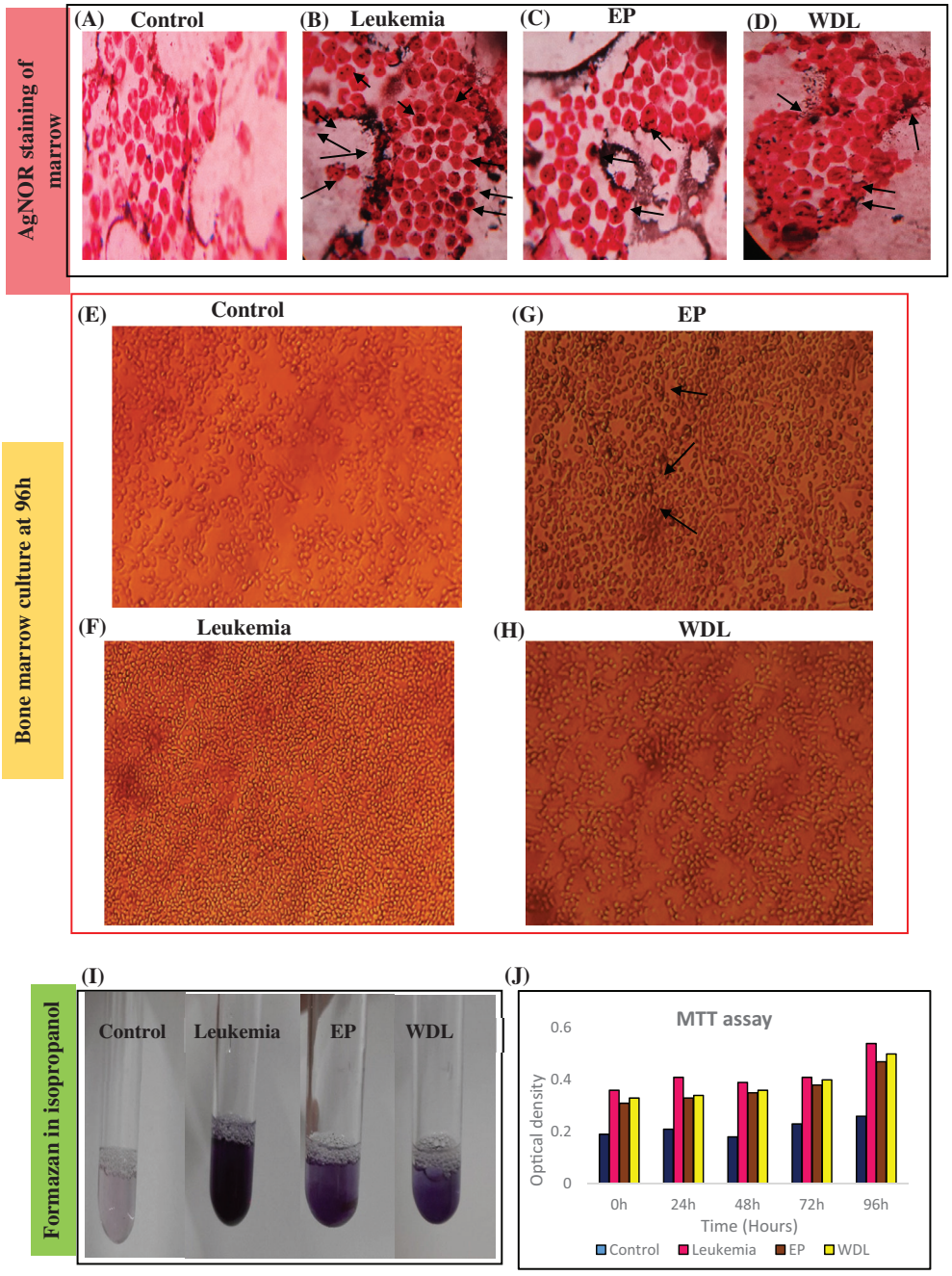

FIGURE 5 Proliferative indices and malignancy conditions pre- and post-treatment. Silver nitrate Nucleolar organizer region staining revealed no malignancy in (A) control, but a sharp rise in the number of nucleolar organizer region (NOR) dots signifying malignancy in (B) leukemic marrow cells. (C) *Eclipta prostrata* (EP) treated, (D) wedelolactone (WDL) treated groups showed significant decrease in the presence of NOR dots in marrow cells. Long-term marrow cell culture of 96 h unveiled normal rate of proliferation in marrow cells of (E) control group. (F) N-N′ethylnitrosourea-induced leukemic marrow cells showed an extremely higher rate of proliferation and stromal cell formation as compared to (G) EP (black arrows depicting apoptotic cells) and (H) WDL treated groups. Growth kinetic study by MTT. (I) Representative image of glass tubes containing marrow cells of each experimental groups after dissolving the formed purple formazan in isopropanol at 96 h interval and (J) representative graph depicting optical densities of cell growth at different time intervals

expression of NLRP3 inflammasome in the BM cells of ENU-induced leukemic mice.[7,53,54] Even though, WDL has been delineated to inhibit NLRP3 inflammasome expression in other disease models, the elaborated mechanism of WDL or the crude extract of EP has never been explored in case of leukemia.[25,26,34] Intriguingly, although we observed a significant downregulation of NLRP3 expression after both EP and WDL treatments separately, the active coumestan indicated a compelling reduction of the NLRP3 expression in the BM cells, in respect of the crude extract. Further, we pursued to explore the remedial influence of the treatments on the adaptor protein of NLRP3 complex, that is, ASC on leukemic BM and found highly elevated expression of ASC molecule in the leukemic condition in contrast to control. Treatment with both EP and WDL could significantly amend the aberrant expression of the adaptor protein ASC, hence authenticating EP and WDL as promising anti-cancer compounds. Next, we aimed to investigate the major pro-inflammatory cytokine IL-1β, which has transpired to be a vital target for hematological malignancies like leukemia, due of its elevated expression and its mounting evidence of being a foe in hematological malignancies.[8,55,56] Inflammasome activation eventually leads to the cleavage of protease caspase-1 and IL-1β maturation to its functional form.[10] Increasing evidences states the anti-inflammatory force of WDL, on the inhibition of IL-1β maturation via caspase-1 activation in various conditions.[26,34,57,58] We observed a spike in cleaved caspase-1 activation and cleaved IL-1β expression in leukemic BM, which was significantly declined in both EP and WDL treated groups

consequently proving their anti-neoplastic effects, however the whole concoction exhibited the maximum effects. Considering the link between inflammasome and leukemogenesis, our study focused on the curative effect of the treatments on the leukemic proliferation and malignant conditions, combined with their anti-inflammasome activity. For instance, various studies in past few decades have reported the anti-metastatic and anti-proliferative properties of EP and WDL.[21,24,32,59,60] Moreover, we carried out AgNOR staining to explore the level of malignancy and we have observed fair mitigation of the malignancy conditions following EP and WDL treatments. In addition, when observed in transwell assay, growth kinetic study and in long-term BM cell culture EP and WDL treatment could significantly attenuate proliferative capacity of leukemic BM cells. Besides, we have investigated the influence of GM-CSF expression pre- and post-treatment, since it is identified for its dual effect of promoting differentiation, mobilization and maturation of myeloid cells, in addition to being the key regulator of IL-1β production.[61] Diseased group showed an abrupt incline in the GM-CSF expression, presumably directing towards the differentiation of leukemic myeloid cells and also generating a persistent inflammatory micro-environment, hence orchestrating to leukemogenesis.[62] EP and WDL treatment significantly declined GM-CSF expression in the leukemic BM cells and interestingly a moderate decrease in the leukocyte population was also observed in treated sections. Taken together, our findings could elaborate the anti-inflammasome and anti-proliferative properties of EP and WDL on leukemic BM cells.

5 | CONCLUSION

In conclusion, our study suggests the environmental pollutant ENU revealed significant leukemogenesis on the bone marrow of ENU-induced leukemia and the crude extract of EP poses to be more overpowering, due to the presence of the concoctions of active compounds as compared to the influence of a single active derivative on leukemic marrow cells. Nevertheless, both EP and WDL can be established as pivotal therapeutic candidates due to the anti-inflammasome and anti-proliferative potentials on the BM of ENU-induced leukemia.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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